POSTER SESSION A

NEOANTIGENS AND CANCER MUTATIONS

A001 / Evaluating prediction strategies for identification of immunogenic mutation-derived neo-epitopes in melanoma

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A number of recent reports point to an important role of mutation-derived neo-antigens in immune recognition of cancer, as predictors of clinical outcome and as potential targets in personalized immunotherapeutic strategies.
Mutagen-induced cancer types as melanoma and lung cancer carry a large number of mutations from which putative neo-epitopes can be predicted. Since the vast majority of mutations are patient specific, identification of neo-epitopes requires prediction and generation of large personalized peptide libraries. Previous reports of neo-epitope identification however have demonstrated that only a minority of peptides (< 1%) elicit T cell recognition at a detectable level. Consequently, there is an unmet need to understand the rules identifying immunogenic neo-epitopes.
In this study we evaluate different neo-epitope prediction approaches in three melanoma patients. Peptide libraries of 200-800 peptides for each patient were generated from whole exome sequencing in combination with HLA binding predictions. A comparison of prediction from autologous tumor cell lines (TCL) and snap-frozen tumor fragments (TF) resulted in an overlap in prediction of 29-81%, 13-68% predicted solely from TCL and 0-5% predicted solely from TF. Furthermore, we included both RNA expression values and immunopeptidome analysis by mass spectrometry as additional tools to identify potential immunogenic neo-epitopes.
T cell recognition in autologous patient material of each personalized peptide library was investigated (ongoing studies) by use of a novel technology based on DNA-barcode labeled MHC multimers, enabling high-throughput screening for >1000 neo-epitope specific T cell populations in a single sample. This broad assessment of neo-epitope reactivity, with minimal preselection, allows evaluation of parameters critical for prediction of immunogenic neo-epitopes.
Identification of precise and effective prediction approaches will provide an important step towards the use of neo-epitopes as therapeutic targets and predictors of response to immunotherapy.

Keywords: neoantigens, peptide prediction, personalized cancer therapy

A002 / Characterization of tumor-associated calreticulin mutants and their effect on antigen presentation by MHC class I

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The phenomenon of antigen processing and presentation aids the immune system in distinguishing between normal and malignant cells. In cancer cells, major histocompatibility complex (MHC)-encoded membrane proteins present tumor- associated antigens that may serve as targets of immunotherapy. Antigen presentation by MHC class I-β2m heterodimers (MHC-I) requires peptides to be loaded onto the complex in the ER. This is facilitated by the peptide loading complex (PLC), of which calreticulin (CRT) is an integral member. CRT associates with MHC-I via the single, conserved, glycan present on MHC-I, drawing it into the PLC to facilitate peptide loading.
CRT is comprised of an N-terminal lectin domain, a proline-rich central P domain, and a negatively charged C-terminal domain (CTD) that also contains an ER retention motif. Two studies have reported a somatic frameshift CRT mutation (CRT_{FS}) associated with myeloproliferative neoplasms (MPNs). The mutation generates a novel C-terminal domain that lacks the ER-retention signal and renders it positively charged. Previous studies in murine cells have shown that full-length CRT is necessary for efficient translocation of MHC-I to the surface of cells. Interestingly, reconstitution of CRT lacking the CTD did not restore MHC-I expression, indicating a role for the CTD in MHC-I expression. We therefore sought to characterize CRT_{FS} and assess its effect on antigen presentation my MHC-I in human cell lines.
We ablated CRT expression in HEK293T cells and found that surface MHC-I levels were lowered in the absence of CRT. This was rescued by the expression of CRT but not CRT_{FS}. While probing the molecular basis for this phenotype, we found that CRT_{FS} is secreted from cells and does not interact with the PLC. Indeed, we find that the PLC is unstable in both CRT-null cells and cells expressing CRT_{FS}. An unstable PLC has impaired peptide-loading function, which affects surface MHC-I levels. Further biochemical characterization of these proteins and their effects on antigen presentation are

underway. We aim to extend this analysis to MPN cell lines to identify tumor-associated antigens as potential immunotherapy targets.

Keywords: myeloproliferative neoplasms, calreticulin, major histocompatibility complex (MHC-I), peptide-loading complex, antigen presentation

A003 / The roles and mechanisms of chromosomal loop domains in directing the recombination of immunoglobulin light chain genes

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The N-terminal variable regions of immunoglobulin (Ig) heavy (IgH) and light (IgL) chains that are involved in specific antigen binding are assembled from germline ***variable (V)***, ***diversity (D),*** and ***joining (J)*** gene segments by V(D)J recombination in developing B lymphocytes. V(D)J recombination is initiated by RAG endonuclease, completed by non-homologous DNA end-joining, and tightly regulated in the contexts of order, lineage, and allelic exclusion. Controlled V(D)J recombination is important beyond its role in generating diverse antibody repertoires against numerous antigens, since dysregulation of V(D)J recombination underlies various immune disorders and oncogenic translocations that are commonly identified in B cell leukemias and lymphomas. Many of such translocations involve IgL chain loci. To provide new insights into mechanisms of chromosomal translocations involving IgL chain loci, we sought to investigate mechanisms of normal V(D)J recombination at IgL loci by developing and applying multiple new approaches. In this study, we focused on elucidating the roles and underlying mechanisms of chromosomal loop domains in directing the recombination of immunoglobulin k light chain locus (Igk). To this end, we firstly developed a novel chromosome conformation capture sequencing method that shows great advantages over classical 4C-seq in improving the chromatin interaction detection sensitivity and identified multiple loop domains mediated by specific sites across the entire Vk region that interact with the CTCF-binding element (CBE)-based ***Cer*** element that lies in the Vk-Jk intervening region. Notably, a significant fraction of these ***Cer***-interacting sites are not related to CBEs but decorated by transcription factors with functions in mediating chromatin interactions that's not clearly defined previously. We further mutated such interacting sites across the Vk region and examined the effects of the mutations on Vk-Jk recombination by employing our recently developed HTGTS repertoire sequencing (HTGTS-Rep-seq) method. Strikingly, the mutation of these interacting sites only specifically diminished the utilization of the Vk that is immediately adjacent to the interacting site, suggesting a specific role for such sites in mediating Vk utilization. And interestingly, in the context of such interacting sites mutations, the RAG initiated off-target activities expanded to the neighboring loop domains, implicating an important role for such loop domains in restricting the mutagenic activities of RAG. Furthermore, the mechanisms by which chromosomal loop domains harness normal V(D)J recombination and prevent potentially oncogenic translocations will be also discussed.

Keywords: V(D)J recombination, immunoglobulin light chain, loop domain

A004 / Identification of unique neoantigen qualities in long-term pancreatic cancer survivors

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Pancreatic adenocarcinoma (PDAC) is a lethal cancer with < 7% of patients surviving past 5 years. T cell immunity has been linked to the exceptional outcome of the rare long-term survivors but the antigens are unknown. To identify these T cell antigens in long-term PDAC survivors, we assembled the largest cohorts of stage-matched short [n=68, median overall survival (OS) 0.8y] and long-term PDAC survivors (n=82, median OS 6y). To enable antigen discovery, we performed a combined analysis of whole exome sequencing, neoantigen prediction, T cell receptor (TCR) Vb-chain sequencing, 9-color multiplexed immunohistochemistry, and tumor transcriptomic analysis. To assess differential neoantigen immunogenicity, we performed neoantigen fitness modeling integrating clonal genealogy, epitope homology, and T cell receptor affinity. To examine in vivo T cell-neoantigen reactivity, we used functional assays in a subset of very long-term PDAC survivors (n=7, median OS 10.5 years). Using these techniques, we found that tumors of long-term survivors displayed 12-fold greater cytolytic $CD3^+CD8^+$Granzyme-B^+ cells, with >94% of intratumoral T cell clones unique to tumors and not shared with adjacent normal pancreatic tissue, suggesting intratumoral antigen recognition. Tumors of long-term survivors exhibited greater TCR repertoire diversity compared to tumors of short-term survivors, implying differential antigenic targets. In examining neoantigens as potential T cell targets, we found that patients with both the highest predicted neoantigen number and the greatest $CD3^+CD8^+$ infiltrates together but not either parameter alone, exhibited the longest survival (median OS not reached vs. 0.8y, P=0.004). In investigating possible unique neoantigen qualities in long-term survivors, a neoantigen quality model conferring greater immunogenicity to dominant neoantigens with homology to microbial epitopes identified long-term survivors independent of confounding variables and chemotherapy (Neoantigen $Quality^{Hi\ vs.\ Low}$ - median OS 8.6y vs. 0.8y; P=0.002) whereas a model ascribing greater immunogenicity to increasing neoantigen number did not (Neoantigen $Quantity^{Hi\ vs.\ Low}$ - median OS 0.8y vs. 1y; P=0.3). Similarly, neoantigen quality, but not quantity, was independently prognostic of survival in a larger, independent cohort unselected by survival (n =166; Neoantigen $Quality^{Hi\ vs.\ Low}$ - median OS 30m vs. 14m; P< 0.0001). Finally, we detected lasting circulating T cell reactivity to both high quality neoantigens and cross-reactive antigens in PDAC survivors, including identical intratumoral clones with specificity to both (n=5 of 7 patients tested). Our results identify neoantigens with unique qualities as T cell targets in PDAC. In a broader sense, we identify neoantigen quality as a biomarker for immunogenic tumors that may facilitate rational application of immunotherapies.

Keywords: Pancreatic cancer, Neoantigens, Microbiome

A005 / Deciphering HLA-I motifs across HLA peptidomes improves neo-antigen predictions and reveals novel properties of HLA molecules

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The precise identification of Human Leukocyte Antigen class I (HLA-I) binding motifs plays a central role in our ability to understand and predict (neo-)antigen presentation in infectious diseases and cancer. Here, by exploiting co-occurrence of HLA-I alleles across ten newly generated as well as forty public HLA peptidomics datasets comprising more than 250,000 peptide-HLA interactions, we show that we can rapidly and accurately identify HLA-I binding motifs and map them to their corresponding alleles without any ***a priori*** knowledge of HLA-I binding specificity. Our approach recapitulates and refines known motifs for more than 50 of the most frequent alleles, uncovers new motifs for 10 alleles that up to now had less than five known ligands and provides a scalable framework to incorporate additional HLA peptidomics studies in the future. The refined motifs improve neo-antigen and cancer testis antigen predictions, indicating that unbiased HLA peptidomics data are ideal for ***in silico*** predictions of neo-antigens from tumor exome sequencing data. The new motifs further reveal novel properties of HLA-I molecules, including cooperativity between different residues in HLA-I ligands, evolution of HLA-I motifs with peptide length and allostery regulating HLA-I binding specificity.

Keywords: Neo-antigen predictions, HLA peptidomics, Computational biology

A006 / Antigen discovery pipeline using mass-spectrometry based immunopeptidomics for the development of personalized cancer immunotherapy

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Cancer immunotherapy reprograms the inherent capacity of cytotoxic T cells to eliminate tumor cells by recognizing molecular entities expressed specifically on tumors but not on normal cells. Recent data show that recognition of mutated neo-antigens plays a key role. The discovery of neo-antigens relies mainly on prediction-based interrogation of the ‚mutanome'. Currently, the performance of HLA-I ligand interaction prediction algorithms is still rather poor for infrequent HLA class I molecules for which binding data is limited, and in general for HLA class II molecules. Mass-spectrometry (MS) analysis of HLA binding peptides eluted from tissue samples is a promising approach to discover the in-vivo presented neo-antigens. Recently, we have developed an in-depth MS-based immunopeptidomics approach combined with exome sequencing analysis to directly identify neo-antigens from human melanoma tumors (Bassani-Sternberg et.al., NatCommun 2016). Four of the eleven neoantigens we identified proved to be immunogenic and tumor-reactive T-cells with specificity for two of the neo-antigens were detected in the patient`s peripheral blood and among the tumor infiltrating T cells.
Currently we are developing and implementing a robust experimental and computational integrative pipeline, compatible with clinical applications, regulations and tight timeframes, for our personalized cancer vaccine programs. Our pipeline allows prioritization and identification of patient specific tumor-associated antigens and neo-antigens directly from tumor tissues by combining our new sensitive and high-throughput immunopeptidomics method with genomics data using novel computational algorithms. MS-based approaches have limited sensitivity and are only applicable to a small fraction of our patient populations due to the large amount of sample that is required (typically $1cm^3$ of tissue). However, while hunting for the neo-antigens, our immunopeptidomics assays produce massive amount of highly valuable data that can be used to better understand the binding properties of HLA-I molecules and the presented peptidome.
In a proof-of-concept study we showed that incorporation of deconvoluted immunopeptidomics data in ligand prediction algorithms improve their accuracy for HLA alleles with few ligands in existing databases (Bassani-Sternberg and Gfeller, J Immunol 2016). We further developed a novel algorithm that can rapidly and accurately identify HLA-I binding motifs and map them to their corresponding alleles, without any ***a priori*** knowledge of HLA-I binding specificity, by taking advantage of co-occurring HLA-I alleles. Our novel approach uncovers new motifs for several alleles that up to now had no known ligands. HLA-ligand predictors trained on such data substantially improve neo-antigen predictions in four melanoma and two lung cancer patients, indicating that unbiased HLAp data are ideal for in silico identification of neo-antigens (Bassani-Sternberg et. al., bioRxiv 2017).

Keywords: Neoantigens discovery, Immunopeptidomics, Personalized cancer vaccines

A007 / Towards improved algorithms for neo-epitope prediction in cancer

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Personalization of immunotherapies such as cancer vaccines and adoptive T-cell therapy will depend on identification of patient-specific neo-epitopes that can be specifically targeted. Efficient methods for identification of neo-epitopes will yield insight into tumor-immune interactions and may improve personalized immune therapy. Current methods for predicting immunogenic mutated peptides, based on features such as HLA binding and gene expression, result in a low rate of validation in screens for reactive T cells. Thus, there is a need for improved prediction methods and new data for optimizing these methods. Data was gathered from published studies, including 1922 potential neo-epitopes originating from single nucleotide variants (SNVs) and screened for T-cell reactivity. 50 of these peptides were recognized by corresponding tumor infiltrating lymphocytes (TILs). We observed that 13 of the 50 immunogenic peptides featured a mutation in an MHC anchor position resulting in a predicted shift from non- / weak-binder in the wild-type peptide to a stronger binder in the mutated peptide. The other 37 immunogenic peptides featured mutations in the presumed T-cell recognition site which do not necessarily undergo this shift. We also applied NetMHCpan 3.0 to the peptides and found that stronger predicted binding is somewhat predictive of immunogenicity of potential neo-epitopes (AUC 74.8%), even though the peptides were pre-selected based on predicted binding affinity. Since the literature data included only SNVs and not indels, and did not take allele frequency or expression into account we believe there is still a need to further generate datasets and develop the predictive methods. We have previously established a platform for neo-epitope extraction and prediction based on tumor sequencing data, MuPeXI (http://www.cbs.dtu.dk/services/MuPeXI/). For several patient cohorts we are

screening for broad libraries of mutation derived peptides (150-1000 per patient) extracted based on MuPeXI, and analyzing the T cell reactivity based on high-throughput screening using DNA barcode-labeled MHC multimers. This data will likely provide novel insight into determinants of immunogenicity of neo-epitopes and define new rules to optimize algorithms for neo-epitope prediction.

Keywords: Prediction, Neo-epitopes, T cell reactivity

A008 / Performance evaluation and optimized decision thresholds for MHC class I binding prediction algorithms

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Numerous different computational methods for the prediction of MHC class I binding epitopes have been developed. These algorithms gained importance with the advent of tumor sequencing programs and checkpoint blockade agents, as mutation-derived neo-epitopes have come into the spotlight as targets for anti-tumor immune responses. The prediction methods are useful tools to asses if a mutation is likely to result in a neo-epitope that binds to the human leukocyte antigen (HLA) molecules of the respective tumor patient. However, there are considerable differences between predicted and actual binders. For this reason we evaluated the prediction performance of nine MHC class I binding prediction algorithms for important HLA alleles by experimental validation of potential epitopes. We predicted the binding of 8-, 9-, 10- and 11-mer peptides derived from HPV16 E6 and E7 to the most common HLA types. Predicted epitopes were synthesized and tested in competition-based cellular binding assays to determine the actual binding affinity. Data from affinity predictions and experimental assessment was used to calculate and analyze receiver operating characteristic (ROC) curves and respective area under the curve (AUC_{ROC}), sensitivity and specificity as measures of prediction performance. No single prediction server outperformed the others, but different prediction servers were found to be best for distinct HLA types and peptide lengths. The analysis revealed that the commonly used decision thresholds are too stringent and miss actual binders. Therefore, we calculated optimized threshold values to increase specificity and sensitivity, individually for each method, HLA allele and peptide length. We provide recommendations for the optimal use of peptide-MHC class I binding prediction methods to increase the chances of predicting actual ligands. This could improve the selection of potential targets for epitope-driven immunotherapy.

Keywords: neo-epitope prediction, MHC class I ligands, epitope prediction performance

A009 / Identification of IDH1R132H-specific T cell receptors from glioma patients and from MHC-humanized mice

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Purpose: Central to an effective immunotherapy for gliomas is the discovery of tumor-specific antigens. One such example are mutations in the gene encoding for isocitrate dehydrogenase 1 (*IDH1*) resulting in the uniform neomorphic enzyme IDH1R132H. We have previously shown that an IDH1R132H-peptide vaccine evokes an MHC class II-restricted T-helper cell response and is therapeutic in an MHC-humanized mouse sarcoma model. Here we aim at identifying IDH1R132H-specific T cell receptors (TCRs) to pioneer IDH1R132H-specific T cell therapy.
Methods and procedures: To identify IDH1R132H-specific TCRs, T cell lines were generated from splenocytes of vaccinated MHC-humanized mice by re-stimulations with autologous $IDH1R132H^{p132-142}$-loaded splenocytes. Subsequently, T cells were sorted for IDH1R132H-specificity exploiting IDH1R132H-specific HLA-DR1-tetramers and IFN-g secretion assays. In addition, we analyzed IDH1R132H-reactive T cells from patients with $IDH1R132H^{+}$ gliomas and IDH1R132H-specific T cell immune responses.
Results: Sequencing analyses of the V-J or V-D-J regions of the *TRA* and *TRB* genes, respectively, revealed dominant TRA and TRB sequences in the established murine T cell lines. Full length murine TCRs were constructed either by pairing dominant TRA and TRB sequences and cloned into expression vectors or delivered as RNAs for functional validation. T cells from glioma patients were expanded *in vitro* and subjected to single-cell-sorting and subsequent TCR sequencing.
Conclusions: Identification of IDH1R132H-specific TCRs may expand the immunotherapeutic spectrum for IDH1R132H-mutated gliomas.

Keywords: glioma, IDH1, T cell receptor

A010 / Immunogenic determinants of tumor neoantigens

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Accumulating preclinical and clinical evidence suggests that tumor-specific mutations can generate neoantigens recognized by T cells and drive protective immunity against cancer. This has resulted in high interest in using neoantigens for personalized cancer vaccination. Since only a small fraction of candidate neoantigens are presented on MHC molecules, and only a further subset of those are immunogenic, prioritization is crucial for personalized cancer vaccination. Next generation sequencing and computational methods have been successfully applied in recent studies to predict neoantigens that may be presented by MHC molecules. However, false positives remain, and incorporating peptide immunogenicity prediction may help to further enrich for immunogenic neoantigens.
We used four mouse models to differentiate between non-immunogenic and immunogenic neoantigens among the mutated peptides that are predicted to bind to MHC-I molecules in these models. We vaccinated naive mice with mutated long peptides and adjuvant to identify the ones that elicit CD8 and/or CD4 T cell responses. As previously published by others, we saw that the majority of the mutated peptides predicted to bind MHC-I were not immunogenic, and that most of the immunogenic peptides elicited a MHC-II restricted T cell response, despite the fact that predictions were for MHC-I binding, not MHC-II. We also investigated properties of the mutant peptides relative to their wild-type counterparts, including binding affinities, differences in amino acid properties, etc., and identified determinants of immunogenicity that may help further prioritize for immunogenic neoantigens.

Keywords: neoantigens, cancer vaccine, immunogenicity

A011 / Novel and shared neoantigen derived from histone 3 variant H3.3K27M mutation for glioma T-cell therapy

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Brain cancers are the leading cause of cancer-related mortality in children. In particular, the median overall survival for children with diffuse intrinsic pontine glioma (DIPG) is less than one year. The majority of diffuse midline gliomas, including over 70% of DIPG, harbor an amino-acid substitution from lysine (K) to methionine (M) at the position 27 of histone 3 variant H3.3. As predicted by the NetMHC3.4 peptide binding algorithm, a 10-mer synthetic peptide encompassing the K27M mutation (H3.3K27M peptide hereafter), but not the corresponding non-mutant peptide, demonstrated an excellent binding affinity to human leukocyte antigen (HLA)-A2 in a competitive binding inhibition assay. Importantly, mass spectrometry demonstrated that the H3.3K27M peptide is indeed naturally processed and presented by HLA class I on the surface of HLA-A2$^+$ glioma cells bearing the H3.3K27M mutation. H3.3K27M-specific CD8$^+$ T cell responses were detected in patients´ peripheral blood mononuclear cells by interferon-γ ELISPOT assay following a 7-day course of in vitro stimulation with the H3.3K27M peptide. Repeated stimulation of HLA-A2$^+$ CD8$^+$ T-cells with the H3.3K27M peptide led to establishment of H3.3K27M-reactive T-cell clones. From one clone with excellent binding to the HLA-A2-H3.3K27M tetramer, cDNA for T-cell receptor (TCR) α- and β-chains were cloned into a retroviral vector. Human HLA-A2$^+$ T-cells transduced with the TCR efficiently killed HLA-A2$^+$ H3.3K27M$^+$ glioma cells in an antigen- and HLA-specific manner. Adoptive cell transfer of TCR-transduced T-cells significantly suppressed the progression of glioma xenografts in mice. Furthermore, alanine-scanning assays demonstrated that there are no known human proteins that share the set of key amino acid residues required for recognition by the TCR, strongly suggesting that the TCR could be safely used in patients without causing off-target toxicity. These data provide us with a strong basis for developing peptide-based vaccines as well as adoptive transfer therapy using autologous T-cells transduced with the TCR. Based on these data, we have initiated a pilot study evaluating the safety and preliminary clinical activities of the H3.3K27M peptide vaccine in HLA-A2$^+$ pediatric patients with H3.3K27M$^+$ glioma.

Keywords: diffuse midline gliomas, neoantigen, T-cell receptor

A012 / Calreticulin mutations induce T cell immunity in myeloproliferative neoplasms

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Somatic mutations in the calreticulin (CALR) gene are key drivers of cellular transformation in myeloproliferative neoplasms (MPN) and are found in about 25% of MPN patients. All CALR mutations identified to date in MPN patients result in the formation of an

altered protein with an identical 36-amino acid sequence in the C-terminus. Interrogation of ***in silico*** peptide binding prediction algorithms suggested that the epitopes from the altered C-terminus can bind to class I and II human leukocyte antigens. Therefore, in this study, we investigated the immunogenicity of the altered CALR C-terminus in both healthy donor and MPN patient peripheral blood mononuclear cells. We found that naïve T cells from healthy blood donors displayed effector functions upon priming with overlapping peptides encompassing the mutated region ***in vitro***. Both $CD8^+$ and $CD4^+$ T cell precursors recognized the mutated-CALR; whereas the corresponding wild type sequence did not induce a T cell response. The mutant-CALR-specific T cells proliferated, upregulated CD137 (4-1BB) and produced interferon (IFN)-γ and tumor necrosis factor (TNF)-α. We also investigated the presence of mutant CALR-specific T cell immunity in MPN patients carrying CALR mutations. We observed that the recognition of mutated-CALR was not abundant in T cells from MPN patients. T cells from 3 out of 16 patients had increased IFN-γ production upon stimulation with overlapping peptides. However, T cells from MPN patients also exhibited increased levels of the programmed cell death (PD)-1 and the cytotoxic T-lymphocyte-associated antigen (CTLA)-4 compared to the healthy controls. Furthermore, the inhibition of PD-1 or CTLA-4 signaling restored mutant CALR-specific T cell responses in some MPN patients. Therefore, we conclude that the scarcity of T cell responses was, at least in part, due to the exhausted state of T cells. Together, these results establish the mutated-CALR as a MPN-specific tumor antigen and provide a rationale for the development of immunotherapies targeting mutated-CALR in MPN patients carrying the mutation.

Keywords: Calreticulin mutations, Cancer neoantigen, Myeloproliferative neoplasms

A013 / Characterization of neoantigen-specific CD8+ T cell immune responses to B16 melanoma inducible by multivalent vaccination

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Mutation-derived tumor antigens (MTA)-alternatively known as "neoantigens"-are a class of tumor antigens (TA) generated by somatic mutations. MTA are thought to be the predominant target of spontaneous and treatment-induced anti-tumor immunity. Direct targeting of MTA with therapeutic vaccination or other approaches is thus an active area of clinical research. However, pre-clinical studies of MTA-specific immunity have been limited in part due to a lack of reproducible targets. Furthermore, properties distinguishing effective MTA-specific immune responses, from less effective responses targeting other types of non-mutated TA have not been identified. To address this deficit we developed a system wherein MTA-specific CD8+ T cell-mediated immunity could be systematically characterized. We performed exome (WES) and RNASeq on the B16F1 and B16F10 melanoma cell lines, as well as matched normal tissue. We identified somatic single-nucleotide substitutions, insertion and deletion (INDEL), as well as larger INDEL and gene translocation events. The identity of B16F10 somatic variants was validated by cross-referencing published WES and RNASeq data. Standard ***in silico*** protocols were used to predict MHC-I binding affinity and off-rate, which were subsequently validated via fluorescent peptide:MHC stability assay. Screening vaccinations were performed with soluble, depot carrier, and nanoparticle-based vaccination protocols. The frequency of responding MTA-specific CD8+ T cells was measured using MHC tetramers generated by UV-mediated ligand exchange, and the surface receptor phenotype of MTA-specific tetramer-positive cells was characterized by flow cytometry and high-dimensional mass cytometry (CyTOF). The function of MTA-specific CD8+ T cells was measured by intracellular cytokine staining, target-mediated degranulation, as well as multiplexed ***in vivo*** cytotoxicity assay. Finally, therapeutic activity of a 6-valent MTA-specific particulate vaccine was assessed using subcutaneous and intravenous B16F10 tumor challenge. In summary, we report the identification and detailed characterization of multiple previously-undescribed MHC-I and MHC-II restricted MTA relevant to the widely-utilized B16F10 model. We assess the utility of proposed ranking criteria vis-à-vis predicting the immunogenicity of putative MTA identified by sequencing. We characterize the phenotype and function of MTA-specific CD8+ T cells—including the first reported characterization by CyTOF. We describe incorporation of MTA into a multivalent vaccine, longitudinal profiling of MTA-specific prime-boost vaccination, as well as the therapeutic response to multivalent MTA-specific vaccination as adjuvant monotherapy. Finally, we describe phenotypic and functional properties of MTA-specific CD8+ T cells associated with therapeutic activity ***in vivo.***

Keywords: Neoantigen, Vaccine, Melanoma

A014 / Major Histocompatibility Complex (MHC) class I-associated phosphopeptides as potential targets for the immunotherapy of cancer

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The identification of tumor-specific antigens provides the basis for the development of an efficient targeted immunotherapy. MHC-class I associated phosphopeptides have been proposed as promising novel tumor antigens. This is because phosphoproteins are abundantly integrated in most signalling pathways and dysregulation of signalling pathways, including aberrant and increased phosphorylation of proteins, represents one hallmark of cancer. These phosphoproteins can be degraded and the derived peptide fragments are presented via MHC-class I molecules on the cell surface of altered cells leading to $CD8^+$ T cell recognition. Using a mass spectrometry approach, until today more than 400 phosphopetides were identified on several different cancer entities, including hepatocellular carcinoma (HCC) and malignant melanoma. Interestingly, most of the identified phosphopeptides were displayed by Human Leukocyte Antigen (HLA)-B7, HLA-B27 or HLA-A2 molecules. HLA-class I presented phosphopeptides were found predominantly on tumorous tissues when compared to adjacent healthy tissues. Notably, many of the underlying proteins play an important role in tumor progression or survival, making them especially interesting for immunotherapeutic strategies. Based on their potential involvement in tumor progression, 37 HLA-A2 and HLA-B7 phosphopeptides were further selected for immunological testing. Peripheral blood lymphocytes from healthy individuals, patients with chronic liver diseases or HCC patients were isolated and stimulated with the phosphopeptides for 7 days followed by intracellular cytokine staining. $CD8^+$ T cell responses against this novel class of tumor antigens were comparable in quantity and quality to those seen against viral epitopes. Phosphopeptide-specific $CD8^+$ T cell responses were found in patients with chronic liver disease and liver cirrhosis but not in patients with HCC. Our results therefore suggest that MHC-class I presented phosphopeptides may be the target of cancer immune surveillance in liver disease and therefore represent an attractive target for future cancer immunotherapies.

Additionally, it was shown that checkpoint blockade beneficially impacts the survival of patients with different cancer entities. Especially for malignant melanoma the efficacy of checkpoint blockade has been demonstrated in several phase III clinical trials and for HCC phase I/II clinical trials looked promising. However, only a fraction of patients experience clinical benefit, but predictive biomarkers are unknown. Phosphopeptides might serve as such predictive markers, as first data indicate that patients responding to checkpoint therapy show phosphopeptide-specific $CD8^+$ T cell responses. Therefore, we are currently evaluating phosphopeptide-specific $CD8^+$ T cell responses in HCC and melanoma patients before and during the course of checkpoint therapy.

Keywords: phosphopeptides, cancer immunotherapy, tumorantigens

A015 / Number of predicted tumor-neoantigens as biomarker for cancer immunotherapies

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Cancer immunotherapies, like immune checkpoint inhibition, aim to boost the immune response towards the tumor. Presentation of somatic mutation-derived neoantigens via HLA molecules on the surface of tumor cells is important for their recognition and destruction by cytotoxic T cells. Concordantly, the quality and quantity of those neoantigens were shown to be of high prognostic and therapeutic value and may therefore serve as biomarkers for the efficacy of cancer immunotherapies.
We established a workflow to identify the individual tumor-specific neoantigens. Somatic variants leading to novel protein sequences (neoantigens) were identified by exome sequencing of normal and tumor tissue. After HLA typing using exome data, computer algorithms were applied to predict those neoantigen-peptides which bind with high affinity to the patient's HLA class I molecules. While the total number of neoantigen-peptides may already be predictive for the efficacy of immune checkpoint inhibition, we also established standardized selection criteria to automatically prioritize the predicted neoantigens for targeting by cancer vaccines or adoptive T cell therapies. Till today more than 100 tumor samples from patients suffering from cancers of

diverse origin have been analyzed for their neoantigen repertoire. Here the summarized results of these analyses will be presented alongside with the established selection criteria for the automated prioritization of the most promising neoantigens for targeted cancer immunotherapies.

Keywords: neoantigens, cancer immunotherapies, target identification

A016 / PDC*vac: a powerful platform for the priming and expansion of Neo-Antigen specific-T cells

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PDC*vac is a patented technology relying on the use of PDC*line, a HLA-A*02:01+ plasmacytoïd Dendritic Cell (PDC) line, as a potent antigen presenting cell to strongly boost T cell responses both in vitro and in vivo. PDC*vac is composed of PDC*line pulsed with any desired HLA-A*02:01 restricted peptide, relevant for targeting a given cancer. Importantly, PDC*line can be also engineered with mRNA or viral vectors to endogenously express any desired antigens.
PDC*vac superior potency and modularity represents a unique solution to overcome all the limitations of conventional therapeutic cancer vaccines. Indeed, PDC*vac shares the advantages of both antigen-based vaccines (homogeneity, cost-effectiveness, scalability) and of classical dendritic cell-based vaccines (optimal DC targeting and loading, efficacy), while in fact being much more potent than either platform.
A strong body of preclinical data has demonstrated its unique ability to induce dramatic T cell expansions from naïve and memory CD8+ cells, against multiple antigens in the context of melanoma, lung cancer or HBV infection, both ***ex vivo*** from cord blood mononuclear cells, patients' PBMC or Tumor Infiltrating lymphocytes (TILs), and ***in vivo*** in an innovative CD34+ humanized mouse model. Moreover, PDC*vac is being evaluated in a first-in-human phase I clinical trial in advanced melanoma.
Here, we aimed at exploiting PDC*vac properties for priming and expanding anti-neoantigen (neoAg) T cell responses from **healthy donor naïve CD8+ T-cells.**
We demonstrated in this study that weekly stimulation by neoAg-pulsed PDC*line leads to sizeable expansion of antigen specific CD8+ T-cells from naïve precursors as soon as 14 days of co-culture, with a powerful expansion as day 21 (Fold increase: 50 to 150 compared to baseline). These results were obtained with cells purified form healthy donors' mononuclear cells and 2 HLA-A*02:01 neoAg selected from the literature and identified in lung cancer or melanoma. This expansion is specific as no other antigen-specific T-cells were expanded. Moreover, these neoAg-specific T cells display functional activity as revealed by the expression of CD107 and IFNg secretion upon stimulation. Importantly, these cells are specific for the mutated form of the peptide and not the wild type form.
Altogether these data demonstrate that PDC*vac represents a powerful tool for assessing the immunogenicity of neo-epitopes ***in vitro*** as well as a powerful vaccine platform for personalized cancer immunotherapies.

Keywords: Neoantigens, plasmacytoid dendritic cell line, cancer vaccine

A017 / EasyFuse - an easy to maintain comprehensive fusion gene detection pipeline

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Genomic aberrations, such as fusion genes or alternative splicing events are important key players in cancer development due to their uniqueness. The originating gene products can provide a selective advantage for the tumor cell and may trigger tumor growth. With the emerging number of next-generation sequencing data sets, especially from transcriptome sequencing (RNA-Seq), it becomes more feasible to study such events in great detail over large cohorts. Despite the great amount of available fusion gene detection tools (>20), it is cumbersome and complicated to install them as they either require many dependencies or are often not well documented and or maintained. In many cases, the software is outdated and not applicable to newer data sets or reference genomes. Also, the results differ by software environment and characteristics of the data set (including quality, read length and sequencing depth). To address these problems, we provide an out-of-the-box pipeline, ***easyFuse***, that (i) integrates top fusion tools, (ii) can be easily installed and runs on a job scheduling system (iii) performs quality control on raw data, (iv) provides reproducible results and (v) offers primers ready for wet-lab confirmation. We first extensively evaluated the performance of the fusion detection tools, which meet the following criteria: application to any genome possible, flexible, high sensitivity on test data and robustness of the algorithm. For this, we used in-house and publicly available data from four cell lines (***SKBR3*** and ***MCF7***) to compare predicted fusion genes with validated ones. The best performing tools were integrated in our pipeline to improve the prioritization of candidates as high confidence true fusion genes and consequently

reduce the number of false positives. To validate our pipeline further, we confirmed the predicted fusion genes including the ones already reported in literature. We have detected 15 new fusion genes in MCF7 and 1 in SKBR3. In summary, with ***easyFuse*** we were able to confirm already known fusion genes, but also detect new ones for two very well studied cell lines. Further, we offer an easy to install, comprehensive pipeline providing a fully detailed prioritization list of fusion genes ready for experimental testing.

Keywords: Genomic aberrations, fusion gene detection, easyFuse

A018 / Deciphering the intra-tumoural T cell receptor repertoire in NSCLC within the lung TRACERx study

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The abundance of tumour infiltrating lymphocytes has previously been associated with clinical outcome in NSCLC. Our group has demonstrated the importance of the clonality of mutations in predicting overall survival in NSCLC and response to checkpoint blockade. There is however very limited information on the impact of mutational burden and intratumoural heterogeneity (ITH) on the TCR repertoire in NSCLC. We sought to assess the T cell repertoire in multi-region tumour specimens, matched normal tissue and peripheral blood of patients with NSCLC as part of a prospective multi-institutional study exploring the mechanisms of NSCLC evolution from diagnosis through to death (Lung TRACERx study). We have previously developed a pipeline for quantitative α and β TCR repertoire analysis utilising molecular barcoding. Here, we report TCR sequencing data from multi-region tumour specimens, histologically confirmed normal lung and PBMC in patients with genetically heterogeneous (high ITH) and homogenous (low ITH) NSCLC. Multi-region NSCLC tumour specimens and matched normal tissue were found to have a similar number of TCRs. The total number of TCRs detected varied in different tumour regions within the same patient and between patients as confirmed by multiplex immunohistochemistry. The five most abundant TCRs found in tumour regions accounted for a greater proportion of the TCR repertoire compared to matched normal tissue regardless of intra-tumoural genetic heterogeneity. The five most abundant TCRs found in tumour regions accounted for a greater proportion of the TCR repertoire compared to normal lung. Tumour repertoires exhibited an oligoclonal TCR repertoire distinct to that seen in normal lung or peripheral blood suggestive of a repertoire of T cells spatially confined to the tumour microenvironment, possibly driven by the presence of tumour antigen. The Jaccard Index of the 100 most abundant TCRs demonstrated a significantly higher degree of overlap in the TCR repertoire of multi-region tumour specimens in low versus high ITH tumours. A lower degree of TCR repertoire overlap was observed between tumour regions and normal tissue or peripheral blood. We observed two classes of intra-tumoural TCRs those that are found at high abundance across different regions of the tumour referred to as ‚pan-tumoural' and those, which were found only in, certain tumour regions referred to as ‚region-specific'. We defined a TCR ITH index based on the ratio of these two classes of TCRs, and correlated this index to the genetic and neoantigen landscape of these patients' tumours. The varying degrees of intra-tumoural TCR diversity in both low and high ITH tumours are indicative of a dynamic intra-tumoural T cell response that may be accounted for by differences in the genetic heterogeneity and mutational burden observed in NSCLC.

On behalf of the Lung TRACERx consortium

Keywords: Lung cancer, TCR repertoire, Neoantigens

A019 / Peptide MHC stability as a better prediction of immunogenicity

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Cancer neo-antigen vaccination holds great promise in the treatment of cancers with high mutational load. Identification of peptide neo-antigens capable of eliciting a CD4 or CD8 T cell response however remains an unresolved obstacle. Affinity based in silico prediction tools such as netMHC or IEDB tends to be overpredictive generating to many false positives. To obtain the full potential of neo-antigen vaccination, novel tools will have to be generated.

Immunitrack is a spinout from the academic group, at the University of Copenhagen, that drove the data generation behind netMHC. Since spinout we have further developed the highly versatile recombinant MHC platform where biotinylated and highly active MHC are used in extremely sensitive assays capable of measuring peptide MHC interactions down to the low nanomolar area.

As a much sought novelty we have developed high throughput MHC I and II stability assays, that can significantly reduce the amount of false positives identified by eg. netMHC. In this study we

will show how our MHC peptide stability assays provides a better prediction of immunogenicity than affinity. In addition we will show how our recombinant platform of biotinylated MHC can be used to generate high numbers of MHC I and II tetramers, enabling an ambitious characterization of e.g. TIL´s .

Keywords: MHC, neo-antigen, netMHC

A020 / Identification of non-exonic immunogenic targets for cancer immunotherapy using a novel proteogenomic approach

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MHC I peptide complexes (pMHCs) are presented at the surface of all nucleated cells and play a central role in CD8 T cell immunosurveillance. pMHCs presented by MHC II^{hi} medullary thymic epithelial cells (mTEC^{hi}) are essential to eliminate self-reactive CD8 T cells in a process called central tolerance. pMHCs unique to cancer cells are referred to as tumor-specific antigens (TSAs). Since they are absent from mTEC^{hi} and other normal cells, TSAs do not induce central tolerance. Despite their clinical relevance, identifying TSAs remains a challenge because of their scarcity and patient-specific nature. To expand the repertoire of TSAs, we developed a proteogenomic approach that enables the identification of TSAs deriving from (i) coding and non-coding regions of the genome, (ii) non-synonymous single-base mutations and more complex rearrangements as well as (iii) endogenous retroelements (ERE). Briefly, we performed RNA-sequencing on EL4 cells, a rearranged murine thymoma, along with two normal controls, mTEC^{hi} and thymocytes. The resulting RNA-sequencing reads were chopped into smaller sequences, called k-mers, to extract the EL4-specific ones (EL4 ≥ 5, Normal = 0) and those coming from ERE overexpressed in EL4 (EL4/Normal ≥ 100). To decipher which of these EL4-specific sequences did generate TSAs, we translated them ***in silico*** and used this as a database for mass spectrometry identification of pMHCs eluted from EL4 cells. This proteogenomic analysis led us to identify 5 TSAs, which all derived from allegedly non-coding sequences. Among them, two were aberrantly expressed (aeTSAs), two derived from an ERE (ereTSAs) and one overlapped an EL4-specific deletion of 7,500 base pairs (mTSA). Although all TSAs elicited T-cell reactivity ***in vitro***, we observed that only immunization against ere or mTSAs allowed partial to complete survival of EL4-bearing mice. We explained this survival gradation (from 20 to 100%) by differences in frequency of TSA-specific T cells in the repertoire of naïve and immunized mice as well as differences in TSA clonality. Finally, since two of our best candidates are ereTSAs, we speculate that their ancestral viral origin may be partly responsible for their strong immunogenic potential. In conclusion, we developed a fast and easy-to-implement proteogenomic platform able to identify the full landscape of TSAs in any given tumor, regardless of its mutational load or complexity. We also demonstrated that TSAs derived from non-coding sequences can be valuable targets for cancer immunotherapy and should therefore expand the repertoire of known TSAs, especially since tumors have a high non-coding-to-coding mutation ratio. Finally, if targeting a single ereTSA can also treat cancer in humans, it will simplify the clinical implementation of personalized immunotherapy because non-mutated ereTSAs should be shared among patients.

Keywords: Proteogenomic, Tumor-specific antigens, T-cell based immunotherapy

A021 / Elucidating the mechanism of RAG tracking and its impacts on V(D)J recombination

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RAG endonuclease initiates V(D)J recombination by binding to the recombination signal sequences (RSSs), introducing DNA breaks between two V(D)J gene segments, and mediating their joining. This process is tightly regulated to generate diverse antigen receptor repertoires and prevent aberrant events that could cause genomic translocations/deletions and lymphoid cancer. RAG can track directionally over chromosomal loop domain in which they reside. Tracking is evidenced by cleavage/joining of short cryptic RSSs lying in convergent orientation to a ***bona fide*** RSS. In order to elucidate the mechanisms of RAG tracking and to obviate potential confounding effects of numerous RSSs and other regulatory elements in antigen receptor loci, I have been constructing an unbiased assay system by utilizing allele-specific barcode, inserting short RSSs in the 1.8-Mb ***c-Myc*** loop domain, and employing high throughput genome-wide translocation sequencing (HTGTS) to quantitatively evaluate recombination frequency in various scenarios. I found that insertion of one single ***bona fide*** RSS is sufficient to initiate RAG tracking in the ***c-Myc*** domain and the recombination between the RSS and its cryptic RSSs seems to follow the 12/23 rule to some degree. Preliminary data suggest that convergent configuration of an RSS pair is preferentially used

compared to same orientation. In addition, the genomic distance between an RSS pair dramatically influences recombination capacity. Interestingly, I also observed a dramatic difference in recombination activity between alleles in a mixed clone.

Keywords: V(D)J recombination, RAG tracking, RSS

A022 / Mutagenicity of urea cycle dysregulation and its implications for cancer immunotherapy

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Although immune checkpoint therapy has led to durable clinical responses in cancer patients, current immunotherapies fail to control neoplasia in many patients. To improve the clinical response, it is critical to define predictive biomarkers for the therapeutic response to immunotherapy. While tumor neo-antigen load and microsatlite instability in tumors have been associated with enhanced response to such therapies, it is clear that additional factors determine immunotherapy success. Specifically, there is growing evidence that metabolic alterations can affect the tumor environment and modulate the immune response; the potential effects of altered cancer metabolism on tumor mutagenesis and immunotherapy remain to be studied.
We and others have shown that silencing of the urea cycle (UC) enzyme ASS1 promotes cancer proliferation by diverting its substrate aspartate toward the CAD enzyme, which mediates the first three reactions in the pyrimidine synthesis pathway. We now demonstrate, by analysis of the TCGA data and cancer cell line experiments, that **UC dysregulation (UCD) is a much wider common metabolic phenomenon that maximizes nitrogen utilization in cancer**, favouring pyrimidine synthesis over urea disposal.
Remarkably, we find that the UCD changes the 1:1 purine (R)-to-pyrimidine (Y) ratio in favour of pyrimidine in cancer cells. Moreover, in analysis of TCGA data and UC perturbed cancer cells, we find that **UCD is significantly associated with a novel and unique pattern of purine-to-pyrimidine transversion mutations across many cancer types** at the *DNA coding (sense) strand*, and this trend becomes stronger and more significant at both the mRNA and protein levels, testifying to its functional importance.
To test whether the mutational bias is associated with better immunotherapy response, we analyzed published data of two large melanoma cohorts. We find that r**esponders of both anti-PD1 and anti-CTLA4 therapy exhibit significantly higher UCD and R->Y mutational bias** than non-responders. We further observe that **the peptides carrying transverse R->Y mutations are preferentially presented as neoantigens in responders independent of mutational load**, promoting UCD as a potential biomarker for the success of immunotherapy.
Collectively, our results show that **UCD is a prevalent metabolic phenomenon in cancer.** Furthermore, **cancer patients harboring tumors with UCD show a striking mutational bias signature favoring pyrimidines and respond significantly better to immunotherapy,** likely due to the formation of **specific nucleotide-biased neoantigens.** Broadly, our findings have important therapeutic implications as they suggest that targeting the unique neoantigen bias associated with UCD may induce a robust and yet specific immune response, that could potentially be used to enhance checkpoint therapy and develop cancer vaccines.

Keywords: Mutagenesis, Cancer Metabolism, Biomarker for Immunotherapy

A023 / NGS data analysis for clinical studies of neoepitope vaccination

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Neoantigens are caused by genomic and transcriptomic alterations such as single nucleotide variants (SNVs), insertions or deletions (INDELs) and fusion transcripts. In addition, neoantigens are both proposed as biomarkers, e.g. for the efficacy prediction of checkpoint blockade inhibition, as well as targets for therapeutic interventions such as vaccination. The detection of neoantigens is enabled by genomic screening techniques, with next generation sequencing (NGS) based exome and RNA sequencing being most prominent. We present a fully automatic data analysis solution for the detection of SNV and INDELs and the subsequent definition of potential neoantigens. The software is designed to enable rapid data analysis, satisfy regulatory demands and for a high detection accuracy; these factors allow the study of neoantigens in interventional clinical trials. The runtime is less than 24h on commodity hardware for a single patient data set. Optional run

modes include NGS based HLA typing. Special emphasis was given to the mitigation of the autoimmunity risk of neoantigens selected for therapeutic vaccination and the optimization of the vaccine sequence. Future updates will include reliable methods for fusion transcript detection, improved run time performance and additional safety features.

Keywords: NGS data analysis, neoepitope vaccination, Neoantigens

A024 / A semi-automated method for the isolation and initial characterization of neoantigen-specific T cell receptors

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Adoptive T cell therapy using tumor-infiltrating lymphocytes (TILs) seems to be most successful if the TILs that are expanded *ex vivo* and reinfused into patients include T cells specific for individual tumor mutations. In addition, the success of checkpoint inhibitors is correlated with the mutational load of tumors indicating that responding tumors carry TILs specific for mutated epitopes displayed by the tumor cells.

Targeting neoantigens for patient-individualized therapy encompasses a number of hurdles. The high number of mutations as potential targets presents the necessity to have in place robust processes for rational, rapid selection and validation of neoantigens as T cell targets. In addition, the assessment of safety becomes paramount given that neoepitopes may differ by only one residue from the normal cellular protein.

Multiple immunotherapy platform technologies were used to investigate neoantigens as future targets for adoptive T cell therapies. In a proof of concept experiment targeting seven different neoantigens ***in vitro***, T cell responses against four of these antigens from peripheral T cells of healthy donors were detected. Using an automated T cell screening platform, a variety of neoantigen-specific T cell clones were isolated and subsequently the T cell receptor (TCR) sequences were identified by targeted Next Generation Sequencing (NGS). After retroviral transfer of the TCR sequences, donor T cells were able to specifically recognize neoantigen-expressing target cells, released cytokines and showed potent cytotoxicity.

In summary, the experiments demonstrate that neoantigen-specific T cell receptors can be isolated from peripheral blood samples of healthy donors and initially characterized in eight to ten weeks using an innovative high-throughput robotics platform.

Keywords: T cell receptor, neoantigen, automation

A025 / Deciphering the T cell response to non-small cell lung cancer

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The efficacy of checkpoint inhibition (CPI) has revolutionised medical oncology, yet clinical benefit remains confined to a fraction of patients. Defining the variables that govern the success of immunotherapy is therefore a prominent challenge in global cancer care. Tumour mutational burden represents one key correlate of CPI efficacy in multiple solid cancers; directly implicating a role for neoantigen reactive T cells (NART) in human tumour immunity.

We recently discovered that truncal neoantigens elicit sensitivity to CPI in Non-Small Cell Lung Cancer (NSCLC). However, it is unclear how the mutational and neoantigenic landscape of NSCLC associates with tumour specific T cell dynamics. Herein, we harness the TRACERX stage I-IIIa NSCLC cohort to decipher the complex interplay between tumour mutational architecture and T cell phenotype, clonality and transcriptome. A sub-sample of TRACERX NSCLC patient TILs, matched PBMC and normal tissue was subjected to high dimensional flow cytometry, multimer screening, (sc)RNAseq and TCRseq analysis in the context of orthogonal WES, multi region tumour genomics, neoantigen prediction and clinical data. Our evidence suggests that tumour- and neo-antigen recognition is linked with profound alterations to the T cell compartment, apparent at the population and single-cell level. We find that tumour associated T cell dysregulation includes, but is not limited to clonal TCR expansion, modified tissue trafficking, molecular reprogramming of TCR signalling, skewed effector-memory differentiation and enhanced exhaustion. Further delineation of the key pathways highlighted by these analyses may inform therapeutic design to resurrect curative NART responses in NSCLC and solid human cancers.

Keywords: NSCLC, Neoantigen, RNAseq

A026 / *In silico* strategies for the support of personalized cancer vaccines: streamlining the identification and prioritization of T cell neo-epitopes

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The wide-spread accessibility of next-generation sequencing has opened the door to personalized cancer therapies targeting mutations expressed by tumor cells. T cell neo-epitopes overlapping with cancer mutations can be harnessed to stimulate focused immune responses against a patient's tumors. However, a large proportion of neo-epitopes selected by current T cell epitope prediction algorithms prove to be non-immunogenic. Poor predictive performance may partially be due to erroneous inclusion of mutated epitopes cross-conserved with self-epitopes recognized by the T cell receptor of regulatory, anergic or deleted T cells. Vaccination with self-epitopes can lead to weak effector responses, active immune suppression, and toxicity due to immune-mediated adverse effects.
We have developed advanced T cell epitope identification and screening tools, EpiMatrix and JanusMatrix, that streamline the selection and prioritization of neo-non-self-epitopes. These state-of-the-art tools have been extensively validated in prospective vaccine studies for infectious diseases [Moise et al. 2015 Human Vaccines & Immunotherapeutics 11:9, 2312-2321]. In a recent application, a poorly immunogenic H7N9 influenza vaccine was engineered to be significantly more immunogenic by removing a regulatory T cell (Treg)-inducing epitope [Liu et al. 2015 Human Vaccines & Immunotherapeutics 11:9, 2241-52; Wada et al. 2017 Scientific Reports 7:1, 1283]. Usage of these tools in the oncology field allows for the prioritization of neo-epitopes exhibiting reduced potential for inducing Tregs, whose activation continues to curtail current cancer therapies.
Newly published elution data offers an unbiased and ideal way of evaluating the accuracy of in silico tools as these datasets are independent of training data used in model development. Analysis of such datasets [e.g. Abelin et al. 2017 Immunity 46, 315-326] shows a 96% agreement between predictions from EpiMatrix and peptides eluted from common Class I HLAs, while only 86% of these sequences are accurately recalled by NetMHC and NetMHCpan. Additional retrospective analyses of a cancer immunogenicity study [Strønen et al. 2016 Science 352(6291), 1337-41] demonstrate that EpiMatrix and JanusMatrix select immunogenic neo-epitopes with 72% accuracy, as compared to 21% accuracy when using public prediction tools. Increased accuracy is primarily explained by the removal of mutated epitopes that present increased potential for Treg activation.
These results demonstrate that EpiMatrix and JanusMatrix focus candidate selection on higher value sequences than conventional algorithms. Neo-epitopes with low Treg activation potential may then be used to support development of personalized therapies including vaccination and in vitro expansion of tumor infiltrating lymphocytes for adoptive cell transfer.

Keywords: T cell, immunoinformatics, vaccine

A027 / Target antigens of TIL reactivity in PDAC

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Pancreatic ductal adenocarcinoma (PDAC) forms the fourth leading cause of cancer related deaths with a 5-year survival rate of less than 5%. Even localized disease can only be partially controlled (2-year recurrence rate of 70%) applying surgery followed by chemotherapy.
It has recently been shown, that PDAC derived tumour-infiltrating lymphocytes (TILs) are capable of recognizing autologous tumor, potentially enabling the use of T-cell based therapies to counter disease recurrence. The nature of the epitopes recognized by tumor-reactive PDA TILs remains to be determined.
In view of the low mutational burden appearing in pancreatic cancer a well characterized and unrestricted screening system monitoring every potential epitope is required.
By modifiying the Lysosome-associated membrane protein 1 (Lamp-1) we developed a tool to adress this issue and simultaneously screen for responses to major histocompatibility complex (MHC)-I and -II restricted antigens in an unbiased fasion. Combining this screening system with patient-derived exome and transcriptome data we aim to identify and characterize (neo) epitopes inducing TIL reactivity.

Keywords: Neoantigens, Adoptive Cell Therapy, Translational Oncology

A028 / Neoantigen vaccination against future tumors in murine models for recurrence and premalignancy

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Development of therapeutic strategies to prevent recurrence in cancer patients or tumor progression in at risk individuals has been challenging, given the long and often unpredictable time to the emergence of the malignant tumors. Neoantigens represent the most potent antigens to induce antitumor immunity, yet the ability

to predict which neoantigens will be expressed in future tumors is at present not an option.

We have previously described a simple and broadly applicable approach to induce neoantigens in tumor cells in situ by inhibiting the Nonsense-mediated mRNA Decay(NMD) process, and more recently mediators of antigen processing like ERAAP, TAP or invariant chain (Ii), using siRNAs that were targeted to tumor cells by conjugation to an oligonucleotide aptamer ligands. Exploiting the ability to induce neoantigens in tumor cells in situ, we describe a novel vaccination strategy targeting potent neoantigens to control the growth of the future tumors whereby patients in remission or individuals at risk are first vaccinated against the NMD or antigen processing downregulation-induced neoantigens and when or if tumor develops the same antigens are induced in the tumor by tumor targeted siRNA inhibition of the corresponding product.

Preclinical studies in mice suggest that the approach has merit. Mice were vaccinated against neoantigens induced by downregulation of TAP in DC in situ using corresponding siRNAs conjugated to a CpG oligonucleotide (ODN). The CpG ODN targeted the siRNAs to DCs and also activated the targeted DC to avoid induction of tolerance against the neoantigens. Vaccination with CpG ODN-TAP siRNA conjugates inhibited subsequently implanted or induced tumors in several tumor models, provided the said neoantigens were induced in the tumor by aptamer-targeted siRNA inhibition of the corresponding product. Vaccination in recurrence models and models for premalignant disease are currently ongoing. The CpG-siRNA and aptamer-siRNA conjugates are simple chemically-synthesized broadly applicable reagents to vaccinate against neoantigens and control the growth of future tumors irrespective of their origin.

Keywords: Neoantigens, Cancer Immunoprevention, Oligonucleotides

A030 / Detection of anti-NPM-ALK CD8$^+$ T-cells in children with NPM-ALK-positive anaplastic large cell lymphoma (ALCL)

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Introduction: Patients with NPM-ALK (Nucleophosmin-Anaplastic Lymphoma Kinase)-positive ALCL mount ALK-specific humoral- and T-cell responses. ALK-specific T-cells were detected by reverse immunology approaches using short ALK-derived peptides. The aim of our study was to analyze ALK-specific CD8$^+$ T-cell responses in a sizeable cohort of patients without HLA preselection, covering the full antigenic spectrum of NPM-ALK and ensuring endogenous processing of the respective peptides. The results of the T-cell response analysis were correlated to the patients´ clinical and biological characteristics. In responsive patients we extended our analyses to the restricting HLA-class I molecules.

Patients and methods: All patients were treated according to NHL-BFM95 or ALCL-99 therapy protocols. The patients were in complete clinical remission, 14 years of age or older, did not receive an allogenic stem cell transplantation and had no sign of infection at the time of analysis. CD8$^+$ or CD3$^+$ T-cells of 29 NPM-ALK$^+$ ALCL patients and 20 healthy controls were stimulated with autologous dendritic cells (DCs) transfected with ***in vitro***-transcribed RNA encoding full-length NPM-ALK. After two weekly restimulations, responder lymphocytes were analyzed with an IFN-γ ELISPOT assay for recognition of ***NPM-ALK***-RNA-transfected DCs. To assess both, antigen reactivity and HLA restriction, responder T-cells were tested for recognition of COS-7 cells co-transfected with the patients´ individual HLA-class I alleles and ***NPM-ALK-cDNA.***

Results: In twelve of 29 NPM-ALK$^+$ ALCL patients (nine of 19 CD8$^+$- and three of ten CD3$^+$-stimulated), NPM-ALK-reactive T-cells were detected and enriched after stimulation with RNA-transfected DCs in peripheral blood samples collected between one and 15 years after treatment. There were no significant differences between patients with or without a detectable T-cell response regarding the median age at diagnosis, median time from diagnosis to analysis, gender, ALK antibody titer, minimal disseminated disease, clinical risk group or histological subtype. Restricting HLA-class I alleles were identified in eight out of eleven NPM-ALK-reactive patients analyzed. In six patients, recognition of NPM-ALK was restricted by HLA-C alleles. No NPM-ALK-reactive T-cells were detected in 20 healthy controls.

Conclusion: Our approach using autologous DC for stimulation without HLA preselection allowed the detection of ALK-specific CD8$^+$ T-cells in the blood of 40% of well characterized and uniformly treated ALCL patients in remission but not in healthy controls. There was no correlation of responses with clinical or biological characteristics. In the majority of NPM-ALK-reactive cases, recognition of NPM-ALK was restricted by HLA-C allele.

Supported by “von Behring-Röntgen Stiftung”.

Keywords: CD8+ T-cell response, NPM-ALK, anaplastic large cell lymphoma

A031 / A pan-HLA predictor of neoantigen processing and presentation to the cell surface

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Currently, neoantigens are often predicted using algorithms predominantly based on knowledge of the key peptide binding affinity difference between HLA alleles. Although HLA binding algorithms predict binding affinity of a peptide to HLA reasonably well, they do not predict processing and presentation of to the cell surface ***(i.e.,*** the immunopeptidome). In fact, only 15% - 20% of "predicted" peptide binders are processed or presented, and therefore contribute to the immunopeptidome. Erroneous predictions may be addressed with time-consuming and laborious experiments, such as mass-spectrometry (MS). However, ***in silico*** predictions may also prove to be very useful in prioritizing therapeutically relevant immunogenic peptides. Previous ***in silico*** studies that predict peptides that are naturally processed and presented to the cell surface, have not excluded entirely the influence of HLA binding in their training or have concentrated on one of the many steps in the antigen processing and presentation pathway (such as TAP transport or proteasome cleavage, ***etc***). Additionally previous antigen processing prediction tools have been trained and are therefore applicable to specific HLA alleles, making it challenging to make predictions for not so well-characterized alleles. Here, we outline a machine learning approach trained on MS elution data that disconnects HLA binding prediction from the prediction of natural processing and presentation of potential neoantigens to the cell surface. We demonstrate here that by negating the effect of HLA binding to the contribution of peptides to the immunopeptidome: our approach, termed ***PanPro,*** may be used to for more accurate neoantigen predictions for any HLA allele in both class I and class II systems. Further, by analysing previously published clinical data we illustrate that the application of ***PanPro*** leads to a significantly improved identification of neoantigen targets for personalized cancer immunotherapy.

Keywords: neoantigen, personalized cancer vaccines, bioinformatics

A032 / Chromosomal alterations predisposing to tumor immunotherapy resistance

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Immune checkpoint blocking therapy has been a breakthrough in treatment of metastatic melanoma, generating impressive clinical response rates. The efficacy of these treatment regimens is based on the high intrinsic immunogenicity of melanoma cells that allows cytotoxic $CD8^+$ T lymphocytes to respond to multiple HLA class I-restricted tumor antigens. Checkpoint blocking antibodies release tumor antigen-specific CTL from intratumoral suppression to secrete cytolytic granules and IFN-gamma (IFNg), both essentially contributing to therapy efficacy. However, primary as well as acquired resistance to therapy is a major problem and the underlying mechanisms are still poorly defined. We demonstrate that IFNg-resistant melanoma variants lacking JAK1 expression evolve in the course of disease. Furthermore, we show that tumor cells acquire T-cell resistance by loss of HLA class I antigen presentation due to B2M deficiency. Studying the underlying genetic alterations we observe that, in both, B2M- as well as JAK1-deficient tumor cells an early acquired chromosomal deletion and subsequent inactivating gene mutation lead to the resistant phenotypes. Thus, in melanoma early chromosomal alterations predispose the IFNg and T-cell resistance development that should be clinically screened for to identify those patients with an enhanced risk of being non-responders to immune checkpoint blocking therapy.

Keywords: immunotherapy resistance, HLA, interferon, genetic alterations

A033 / HLA-A24 ligandome analysis identifies a neoantigen that elicits strong CTL responses

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Most colorectal cancer patients do not benefit from checkpoint-blockade immunotherapy, however those exhibiting the microsatellite instability (MSI) phenotype with a higher mutation rate is a notable exception, implying host CTL-surveillance often recognizes neoepitopes that arise from genomic mutations. Here, we analyzed and compared the landscapes of HLA-A24 peptides presented by MSI (HCT15/β2m) and MSS (SW480 and Colo320) colorectal cancer cells. The analysis using mass spectrometry detected a novel neoepitope and its wild-type counterpart in HCT15/β2m cells and other MSS-colorectal cancer cells, respectively. A somatic gene mutation of the AP2S1 gene (c. 258 C>G) was responsible for the amino acid substitution (N86K), converting the wild-type ANF9 into the neoepitope AKF9. Structure modeling of the peptide-HLA-A24 complexes predicted that the N>K substitution at P8 of the peptide was accessible to T-cell receptors. In fact, AKF9 readily elicited CTL responses, which discriminated AKF9 from ANF9 and exhibited considerably high cytotoxicity against HCT15/β2m cells. Thus, the HLA-ligandome analysis provides a comprehensive way to screen naturally-processed neoantigens as well as demonstrates their therapeutic potential.

Keywords: Neoantigen, HLA-ligandome analysis, CTL

A034 / Induction of broad neoantigen-reactivity to challenge the dynamic neoantigen landscape

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The clinical success of T cell checkpoint blockade and adoptive T cell therapy has been illustrated to rely on recognition of neoantigens. Therefore, numerous strategies aiming to identify genetically defined clonally expressed neoantigens are being applied in order to selectively enhance T cell reactivity against these neoantigens. These approaches assume that neoantigen availability remains constant during disease progression.
However, we analyzed neoantigen stability in stage IV melanoma patients that were successfully treated with adoptive T cell transfer and showed that the availability of neoantigens could be lost due to reduced expression or selective loss of the mutated allele. These data show that neoantigen expression is dynamic and changes under T cell pressure.
In our ongoing studies we further investigated strategies to induce neoantigen-specific T cells for possible therapeutic application. To this end synthetic long peptides (SLP) harboring previously identified immunogenic neoantigens or whole autologous tumor fragments were used to stimulate peripheral blood mononuclear cells from the corresponding melanoma patient. Bulk T cell cultures obtained after repeated stimulations were shown to contain neoantigen-specific cells that also recognized autologous tumor cells.
These data suggest that tumor cell fragments or neoantigen SLP can be used for therapeutic purposes including vaccination or in vitro induction of neoantigen- and tumor-specific T cells for adoptive cell therapy. In order to avoid the evolution of immune escaped tumor variants induction of broad neoantigen-specific T cell-reactivity is required. This calls for identification of multiple neoantigens as well as monitoring their expression on progressing tumor lesions to keep pace with the dynamic neoantigen landscape.

Keywords: neoantigen, synthetic long peptide, melanoma

ONCOLYTIC VIRUSES

A035 / Heterologous prime-boost vaccination approach using Measles Virus as an oncolytic prime vector for the Maraba virus boost vaccine

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Oncolytic viruses (OVs) are considered as immunotherapeutic agents. However, generating a robust and specific anti-tumor immune response remains a challenge.
Inspired by vaccination protocols, the oncolytic heterologous prime-boost strategy has been developed, using two different OVs expressing the same tumor-associated antigen (TAA): one to prime a specific TAA immune response and the other to boost it. Therefore, the immune response would be directed towards the TAA rather than towards the virus and a large population of memory CD8 T cells against the tumor will be generated. This strategy is under clinical investigation using a replication-deficient adenovirus followed by the oncolytic Maraba virus (MG1).
We aim at strengthening this strategy by using the replication competent oncolytic measles virus (MeV) as a priming vector rather than a non-replicating adenovirus. We hypothesize that the T cell priming with MeV is more efficient due to MeV-mediated tumor lysis that generates an inflammatory environment, promoting tumor antigen presentation. We generated an MeV encoding fused epitopes of E6 and E7 proteins of serotypes 16 and 18 of Human Papillomavirus (HPV), which is the causative agent for different cancers. E6 and E7 proteins are foreign viral antigens, therefore naturally immunogenic unlike most autologous TAAs. Furthermore, these oncoproteins interfere with the type I interferon responsiveness, making HPV^+ tumors susceptible targets for OVs. After characterization of MeV-E6E7, we will evaluate its therapeutic potential as a priming vector used in combination with MG1-E6E7 in an immunocompetent mouse model bearing syngenic tumors.
This work has direct implications towards the development of clinical trials with MeV-E6E7/MG1-E6E7 prime-boost treatment of HPV^+ cancers.

Keywords: oncolytic viruses, measles virus, oncolytic prime-boost vaccination strategy

A036 / The development of a heterologous prime-boost oncolytic vaccine regimen with an improved CD8 T cell response to pancreatic cancer tumor antigens

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Pancreatic cancer (PDAC) is one of the highest fatality cancers with a survival rate under 10%. Although immune modulating therapies have had a dramatic impact in other cancer types the efficacy of this approach has been limited in PDAC. Neoadjuvant administration of a prime-boost oncolytic vaccine targeting tumor associated antigens (TAA) have been shown to overcome tolerizing effects of the tumor microenvironment and potentiate tumor-specific immune responses leading to improved patient prognosis. Panc02, a murine PDAC cell line which recapitulates many aspects of PDAC tumor growth ***in vivo,*** was used to investigate the efficacy of a heterologous prime-boost vaccination strategy as this line was found to be highly susceptible to oncolytic virus infection and expressed a pancreatic TAA (pTAA). However, a standard vaccination protocol in non-tumor bearing mice did not result in a measurable IFNγ+ T cell response to representative pTAA peptides (< 0.05% IFNγ+ CD8 T cells in no stim vs peptide) suggesting the presence of a tolerizing mechanism. To investigate this possibility directly, we next investigated the effects of depleting regulatory T cells (CD4+ Foxp3+) through low dose cyclophosphamide (100mg/kg) combined with a CD25 depleting antibody. This approach resulted in the complete depletion of regulatory T cells (2.5% Foxp3+ vs 0.0% Foxp3, p=0.03) and an increased number of IFNγ+ T cells following non-specific stimulation with PMA/Ionomycin (8.82% vs 4.22%, p=0.0037). Using the depletion approach in combination with our vaccination strategy, a robust immune response against a self-antigen (4.06% IFNγ+ CD8 T cells) was elicited. Importantly, this novel strategy lead to a significant increase in antigen reactive T cells compared to the leading therapeutic strategy currently under clinical investigation. The therapeutic efficacy of this approach is being explored in an orthotopic model of pancreatic cancer. An oncolytic vaccine platform has the potential to promote tumor clearance in patients diagnosed with pancreatic cancer, leading to increased survival times.

Keywords: Oncolytic viruses, Cancer Vaccines, Pancreatic cancer

A037 / CD40L-armed oncolytic viruses activate dendritic cells, control tumor growth by oncolysis and induce CD40-mediated apoptosis in CD40+ tumor cells

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LOAd703 is an oncolytic adenovirus encoding a trimerized CD40L and 4-1BBL. The purpose of this study was to evaluate the sensitivity to LOAd703 infection across multiple tumor types and to evaluate its capacity to stimulate the immune system. CD40-CD40L signaling is a pleiotropic pathway of great interest in cancer immunotherapy. CD40 stimulation of immune cells, such as antigen-presenting cells, ultimately drives a Th1-type anti-tumor response with activation of cytotoxic T cells. Nevertheless, CD40 stimulation on tumor cells can lead to enhanced tumor cell apoptosis. 4-1BBL potentiates activation of antigen-presenting cells and acts directly on both T cells and NK cells to promote their expansion. The LOAd platform consists of oncolytic adenoviruses encoding different immunomodulatory genes. Most encode a trimerized form of CD40L (TMZ-CD40L) alone (LOAd700) or in combination with other molecules like 4-1BBL (LOAd703). In the current study, the viruses were evaluated for oncolytic capacity ***in vitro*** (MTS assay) and ***in vivo*** (xenograft models) in a wide range of solid malignancies including pancreatic cancer (Panc01), bladder cancer (T24), lung cancer (H727), ovarian cancer (SKOV3) and colorectal cancer HT29). Further, activation post infection of human monocyte-derived immature dendritic cells (DCs) was evaluated by flow cytometry and proteomics. LOAd viruses effectively induced oncolysis in all cell lines both ***in vitro*** and in xenograft mouse models. Interestingly, the CD40+ urinary bladder cancer cell line T24 showed a significantly reduced viability ***in vitro*** after infection with TMZ-CD40L-expressing LOAd700 compared to the empty control virus, LOAd(-). To rule out the oncolytic effect, T24 cells were co-cultured with LOAd-infected DCs. Co-culture with DCs expressing TMZ-CD40L led to an increased induction of apoptosis compared to co-culture with LOAd(-) infected DCs. ***In vivo***, all LOAd viruses could reduce T24 tumor growth but when TMZ-CD40L was expressed (e.g. in the LOAd703 group), tumor control was faster, and at end point, only 1/5 animals had tumor growth compared to 3/5 in the LOAd(-) treated group. Hence, CD40 signaling led to additional growth control in CD40+ tumors. Models with CD40- tumor cells (Panc01, SKOV3) responded similarly to both control virus and virus encoding TMZ-CD40L. Human DCs infected with LOAd700 or LOAd703 upregulated maturation markers including CD83, CD86 and CD70 compared to LOAd(-) infected DCs. Further, the LOAd700- or LOAd703-matured DCs produced cytokines, co-stimulating factors and chemokines consistence with a Th1

response. In conclusion, oncolytic viruses encoding for TMZ-CD40L can induce activation of DCs and at the same kill tumor cells by oncolysis. In CD40+ tumor cells LOAd viruses have an increased killing capacity due to the combination of oncolysis with CD40L-induced apoptosis. Hence, CD40+ solid malignancies may be of high interest for TMZ-CD40L-encoding LOAd viruses.

Keywords: adenovirus, CD40L, 4-1BBL

A038 / Targeting post-surgical breast cancer metastasis with combined natural killer T cell and oncolytic virus therapies

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Natural killer T (NKT) cells are a population of immune cells that have been shown to limit primary tumor growth and target distant metastatic disease in a number of animal models. We have shown that NKT cell activation improves survival in a model of post-surgical metastatic breast cancer. We have expanded this work to determine whether NKT cell activation can be safely combined with oncolytic virotherapy to improve outcomes further. Vesicular stomatitis virus ΔM51 (VSV-ΔM51) infected 4T1 mammary carcinoma cells in culture and increased their potential immunogenicity by increasing the exposure/release of MHC I, calreticulin, HMGB1 and ATP. In addition, VSV-ΔM51 enhanced the expression of cytokines (***cxcl9, cxcl10, cxcl11, tnf, ifnα***) that enhance the recruitment and activation of immune cells. 4T1 cells were inoculated into the 4th mammary fatpad of BALB/c mice. Tumors were resected at day 12, and mice were treated intravenously with 5x10^{8} pfu of VSV-ΔM51 on days 13, 15 and 17. On day 18, NKT cells were activated by transfer of 5 x 10^{5} dendritic cells loaded with the glycolipid antigen α-galactosylceramicde (α-GalCer). Treatment with VSV or α-GalCer-loaded dendritic cells as individual treatments reduced metastasis and prolonged survival. However, survival was significantly increased when the treatments were combined. Sustained immunological memory was induced as tumour growth was inhibited in surviving mice following re-challenged with 4T1 cells. Our results demonstrate that NKT cell activation therapy can be combined with oncolytic virus therapy to enhance protection against tumor metastasis and recurrence.

Keywords: Oncolytic virus therapy, Adoptive cell therapy, Breast cancer

A039 / CD30-targeted oncolytic viruses as novel therapeutic approach against Hodgkin Lymphoma

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Hodgkin lymphoma (HL) is a hematopoietic malignancy with a characteristic cellular composition. The tumor mass is made up of infiltrated lymphocytes and other cells of hematologic origin but only very few neoplastic cells (< 2%) that are identified by the diagnostic marker CD30. While most patients can be cured by standard therapy, approximately 20% relapse, suffer from progressive disease and develop secondary cancers. Here, we suggest a novel therapeutic concept relying on oncolytic viruses that selectively destroy the CD30-positive HL tumor cells.

Relying on a recently described CD30-specific scFv, which we displayed on the measles virus (MV) hemagglutinin for lentiviral vector targeting, we have generated CD30-targeted MV (MV-CD30) and vesicular stomatitis virus (VSV-CD30). For VSV-CD30 the VSV glycoprotein G reading frame was replaced with the CD30-targeted MV glycoproteins. Both viruses were found to be highly selective for CD30-positive cells as demonstrated by infection of co-cultures of target and non-target cells as well as through blocking infection by soluble CD30. Notably, VSV-CD30 replicated significantly faster to much higher titers than MV-CD30 and resulted in a more rapid and efficient killing of cultivated HL cells. Data from mouse tumor models show that intratumorally as well as systemically injected VSV-CD30 replicated in s.c. HL xenografts and significantly slowed down tumor growth resulting in a substantially prolonged survival of tumor-bearing mice. Taken together, the data support further preclinical testing of VSV-CD30 as novel therapeutic agent for the treatment of HL.

Keywords: Oncolytic VSV, Retargeting, Hodgkin Lymphoma

A040 / Oncolytic reovirus infection is facilitated by the autophagy machinery

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Mammalian Reovirus is a double-stranded RNA virus that selectively infects and lyses transformed cells, making it an attractive oncolytic agent. Despite clinical evidence for anti-tumor activity, the efficacy as a stand-alone therapy is limited. In the search for therapeutic combination strategies, we are exploring which cellular pathways are important for reovirus replication and oncolysis. We found that reovirus induces autophagy in several cell lines, evident from the formation of Atg5-Atg12 complex, LC3 lipidation, p62 degradation, the appearance of acidic vesicular

organelles, and LC3 puncta. Furthermore, autophagosomes were observed in electron microscopic images of reovirus-infected cells. Using UV-inactivated reovirus, we demonstrate that the induction of autophagy is facilitated by a productive reovirus infection. Importantly, knock-out cell lines for specific autophagy-related genes revealed that the expression of distinct members but not all of the autophagy machinery facilitates reovirus replication. These findings highlight a central and non-canonical role for this cellular process in reovirus infection and contribute to a better understanding of reovirus-host interactions.

Keywords: oncolytic reovirus, autophagy, replication

A041 / Oncolytic vesicular stomatitis virus expressing a reovirus fast protein as tumor treatment by enhancing anti-tumor immunity

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Reovirus FAST proteins are the smallest known viral fusogens and efficiently induce cell-cell fusion and syncytium formation in multiple cell types. Syncytium formation enhances cell-cell virus transmission and may also induce immunogenic cell death, a form of apoptosis that stimulates immune recognition of tumour cells. These properties suggest that FAST proteins might serve to enhance oncolytic virotherapy. Oncolytic viruses (OVs) constitute a form of cancer immunotherapeutic exhibiting promising results in a broad range of tumor types and Vesicular Stomatitis Virus (VSVd51) is an oncolytic viral platform that has shown a good therapeutic window in various studies and models.
The oncolytic activity of recombinant VSV encoding the p14 FAST protein (VSV-p14) was compared to a similar construct encoding GFP (VSV-GFP) in ***in vitro*** and ***in vivo***. Compared to parental VSV, VSV-p14 exhibited increased oncolytic activity against MCF-7 and 4T1 breast cancer spheroids in culture, and reduced primary 4T1 breast tumour growth ***in vivo***. VSV-p14 prolonged survival in both primary and metastatic 4T1 breast cancer models, and in a CT26 metastatic colon cancer model. As with VSV-GFP, VSV-p14 preferentially replicated ***in vivo*** in tumors and was cleared rapidly from other sites. Furthermore, VSV-p14 increased the numbers of activated splenic CD4, CD8, NK, and NKT cells, and increased the number of activated CD4 and CD8 cells in tumors.
FAST proteins may therefore provide a multi-pronged approach to improving oncolytic virotherapy via syncytium formation and enhanced immune stimulation.

Keywords: Vesicular stomatitis virus, Reovirus p14-Fast, Cancer Immunotherapeutic

A042 / Preclinical testing of HSV-1 based oncolytic viruses for lung tumor therapy

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For immunotherapy oncolytic viruses are particularly interesting, which selectively kill tumor cells and as such have shown a promise for the treatment of cancer. The usage of the first HSV based-oncolytic virus Imlygic® is encouraging, but there is still a considerable need for optimization. We aimed to increase the efficacy of the oncolytic activity of an HSV-1 based oncolytic virus by genetically introducing different genes for immune modulation and for targeting optimization of tumor therapy. Thus, the virus-mediated oncolysis is combined with immunotherapy in one virus vector and an effective destruction of tumors as well as metastases is possible.
The combination of viral- and immunotherapy requires the establishment of preclinical models, which are suitable to study therapy effects on primary tumors and metastases. As a proof of concept the lung carcinoma, which is associated with a high mortality rate, is to be established as preclinical mouse model. For this purpose, reporter cell lines for tumors and metastases (A549 and HCC827) will be generated, by stably expressing the reporter firefly-luciferase in order to be detected by a highly sensitive light camera (IVIS-BLI) ***in vivo.***
In the future, the described mouse model will be used to estimate the oncolytic virus amount for the application into other tumor entities particularly solid tumors. In order to meet the requirements of modern therapies, there are increasing attempts to test genetic modified viruses in humanized mouse models. Thus, humanized mouse models will be combined with a matched human tumor, to test on the modulation of the immune system to enhance the tumor lysis to enhance the tumor therapy.

Keywords: Oncolytic virus, Immunotherapy, lung carcinoma

A043 / Large genomic deletions of a novel oncolytic Vaccinia virus increase safety, efficacy and cancer immunogenicity

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The heterogeneous nature of cancer cells helps explain why chemotherapy treatments, which typically have a single molecular target, often fail to improve patient survival. An alternative treatment is the use of oncolytic viruses which are able to control tumor growth through multiple different mechanisms. We have previously shown that Pexa-Vec (formerly JX-594), a Vaccinia virus (VacV) currently in Phase III clinical trials, can be safely administered to cancer patients. We believe a successful 2nd generation VacV-based oncolytic will be able to replicate faster in the tumor and have greater immunostimulatory properties.
By co-infecting mono-layers of cancer cells with multiple wild-type Vaccinia we have identified which strain is able to replicate the fastest and out-compete other strains in tumor samples. We have kept the "oncolytic engine" of this strain but deleted over 10% of the genome responsible for mainly immunosuppressive functions and other non-oncolytic factors.
This deletion does not affect the replication kinetics of our virus. In fact, this deleted viruses kills cancer cells much faster with up to 6 times more tumor cell death. In vivo, the deleted virus does not cause sickness or weight loss independent of delivery method, being much safer than its parental wild-type strain. We have shown this virus to have comparable efficacy with other Vaccinia strains in a variety of tumor control models. When using an infected cell vaccine platform, our virus can immunize mice against B16 tumors while other Vaccinia fail. Furthermore, when combined with FDA approved immune checkpoint inhibitors, treatment with this virus can cure murine tumors.
Unlike Pexa-Vec, our large deletion mutant does not suppress activation of the immune system and thus the combination of enhanced cancer cell killing, improved immune stimulation and ability to rapidly replicate and spread in tumors suggests that our new variant will be a superior oncolytic virus. This 2nd generation VacV-based oncolytic has shown great promise in various models enhancing therapeutic effect and is scheduled for clinical trials within the next 2 years. Studies in a variety of in vitro and in vivo assays will be presented illustrating the strategy we have used to create an optimal oncolytic virus platform.

Keywords: Oncolytic Virus, Cancer Research, Immune Checkpoint Inhibitors

A044 / Type I IFN responsiveness of melanoma is cell state dependent and can be both harnessed and suppressed to enhance oncolytic virotherapy efficacy

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Oncolytic virotherapy is a new promising approach to treat malignant melanoma. Tumor cells are often permissive for viral infection and oncolysis due to their active metabolism and decreased responsiveness to type I interferons (IFN-I). However, also IFN-I responsive tumors may be suitable targets for oncolytic virotherapy, as local IFN-I responses have often been associated with anti-tumor immunity. The underlying mechanisms accounting for the differences in IFN-I responsiveness of melanoma are poorly understood. The aim of this work was to analyze the responsiveness of a collection of human and mouse melanoma cell lines to IFN-I utilizing an oncolytic Semliki Forest virus expressing EGFP (SFV-VA7-EGFP). Human melanoma cells with a spectrum of phenotypes ranging from very melanocytic (MITFhigh) to poorly differentiated (MITFlow) were screened for their IFN-I responsiveness by treatment with varying concentrations of IFN-I followed by infection. The infection kinetics were monitored with fluorescence and bright field microscopy over 72h, after which the net result of cell proliferation was quantified using crystal violet staining. All 16 human melanoma cell lines were susceptible to high (MOI=1) and medium (MOI=0.01) doses of SFV VA7-EGFP without IFN-I pretreatment resulting in significant cell death. When focal ***in vivo*** infection patterns were simulated ***in vitro*** by inoculating 10 infectious viral particles per well, half of the cell lines underwent efficient oncolysis. Following IFN-I pretreatment, healthy primary melanocytes were readily protected from infection, whereas all melanoma cell lines had, to varying degree, lowered antiviral type I IFN responsiveness. Melanoma cell lines, which had retained partial responsiveness to IFN-I displayed a basal IFN-I signature in a bioinformatic analysis. Interestingly, the one quarter of the melanoma cell lines with poorest IFN-I responsiveness were all melanocytic (MITFhigh), suggesting a potential link between the differentiation status and the responsiveness to type I IFNs. Supporting the hypothesis, MITF overexpression utilizing a tet-ON system in MITFlow Mamel65 human melanoma cell line completely abrogated their type I IFN responsiveness allowing productive SFV-VA7-EGFP infection and oncolysis. To test the hypothesis that both suppressing and harnessing the type I IFN responses may be utilized to benefit oncolytic virotherapy, we treated HCmel12 mouse melanomas with SFV-VA7-EGFP in combination with antibodies targeting either the type I IFN

receptor or an immunosuppressive PD1 receptor on T cells. While both approaches were found to enhance oncolytic virotherapy efficacy, local IFN-I signaling blockade in the tumors before virus administration resulted in marked toxicities suggesting peripheral spread of the infection.

Keywords: Oncolytic virotherapy, Melanoma, Type I IFN

A045 / Incorporation of the type-I interferon decoy receptor B18R in a microRNA-detargeted Semliki Forest virus improves oncolytic capacity but promotes neurotoxicity

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Oncolytic Semliki Forest virus (SFV) has been suggested as a potential candidate for the treatment for glioma and neuroblastoma. However, oncolytic capacity of SFV is restricted by the type-I antiviral interferon (IFN-I) response. The aim of this study is to increase the oncolytic capacity of a microRNA target tagged SFV against glioblastoma by arming it with the IFN-I decoy receptor B18R (SFV4B18R-miRT), which is a receptor encoded by Vaccinia virus to overcome IFN-I response during its infection. Expression of B18R by SFV4B18RmiRT aided neutralization of IFN-I by reducing STAT-1 phosphorylation and improved virus spread in plaque assays. B18R expression by SFV4 also increased its oncolytic capacity ***in vitro*** against murine glioblastoma (CT-2A) regardless of the presence of exogenous IFN-β. SFV4B18RmiRT treatment decreased tumor burden in mice with syngeneic orthotopic glioma (CT-2A) compared to SFV4miRT. However, some mice injected with SFV4B18RmiRT developed severe neurological symptoms due to virus replication in healthy brain. No neurotoxicity and virus replication in brain was observed when only SFV4miRT was administered, which in accordance with previous reports. In summary our results indicate that the oncolytic capacity of SFV4 was improved ***in vitro*** and ***in vivo*** by incorporation of B18R but neurotoxicity of the virus was increased, possibly due to loss of microRNA targets.

Keywords: Glioma, Oncolytic virus, Type-I interferons

A046 / Oncolytic chimeric vesicular stomatitis virus pseudotyped with LCMV-GP for lung cancer therapy

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Treatment options for advanced lung cancer remain limited. Recently, checkpoint inhibitor immunotherapies have shown significant clinical benefit, though only to a subset of patients. Oncolytic viruses (OV) that preferentially replicate in and kill tumour cells can enhance antitumor immunity through various means and may act synergistically with other immunotherapies. Among currently studied oncolytic viruses, vesicular stomatitis virus pseudotyped with LCMV glycoprotein (VSV-GP) is a particularly promising candidate due to its fast mode of action, high titer production, absence of pre-existing immunity and broad tumor tropism. Here, we tested the use of oncolytic VSV-GP for the treatment of lung cancer in a number of human and mouse tumor models.

VSV-GP was found to efficiently infect and lyse most of the cell-lines ***in vitro***. However, analysis of the innate immune response of lung cancer cells to VSV-GP revealed sensitivity to type I interferons (IFN) and induction of an antiviral state in many of the cell types. Conversely, only human lung cancer tumors defective in their IFN response were highly susceptible to VSVgp treatment in vivo. To assess whether the same correlation holds true in syngeneic mouse models we generated a variant of mouse LLC lung cancer cells with weakened antiviral response by knocking out interferon receptor 1 (IFNAR1), resulting in significantly enhanced oncolytic activity of VSV- GP in vitro. ***In vivo***, virus replication increased significantly, though the therapeutic outcome in a subcutaneous syngeneic model improved only marginally, suggesting that other factors may be involved in resistance to VSV-GP therapy.

To visualize virus activity ***in vivo***, xenograft and syngeneic s.c. tumors were analyzed for bioluminescence activity after intratumoral or systemic treatment of luciferase-expressing VSV-GP. Virus replication was limited to tumor tissue only and recorded for several days post infection. Importantly, studies on syngeneic models with bilateral tumors revealed successful tumor-to-tumor spread of virus after unilateral intratumoral injection in immune-competent hosts.

Ongoing studies are currently addressing the interaction of the triad VSV-GP - immune system - tumor to identify resistance factors and improve therapeutic outcome of VSV-GP in lung cancer.

Keywords: oncolytic virus, lung cancer, interferon

A047 / Immunostimulatory and oncolytic properties of rotavirus can overcome resistance to immune checkpoint blockade therapy

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Background: Immune checkpoint targeted therapies against PD-1, PD-L1 and CTLA-4 are currently revolutionizing cancer care. However, only a minority of patients generate objective tumor responses with these treatments. Therefore, new therapeutic interventions are needed to increase the immunogenicity of tumors in order to overcome the resistance to immune checkpoint blockade therapy. Pattern recognition receptors (PRR) such as toll-like receptor agonists have been shown to overcome resistance to immune checkpoint targeted therapy in pre-clinical models. Besides their intrinsic ability to stimulate PRR, the oncolytic properties of common viruses can be exploited also for the priming of anti-tumor immune responses. Several oncolytic viruses (OVs) are currently in clinical development for cancer immunotherapy, and the first Herpes derived OV (T-VEC) has been recently approved for the treatment of metastatic melanoma. However the routine implementation of these therapies is limited by the ongoing regulations on GMOs.

Hypothesis: Can anti-infectious vaccines be used as a source or PRR agonists and/or oncolytic viruses?

Results: We confirmed that commercially available anti-infectious vaccines do have PRR agonist properties. More interestingly, we discovered that rotavirus vaccines also have oncolytic properties. These attenuated viruses can directly kill cancer cells with features of immunogenic cell death such as upregulation of calreticulin on dying cancer cells but doesn't have cytotoxicity on healthy primary fibroblast. Moreover, they have pro-inflammatory properties and can activate the NF-KB pathway in a toll-like receptor and IRF3 independent manner. These ***in vitro*** biological properties translate into ***in vivo*** anti-tumor activity. Intra-tumoral rotavirus therapy has anti-tumor effects which are mainly immune mediated as demonstrated by their weaken activity in NSG mice. Interestingly, in immunocompetent syngeneic murine tumor models of neuroblastoma and lymphoma, intra-tumoral rotavirus therapy can overcome resistance to immune checkpoint targeted therapy and in particular synergies with anti-CTLA4. This therapeutic effect relied on specific modifications of tumor immune infiltrates and immune activation pathways. Intratumoral rotavirus vaccines was associated to an increase of myeloid infiltrating cells expressing up-regulated level of CD86 in the tumor microenvironment, and upregulation of activation markers such as OX40/CD137 on T cells.

Significance & Impact: Rotavirus vaccines are clinical grade products, including for children. Therefore, ***in situ*** immunization strategies with intra-tumoral attenuated rotavirus can be implemented quickly in the clinic including in pediatric cancers. Intra-tumoral priming of the anti-tumor immunity with oncolytic and immunostimulatory rotavirus vaccines could be a feasible strategy to overcome resistance to anti-PD-1/anti-CTLA-4 therapy in patients with cancer.

Keywords: Oncolytic virus, Immunomodulation, cancer immunotherapy

A048 / Targeted BiTE expression by an oncolytic vector augments therapeutic efficacy against solid tumors

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Bispecific T cell engagers (BiTEs) directly crosslink T cells to tumor cells, resulting in T cell cytotoxicity against the bound target cells. This approach has achieved compelling clinical success in the treatment of B cell malignancies. However, BiTEs have yet to prove therapeutic success in clinical trials against solid tumors. In addition, systemic administration of BiTEs can cause severe and even fatal side effects.

We hypothesize that encoding BiTEs in oncolytic measles vectors (MV-BiTE) improves therapeutic efficacy against solid tumors. Viral tumor tropism allows for tumor-restricted BiTE expression, increasing local BiTE concentrations while minimizing systemic side effects. MV oncolysis not only results in tumor debulking, but also the accumulation of danger- and pathogen-associated molecular patterns as well as the release of tumor antigens during immunogenic cell death. Therefore, we further hypothesize that the combined immunomodulatory facets of MV oncolysis and BiTE therapy are superior to each agent alone and able to set the stage for a durable antitumor immune response.

To test these hypotheses, we engineered oncolytic measles virus constructs to encode BiTEs targeting either human CD20 or CEA as tumor-associated antigens along with human and murine CD3, respectively. Replication capacity and oncolytic efficacy of MV-BiTEs are not impaired compared to the parental vector. In addition, expression and functionality of MV-encoded BiTEs were validated ***in vitro.*** BiTEs specifically bind to relevant target antigens,

as verified by sandwich and competitive ELISAs, pull-down assays and flow cytometry. In co-cultures with freshly isolated PBMCs or murine T cells, BiTEs mediate specific tumor cell lysis and induce secretion of TH1 effector cytokines. Therapeutic efficacy of MV-BiTE was demonstrated in a novel, syngeneic tumor model of B16 cells stably expressing human CD20 as BiTE target antigen and human CD46 as measles virus entry receptor. Treatment with MV-BiTE delayed tumor progression and significantly prolonged survival in fully immunocompetent mice, compared to MV encoding a non-relevant BiTE targeting CEA and administration of purified BiTE, respectively. Therapeutic effects were associated with increased T cell infiltration and activation. Furthermore, we validated MV-BiTE efficacy and safety in a unique humanized model of colorectal carcinoma with CEA-expressing human primary tumor spheres and transfer of human PBMCs.
In conclusion, the use of oncolytic measles virus as a vector for targeted expression of BiTEs resulted in enhanced therapeutic efficacy in syngeneic and xenograft models of solid cancer. Our results indicate potential for future clinical translation.

Keywords: Oncolytic virus, Bispecific T cell engagers, Preclinical models

A049 / Oncolytic vesicular stomatitis virus pseudotyped with the glycoprotein of lymphocytic choriomeningitis virus for the treatment of prostate cancer

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Treatment of prostate cancer (PCa) that relapses after conventional therapy remains a serious medical challenge. Here we propose the use of VSV-GP, a novel and potent chimeric oncolytic virus (OV) based on the vesicular stomatitis virus (VSV) carrying the glycoprotein of lymphocytic choriomeningitis virus (LCMV), for the treatment of PCa. OVs that preferentially replicate in and kill tumor cells represent an innovative treatment option for cancer patients after failure of common therapeutic strategies. Through cell lysis, OV set free tumor antigens, which in combination with the OV adjuvant effect unleashes a strong anti-tumor immune response. We used prostate cancer cell lines and primary cultures from patient samples to test the efficacy of VSV-GP in prostate cancer. We analyzed oncolytic efficiency as well as the role of the innate immune response in therapy outcome. VSV-GP was further tested *in vivo* using different xenograft models of both local and metastatic tumors.
VSV-GP exhibited high oncolytic efficiency *in vitro*, efficiently killing the majority prostate cancer cell lines tested. Further analysis of resistance factors revealed that VCaP and TRAMP-C1 were able to mount an interferon type-I (IFN-I)-induced antiviral response, while defects in the IFN-I signaling pathway were found in VSV-GP susceptible cell lines. Results in cell lines were confirmed in primary cultures derived from patients who had undergone radical prostatectomy.
In our *in vivo* studies, intratumoral treatment with VSV-GP in two different subcutaneous (s.c.) models resulted in high response rates which ranged from stable disease to complete tumor remission. Intratumoral treatment of localized tumors in a syngeneic model resulted in tumor growth delay and significant increase of the median survival. In addition, systemic treatment of localized s.c. tumors resulted in 100% tumor remission. Finally, a single intravenous administration of VSV-GP in a bone metastasis prostate cancer model was sufficient to cause tumor remission in all treated mice.
VSV-GP is a promising novel therapeutic option for the treatment of prostate cancer. To optimize the efficiency of VSV-GP, further studies will be necessary to better understand how the oncolytic effect, the IFN-I response and anti-tumor immune response interact and what strategies will result in enhanced therapeutic outcome.

Keywords: VSV-GP, Prostate cancer, IFN-I

A050 / Oncolytic measles virus for local delivery of antibodies targeting the PD-1/PD-L1 axis

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Blockade of negative T cell regulation through the PD-1/PD-L1 interaction using specific antibodies is now an established treatment option for different cancer types. Nevertheless, most of the patients receiving systemic therapy with antibodies against PD-1/PD-L1 do not experience clinical benefits and treatment is associated with considerable toxicities. To address these limitations, we propose the use of oncolytic measles vaccine strain vectors (MV) encoding PD-1/PD-L1 blocking antibodies to allow targeted local delivery of these immunotherapeutics. In addition to an improved safety profile, we expect enhanced therapeutic efficacy by combining the virus-mediated oncolytic vaccination effect with a local blockade of negative T cell signaling.
We have previously developed MV vectors encoding an antibody against murine PD-L1 (MV-anti-PD-L1). These vectors demonstrated

improved therapeutic efficacy in terms of extended survival of the animals in the immunocompetent murine MC38cea and B16-CD20 tumor models. PD-L1 blockade is considered to have improved safety, but impaired therapeutic efficacy in comparison to direct PD-1 blockade on T cells in a setting of systemic administration. Thus, we hypothesized that MV encoding an antibody against the PD-1 receptor would have an enhanced therapeutic efficacy in comparison to the MV-anti-PD-L1 without compromising safety. To this end, we developed a novel oncolytic MV Schwarz vaccine strain virus encoding an antibody against murine PD-1 (MV-anti-PD-1). MV-anti-PD-1 was characterized ***in vitro***, demonstrating secretion of the inserted anti-PD-1 in the supernatant of infected cells. MV-expressed anti-PD-1 specifically bound to PD-1 on the surface of activated murine cytotoxic T lymphocytes. We will present data on therapeutic efficacy of MV-anti-PD-1 and MV-anti-PD-L1 in the MC38cea tumor model. Moreover, modulation of the tumor immune environment after treatment with both vectors is compared assessing the impact on the intratumoral cytokine profile as well as on tumor infiltrating lymphocyte subpopulations and their activation status. These results will contribute to the understanding of the immunomodulatory aspects of PD-1/PD-L1 blockade in the context of oncolytic measles virus therapy, facilitating clinical translation of this therapeutic option. Most importantly, a phase I/II clinical study for combination of oncolytic MV and PD-1 blockade is currently in preparation at the National Center for Tumor Diseases in Heidelberg, Germany.

Keywords: Oncolytic measles virus, PD-1/PD-L1 blockade, Immunomonitoring

A051 / A novel CD40L/4-1BBL-encoding oncolytic virotherapy for CD46+ B-cell lymphoma and multiple myeloma

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Immunostimulatory gene therapy may be of interest to prime lymphoma lesions prior CAR T cell or checkpoint blockade therapy. LOAd703 is a tumor-selective oncolytic adenovirus (serotype 5/35) encoding two immunostimulatory transgenes (trimerized membrane-bound CD40L and 4-1BBL). We have previously shown the immunostimulatory effect of LOAd703 in pancreatic cancer models and a clinical trial enrolling patients with pancreatic cancer is ongoing. LOAd703 can potentially be used in a variety of cancers but many adenoviruses have failed to show efficacy in B-cell malignancies. The LOAd viruses infect cells via CD46, which should enable virus infection of B cells. Herein, we investigated the effect of LOAd703, and other viruses within the LOAd platform, in models of human B-cell malignancies with a focus on B-cell lymphoma. A panel of human B-cell lymphoma cell lines (Karpas-422, BC-3, Daudi, DG-75, U-698) and a multiple myeloma cell line (RPMI-8226) was selected. All cell lines were confirmed to express CD46 by flow cytometry, but expression levels varied between the cell lines (8-95% $CD46^+$ cells). LOAd703 infection resulted in 4-1BBL transgene expression 48 hours post infection in all cell lines except for Daudi, which could be due to inefficient infection because of low CD46 expression (8%). However, CD40L expression could only be detected in 3/6 cell lines (Karpas-422, BC-3 and RPMI-8226) indicating that CD40L surface expression might not be feasible in certain malignant B cells. The killing capability of LOAd viruses was analyzed using CellTiter96®AQueous One Solution Cell Proliferation Assay. The viability of Karpas-422, BC-3 and RPMI-8226 was significantly reduced by LOAd infection, which agrees with the high CD46 expression level in these cell lines. Killing of the cells was due to oncolysis as viral replication after infection was verified with real-time PCR detecting virus genome copies. Furthermore, LOAd703 infection could induce a more immunogenic phenotype in the tumor cells as shown by flow cytometry. Several molecules that facilitate recognition and killing by the immune system were upregulated 48 hours post infection. These included co-stimulatory molecules like CD80 and CD86, MHC molecules as well as the death receptor Fas and the adhesion molecule ICAM-1. Supernatants from infected cells will be analyzed for inflammatory molecules by multiplex analysis. In conclusion, LOAd oncolytic adenoviruses infected and replicated in $CD46^+$ malignant B cells. LOAd703 encoding immunostimulatory transgenes induced transgene expression and subsequently an immunogenic phenotype in infected cells. LOAd703 is an interesting immunotherapy for $CD46^+$ B-cell lymphoma and multiple myeloma.

Keywords: Adenovirus, B-cell lymphoma, Multiple myeloma

A052 / Preclinical studies of oncolytic adenovirus 3 coding for CD40L as an enabler of dendritic cell therapy

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Dendritic cells are considered as principle initiators of the immune system and are uniquely suited for cancer immunotherapy. However, tumor induced immunosuppression impairs the biological function of dendritic cells. Therefore, outcomes of human clinical trials with the DC therapy have often been disappointing. Oncolytic adenovirus, interestingly, have been shown to enhance the release of tumor-specific antigens and trigger danger signals at the tumor site thus activating anti-tumor immune response. To optimize the activation of the transferred dendritic cells, we armed adenoviruses with CD40 ligand (CD40L), best known for its capacity to initiate multifaceted signals in dendritic cells, leading to the activation of cytotoxic T-cells. Therefore, we generated a novel virus Ad3-hTERT-CMV-hCD40L which is fully serotype Ad3 and expresses human CD40L, a potent stimulator of dendritic cells, for induction of antitumor efficacy. The viral particles are produced in 293 cells using standard calcium phosphate method. The functionality of the virus is studied by infecting different cell lines with different amount of viral particles and measuring the proportion of surviving cells with MTS assay. Moreover, human and animal data have shown the ability of Ad3 to successfully reach tumors through the intravenous route. To deeply dissect if CD40L-encoding adenovirus can modulate the tumor microenvironment, we generated a murine version of the virus (Ad5/3-CMV-mCD40L). In syngeneic studies in immunocompetent model, DC therapy in combination with Ad5/3-CMV-mCD40L showed potent antitumor activity. The improved therapeutic effect by the adenovirus expressing CD40L and DCs combination treatment correlated with induction of the T-helper type 1 cytokines, increased numbers of tumor infiltrating lymphocytes, and the reduction of immunosuppression in the tumor stroma. The combination of our Ad3-hTERT-CMV-hCD40L virus and human dendritic cells is further evaluated in mice humanized with human peripheral blood mononuclear cells (PBMCs). We have shown that the hCD40L armed adenovirus is a promising way to amplify the potency of dendritic cell vaccine to initiate innate immunity. Thus, eventually the long lasting adaptive immunity against cancer. In conclusion, our results suggest that hCD40L- armed adenovirus act as a self-sensing and immune boosting system that can alter the immunosuppressive tumor microenvironment and also as a way to improve DC therapy. Thus, these findings support the development of clinical trials where dendritic cell therapy is enhanced with oncolytic adenovirus.

Keywords: Oncolytic adenovirus, CD40L, Dendritic cells

ADOPTIVE CELL THERAPY

A053 / Redirection of human T cells to tumor cells via nanobody-based target modules using the universal chimeric antigen receptor system

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In general, adoptive transfer of chimeric antigen receptor (CAR)-expressing T cells has an impressive immunotherapeutic potential. However, due to the time-consuming establishment of new CAR constructs, the risk of life-threatening side effects, and the lack of control mechanisms once infused into patients, we developed a switchable modular CAR platform technology termed UniCAR. The UniCAR system is composed of two individual components, the universal signal-transducing UniCAR and an exchangeable target module (TM). In contrast to conventional CARs, the single-chain fragment variable (scFv) of the UniCAR binds to a small peptide epitope, which is physiologically not accessible on intact cells. The cross-linkage to tumor cells is mediated via TMs comprising the antigen-specifity and the epitope recognized by the UniCAR. Consequently, UniCAR-engrafted T cells are inert in the absence of redirecting TMs and only switched on in their presence. In addition to the increased safety, the modular structure enables a flexible targeting of different tissue antigens. New TMs can be easily constructed just by fusing the UniCAR epitope to a targeting entity. So far we produced a series of functional scFv-based TMs against different tumor-associated antigens like PSCA, PSMA, GD2 and CD33.

Here we demonstrate that TMs can alternatively contain a nanobody (nb) domain instead of an scFv. Nbs are derived from camelid heavy-chain antibodies, consist of a single variable domain and form the smallest known antigen binding fragments. For redirection of UniCAR T cells to epithelial tumors the frequently overexpressed EGFR is a suitable target antigen. Thus, we generated a nb-based a-EGFR TM. As shown by ***in vitro*** assays with

EGFR$^+$ tumor cell lines, the novel TM efficiently activates UniCAR T cells in a strictly target-specific manner and induces the release of pro-inflammatory cytokines. Furthermore, the data reveal that the a-EGFR TM triggers a highly potent tumor lysis at low pM concentrations and redirects UniCAR-engrafted T cells to tumor cells in immunodeficient mice. Using dynamic PET analysis we observed a short half-life of the TM and could confirm its release from UniCAR-TM-complexes. Thereby, it is possible to precisely dose the TM concentration and to rapidly switch the system off in case of adverse side effects. Finally, the a-EGFR TM also offers the possibility to image the tumor during therapy. For an increased anti-tumor response we additionally generated a bivalent a-EGFR-EGFR TM that shows improved *in vitro* and *in vivo* functionality compared to the monovalent construct.
In summary, we established a novel mono- and bivalent nb-based TM for EGFR-specific recruitment of UniCAR T cells which results in an efficient, target-specific and -dependent killing of EGFR$^+$ tumor cells. Thus, we could prove that instead of scFvs also other binding moieties can be used and confirmed the high flexibility of the modular UniCAR platform.

Keywords: CAR T cell therapy, nanobodies, EGFR-targeting

A054 / T cell delivery of immune-stimulatory cytokines to enhance cancer immunotherapy

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Adoptive cell therapy using TCR-engineered T cells is an exciting area of research and has emerged as a promising strategy for treating cancer patients. However, the effector function of TCR-engineered T cells can be tuned down by local mechanisms of tumor-associated immunosuppression. The potential of cytokines to reverse local immune suppression and enhance tumor immunity has been described in the past. The main aim of this study was to engineer T cell specificity as well as effector cytokine production as a strategy to enhance cancer immunotherapy. This was achieved by combining TCR gene transfer with genetic engineering to achieve IL-12 and IL-27 production in therapeutic T cells. In order to circumvent previously described toxic side effects observed with systemic IL-12 delivery, a tet-regulated gene expression system was utilized to regulate cytokine production by engineered T cells *in vivo*. Adoptive transfer of TCR-redirected T cells expressing regulated IL-12 in B16F10 melanoma-bearing mice resulted in an enhanced accumulation of transferred CD8$^+$ T cells in the tumor and in a change of the innate immune cell composition in the tumor microenvironment. Importantly, regulated IL-12 delivery resulted in enhanced therapeutic efficacy of the transferred T cells without causing systemic toxicity. IL-27 delivery in engineered T cells also showed some effectiveness when combined with TCR gene therapy, although the therapeutic benefit of IL-27 was inferior to IL-12. Interestingly, IL-12 and IL-27 overexpression in TCR-modified T cells significantly enhanced the levels of TCR surface expression in the tumor compared to their pre-transfer levels of TCR. The data in this study demonstrate the potency of additional genetic manipulation to tailor the TCR-redirected T cell effector function which can result in a substantial enhancement in their therapeutic efficacy, and thus, enhanced antitumor immune response.

Keywords: Cancer immunotherapy, TCR gene therapy, T cell engineering

A055 / Tandem TCR T cells targeting MDM2 and p53 in combination with checkpoint inhibitors as a novel approach for cancer immunotherapy of Multiple Myeloma

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Adoptive cell transfer (ACT) using genetically modified T cells with T cell receptor (TCR) has achieved tremendous success in the treatment of cancer. The therapeutic results recently obtained with checkpoint inhibitors have opened a new era in the field of cancer immunotherapy. Yet, clinical responses are still often observed either transiently or in a minority of patients. Tumor antigen escape relapses have emerged as a major problem in ACT therapy. Overexpression/mutation of the human homologous of the murine double-minute 2 protein (MDM2) and the tumor suppressor p53 are often associated with enhanced proliferation and survival of Multiple Myeloma (MM) cells and resistance to standard therapy. Here, we evaluated the therapeutic potential of combining two high-affinity A2.1-restricted murine TCRs specific for MDM2 and p53 as a novel approach to prevent antigen-loss escapes in a xenograft model of MM.
MDM2- and p53-specific TCR were modified by codon optimization, addition of a second disulfide bond between TCR α- and β-chain constant domains and cloned into a 2A-based bicistronic retroviral vector. Human T cells from healthy donors were retrovirally transduced with the optimized MDM2- and p53-specific TCRs and expression levels were analyzed by flow cytometry. MDM2 and p53 protein expression in MM cell lines was determined by Western

blot. The therapeutic efficacy of MDM2 and p53 TCR redirected T cells was evaluated in NOD-scid IL2R gamma chain^{null} (NSG) mice engrafted with A2.1-expressing NCI-H929 MM cell line.
Freshly extracted tumor cells from tumor-bearing animals exhibited a down-regulation of MDM2 expression and concomitantly an up-regulation of p53 expression which was associated with a lower cytolysis by MDM2 TCR T cells ***in vitro***. Accordingly, MDM2 and p53 TCR transduced T cells showed superior anti-tumor response ***in vivo*** compared to single TCR treatment, demonstrating the need to target multiple MM antigens to circumvent antigen-loss escapes. In addition we observed a strong up-regulation of PD-L1 expression in tumor cells ***in vivo*** and enhanced PD-1 expression in tumor-infiltrating lymphocytes (TILs) which may limit the efficacy of antigen-specific TILs. ACT experiments combined with anti-PD-1 inhibitor (nivolumab) demonstrated the synergistic therapeutic potential of this approach as compared to single agent. Yet, it did not result in complete tumor eradication suggesting that targeting one single immune checkpoint receptor is not sufficient to drive a full anti-tumor response. Blockade of additional immune checkpoint receptors (i.e. PD-L1, LAG-3 or TIM-3) or targeting SLAMF7, a receptor expressed on normal plasma cells and MM cells, and currently tested in an early-phase study in patients with MM could enhance the efficacy of ACT therapy.
Checkpoint blockade has demonstrated synergistic potential in our ACT experimental MM model and forms the basis for a novel multi-modal immunotherapeutic combination treatment for multiple myeloma.

Keywords: adoptive cell transfer, checkpoint blockade, preclinical model

A056 / CAR T cells specific for CD19 are redirected to kill CD19 negative tumors

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Remarkable progress has been made in the treatment of relapsed/refractory Acute Lymphocytic Leukemia and Non-Hodgkin's Lymphoma with CAR-CD19 T cells. In contrast, progress against CD19-negative hematological cancers and solid tumors has been modest. CAR T cells directed to solid tumor antigens typically expand for a limited time, and then abruptly die off, suggesting activation-induced cell death triggered by lack of antigen. Indeed, insufficient antigen is an unappreciated hurdle to effective CAR-T therapeutics for solid tumors. We present a novel strategy to utilize the hematopoietic expression of CD19 to provide consistent to promote CAR expansion, efficacy and persistence. The strategy, called **I**MPACTtm (**I**ntegrated **M**odules o**P**timize **A**doptive **C**ell **T**herapy), uses modular fusion proteins, and can therefore be applied to diverse antigens and tumor types while retaining the well-established advantages of CAR T cells directed to CD19.
In order to establish this system we used FMC63-derived CAR19 T cells and soluble fusion proteins. We cloned human CD19 extracellular domain protein in frame with the anti-Her2 trastuzumab-derived Vh/Vl sequence and a His tag. This module encodes an IMPACT^{tm} fusion protein (FP): CD19-anti-Her2-scFv. This protein and two control proteins (CD19 alone; and part of the extracellular CD22-anti-Her2-scFv FP) were generated by transient transfection followed by anti-His tag chromatography and SEC purification. In vitro cytotoxicity studies showed robust redirected killing of HER2+ (CD19-) tumor cells by CAR19 T cells that was specifically mediated by the CD19-anti-Her2 scFv FP. A tumor xenograft model was used to demonstrate effective killing in vivo when CAR19 T cells and purified CD19-anti-Her2 scFv FP were injected into immunodeficient mice carrying HER2+ ovarian carcinoma SKOV3 cells. Finally, we used a lentiviral vector to express both the CAR19 and the CD19-anti-Her2-scFv FP within the CAR-T cell; these were able to directly kill Her2+ tumor cells.
We conclude that a CD19-anti-HER2-scFv FP was able to redirect the cytotoxicity of CAR19 T cells in order to kill HER2+ solid tumor cells. The IMPACT^{tm} technology is modular: FPs diverse protein domains paired with distinct scFvs, and allowing CAR T cells to CD19 or other targets to be used to kill any tumor. Programs redirecting CAR19 T cells to CD20, CD22, BCMA, ROR1 and other antigens are underway.

Keywords: chimeric antigen receptor, CD19, Her2

A057 / Bisphosphonate loaded calcium phosphate nanoparticles for γδT mediated cancer immunotherapy

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γδT cell mediated immunotherapy is a potential therapeutic approach against cancer that activates a subset of T cells called γδT cells. Efficient γδT cell activation can lead to anticancer effector mechanisms that include release of perforin and granzymes, expression of ligands (CD95, TRAIL) that engage death receptors, high clonal frequency and secretion of IFNγ. Although the potential of γδT cells is promising for cancer immunotherapeutic applications, there are limited reports on significant improval of overall survival in cancer patients. Activation of γδT cells for cancer

immunotherapy is clinically carried out using a class of drugs called bisphosphonates. Patients either receive an intravenous (I.V.) injection of drug, bisphosphonate, or injection of ***ex vivo*** activated bisphosphonate treated autologous γδT cells (adoptive therapy). The major limitation in obtaining an effective antitumor response is the insufficient ***in vivo*** activation of γδT cells. One of the reasons behind this is the short plasma half-life of bisphosphonates due to its strong adsorption to calcium phosphate component in human skeletal system. This will limit the drug uptake by monocytes as well as tumor which is the prerequisite for γδT cell activation. In the present work, we developed bisphosphonate tagged calcium phosphate nanoparticles, that could be utilized for enhancing γδT cell activation compared to free drug by altering the biodistribution of drug to enhance monocyte uptake in peripheral blood or lymph nodes. Size of the nanoparticle-drug conjugate analysed using dynamic light scattering showed ~100 nm which was confirmed using transmission electron microscopy. Zeta potential analysis gave a surface charge of -15mV owing to the adsorbed bisphosphonate ions on nanoparticle surface. We optimized the weight percentage of the drug to nanoparticle concentration for efficient conjugation as 1: 10 (wt%). Stability analysis (using dynamic light scattering) at the optimized drug to nanoparticle ratio showed excellent stability for a period of one week. Cell viability analysis (MTT) on human peripheral blood mononuclear cells (PBMC) showed that the drug conjugated nanoparticle treated cells gave enhanced (by 20%) T cell proliferation compared to free drug treated cells. Flow cytometric analysis on PBMC using γδT cell receptor and CD3 also confirmed the enhanced proliferation of γδT cells treated with nanoparticle-drug conjugates compared to cells treated with free drug. We also confirmed efficient uptake of nanoparticle drug conjugates by RAW macrophages by prussian blue staining of the nanoparticle (doped with $Fe3^{+}$) treated cells. Release of IFNγ, perforin, granzymes and γδT cell mediated cytotoxicity were also measured ***in vitro*** prior to ***in vivo*** experiments. Thus we optimized a nanoparticle-bisphosphonate conjugate of size ~ 100 nm and excellent stability with the ability to activate γδT cells for potential cancer immunotherapeutic applications.

Keywords: γδT cells, cancer immunotherapy, nanoparticles

A058 / TRuC™-T cells targeting CD19 or mesothelin demonstrate superior antitumor activity in preclinical models compared to CAR-T cells

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T cells expressing chimeric antigen receptors (CARs) have demonstrated impressive clinical benefit in certain hematological malignancies but so far struggled to show efficacy in solid tumors. We believe this is because CAR constructs bypass T cell receptor (TCR) complexes and therefore fail to initiate the entire signaling cascade that naturally occurs when TCRs bind their antigen on cancer cells. To overcome this limitation, we have developed TRuC™ technology (T Cell Receptor Fusion Constructs) that have antigen binding domains recombinantly fused to subunits of the intact TCR complex. We show that TRuC™ variants become an integral part of the TCR complex and allow activation of the natural signaling cascade. Upon T cell activation, TRuC™ variants were able to trigger CD3ε phosphorylation and showed a broader and only partially overlapping gene expression profile compared to CAR-T cells. CD19-specific TRuC™-T cells potently killed tumor cells ***in vitro*** while releasing less cytokines than respective CD19-CAR-T cells. In a subcutaneous RAJI xenograft model, CD19-specific TRuC™-T cells eliminated tumors and significantly increased the survival of mice while 28zeta or 4-1BB CD19-specific CAR-T cells were less active. To show that TRuC™-T cells are also efficacious with solid tumor targets, we developed TRuC™-T cells targeting mesothelin, which is highly expressed in a range of solid tumor types. A single dose of MSLN TRuC™-T cells completely eradicated primary tumors without relapse. In contrast, T cells expressing a 28ζ CAR construct failed to control tumor growth in the majority of mice. TRuC™ cells not only cleared the primary tumor, but also protected mice from a later re-challenge. Our TRuC™ variants power T cells through the entire TCR without requiring additional costimulatory domains and reprogram T cells to recognize tumor surface antigens in a non-MHC-restricted fashion. We believe that these features make the TRuC™ platform a superior approach to treat cancer with engineered T cells.

Keywords: TCR therapy, mesothelin, solid tumors

A059 / Patient-derived xenograft (PDX) models of human malignancies

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Despite advances in surgical techniques, chemotherapeutics, and radiotherapy, the 5-year survival rate for HNSCC patients remains at a disappointing 50%, stressing the need for new and more effective treatments. Recently, cancer immunotherapies involving both immune

checkpoint blockade (ICB) and adoptive cell transfer of tumor infiltrating lymphocytes (TIL) have emerged as potent treatment options for advanced malignancies, and are able to occasionally produce durable and complete responses. The efficacy, reliability, and safety of these approaches in HNSCC, would be greatly improved if ACT and ICB could be optimized in preclinical patient-derived xenograft (PDX) models in which human tumor cells are engrafted into immunodeficient mice. Using conditional cell reprogramming we have successfully developed a patient-derived xenograft (PDX) platform where HNSCC cell lines are established from small tumor biopsies with concomitant isolation and expansion of autologous TIL from the same specimen. These HNSCC cancer cell lines engraft and grow progressively in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice, recapitulate the primary tumor histology and genetic profile, and are recognized by autologous TIL. To the best of our knowledge, this represents the most comprehensive HNSCC model that has been developed for the preclinical testing of immune therapies to date.

Keywords: Patient-derived xenograft, human malignancies, tumor infiltrating lymphocytes

A060 / CRISPR screen reveals critical role for the citric acid cycle during T cell activation

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T cell activation is accompanied by a rapid rewiring of metabolic networks. While resting T cells are thought to meet their energetic demands largely through oxidative phosphorylation in the mitochondria, activated T cells are thought to rely heavily on glycolysis in the cytoplasm to both generate energy and provide the biosynthetic material needed to support rapid proliferation. Outside of this binary view of T cell energetics, little is understood about the wider network of metabolic pathways that underlie T cell activation. To address this, we undertook a targeted CRISPR screen in primary mouse T cells using a sgRNA library against all genes encoding metabolic enzymes and cellular transporters. In addition to finding that T cells continue to rely on mitochondrial ATP production after activation, we find that the citric acid cycle (CAC) is also essential for producing metabolic intermediates that act outside of its classic role as an oxidative loop. We identify the aspartate-malate shuttle and the citrate-malate shuttle as key interchanges for reducing agents between the mitochondria and cytoplasm, necessary for fueling glycolysis and the electron transport chain. Moreover, we show that intermediates of the CAC play an important role in the epigenetic reprogramming of activated T cells. Collectively these findings illustrate the dynamic interplay between the cytoplasm and the mitochondria during T cell activation and highlight the role of the CAC as a critical point of crosstalk for this process.

Keywords: CRISPR screening, T cells, Metabolism

A061 / NKG2A represents an important immune checkpoint for human cytokine-induced memory-like NK cells in patients with acute myeloid leukemia

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Natural killer (NK) cells are an emerging cellular immunotherapy for patients with acute myeloid leukemia (AML); however, the best approach to maximize NK cell anti-leukemia potential is unclear. Paradigm-shifting reports have shown that NK cells exhibit “memory-like” properties following hapten exposure, virus infection, or combined cytokine pre-activation. We and others have established that human cytokine-induced memory-like NK cells display enhanced re-stimulation responses to numerous activating stimuli, including tumor target cells. We have translated this finding into a potential cellular therapy for rel/ref AML patients, and have completed the dose escalation of a phase 1/2 study. We demonstrated that donor memory-like NK cells expand in patients’ blood and bone marrow and retain enhanced functionality ex vivo. Further, of the 11 evaluable AML patients, we have observed 7 clinical responses, including 6 patients with CR/CRi. Since NK cell recognition depends on signals from multiple activating and inhibitory receptors, we developed mass cytometry panels to immunophenotype and track the diversity and effector functions of these human in vivo-differentiated memory-like NK cells. Multidimensional analyses revealed a memory-like phenotype: CD56^{hi} CD11b^{lo} CD62L^{+} NKG2A^{hi} NKp30^{hi} Ki-67^{+} which was distinct from conventional baseline NK cells. Furthermore, Citrus analyses revealed that higher NKG2A expression significantly correlated with treatment failure. NKG2A is an inhibitory receptor that binds HLA-E, a non-classical MHC-I molecule that is expressed abundantly on many normal tissue types as well as tumors, including AML. Based on the observation that NKG2A is upregulated on memory-like NK cells and the intensity of NKG2A on memory-like NK cells correlated with patient responses, we hypothesized that NKG2A/HLA-E interactions in vivo represent a major barrier to memory-like NK cell responses. In order to interrogate the role NKG2A may play in limiting cytokine-induced memory-like NK cell responses, we stimulated in vitro differentiated ML cells with K562 AML

tumor line forced to express HLA-E (K562-E) in the presence of isotype or anti-NKG2A blocking antibody. Increased IFN-γ and TNF production by NKG2A-blocked ML NK cells was detected, compared to isotype-treated ML NK cells. We performed a similar stimulation assay using primary AML blasts as targets, which express a variable level of HLA-E. Again, ML NK cells treated with NKG2A blockade produced significantly more IFN-γ than isotype treated ML NK cells. Studies examining NKG2A checkpoint blockade on in vivo-differentiated ML NK cells from AML patients are ongoing. In conclusion, in vivo-differentiated cytokine-induced memory-like NK cells are phenotypically distinct from conventional NK cells. Further, targeting NKG2A expressed on ML NK cells in vivo has the potential to enhance the clinical efficacy of memory-like NK cell therapy for patients.

Keywords: Memory-like Natural Killer Cells, Adoptive Immunotherapy, Acute Myeloid Leukemia

A062 / A T-cell receptor recognizing MAGE-A4 in context of allogeneic HLA-A2 targets cancer cells

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We aimed at identifying a T-cell receptor (TCR) specific for a peptide from MAGE-A4 restricted by HLA-A*02:01, expressed by 50% of Caucasians, for potential use in cancer immunotherapy with adoptive transfer of genetically engineered T cells. MAGE-A4 is a cancer/testis antigen of interest for cancer immunotherapy as it is shared among patients with several cancer types. High MAGE-A4 expression levels are found in tumors of the urogenital tract, melanoma, lung cancer and head and neck cancer. In contrast, MAGE-A4 expression in healthy tissues is restricted to testes and ovaries. Since the antigen is intracellular, it cannot be targeted by a therapeutic antibody or chimeric antigen receptor (CAR). In contrast, peptides from intracellular antigens can be recognized by T-cell receptors when presented in context of HLA molecules on the surface of cancer cells. MAGE-A4 is weakly immunogenic in cancer patients, and MAGE-A4 specific cytotoxic T cells are rarely found following *in vitro* stimulation or *in vivo* vaccination. The reason might be depletion of self-reactive T cells during negative thymic selection. To circumvent this, we used our previously described *in vitro* approach to stimulate high-avidity T cells reactive to self-peptides by presentation in context of foreign/allogeneic HLA. In brief, monocyte-derived dendritic cells from HLA-A2 negative healthy donors were transfected with HLA-A2-encoding plasmid DNA, pulsed with MAGE-$A4_{230-239}$ peptide and then co-cultured with autologous CD8 T cells. The approach yielded T-cell clones reactive to the HLA-A*02:01-restricted MAGE-$A4_{230-239}$ epitope. The identified clones were able to lyse HLA-A2 positive peptide-loaded target cell lines and MAGE-A4-expressing melanoma cell lines in Cr^{51}-release cytotoxicity assays. In contrast, HLA-A2 negative melanoma cell lines were not lysed. From these clones, we identified a HLA-A*02:01 MAGE-$A4_{230-239}$-targeting TCR. Retroviral transduction of the TCR led to high expression levels (50-85%) in healthy donor peripheral blood T cells. CD8 T cells expressing the introduced TCR responded to HLA-A2 positive cell lines pulsed with low concentrations of peptide by CD137 or CD107 expression and IFNɣ secretion. Furthermore, TCR expressing CD8 T cells also responded to tumor cell lines endogenously expressing MAGE-A4, including melanoma and cervical cancer cell lines. Also HLA-A2 positive cell lines with low expression of MAGE-A4 evoked corresponding responses, whereas cell lines lacking HLA-A2 or MAGE-A4 did not. In conclusion, our newly obtained MAGE-$A4_{230-239}$-targeting HLA-A*02:01-restricted TCR might be suitable for cancer immunotherapy with transgenic T cells, warranting further studies on specificity and efficacy.

Keywords: Adoptive T cell therapy, MAGE-A4, T cell receptor

A063 / Novel phosphopeptide-specific TCRs for cancer cell therapy

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Phosphoproteins originating from de-regulated post-translational modifications are critical determinants for cancerous cell transformation, are processed to phosphopeptides presented by MHCs, and have the capacity to promote cancer-specific T cell responses. Here, for the first time, we report the isolation of native, fully-human TCRs directed against cancer-specific phosphopeptide targets from central memory T cells of healthy individuals. These TCRs were recovered using two proprietary platform technologies: a) a primary T cell expansion wherein phosphopeptide-specific cognate TCRαβ pairs were identified by functional screening and/or NGS-based sequencing; and b) a TCR display platform comprising the generation of α and β chain

libraries from the donor PBMCs without previous target-specific stimulation, followed by several rounds of TCR enrichment for phosphopeptide target-specific binding.
Importantly, we demonstrate that these native TCRs from the central memory compartment are highly potent at killing tumor cell lines expressing their cognate phosphopeptide-MHC. Phosphopeptide recognition was highly sequence-specific and depended in particular on the phosphoseryl moiety.
The discovery of healthy donor-derived memory phosphopeptide-specific TCRs provides evidence of prior immunological exposure to phosphopeptides. It also provides TCRs with a preferential safety profile as they have undergone immune selection in the absence of overt auto-reactivity within the donor. Thus, phosphopeptide-specific TCRs constitute prime candidates for clinical development.

Keywords: TCRs, Phosphopeptides, Cancer cell therapy

A064 / Augmentation of natural killer cell cytotoxicity by *ex-vivo* cell expansion and anti-GD2 antibody-dependent cellular cytotoxicity as immunotherapy for soft-tissue sarcoma

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Background: Dysfunction of Natural Killer (NK) cells can be found in patients with different cancers, hinting at tumor-induced immunotolerance. Patients with advanced soft-tissue sarcoma (STS) show impaired NK-specific cytotoxicity compared to NK cells from healthy donors (Bücklein et al., Oncoimmunology 2016). Reversion of this impairment might be a promising strategy to eradicate quiescent malignant cells as origin of relapse. We therefore aimed to restore NK-cell mediated cytotoxicity by ***ex vivo*** expansion and activation in a long-term cell culture system. Additionally, we employed a tumor antigen-specific antibody to induce antibody-dependent cellular cytotoxicity (ADCC) against STS cell lines, and tested the combination of both immunomodulatory approaches for synergistic effects on anti-tumor immunity.
Methods: NK-specific cytotoxicity was assessed in standard ^{51}Cr release assays against K562 cells. NK cell expansion and augmentation of cytotoxicity was evaluated after long-term culture in an Interleukin 2-containing bioreactor. Expression of NK cell receptors and effector molecules was analyzed by flow cytometry. Additionally, effects of ch14.18, an Anti-GD2 antibody directed against an antigen frequently expressed on STS, on NK cytotoxicity were tested against sarcoma cell lines with diverging GD2 expression (TC-71, SW872, SKUT-1).
Results: Long-term expansion of NK cells of patients (n=4) and HD (n=3) resulted in cell counts of up to 90-fold of baseline, and led to a significant increase (up to 25-fold) in cytotoxicity against K562 and STS cell lines. Flow cytometry revealed an altered expression of NK cell receptors (e.g. NKp46, CX3CR1) of patient-derived NK cells compared to healthy donor NK cells as possible contributors to the abrogated NK-specific cytotoxicity. Cell expansion lead to an upregulation of activating receptors (e.g. NKG2D). Addition of ch14.18 resulted in an increase in cytotoxicity of unstimulated NK cells of healthy donors and patients against GD2-expressing STS cell lines of up to 7-fold. For expanded NK cells of healthy donors, no synergistic effects for the use in combination with ch14.18 were seen.
Conclusions: Reversing tumor-induced NK cell dysfunction by reconstitution of NK-specific cytotoxicity might be a promising immunotherapeutic strategy. Bioreactor-based long-term cell expansion and use of anti-GD2 antibodies stimulate NK cell-mediated anti-STS cytotoxicity ***in vitro.*** Although no synergistic effects between both methods were seen, ***ex vivo*** NK cell expansion and anti-GD2 ADCC are complementary methods for the treatment of STS and warrant further evaluation.

Keywords: Natural Killer cells, Antibody-dependent cellular cytotoxicity, Soft-tissue sarcoma

A065 / Reconstitution of an evolutionary conserved motif of membrane immunoglobulin in transmembrane regions of alpha and beta TCR chains prevents formation of mixed dimers and improves the functional activity of transduced T cells

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Adoptive transfer of T lymphocytes (ACT) engineered with T cell receptors (TCR) of known anti-tumor specificity has proven to be an effective therapeutic strategy. However, one major constraint of ACT is the formation of mispaired TCRs, caused by random pairing between endogenous and transduced alpha and beta TCR chains. These mixed dimers may reduce the efficacy of the genetically modified primary T cells or may result in unpredictable specificities when transferred into patients.
Here we report that by introducing aminoacidic mutations in the transmembrane regions of both alpha and beta chains, reproducing the motif stabilizing heavy chains Ig transmembrane interactions, we promote in the mouse system the correct pairing of exogenous TCR, inhibiting the formation of mixed dimers with endogenous

chains. Molecular modeling analysis showed correct pairing of mutated TCR and absence of dimerization between mutated and wild type chains. Moreover, reconstitution of both mutated alpha and beta chains in mouse hybridoma T cells conferred TCR expression and functional antigen specificity, while transduction of combined wild type and mutated alpha or beta chains did not result in TCR expression.Furthermore, FRET analysis on mouse splenocytes demonstrated higher efficiency upon transduction of mutated TCR chains respect to wild type TCR chains. Importantly, the same strategy was proven efficacious in human TCRs. This was demonstrated using tumor associated antigens specific TCRs with different intrinsic interchain pairing properties. Human ex vivo isolated T cells transduced with the mutated TCRs showed correct pairing, higher expression and higher cytotoxic function with respect to T cells transduced with wild type TCR. Overall, this strategy, promoting the correct pairing of the transferred exogenous alpha and beta TCR chains, and improving the functional activity and safety of the genetically modified T cells, may be applied in human ACT formulations.

Keywords: T cell receptor, transmembrane regions, mixed dimers

A066 / Generation of tumor-reactive T-cells cultured from non-small cell lung cancers

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Adoptive transfer of ex vivo expanded tumor infiltrating lymphocytes (TILs) is a potent therapy for melanoma. The impressive results of 20% complete remission in treated patients prompted us to explore whether patients with other solid tumors could also benefit from this treatment. Here we investigated the composition of the tumor infiltrate and whether tumor-reactive T-cells could be cultured from non-small lung cancers (NSCLC). We determined the tumor specific infiltrates by comparing lymphoid and myeloid cell infiltrates from NSCLC tumor tissue and non-tumor tissue from the same lobectomy. Tumor tissue contained a higher percentage of infiltrating $CD4^+$ and $CD8^+$ T-cells than adjacent non-malignant tissue from the same patient. These tumor-infiltrating T-cells expressed increased levels of activation markers HLA-DR and PD-1. In addition, tumor tissue was significantly enriched for myeloid cells, B-cells expressing PD-L1 and regulatory T-cells, suggesting an immune suppressive tumor environment. We were able to grow substantial amounts of TILs from the tumor digest from all NSCLC patients. Importantly, these TIL-cultures contained tumor-reactive T cells in 9 out of 14 patients as shown by tumor specific up-regulation of IFN-γ production in T-cells after co-culture with tumor digest. The presence of tumor-reactivity in the majority of analyzed NSCLCs underscores the relevance of generating immunereactive tumor-specific TILs for immunotherapy.

Keywords: TIL culture, Non Small Cell Lung Cancer, Cancer Immunotherapy

A067 / Dual-switch GoCAR-T cells: Dual molecular switches to control activation and elimination of CAR-T cells to target $CD123^+$ cancer *in vivo*

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Background: Improvement of chimeric antigen receptor (CAR)-T immunotherapies may require controlled activation and termination of the T cells when transfused into patients. Here we present two independently regulated molecular switches that can elicit specific and rapid induction of cellular responses upon exposure to their cognate ligands. Cell activation is controlled by the homodimerizer rimiducid that triggers signaling cascades downstream of MyD88 and CD40 via an engineered protein termed iMC. A rapamycin-controlled pro-apoptotic switch (iRC9), that induces dimerization of caspase-9 mitigates possible toxicity from excessive CAR-T function. When combined with a first generation CD123-specific CAR, these molecular switches allow for specific and efficient regulation of engineered T cells to control Acute Myelogenous Leukemia (AML) *in vitro* and *in vivo.*

Methods & Results: T cells were activated and co-transduced with CD123 GoCAR (pSFG-iMC.2A-CAR.ζ) and RapaCIDe (pSFG-iRC9.2A-ΔCD19) vectors to generate Dual-switch (DS) GoCAR-T cells. Combined transduction of RapaCIDe and CD123 GoCAR vectors into T cells did not adversely affect the antitumor efficacy of GoCAR-T cells, which eliminated $CD123^+$ THP1 tumor cells in a co-culture assay at 1:10 effector to target ratio but not CD123-HPAC tumor cells (4.49% THP1-e***GFPfluc*** cells remained in GoCAR-modified cultures treated with 1 nM rimiducid versus 4.23% for DS GoCAR-T). When challenged in a THP1-eGFP***fluc*** tumor-bearing mouse model, activation of the on-switch by rimiducid treatment of mice implanted with either GoCAR-T or DS GoCAR-T cells enhanced tumor killing (bioluminescence imaging) and T-cell expansion (splenocyte flow cytometry and VCN analyses). Deployment of the off-switch induced fast (½ V_{max} ~ 8 hours) and efficient elimination of T cells (DS GoCAR-T = 77.6% $AnnV^+$ versus NT = 2.2% treated with 1 nM rapamycin) in a caspase-3 activation assay with real-time monitoring by the IncuCyte as well

as annexin V detection by flow cytometry. Importantly, the off-switch is insensitive to high rimiducid concentration (the on-switch regulator), thus avoiding cross talk between the two molecular switches. ***In vivo*** assessment of the suicide switch was performed with eGFP***luciferase*** (eGFP***Fluc***)-labeled CD123 DS GoCAR-T cells in NSG mice. Rapamycin, but not rimiducid, treatment efficiently eliminated DS GoCAR-T cells within 24 hours in NSG mice, which is similar to the clinically validated rimiducid-regulated CaspaCIDe switch.

Summary: DS GoCAR-T, a novel platform comprising a first-generation CAR combined with regulated activation and apoptotic signaling elements, effectively controlled tumor growth and T-cell expansion and elimination ***in vitro*** and ***in vivo.*** This dual switch technology provides a user-controlled system for managing persistence and safety of tumor antigen-specific CAR-T cells.

Keywords: CAR-T, chemical induction of dimerization, safety switch

A068 / HLA-A2 mediates self-reactivity in tumor-specific CD8 T cells expressing very high affinity TCRs

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Background and Rationales: T cell receptor (TCR) affinity for its cognate antigen (pMHC) is a key parameter controlling the activation and effector functions of CD8 T cells. Several studies have shown the existence of an optimal window of TCR-pMHC affinities for maximal T cell response. Strikingly, further increase in TCR affinity leads to severe functional declines. Using a panel of human CD8 T cells engineered with incremental affinity TCRs against the cancer testis antigen NY-ESO-1, we recently reported that SHP-1 phosphatase and PD-1 inhibitory receptor were involved in the hyporesponsiveness state observed in the cells expressing very high affinity TCRs. Since the gain in affinity (K_D < 1 uM) was mainly related to amino acid changes in the CDR2 loops, known to interact with the HLA-A2 backbone, we hypothesized that this may trigger, in the very high affinity T cells, weak but chronic TCR-HLA-A2 binding interactions, even in the absence of specific antigen, eventually resulting in a tolerance-related state.

Results: To test this hypothesis, we transduced HLA-A2^{pos} or HLA-A2^{neg} primary CD8 T cells with our panel of affinity-improved TCRs. Upon transduction of very high affinity TCRs and in the absence of cognate antigen, HLA-A2^{pos} CD8 T cells rapidly (< 10 days) up-regulated the activation marker CD69, as well as the inhibitory receptors PD-1 and TIM-3, while no difference of expression was observed for PD-L1, PD-L2, TIGIT, or NKG2A. By contrast, no or only marginal modulation of these markers were seen in HLA-A2^{neg} transduced CD8 T cells. At the functional level, these early phenotypical changes were accompanied by a severe impairment of the basal proliferative capacity of HLA-A2^{pos}, but not HLA-A2^{neg} CD8 T cells, while reduced CD28 surface expression occurred later in culture. Intriguingly, when T cell potency was assessed upon antigen-dependent TCR triggering, both very high affinity HLA-A2^{pos} and HLA-A2^{neg} CD8 T cells were functionally impaired, which may be explained by the productive hit rate model. Importantly, this NY-ESO-1 antigen-specific impairment was TCR-dependent. Indeed, engineered CD8 T cells expressing dual virus- and very high affine tumor-specific TCRs retained their functional competence when stimulated with the virus epitope.

Conclusions: Our results suggest that HLA-A2 self-reactivity is involved in the acquisition of this early activation cell phenotype, which subsequently evolves into an anergy-like state in the very high affinity CD8 T cells. Moreover, the mechanisms underlying this high affinity-associated loss in function might directly be regulated at the individual TCR level rather at the cellular level.

Keywords: NY-ESO-1, HLA-A2, TCR affinity

A069 / Redirection of human T lymphocytes armed with on/off switchable universal chimeric antigen receptors against various malignant cells

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Recently, chimeric antigen receptor (CAR)-expressing T cells have shown tremendous clinic effects in several cancer patients. However once those genetically modified T cells are adoptively transferred in a patient their reactivity cannot be controlled in case of life-threatening side effects or tumor alterations including antigen loss occur. These limitations encouraged us to develop an on/off switchable universal CAR (UniCAR) platform.

As an optimization of conventional CARs, UniCARs do not bind to a cell surface antigen. In contrast their extracellular single-chain fragment variable (scFv) is redirected to the short peptide epitope E5B9 that is physiologically not presented on the surface of living cells. Consequently the UniCAR T cells are inert. Only in the

presence of a target module, that exhibits the E5B9 and binds to a tumor surface target, the UniCAR T cells can be cross-linked to tumor cells and thus get activated to kill them. Recently, we have produced a series of monospecific and bispecific target modules against a series of tumor-associated antigens including PSCA, PSMA, CD33, CD123, GD2, and EGFR.

Here we demonstrate *in vitro* as well as in experimental mice that all these target modules are able to efficiently redirect UniCAR T cells against tumor cells in a strictly target-dependent and target-specific manner. Tumor cell killing occurred at pM target module concentrations and the killing efficacy of UniCAR T cells was comparable to conventional CAR T cells. As measured by ELISA and/or flow cytometry-based multiplex assays redirected UniCAR T cells released pro-inflammatory cytokines including for example TNF, IL-2 and IFN-γ but not IL-6. Bispecific tumor targeting mediated superior tumor cell killing effects than the usage of monospecific target modules whereas the amount of released pro-inflammatory cytokines was not increased. Finally, we have proven that redirected UniCAR T cells can kill luciferase-positive tumor cells in immunodeficient mice. In agreement with the UniCAR concept, target modules showed a very short half-life in peripheral blood, could accumulate in established tumors and were released from UniCAR-target module-complexes in a concentration-dependent manner as measured by dynamic PET analysis in mice.

In summary, we established a controllable UniCAR platform for tumor immunotherapy. The reactivity of UniCAR-armed T cells can be switched on and off in the presence or absence of target modules and can be regulated in a dose-dependent manner providing an improved safety of the CAR technology. Moreover a variety of different target modules against a series of different tumor targets can be introduced in the UniCAR platform supporting its high flexibility.

Keywords: tumor immotherapy, T cell retargeting, chimeric antigen receptor

A070 / Enrichment and separate expansion of tumor-infiltrating CD8 T cells expressing PD-1 improves the efficacy of adoptive therapy

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At a time when great efforts are being made to identify the tumor antigens recognized by the patient's lymphocytes, the use of distinctive biomarkers of tumor-specific T cells may allow the development of tumor-specific cancer therapies without knowing the specific antigen targeted. In this sense, recent studies in melanoma patients have found that most antitumor reactivity is harbored by the PD-1$^+$ CD8 TIL subset, suggesting that cells derived from PD-1$^+$ TILs can be used in adoptive T-cell therapy (ACT). However, no study thus far has evaluated the antitumor activity of PD-1-selected TILs *in vivo*. Using mouse models of solid and hematological tumors, we have investigated the therapeutic potential of PD-1-selected CD8 TILs. We show that tumor-specific CD8 T-cell populations identified by MHC-I-tetramer staining were almost exclusively assigned to the PD-1$^+$ TIL compartment. Importantly, despite the high proportion of tumor-reactive T cells present in bulk CD8 TILs before expansion, only T cell products derived from sorted PD-1$^+$, but not from PD-1$^-$ or bulk CD8 TILs, specifically recognized tumor cells. The original TCR repertoire was more faithfully preserved in the T-cell product derived from PD-1-selected TILs than in that obtained from total CD8 TILs, as assessed by tetramer staining and CDR3-spectratyping analysis. The fold-expansion of PD-1$^+$ CD8 TILs was 10 times lower than that of PD-1$^-$ cells, suggesting that outgrowth of PD-1$^-$ cells was the limiting factor in the tumor specificity of cells derived from bulk CD8 TILs. The highly differentiated state of PD-1$^+$ cells was likely the main cause hampering *ex vivo* expansion of this subset. *In vivo*, only cells expanded from PD-1$^+$ CD8 TILs contained tumor progression, and their efficacy was enhanced by PDL-1 blockade. Overall, our data provide a rationale for the use of PD-1-selected TILs in ACT.

Keywords: Adoptive cell therapy, tumor-infiltrating lymphocytes, PD-1 based sorting

A071 / Adoptive immunotherapy with Cytokine Induced Killer cells and targeted therapy in a preclinical model of metastatic melanoma

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Purpose: of this study is to explore the preclinical anti-melanoma activity of Cytokine Induced Killer (CIK) cells in association with BRAF inhibitors (BRAFi), including the targeting of putative melanoma cancer stem cells (mCSC) that may play a relevant role in disease relapse.

Adoptive immunotherapy with CIK cells is a new emerging cancer treatment. An important feature of CIK cells is their MHC independent tumor killing based on the interaction of their NKG2D receptor with specific tumor ligands (MICA/B, ULBPs).
The ***experimental design*** planned to set a preclinical autologous immunotherapy model with mMel and CIK cells generated from 6 patients harboring BRAF V600E/K mutations, to study the killing activity of combined treatments. Moreover, to visualize melanoma Cancer Stem Cells (mCSCs) we transduced tumor cells with a lentiviral CSC-detector vector encoding enhanced Green Fluorescent Protein under control of the stem-gene oct4 promoter. We treated mMel cultures with BRAFi, evaluated in vitro the presence of residual mCSCs potentially responsible of resistance, and explored their susceptibility to immunotherapy. Selected in vivo experiments were set up to confirm in vitro results.
Treatment with therapeutic doses (IC50) of BRAFi were active ***in vitro*** against mMel but determined a relative enrichment in the rate of mCSCs (1.5 fold) compared to untreated controls ($p< 0.0001$). The rate of mCSCs was instead not affected following effective treatment with CIK cells. The sequential immunotherapy with CIK cells effectively killed mMel and mCSCs surviving after BRAFi; the rate of tumor killing ranged between 90 (E/T=40:1) and 20% (E/T 1:3). CIK cell target molecules were expressed at comparable levels in both mMel and mCSCs, without any apparent modulation by BRAFi. We did not observe upregulation of immune checkpoint ligands (PD-L1/2) in mMel residual after treatments with CIK cells. ***In vivo*** infusion of autologous CIK cells or BRAFi into mice bearing xenografts from 3 distinct melanoma demonstrated significant antitumor responses ($p=0.001$). The sequential BRAFi-immunotherapy resulted in superior antitumor activity compared to each treatment alone.
In **conclusions**, our findings support the hypothesis that the combination of target- and immune-therapy with CIK cells is active against mMel, including the relevant subset of mCSCs. CIK cells could be an effective therapeutic alternative/integration to checkpoint inhibitors against melanoma with defective MHC-machinery, supporting their exploration in clinical trials.

Keywords: Melanoma, Citokine Induced Killer, Cancer Stem Cells

A072 / Adoptive transfer of CMV- and EBV-specific peptide-stimulated T cells after allogeneic stem cell transplantation: First results of a phase I/IIa clinical trial [MULTIVIR-01]

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Background: Reactivation of CMV and EBV negatively impacts on outcome after allogeneic stem cell transplantation (aSCT). Specific antiviral therapy is only available for CMV. With the exception of ganciclovir all drugs are being used off-label. 40-50% of patients reactivate CMV following aSCT. For the 20-30% of patients reactivating EBV, only the use of rituximab is available to control EBV. Rituximab leads to long term B-cell depletion requiring frequent administration of immunoglobulins. To cover the unmet medical need of CMV and EBV control after aSCT, we set up a prospective randomized controlled phase I/IIa multi-center clinical trial to evaluate the preventive and preemptive adoptive transfer of CMV/EBV-specific T cells in patients after aSCT (EudraCT number 2012-004240-30). The multi-center trial is currently recruiting.
Methods: For manufacturing of the cell product two peptide pools (CMV and EBV) each covering 17 well-defined HLA class I and class II epitopes for stimulation of donor derived PBMC are used. To avoid a second leukapheresis of the donor, CMV- and EBV-specific T cells are preferentially expanded from a small fraction of the stem cell graft. A strong expansion of virus-specific T cells could be observed for the first products analyzed by flow cytometry with HLA class I multimers. Reconstitution and cell counts of leukocytes after aSCT are monitored for both treatment and control group. To obtain further insights in the expansion of transferred T cells, the TCR beta (TCRβ) repertoire of the T-cell product before and after adoptive transfer in the patient is monitored by high throughput sequencing.
Study design: After recruitment patients are randomized in intervention or control group. Patients of the intervention group receive 3 applications of virus specific T cells (5x10e4/kg bodyweight) starting the first adoptive transfer 30 days after allogeneic stem cell transplantation. Cells are injected as preventive, preemptive, or also as therapeutic treatment. Patients are monitored for occurrence of GvHD, for viral load as well as for immune-reconstitution, especially of virus-specific T cells.
Results: So far, 19 patients have been randomized. The reconstitution of virus-specific T cells of treated patients looks encouraging after transfer. The immunomonitoring of 10 included patients is completed. New CMV- and EBV-specific TCRβ-sequences could be identified and tracked. Our first observations show promising results regarding feasibility and efficacy of our approach under clinical trial conditions.

Keywords: Cell therapy, CMV and EBV, allogeneic stem cell transplantation

A074 / Targeting liver cancer stem cells is vital for anti GPC-3 treatments in hepatocellular carcinoma

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Background and aims: Liver cancer stem cells (LCSCs) are thought to be responsible for the initiation of hepatocellular carcinoma (HCC) and are closely associated to tumor recurrence and patients survival. GPC-3 has been used as a specific tumor antigen in generating chimeric antigen receptor T (CAR-T) cells for HCC. We identified that the level of GPC-3 expression is negatively correlated with cell stemness, suggesting targeting GPC-3 alone may not be sufficient to achieve ideal therapeutic goals, as LCSCs will likely escape the treatment. The aim of this study is to investigate the rationale of simultaneously targeting GPC-3 and CD133 in liver cancer therapy.
Methods: Online datasets GSE14520 and GSE14897 were analyzed by R 3.3.3 and SPSS 23.0. The expression of GPC-3 and CD133 were detected by Western blot and qPCR in LCSCs derived from HCC cell line (SK-HEP-1). Immunohistochemical staining was performed to examine the expression pattern of GPC-3 and CD133 in human HCC tissues.
Results: Based on GSE14520 dataset, no significant association was found between the expression level of GPC-3 and the rate of tumor recurrence and patient survival, whereas high level of CD133 expression was significantly associated with high likelihood of tumor recurrence and worse survival. Based on GSE14897 dataset, differentiated H9 cells (a human embryonic stem cell line) express high level of GPC-3 whereas in the undifferentiated H9 cells GPC-3 expression is extremely low. Significantly low level of GPC-3 but high level of CD133 were identified in LCSCs. CD133+ LCSCs are largely located at the tumor invasive front. No co-localization of GPC-3 and CD133 was found.
Conclusions: CD133 but not GPC-3 are correlated with the rate of tumor recurrence and patients survival. GPC-3 is negatively correlated with cell stemness. We speculate that successful GPC-3 based CAR T cell therapy would require a simultaneous targeting of LCSC markers such as CD133.

Keywords: liver cancer stem cell, anti GPC-3 treatment, CD133

A075 / T cell recognition of large T and small T antigen in Merkel cell polyomavirus-associated cancer

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Merkel Cell Carcinoma (MCC) is a rare but aggressive human skin cancer induced by Merkel Cell Polyomavirus (MCPyV). The virus is commonly found in humans, but the oncogenic transformation requires two mutation events, often inflicted by UV light, which allow the clonal integration of the viral genome into the host genome and the translation of the two viral oncogenes large T (LTA) and small T antigen (STA). The viral origin of the cancer makes it potent for immunotherapy. Recently, the use of PD-1 checkpoint inhibitors became FDA approved. However, not all patients are able to mount an immune response. Instead adoptive transfer of MCPyV-reactive T cells is an attractive strategy for this patient cohort.
We have previously identified several T cell epitopes from the MCPyV-derived proteins LTA, STA and viral capside protein1 (VP1), restricted to HLA-A01, -A02, -A03, -A11, -A24 and B07. Here we aim to expand the knowledge about T cell epitopes by including a broader range of HLA restrictions (HLA-B08, -B35 and -B44).
We analyzed 49 patients' *peripheral blood mononuclear cells (PBMC) using a platform consisting of a peptide-MHC-based enrichment, which allowed detection of low frequency T cell clones.* Followed by detection of T cell reactivity against the MCPyV-derived epitopes using combinatorial color-encoding of MHC class I multimers. Three patients tumor infiltrating lymphocytes (TIL) were analyzed directly ex-vivo for detection of T cell reactivity against the MCPyV-derived epitopes.
In 32 of the patients' PBMC samples we identified a total of 60 MCPyV-directed T cell responses against 34 different peptides. In all of the three TIL samples we identified a total of five MCPyV-directed T cell responses against five different peptides. Epitope targets embedded in the LTA and STA proteins where solely observed in the patient samples compared to a cohort of healthy donors. Demonstration of peptides processing and presentation of the detected epitopes in MCC tumor cell lines and capability of eliciting a functional CD8 T cell responses is still ongoing.
Epitopes that is presented on MCC tumor cell lines and provoke an immune response could be characterized as a true CD8 T cell epitope target with potential of being used in immunotherapy. The strategy with adoptive transfer of MCPyV-reactive T cells as mono- or combination therapy would be a personalized therapy based on the patient's HLA type. Including additional HLA restrictions would therefore benefit a larger cohort of MCC patients.

Keywords: Merkel Cell Carcinoma, CD8 T cell recognition, Adoptive T cell transfer

A076 / T cells engineered with T-Cell Antigen Coupler (TAC) receptors display robust efficacy against solid and liquid tumor xenografts in the absence of any toxicity

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Engineering T cells with chimeric antigen receptors (CARs) is an an effective method for directing T cells to attack tumors in an MHC-independent manner. CARs aim to recapitulate T cell signaling by incorporating modular functional components of the TCR and co-stimulatory molecules. We designed an alternate chimeric receptor that retains MHC-independent antigen recognition but signals through the native TCR. Our novel chimeric receptor, named the T-cell Antigen coupler (TAC), is a membrane-anchored receptor that co-opts the TCR and co-receptor in the presence of tumor antigen.

In vitro testing has demonstrated robust and specific cytokine production and cytotoxicity by TAC-engineered human T cells (TAC-T cells) directed against either CD19, BCMA or HER2. Unlike CAR-T cells, TAC-T cells showed no upregulation of checkpoint receptors at the end of the manufacturing process. Further, TAC-T cells were enriched for memory markers CCR7 and CD62L relative to CAR T cells. Using a combination of functional assays and transcriptional profiling, we observed tonic signaling in CAR-T cells, similar to previous reports. In contrast, TAC-T cells revealed no evidence of tonic signaling consistent with the design of the receptor to mimic the natural T cell activation process.

In vivo, TAC-T cells revealed strong activity in variety of xenograft models, including liquid (Raji, NALM-6, KMS-11) and solid tumors (MDA-MB-231, OVCAR-3). Head-to-head studies in the OVCAR-3 tumor model using HER-2 as the target demonstrated that TAC-T cells outperformed T cells engineered with either CD28- and CD137-based CARs demonstrating both increased anti-tumor efficacy and reduced toxicity. Histological analysis revealed that TAC-T cells rapidly infiltrated the OVCAR tumors within days whereas the CAR-T cells poorly infiltrated the tumor, even at 7 days post-administration. The TAC-T cell infiltrate was dominated by Ki-67+ CD8+ T cells, confirming both infiltration and local expansion. In contrast CAR-T cells expanded in non-tumor tissues, such as the heart, lung and sometimes the liver. Notably, CAR-T cells expansions in non-tumor tissue were dominated by Ki-67+ CD4+ cells and associated with a massive cytokine storm and severe toxicity, including death. No toxicities were observed in mice treated with TAC-T cells, even at doses that produced complete tumor regression.

These differences in functional characteristics, anti-tumor efficacy and toxicity highlight the biological differences of TAC and CAR receptors and indicate that TAC-T cells may have a superior therapeutic index relative to CAR-T cells.

Keywords: T-Cell Antigen Coupler, Adoptive Cell Therapy, solid and liquid tumors

A077 / Generation and phenotypical analysis of CD19-specific CAR T cells from healthy donor and patient lymphocytes using IL-7/IL-15 or IL-2

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Introduction: During recent years, CAR T cell (CART) therapy has become a promising treatment option for cancer patients. However, reproducibility and safe generation of CART must be guaranteed for clinical application. We investigated how the cellular composition of CART from either healthy donors (HD) or untreated CLL patients was influenced by different culture conditions, i.e. IL-7/IL-15 vs. IL-2, and focused on the specific phenotype of the resulting CART preparation, being composed of naïve (T_N), central / effector memory (T_{CM}/T_{EM}) and effector (T_E) cells.

Methods: We generated CART by transducing human PBMCs with a CD19.CAR-CD28-CD137zeta 3rd generation retroviral vector under two different stimulating culture conditions - anti-CD3/anti-CD28 antibodies with the addition of either IL-7/IL-15 or IL-2. CART cultivation lasted for 20 days. Cytotoxic ability of the generated CART was assessed by a standard 4h chromium (Cr-51) release assay. Multi-parametric flow cytometry was performed using a FACS LSR device. 25 healthy donors and 15 patients were screened.

Results: CART were generated from 8 different donors. Stimulation with IL-2 was responsible for a lower CD4:CD8 ratio at the end of culture compared to IL-7/IL-15 (0.4 vs. 0.9, respectively). For both HD and patients, T_{CM} and T_E subsets evolved similarly and independent of IL-7/IL-15 or IL-2. IL-2 was responsible for an overall increase in T_{EM}, HLA-DR+, CD56+ and CD4+ T_{Reg} cells, whereas IL-7/IL-15 stimulated T_N, T_{SCM} (naïve CD27+CD95+) and CD62L+ CART. Importantly, the naïve and effector memory CART subtypes of patients evolved differently from those of HD (T_N: $p<$

0.01 and T_{EM}: $p < 0.03$): for patients, T_N decreased and stayed < 5% until day 20, even after stimulation with IL-7/IL-15 (T_N for HD: 45% on day 17), while T_{EM} became the main subpopulation (>50%). For HD and patients, stimulation with IL-2 led to the highest lysis of CD19+ Daudi cells by CART ($p < 0.03$) for both fresh as well as freshly thawed cells (55% vs. 35% lysis, respectively). TNF-α and IFN-γ secretion was assessed by intracellular staining at day 15 of culture and reached for both cytokines >45% by CD8+ CART from HD and patients.

Conclusion: We have established a combination of immunophenotyping and cytotoxicity assays for CART. Interestingly, the evolution of the CART subpopulations was similar for HD and patients except for their T_N and T_{EM} subtypes. Correlation of these data with the clinical outcome of patients receiving the corresponding cell products will allow optimization and standardization of CART therapy.

Keywords: Chimeric Antigen Receptor, CD19, Immunotherapy

A078 / TGF beta 1- reprogrammed myeloid-derived suppressor cells lose immunosuppressive function and acquire Fas-dependent tumor killing activity

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Myeloid-derived suppressor cells (MDSC) are induced from bone marrow precursor cells by cancer-mediated pro-inflammatory signals, and play an important role in tumor immune evasion. TGF-β1 is a highly pleiotropic cytokine abundantly expressed in the tumor microenvironment; while primarily immunosuppressive effects of TGF-β1 on tumor, lymphocytes, and macrophages are well-described, little is known about the direct effects of TGF-β1 on MDSC development. CD11b+ MDSC derived ***ex vivo*** from mouse bone marrow with tumor-conditioned medium upregulated inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), and arginase, and efficiently suppressed T cell proliferation. MDSC derived under identical conditions but in the presence of TGF-β1 (myeloid-derived non-suppressor cells: TGFβ-MDSC[AS1] [PJ2] [PJ3]) acquired a more macrophage/monocyte-like phenotype by cytospin. TGFβ-MDSC failed to upregulate iNOS and lost the ability to suppress T cell proliferation. Conversely, they gained expression of maturation and costimulatory molecules, and acquired enhanced antigen presentation capability. TGFβ-MDSC upregulated FAS-ligand expression, leading to FAS-dependent killing of MTEC HPV+ murine head and neck cancer cells and 3-dimensional tumor spheroids. While intratumoral injection of conventional MDSC into established MTEC tumors reversed the beneficial anti-tumor effects of external beam radiotherapy, the combination of TGFβ-MDSC and radiotherapy led to regression and long-term tumor control. TGFβ-MDSC derived from human PBMC with either tumor supernatants or cytokines in the presence of TGF-β1 also developed tumor killing activity and lost immunosuppressive function, associated with downregulation of PD-L1. In summary, induction in the presence of TGF-β1 causes myeloid precursor cells otherwise destined to become immunosuppressive MDSC to take on a novel phenotype, with loss of ability to suppress T cell proliferation and acquisition of FAS-dependent killing activity capable of durable tumor control in combination with radiotherapy.

Keywords: Myeloid derived suppressor cells, tumor killing, T cell proliferation

A079 / Targeting BCL2 ablates host natural killer cells to permit alloSCT engraftment with reduced host conditioning and preventing GVHD

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Allogeneic haematopoietic stem cell transplantation (alloSCT) is used to treat over 15,000 patients with acute myeloid leukemia globally per year. Donor immune-mediated graft-versus-leukemia (GVL) effect can prevent AML relapse however alloSCT is limited by significant toxicity related to conditioning intensity, immunosuppression, opportunistic infections and graft-versus host disease (GVHD). Reducing the conditioning intensity to minimize GVHD has not proven successful likely due to poor elimination of host cytotoxic lymphocytes capable of rejecting the MHC-I mismatched graft. Here we demonstrate a novel host conditioning approach combining reduced irradiation with host natural killer (NK) cell ablation that permits efficient donor reconstitution and GVL without resulting in GVHD. Reduced host conditioning (< 12Gy) alone is not compatible with alloSCT engraftment. Here we show that NK cell specific deletion of ***Bcl2*** or pharmacological inhibition of BCL2 impairs radio-resistant NK cell rejection of alloSCT engraftment in hosts receiving non-lethal irradiation (8Gy). Ablating host NK cell function via BCL2 antagonism prior to non-lethal irradiation allows long-term alloSCT engraftment, efficient GVL and importantly, no signs of GVHD. In contrast, control mice or those

receiving vehicle and 8Gy failed to show any alloSCT engraftment. The rate of long-term alloSCT engraftment improved significantly when both MCL1 and BCL2 were genetically targeted in NK cells or antagonized ***in vivo*** with pharmacological inhibitors. Since long-term donor engraftment is essential for GVL, we propose that the translation of our data to human AML will dramatically improve leukemia-free survival rates and eliminate toxicities associated with more intense conditioning such as GVHD.

Keywords: leukaemia, stem cell transplantation, natural killer cell

A080 / Oxidative stress inducible factor induced by tumor environment suppresses anti-tumor activity of cytotoxic CD8⁺T cells

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The anti-tumor immune responses in cancer are significantly inhibited. Especially, it has been shown that cytotoxic activity of $CD8^+$ cytotoxic T lymphocytes (CTLs) was diminished in tumor. The immunosuppressive tumor microenvironment is a major obstacle to effective anti-tumor immunotherapy. Recently, it has been shown that tumor-induced myeloid-derived suppressor cells (MDSC) play a critical role of immune suppression in tumor-bearing individuals. The studies demonstrated that MDSC suppressed anti-tumor response of effector T cells through production of reactive oxygen species (ROS). The ROS induces oxidative stress which is able to activate related-transcription factor and regulate a battery of genes that attenuate oxidative stress. It is easy to imagine that oxidative stresss condition may affect function of immune cells infiltrated into tumor mass. We have thus questioned how oxidative stress affects and what roles of oxidative stress inducible factor (OSIF) are in anti-tumor responses of cytotoxic CD8+ T cells. First, tumor infiltrating T cells (TILs) were sorted from tumor mass to investigate the alteration of OSIF expression. Interestingly, we found that mRNA levels of OSIF are significantly increased in TILs compared to control T cells. In order to establish a relationship between T cell activity and OSIF levels, $CD8^+$T cells are serially stimulated. OSIF expression and their targets are serially downregulated as increase in T cell activity. We further confirmed that IFN-γ and Granzyme B production in OSIF-deficient $CD8^+$ T cells was dramatically enhanced. In addition, Adoptive transfer of OSIF-deficient $CD8^+$ T cells more efficiently controlled tumor growth and metastasis than those of wild-type $CD8^+$ T cells. Collectively, OSIF negatively regulates CTL responses by tumor specific high level of ROS. Our findings offer novel insights into how immune response and oxidative stress is integrated in tumor cells, and we highlight OSIF as a powerful candidate of molecular targets to optimize effector T cells for adoptive immunotherapy of cancer.

Keywords: Oxidative stress inducible factor, Cytotoxic CD8+ T cells, Adoptive immunotherapy

A081 / MISTRG: An improved version of mice with humanized immune system to test the efficacy of transplantable T cell therapy protocols against solid tumors

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Transfusion of ***in-vitro*** expanded T cells isolated from tumors (Tumor-Infiltrating lymphocytes or TILs), back into patients is a promising form of therapy for yet untreatable types of cancer, such as melanoma. Although significant progress has been achieved in optimizing ***in-vitro*** TIL cultures, many parameters affecting T cell responses against tumors cannot be approximated ***in-vitro***. Therefore, we aim to establish and characterize a human immune system mouse model to study TIL responses to tumors ***in vivo***. To this end, we will use the newly developed "MISTRG" mouse model, which allows superior development of a human immune system, that includes both lymphocytes as well as functional myeloid and NK cells at close to physiological levels. Importantly, these mice allow establishment of human tumors that are histologically similar to human tumors in terms of vascularization and infiltration of myeloid cells. This model therefore more reliably mimics human tumors than traditional models using NSG mice. We will use this new model to engraft patient-derived non-small cell lung carcinoma (NSCLC) tumor cells and subsequently test ***ex-vivo*** expanded TILs in an autologous setup. To do so, tumor cells will be isolated directly from patient biopsies and expanded ***ex-vivo*** as organoid culture, cell line culture, or ***in-vivo*** in a xenograft model. We will use this model to study parameters that determine the outcome of TIL therapy and to develop and test different treatment modalities.

Keywords: Tumor infiltrating lymphocytes, solid tumor, immunotherapy

A082 / The costimulatory domain in CAR T cells determines the resistance to immunosuppression by regulatory T cells

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Chimeric antigen receptor (CAR)-modified T cells are intensively studied for their application in cancer patients and already proved incredible success in clinical trials. However, the choice of the intracellular signaling domain integrated into the CAR architecture can largely influence T cell function and fate, as already shown *in vitro* and *in vivo*. Moreover, especially within solid tumors regulatory T cells (Tregs) play an important role in establishing an anti-inflammatory milieu and suppressing effector cells. Consequently, endogenous Tregs might impair CAR-engrafted T cells and thereby affect treatment outcome of cancer patients. Therefore, it is of large interest to investigate the responsiveness of T cells comprising CARs with different intracellular signaling domains on Treg suppression.

To address this question, we isolated $CD4^+CD25^-$ conventional T cells (Tconv) and genetically modified them to express a universal CAR (UniCAR) construct as part of our previously developed UniCAR platform technology. In contrast to conventional CARs, UniCARs are indirectly linked to their target cells via a separate antigen-specificity providing target module (TM), which allows a flexible application of UniCAR-engrafted T cells against a wide range of tumor-associated antigens. It also enables a modulation of T cell activity between an "on" and "off" status. To compare the influence of different intracellular costimulatory signals, we designed UniCARs containing either a CD3ζ, CD28-CD3ζ or CD137-CD3ζ domain.

By using a lentiviral gene transfer system for genetic modification, transduction rates of more than 80 % were achieved. Upon TM-mediated activation via the UniCAR, Tconvs containing UniCAR28/ζ produced significantly higher amounts of the pro-inflammatory cytokine TNF and the growth-related cytokine IL2 than UniCAR137/ζ- or UniCARζ-engrafted cells. To investigate the impact of Tregs, Tconvs containing the individual UniCAR constructs were cultured in the presence of expanded, autologous $CD4^+CD25^+CD127^{low}CD45RA^+$ Tregs for 96h. On the one hand, Tregs were pre-stimulated with anti-CD3/CD28 beads to mimic polyclonal activation via the endogenous TCR. On the other hand, an antigen-specific stimulation was achieved by engrafting Tregs with UniCARs. In both cases, UniCAR-armed Tconvs showed a distinct responsiveness on Treg suppression in dependence on the intracellular signaling domain. We observed, that in contrast to UniCAR28/ζ-armed Tconvs, UniCAR137/ζ- and UniCARζ-engrafted cells could be substantially repressed by Tregs. In summary, we could demonstrate that Tconvs containing UniCARs with different intracellular signaling domains display not only a distinct cytokine secretion profile but also a disparate resistance against Treg suppression. These data indicate, that the chosen costimulatory signal has an impact on both the efficacy and the safety of a cancer treatment conducted with genetically modified CAR T cells.

Keywords: tumor immunotherapy, CAR design, Treg suppression

A083 / A phase I/II clinical trial evaluating feasibility and safety of MAGE-A1-specific T cell receptor gene therapy

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Previously, we reported the isolation of an optimal-affinity TCR, T1367, against the cancer/testis antigen MAGE-A1 (Obenaus, et al. 2015). T cells, transduced with T1367, showed high activity against MAGE-A1-expressing cells with no notable off-target toxicity.

We established a GMP-compliant manufacturing process of TCR-modified T cells at the Experimental and Clinical Research Center of MDC/Charité. This manufacturing process enables us to transduce T cells with GaLV-pseudotyped gamma-retroviral vectors, reaching transduction rates of up to 60% with a mean vector copy integration number per cell of less than 5. We could expand the T cells to numbers of up to $1x10^{10}$ $CD8^+$ cells with the majority of the cells exhibiting a stem cell memory phenotype. In June 2017 we have filed an application for the manufacturing license.

The GMP-compliant T cells were comparable to T cells generated for preclinical studies regarding their efficacy and toxicity profile

in vitro. Specifically, the T cells had a similar high affinity for the MAGE-A1 epitope while not being cross-reactive to other HLA alleles, or primary cells from several tissues.
A single-arm, open-label Phase I/IIa clinical trial in cooperation with the hematology department of the Charité was initiated, in order to evaluate the feasibility, tolerability and safety of adoptive T cell therapy with T1367-modified T cells in patients with relapsed/refractory multiple myeloma. As a secondary objective, anti-myeloma activity will be measured. This study will include five cohorts: four cohorts will receive different dose regimen of T1367 T cells (10^5 - 10^8 cells/kg) after lymphodepleting chemotherapy with cyclophosphamide and fludarabine. In the 5th cohort, T1367 T cells will be given after high-dose melphalan treatment and standard autologous stem cell transplantation. The clinical trial application has been finalized and the study is expected to start recruiting in December 2017.

Keywords: TCR, MAGE, multiple myeloma

A084 / Soluble common γc receptor attenuates survival and anti-tumor responses of cytotoxic CD8⁺ T cells via dampening IL-2 and IL-15 signaling

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Previous studies have shown that soluble common γ-chain (sγc) modulates CD4+ T cell immunity with antagonistic functions in γc cytokine signaling. However, role of sγc in functional properties of effector $CD8^+$ T cells is poorly defined. In this study, we report a new mechanism by which the anti-tumor activity of mouse $CD8^+$ T cells is suppressed in sγc of their own producing. While sγc significantly inhibits cytotoxicity of $CD8^+$ T cells, blocking sγc production by genetic modification leads to potentiated effector function and enhanced proliferation of $CD8^+$ T cells as well as establishes persistent $CD8^+$ T cells. This is due to the modulation of IL-2 and IL-15 signaling, which is required for expansion and survival of $CD8^+$ T cells as well as optimal cytotoxic activity. Adoptive transfer of sγc-deficient $CD8^+$ T cells more efficiently controlled tumor growth and metastasis than those of wild-type $CD8^+$ T cells. Block of IL-2 and IL-15 signaling by sγc attenuates the capacity of $CD8^+$ T cells to mount an optimal response to tumor, with both quantitative and qualitative effects on antigen-specific $CD8^+$ T cells. These results could have critical implication for generation and expansion of optimal effector T cells for adoptive immunotherapy of cancer or infections.

Keywords: Soluble common γ-chain (sγc), Cytotoxic CD8+ T cell, IL-2 and IL-15 signaling

A085 / T cell maturation stage prior to and during GMP processing informs on CAR T cell expansion in patients

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Autologous T cells were genetically modified to express a Chimeric Antigen Receptor (CAR) directed towards carboxy-anhydrase-IX (CAIX) and used to treat patients with CAIX-positive metastatic renal cell carcinoma. In the present study, we questioned whether the T cell maturation stage in the pre-infusion product affected CAIX CAR expression and function ***in vitro*** as well as ***in vivo*** CAR T cell numbers and expansion. During the 14 days expansion of CAR T cells prior to administration, we observed shifts from a predominant CD4 to a CD8 T cell phenotype, and from a significant fraction of naïve to central effector T cells. Surface expression of the CAR was equally distributed among different T cell subsets and T cell maturation stages. During T cell culture days 14 to 18 (which covered patient treatment days 1 to 5), T cells demonstrated a decline in CAR expression level per cell irrespective of T cell maturation stage, although the proportion of CAR positive T cells and CAR-mediated T cell effector functions remained similar for both CD4 and CD8 T cell populations. Notably, patients with a higher fraction of naïve CD8 T cells at baseline (prior to genetic modification) or central effector CD8 T cells at 2 weeks of CAR T cell culture demonstrated a higher fold expansion and absolute numbers of circulating CAR T cells at 1 month after start of therapy. We conclude that the T cell maturation stage prior to and during CAR T cell expansion culture is related to ***in vivo*** CAR T cell expansion.

Keywords: Renal cell cancer, Chimeric Antigen Receptor, Immune monitoring

A086 / CAR against Ig kappa light chain as an alternative to CD19 CAR against B-cell malignancies

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Chimeric antigen receptor (CAR) based immunotherapy is coming under the spotlight in the cancer treatment. This is mainly due to the success of CAR T cells targeting B-lymphocyte antigen CD19, which has led to astonishing results in clinical trials. Considering that all B cells express CD19 antigen, CAR-T cells eliminate all B

cells, including non-malignant B cells. Therefore, the patients suffer from impaired humoral immune response, specifically B-cell aplasia and hypogammaglobulinemia, which might increase susceptibility to severe infections. Another problem is related to the target itself. Accumulation of data demonstrates the possibility of immune escape by down regulation of CD19 or alternative splicing variant which becomes resistant to standard CD19 CAR. There is, therefore, a need for alternative targets. Taking into account that most B-cell lymphomas and chronic lymphocytic leukemia cells have a clonally restricted expression of Immunoglobulin (Ig) light chains, either Ig-kappa or Ig-lambda, Ig-kappa+ tumor cells can be targeted while sparing normal Ig-lambda+ B-cells. Hence, Ig-kappa CAR T cells could provide lower on-target toxicity than CD19 CAR T cells and would be expected to improve the life quality of the patients. To this end, we isolated the sequence encoding the antigen-binding parts of an anti-Ig kappa antibody and designed a second generation CAR construct (IGK CAR). Initial studies using single chain Fv fused to human IgG confirmed the specific binding to Ig-kappa+ target cells. We then performed RNA electroporation of IGK CAR into expanded peripheral blood T cells. Our ***in vitro*** data indicated that Ig-kappa targeting was efficient regarding specificity, cytokine response and killing capacity against kappa expressing B-cell lines such as BL-41 and REC-1, whereas no response was observed against lambda positive B-cell lines such as Granta-519. We compared IGK CAR with the clinical CD19 CAR (fmc63) and observed similar potency in target killing. Targeting Ig-kappa raised the imported question of whether the IGK CAR killing efficiency would be affected by free immunoglobulins present in human serum or not. Previous reports showed a decreased killing efficiency in the presence of human serum. Our observations confirmed that killing efficiency of IGK CAR decreased in the presence of human serum or serum purified IgGs whereas the killing efficacy of CD19 CAR was not affected. Additionally, our data indicated that the sensitivity is reversely correlated with the target's Ig-kappa expression levels. Currently, we are aiming to eliminate the inhibitory effects of serum by developing novel IGK CAR constructs and test efficacy of these constructs ***in vivo***. Taken together, our ***in vitro*** data demonstrate that targeting Ig-kappa in clonally restricted B-cell malignancies might represent an efficient or complementary alternative to CD19 CAR.

Keywords: Chimeric antigen receptors, B cell malignancies, Kappa light chain

A087 / Development of a novel chimeric antigenic receptor (CAR) against glycoprotein B (gB) of human cytomegalovirus (HCMV)

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Human cytomegalovirus (HCMV, ***Herpesviridae***) possesses a great threat to infected patients undergoing hematopoietic stem cell transplantation. Severe immunosuppression in these patients can lead to viral reactivation and potential life-threatening disseminated infections. Seropositive patients receiving graft from seronegative donors have the highest risk of HCMV reactivation as they receive a graft that does not contain HCMV-specific T cells. Therefore, they cannot suppress the replication of the virus and control the infection. Adoptive cell therapy using T cells with chimeric antigenic receptors (CAR) specific to HCMV molecules could represent a new approach for the treatment of these patients.

We have developed a novel CAR based on a broadly neutralizing antibody recognizing the conserved AD2 epitope of the HCMV envelope glycoprotein B (gB). gB is located on the surface of infected cells after viral fusion with the cell membrane and during the virion assembly, making it a possible target for CAR.

The CAR against gB (gBCAR) was constructed and its expression was verified by western blotting. Lentiviral transduction system or PiggyBac transposon system were used to introduce the construct into T cells derived from PBMCs of seronegative healthy donors. Flow cytometry analysis showed expression of gBCAR on T cell surfaces (gBCAR T cells). For functional testing of the gBCAR T cells, human lung embryonic fibroblasts (LEP) infected with Ad169 strain of HCMV (HCMV-LEP) were used as target cells. Expression of gB on the surface of these cells was verified by flow cytometry 96 h.p.i. Incubation of HCMV-LEP with gBCAR T cells was carried out for 24 hours (72 h.p.i. to 96 h.p.i.). Supernatants from the incubations were analyzed by ELISA for the presence of interferon γ (IFNγ). A significant increase of IFNγ concentration was seen in samples from incubation of gBCAR T cells with HCMV-LEP when compared to the incubation of non-transfected T cells with HCMV-LEP or gBCAR T cells with uninfected LEP. Flow cytometry analysis of gBCAR T cells from the incubation was also performed.

To conclude, we have shown that our CAR against gB is expressed and exposed on the surface of transfected T cells. We have established a target cell system comprised of LEP infected with HCMV that allows us to test functionality of the gBCAR. Our initial results suggest that gBCAR T cells specifically recognize HCMV infected cells and produce IFNγ.

This project is supported by grant 15-34498A of the Ministry of Health, Czech Republic.

Keywords: human cytomegalovirus (HCMV), chimeric antigenic receptor (CAR), glycoprotein B (gB)

A089 / The unrevealed function of p53 isoform Δ133p53 as a potent transcriptional regulator of T-cell replicative senescence

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Adoptive transfer of genetically modified T lymphocytes with tumor antigen-specific T-cell receptor (TCR) or chimeric antigen receptor (CAR) has proven efficacy in cancer immunotherapy. However, in many patients the overall benefit is still limited due to various tumor escape mechanisms. Cell damage and metabolic/hypoxic stress in the tumor microenvironment can lead to T cell senescence, a state characterized by proliferation arrest, phenotypic changes and a dysfunctional anti-tumor response. The tumor suppressor *TP*53 is a master molecule in controlling the cell cycle and also regulates cell damage response and senescence. *TP*53 gene is expressed as several isoforms, however, their roles in T cells in general and in antigen-specific T cell function in particular is not known. To address this, we explored the role of two defined p53 isoforms for their ability to regulate T cell function. Human T cells form healthy donors were retrovirally co-transduced with either p53b or D133p53 isoforms, together with a tumor antigen-specific TCR. Modified T cells were characterized for the expression of key activating/inhibitory molecules, homing markers and their proliferation capacity by flow cytometry. The effector functions (i.e. cytokine secretion and antigen-specific killing capacity) were assessed by Luminex immunoassay and long-term tumor colony-forming assay, respectively. T cells overexpressing the D133p53 isoform revealed reduced levels of the inhibitory receptor Programmed cell death 1 (PD-1), T-cell immunoreceptor with Ig and ITIM domain (TIGIT) and CD160, as well as upregulation of the chemokine receptor CXCR3 while the p53b-modified cells exhibited an increased expression of PD-1 and downregulation of CXCR3. Furthermore, after several weeks in culture, control T cells reached cellular senescence, characterized by poor proliferation, while, D133p53-expressing cells remained proliferative, showed superior cytokine secretion levels and enhanced tumor-specific killing capacity. In a mouse osteosarcoma xenograft model, mice receiving D133p53-modified T cells showed a prolonged overall survival compared to control animals. Overall, these results revealed a critical role of Δ133p53 as a new transcriptional enhancer of T-cell effector function and could represent a novel approach to improve current adoptive T cell-based therapies by circumventing tumour-mediated T-cell replicative senescence.

Keywords: t cell, senescence, p53 isoforms

A090 / Cytokine-induced killers redirected with a CD44v6 CAR effectively kill soft tissues sarcomas

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Purpose of the study is to explore the anti-sarcoma activity of cytokine-induced killer (CIK) cells engineered with a chimeric antigen receptor (CAR) against the isoform variant 6 of adhesive receptor CD44 (CD44v6). CD44v6 is a tumor-promoting antigen associated with aggressiveness and worse prognosis in various tumors including sarcomas. CIK cells are ex vivo expanded T lymphocytes endowed with MHC-independent antitumor activity. We aimed at generating bipotential killers, combining the CAR specificity with the intrinsic tumor-killing ability of CIK cells
The experimental design was based on CAR-CIK generated from STS patients by transduction of CIK precursors with a lentiviral vector encoding for anti-CD44v6-CAR containing a CH2CH3 spacer, CD28 signaling and HSV-TK inducible suicide switch (CAR+CIK). The anti-sarcoma activity of CAR+CIK was assessed in vitro against multiple histotypes of patient-derived STS and in vivo within a STS xenograft model.
We found that CD44v6 was expressed in 11/27 (41%) of STS analyzed. Anti-CD44v6 CAR+CIK were successfully generated from 12 STS patients. The mean transduction efficiency was 67%±3, CAR+CIK were mostly effector memory and the phenotype was comparable with unmodified controls.
CAR+CIK efficiently killed STS (including autologous targets, n=4). The killing activity was significantly higher compared with unmodified CIK, especially at low effector/target ratios: 98% vs 82% (E/T=10:1), 88% vs 41% (1:1) and 68% vs 26% (1:4), (n=43; $p< 0.0001$). Specificity of tumor killing was confirmed by blocking experiments with anti-CD44v6 antibody. CAR+CIK produced higher amounts of IL6 and IFN-γ compared to controls. CAR+CIK were highly active when intravenously infused in mice bearing subcutaneous STS xenografts. Significant delay of tumor growth ($p< 0.0001$) and tumor infiltration were observed, without any toxicity.
Ours is the first report of CAR+CIK cells against sarcomas, demonstrating that CAR redirection highly improves CIK's innate tumor killing ability. CIK may be considered a valuable platform for the translation of CAR-based strategies to challenging field of solid tumors. Our findings support the clinical exploration of CAR+CIK against incurable STS and CD44v6 as a new CAR target in this setting.

Keywords: CIK, Chimeric Antigen Receptor, Soft Tissue Sarcoma

A091 / Diverse T cell receptor repertoire with specificities for tumor antigens are identified from a cervical cancer patient after cellular immunotherapy

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Adoptive transfer of antigen-specific T cell receptor (TCR)-engineered T cells, TCR-T therapy, has shown significant efficacy in clinical trials against cancer. A cervical patient with bone metastasis was treated with Multiple-Antigens Stimulating Cellular Therapy (MASCT), a combination of multiple-antigens loaded dendritic cells (DCs) and autologous T cells stimulated by these DCs. After treatment the patient showed partially response and remained stable disease for 18 months. Moreover, boosted specific immune responses were detected in patient's periphery blood by IFNγ-Elispot assay against various tumor antigens, such as CEA, RGS5 and HPV18/58.The clinical benefits of this patient indicated that T cells with diverse tumor specificities expanded ***in vivo*** and played an important role to control tumor, which made them good sources to isolate safe and effective TCRs for TCR-T therapy targeting multiple antigens. Given that, patient's PBMCs were stimulated ***in vitro*** with autologous DCs pulsed with one of selected tumor peptides including CEA, RGS5 and HPV18 E7. Sixteen days later, IFNγ-secreting T cells upon peptide stimulation were enriched by using magnetic beads and analyzed for TCR alpha/beta repertoire via NGS. The procedure was repeated three times with a 3-month interval. The patient's immune response against these 3 tumor antigenic peptides remained strong. The TCR repertoire NGS data reveals that the enriched tumor-specific T cells harbor several hundred unique TCR alpha/beta clonotypes. Some of these tumor-specific TCR clonotypes were detected at two or three time points. In total we have identified repeatedly 35/56, 26/25 and 4/18 unique TCR alpha/beta clonotypes with specificities against CEA, RGS5 and HPV18 E7, respectively. Furthermore, a dominant specific TCR alpha/beta clonotype with a high frequency range (16.9%-56%) has been identified for each selected antigen. In conclusion we have successfully identified diverse specific TCR alpha/beta clonotypes targeting selected tumor antigens from a MASCT-benefited cervical patient, which may be good candidates for developing safer and more effective TCR-T therapy targeting multiple

antigens. Several TCR alpha/beta clonotypes are being further investigated in terms of alpha/beta pairing, peptide specificity as well as tumor recognition for potential clinical application.

Keywords: NGS TCR sequencing, diverse tumor-specific TCRs, multiple tumor antigens

A092 / Targeting VEGFR2 with CAR-T cells for immunotherapy by using a high throughput antibody phage display platform

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Overexpression of VEGF and its receptor VEGFR2 are often associated with angiogenesis, survival, invasion, metastasis, recurrence, and prognosis in human cancers. Targeting VEGFR2 by monoclonal antibodies has been approved as treatments for certain indications in lung, stomach and colorectal cancers. In addition to targeting therapies, the combination of immune checkpoint blockades and chimeric antigen receptor T cells (CAR-T) has started to be tested for therapeutic efficacy in different solid tumors. However, combining VEGFR2 targeting and CAR-T cells for cancer therapy have not been verified. In this study, the anti-VEGFR2 single chain fragment variable (scFv) antibodies were screened by using an automated high throughput phage display system. 72 clones with various CDR sequences were selected and binding affinity of these clones to VEGFR2 were in the range of 10^{-8}M to 10^{-10}M. In addition, 16 clones can specifically inhibit VEGFR2-mediated proliferation of HUVEC cells in vitro. To further test the effect of anti-VEGFR2 antibodies within the CAR-T system, the anti-VEGFR2 scFvs were fused with second-generation chimeric antigen receptors to generate VEGFR2-CAR-T cells. ***In vitro*** cell toxicity assays showed a 50~60% of VEGFR2-overexpressed FS293 cells to be killed by VEGFR2-CAR-T cells. Our data suggest that targeting VEGFR2 with CAR-T cells can be a novel strategy for applications in cancer immunotherapy.

Keywords: VEGFR2, Phage display, CAR-T

A093 / A switchable universal chimeric antigen receptor (UniCAR) T cell platform for treatment of hematopoietic malignancies

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The clinical success of T cells engineered to express CD19-specific chimeric antigen receptors (CAR-T) against B cell malignancies has raised much hope that cell therapy with genetically engineered autologous T cells could provide cure for many late stage cancer patients with unmet medical need. However, serious limitations of conventional monospecific CAR therapies include on-target off-tumor toxicity, therapy-induced antigen-loss and severe cytokine release syndrome (CRS) in response to high tumor burden.
In order to overcome these limitations, we recently introduced a universal CAR (UniCAR) platform technology [1-3], separating antigen recognition and receptor signaling properties into two individual parts. In this system, T cells are engineered to express a non-reactive universal CAR (UniCAR-T). Temporal controlled activation of UniCAR-T is provided by individual antigen-specific targeting modules (TMs). Major advantages of this technology include an increased patient safety due to its intrinsic "switch on/switch off" mechanism while maintaining high anti-tumor efficacy of CAR-T. Moreover, the platform approach offers high flexibility, as TMs can be combined or exchanged for each other preventing the development of tumor escape variants. Here we report on the successful ***in vitro*** and ***in vivo*** retargeting of primary human T cells engineered to express UniCARs against acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) using CD123- and CD19-specific TMs alone or in combination. Anti-tumor reactivity of TM-directed UniCAR-T against leukemic cell lines and patient derived AML or ALL samples was observed even at picomolar concentrations of CD19- or CD123-specific TMs. In these experiments UniCAR-T demonstrated a comparable efficacy like conventional CAR-T. However, temporally restricted activation of UniCAR-T had the advantage to spare healthy cells with low antigen expression levels whereas constitutive activated conventional CAR-T attacked both malignant and healthy cells.
In conclusion, the modular UniCAR technology maintains high anti-tumor specificity and efficacy of CAR-T, while adding precise control of UniCAR-T reactivity combined with target flexibility.

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Keywords: Immunotherapy, Leukemia, Switchable CAR T cells

A094 / Novel target module for redirecting T lymphocytes against sialyl-Tn expressing cancer cells via the modular UniCAR platform

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The development of antibody-based therapies has been driven by progress in the immune response field associated to the discovery and recognition of cancer-specific antigens. T cells genetically engineered to express chimeric antigen receptors (CARs) represent a promising approach in cancer immunotherapy. Nevertheless, drawbacks associated with CAR T cells therapies include on-target off-tumor effects or severe cytokine release syndrome. Recently, a universal CAR (UniCAR) platform was developed as a novel approach to increase clinical safety while maintaining the efficacy of CAR T cell therapy. In the UniCAR system, antigen-binding specificity and signaling features are two distinct entities, in which the antigen specificity is provided by specific targeting modules (TMs) to redirect UniCAR T cells in an individualized time- and target-dependent manner. A set of monospecific and bispecific target modules has been successfully developed targeting different tumour antigens, such as PSCA, PSMA, CD123, CD33 and EGFR. Given this, the aim of this work was to develop and assess the potential therapeutic application of a UniCAR system targeting the cell-surface tumor-associated carbohydrate antigen sialyl-Tn (STn). This target is particularly interesting due to its expression in several types of cancer and absence in normal healthy tissues, being associated with tumor invasiveness and metastasis. In addition, it was shown that STn-expressing cancer cells are prone to cause immune tolerance, supporting the potential use of this tumor specific antigen as a target for immunotherapy. Therefore, a novel anti-STn monoclonal antibody (mAb) - L2A5 - was developed and characterized using a wide range of techniques. Particularly, the high specificity of this antibody towards the STn antigen was demonstrated using glycan microarray technology and tumor specificity was proven by immunohistochemistry (IHC). Using the variable sequences of the L2A5 mAb, a novel TM was created to retarget T lymphocytes against STn-expressing cancer cells via the modular UniCAR system. Binding and specificity of the TM to STn positive cancer cell lines was assessed by flow cytometry and most importantly, efficiency of the TM to retarget UniCAR T cells towards these specific cancer cells was observed using cytotoxicity assays. Overall, the results demonstrate the efficacy of this novel TM to retarget UniCAR T cells against STn-expressing cancer cells along with mediating an effective target-specific and target-dependent tumor cell lysis. These findings evince for the first time, the therapeutic potential of combining this innovative and flexible modular UniCAR platform with antibodies specifically targeting sialylated tumour-associated antigens and further application in the treatment of a wide range of cancer types.

Keywords: Sialyl-Tn (STn) antigen, CAR T cell therapy, Novel Cancer Immunotherapies

A095 / *In vitro* generation of HCMV specific T-lymphocytes using dendritic cells electroporated with *in-vitro* transcribed mRNA

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Introduction: Reactivation of human cytomegalovirus (HCMV) in leukemia patients after hematopoietic stem cell transplantation (HSCT) increases their morbidity and mortality during the first year after HSCT. The risk of the disease is even higher for HCMV-seropositive recipients receiving graft from HCMV-seronegative donors (R+D-). Our aim is to produce functional HCMV specific Tlymphocytes ***in vitro*** and use it for adoptive transfer in these patients. As a source of HCMV antigens we use dendritic cells (DC) electroporated with ***in-vitro*** transcribed mRNA (IVT-mRNA). In this work we focused on choosing the best HCMV antigen or a combination of antigens and costimulatory molecules for stimulation of HCMV seropositive donor T-lymphocytes.
Materials and methods: Dendritic cells were cultivated from monocytes in presence of IL4 and GMCSF. After 5 days of cultivation, DC were matured with monophosphoryl lipid A and IFN-γ, electroporated with IVTmRNA encoding modified HCMV antigens and mixed with lymphocytes of HCMV seropositive donor. T-cells were cultured and the response was subsequently analyzed with ELISPOT IFN-γ.
Summary of the data: We prepared IVT mRNA for HCMV tegument protein pp65 wt or synthetic pp65m with modifications such as nuclear localization signal deletion, insertion of LAMP-

1 or Pan-DR-epitope sequences. We also prepared mRNA for HCMV envelope protein gB and immediate-early protein IE-1, as well as for costimulatory molecules OX40L and 4-1BBL. From immunological assays it is clear that T-cells are better stimulated with DC electroporated with IVT-mRNA Sigpp65mLAMP than with other mRNA. Combination of HCMV antigens and costimulatory molecules IVT-mRNA will be analyzed soon.

Conclusion: Our immunological ***in vitro*** experiments comparing stimulating capacity of DC expressing different HCMV antigens revealed that DC electroporated with HCMV mRNA increased HCMV specific Tcell response.

This work is supported by grants 17-31593A and NV15-34498A of Czech health research council (AZV ČR).

Keywords: human cytomegalovirus, in-vitro transcribed mRNA, dendritic cells

A096 / Functionality Assessment of the MACS GMP T Cell TransAct, a robust and potent polyclonal T cell activation and expansion tool for clinical scale manufacturing of gene-modified T cells

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The clinical success of adoptive T cell transfer therapy is resulting in growing enthusiasm as indicated by the ever-increasing number of clinical trials and the involvement of large pharmaceutical companies. The stimulation reagents commonly used to activate T cells, e.g. for genetic manipulation and expansion, are soluble anti-CD3 ± anti-CD28 antibodies that require accessory cells such as antigen presenting cells (APCs). The resulting T cell expansions are often variable and very dependent on the quality of the "feeders" used. Alternatively, large beads (2-5 µm) coated with agonistic anti-CD3 and anti-CD28 antibodies are used successfully. However, cell manufacturing processes using such reagents entail manipulations that are suboptimal to implement cell therapy for large numbers of patients.

Aiming to streamline the safe and robust clinical manufacturing of gene-engineered T cells, we have developed a cGMP compliant stimulation reagent, the MACS GMP T Cell TransAct, which allows potent polyclonal T cell activation prior to gene-modification (lenti- and retroviral) in absence of feeder cells and which can be easily integrated into a closed manufacturing process.

The MACS GMP T Cell TransAct is a soluble polymeric nanomatrix conjugated to humanized CD3 and CD28 agonist. It can be sterile filtered and is biodegradable. In contrast to the large beads previously used in the field, MACS GMP T Cell TransAct therefore presents several advantages: "debeading" steps are no longer required resulting in simplification of the manufacturing process. Also, as the reagent is soluble, there is no more critical dependence on the bead to cell to surface density ratio which enables more reliable and flexible stimulation conditions of the T cells with a large range of T cell densities.

Here we demonstrate the functionality of the reagent in small scale experiments as well as in an automated setting, using the CliniMACS Prodigy and the TCT application. Comparable results to commonly used polyclonal activation reagents as well as potent stimulation and sufficient expansion in different cultivation media indicated the broad applicability of the MACS GMP T Cell TransAct. In addition potent gene-modification, using lenti- or retroviral gene-modification can be shown, using manual or automated approaches. Interestingly, TransAct also enables efficient non-viral modification without having to be removed prior to electroporation.

Overall the MACS GMP T Cell TransAct is a safe and robust product which enables potent T cell activation in different settings. In combination with the commercially available TCT process, it is capable of dealing with highly diverse cell sources and through the automated manufacturing process for gene-modified T cells yields a consistent drug product suitable for adoptive T cell therapy.

Keywords: polyclonal T cell stimulation, gene-engineered T cells, automated

A097 / Automated manufacturing of gene-engineered T cells under serum free conditions

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Automated manufacturing of gene-modified T cells for adoptive T cell therapy requires robust and reproducible processes that use materials and reagents that must fulfill strict safety requirements and that can be difficult to obtain in large enough quantities. GMP compliant human AB serum is one of such reagents, which is, in most cases, used for potent T cell expansion in culture systems. Therefore, improving methods to generate sufficient numbers of gene-engineered T cells suitable for clinical use, independent of human AB serum, is essential for the commercial scalability of automated cell manufacturing processes.

Here we show that the T Cell Transduction (TCT) process developed on the CliniMACS Prodigy platform, enables the robust manufacturing of gene-modified T cells without the need for serum supplementation when using the TexMACS GMP Medium. Furthermore, implementation of a humanized recombinant activation reagent, MACS GMP T Cell TransAct, allows for process

simplification whereby the "bead removal" step is obsolete. The results obtained were comparable to standard process, using 3% human AB serum supplementation with respect to T cell numbers, viability and phenotype. Interestingly, we observed that lentiviral transduction in absence of serum led to increased efficiency of gene-modification and therefore yielded increased CAR T cell numbers. Additionally, CAR T cells generated using the automated serum free process were shown to be fully functional in vitro as well as in vivo.
Accordingly, these improvements are another step towards simplified fully automated treatment of a large number of patients up to a commercial scale.

Keywords: serum free, automated, gene-engineered T cells

A098 / Self-antigen-specific T cells express a regulatory phenotype and anti-tumor activity

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Inhibition of the regulatory immune system has focused on improvement of overall survival in cancer patients. Regulatory T cells (T_{reg}) contribute to suppression of anti-tumor immunity and exist in both CD4 and CD8 T-cell subsets. We have shown that down-regulation of the transcription factor Helios increases anti-tumor immune responses by disrupting CD4 T_{reg} stability and inducing conversion into effector T cells. Helios is also the canonical transcription factor of the CD8 lineage of regulatory T cells. T_{reg} recognize self-antigens, including tumor-associated antigens, and induce immune tolerance. However, the contribution of antigen-specific T_{reg} in cancer immunity remains unclear. Here we have identified T cell receptors (TCRs) from T_{reg} specific for Qa-1/Fam49b $_{p190-198}$, a highly-conserved peptide, and analyzed their potential role in cancer.
Qa-1/Fam49b $_{p190-198}$-specific T cells were obtained by tetramer-dependent detection within the $CD8^+CD44^+CD122^+Ly49^+$ T_{reg} population from peptide immunized mice. TCR cDNAs were amplified from single $tetramer^+$ T_{reg} via RT-PCR and inserted into retroviral expression vectors. Tetramer binding and antigen-responsiveness were assessed using the hybridoma cell line $58\alpha^-\beta^-$ transduced with each TCR gene. We also generated TCR retrogenic mice to test clonal T cells for their ability to kill EL4 tumors in mice. We obtained 11 different T cell receptor genes and identified a low-affinity TCR and a high-affinity TCR. The high-affinity TCR recognized activated lymphocytes as well as EL4 tumor cells, suggesting endogenous antigen presentation by immune cells and cancer cells. In the TCR retrogenic model, the high-affinity TCR displayed characteristics of Treg as judged by Ly49 and Helios expression. Transfer of high-affinity but not low-affinity retrogenic T cells into EL4 tumor-bearing mice resulted in reduced tumor growth.
Fam49b$_{p190-198}$-specific T cells may have a function to detect and eliminate early stage of cancer since this antigen peptide is expressed by transformed cells due to dysfunctional endoplasmic reticulum aminopeptidase. In addition, our studies open the possibility that anti-tumor immune responses can be enhanced by utilizing CD4 Treg and CD8 Treg as effector cells. These findings may yield potential therapeutic approaches utilizing T_{reg} to treat cancer.

Keywords: regulatory T cells, EL4, T cell receptor

A099 / Generation of tumor-specific NK cells by differentiation of CAR-gene transduced hematopoietic progenitors

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Natural killer (NK) cells hold promise for adoptive cancer immunotherapy. Like T cells, the antitumor activity of NK cells can be enhanced by expression of chimeric antigen receptors (CARs) that facilitate selective recognition and killing of malignant cells. CARs consist of an extracellular single-chain antibody fragment (scFv) for recognition of a cell surface antigen, linked to an intracellular signaling moiety such as CD3ζ or CD3ζ fused to a costimulatory protein domain. The engagement of CARs on NK cells triggers antigen-specific lysis of target cells, hence bypassing the need for the activation of endogenous cytotoxicity receptors.
For adoptive immunotherapy, NK cells are usually isolated from peripheral blood and expanded ***ex vivo*** with cytokines before infusion into patients. Experimentally, NK cells have also been derived from hematopoietic stem cells (HSCs) by ***ex vivo*** differentiation following different protocols. CAR NK cells may be generated from CAR gene transduced HSCs following a similar approach. To explore this strategy, we established a protocol for ***ex vivo*** expansion and subsequent differentiation of $CD34^+$ HSCs into NK cells. Here, mobilized human $CD34^+$ HSCs isolated from peripheral blood of healthy donors were cultured ***ex vivo*** in a specific cytokine mix to allow preferential generation of NK cells. We observed the appearance of $CD56^+$ NK cells starting between days 23-27 of the culture period, and the percentage of these cells in the cell pool increased over time. Additionally, various NK

cell-associated surface receptors were found to be expressed by the ***ex vivo*** generated $CD56^+$ cells, which were functionally active as confirmed in cytotoxicity assays using K562 tumor cells as targets. To restrict CAR expression to NK cells developing during the differentiation process, in parallel we constructed a lentiviral vector encoding an ErbB2 (HER2)-specific CAR under the control of an NK-specific NCR1 promoter. We found lineage-specific activity of the CAR construct in established human NK cell lines, while no CAR expression was detected in cells of B-cell or myeloid origin. In a pilot experiment, $CD34^+$ cells transduced with this vector and cultured ***ex vivo*** differentiated into ErbB2 CAR-expressing NK cells, albeit at low frequency. Importantly, the ***ex vivo*** generated CAR NK cells were functionally active, displaying enhanced cytotoxicity against ErbB2-expressing tumor cells. Ongoing work aims at optimizing the transduction protocol to improve the yield of CAR NK cells, and thereby allow further functional studies.

Keywords: Natural killer cells, chimeric antigen receptor, hematopoietic stem cells

A100 / Fully automated clinical grade isolation of CD141 (BDCA-3)+ dendritic cells for the generation of vaccines that stimulate antigen specific CTL responses

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It is established that CD8-alpha+ dendritic cells (DCs) in the mouse constitute a subpopulation that excels in cross-presentation. This cross-priming capacity of CD8-alpha+ DCs is essential for the initiation of effective cytotoxic T cell (CTL) responses against tumors. An equivalent subset of professional cross-priming DCs (XP-DCs) has recently been defined in humans as lineage negative, MHC class II high, CD141+, XCR-1+, CLEC9A+ DCs.
Here we demonstrate that XP-DCs isolated using a fully automated clinical grade process take up, process and present antigens and are thereby able to stimulate T-cells in an antigen-specific manner.
To this end XP-DCs were isolated from CMV-seropositive donor in a two-step procedure using the CliniMACS Prodigy instrument and the dedicated LP141-Predepletion and LP141-Enrichment software. To analyze antigen specific stimulation enriched XP-DCs were then incubated with CMV-derived PepTivator (peptide pools of pp65 and IE-1) and simultaneously activated by the means of TLR3-agonist poly I:C. Activated and antigen loaded XP-DCs were then cocultured with CellTrace Violet-labeled autologous CD8+ T cells. After 10 days antigen specific T cell proliferation was assessed according to reduction of the CellTrace Violet staining intensity and CMV-Tetramer staining. Additionally, IFNgamma and TNFalpha production in restimulated cells was probed by intracellular staining.
XP-DCs upregulated activation markers, produced a specific set of cytokines including IFN-lambda upon stimulation with poly-I:C and efficiently presented antigens to T-cells. Antigen specific T cells stimulated by activated and antigen-loaded XP-DCs proliferated in an antigen specific manner as shown by increasing cell number and frequency of tetramer positive cells. Antigen specificity of the expanded cells was further confirmed by intracellular cytokine staining upon restimulation with either the CMV-derived antigen or an irrelevant antigen for comparison. Expanded CD8+ T cells produced IFN-gamma and TNF-alpha, but no IL-4 whereas T-cells restimulated with irrelevant antigen showed negligible cytokine production. Altogether we developed the first clinical grade process for the manufacturing of the next generation XP-DC based vaccines that are capable of stimulating antigen specific cytotoxic T cells.

Keywords: CD141 / BDCA-3, Dendritic cells, T cell stimulation

A101 / Engineering antigen-specific natural killer cells via T cell receptor gene transfer: a novel source for adoptive immunotherapy

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Recent decades have witnessed extraordinary improvements in the use of cellular immunotherapy against malignancies. A commonly used approach is the use of cytotoxic T lymphocytes (CTLs) for adoptive transfer. The technology of T cell receptor (TCR) gene therapy has improved this approach by supplying large populations of antigen-specific T cells genetically modified to express TCR specific to epitopes of tumor antigens. However, mispairing of endogenous and transferred TCR subunits constitutes a bottleneck in the development of effective and safe TCR gene therapies.
In this study, we use NK cells for TCR gene therapy. Our results show that lentiviral transfer of the CD3δ, CD3γ, and CD3ε chains along with TCR α/β gene delivery to NK-92 or YTS cells enables the functional expression of a TCR specific to tyrosinase-derived peptide $Tyr_{368-379}$ in complex with HLA-A2. We observed that neither the TCR α/β heterodimer, nor the CD3 subunits have the capacity to transport to the cell surface alone but can only form a stable complex when all components are present and no transfer of CD3ζ chain is necessary since NK cells inherently express this gene.

Introduction of a functional TCR complex to NK cells was shown to successfully equip NK cells with the capacity of carrying out MHC dependent antigen-specific cytotoxicity and triggers secretion of pro-inflammatory cytokines upon target cell contact.
Our strategy does not only have the potential to open up a new chapter in the field of cancer immunotherapy but also provides an alternative solution for the mispairing problem observed in TCR gene therapy.

Keywords: Natural Killer Cells, TCR gene therapy, T cell receptor

A102 / Establishment of an animal toxicity model for the prediction of clinical utility of CAR T cell therapies and technologies

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Chimeric antigen receptor (CAR) T cells have shown unprecedented efficacy in the treatment of hematologic malignancies that are non-responsive to conventional therapies. Yet the translation of this treatment modality to the solid tumor setting remains challenging. Due to the lack of truly tumor-specific antigens, damage of healthy tissue expressing the targeted antigen poses a serious adverse effect. In fact, early attempts in treating solid tumors by CAR T cells resulted in life-threatening or even lethal toxicities as a result of on target/off-tumor effects. With increasing experience in the clinic, it has become apparent that the current standard animal testing approach does not allow for proper assessment of CAR T cell-related side effects. In this context, various research questions are still open in regard to the optimal CAR strategy design, CAR T cell dosing and subset choice as well as the prediction and treatment of those emerging novel toxicities.
Aiming for the development of safer CAR T cell therapies, we established an ***in vivo*** on target/off-tumor CAR T cell toxicity model. The glycolipid SSEA-4 was used as target antigen as its expression has been shown on some normal tissue but a broad array of solid tumors. Due to the correlation of antigen expression between the human and mouse organism a preclinical on target toxicity profiling is possible. By using a human/mouse cross-reactive scFv and varying the spacer region of the receptor, we generated different CAR constructs that showed different ***in vivo*** toxicity kinetics thus highlighting the modulatable T cell effector function based on CAR design. Current steps now focus on ***in vivo*** tracking of CAR T cells in mice, the biodistribution as well as the ***in vivo*** proliferative capacity of T cells bearing CARs with different architecture. This will then allow a thorough comparison of currently described strategies to minimize on target/off tumor toxicities and aid improving the safety profile and clinical outcomes of future CAR T cell therapies.

Keywords: CAR T cell therapy, CAR design, toxicity

A103 / *In vivo* generation of CAR-T cells mediated by a CD8-targeted lentiviral vector

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T cells modified with CD19-specific chimeric antigen receptors (CARs) result in significant clinical benefit for leukemia patients. However, production of CAR-T cells requires extensive and time-consuming procedures of cell isolation, sorting, transduction and ***in vitro*** expansion of T cells. Receptor-targeted lentiviral vectors (LV), which transfer genes selectively into particular types of lymphocytes may enable direct ***in vivo*** CAR gene delivery, thus simplifying the process.
The CD8-targeted LV transferred reporter genes specifically into lymphoid tissues and selectively into $CD8^+$ T cells when administered intravenously into NSG mice engrafted with human lymphocytes. CAR-T cells generated by ***ex vivo*** transduction of huPBMCs using the CD8-LV specifically killed target cells and proliferated efficiently upon antigen exposure. For the ***in vivo*** generation of CAR-T cells NSG mice were i.p. injected with $CD19^+$ Raji-luc cells, followed by human PBMC injection and finally CD8-LV delivering the CD19-CAR. One week after vector injection mice were sacrificed and peritoneal cells as well as spleen and blood cells were analysed by flow cytometry. Interestingly, vector-treated mice contained substantially more $CD8^+$ cells than control animals. Notably, about 20-50 % of the $CD8^+$ cells, isolated from the peritoneum were CAR-positive, while no CAR cells were detected in the $CD8^-$ cell fraction. Moreover, the control mice contained about 0.3 % $CD19^+$ cells floating in the peritoneal cavity, while no $CD19^+$ cells could be detected in vector-treated mice, indicating that the ***in vivo*** generated CAR-T cells were functionally active.
Ongoing activities focus on investigating the anti-tumor potential of ***in vivo*** generated CAR-T cells and transferring the system to $CD34^+$ humanized mice, better resembling the generally non prestimulated immune system in a clinical relevant situation.

Keywords: CAR-T cell, Receptor-targeted lentiviral vector, In vivo gene delivery

A104 / Effectivity of a switchable chimeric antigen receptor platform in a pre-clinical solid tumor model

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T-cells engineered to express chimeric antigen receptors (CARs) can target surface antigens in an MHC-independent manner. CAR T cells have been successfully applied in treating hematological malignancies, whereas targeting solid tumors remains challenging. Major hurdles are off-tumor/on-target effects and the immunosuppressive microenvironment of solid tumors. Strategies that increase the controllability of CAR T cells to manage toxicities associated with effector cell activity are a prerequisite for improving the applicability of this technology. The newly described universal CAR (UniCAR) platform allows a precise control of CAR T cells by splitting the antigen binding domain and the signaling domain of conventional second-generation CARs into two individual components: (i) The effector module is a modified T cell expressing the UniCAR with a binding moiety to an epitope derived from a human nuclear protein. (ii) The soluble targeting module (TM) is composed of a single chain variable fragment against a tumor associated antigen (TAA) fused to the epitope which can be recognized by the UniCAR. The activity of this platform is strictly dependent on the availability of all three components: target cells expressing the TAA, UniCAR T cells and TM. Herein, the UniCAR platform was validated for its capacity to treat solid tumors ***in vivo***. Castration-resistant prostate cancer was chosen as a disease model and prostate stem cell antigen (PSCA), a non-tumor specific antigen, was selected as the target. Next, two ectopic xenotransplantation models of castration-resistant prostate cancer were established in NSG mice. In both the low and high tumor burden model, short-term administration of TM significantly delayed tumor growth and prolonged the survival of tumor-bearing mice. Additionally, responses to the activity of the UniCAR platform were assessed by monitoring phenotypic and functional changes of effector and target cells ***in vitro*** and ***in vivo***. Data obtained from these studies demonstrate that UniCAR T cells could long-term infiltrate the tumor tissue and the proportion of infiltration was significantly increased directly linked to the injection of TM. Furthermore the upregulation of immune-inhibitory checkpoint molecules such as PD-L1 and PD-L2 was observed following UniCAR T cell activation. Analysis of a wide range of cytokines implied an exhaustion state of UniCAR T cells.

In conclusion, the UniCAR platform is able to specifically mediate potent anti-tumor activity against a PSCA-expressing solid tumor ***in vitro*** and ***in vivo***. However, the activity of the UniCAR platform is associated with increased expression of inhibitory pathways which might have limited the effectivity of UniCAR T cells. Targeted immunotherapies (e.g. immune checkpoint blockade) in combination with the UniCAR platform might help to further improve the efficacy of this approach.

Keywords: Chimeric antigen receptor, solid tumors, immune checkpoints

A105 / NKP30-CAR redirected human T lymphocytes induce potent antitumor immunity to leukemia cell lines and patient-derived acute myeloid leukemia in NSG xenograft models

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Chimeric antigen receptor (CAR)-redirected T cells have evolved as an effective adoptive cellular therapy (ACT) for leukemia and solid cancer. In particular, clinical trials using CD19 CAR expressing T lymphocytes to combat $CD19^+$ lymphomas demonstrate compelling results. However, suitable antigens for ACT to acute myeloid leukemia (AML) are still warranted as e.g. CD33 and CD123 CAR^+ T cells recognize not only AML blasts but also normal hematopoietic stem cells. In contrast, B7H6, a member of the B7 family, is frequently expressed on various tumor cells including AML blasts while not detectable on normal tissues, and is recognized by the natural killer (NK) cell activating receptor NKp30. Moreover, NKp30 recognizes human leukocyte antigens (HLA)-B-associated transcript 3, a nuclear factor that is secreted and translocated to the cell surface in stressed and transformed cells. In the current study, we thus explored human NKp30-CAR redirected T cells for inducing effective antileukemic immunity to the leukemia line K562 and primary AML blasts in NSG xenograft mouse models following ACT. PBMCs or MACS® purified human T cells were polyclonally stimulated and reprogrammed with a CAR composed of the extracellular region of the NKp30 receptor fused to the CD3ζ chain signaling domain (kindly provided by Dr. S. Klobuch, Dept. of Medicine 3, University Regensburg, Germany) by retroviral gene transfer. Upon transduction and selective expansion NKp30 expression was determined by flow cytometry. IFN-γ ELISPOT analyses and cytotoxicity assays were performed to assess antileukemic responses in vitro and K562 or primary AML blast engrafted NSG mice were used to evaluate therapeutic efficacy after transfer of NKp30-CAR T cells. Expression of B7H6 on target cells was confirmed by RNA-based RT PCR.

Following transduction and puromycin selection ≥90% of $CD3^+$ T cells expressed the NKp30 CAR. In addition, most T cells displayed an effector-memory phenotype. Upon coculture with the B7H6 expressing targets such as K562 and HL-60 (myelogenous leukemia cell lines), NALM 16 (pre-B-ALL) and patient-derived AML samples NKp30-redirected T cells elicited potent IFN -γ release and exhibited specific cytolytic activity to both leukemia lines and primary AML blasts in vitro while the B7H6 negative myeloma line U266 was not recognized. Moreover, adoptive transfer of of 1 - $5x10^6$ HLA-matched, NKp30-CAR redirected $CD3^+$ T cells into NSG mice showing up to 5% engraftment of patient derived AML blasts and thus resembling a clinically relevant minimal residual disease status at time of ACT resulted in clear leukemia regression.
Thus, these studies demonstrate that human T lymphocytes can be successfully redirected to acute leukemia by NK cell activating receptor based CARs such as the NKp30-CAR. As its ligand B7H6 has not been reported to be expressed on $CD34^+$ HSC, this antigen might be an interesting target for ACT to AML.

Keywords: Adoptive cellular therapy, Acute myeloid leukemia, NK-cell activating receptor NKp30

A106 / Heterogeneity and dynamics of the tumor-infiltrating lymphocyte repertoire in melanoma and pancreatic cancer patients

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The presence of tumor-infiltrating lymphocytes (TIL) is associated with prolonged survival in many cancers, and harnessing of the T-cell response through checkpoint inhibition or infusion of ex vivo expanded TILs can result in tumor regression.
We characterize the TIL repertoire by T-cell receptor (TCR) deep sequencing and functional analysis in patients with melanoma, an immunogenic tumor with high mutational load, and pancreatic ductal adenocarcinoma, a tumor that is difficult to penetrate and has low- intermediate mutational load.
The TIL TCR repertoire is dominated by highly frequent CDR3 sequences that can be up to 10.000-fold enriched in tumor as compared to peripheral blood, suggesting in situ proliferation in response to tumor-derived antigens.
Intra-tumoral and intra-patient heterogeneity is significant and highly individual: TIL repertoires from multiple regions of the same tumor show an overlap between 8.4-100% (n=8 patients). TILs from multiple lesions within the same patient share between 0-70% of TCRs (n=10) and tend to overlap less if biopsies are not acquired concurrently, indicating a continuous turn-over or reshaping of TIL composition. Notably, repertoire-sharing is always most prominent among the largest TIL clones, possibly explained by efficient migration/re-cirulcation of some clones, or their maintenance by ubiquitously expressed (tumor-)antigens.
Importantly, TIL repertoire composition undergoes drastic shifts during in vitro expansion in >50% of patients (n=20), possibly depleting the most relevant clones from cultures used for in vitro assays and patient treatment.
Our findings therefore call for careful sampling and optimized culture conditions for TIL infusion products and illustrate the need to probe T-cell reactivity directly ex vivo. The heterogeneity of the TIL response in cancer patients implies that therapeutic efficacy of TCR gene therapy using tumor-dominant TCRs could be more consistent than TIL therapy.

Keywords: tumor-infiltrating lymphocytes, T-cell receptor deep sequencing, tumor heterogeneity

A107 / Crk adapter proteins coordinate LFA-1 dependent cytoskeletal responses and mechanosensing in T cells

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Allogeneic hematopoietic stem cell transplants (HSCT) are used to treat many malignancies, but the prevalence of graft-versus-host disease (GvHD) limits their overall success. Manipulating T cell trafficking has emerged as an effective countermeasure, yet downstream integrin signaling pathways have yet to be targeted. We previously found that T cells lacking the adapter proteins Crk and CrkL exhibit a robust anti-tumor response while causing little GvHD. Here, we show that T cells from mice lacking both Crk and CrkL exhibit defects in LFA-1 induced actin polymerization and cell spreading. Crk/CrkL deficient T cells fail to form an actin-rich leading edge, and migrate more slowly and less directionally on ICAM-1 than WT controls. In addition, we find that while WT T cells respond to changes in substrate stiffness with altered spreading and actin dependent phosphorylation of the force-sensitive protein CasL, this response is defective in Crk/CrkL deficient T cells. Analysis of LFA-1 dependent signaling pathways reveals that Crk proteins interact with and promote the tyrosine phosphorylation of Cbl family ubiquitin ligases in T cells spreading on immobilized ICAM-1. This phosphorylation event allows Cbl to serve as a scaffold for the PI3K p85 regulatory subunit, thereby promoting PI3K dependent cytoskeletal remodeling. These findings

are consistent with a model in which LFA-1 engagement triggers a cytoskeletal regulatory signaling cascade involving Crk proteins, Cbl, and PI3K, thereby setting into motion actin-driven force production needed for CasL-dependent mechanotransduction. Importantly, these data provide insight into integrin signaling that could be used to manipulate T cell trafficking.

Keywords: T cell, Trafficking, Migration

A108 / Adenoviruses armed with TNFα and IL-2 increase efficacy of adoptive cell therapy in the absence of lymphodepleting preconditioning

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The clinical success of conventional adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TILs) relies on preconditioning with lymphodepleting chemotherapy. This strategy is able to potentiate the anti-tumor efficacy of transferred T cells by, increasing their infiltrative capacity in tumors and modulating the tumor microenvironment to become less immunosuppressive. Unfortunately, systemic lymphodepleting chemotherapy is exceptionally toxic, often resulting in severe adverse events in cancer patients, including mortality. Oncolytic adenoviruses, however, are well tolerated and when engineered to express Tumor Necrosis Factor alpha (TNFα) and Interleukin-2 (IL-2) they can improve the outcome of ACT through local immune modulation of the tumor microenvironment. Thus, this strategy might represent a safer alternative to standard lymphodepleting preconditioning regimens.
Experiments were carried out to determine the safety and efficacy of adenoviruses expressing TNFα and IL-2 with ACT in the absence of lymphodepleting preconditioning. To this end, we used the syngeneic Syrian hamster Hapt1 pancreatic subcutaneous tumor model infused with TILs and, the syngeneic CB57BL/6 mouse B16.OVA melanoma subcutaneous tumor model infused with OVA-specific T cells. Prior to ACT, animals were administered with oncolytic serotype 5/3 chimeric (hamsters) or replication-deficient serotype 5 (mice) TNFα- and IL-2-expressing adenoviruses or were administered with a cyclophosphamide and fludarabine lymphodepleting regimen. While hamsters are semi-permissive for human adenoviruses and allow us to study the oncolytic capability, mice allow detailed characterization of the immune repertoire responsive to the TNFα and IL-2.
In both animal models, the anti-tumor control and efficacy of ACT is similarly improved by lymphodepleting preconditioning or adenovirus regimens. The latter resulted in upregulation of mature antigen-presenting cells and NK cells, while tumors from both treatment regimens featured an increased infiltration of OVA-specific T cells and CD8+ T cells. Of note, and as seen in humans, there were severe degenerative changes in the cardiac muscle and pulmonary blood vessels in animals that were treated with lymphodepleting chemotherapy. No degenerative changes were observed in adenovirus treated groups.
This study demonstrates that adenoviruses expressing TNFα and IL-2 can successfully replace high dose preconditioning chemotherapy frequently used in ACT. The absence of toxic side-effects and a positive modulation in the tumor immune composition suggest safety and efficacy advantages over traditional ACT strategies. Importantly, this preclinical data provides a rationale to be further explored in a Phase I clinical trial.

Keywords: Oncolytic adenoviruses, Adoptive cell therapy, Lymphodepleting preconditioning

A109 / Epigenomic analysis implicates BATF signaling as a critical pathway driving CD81-mediated naive T cell costimulation

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Distinctive T cell costimulatory signals uniquely influence T cell phenotype and function and can critically influence the efficacy of a T cell therapeutic product. We employ epigenomic analysis to derive mechanistic insight into the T cell signaling pathways explicitly driven by CD81-mediated costimulation. T cell adoptive immunotherapy strategies traditionally rely upon ex vivo CD3/CD28 mediated T cell activation to facilitate efficient genetic engineering. CD3/CD28 antibody mediated T cell activation preferentially activates memory T cells as compared to naive T cells. Naive derived T cells have demonstrated superior persistence and anti-tumor activity compared to memory derived cells in adoptive T cell pre-clinical murine models. We previously identified an alternative costimulatory signal, CD81, that when combined with CD3 and CD28 signaling acts as a powerful activator of naive T cells and resultantly skews viral-mediated T cell engineering towards the naive T cell subset. CD81 is a tetraspanin, known to co-localize with CD4 and CD8 on T cells. The mechanism behind CD81's ability to enhance naive T cell activation has yet to be resolved. We employed epigenomic profiling to interrogate the dynamic changes of chromatin accessibility in naive T cells activated with CD3/CD28/CD81 signaling as compared to dual CD3/CD28 signaling. We isolated healthy donor naive T cells and differentially activated cells with CD3, CD28 +/- CD81 specific monoclonal antibodies.

Following 24 hours of activation, we utilized assay for transposase-accessible chromatin using sequencing (ATAC-seq) to investigate differences in chromatin accessibility between the two activation conditions. We identified that the addition of CD81 mediated costimulation induces accessibility of >500 additional chromatin sites that remain inaccessible with CD3/CD28 activation. Analysis of these specific sites revealed that 53.13% of these sites are related to the BATF transctiption factor. BATF signaling has been described to be vital to both Th17 and T follicular helper cell development and has been shown to play a critical role at the interface of naive T cells transitioning to effector T cells. BATF signaling has also been implicated as the pathway driving PD-1 mediated exhaustion. ATAC-seq data demonstrated increased accessibility of TIM3, LAG3, CTLA4 and PD1 in naive T cells activated with additional CD81 costimulation. These markers, although commonly associated with exhaustion, are also established markers of T cell activation. We conclude that activating naive T cells with CD81 additional to CD3 and CD28 impacts T cells at a protein level as well as an epigenomic level. We establish BATF as a critical pathway driving CD81-mediated naive T cell costimulation and suggest CD81 as a novel T cell costimulator that may enhance current adoptive immunotherapy strategies.

Keywords: Adoptive Cell Therapy, T cell signaling, Epigenomics

A110 / CD37-targeted CAR T cells as an alternative to CD19-directed CAR T cells for the treatment of B-cell non-Hodgkin Lymphoma

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CD37 is a tetraspanin that is widely expressed on the surface of mature B cells. Due to its high level expression across all subtypes of B-cell non-Hodgkin lymphoma (B-cell NHL), CD37 is one of many potential antibody targets for B-cell malignancies. Since adoptive immunotherapy using CAR gene-modified T-cells has generated impressive clinical responses in B-cell malignancies, we designed a novel second-generation CAR that redirects T-cell specificity towards CD37. Initial testing showed that CD37-directed human peripheral blood CAR T cells potently killed $CD37^+$ B-cell NHL cell lines such as SU-DHL-4, BL-41, Mino and U-2932. We compared the anti-lymphoma activity of CD37-specific CAR T cells with that of CD19-specific CAR T cells and found no difference except for U-2932 cells which were more susceptible to CD37-specific CAR T cells, in concordance with high CD37 expression and low CD19 expression in these cells. The cell line U-2932 was originally derived from a patient with ABC-type of diffuse large B-cell lymphoma (DLBCL), who suffered from many relapses after multiple chemo- and radiotherapy regimens. Assessment of expression of CD19 and CD37 as well as other B-cell antigens such as CD20, CD22 and CD23 demonstrated a dramatic variation in their expression across the B-cell NHL cell lines. Genetic heterogeneity is common in B-cell NHLs and loss of or reduced expression of CD19 has already been reported in some B-cell NHLs. Moreover, extensive chemo- and radiotherapy regimens could potentially contribute to the loss of or reduced CD19 expression. Therefore, we screened B-cell NHL patient tumors for loss of or reduced expression of particular B-cell antigens, including CD19, CD20 and CD37 and found dramatic variations in the expression of these antigens between patient tumors. This type of screening can help us to identify the patient groups that are likely to benefit from CD19-, CD20- or CD37-targeted therapies. In summary, our findings suggest that CD37-directed CAR T cells can be used as an alternative to CD19-targeted CAR T cells, especially when CD19 expression is lost or reduced in patients' tumor cells.

Keywords: CAR T cells, B-cell non-Hodgkin Lymphoma, CD37

A111 / Comparison of tumor lysate preparations for the expansion of tumor-specific T cells

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The adoptive transfer of tumor-specific T cells, known as adoptive cell transfer (ACT), is a promising novel approach for the treatment of colorectal cancer. This immunotherapy aims to stimulate complete and long-lasting anti-tumor immune responses via the isolation and ***ex vivo*** expansion of tumor-reactive T cells. However, the ability to select and enrich for tumor-specificity in culture is a major challenge limiting the clinical application of ACT for the treatment of solid tumors. Antigen presenting cells (APC) loaded with autologous tumor lysates can serve as a platform for the ***ex vivo*** expansion of tumor-specific T cells. This approach offers the advantage of expanding a broad repertoire of T cells without the need to identify specific tumor antigens. It has been demonstrated that the nature of tumor cell death dictates subsequent antitumor responses and that the immunogenicity of lysate preparations can be enhanced by stressing tumor cells prior to lysis. This study compared the immunogenicity of three different lysate preparations; necrotic freeze-thaw (FT), hypochlorous acid-oxidized (HOCl-Ox) and heat-killed (HK). Tumor lysates were prepared

from MC38 tumor cells, which were either oxidized with 300 µM hypochlorous acid, heat-killed at 50°C or left untreated prior to undergoing freeze-thaw lysis. Bone marrow-derived dendritic cells (BMDC) were cultured in the presence of tumor lysate preparations at various concentrations to assess their effects on viability, maturation and cytokine production. Whole lysates, especially HOCl-Ox preparations, were detrimental to BMDC viability at total protein concentrations greater than 50µg/mL, while the soluble fractions of all lysate preparations were well tolerated at protein concentrations of up to 200µg/mL. All tumor lysates induced modest upregulation of CD80 and CD86 compared to untreated controls, but failed to induce significant CD40 upregulation and IL-12 production, which could be observed in LPS-treated BMDC. When lysate-stimulated BMDC were co-cultured with naïve $CD4^+$ and $CD8^+$ T cells, marked differences in T cell activation were observed, as measured by CFSE proliferation, CD69 expression and IFNγ production after 72 hours. Specifically, BMDC stimulated with HK lysate induced robust $CD8^+$ T cell proliferation, CD69 upregulation and IFNγ secretion, the extent of which was not matched by any other lysate preparation. These findings indicate that damage-associated molecular patterns (DAMPs) produced by tumor cells during heat-killing greatly enhanced the uptake and cross-presentation of tumor antigens. Thus, heat-killed tumor lysate preparations may represent an immunogenic source of tumor antigen that can facilitate the expansion of tumor-specific T cells for use in ACT.

Keywords: Tumor lysate, Adoptive cell transfer, MC38

A112 / Cellular and molecular mechanisms controlling *in vivo* acquisition of cytotoxic phenotype by tumor-reactive $CD4^+$ T cells

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$CD4^+$ T cells, the main orchestrators of the adaptive immune response, appear to not only have helper or regulatory potential but potent cytotoxic activity with capacity to mediate rejection of large established tumors. The latter function of this subset has been underestimated for some time. Despite the fact that $CD4^+$ T cells with lytic activity have been identified in mice and humans in multiple diseases from viral infection to leukemia, it remains unclear whether they represent a stable lineage of $CD4^+$ effector cells and which factors drive their differentiation. We therefore sought to comprehensively characterize both transcriptome and proteome of in vivo generated cytotoxic tumor-infiltrating $CD4^+$ T cells in several mouse tumor models in order to define molecular events required for the development of this T cells subset. The analysis of melanoma-specific $CD4^+$ Trp1 cells transferred to lymhodepleted tumor-bearing mice revealed their highly plastic phenotype as they co-express Th1 and Th2 master regulators, T-bet and GATA-3, respectively and secret Granzyme B along proinflammatory cytokines. We identified polyfunctional effector $CD4^+$ T cells of similar phenotype among OT-II cells infiltrating OVA-expressing tumor in adoptive cell transfer setup and within polyclonal $CD4^+$ T cell compartment infiltrating immunogenic sarcoma. In contrast to classical $CD8^+$ cytotoxic T cells effector $CD4^+$ T cells did not, however, express Eomesodermin in tumor microenvironment. Furthermore, those polyclonal effector $CD4^+$ T cells do not express chemokine receptors associated with Th1 phenotype. These data suggest that $CD4^+$ cytotoxic cells can develop through separate development pathway which may not overlap with the T-bet dependent Th1 differentiation program.

Keywords: cytotoxic CD4 T cells, Granzyme B, Th1 cells

A113 / Immunogenic chemotherapy markedly enhances the efficacy of ROR1 CAR T cells in an autochthonous mouse model of lung adenocarcinoma

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Immunotherapy using chimeric antigen receptor (CAR)-modified T cells has impressive efficacy in hematological malignancies but has been less effective in solid tumors such as lung cancer, which is the leading cause of cancer mortality worldwide. Prior studies of CAR T cells for solid tumors relied primarily on transplantable and xenogeneic models, which do not replicate the complex tumor microenvironment of human disease. To develop a CAR T therapy model for lung cancer, we adapted the $Kras^{LSL\text{-}G12D/+}$ $p53^{fl/fl}$ (KP) mouse model to express the tumor-associated antigen ROR1, which is highly expressed in human lung adenocarcinoma but absent from vital adult tissues. We infected KP mice intratracheally with Cre-expressing lentivirus to induce deletion of p53 and activation of oncogenic $Kras^{G12D}$ in lung epithelia, mimicking the two most common mutations in non-small cell lung cancer. The lentivirus also encoded ROR1, resulting in expression of ROR1 in all tumors. This model replicates initiation and progression of human lung adenocarcinoma, including infiltration by myeloid cells and $CD4^+$ Tregs. Adoptive transfer of ROR1 CAR T cells into mice bearing established lung tumors significantly reduced tumor growth for the first 6 weeks of treatment. ROR1 CAR T cells were present in some tumors at 5-fold higher number relative to control T cells and up-regulated markers of activation and proliferation including PD-1, CD25, CD69, and Ki-67 by 2 weeks after transfer. Whereas all tumor nodules grew steadily in control mice and

had minimal T cell infiltration, a fraction of tumors in ROR1 CAR T cell treated mice had strong T cell infiltration and regressed or showed minimal growth. However, CAR T cells were not able to infiltrate all tumor nodules and survival was not improved. Bioluminescence imaging of luciferase-labeled CAR T cells revealed that the majority of CAR T cells, which were CXCR3+, resided in spleen and lymph nodes rather than tumors, perhaps due to the minimal production of the CXCR3 ligands CXCL9 and CXCL10 by lung tumors. Consistent with the impairment of tumor infiltration by CAR T cells, combination therapy with anti-PD-1 did not enhance antitumor activity despite the expression of PD-1 on CAR T cells and PD-L1 on tumor-associated myeloid cells. We next tested whether combination therapy of anti-PD-L1 with oxaliplatin and cyclophosphamide (Ox/Cy), which induces immunogenic cell death and may locally activate CXCL9/10 expression, could improve CAR T cell recruitment to tumors. Indeed, we found that ROR1 CAR T cells were able to infiltrate and induce dramatic regression of a significantly larger fraction of tumor nodules in KP mice pre-treated with Ox/Cy and anti-PD-L1 compared to non-pre-treated mice, or to mice given Ox/Cy, anti-PD-L1, and control T cells. These data indicate that immunogenic chemotherapy can enhance CAR T cell activity against solid tumors, suggesting this model will be useful to identify combinations for clinical translation.

Keywords: ROR1, CAR, lung adenocarcinoma

A114 / T cell receptor (TCR) fingerprinting through molecular encoding of pMHC multimers

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Adoptive cell transfer (ACT) of TCR-transduced T cells has shown strong tumor rejection capacity of the TCR-transduced T cells, but also an inherent risk of cross reactivity that can induce lethal side effects. Hence, it is crucial to understand the precise recognition element of TCRs to increase ACT efficacy but also, to foresee any potential cross-recognition of endogenous epitopes prior to clinical investigations.

Here, we present a novel tool that allows description of an affinity-based hierarchy of pMHC interaction using large libraries of peptide variants of the originally identified recognition element. We make use of DNA-barcode labeled MHC multimers that allow simultaneous screening for T cell recognition of multiple (> 1000) different peptide specificities in a single sample. Importantly, the relative contribution of different pMHC in the TCR interaction can be assessed using this technology - a feature not possible by conventional flow-based MHC multimer analyses.

We have previously characterized $CD8^+$ T cell responses of Merkel cell Carcinoma patients (MCC), directed towards the Merkel Cell Polyomavirus-encoded Large T Antigen (LTA). From these MCC responsive T cells we identified and sequenced two TCRs recognizing LTA-derived epitopes in the context of HLA-B*0702 and HLA-A*2402, respectively.

To characterize the recognition profiles of these TCRs we generated libraries of 191 peptides including 19 different amino acid substitutions for each position of the original LTA-derived sequence, and additional N- and C-term frame shift and length variants based on the full TLA sequence. DNA-barcode labelled MHC multimers were generated, mixed and subjected to interaction with TCR-transduced T cells. Based on the interaction hierarchy of these pMHC multimers we build a recognition profile of each TCR, showing that position 3,5,6 and 7,8,10 were essential for TCR recognition of the HLA-B*0702 and HLA-A*2402 restricted TCR, respectively. The TCR recognition profile was independent of peptide-HLA binding affinity.

Additional, we screened 12 $CD8^+$ T cell clones from MCC patients restricted to a prominent epitope of Merkel cell polyomavirus (MCPγV). In the context of HLA-A*0201, these T cell clones recognized the same peptide sequence but showed high diversity in their TCR sequences, functional avidity and their capacity to infiltrate tumor tissue. Using a library of 172 DNA-barcoded multimers of amino acid substituted peptides, we found significant diversity among the recognition profiles of the clones, correlating to their TCR sequences and functional avidity.

In summary, these data provide proof-of-concept for the use of a novel technology to identify TCR recognition profiles, and may prove highly valuable for the assessment of TCRs prior to clinical investigations.

Keywords: TCR Fingerprint, DNA-barcode based multiplex technology, Adoptive cell transfer

A115 / Enrichment and detection of T cells reactive to multiple tumor-associated antigens in prostate cancer patients based on peptide-mediated lymphocyte stimulation and detection via activation-induced externalization of CD107a

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Enrichment and detection of T cells reactive to multiple tumor-associated antigens (TAAs) with a sufficient functional avidity is the key step to development of efficient adoptive T cell transfer-based (ACT) immunotherapy of multiple cancers. Although many TAAs have been identified for many types of cancer the actual repertoire and functional avidity of T cells reactive to these TAAs in individual patients is however highly diverse and therefore difficult to determine. In this study we show a strategy for enrichment and detection of TAAs-reactive T cells in individual prostate cancer (PCa) patient´s lymphocytes using a pool of multiple TAAs-derived peptides in combination with activation-induced externalization of a T cell cytotoxicity marker, CD107a. We initially observed that untreated lymphocytes of biochemically relapsed (BR, n=14) or hormone refractory (HR, n=12) PCa patients had no detectable T cell populations that externalized CD107a in response to stimulation with a pool of peptides derived from six known PCa TAAs - PSA, PAP, NY-ESO-1, MAGE-A1, MAGE-A3 and MAGE-A4. However, when such stimulated lymphocytes were cultured for two weeks in the presence of IL-2 and then re-stimulated, half of BR and HR patients were enriched with the TAAs-reactive $CD8^+$ T cells as identified by the activation-induced externalization of CD107a. No such reactive $CD8^+$ T cell populations were detected when the patient´s lymphocytes were not stimulated with the TAAs-derived peptides prior to the culturing. When the responding lymphocytes were stimulated with individual TAAs-derived peptides a unique repertoire of $CD8^+$ T-cell reactivity to these peptides was found. Further analysis also revealed a broad functional avidity of the enriched TAAs-reacting $CD8^+$ T cells. Collectively, our enrichment and detection strategy allows a fast evaluation of the repertoire and functional avidity of reactive T cells to multiple TAAs for individual PCa patients. This may help better personalize T cell production for ACT PCa immunotherapy and monitor its efficacy.

Keywords: T cells, tumor-associated antigens, CD107a

A116 / NK cell anti-tumor efficacy in multiple myeloma patients before and after autologous stem cell transplantation

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Natural Killer (NK) cells are innate lymphocytes with a strong anti-tumor ability. In tumor patients, such as multiple myeloma (MM) patients, an elevated number of NK cells after stem cell transplantation (SCT) seems to correlate with a higher overall survival (OS) rate. We recently showed that, at an early time point after auto-SCT (leukocytes >1000/µl), CD56bright CD16-/+ NK cells represented the main lymphocyte subset. These cells, considered to be immature, expressed maturation markers and exerted important cytotoxic functions, when challenged with K562 cells, indicating a potential advantage for patients' health. With the aim of the NK cell use for adoptive cell therapy, we also addressed the cytotoxicity of patient-derived, cytokine-stimulated NK cells against MM cells at specific time points (TPs): at diagnosis, before and after auto-SCT. We detected changes in NK cell phenotype as well as cytotoxic function before and after expansion. Remarkably, after cytokine stimulation patients' NK cell did not significantly differ from healthy donors' NK cells and also patients' NK cells showed a highly activated phenotype and were able to significantly enhance the lysis of MM cells. In a smaller cohort of MM patients we were able to isolate autologous tumor cells and we could show that our activation protocol was able to significantly improve even the lysis of autologous tumor cells, suggesting a potential use of NK cells as adoptive therapy for MM patients.

References: Jacobs B, Tognarelli S, Poller K, Bader P, Mackensen A and Ullrich E (2015) NK Cell Subgroups, Phenotype, and Functions After Autologous Stem Cell Transplantation. Front. Immunol. 6:583. doi: 10.3389/fimmu.2015.00583

Keywords: Natural Killer Cells, Multiple Myeloma, Adoptive Cell Therapy

A117 / A two-chain chimeric antigen receptor (CAR) exhibits superior effector functions due to combinatory chain pairing as a prerequisite for tumor antigen recognition

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Chimeric antigen receptor (CAR) based cellular immunotherapy has proven to be a powerful approach to target hematological malignancies in recent years which we aim at to achieving for solid tumor entities.
We are focusing on the development of alternative antigen receptor designs with a better perspective towards physiologic T-cell signaling. Our prototypic CAR evolved from a novel single chain T-cell receptor (TCR) scaffold, where a single chain variable fragment (scFv) specific for a tumor associated antigen, is fused to the murine TCR Cbeta constant domain and is coexpressed with an autonomous murine Calpha to assemble to a fully active scTCR/CD3- or correspondingly, to a scCAR/CD3-complex (Voss, CIMT 2011). We hypothesized that this format may be superior to classical CARs, since it does not short-circuit the T-cell signaling machinery upon antigen encounter by artificially fused CD3zeta. The tight-junction proteins Claudin 6 and 18.2 overexpressed in a multitude of solid cancers served as model tumor antigens. However, the risk of human anti mouse (HAMA) reactivities prompted us to avoid murine C-domains in this CAR design. Hence, we took advantage of the strong interaction between VH and VL domains of an antibody which, as we hypothesized, may supersede the dependency on murine C-domain pairing. We created a bivalent “inter-chain combinatory CAR” by means of a non-functional VH-VH domain order fused to human Calpha and in the same way, a non-functional VL-VL domain order to human Cbeta. Antigen recognition of this receptor strictly depends on combinatory chain pairing, here primarily mediated by the V-domains connected in tandem to fortify antigen affinity. IFNgamma secretion was much higher as for a bivalent “intra-chain non-combinatory CAR” (VH-VL-Cbeta + VH-VL-Calpha).
Most intriguingly, this CAR architecture exhibited superior reactivity towards very low doses of tumor antigen and low effector:target ratios in comparison with a classical 2nd generation CAR. The combinatory chain design could be extended to different invariant receptor frameworks able to couple with the CD3 components and even to classical CAR backbones which allows to consider the integration of costimulatory signals into a combinatory CAR. We speculate that besides combinatory chain pairing mediated by the peculiar V-domain arrangement, the momentum of antigen binding across both chains supports chain pairing and recruitment of endogenous CD3 and ultimately, leads to superior T-cell signaling. The exquisite effector function of this novel CAR became prominent in a set of cytotoxicity-, proliferation-, and IFNgamma ELISA-experiments, but also in a tumor spheroid based cytotoxicity assay. Currently, we are assessing the expression profiles of biomarkers and cytokines compared with a classical CAR in flow cytometry and dotblot ELISA systems. In an early syngeneic CT26 colon cancer in vivo model we observe significant tumor growth delay and long term T-cell persistence.

Keywords: T-cellular immunotherapy, chimeric antigen receptor, physiologic T-cell signaling

A118 / Generation of high-avidity, WT1-reactive CD8⁺ cytotoxic T cell clones with anti-leukemic activity by streptamer technology

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Allogeneic stem cell transplantation (SCT) is an effective treatment modality for patients with acute myeloid leukemia (AML). Efforts to decrease the risk of graft-versus-host disease (GvHD) as a major cause of transplant-related mortality by depleting T cells from the graft resulted in an increased incidence of AML relapse. These observations revealed that donor T cells representing the mediators of GvHD are also capable of inducing therapeutic graft-versus-leukemia (GvL) effects. Donor lymphocyte infusion (DLI) has been established as a potentially benefical treatment option for AML patients relapsing after allogeneic SCT. However, DLI is often associated with significant GvHD.
One strategy to augment graft-versus-leukemia effects without promoting GvHD is the adoptive transfer of high-avidity CD8⁺ T cell clones recognizing AML-associated antigens. In the present studie, we explored the suitability of a novel strategy to generate high-

avidity, Wilms' tumor antigen 1 (WT1)-specific $CD8^+$ T cell clones. $CD8^+$ T cells were stimulated with antigen-presenting cells loaded with the human leukocyte antigen (HLA)-A*02:01-restricted $WT1_{126}$ peptide. Expanded $WT1_{126}$ peptide-specific $CD8^+$ T cells were markedly enriched by streptamer technology. Three $WT1_{126}$-specific T cell clones were equipped with high-avidity T cell receptors and efficiently lysed AML blasts. These results indicate that the streptamer-based enrichment of tumor peptide-specific $CD8^+$ T cells prior cloning is an appropriate strategy to generate high-avidity $CD8^+$ T cell clones with tumor-directed activity.

Keywords: antigen-specific T cell clons, streptamer technology, Wilms' tumor antigen 1 (WT1)

A119 / A PRAME-specific TCR for adoptive T cell immunotherapy of cancer

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Adoptive T cell therapy using transgenic T cell receptors (TCR) has been demonstrated to be a promising immunotherapeutic approach for the treatment of cancer. In contrast to significant progress in process development and clinical product design, the identification of relevant targets as well as the isolation of promising tumor-specific TCRs for the use in adoptive T cell therapy remains challenging. Here, we describe the isolation and characterization of a PRAME-specific, HLA-A*02:01-restricted TCR using an autologous ***in vitro*** priming approach.
Mature dendritic cells were transfected with full-length PRAME ***in vitro***-transcribed ***(ivt)*** RNA and used to activate T cells expressing TCRs specific for PRAME-derived epitopes within the autologous repertoire of healthy HLA-A*02:01-positive blood donors.
MHC multimer technology and T cell activation markers were utilized to select and isolate antigen-specific T cell clones that were subsequently analyzed for their respective TCR sequence using Next Generation Sequencing (NGS). PRAME-specific TCR sequences were reconstructed in a γ-retroviral vector system that enables transduction and stable expression in adequate effector cells. Resulting TCR-modified T cells were thoroughly analyzed to evaluate multiple safety and efficacy parameters using a dedicated set of state-of-the-art ***in vitro*** assays and innovative ***in silico*** tools. The lead TCR T4.8-1-29 was found to be highly specific for a PRAME-derived epitope presented on HLA-A*02:01. TCR-transgenic effector T cells expressing this T4.8-1-29 show high natural avidity for the target epitope without the need for further manipulation, e.g. no need for functional efficacy enhancement using TCR affinity maturation. Only epitope-positive target cells expressing the respective HLA restriction element, i.e. peptide-loaded T2 cells, PRAME-transfected antigen presenting cells (APC) and PRAME-positive tumor cells, induced cytokine secretion as well as specific lysis by T4.8-1-29-expressing effector T cells. Thorough safety testing, on the other hand, revealed a favorable preclinical TCR profile showing no signs of potential on-target/off-tumor or off-target toxicity.
Using an innovative TCR isolation and characterization platform a TCR with natural high avidity for a PRAME-derived epitope was isolated showing potent efficacy and a favorable safety profile that can potentially be further evaluated in clinical trials.

Keywords: adoptive T cell transfer, tumor antigen, T cell receptor

A120 / Using antigen-specific B cells to combine antibody and T cell-based cancer immunotherapy

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Cancer immunotherapy by therapeutic activation of T cells has demonstrated significant clinical potential. The most promising approaches include checkpoint inhibitors and chimeric antigen receptor T cells. Here, we report the development of an alternative strategy for cellular immunotherapy that combines induction of a tumor-directed T cell response and antibody secretion without the need for genetic engineering. CD40 ligand stimulation of murine tumor antigen-specific B cells, isolated by antigen-biotin tetramers, resulted in the development of an antigen-presenting phenotype and the induction of a strong tumor antigen-specific T cell response. Differentiation of antigen-specific B cells into antibody-secreting plasma cells was achieved by stimulation with interleukin-21, interleukin-4, anti-CD40 and the specific antigen. Combined treatment of tumor-bearing mice with antigen-specific CD40-activated B cells and antigen-specific plasma cells induced a potent therapeutic anti-tumor immune response resulting in remission of established tumors. Importantly, human CEA or NY-ESO-1-specific B cells were detected in tumor draining lymph nodes and were able to induce antigen-specific T cell responses in vitro indicating that this approach could be translated into clinical applications. Our results describe a novel technique for the exploitation of B cell effector functions and provide the rationale for their use in combinatorial cancer immunotherapy.

Keywords: Antigen-specific B cell, Antigen presentation, Cancer immunotherapy

A121 / Tumor-antigen specific immune responses in ovarian cancer

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The human immune system can mount responses to eradicate advanced tumors via T cell recognition of tumor-antigens. However, what the effector T cells see on the surface of immune-sensitive solid tumors, such as ovarian cancer, is largely unknown.
We have previously shown that tumor-infiltrating T cells recognize frequently autologous ovarian cancer cells in vitro. Therefore, this project is investigating which molecules are seen by ovarian tumor-infiltrating T cells. Further, with bioinformatics tools and ex vivo screenings, we selected 60 peptides covering 13 proteins of relevance in ovarian cancer. Immune responses to relevant peptides were analyzed by IFN-γ ELIspot. Preliminary results showed responses to peptides derived from MUC16 (CA125) protein.
The knowledge obtained from this study will contribute to a better understanding of the interaction between ovarian tumor cells and lymphocytes in the tumor microenvironment.

Keywords: Ovarian cancer, Tumor-antigens, Tumor-infiltrating lymphocytes

A122 / Targeted NK cells display potent activity against glioblastoma and induce protective antitumor immunity

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Significant progress has been made over the last decade towards realizing the potential of natural killer (NK) cells for cancer immunotherapy. In addition to donor-derived primary NK cells, also continuously expanding cytotoxic cell lines such as NK-92 are being considered for adoptive cancer immunotherapy. High cytotoxicity of NK-92 has previously been shown against malignant cells of hematologic origin in preclinical studies, and general safety of infusion of NK-92 cells has been established in phase I clinical trials. To enhance their therapeutic utility, here we genetically modified NK-92 cells to express a chimeric antigen receptor (CAR), consisting of an ErbB2-specific scFv antibody fragment fused via a linker to a composite CD28-CD3 zeta signaling domain. GMP-compliant protocols for vector production, lentiviral transduction and expansion of a genetically modified NK-92 single cell clone (NK-92/5.28.z) were established. Functional analysis of NK-92/5.28.z cells revealed high and stable CAR expression, selective cytotoxicity against ErbB2-expressing but otherwise NK-resistant tumor cells of different origins in vitro. Ongoing work focuses on the development of these cells for adoptive immunotherapy of ErbB2-positive glioblastoma (GBM). We evaluated the activity of NK-92/5.28.z cells against a panel of GBM cell lines and primary GBM cultures and demonstrated selective in vitro cell killing that was dependent on ErbB2 expression by the target cells. Potent in vivo antitumor activity of NK-92/5.28.z was observed in orthotopic GBM xenograft models in NSG mice, leading to a marked extension of symptom-free survival upon repeated stereotactic injection of CAR NK cells into the tumor area. In immunocompetent mice, local therapy with NK-92/5.28.z cells resulted in cures of transplanted syngeneic GBM in the majority of animals, induction of endogenous antitumor immunity and long-term protection against tumor rechallenge at distant sites. Our results suggest adoptive transfer of ErbB2-specific NK-92/5.28.z cells as a promising new immunotherapy approach for GBM. Preparations for a phase I clinical trial investigating NK-92/5.28.z cells as a treatment for recurrent ErbB2-positive GBM are ongoing.

Keywords: Chimeric antigen receptor, natural killer cells, ErbB2

A123 / Primary human T cells engineered with alpha fetoprotein-specific T cell receptor genes effectively kill hepatocellular carcinoma cells and generate antitumor effect in treating HCC xenografts in NSG mice

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Liver cancer is the 6th most common cancer and 4th leading cause of cancer death worldwide. Hepatocellular carcinoma (HCC) is the major form of liver cancer with no effective therapy. Genetic modification with T cell receptors (TCR) specific for HCC-associated tumor antigens, such as human alpha fetoprotein (hAFP), can potentially redirect the human T cells to recognize and kill HCC tumor cells to achieve antitumor effect. In this study, we report that immunization with recombinant lentivector and hAFP peptide is able to activate a population of CD8 T cells in HLA-A2 transgenic mice that recognize the HLA-A2 presented $hAFP_{158}$ epitope on human HCC tumor cells. Adoptive transfer of the $hAFP_{158}$-specific CD8 T cells derived from immunized HLA-A2 mice could eradicate large human HepG2 tumor xenografts in the immunocompromised

NSG mice. T cell hybridomas were then established from the mouse splenic CD8 T cells with strong antitumor effect. Next, 3 sets of paired TCR α and β chain genes were identified from 5 T cell hybridomas. Expression of the murine TCR genes enabled primary human CD8 and CD4 T cells to bind HLA-A2/$hAFP_{158}$ Tetramer. In addition, the TCR gene modified human CD8 and CD4 T cells could specifically recognize HLA-A2+ hAFP+ HepG2 tumor cells and be stimulated to produce effector cytokines of IFNg and IL-2. The TCR gene modified human CD8 T cells could also effectively and specifically kill HLA-A2+ hAFP+ HepG2 tumor cells. Adoptive transfer of the primary human T cells engineered with the TCR genes generated significant antitumor effect to treat HCC tumor xengografts in NSG mice. Our data suggest that these novel $hAFP_{158}$-specific TCR genes have a great potential to modify patient's autologous T cells and redirect them to recognize and kill HCC tumor cells and generate therapeutic antitumor effects in HCC patients.

Keywords: Hepatocellular Carcinoma, T cell receptor engineering, Adoptive cell immunotherapy

CHECKPOINT BLOCKADE THERAPY

A124 / Exhausted T cells partially recover some memory properties upon antigen elimination during chronic infection

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T-cell exhaustion is a hallmark of immunological failure to control cancer and chronic infection. Blocking inhibitory receptors such as PD-1 can re-invigorate exhausted T cells (T_{EX}), but clinically many patients fail to achieve durable tumor control. In addition, re-invigoration of T_{EX} by checkpoint blockade only restores effector functions temporarily, and fails to establish durable immunological memory. A key "signal" needed for immunological memory is rest from antigenic stimulation, a signal often lacking during chronic infections and cancer. ***Thus, a deeper understanding of other mechanisms underlying reversal of T-cell exhaustion is needed, and we need a better understanding of how to convert T_{EX} into durable functional cells.*** To address these gaps in knowledge, we investigated whether T_{EX} become "reprogrammed" into more functional effector or memory-like T cells following non-immunological antigen elimination during chronic infection. Using the LCMV model we adoptively transferred T_{EX} from mice chronically-infected with the clone 13 strain to antigen-free mice. As a control, memory T cells (T_{MEM}) from mice that cleared an acute infection by LCMV Armstrong strain were also examined. Thus, we sought to determine; the cellular profiles, recall capacity, and population dynamics of recovering T cells (T_{REC}). ***Our data support the notion of partial recovery of some memory properties upon cure of infection, though other aspects of T_{EX} biology do not seem corrected simply by curing chronic infection***. Specifically, the level of expression of PD-1 diminished upon antigen elimination, and the percentage of CD127+ virus-specific CD8 T cells increased, although not reaching the levels of T_{MEM}. Additionally, the functionality of recovering T_{EX} was improved, but also remained significantly lower than T_{MEM}. We then tested the recall capacity upon challenge infection. Although T_{EX} retained some capacity to expand upon re-exposure to the antigen and showed improved simultaneous production of the effector cytokines interferon-γ and TNFα, T_{EX} were substantially less effective in recall capacity compared to functional T_{MEM}. Collectively, these results suggest that upon curing chronic viral infection T_{EX} acquire some effector and memory properties including recovery of an effector/memory-like phenotype, improved functionality, and improved recall capacity.

Nevertheless, these properties remain defective in T_{REC} compared to optimal T_{MEM} generated following cure of acute infection. Our ongoing studies are investigating whether these changes are linked to selective recovery of a specific subset of T_{EX}, and whether improvements are accompanied by changes in the molecular pathways of these recovering T cells. We expect these studies to enhance our understanding of the molecular mechanisms of T_{EX} recovery, and to identify candidate transcriptional circuits differentially regulated in T_{REC} that could represent novel therapeutic targets to reverse immune exhaustion.

Keywords: Exhausted T cells, Immunological recovery, Novel immunotherapeutic targets

A125 / Analysis of the tumour microenvironment to inform the design of immune modulatory antibodies

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Modulation of inhibitory and stimulatory receptors on tumour-reactive lymphocytes has emerged as a promising approach in cancer immunotherapy. Recent pre-clinical evidence has shown that some monoclonal antibodies (mAbs) targeting these molecules (i.e., anti-CTLA-4, -GITR and -OX40) act not only by blocking or stimulating their target on effector T cells but their activity also relies on their ability to deplete tumour-infiltrating regulatory T cells (Treg) cells through Fc-gamma receptor (FcγR)-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). We studied the expression landscape of immune modulatory receptors on tumour-infiltrating T cells (TILs) and of FcγRs on tumour-infiltrating myeloid cell subsets in murine and human tumors in order to inform the design of antibodies targeting CD25 and CTLA-4. We demonstrated that these targets are largely restricted to Treg cells relative to Teff cells both in murine and human tumors. In the case of anti-CD25, we showed that in mice depletion of tumour-infiltrating Treg cells required selection of an antibody isotype with optimal activatory to inhibitory FcγR-binding ratio, which led to improved control of established tumors and a potent synergistic effect in combination with programmed death (PD)-1 blockade. Similarly, only anti-CTLA-4 with depleting activity are therapeutically active in mice. To understand the translation of this into the human setting, we employed mice expressing human FcγRs and chimeric anti-murine CTLA-4 mAbs with human IgG variants displaying different binding affinities to human FcγRs. In this context, we found that the therapeutic efficacy of anti-CTLA-4 mAbs depends on a double mechanism of action involving both blocking of the target and FcγR-mediated Treg cell depletion in the tumour.

Keywords: immune regulatory antibodies, regulatory T cells, tumor microenvironment

A126 / Enabling Immune checkpoint blockade with oncolytic viruses armed with TNFa and IL-2

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Immune checkpoint blockade became a revolutionary tool to unleash patient's immune system against their malignancies. Even if the approach is highly promising, only a subset of patients take benefit from it leaving room for improvement. One of the biggest difficulties to make the therapy work appears when the immune system is not able to detect or penetrate the tumor. We hypothesize that oncolytic viruses are an attractive way to make immune checkpoint blockade work where in those "cold tumors" and to improve it where it works. Due to their nature, adenoviruses are recognized by different members of the innate defenses which will generate an increased attention from the immune system to the tumor. Furthermore, our viruses are engineered to express tumor necrosis factor alpha (TNFα) and interleukin-2 (IL-2), two cytokines to improve the trafficking to the tumor and enable T-cell mediated responses in the tumor.
An ***in vivo*** model of subcutaneous melanoma (B16.OVA) was used to study the antitumor efficacy of the previously described scenario where. First, the combination was studied for the tumor growth control and survival (n=75). The second one (n=48) focused on studying the combination versus the virus as monotherapy and to collect biological samples to study the immune status of the tumors. Double therapy provide benefit on tumor growth control when compared with immune checkpoint blockade ($P< 0.0001$) or viral treatment ($P< 0.01$) as monotherapies. The same kind of beneficial results pointed the combination group after analyzing overall survival ($P< 0.01$ and $P< 0.05$). When Studying the tumors, the double virus plus checkpoint blockade showed a statistically significant ($P< 0.05$) increase in tumor specific CD8+ cells, in non-Treg CD4+ cells while the Treg population didn't increase. The virus treatment also increases the expression of PD-1 on effector T cells, giving a plausible explanation for the benefits of the combination. More data regarding the tumor microenvironment will be analyzed. In this study we tested viruses specifically designed to potentiate T cell therapies such as checkpoint blockade in a preclinical set-up. The effect of the combination showed a polarization of the immune compartment towards an antitumor phenotype protected against anergy thanks to the use of checkpoint inhibitors. Significant benefits were obtained when genetically modified adenovirus and PD-1 blockade were used as each other's companions, making this combination an appealing approach to move towards clinical trials.

Keywords: Checkpoint blockade, anti-PD-1, Oncolytic adenovirus

A127 / Myeloid heme oxygenase-1 is an innate immune checkpoint in cancer

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Blockade of immune checkpoint receptors PD-1 and CTLA-4 has resulted in unprecedented clinical responses in the treatment of cancer. However, as a significant number of patients are unresponsive or only respond transiently to current therapies, other immune checkpoints might still prevent immune-mediated tumor regression even in the absence of CTLA-4 and PD-1 signaling. Heme oxygenase-1 (HO-1) is an enzyme that is responsible for the breakdown of heme to generate the biologically active products biliverdin, ferrous iron and the immune suppressive molecule carbon monoxide. HO-1 is expressed in a variety of human cancers. We studied HO-1 in a spontaneous murine model of mammary adenocarcinoma (MMTV-PyMT). In this model HO-1 is primarily expressed by tumor associated macrophages. Pharmacological inhibition of HO-1 had no effect on tumor growth as the tumor microenvironment was almost devoid of $CD8^+$ T cells. We therefore stimulated an anti-tumor $CD8^+$ T cell infiltrate using the chemotherapeutic 5-fluorouracil, which on its own did not affect tumor growth. However, in this context, concurrent pharmacological inhibition of HO-1 resulted in tumor regression and sustained immunological control of tumor growth. Pharmacological inhibition of HO-1 allowed an increased number of tumor-infiltrating $CD8^+$ T cells to maintain their effector functions, indicating that HO-1 was an innate immune checkpoint in this model. Genetic inactivation of HO-1 in myeloid cells paralleled that seen with pharmacological targeting of the enzyme and demonstrated that the myeloid cells were the biologically relevant source of HO-1. We tested the relevance of our findings in the clinical setting and observed that in breast cancer patients receiving chemotherapy, high HO-1 expression was associated with poor prognosis. Microarray data from breast cancer patients also revealed that HO-1 could be co-expressed with the clinically targeted checkpoint molecules PD-L1 and PD-L2. As PDLs were also present in MMTV-PyMT tumors, we compared the efficacy of therapeutically targeting PD-1 and HO-1. In this model, targeting HO-1 had a significantly greater effect on tumor growth than PD-1 blockade, highlighting the hierarchical structure of immune checkpoints within the tumor microenvironment. These results suggest that HO-1 should be considered as an immune checkpoint and that its activity could influence the efficacy of current immune checkpoint blockade therapies.

Keywords: Heme oxygenase-1, Macrophage, Breast cancer

A128 / Strengths and limitations of NSG humanized mouse as pre-clinical tool to evaluate immune checkpoint blockade

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The blockade of immune checkpoints with antibodies anti-CTLA-4, anti-PD1 and anti-PD-L1, has given impressive clinical results but the response rate remains low. Therefore, it is essential to gain knowledge on their mechanism of action, identify prognostic and predictive biomarkers of response and toxicity, and prioritize the test of therapeutic approaches based on the combination of Abs with other therapies. However, today there is an unmet need of translatable in vivo mouse tumor models to do such mechanistic and pre-clinical studies. Here, we developed a humanized mouse model in which NOD SCID γc-/- (NSG) mice, which lack T, B and NK cells are grafted with human immune cells and tumors. We tested human tumors from different origins: ATCC tumor cell lines, cell lines that we derived from patient´s invaded tumor draining lymph nodes or patient derived xenografts (PDX); and human immune cells from healthy donors peripheral blood mononuclear cells (PMBCs) or from lymph nodes. To set up the model, we tested several variables, including the number and quality of immune cell injected and the sequence of injection of tumor and immune cells. We have observed that mainly lymphocytes (CD8, CD4, Tregs and NK) reconstitute the mice and readily infiltrate the tumor, modifying the level of checkpoint molecule expression on tumor cells. Major limitations of this model are: i) that the injected T cells induce xeno-Graft versus Host Disease (GvHD), and ii) that the human myeloid compartment does not engraft in NSG mice. To solve these two problems, we evaluated two improved mouse strains. First, to avoid GvHD we tested the NSG β2m-/- mouse, which effectively lead to a significant later onset of GvHD. Second, to improve the homeostasis of the human myeloid cells present in the injected samples (PBMCs or lymph node cells) we used the NSG-SGM3 mice, which has been genetically modified to express human IL-3, GM-SCF and SCF. However, the latter strain was not associated with better myeloid reconstitution. Finally, we succeeded to set up conditions that allowed us to test the therapeutic effect of an anti-PD1 antibody (Nivolumab). We screened the sensitivity to anti-PD1 antibody of a dozen of lung PDXs from Non Small Cell Lung Cancer, and observed 30 % of responses, similar to the response rates observed in humans. In all, we have defined optimized conditions allowing the evaluation to anti-PD1 mAb immunotherapy in humanized mice and defined the pros and cons of the model.

Keywords: Humanized mouse, Immunotherapy, Nivolumab

A129 / CIS is a potent checkpoint in NK cell homeostasis and NK cell-mediated tumor immunity

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The detection of aberrant cells by natural killer (NK) cells is controlled by the integration of signals from activating and inhibitory ligands and from cytokines such as IL-15. We identified cytokine-inducible SH2-containing protein (CIS, encoded by ***Cish***) as a critical negative regulator of IL-15 signaling in NK cells. ***Cish*** was rapidly induced in response to IL-15, and deletion of ***Cish*** rendered NK cells hypersensitive to IL-15, as evidenced by enhanced proliferation, survival, IFN-γ production and cytotoxicity toward tumors, in vitro. This was associated with increased JAK-STAT signaling in NK cells in which ***Cish*** was deleted. ***Cish***$^{-/-}$ mice were resistant to melanoma, prostate and breast cancer metastasis ***in vivo***, and this was intrinsic to NK cell activity, uncovering CIS as a potent intracellular checkpoint in NK cell-mediated tumor immunity, In addition, subtle changes in ***Cish***$^{-/-}$ mice were observed ***in vivo***, including an increase in the mature subset of NK cells as well as increased expression of cell cycle markers in NK cells. This phenomenon was conserved in mixed bone marrow chimeras, as well as a significant increase in the ***Cish***$^{-/-}$ NK cell population (all other lineages remained 1:1) after mice were reconstituted. The changes observed in steady state ***Cish***$^{-/-}$ NK cells also manifested in a lower activation threshold, evidenced by the redundancy of exogenous IL-15 to induce augmented production of inflammatory cytokines and cytotoxicity by ***Cish***$^{-/-}$ NK cells when stimulated ***ex vivo***. These data suggest that ***Cish*** deletion may affect NK cell homeostasis ***in vivo***, reduce the activation threshold for NK cells and is a step towards breaking NK cell tolerance ***in vivo***.

Keywords: CIS, NK cell, checkpoint

A130 / Combined blockade of PD-L1 and NKG2A checkpoints enhances anti-tumor CD8+ T cell response

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Inhibitory CD94-NKG2A receptors (NKG2A) are expressed on subsets of cytotoxic NK cells, gd and CD8$^+$ T cells. The ligand of NKG2A is HLA-E (Qa-1^b in mice), a non-classical major histocompatibility complex (MHC) class I molecule. HLA-E is frequently upregulated on cancer cells of several solid tumors, providing a negative regulatory signal to tumor-infiltrating lymphocytes (TILs). Blockade of this immune checkpoint pathway with anti-NKG2A monoclonal antibodies (mAbs) enhances NK cell responses toward tumor cells in vitro and in humanized mice. We recently reported that anti-NKG2A mAb potentiates PD-1 checkpoint blockers in a syngeneic mouse tumor model with nearly a doubling in the rate of complete responses compared to PD-1 blockade only, and improves survival. In this model, subcutaneous injection of A20 cells into BALB/c mice results in induction of Qa-1^b expression and upregulation of PD-L1 expression on tumor cells. Both NK and CD8$^+$ T cells are involved in tumor rejection in response to anti-NKG2A and anti-PD-1/PD-L1 blocking mAbs. However, the exact mechanisms are not clear yet. Here, we further investigated the effects of in vivo and ex vivo targeting of both pathways, with the emphasis on their effects on anti-tumor CD8$^+$ T cell response. PD-L1 and Qa-1^b are both expressed on subcutaneous A20 tumor cells and on infiltrating immune cells. To explore whether both molecules contribute directly to the protection of the tumor cells, we used CRISPR/cas9 technology to knock out each of them. Deletion of either Qa-1^b or PD-L1 in A20 cells significantly delayed tumor growth in mice supporting a role for both molecules in immune-escape of A20 tumor growth in this model.In A20 tumors, around 50% of NK TILs expressed NKG2A, whereas PD-1 expression was mainly restricted to T cells. Moreover, CD8$^+$ TILs expressing high levels of PD-1 co-expressed high levels of NKG2A, raising the possibility that NKG2A blockade may potentiate PD-1/PD-L1 blockers by directly enhancing CD8$^+$ T cell-mediated killing of A20 tumors. To test this possibility, we developed an ex vivo assay that allowed to monitor activation of NKG2A$^+$ and/or PD-1$^+$ CD8$^+$ TILs, in response to in vitro treatment with anti-NKG2A or anti-PD-1 mAb, or the combination of both. CD8$^+$ T cell activation was measured by induction of CD107, a marker of degranulation. Combined PD-L1 and NKG2A blockade induced significant increases in CD107 mobilization by NKG2A$^+$PD-1$^+$CD8$^+$ TILs in response to A20 cells when compared to anti-NKG2A or anti-PD-L1 mAbs alone.Together, these data indicate that blocking NKG2A in conjunction with PD-L1 checkpoint blockers could enhance anti-tumor efficacy of CD8$^+$ T cells, as well as providing coordinate enhancement of NK cell and CD8$^+$ T cell activities, further supporting the rationale for ongoing clinical trials with this combination.

Keywords: CD94-NKG2A, PD-1/PD-L1, Cancer

A131 / Mechanisms of response and resistance to immune checkpoint blockade in experimental glioma

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Although checkpoint inhibitors are now implemented into the standard therapy of an increasing number of tumor entities, gliomas seem resistant to checkpoint inhibition. Recent evidence from a randomized clinical trial did not show a therapeutic benefit in an unselected population of patients with recurrent glioblastoma. This project investigates the mechanisms of response and resistance to checkpoint blockade targeting CTLA-4 and PD-1 in an experimental syngeneic glioma model to improve efficacy and provide biomarkers for therapy response. We observed increased numbers of tumor infiltrating T cells (TILs) in responder compared to non-responder mice. Moreover, CD8 TILs of responder mice showed less diversity in their TCRβ repertoire, suggesting a clonal expansion of tumor reactive clones. Interestingly, depletion of CD4 T cells resulted in a complete abrogation of the therapeutic effect of immune checkpoint blockade, indicating a key role of CD4 T cells for the therapeutic response. As CD4 T cells interact primarily with myeloid cells in the tumor microenvironment, we further assessed the myeloid compartment in GL261 gliomas in response to checkpoint blockade. Here, responder mice showed lower frequencies of macrophages ($CD45^{high}Cd11b^{+}$), while microglia cells ($CD45^{low}Cd11b^{+}$) were the predominant $CD11b^{+}$ antigen presenting subset among $CD45^{+}$ immune cells. Contrary, non-responder mice showed higher frequencies of tumor associated macrophages which expressed high levels of PD-L1, CD38 and CD73 suggesting the presence of a suppressive myeloid cell compartment. We hypothesize that these macrophages might inhibit T cell proliferation and cytokine production by PD-L1-CD80 interaction and adenosine production. In order to assess the relevance of PD-L1 mediated resistance, PD-L1 was blocked in addition to PD-1+CTLA-4 blockade. Strikingly, addition of PD-L1 blockade was able to increase response rates of PD-1+CTLA-4 blockade. Our study suggests a novel mechanism of resistance to checkpoint blockade targeting PD-1 and CTLA-4 by a suppressive tumor associated macrophage subset in glioma and provides a rational for combinatorial therapy strategies to overcome resistance to checkpoint blockade.

Keywords: glioma, immune checkpoint blockade, tumor-associated macrophages

A132 / The role of tumor-draining lymph nodes in the therapeutic effect of immune-modulating antibodies

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Cancer therapy with Immune-modulating antibodies, such as CTLA-4 and PD-1 blockers, has shown great therapeutic success, and has been approved for treatment of patients over the last decade. Local administration of immune-modulating antibodies, such as against CD40 or CTLA-4, is an attractive strategy to limit treatment-induced side-effects and decrease dosing, without loss of therapeutic efficacy, as we, and others, have shown in previous publications. It also indicates that factors targeted by these antibodies are mainly residing in the tumor-area. The tumor area consists of the tumor, the microenvironment with immune infiltrating cells, and the tumor-draining lymph nodes(LN). Tumor-draining LN have a controversial role in tumorimmunology, since they are often resected for diagnostic purposes.
We now focus on the role of tumor-draining LN for the success of this local administration therapy. We surgically resected tumor-draining LN (inguinal and axillary) in mice bearing subcutaneous tumors in the flank, and subsequently treated with local antibody therapy. This way we determined the contribution of the sentinel LN to the treatment efficacy. In mice bearing MC38 colon carcinoma tumors, local administration of a low dose of agonistic anti-CD40 was very effective. In these mice, resection of the tumor-draining LN did not hamper the treatment efficacy, indicating that the cells activated by this antibody are mainly located in the tumor-microenvironment. By depleting T cell subsets we determined that CD8+ T cells are crucial for the treatment effect. In a model with a local and a distant tumor, LN resected mice treated locally with anti-CD40 antibody were still capable of inhibiting the outgrowth of the distant tumor, indicating that a systemic CD8+ T cell response is activated regardless of the presence of lymph nodes. In the same model, we treated mice with PD-1 blocking antibodies. PD-1 blocking antibody was capable of activating an effective tumor-eradicating response, but when tumor-draining LN were absent, the treatment effect was gone. We are currently validating our hypothesis that CD40 antibody activates tumor-infiltrating CD8+ T cells, whereas the therapeutic effect of PD-1 blocking antibody is dependent on influx of cells from LN.

Keywords: Checkpoint therapy, sentinel lymph node, preclinical

A133 / Immune checkpoints are discordantly regulated by TGF-b1 and class I HDACs

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Chronic antigen stimulation, common in both cancer and persistent viral infection, leads to the development of a dysfunctional T cell state called exhaustion. Exhausted T cells are characterized by a loss of effector function and expression of immune checkpoints, such as PD-1 and TIM-3. TGF-β1, a potent immunomodulatory cytokine prevalent in the tumor

microenvironment, has been shown to upregulate the expression of PD-1 but its role in modulation of other immune checkpoints is poorly understood. As such, we hypothesized that TGF-β1 regulates the expression of other immune checkpoint molecules in a similar manner. Using NanoString gene expression profiling of human CD8+ T-cells activated ***in vitro***, we observed two distinct clusters of immune checkpoint molecules that are discordantly regulated by TGF-β1. Surprisingly, while PD-1 expression was distinctly upregulated by TGF-β1, the expression of CTLA-4, LAG-3, TIM-3, and TIGIT were coordinately downregulated. Mechanistically, this discordant regulation was dependent on Smad3, a downstream transcription factor of the TGF-βR complex, in cooperation with class I histone deacetylases (HDAC). Inhibition of either Smad3 or class I HDAC was able to enhance the expression of immune checkpoints that were downregulated by TGF-β1. Treatment with Smad3 inhibitor in combination with entinostat (class I HDAC inhibitor), resulted in maximal increases in TIM-3, CTLA-4, LAG-3, and TIGIT expression. Each cluster had a unique molecular signature that corresponded with independent cytokine signatures. ***In vivo***, mice were infected with Lymphocytic Choriomeningitis Virus (LCMV), and treated with either entinostat or with vehicle control. Treatment with entinostat during the initial priming phase of the infection yielded long-term and high expression of TIM-3, CTLA-4, and LAG-3. Upregulation of these immune checkpoints was also associated with production of significantly more granzyme B and enhanced target cell lysis in an ***in vivo*** CTL assay. $CD8^+$ T cells isolated from entinostat treated mice showed a greater proportion of KLRG1+ short-lived effector cells and a lower proportion of CD127+ memory precursors. In conclusion, we have found in both human and mouse $CD8^+$ T cells that there are two clusters of differential regulation of immune checkpoint expression. These patterns are regulated by a signaling axis composed of TGF-β1, Smad3, and class I HDACs. Inhibiting class I HDACs during T cell priming programs these cells for different patterns of checkpoint expression and is associated with significantly increased cytolytic function. These results suggest that combinations of checkpoint inhibitors and epigenetic modifying agents may be able to reprogram T cells in cancers where checkpoint inhibitors alone have been unsuccessful.

Keywords: PD-1, TIM-3, TGF-β1

A134 / Combination of PD-1/PD-L1 blockade and targeting of a tumor-specific carbohydrate antigen in a novel trifunctional antibody as a strategy to improve anti-PD-L1 therapy

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PankoMab (PM) is a humanized monoclonal antibody targeting the novel carbohydrate-induced conformational epitope TA-MUC1 which is expressed on a variety of high prevalence carcinomas, but is virtually absent on normal tissues. Its key modes of action include ADCC and phagocytosis. Antibodies blocking the PD-1/PD-L1 axis are able to restore T cell functions thereby enhancing the antitumor immunity, but long lasting responses are only seen in a subset of patients. We developed a trifunctional antibody which combines the PM specificity for TA-MUC1 and PD-L1 targeting with a functional Fc part in one molecule (PM-PDL). By focusing the PD-1/PD-L1 blockade to the tumor and combining different modes of action, PM-PDL has the potential to increase efficacy and broaden the patient coverage giving additional benefit compared to the respective monospecific antibody. As Fc engagement and ADCC seem to be important mechanisms including for anti-PD-L1 therapy we investigated the effects of Fc glyco-optimization in relevant ***in vitro*** assays.

Using the human expression platform GlycoExpress we generated two glycosylation variants of PM-PDL: a normal fucosylated variant and a fucose-reduced counterpart. Binding to PD-L1, TA-MUC1 and FcγRIIIa was assessed using ELISA, flow cytometry and AlphaScreen technology. ADCC activity was tested against cancer cells and primary immune cells in cytotoxicity assays. Mixed lymphocyte reactions were performed to compare their capacity to induce T cell activation.

Both glycosylation variants retained the TA-MUC1 binding properties of PM and showed effective blocking of PD-L1/PD-1 interaction. The de-fucosylated variant was characterized by increased FcγRIIIa binding resulting in enhanced ADCC against $TA\text{-}MUC1^+$ and $PD\text{-}L1^+$ cancer cells. No obvious antibody-mediated lysis of primary B cells and monocytes was observed even with the de-fucosylated antibody. Furthermore, PM-PDL induced T cell activation determined by increased IL-2 secretion and enhanced surface expression of activation markers. Interestingly, IL-2 secretion was comparable between both glycosylation variants and PD-1/PD-L1 targeting reference antibodies, whereas CD8 T cell activation measured via CD25 and CD137 expression was most effectively induced by the fucose-reduced variant.

This study demonstrates that a novel tri-functional checkpoint antibody combines the advantages of two highly effective targeting strategies within one molecule with respect to PD-1/PD-L1 blockade leading to activation of T cells and ADCC against

cancer cells. De-fucosylation further increased both effects, but showed no negative effect regarding killing of PBMC subsets underlining the potential of glyco-optimization to enhance not only cytotoxic but also immunomodulatory antibody effector functions.

Keywords: PD-1/PD-L1 axis, Tri-functional antibodies, Glyco-engineering

A135 / High NKT cell frequency and CD25+ NK cells in peripheral blood correlate with positive response to PD-1 inhibitors in metastatic melanoma patients

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Anti-PD1 antibodies (Pembrolizumab, Nivolumab) are immune-checkpoint inhibitors designed to reactivate T cells against cancer. Anti-PD1 therapy has shown great promise in various cancer types, but not all patients are responding. The effects of anti-PD1 therapy on other cytotoxic lymphocytes, such as NK cells which are able to kill cancer cells, have not been studied in detail. Therefore, we aimed to find potential predictive markers of treatment response and discover how anti-PD1 affects patients' NK cells *in vivo.*
Peripheral blood samples from metastatic melanoma patients (n=17) were obtained before the start of the treatment and 1 month after initiation of treatment. The immunophenotype of NK and T cells was analyzed with multicolor flow cytometry. Serum cytokines were measured using Olink multiplex inflammation panel, and the complete blood count (CBC) data was obtained from the hospital database. In addition, plasma cytomegalovirus (CMV) IgG levels were measured with VIDAS® CMV IgG assay.
The CBC indicated that positive responders (R) had a trend of higher lymphocyte (36% vs. 28%, p=0.1) and higher eosinophil (4% vs. 2.5%, p=0.01*) percentage before and during treatment when compared to patients with progressive disease (PD). In contrast, the proportion of neutrophils (59% vs. 49%, p=0.01*) was higher in PD than in R cohort before and during treatment. Immunophenotyping of lymphocyte subpopulations revealed that R patients had more NKT cells (7.7% vs. 2.7%, p=0.02*) than PD patients. Interestingly, responder patients' NK cells had a tendency of expressing higher levels of CD25, DNAM, and CCR7 compared to PD patients before initiation and after 1month of treatment. High expression of CD25 has previously been linked to high cytotoxic function and proliferation of NK cells. After 1 month of therapy an increasing trend of CD25 expressing NK cells was observed in responder patients (14% increase, p=0.09), but not in PD. Further, the cytokine assay indicated that anti-PD1 treatment increases the levels of CXCL9 (309 vs. 638, p=0.008**), CXCL11 (30 vs. 62, P=0.01**), and IL-12B (27 vs. 41, p=0.01**) in the serum after 1 month of treatment (protein levels presented as arbitrary units of normalized protein expression, NPX, on Log2 scale). As CMV infection is known to activate CD25 expression on NK cells, we wanted to study CMV seropositivity status in our patients. Our results indicated that before the initiation of treatment there was a positive correlation between CMV IgG and CD25+ expressing NK cells in CMV-positive patients (p=0.02*).
Our preliminary results suggest that increased amount of NKT cells in blood and CD25 expressing NK cells are related to the response to PD-1 inhibitors. Thus, in addition to T cells also NK and NKT cells may play a role in the antitumor response induced by PD-1 inhibition. Hence, further analysis is needed to discover the mechanism and fundamental role of NK and NKT cells in positive treatment response.

Keywords: Anti-PD1, Melanoma, NK cell

A136 / PD-L1 signaling regulates proliferation and activation of tumor-associated macrophages

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The goal of this study was to investigate the role of PD-L1 signaling in regulating the function of tumor-associated macrophages and the effects of PD-L1 antibody blockade. We recently reported that PD-L1 expression by tumor-associated macrophages is regulated by locally-produced TNF-a, but the effects of PD-L1 signaling on the function of tumor macrophages has not been previously reported. Moreover, the effects of PD-L1 blockade with therapeutic antibodies on tumor macrophage populations and their numbers and activation states in vivo have not been investigated.
The in vitro effects of PD-L1 blockade on macrophage proliferation, survival and activation were assessed using bone marrow derived macrophages, together with studies of macrophages derived from PD-L1$^{-/-}$ mice. Studies in tumor-bearing mice treated with therapeutic PD-L1 antibodies investigated the effects of treatment on tumor macrophage numbers and activation status. The effects of PD-L1 blockade on macrophage gene expression patterns were also assessed using gene array.
Treatment of macrophages in vitro with PD-L1 antibody or with soluble PD-1 triggered macrophage proliferation and increase survival, and was also associated with macrophage spreading and activation. In addition, macrophages incubated with PD-L1 antibody spontaneously upregulated production of inflammatory

cytokines and expression of co-stimulatory molecules. Numbers of tumor-associated macrophages in mice treated with PD-L1 antibody were significantly increased and these macrophages expressed an activated phenotype. Combined treatment with PD-L1 and PD-1 antibodies remarkably controlled tumor growth compared to treatment with either antibody alone, suggesting non-redundancy of the two pathways. Gene expression profiling revealed upregulation of genes associated with inflammatory macrophage functions and downregulation of genes coding for macrophage migration and adherence.
These findings suggest that treatment with PD-L1 targeted antibodies elicits immunological effects on tumor macrophages that are distinct and independent of effects of blocking the T cell PD-1/PD-L1 interaction. The results suggest that constitutive signaling by PD-L1 maintains an immune suppressive macrophage phenotype, and that blockade of this signaling pathway by PD-L1 antibodies reverses the effect. Furthermore, these findings indicate that blocking PD-L1 constitutive signaling by tumor macrophages induces additional antitumor effects when combined with PD-1 blockade.

Keywords: PD-L1, macrophage, tumor

A137 / Investigation of the immune infiltrate of metastatic melanoma under immune checkpoint inhibition

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Tumor infiltrating lymphocytes (TIL) play a crucial role in the therapeutic impact of immune checkpoint blockers. We investigated metastases from 64 patients with metastatic melanoma before and during treatment with immune checkpoint blockers (i) immunohistochemically, (ii) with TCR repertoire profiling and (iii) analysis of the transcriptome. The patients were treated with ipilimumab (n=28) or pembrolizumab (n=24) or ipilimumab/nivolumab (n=11); half of them had a disease control (response or stable disease), the other half progressed as best response to treatment. In contrast to previous reports immunohistochemical analysis of the immune infiltrate revealed no significant difference in the number of CD8+ TILs in pretreatment samples of responders and non-responders. Instead, the number of CD4+ TILs including regulatory T cells (Treg; FoxP3) and PD-1+ cells was higher in responders receiving pembrolizumab. Ipilimumab responders had significantly more M1 macrophages pretreatment (CD68, iNOS). Samples taken about 6 weeks after start of the immune checkpoint blocker showed a significant higher number of immune cells in responders through all T cell subsets (CD3,4,8,FoxP3, PD-1), B cells (CD20) as well as macrophages (CD68, CD163, iNOS). TCR repertoire profiling by deep TCR sequencing demonstrated that responders develop a more diverse repertoire under treatment ($p=0.05$). Pretreatment samples as well as the size of the top 10 TCR clones (clonality) posttreatment did not differ significantly in responders and non-responders. By RNA sequencing no differential expression profiles between responders and non-responders was found pretreatment. However, with treatment responders revealed a differential gene expression compared to pretreatment samples with an upregulation of MHC molecules, TNF family members and different apoptosis-inducing genes and a downregulation of c-myc and CDK2/4. There was no differential gene expression in non-responders pre- and posttreatment. In conclusion, pretreatment metastases from responders and non-responders reveal subtle differences in the immune infiltrate as the number of Treg and PD-1+ cells in pembrolizumab responders and the number of M1 macrophages in ipilimumab responders. With treatment responding patients have significant higher numbers of immune cells including T- and B- lymphocytes as well as macrophages and develop a more diverse TCR repertoire. RNA sequencing revealed a differential expression with treatment only in responding patients.

Keywords: melanoma, immune checkpoint blocker, response

A138 / Prediction of efficacy of immune checkpoint inhibitors using an immune checkpoints gene signature in non-clinical sensitive and resistant models

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Immune checkpoint inhibitors (ICIs) such as Nivolumab and Ipilimumab are currently used in cancer immune therapy. Even though ICIs exhibit superior efficacy and long-term effectiveness, response rate is not high. Therefore, the prediction methods and markers of ICI efficacy are desired. However, comprehensive information about non-clinical models for sensitive and resistance to ICIs has not been reported, which inhibits translational

research in development of prediction methods of ICIs efficacy in the clinic. The aim of this study is to systematically evaluate the relevance between immune checkpoint gene signature (ICG-Sig) in 14 mouse tumors and pharmacological effect of ICIs such as anti-PD-1/PD-L1 antibodies (Abs) in the models and to identify characteristics of sensitive and resistance to ICIs.
Tumor bearing mice were developed by s.c. inoculation with 3 colon cancers, 3 breast cancers, 2 lung cancers, 2 melanomas, osteosarcoma, fibrosarcoma, T-lymphoma, or mastocytoma. We have evaluated 10 immune checkpoint genes related to immune suppression such as programmed cell death 1 (PD-1), PD-L1, and cytotoxic T lymphocyte antigen-4 (CTLA-4). The gene expressions among tested models were relativized and scored in radar charts (ICG-Sig), which indicated the dependence of a tumor on immune checkpoint genes. According to ICG-Sig, MC38, colon cancer, mainly depended on PD-1 suggesting that PD-1 inhibition was an effective strategy to inhibit tumor growth of MC38. On the other hand, melanoma B16F10 showed almost negligible expression of the genes, which suggested B16F10 was PD-1/PD-L1 negative and a non-responsible model to ICIs. Since most genes were highly expressed in colon CT26 or breast 4T1 tumors, CT26 and 4T1 seemed to be resistant models to PD-1 Ab and a combination strategy might be required to induce pharmacological effect of ICIs on tumor growth.
Based on the ICG-Sig, we evaluated the pharmacological effect of anti-PD-1 or PD-L1 Abs on tumor growth in the models. Tumor bearing mice were treated with control IgG, anti-PD-1, or PD-L1 Abs, and tumor growth was observed. As we expected, MC38 and other predicted sensitive models were actually sensitive to anti-PD-1 Ab or anti-PD-L1 Ab. On the other hand, tumors that were expected to be non-responsible or resistant to PD-1/PD-L1 Abs such as B16F10, 4T1 and CT26 did not response to PD-1/PD-L1 Abs.
These results demonstrated that ICG-Sig based on the systemic evaluation of 10 immune checkpoint genes among 14 tumor models provided information in terms of dependence upon immune checkpoints in tumors, prediction of the effect of ICIs on tumor growth, and ICIs that should be selected. The strategy based on the ICG-Sig is a useful method for prediction of ICIs' efficacy, and could be applicable to the clinic.

Keywords: immune checkpoint inhibitors, PD-1, sensitive and resistant mouse models

A139 / Tumor-intrinsic RIG-I signaling promotes anti-CTLA-4 checkpoint inhibitor-mediated anticancer immunity

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Strong inter-individual variation in clinical response to checkpoint inhibitors such as anti-CTLA4 remains a major challenge. In a retrospective analysis, expression of viral defense genes in human melanomas including the cytosolic RNA receptor RIG-I (***DDx58***) has been associated with clinical benefit to CTLA-4 blockade. However, possible interconnections of these two distinct pathways remain unknown. Using CRISPR/Cas9 technology to generate B16 melanoma cells lines that lack nucleic acid receptors or downstream signaling molecules (RIG-I, STING, IRF3/7) together with available genetically deficient mouse models, we addressed the importance of nucleic acid receptor signaling in both tumor and host cells for the efficacy of anti-CTLA-4 immunotherapy. We here provide experimental proof that anti-CTLA-4 immunotherapy relies on tumor cell-intrinsic RIG-I but not STING signaling. Following anti-CTLA-4 treatment, tumor-intrinsic RIG-I signaling critically impacts on cross-presentation of tumor-associated antigen by $CD103^+$ dendritic cells, the expansion of tumor antigen-specific $CD8^+$ T cells and ultimately the accumulation of $CD8^+$ T cells within tumor tissue. Consistently, therapeutic targeting of RIG-I with 5′-phosphorylated-RNA in both tumor and non-malignant cells potently augmented the efficacy of CTLA-4 checkpoint blockade. These processes were additionally dependent on host STING, MAVS and type I interferon signaling and were closely linked to RIG-I-mediated tumor cell death. In summary, our study identifies both tumor- and host-intrinsic RIG-I signaling as fundamental requirements for anti-CTLA-4-mediated antitumor immunity. Our data predict that targeting RIG-I may serve as a basis for the development of new combination strategies to increase the response rate of checkpoint inhibitor-based immunotherapy, particularly in individuals that do not have a sufficient spontaneous antitumor T-cell immune response.

Keywords: Immune checkpoint blockade, Nucleic acid receptors, Immunogenic cell death

A140 / Novel antibody derivative locally blocking the PD-1/PD-L1 immune checkpoint in Acute Myeloid Leukemia

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Programmed cell death-protein 1 (PD-1) is an inhibitory T cell receptor crucial for maintaining T cell homeostasis during long-term inflammation. Its main ligand, Programmed cell death-ligand 1 (PD-L1), is expressed in hematopoietic and non-hematopoietic tissue and is upregulated under inflammatory conditions to dampen the T cell response and induce tolerance. Different types of cancers, including Acute Myeloid Leukemia (AML), take advantage of the PD-1/PD-L1 immune checkpoint and upregulate PD-L1 expression inducing T cell dysfunction and immune escape. In recent years, the application of PD-1/PD-L1 checkpoint inhibitors has been proven to be intriguingly successful in the clinics even raising hope for patients with an advanced cancer status. Yet, a common drawback of these currently applied blocking agents is the risk of interfering with PD-L1 expressed on healthy cells, thus causing side effects due to autoimmune toxicity. To decrease these immune-related adverse events, we developed a novel molecule called “local inhibitory checkpoint antibody derivative” (liCAD). The liCAD scaffold is composed of three different domains, each with a distinct function: (1) tumor cell targeting, (2) T cell recruitment and (3) immune checkpoint blocking. Similar to the Bispecific T cell Engager (BiTE) format, two moieties are single-chain variable fragments (scFv) against the AML antigen CD33 and the T cell co-receptor CD3. The N-terminally engrafted immune checkpoint blocking module consists of the endogenous extracellular domain of human PD-1 ($PD1_{ex}$), which has a naturally occurring low affinity for PD-L1. With this strategy, we aim to combine high-affinity tumor targeting with low-affinity checkpoint blockade limited to the cytolytic synapse. We evaluated the liCAD regarding its ability to block PD-L1, its influence on T cell activity and redirected tumor cell lysis as well as the risk to cause on-target off-leukemia adverse effects ***in vitro***. Furthermore, we performed ***in vivo*** experiments using a murine xenograft model. The liCAD revealed blockage of PD-L1 accessibility and induced efficient lysis of PD-L1 positive AML cells in vitro and ***in vivo***. Apart from that, it enabled selective eradication of PD-L1/CD33 double positive cells. Therefore, our findings suggest the liCAD format as a promising novel strategy to prevent side effects in cancer immunotherapy.

Keywords: PD-1/ PD-L1, immunotherapy, Acute Myeloid Leukemia

A141 / A novel non-competitive and non-brain penetrant adenosine A_{2A} receptor antagonist designed to reverse adenosine-mediated suppression of anti-tumor immunity

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High levels of extracellular adenosine in the tumor microenvironment play a significant role in tumor immune evasion by suppressing Th1 cytokine production and the activation and cytolytic activity of T cells and NK cells. We defined the receptor(s) mediating adenosine activity on selected immune cells found within the tumor microenvironment and characterized a novel immuno-oncology-dedicated A_{2A} antagonist optimized to overcome immune suppression despite the high adenosine concentrations found in tumors.

We first explored the expression of the four adenosine receptors in primary human immune cells by nanostring. A_{2A} was the main adenosine receptor expressed by CD4 and CD8 T lymphocytes and monocytes, and the only one in mature monocyte-derived dendritic cells and NK cells. A_{2B} mRNA were not abundant in T cells and monocytes, while A_1 and A_3 mRNA were not detected in any cell type. We further studied A_{2A} functions in primary human T lymphocytes and monocytes. Selective A_{2A} agonists such as CGS-21680 strongly suppressed cytokine production by activated T lymphocytes and monocytes, highlighting the role of A_{2A} as the main receptor mediating adenosine signaling in these cells.

In parallel, we undertook a comprehensive characterization of extracellular adenosine concentrations. Using intratumoral microdialysis of 10 patient-derived xenografts (PDX) from 4 different indications (kidney, lung, pancreas, head and neck carcinomas), we measured adenosine concentrations in the range of 0.5 to 13 µM (median: 5.5 µM), significantly higher than the 0.1-0.2 µM reported in normal brain tissue. In a complementary fashion, we showed that A_{2A} antagonists initially designed for Parkinson’s disease but recently repurposed for immuno-oncology dramatically loose potency in a high adenosine environment. We therefore developed EOS100574, a novel non-brain penetrant and non-competitive inhibitor of A2A, with sub-nanomolar Ki and acceptable selectivity versus A_1, A_{2B}, and A_3 receptors. Our compound potently inhibited A_{2A} signaling in human T lymphocytes independently of adenosine concentrations (IC_{50} in a T cell cAMP assay = 2.8 and 5.5 nM at 1 and 100 µM adenosine, respectively), and rescued cytokine production in the presence of high concentrations of A_{2A} agonists. EOS100574 potently reversed AMP-mediated T

cell suppression, rescued TNFα production in primary human monocytes treated by A_{2A} agonists, and increased CD8 T cell cytotoxicity in a co-culture assay of effector CD8 T cells and target cancer cells.
iTeos' non-competitive and non-brain penetrant A_{2A} receptor antagonist is uniquely designed to address the challenge of counteracting elevated adenosine concentrations in tumors in order to restore antitumor immunity.

Keywords: A2A receptor antagonist, non-brain penetrant, anti-tumor immunity

A142 / Pre-clinical efficacy evaluation of agonistic CD137 therapeutic antibodies in human CD137 knock-in mouse model (HuGEMM™)

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Despite huge clinical success of PD1/PD-L1 therapeutic antibodies in multiple tumor types, overall response rate is still low. Growing efforts have been fostered around priming and activating T cells through agonist CD137 antibodies as combinatory strategies to improve tumor killing activities of T cells. Multiple agonistic CD137 antibodies and PD-L1/CD137 bi-specific antibodies are currently under development, which were expected to become important new immuno-oncology therapeutics in clinic. However, lack of pre-clinical animal models for testing in vivo efficacies of these human therapeutic antibodies has hindered the research process.
We have engineered CD137 HuGEMM™, i.e., human CD137 knock-in C57BL/6 mice expressing chimeric human/mouse CD137 composed of human extracellular and transmembrane domains (Exon 4-7), with intact mouse signal peptide and intracellular domain. FACS analysis of splenocytes derived from homozygous knock-in mice showed that CD3/CD28 stimulated T cells only express chimeric CD137 reactive to human CD137 antibodies, but not mouse CD137; while heterozygous mice express both chimeric and mouse CD137. To evaluate the efficacy of human agonistic CD137 antibodies, we engrafted the heterozygous or homozygous CD137 HuGEMM™ mice with engineered syngeneic MC38 cells expressing human PD-L1 (MC38 HuCELL™). We found that human CD137 antibody, Urelumab analog, showed modest anti-tumor activities as single agent; but exhibited much improved efficacies when combined with anti-PD-L1 therapeutic antibody or mouse surrogate anti-PD1 antibody. Interestingly, when we grafted CT26 syngeneic tumor cells in heterozygous CD137 HuGEMM™ mice in Balb/c x C57BL/6 F1 background, and treated with CD137 antibodies, both mouse and human antibodies showed robust efficacies. Taken together, development of CD137 HuGEMM™ provides an urgently needed model for pre-clinical efficacy evaluation of human CD137 therapeutics, as well as PD1/PD-L1 combos. In the meantime, we are also engineering & validating other HuGEMM™ models with humanized checkpoint targets, including OX40, GITR, TIM3, LAG3, etc. Double knock-in HuGEMM™ models in combination with PD1 are also under development. These humanized mouse models provide tools that can directly evaluate human therapeutic antibodies, and may expedite the process for new therapies to reach clinic.

Keywords: CD137, Humanized model, Efficacy

A143 / Optimizing monoclonal antibodies (mAbs) to PD-L1 for the immunohistochemical analysis of formalin-fixed paraffin embedded (FFPE) specimens on automated stainer platforms

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PD-L1 in situ protein expression analysis in archival pathological specimens is one of the most pressing topics of current tumor immunology research. Various serological reagents have been generated in order to assess the presence of PD-L1 in formalin-fixed paraffin embedded tissue samples. There is plenty of confusion which mAbs to use. Moreover there are reagents offered as companion drugs which are suggested or mandatory for PD-L1 evaluation in the context of certain anti-PD1/PD-L1 drugs. Moreover, some of these reagents are tight to instrumentation and automated stainer platforms, not commonly in use.
While various studies have analyzed the correct morphological interpretation of immunostained and factors such as interobserver variability, considerably fewer studies have address the development of proper immunohistochemical protocols for the various available antibodies.
In the present analysis we have analyzed the following reagents: mAb E1L3N (Cell Signaling), mAb 22C3 (Agilent), mAb 28-8 (Abcam), mAb E1J2J (Cell Signaling), mAb 405.9A11 (Cell Signaling). The following automated stainer platforms were employed: Leica Bond-3, Ventana Benchmark Ultra and for reference sets DAKO Link48. Various sets of tissues were analyzed such as normal tissue panels including placenta, tonsil, spleen. Furthermore, tumor panels comprising of 20 NSCLC cases and 30 melanomas were analyzed. Various antigen retrieval methods comprising of different buffer solutions, heating times and protocols were employed.
All antibodies showed different characteristics and different working conditions. Most difficult was the adjustment of mAb 22C3 and 28-8 outside their recommended stainer platform (Link48).

However, congruent staining could be achieved with mAbs E1L3N, 28-8, and 22C3 using a Leica-Bond-3 automated stainer platform producing comparable immunohistochemical staining for all three reagents.
We will demonstrate and explain the details of these protocols and ways to optimize the staining for these reagents outside their companion diagnostic immunohistochemical test kits!

Keywords: PD-L1, IHC in FFPE, optimizing IHC protocols

A144 / High density of PD-1-positive T cells are associated with immune evasion in DNA mismatch repair-deficient colorectal cancers

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Lynch syndrome-associated cancers show microsatellite instability (MSI) and accumulate high numbers of mutations at repetitive sequence stretches as a consequence of DNA mismatch repair (MMR) deficiency. The high mutational load of MMR-deficient cancers leads to the generation of multiple highly immunogenic frameshift peptide (FSP) neoantigens. MMR-deficient cancer cells can grow out to clinically manifest cancers, if the local T cells in their environment get exhausted. Therefore, a subset of MMR-deficient cancer patients responds particularly well to treatment with immune checkpoint inhibitors such as anti-PD-1 antibodies. Alternatively, MMR-deficient cancer cells that have undergone immune evasion due to loss of HLA-mediated antigen presentation may grow out irrespective of local T cell surveillance. In order to analyze whether HLA-related immune evasion in MMR-deficient cancer is related to the local immune cell activation status we evaluated mutations of the ***Beta2-microglobulin (B2M)*** gene and the HLA class II-regulatory genes ***RFX5*** and ***CIITA*** in MMR-deficient colorectal cancer specimens (n=53). Tumor-infiltrating lymphocytes (CD3-positive T cells, PD-1-positive T cells) were quantified using a semi-automated system and related to immune evasion status. PD-1-positive T cell infiltration was significantly higher in ***B2M***-mutant (mt) compared to ***B2M***-wild type (wt) tumors (median: 22.2 cells per 0.25 mm^2 in B2M-mt vs. 2.0 cells per 0.25 mm^2 in ***B2M***-wt, Wilcoxon's rank sum test p=0.002). Increasing PD-1-positive T cell infiltration was significantly related to an increased likelihood of ***B2M*** mutation and loss of HLA class I antigen expression (OR=1.81). In contrast, HLA class II antigen expression status was not related to the proportion of PD-1-positive lymphocytes, but significantly associated with enhanced overall T cell infiltration. These results suggest that immune evasion mediated by ***B2M*** mutation-induced loss of HLA class I antigen expression predominantly occurs in an environment of activated PD-1-positive T cell infiltration, supporting the validity of the immunoediting concept in MSI colorectal cancers. If ***B2M*** mutations interfere with anti-PD1/PD-L1 therapy success, we predict that resistance towards anti-PD1 therapy may - counterintuitively - be particularly common in MSI cancer patients with high PD-1-positive T cell infiltration.

Keywords: immune checkpoints, immune evasion, DNA mismatch repair deficiency

A146 / Computational discovery and experimental validation of CGEN-15032 as a novel target for cancer immunotherapy

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Antibody blockade of CTLA4 and PD-1 immune checkpoints emerged as an effective treatment modality for cancer. However, most patients do not achieve sustained benefit, suggesting a need for targeting of additional immune checkpoints. Towards prediction of novel immune checkpoints, we developed a set of computational tools including gene structure alignment for the identification of functional homologs for B7/CD28 genes in the absence of sequence similarity. This discovery platform has been tested and validated extensively and has demonstrated its validity by identifying novel immune checkpoints such as TIGIT and PVRIG, which are currently in preclinical development by Compugen. This predictive platform was utilized to identify an additional immuno-modulatory target, designated CGEN-15032.

CGEN-15032 is expressed on cancer cells and on the myeloid component of immune infiltrate within the tumor microenvironment. Several human and mouse in vitro experimental systems have demonstrated an immune-modulatory effect for CGEN-15032. Ectopically expressed CGEN-15032 dampened anti-melanoma activity of tumor-infiltrating lymphocytes, derived from melanoma patients. In line with the effect observed in human system, over expression of murine CGEN-15032 in artificial antigen-presenting cells resulted in reduced activity of TCR-transgenic DO11.10 T cells. These functional data, in conjunction with the expression of 15032 on myeloid and tumor cells, suggest that 15032 is a ligand and part of a novel pathway that inhibits T cell function.

To study the immuno-modulatory function of CGEN-15032 and its role in anti-cancer immunity, we generated CGEN-15032 knock-out (KO) mice. MC38 tumors grew slower in CGEN-15032 KO relative to wild-type mice (in average 47%, $p< 0.01$). Furthermore, a combinatorial treatment of CGEN-15032 deficient mice with anti-PDL1 antibody resulted in tumor growth inhibition compared with anti-PDL1 treated wild-type mice (in average 40%, $p< 0.05$).

Taken together, these data provide experimental validation of our computational discovery approach and highlight CGEN-15032 as an attractive target for cancer immunotherapy.

Keywords: immune checkpoint, novel target, myeloid

A147 / PD-L1 expression on cancer cells may inhibit T-cell function and regulate response to anti-PD1 in a mouse orthotopic liver cancer model

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Anti-programmed cell death-1(PD-1) treatment has shown promising antitumor efficacy in patient with advanced hepatocellular carcinoma(HCC). It is unknown whether expression of PD-L1 in tumor cells, which occurred in about 20% of the HCC patients, may help predict the treatment response. To explore the effects of PD-L1 expression on HCC immune microenvironment, we established an orthotopic HCC mouse model with PD-L1 expression by transfecting PD-L1 plasmid (MR203953; Origene Technologies, Rockville, MD) into BNL-MEA liver cancer cells and selecting subclones with stable PD-L1 expression. The in vitro growth characteristics of parental and PD-L1-expressing BNL-MEA cells were compared by MTT assay, 3D proliferation assay and colony formation assay. Cells were implanted into the subcapsular space of BALB/c mice and the in vivo growth characteristics were evaluated by microbubble-enhanced ultrasound and animal survival. These results indicated that PD-L1 expression doesn't affect the growth characteristics of BNL-MEA cells in vitro or in vivo. PD-L1-expressing tumors may recruit more CD8+ T cells but down-regulate expression of genes related to T cell activation in the tumor microenvironment, including CD8a, ZAP70 and Cd3e, as demonstrated by using the Nanostring nCounter® PanCancer Immune Profiling Panel and qRT-PCR. Co-culture of

PD-L1 expressing BNL-MEA cells with CD8+ T cells reduced T cell proliferation and expression of cytokines IFN-γ and TNF-α. The above data suggested that PD-L1 expression may be associated with T cell dysfunction in the liver tumor microenvironment. Tumors with PD-L1 expression showed better response to anti-PD1 therapy and depletion of CD8+ T cells abolished the anti-tumor effect. The difference in treatment response between parental and PD-L1 expressing tumors disappeared when combination of anti-PD1 and sorafenib was given. In conclusion, PD-L1 expression in HCC cells may inhibit T-cell function in the liver tumor microenvironment. Anti-PD1 antibodies facilitate activation of CD8+ T cells, thus exhibit anti-tumor activity.

Keywords: PD-L1, Hepatocellular carcinoma(HCC), CD8+ T cell

A148 / Dendritic cells dictate the responsiveness to PD-L1 blockade in cancer

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Inhibitors of the PD-L1/PD-1 axis have demonstrated therapeutic efficacy across a broad range of human cancers, suggesting its fundamental importance in restraining pre-existing anti-cancer T cell immunity. While the focus of studies addressing underlying biologic mechanism has been on understanding how PD-L1 expressed by tumor cells and tumor infiltrating immune cells binds to PD-1 on pre-existing anti-cancer T cells to inhibit them, durable responses and overall survival benefit of PD-L1/PD-1 inhibitors have not been limited to patients expressing PD-L1/PD-1 in the tumor microenvironment, suggesting an incomplete understanding of how these therapies affect immunity. Here, we demonstrate how PD-1 expression on immature DCs restrains DC maturation, which is reversed with PD-L1 pathway blockade in both human and mouse models ***in vitro*** and ***in vivo***. Upon maturation of DCs, PD-1 expression is down-regulated. However, PD-L1 expression increases, which leads to binding of PD-L1 to B7.1 on the surface of DCs, sequestering B7.1 and preventing binding of B7.1 to CD28 on T cells. Administration of PD-L1 antibodies, but not PD-1 antibodies relieve the B7.1 sequestration, enabling further co-stimulation of anti-cancer T cells through B7.1/CD28 interaction. Patient tumor specimens from clinical studies of atezolizumab, an anti-PD-L1 therapeutic, in renal cell carcinoma (RCC, n=56) and non-small cell lung cancer (NSCLC, n=188) further demonstrate a strong correlation between a cross-presenting DC gene signature and improved overall survival from atezolizumab treatment (RCC: HR=0.38, p=0.029; NSCLC: HR=0.54, p=0.037). In conclusion, this represents the first demonstration of how the PD-L1/PD-1 pathway biologically inhibits DCs, and functions as an immune checkpoint in anti-cancer T cell priming and activation. Treatment with PD-L1 pathway inhibitors can relieve this inhibition by promoting DC maturation and function, leading to generation of new immunity that may not be measured by PD-L1 expression in baseline tumors.

Keywords: PD-L1, PD-1, Dendritic cells

A149 / Combining anti-VEGF and anti-PD-L1 therapy prolongs overall survival in small cell lung carcinomas

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Metastatic lung cancer is the leading cause of smoking- and cancer-related deaths worldwide. Small cell lung cancer (SCLC) accounts for approximately 14 % of newly diagnosed lung cancer cases, is driven by lesions in ***RB1*** and ***TP53*** and represents the most aggressive pulmonary carcinoma. Current chemotherapies are initially effective in patients suffering from SCLC, however tumors rapidly reoccur and patients die within only a few months. Results from KEYNOTE-028 were presented on the ASCO conference. Here, programmed death ligand 1 (PD-L1) positive late stage SCLC patients received a high-affinity, humanized monoclonal programmed death receptor 1 (PD-1) antibody. They showed response rates of up to 25 % which is remarkable for SCLC, since targeted therapies for this tumor entity are rare. However, most SCLC patients harbor a primary resistance or acquire resistance during treatment by an immune suppressive microenvironment or other resistance mechanisms triggered by the tumor cells themselves. Thus, there is a critical need to combine immune checkpoint inhibitors with other therapies to overcome these resistances.
We implemented a combined therapy concept including anti-VEGF and anti-PD-L1 monoclonal antibody therapy for SCLC in an autochthonous mouse model. As a read out, we used X-ray computed tomography, flow cytometry and end point immunohistochemistry.

We found that combined anti-VEGF/anti-PD-L1 treatment synergistically improved clinical outcome, compared to anti-PD-L1 and anti-VEGF monotherapies, and enhanced T cell infiltration into the tumor. Moreover, we identified in murine SCLC tumors which acquired resistance to anti-PD-L1 monotherapy, a significant increase in PD1+/TIM3+ cytotoxic T cells. This exhausted T cell phenotype was significantly reverted upon combined anti-VEGF/anti-PD-L1 therapy. In line with the results obtained from our mouse model, we found a similar TIM3+ T cell phenotype in peripheral blood mononuclear cells of SCLC patients with acquired adaptive resistance to PD1/PD-L1 blockade. Mechanistically, we show that VEGF-A triggers co-expression of TIM3 on T cells indicating an immunosuppressive function of VEGF in SCLC patients during anti-PD1/PD-L1 therapy resistance.
Taken together, there is evidence for a substantial benefit of implementing combined anti-angiogenic and anti-immune checkpoint therapy approaches in the clinic in order to overcome acquired resistances especially in case of SCLC.

Keywords: lung cancer, checkpoint blockade, acquired resistance

A150 / Effects of anti-CTLA-4 and anti-PD-1 on memory T-cell differentiation and resistance to tumor relapse

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The FDA has begun to expand the approved uses of immune checkpoint blockade antibodies targeting CTLA-4 and PD-1. Blocking either checkpoint relieves the negative regulation of T-cells resulting in significant responses in patients with cancer. Data has now begun to emerge regarding differences between these two therapies. While αPD-1 therapy has a greater response rate (~30% vs. 11%) according to RECIST criteria, recent reports have suggested responses to αPD-1 may not be as durable as αCTLA-4. 25% of patients who initially responded to αPD-1 have tumor relapse within 24 months. In contrast, the 3-year survival rate of patients treated with αCTLA-4 is at least ~25% suggesting a durable response. Previous studies in bacterial or chronic LCMV infectious models have shown that αCTLA-4 can increase CD8+ memory T-cell formation, whereas genetic ablation of PD-1 on T-cells often promotes the terminally differentiated exhausted CD8+ T-cell phenotype, while attenuating memory T-cell formation. However, the mechanism which leads to relapse following αPD-1 treatment in tumor models is not clear. The goal of this project is to understand how immunotherapies shape memory T-cell formation and how that relates to the mechanism of tumor relapse.
To test whether αCTLA-4 or αPD-1 can induce a better memory T-cell response, mice were vaccinated with irradiated B16F10 murine melanoma cells and treated αCTLA-4 or αPD-1. Mice were re-challenged with B16F10 80 days after vaccination. Although both αCTLA-4 and αPD-1 improved tumor rejection compared with controls, αCTLA-4 treated mice exhibited superior tumor control compared to αPD-1 ($p< 0.0005$) suggesting the memory T-cell response mediated by αCTLA-4 is more durable. In order to test whether this memory T-cell response is antigen-specific, mice were re-challenged with unrelated MC38 or 3LL cancer cells. The antibody treated groups did not show improved antitumor effect compared with vaccine control ($p>0.5$). To test whether the frequency of memory T-cells recruited to the re-challenged tumor could affect memory T-cell response, antigen-specific pmel-1 T-cells were infused to mice following vaccination with αCTLA-4 or αPD-1. Our result suggested that there were more tumor-infiltrating pmel-1 T-cells in the αCTLA-4 treated group compared to the αPD-1 treated group ($p< 0.05$). In order to augment the durability of αPD-1 treatment, αPD-1 was combined with αCTLA-4 following vaccination. The combined treatment group has superior antitumor response compared to that with αPD-1 ($p< 0.000003$) and overlapped with the αCTLA-4 treated group ($P>0.05$) during re-challenge, indicating that the effect of the combined treatment is dominated by αCTLA-4.
Collectively, our studies facilitate the design of combination immunotherapy treatments that enhance both response rates and generation of memory T-cells to prevent relapse.

Keywords: anti-CTLA-4, anti-PD-1, memory T-cells

A151 / Establishment and characterization of a PDX based preclinical platform for checkpoint inhibitor testing and development of translational biomarker

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Patient-derived tumor xenografts (PDX) play a major role in the development of new cancer therapies. However, one major drawback of PDX is the lack of an immunological competent host. To overcome this hurdle, the use of humanized mouse models is gaining more and more importance. The current project aims to establish a drug screening workflow bridging between innovative mouse models and biomarker development. A total of 69 NOG (NOD/Shi-scid/IL-2Rγnull) mice were engrafted with CD34+ hematopoietic stem cells. Thereafter, tumor material from 11 different lung cancer patient derived xenograft models

was implanted subcutaneously. Individual mice were treated with α-CTLA-4, α-PD-1 or the combination thereof. With n=1 per treatment arm and model the study design followed the screening approach of the single mouse trial (SMT). Infiltration of human immune cells and PDL-1 expression was detected by flow cytometry (FC) and immunohistochemistry (IHC) in hematopoietic organs and tumor tissue. A computerized analysis for digitized whole-slide images of the samples was used to quantify the results using color classification and morphological image processing techniques. All 3 treatment arms displayed a discrete activity pattern throughout the PDX panel. Tumor models with high tumor infiltrating lymphocyte (TIL) rates in the donor patient material tended to be more sensitive towards treatment as models with low rates. PDX models being sensitive towards checkpoint inhibitor treatment (responders) displayed a higher percentage of DAB+ nuclei in huCD45 IHC stains than non-responder models as determined by image analysis. Irrespective thereof, in responders as well as non-responders the treatment with checkpoint inhibitors enhanced the percentage of huCD45 cells. PDL-1 expression, determined by IHC, of the tumor cells was influenced by the immune status of the murine hosts. Low expressers showed a distinct upregulation in humanized mice as compared to conventional NOG. Models already exhibiting a very high expression in conventional mice depicted no further increase. In humanized mice the PDL-1 expression was significantly higher ($p < 0.05$, t-test) in responders vs non-responders. Whole-slide image analysis of the H&E stains revealed an increase of the stromal compartment proportion in the tumor tissue under treatment in responder models. In non-responder models the ratio between tumor and stroma was not influenced by drug treatment. The use of PDX based humanized mouse models in a SMT format allows screening approaches in complex mouse models. The combination with a comprehensive image analysis tool enables additional read-outs to quantify antitumoral activity of immune modulatory compounds. The latter can be used to identify translational biomarker candidates in the preclinical setting.

Keywords: humanized mice, PDX, checkpoint inhibitor

A152 / Vitiligo susceptibility as predictor for immune checkpoint inhibitor response in melanoma patients; a new angle toward personalized treatment

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Immune checkpoint inhibitors (ICI) are a new class of cancer therapies targeting CTLA-4 or PD-(L)1 molecules on T cells and can confer impressively long-term survival in advanced melanoma patients, but also severe and potentially life-threatening side-effects. However, response rates remain low to moderate (< 20% for ipilimumab and 30-40% for PD-1 inhibitors, or up to 60% for a combination of anti CTLA-4 and PD-(L)1 therapy) at high therapy costs.

This puts a significant burden on the patient's quality of life, especially if the treatment is ineffective. There is no biomarker yet with sufficient power to predict response to immune checkpoint blockade. It would be considerable in identifying reliable predictive (bio-)markers to select patients who would benefit from anti CTLA-4 or PD-(L)1 treatment and reduce the number of patients receiving immune checkpoint inhibitors without benefit and treatment delay due to unresponsiveness.

A large systematic review has demonstrated that melanoma patients who develop vitiligo during immunotherapy have a significantly longer progression-free and overall survival than patients without vitiligo. Recently, vitligo susceptibility gene profile has been identified.

The purpose of this study is to conduct vitiligo SNP genotyping using an illumina Infinium® global screening array from Illumina® and develop a genetic risk score for ICI response in melanoma that allows for implementation in clinical setting using a multicenter retrospective and prospective cohort study.

We are gathering 1410 patients (850 in an index cohort and 660 in a validation cohort) who are/have been treated with CTLA-4 or PD-(L)1 immune checkpoint inhibitors. In these patients, we will analyse 55 SNPs (48 genome-wide significant and 7 suggestive vitiligo susceptibility loci) in 30 vitiligo susceptible genes. We calculate the predictive value of the entire set of SNPs using genetic risk scores, estimated by the number of minor alleles (0-55) per patient. The differences in number of minor alleles in responder versus non-responders will be tested using a t-test (or Mann Whitney test, in case in non-parametric data). Correlations

of the genetic risk score with survival will be analysed by logistic or Cox regression analyses, corrected for confounding factors.
This project will reveal whether vitiligo genetic susceptibility SNPs are associated with clinical response and/or survival upon anti CTLA-4 or PD-(L)1 therapy and which SNPs show superior associations. Based on these data, a genetic screening consisting of a subset of genes mostly associated with responsiveness will be defined that can be tested further for its value as predictive genetic biomarker to select patients with optimal therapeutic benefit of ICI.

Keywords: Immune checkpoint inhibitors, genetic profile, response to treatment

A153 / Engineering a 4-1BB agonist antibody with enhanced stability, affinity and T cell activation properties

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4-1BB is a member of the tumor necrosis factor superfamily and plays a critical role in activation of cytotoxic T cells to promote anti-tumor immunity. As a co-stimulatory molecule, it is ideally suited for activation via agonist therapeutic antibodies in order to reactivate the immune system and promote cytolytic activity through CD8+ T cells. However, chronic activation of 4-1BB may have detrimental effects on immune cells, such as CD4+ T cell depletion in animal models. Agonist antibodies against 4-1BB have been tested in the clinic and although the agent was well tolerated, up to 15% of patients had grade 3 or higher treatment related adverse events associated with elevated liver enzymes, suggesting potential safety concerns at higher doses. Hence, it may be necessary to temper activation of 4-1BB such that the pathway is not chronically hyper stimulated. Our approach has been to develop agonist antibodies against 4-1BB that have been engineered to represent varying degrees of functional activity and affinity for the target in order to leverage the benefit of 4-1BB agonism without over-stimulating the pathway. In one example, we generated two antibodies, 093 and 094 with very similar binding specificity for 4-1BB on cells and by ELISA that do not block ligand binding. Activation in an NFkB luciferase reporter assay and IL-2 secretion from T cells was similar between the two clones but significantly weaker than that induced by the anti-4-1BB benchmark antibody Urelumab . The physical properties of antibody 093 were far inferior to clone 094; hence we switched the heavy chain of 093 with that of 094 which led to a marked improvement in purity by size exclusion chromatography (SEC) and capillary isoelectric focusing (cIEF). The antibody with the switched heavy chain also had functional improvements in T cell activation as measured by IFNγ secretion. Clone 093 had weak potency and the maximum IFNγ secretion was 2 fold lower than Urelumab. Similarly, clone 094 had potency that was 7 fold weaker than Urelumab and 1.5 fold lower maximum secretion of IFNγ compared to this benchmark. Of note, the potency of the 093 light chain/094 heavy chain hybrid antibody had potency similar to Urelumab but 2.5 fold lower maximum secretion of IFNγ relative to Urelumab. With this set of antibodies and additional clones that are under evaluation, there is potential to temper activation through the 4-1BB pathway which may enable in vivo efficacy in tumor models through expansion of CD8+ T cells while allowing other critical immune cells to survive.

Keywords: 4-1BB, T cell Activation, Agonist Antibody

A154 / A single institution experience of anti-PD-1 antibody in cutaneous squamous cell carcinoma (cSCC)

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Background: cSCC represents one of the most common malignancies but there is no standard of care therapy for unresectable or metastatic disease. Checkpoint inhibitors, such as anti-PD-1 antibodies (PD-1 Ab), have shown promising efficacy in cutaneous malignancies including melanoma and Merkel cell carcinoma, but their efficacy in cSCC is unknown. Here we report our institutional experience of off-label PD-1 Ab use in patients (pts) with unresectable or metastatic cSCC.
Methods: We performed a retrospective analysis of clinical data of pts with cSCC who were treated with either nivolumab or pembrolizumab at Massachusetts General Hospital Cancer Center. Patient characteristics and treatment responses were summarized and analyzed by descriptive statistics.
Results: A total of 13 pts with cSCC treated with at least 2 doses of PD-1 Ab were identified. Eight pts were treated with pembrolizumab and 5 with nivolumab. Median age was 73 years (range 18-90). Nine pts (69%) were male and 11 (85%) were white. The head and neck was the most common area of primary tumor (8/13, 62%), while the remainder arose in the extremities. The cohort had diverse underlying risk factors; 2 pts have xeroderma pigmentosum, 2 pts have Marjolin's ulcer, and 3 pts have a history of leukemia/lymphoma treated with chemoimmunotherapy. Five pts (38%) had cytotoxic chemotherapy prior to PD-1 Ab treatment. Five pts had molecular testing: TP53 alteration was present in all 5. PIK3CA, BRCA2, NF1, CDKN2A, and TERT promoter variations were detected in 2 pts each.
At a median follow-up of 9 months (range 0.7-13), 12 pts were evaluable for treatment response: 6 pts (50%) achieved objective

responses with 1 complete response. Five pts had stable disease and 1 pt had primary resistance. The majority of responses were observed within 10 weeks from treatment initiation (mean 10.5, range 6.3-15.3). Pseudo-progression was observed in 1 pt: a pulmonary metastatic lesion increased on the first assessment from 7.5 x 4.4 cm to 9 x 5 cm but subsequent imaging showed a dramatic decrease in size to 1.5 x 1.6 cm. Estimated 12-month progression-free survival (PFS) rate was 52.4 % and median PFS was not reached. Seven pts remain on treatment. Two pts discontinued treatment due to immune-related adverse events (irAE) (colitis and dermatitis) but both pts have ongoing response off-treatment. Treatment was generally well-tolerated: 3 pts (23%) had grade 3-4 toxicities and 2 pts with irAE required immune suppressive treatment.

Conclusion: Our single institution experience with PD-1 Ab therapy in pts with cSCC shows promising activity with a high response rate and durable responses. The treatment was well-tolerated in most patients with no unexpected AEs. Molecular analysis of these pts to identify potential predictive biomarkers is ongoing. PD-1 Ab therapy merits further study in pts with cSCC.

Keywords: Cutaneous squamous cell carcinoma, anti-PD-1 antibody, Antitumor efficacy

A155 / Novel antibody blocking the CD47-SIRPα immune checkpoint as an improved strategy for Acute Myeloid Leukemia immunotherapy

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The cell surface protein CD47 is ubiquitously expressed on many cells in the body and functions as a "marker of self". Upon interaction with its myeloid-specific receptor Signal Regulatory Protein alpha (SIRPα), a "don´t eat me" signal that inhibits phagocytosis is delivered to phagocytic cells. Notably, CD47 is overexpressed on a variety of tumor cells, including Acute Myeloid Leukemia (AML) cells, thus conferring immune resistance. Recent studies have shown that blocking the CD47-SIRPα signaling pathway with CD47-directed antibodies enhances phagocytosis and increases antitumor immune effects. The expression of CD47 on healthy cells, however, creates an antigen sink and potential sites of toxicity and therefore limits the efficacy of CD47-directed therapies. In order to inhibit the interaction between CD47 and SIRPα at the tumor site, we developed the so-called "local inhibitory checkpoint monoclonal antibodies" (licMABs). LicMABs were created by grafting the endogenous N-terminal Ig domain of SIRPα onto the variable light chain of an IgG1 antibody targeting CD33, a surface antigen expressed in AML. We evaluated these novel therapeutic agents based on their binding specificity, cytotoxic effect and ability to promote phagocytosis of AML cells *in vitro*. Due to the high affinity for CD33 and the endogenous low affinity for CD47, licMABs selectively bind CD33-expressing cells even in the presence of a large CD33-negative CD47-positive antigen sink. Moreover, licMABs stimulate phagocytosis of AML cells and eliminate primary, patient-derived AML cells. Collectively, our findings qualify licMABs as a promising therapeutic approach to confine the benefit of disrupting the CD47-SIRPα axis to tumor antigen-expressing cells.

Keywords: Therapeutic antibody, CD47 - SIRPα, Acute Myeloid Leukemia

A156 / Cyclooxygenase-2 inhibitors and anti-PD-1 blockade synergistically promote tumor rejections in cancer models poorly responsive to mono-therapy

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To combat immune recognition and regulation, tumors have evolved a plethora of evasive mechanisms, many of which constitute immunotherapeutic targets. Expression of cyclooxygenase (COX)-2 and its main downstream enzymatic product, the inflammatory lipid prostaglandin E2 (PGE_2), is a common feature of several cancers. We have previously shown intra-tumoural expression of the COX-2/PGE_2 pathway is associated with a drastic shift in the inflammatory profile at the tumour site, favoring immune evasion and tumour progression. We therefore hypothesized that therapeutically targeting the COX-2/PGE_2 pathway would enhance the efficacy of immune checkpoint blockade against tumors resistant to this treatment modality. Co-administration of systemic anti-PD-1 antibody and selective COX-2 inhibitors to mice with established tumors resulted in full tumor rejections in a large proportion of mice otherwise unresponsive to either single-therapy. Likewise, anti-PD-1 blockade led to complete responses in mice bearing tumors formed by cancer cells genetically engineered to lack COX-2 expression but not by COX-2-competent control cells. The synergy between anti-PD-1 and COX-2 inhibition was conserved across multiple cancer models and using different COX-2 inhibitors, suggesting tumour-cell derived

PGE_2 is a dominant mechanism of immune escape. In conclusion, our data indicates COX-2 inhibitors act synergistically with immune checkpoint blockade therapy and implies that up-regulation of the COX-2/PGE_2 pathway could constitute a mechanism of intrinsic and/or acquired resistance to immunotherapy.

Keywords: Cyclooxygenase-2, Checkpoint blockade therapy, Immune evasion

A157 / Modeling melanoma response to immune checkpoint blockade for the functional study of neoantigens in mice

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Metastatic melanoma is notoriously heterogeneous and aggressive, accounting for most skin-cancer-related deaths. Treatment of late-stage melanoma is extremely challenging due to frequent resistance to conventional and targeted therapies, resulting in < 10% overall survival of patients with distal metastasis. Recent development of Immune Checkpoint inhibitors (ICi), such as antibodies against CTLA-4 and PD-1/PD-L1, has led to unprecedented durable responses in 20-30% of melanoma patients. High mutational and neoantigen load have been shown correlated with ICi efficacy in melanoma and other cancers. However, mechanistic studies are limited in humans and the molecular determinants of ICi response are mostly unknown. To recapitulate human melanoma heterogeneity, we developed three carcinogen-induced syngeneic mouse models, harboring distinct genetic modifications: (i) UV-induced melanomas in $Braf^{V600E/+}$; $Pten^{flox/+}$; $Cdkn2a^{flox/+}$; Tyr-Cre^{ERT2}-tg mice (UBPC), (ii) DMBA-induced melanomas in HGF-tg; $Cdk4^{R24C}$ mice (DHC) and (iii) UV-induced melanomas in HGF-tg mice (UH). While UH and DHC melanomas demonstrated high or partial sensitivity to anti-CTLA-4, respectively, UBPC exhibited intrinsic resistance. We hypothesized that effective anti-CTLA-4 therapy depends upon the immunogenic properties of melanoma cells. Using ***in vivo*** vaccination assays we confirmed that high immunogenicity correlates to greater anti-CTLA-4 response. Notably, all 3 models had similar expression of the antigen presentation related genes (e.g. Tap1 and MHC-I) and showed no difference in cytotoxic T-cell reactivity assays, indicating they exhibited functional antigen presentation pathway. Moreover, exome and RNA sequencing analyses revealed similar mutational and neoantigen load in the three models, albeit with no common expressed mutations. These results led us to hypothesize that specific neoantigens in UH cells may contribute to their sensitivity to anti-CTLA-4. To test this, we predicted MHC-I/-II binding of UH mutated epitopes ***in silico*** and generated a "neo-epitope" library which was transduced into UBPC non-responder cells. Future studies will identify the neo-epitopes lost from tumors upon anti-CTLA-4 treatment, representing determinants of therapeutic success. We anticipate that our study will provide insight into the role neoantigens play in melanoma immunotherapy responses, offering a platform for identifying individuals who will respond to ICi.

Keywords: Preclinical mouse models, Neoantigens, Melanoma

A158 / Phase 1 study to evaluate safety and efficacy of ipilimumab + nivolumab + external beam radiotherapy in patients with metastatic melanoma

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Immunotherapy with checkpoint blocking antibodies has led to progress in metastatic melanoma with FDA-approved drugs, including the combination of ipilimumab (IPI), a cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blocking antibody, and nivolumab (NIVO), a programmed cell death-1 (PD-1) blocking antibody. Although radiotherapy (RT) is primarily used as local palliative therapy in metastatic melanoma, it also possibly affects systemic antitumor immunity. Preclinical data suggest that RT alters the tumor microenvironment and renders tumor cells more susceptible to immunologically-mediated disease regression. These preclinical immunologic effects of RT have been shown to vary by RT dose and fractionation. This ongoing Phase 1, open-label, multicenter study (NCT02659540) is the first clinical trial in patients to evaluate the triple combination of IPI + NIVO + RT using 2 different dose/fractionation schemes of RT. The study is designed to evaluate the safety, efficacy, and immunologic effects of IPI + NIVO + RT in 18 patients with unresectable stage IV melanoma. Patients must have 1 melanoma metastasis that can be safely irradiated for palliative purposes and at least 1 measurable lesion that will not be irradiated. Patients receive concurrent IPI (3 mg/kg) and NIVO (1 mg/kg) every 3 weeks for 4 treatments, followed by NIVO monotherapy (240 mg every 2 weeks), with RT initiated between the first and second doses of IPI + NIVO. In Cohort A, the irradiated metastasis receives a conventionally fractionated RT dose of 30 Gy in 10 fractions of 3 Gy each over 2 weeks. Prior experience with IPI + NIVO combination therapy was associated with a treatment-related Grade 3/4 toxicity rate of approximately

53%. Given the addition of RT to the combination regimen, if ≤7 of 9 patients (78%) in Cohort A experience Grade 3/4 drug- or radiation-related adverse events, Cohort A safety is deemed acceptable and Cohort B enrollment opens. In Cohort B, the irradiated metastasis receives a hypofractionated RT dose of 27 Gy in 3 fractions of 9 Gy each over 2 weeks. The primary endpoint for the study is safety according to the National Cancer Institute Common Terminology Criteria for Adverse Events. Secondary endpoints are objective response rate and disease control rate evaluated at Weeks 12 and 18 per the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 and immune-related RECIST, duration of response, progression-free survival, and overall survival. Exploratory endpoints include correlative studies of immunological effects. Enrollment opened on 05 August 2016. As of 07 June 2017, 10 patients are enrolled. Enrollment is ongoing.

Keywords: ipilimumab + nivolumab, radiotherapy, metastatic melanoma

A159 / Tumor-specific delivery of αPD-1 checkpoint-inhibitor by receptor-targeted AAV

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Antibodies directed against immune checkpoints have revolutionized cancer therapy with now several products on the market that substantially prolong survival of melanoma and lung cancer patients. ***Nivolumab*** is directed against PD-1 (programmed death-1) thereby preventing the inactivation of T cells by cancer cells expressing the PD-1 ligand PD-L1. While the clinical response is at least in some cancer patients impressive, therapy is often associated with serious immune-related adverse events. Therefore, the aim of this study is to specifically deliver αPD-1 precisely to sites of tumor growth and thus reduce side effects and enhance the therapeutic benefit.

As vehicle for the delivery of checkpoint inhibitors tumor-targeted AAV vectors are used. We have recently shown that Her2/neu-targeted AAV vectors (Her2-AAV) selectively transduce Her2/neu-positive tumor cells after i.v. injection into immunodeficient mice. In the present study, we first assessed tumor-targeting in an immunocompetent mouse model. ***In vivo*** imaging analysis revealed that non-targeted AAV2 transduced mainly liver while Her2-AAV successfully targeted tumor cells after systemic injection into BALB/c mice bearing subcutaneously growing RENCA-Her2/neu-tumors. When equipped with the coding sequence for a mouse PD-1 specific miniantibody (scFv-Fc fusion protein), the inhibitor was readily detectable in the supernatant of tumor cells transduced with the resulting Her2-AAV$^{\alpha PD\text{-}1}$ and showed specific binding to its target antigen PD-1. Upon systemic administration of Her2-AAV$^{\alpha PD\text{-}1}$ into the aforementioned tumor mouse model, αPD-1 miniantibodies were mainly produced in tumor tissue. Levels in liver were at least 10-fold lower. In contrast, mice injected with non-targeted AAV2$^{\alpha PD\text{-}1}$ revealed the highest level of αPD-1 miniantibody in liver. AAV-constructs were next designed in order to deliver the entire sequence of the therapeutic antibody ***Nivolumab. In vitro*** analyses revealed successful expression upon transduction of MDA-MB-453 cells as well as reliable binding and functionality of AAV-encoded ***Nivolumab*** to a human PD-1-expressing T cell line.

In conclusion, we showed that tumor-specific delivery of checkpoint inhibitors can be achieved with receptor-targeted AAV vectors. The vectors are now available for preclinical studies to evaluate their antitumoral activity in combination with chemo- or immunotherapy.

Keywords: Immune checkpoint blockade, Adeno-associated viral vector, Gene therapy

A160 / Emergence of high avidity Melan-A specific neoclonotypes as a marker of anti-PD-1 clinical efficacy

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Therapeutic strategies using anti-PD-1 antibody reported unparalleled effectiveness for cancer immunotherapy. Understanding immune mechanisms involved in clinical benefit is crucial to improve patients' management.

The specificity of CD8 T cells reinvigorated upon PD-1 blockade in cancer patients remains largely elusive. Previous studies mainly focused on neoantigen-specific T-cell repertoires as a high mutational load has been previously associated with clinical benefit. Nonetheless, even if neoantigen-specific T cells are of great interest for anti-tumor responses, the entire tumor-specific T cell repertoire is a potential target of anti-PD-1 therapy.

Despite its negative role in anti-tumor immunity, PD-1 first identifies reactive tumor-specific T-cells. We previously demonstrated that PD-1^{pos} melanoma specific T-cell clones exhibited a better functional avidity than their PD-1^{neg} counterpart. We further documented ***in vitro*** that PD-1 blockade during the selection and amplification process of melanoma specific T-cells from patients' PBMC, resulted in the proliferation of specific T-cells with a biased TCR Vbeta repertoire exhibiting a better functional avidity (***Simon et al., Oncoimmunol, 2015***).

We assumed that this bias in antigen specific T-cell repertoire also occurs ***in vivo*** for patients treated with anti-PD-1 antibody. We compared Melan-A specific T-cell repertoire diversity from melanoma patients before and after anti-PD-1 therapy. We documented, for all patients tested, a bias in Melan-A-specific T-cell repertoire after treatment with the preferential emergence of new Melan-A specific T cell clonotypes (neo-clonotypes, not detectable before therapy) in responding patients, with a monoclonal expansion in patients with a complete response. These neo-clonotypes could be identified by a sustained co-expression of PD-1 and TIGIT receptors and displayed a higher functional avidity for their cognate antigen than clonotypes not amplified upon anti-PD-1 therapy.
Thus, in addition to the emergence of neo-antigen specific T-cells previously documented upon anti-PD-1 therapy, this work is the first demonstration of PD-1 induced changes within a vast T-cell repertoire specific for a shared melanoma antigen and describes the emergence of high avidity Melan-A specific neo-clonotypes as a surrogate marker of treatment efficacy. In contrast to neoantigen specific T-cell repertoire that are mostly patient-specific, such a vast and shared specific T-cell repertoire would be of great interest for the early immune follow-up of anti-PD-1 treated patients.

Keywords: anti-PD-1, neo-clonotypes, immune follow-up

A161 / Multimodality mapping and analysis of the CD38 expression landscape in various human cancers

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CD38 is emerging as an exciting immune oncology target. Pervasive expression of this pleiotropic, membrane-bound enzyme in multiple myeloma spurred the development of antibodies (Daratumumab, Isatuximab, Mor202) targeting CD38 directly on tumour cells but emerging data suggests a broader, multifaceted immune regulatory role for CD38. There is, however, a paucity of data on the expression of this target in human cancer. We took an unbiased approach gathering data to show CD38 distribution in in various cancers.
Using mRNA expression libraries, immunohistochemistry and flow cytometry CD38 expression was mapped out with a view to interrogating the complex dynamic that exists between CD38 activity and that of other known and unknown drivers of the immune system.
Bioinformatic sampling of the cancer genome atlas led to inferences about both tumor immunity and the relative expression of CD38 among various lymphoid subsets. Specific hematopoietic signatures were used to deconvolve the mRNA data and identify CD38 expression among various immune and primary tumor cells. CD38-anchored clustering procedures revealed striking and potentially non-obvious similarities between key cell types. Key differences between different tumor types were found according to their constituent immune populations. Phenotypic roles for CD38 were investigated using simple, linear correlation between expression of known drivers such as Foxp3 and PD-1/PD-L1 and also by association with known cell types.
Immunohistochemical and flow cytometry techniques confirmed CD38 expression across common cancer types including Central nervous system, Esophagus, Stomach, Liver, Colon, Rectum, Lung, Bladder, Heart, Kidney, Thyroid, Pancreas, Uterus, Skin, Breast, Ovary, Prostate and testis.
CD38 expression was largely confined to the leucocyte subset in tumor tissue, and rarely found on tumor cells, with the exception of tumors with previously published CD38 expression such as prostate.
CD38 expression among immune cell subsets was found to be variable on natural killer cells, macrophages and T and B (effector and suppressor) cells, many of which differed notably by their pattern of expression of CD38 from one tumor type to the next. Correlations with CD38 expression and genuine checkpoint inhibitors were generally positive.
We conclude that CD38 mapping reveals a complex leucocytic architecture in tumors. We are currently performing high-dimensional cytometry and multiplex IHC analyses on multiple tumor types to further characterize the distribution of this novel target on effector and regulatory cells. Our analyses confirm the positioning of CD38 as a new immune oncology target and indicate that CD38 targeting agents could have immune modulatory properties that extend beyond direct killing of CD38 expressing tumor cells.

Keywords: CD38, checkpoint, mapping

A162 / Identification of CMTM6 and CMTM4 as PD-L1 protein regulators

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The clinical benefit in patients with diverse types of metastatic cancers that is observed upon blockade of the PD-1 - PD-L1 interaction has highlighted the importance of this inhibitory axis in the suppression of human tumor-specific T cell responses.

In spite of the key role of PD-L1 expression by cells within the tumor micro-environment, our understanding of the regulation of the PD-L1 protein is limited. Using a haploid genetic screen, we here identify CMTM6, a poorly described type 3 transmembrane protein of previously unknown function, as a regulator of the PD-L1 protein. Interference with CMTM6 expression results in impaired PD-L1 protein expression in all tumor cell types tested and also in primary human dendritic cells. Furthermore, through both a haploid genetic modifier screen in CMTM6 deficient cells and genetic complementation experiments, we demonstrate that this function is shared by its closest family member CMTM4, but not by all other CMTM members tested. Notably, CMTM6 increases the PD-L1 protein pool without affecting PD-L1 transcript levels. Rather, we demonstrate that CMTM6 is present at the cell surface on tumor cell lines and patient samples, associates with the PD-L1 protein and reduces its ubiquitination, and increases PD-L1 protein half-life. Consistent with its role in PD-L1 protein regulation, T cell inhibitory capacity of PD-L1 expressing tumor cells is enhanced by CMTM6. Collectively, our data reveal that PD-L1 relies on CMTM6/4 to efficiently carry out its inhibitory function, and suggest potential new avenues to block this pathway.

The first 3 mentioned authors contributed equally to this work.

Keywords: PD-L1 protein regulator, CMTM6/CMTM4, Haploid genetic screen

A163 / Efficacy of combined PD-1/CTLA-4 blockade in melanoma brain metastases requires extracranial disease and enhanced CD8+ T cell trafficking to the brain

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Inhibition of immune checkpoints Programmed Death 1 (PD-1) and Cytotoxic T-lymphocyte Associated Protein 4 (CTLA-4) on T cells results in durable anti-tumor activity in melanoma patients. Despite high frequency of melanoma brain metastases (BrM) and associated poor prognosis with a median survival time below one year, the activity and mechanisms of immune checkpoint inhibitors in BrM remain elusive. Notably, BrM in patients mostly coincide with metastases outside the brain and to mimic this clinical situation, we established a tumor transplantation model bearing extracranial (subcutaneous) plus intracranial tumors. Combined PD-1/CTLA-4 blockade, rather than monotherapies, significantly inhibited intracranial tumor growth and prolonged survival. Strikingly, the extracranial tumor was required for intracranial anti-PD-1/anti-CTLA-4 efficacy, as the therapy was ineffective in a model bearing intracranial tumor alone. The therapeutic efficacy also required CD8+ T cells. However, PD-1/CTLA-4 blockade failed to enhance CD8+ T cell activation and expansion within intracranial tumors, and intracranial anti-PD-1/anti-CTLA-4 activity thus relied on the enhanced recruitment of CD8+ T cells that have been activated/released from immune checkpoint inhibition outside the brain. A drastic (~14-fold) increase in homing of CD8+ T cells to intracranial tumors was observed following PD-1/CTLA-4 blockade, concomitantly with marked upregulation of IFNγ and T cell entry receptors ICAM-1 and VCAM-1 on tumor vasculature. Our study indicates that extracranial activation/release of CD8+ T cells from PD-1/CTLA-4 inhibition and upregulation of T cell trafficking determinants in the brain are paramount to the intracranial anti-PD-1/anti-CTLA-4 activity, suggesting augmentation of these processes as an immune therapy-enhancing strategy in metastatic brain cancer.

Keywords: Anti-PD-1, anti-CTLA-4, melanoma brain metastases, T cell trafficking

A164 / GITR agonists enhance the functionality of tumor-infiltrating T cells in hepatocellular carcinoma and liver metastases of colorectal carcinoma

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Effective cancer therapeutics are in high demand for hepatocellular carcinoma (HCC) and liver metastases of colorectal carcinoma (LM-CRC). Check-point inhibitors blocking e.g. PD-1:PD-L1 interaction, which are approved for several other cancer subtypes, have not shown durable responses in the majority of HCC and CRC patients. Next generation immune-therapeutics for cancer include agonist antibodies of costimulatory receptors like 4-1BB, OX-40 and GITR. This research evaluates a proof of concept that costimulatory agonists may be effective for HCC or CRC immune-therapy. Previously, we demonstrated that agonistic targeting of GITR on $CD4^+FoxP3^+$ regulatory T cells (Treg) isolated from HCC and LM-CRC tumors could reduce their suppressive capacity. Here, we studied whether agonistic targeting of GITR could reinvigorate functional responses of tumor-infiltrating lymphocytes (TIL) from HCC and LM-CRC patients. We used paired samples of leukocytes freshly isolated from peripheral blood, tumor-free liver tissues (TFL) and resected liver tumors of 23 patients with HCC and 33 patients with LM-CRC.

First, we measured GITR expression on T cells by flow cytometry. We found that expression of GITR was significantly higher on $CD4^+FoxP3^+$ Treg in TIL in primary as well as secondary liver tumors as compared with those in blood and TFL, and compared with $CD4^+FoxP3^-$ T helper cells and $CD8^+$ cytotoxic T cells in blood, TFL, and TIL. On average, 30% (range: 6-86%) and 40% (range: 16-85%) of Treg in HCC and LM-CRC TIL respectively, expressed GITR.
To determine the effect of GITR ligation, we performed ***ex vivo*** proliferation assays (at least n=5) with TIL from LM-CRC and HCC in the absence or presence of recombinant GITR-ligand (GITRL). Addition of GITRL to TIL derived from both LM-CRC and HCC patients increased proliferation of $CD4^+$ and $CD8^+$ TIL and enhanced IFN-γ production upon stimulation with anti-CD3/CD28 antibodies. In addition, GITRL enhanced proliferative responses of HCC-derived $CD4^+$ and $CD8^+$ TIL to the tumor antigens Glypican-3 and MAGE-C2 presented by mRNA-transfected autologous B cell blasts.
As monoclonal antibodies are more amenable to therapeutic development than recombinant proteins, we assessed the effect of a humanized agonistic antibody against GITR (10H2#13, Pfizer). Similar to GITRL, the anti-GITR antibody enhanced the ***ex vivo*** proliferation and cytokine production of TIL derived from HCC and LM-CRC in both polyclonal and tumor antigen-specific assays.
Conclusions: GITR expression is up-regulated on $CD4^+FoxP3^+$ Treg in the tumors of HCC and LM-CRC patients. Stimulating GITR by GITRL or anti-GITR antibody enhanced proliferation and cytokine production of $CD4^+$ and $CD8^+$ TIL. Thus, GITR may be a promising target for immunotherapy in HCC and LM-CRC.

Keywords: GITR, Hepatocellular carcinoma, Colorectal carcinoma

A165 / Targeting tumor Cdk5 enhances immune sensitivity by diminishing interferon-gamma induced PD-L1 response and enhances efficacy of anti-PD-L1 blockade

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Emerging data support a critical role for PD-L1/PD-1 signaling in tumor immunity, with some therapy-refractory cancers dramatically responding to checkpoint blockade. However, despite this promising development a significant number of patients do not benefit, and additional cellular and molecular mechanistic understandings of signaling among tumor cell-tissue stroma-immune cells must be sought to further improve current therapies, to provide strong scientific basis for combination trial designs, and to circumvent undesirable side effects shown to be limiting and even life-threatening in some cases. Furthermore, tumor biopsies revealed that most pediatric and certain adult cancers do not express PD-L1 or express it at a low level, causing skepticism for potential efficacy of immune checkpoint blockade in these tumors. Recently, we reported that Cyclin-dependent Kinase 5 (Cdk5) has a surprising role in regulating tumor Programmed Death-Ligand 1 (PD-L1) in response to interferon-gamma (IFNγ) in a murine medulloblastoma (MB) model derived from ***Ptch$^{+/-}$/p53$^{-/-}$*** mice. $Cdk5^{def}$ MB solicits $CD4^+$ T cell-dependent rejection linked to enhanced tumor microenvironment (TME) IFNγ contents, increased PD-L1 expression in lymphoid and myeloid cells, and a blunted tumor PD-L1 expression in response to IFNγ in p53 and SHH altered MB and rhabdomyosarcoma. We have now gathered new data showing time-dependent efficacy of anti-PD-L1 blockade in $PD\text{-}L1^{def}$ MB. 5x10e4 $PD\text{-}L1^{def}$ or $Cdk5^{def}$ MB resulted in a 50% tumor incidence as compared to 100% tumor incidence with wild type MB. Weekly administration of anti-PD-L1 antibody to mice bearing $PD\text{-}L1^{def}$ MB starting at 7 days following tumor inoculation resulted in 100% tumor rejection, while injection of antibody treatment starting on day 0 resulted in only 30-50% tumor-free incidence. A significant reduction in wild type MB tumor growth can also be achieved by administration of a high-potency Cdk5 inhibitor via oral gavage, with resultant tumors showing an altered PD-L1, MHC-I and MHC-II response to IFNγ in vitro after culturing for 10 days in the absence of additional Cdk5 inhibitor exposure. Our data support the view that the ***absence of tumor PD-L1 does not preclude the potential utility of checkpoint blockade, as PD-L1 expression is a highly dynamic process that is critically affected by the tumor-immune interplay at the tissue microenvironment,*** and alternative cellular mechanisms of how PD-L1/PD1 interaction enhances immune function among non-tumor cells need be further examined. Our observations establish a functional link between an increasingly important serine/threonine kinase in cancer to immune checkpoint regulation and other aspects of tumor immunity, provide the scientific rationale for carefully timed application of checkpoint blockade in $PD\text{-}L1^{lo}$ tumors, and allow the pursuit of pharmacologic Cdk inhibitor to modulate tumor sensitivity for immunotherapy.

Keywords: Cdk5, PD-L1, Interferon-gamma

A166 / Characterisation of TCR Vβ repertoire in anti-tumour responses

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Background: Anti-PD-1 therapy has achieved clinical success in patients with various types of tumours. However, not all patients benefit and the underlying mechanism for this differential response is not clearly understood. Previous studies using the murine CT26

tumour model have identified that tumour protective GSW11-specific CD8+ T cells show differential phenotypes in mice that respond compared to those that do not, following anti-PD-1 therapy. In mice that respond, GSW11-specific T cells show lower affinity to GSW11 tumour antigen. Therefore, this difference in response may reveal a potential T cell receptor (TCR) signature that can be used to predict clinical responses. By examining the sequences of TCR in CT26 mice, we were able to characterise the clonality of the anti-tumour responses and provide a baseline for future experiments.

Methods: Mice were challenged with CT26 tumour and CD8+ cells were extracted from tumours, tumour draining lymph nodes (tdLN) and spleens. PCR-based spectratyping was used to characterise the Vβ usage of extracted cells.

Results: Following the establishment of a reliable PCR spectratyping protocol to amplify Vβ segments, we analysed CT26 challenged mice. Comparison of tumours to tdLN and spleen revealed a more focused Vβ usage in tumours compared to more distal tissues. Studying the clonality of TCR in detail, we also observed that tumour tissues have a more clonal TCR repertoire compared to other tissues. These findings indicate that the tumour is likely to be exerting selective pressure on the CD8+ T cells, defining the predominant clonality present.

Conclusion: The differential responses observed following anti-PD-1 treatment is not yet fully understood. In our model we were able to characterise the clonality of anti-tumour responses, identifying that CD8+ T cells repertoires are more clonal in the tumour. This finding lays the foundation for on-going further experiments in discovering a TCR signature for anti-PD-1 treatment responsiveness.

Keywords: TCR clonality, Anti-PD-1, Immunological landscape

A167 / Mass cytometry identifies PD1 and LAG3 immune checkpoints as promising targets for dual immunotherapy in the treatment of chronic lymphocytic leukemia

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Chronic lymphocytic leukemia (CLL) represents the most frequent leukemia in the western world. CLL progression is highly dependent on complex interactions within the tumor microenvironment (TM) composed of immune cells, stromal cells and other non-malignant cells and is associated with poor immune cell effector functions and dysfunctional anti-tumor immune responses. Immunotherapy tends to restore immune cell functions by modulating the cross-talk between cells within the TM, for example by immune checkpoint blockade. Despite recent advances in CLL treatment targeting the TM, CLL remains an incurable disease.

The aim of our study was to provide a comprehensive and deep insight into the CLL microenvironment to identify and characterize novel potential targets for an immunotherapeutic approach in a murine model of CLL. For this purpose, we performed adoptive transfer (AT) of murine splenocytes (Eμ-TCL1 model of CLL) for immune profiling of healthy and sick AT-TCL1 mice by single-cell mass spectrometry. A panel of 35 antibodies was designed to phenotype immune cells and to detect intracellular cytokines. Immunophenotyping revealed important phenotypic differences between normal B cells and CLL cells as well as relevant alterations in the composition of lymphoid and myeloid cell populations within the TM in AT-TCL1 mice. These alterations are associated with dysfunctional immune reactions and CLL cell migration. More precisely, in addition to exhausted CD8+ T cells, we identified a significant increase in the number of highly suppressive KLRG1+ regulatory T cells, of patrolling monocytes and of CD8+ dendritic cells known to produce less cytokines and to induce a weak T cell proliferation. We also detected an upregulated expression of the immune checkpoints Programmed cell Death 1 (PD1) and Lymphocyte Activation Gene 3 (LAG3) on most of immune cell populations within the TM.

Following this, AT-TCL1 mice were treated with antibodies blocking the pathways of PD1 and LAG3 or with respective isotypes. The disease was followed-up by blood analysis and then splenocytes were analyzed by mass cytometry. In contrast to single therapy, dual PD1/LAG3 blockade in AT-TCL1 mice effectively controlled CLL development in the spleen and in peripheral blood, as shown by the reduction in spleen weight and CD5+CD19+ cell number and percentage in both spleen and blood. Furthermore, alterations in the composition of cells within the TM induced by CLL were resolved pointing to a restoration of an anti-tumor immune response.

In conclusion, we extensively characterized cell populations within the CLL TM and detected an upregulation of the immune checkpoints PD1 and LAG3 associated with poor immune effector functions. Thus, the dual PD1/LAG3 blockade could have potential benefits in CLL to restore a functional anti-tumor immunity.

Keywords: Chronic lymphocytic leukemia, Mass cytometry, Dual anti-PD1 and -LAG3 blockade

A168 / Immune checkpoint modulation by urease-mediated alkalization

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Immune checkpoint proteins, such as cytotoxic T lymphocyte antigen (CTLA)4 and programmed death (PD)1, downregulate T cell responses in order to prevent autoimmunity and inflammation. However, tumor cells that overexpress the ligand for PD1, PDL1, inhibit the activity of local T cells, providing a survival benefit to the tumor cells. Antibodies that target PD1 (nivolumab, pembrolizumab) increase T cell activity and have provided remarkable outcomes in melanoma and lung cancer patients, and are now approved for clinical use. In this study, we describe a novel method to reactivate T cells by reducing PDL1 expression on tumor cells, using the previously described antibody-urease conjugate, LDOS47. LDOS47 is currently in Phase I/II testing for treatment of non-small cell lung cancer. It is prepared by conjugating urease to the camelid single domain antibody specific for human CEACAM6. The immunoconjugate specifically targets and delivers urease to CEACAM6-expressing cancer cells, where the urease enzyme converts urea into ammonia. The ammonia increases the pH of the tumor microenvironment ***in situ***. In this study, LDOS47 and urea were used to increase the extracellular pH of culture media for ***in vitro*** studies. The breast cancer cell line MDAMB231 was used as it is known to express high levels of PDL1 upon induction with IFNγ, which was confirmed by flow cytometry. Treatment of IFNγ-simulated MDAMB231 cells with LDOS47/urea restored PDL1 expression to levels observed on unstimulated cells. IFNγ-stimulated MDAMB231 cells were then treated with lactic acid, to reduce the pH of the tissue culture medium to mimic the low pH observed in the tumor microenvironment ***in vivo***. Levels of PD-L1 were significantly increased by lactic acid treatment compared to either untreated or IFNγ-treated cells. Once again, addition of LDOS47/urea restored PDL1 levels to that observed on untreated tumor cells. We have expanded this study to include additional cell lines, and observe that not all cell lines are sensitive to lactic acid-induced PDL1 upregulation (including the SKOV3 ovarian cancer cell line). Work is ongoing to identify the factors responsible for this response, such as pH regulators, in order to understand the underlying mechanism. We previously showed that Jurkat T cells incubated in acidic tissue culture media had impaired activity, which could be partially restored by treatment with LDOS47/urea. Thus, LDOS47 could potentially modulate the cancer immune response through multiple mechanisms: by reducing PDL1 expression on tumor cells and by enhancing proliferation and cytokine production of T cells.

Keywords: PD-1, immune checkpoint, tumor microenvironment

A169 / LAG-3 and PD-L1 inhibitors boost the immune function of tumor-infiltrating T cells in mismatch repair-proficient liver metastasis of colorectal cancer

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Targeting the PD-1/PD-L1 co-inhibitory pathway is a promising novel treatment for several types of cancer including mismatch repair (MMR)-deficient colorectal cancer (CRC), but not effective in MMR-proficient CRC. Liver metastasis (LM) develops in 40%-50% of patients with MMR-proficient CRC, and is a leading cause of CRC-related death. Therefore, we determined which co-inhibitory pathways can be targeted to enhance the function of tumor-infiltrating lymphocytes (TIL) in MMR-proficient LM of CRC (LM-CRC). The expression of co-inhibitory receptors and their ligands was measured on leukocytes freshly isolated from paired resected metastatic liver tumors, tumor-free liver tissues (TFL), and blood of patients with MMR-proficient LM-CRC. The effects of blocking co-inhibitory pathways on TIL responses were studied in ***ex vivo*** assays. Finally, the intra-tumoral expression of co-inhibitory molecules was compared among MMR-proficient LM-CRC, peritoneal metastasis from CRC (PM-CRC) and primary CRC tissues. PD-1, TIM-3 and CTLA-4 were higher expressed on $CD4^+$ T helper cells, while PD-1, LAG-3 and TIM-3 were higher expressed on $CD8^+$ cytotoxic T cells in LM-CRC tumors than in TFL and blood. Antigen-presenting cells in tumors expressed their ligands. LM-CRC TIL expressing those co-inhibitory receptors showed a more activated phenotype but comparable or reduced cytokine production compared to the TIL without co-inhibitory receptor expression. Importantly, blocking LAG-3 or PD-L1 with neutralizing antibodies increased proliferative and cytokine responses of LM-CRC TIL to both polyclonal and autologous tumor antigen stimulation. Interestingly, TIL of MMR-proficient LM-CRC revealed higher expression of several co-inhibitory receptors than TIL of MMR-proficient primary CRC tissues or TIL of MMR-proficient PM-CRC. Increased co-inhibitory receptor expression on LM-CRC TIL, possibly related to the tolerogenic liver environment, suggests that MMR-proficient LM-CRC may respond better to immune checkpoint inhibitors than MMR-proficient primary CRC or PM-CRC. Blocking LAG-3 or PD-L1 enhances ***ex vivo*** function of intra-tumoral T cells in MMR-proficient LM-CRC. Therefore, these two co-inhibitory pathways may be promising immunotherapeutic targets for the most prevalent secondary liver cancer.

Keywords: mismatch repair, colorectal cancer, tumor-infiltrating T cells

IMMUNOMONITORING AND BIOMARKERS

A170 / A mathematical model for predicting the dynamics of tumor-reactive lymphocytes in murine melanoma model

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In recent years immunotherapeutic approaches with the goal to harness the patient's T cell response have become a therapeutic option for several cancers. Still, the underlying mechanisms of the antitumor immune response are poorly understood which is reflected in the unpredictability of the individual patient's response to a particular immunotherapeutic treatment.
To further our mechanistic insights, we are devising a mathematical model of the anti-tumor T cell response in the B16-OVA murine melanoma model. Following inoculation of the B16-OVA tumor cells and the transfer of naïve OT-I CD8 T cells which are able to recognize an OVA-derived peptide, the OT-I proliferation was monitored using the cell dye Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) and cell numbers in all relevant compartments were determined (tumor, blood, draining and non-draining lymph nodes, spleen). In conjunction with the mathematical modeling, these data allowed us to quantify the time-dependent proliferative activity of the tumor-infiltrating lymphocytes in these compartments and to determine T cell differentiation as well as their subset-dependent migration behavior.
Our results indicate that the proliferative activity of the transferred OT-I cells in the draining lymph nodes depends on the differentiation marker CD27 and highly proliferated OT-Is could be found in the tumor. Interestingly, the model predicts ongoing strong proliferation of the T cells following tumor infiltration; model variants that aim to explain the measured number of divisions in the tumor solely by preferential emigration of highly proliferated T cells from the lymph node fail to describe the data. Based on this model, we are currently studying the effects of immunotherapeutic treatments on T cell dynamics.

Keywords: mouse model, mathematical model, CD8 T cell

A171 / Cancer-germline antigens discriminate clinical outcome to CTLA4 blockade

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Because CTLA4 immune checkpoint blockade is clinically effective only in a subset of patients with metastatic melanoma, we sought to interrogate and identify transcriptional determinants of clinical outcome specific to CTLA4 blockade. We analyzed RNA sequencing data from three previously reported clinical studies comprising four clinical treatment cohorts as well as both transcriptomic and DNA methylation data from The Cancer Genome Atlas. We further validated and extended our findings using RNA expression (RT-qPCR), DNA methylation (methylation-specific PCR), and protein-based (immunohistochemistry,immunofluorescence) techniques. Here, we identify a cluster of 8 cancer-germline antigens, located within a narrow 75 kb region of chromosome Xq28, that predicts resistance uniquely to blockade of CTLA4, but not PD1. We validate this gene expression signature in an independent anti-CTLA4-treated cohort and show its specificity to the CTLA4 pathway with two independent anti-PD1-treated cohorts. This signature was an independent risk factor for post-ipilimumab progression. Consistent with known epigenetic regulation of these genes, we demonstrate significantly decreased promoter methylation of MAGE-A3/A6 in resistant tumor samples. Furthermore, analysis of TCGA melanoma methylome data revealed global hypomethylation associated with high expression of this specific cancer-germline signature. Two of four significantly demethylated gene pathways were also upregulated in primary resistant samples in the discovery cohort, further implicating epigenomic dysregulation in primary resistance to anti-CTLA4 therapy. Finally we identify the damage-associated molecular pattern (DAMP) molecule HMGB1 as an in vitro target of ubiquitination by the MAGEA-TRIM28 ubiquitin ligase and show its mutual exclusivity with MAGE-A in vivo. Our findings suggest that degradation of the danger signal HMGB1 by

the MAGEA-TRIM28 ubiquitin ligase may mediate resistance to CTLA4 blockade via suppression of immunogenic cell death. These findings identify a unique pathway of primary resistance to CTLA4 blockade that could be exploited for therapeutic synergy with CTLA4 inhibitors.

Keywords: melanoma; ipililmumab, MAGE-A, HMGB1

A172 / Relevance of CA125, platelet count and neutrophil to lymphocyte ratio in the diagnosis and follow-up of ovarian cancer patients

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Objective: It has been shown that the immune system of ovarian cancer patients changes during the disease course. Literature has demonstrated correlations between commonly used variables in the clinic such as CA125, neutrophil to lymphocyte ratio (NLR), thrombocytosis and survival. This study explores these variables in ovarian cancer patients, and whether a correlation exists between these clinical variables and the immune system.
Methods: Serum samples of 52 ovarian cancer patients were collected at diagnosis and were retrospectively analysed for clinical characteristics, clinical parameters (NLR, CA125, platelet count, eosinophils, white blood cells (WBC)) and immune profile [IL-4 (interleukin), IL-10, IL-13, IL-17, TGF-β (transforming growth factor), arginase, IFN-γ (interferon gamma), VEGF (vascular endothelial growth factor), Gal-1 (galectin) and CCL-2 (chemokine (C-C) motif ligand 2)].
Results: Increasing NLR (p=0.0113), WBC (p= 0.003) and the absolute number of neutrophils (p=0.0013) were significantly correlated with decreasing overall survival. Increasing platelets were significantly correlated with increased risk of recurrence (p=0.0137). Eosinophil count was not correlated with survival. Platelet count was significantly correlated with IL-10 (p= 0.0001) and TGF-β (p= 0.029), NLR with arginase (p= 0.0482), high levels of CA125 (>105 kU/L) with IFN-γ (p=0.029) and the percentage of neutrophils with VEGF (p= 0.0104). FIGO stage III and IV were significantly correlated with thrombocytosis (p= 0.034).
Conclusions: Increasing amounts of blood platelets, CA125, NLR and neutrophils seem to be correlated with an inferior immune profile. This has important clinical relevance, as these variables can be used to select patients for clinical trials with immunotherapeutic drugs. For example, in ovarian cancer it was already observed that CA125 < 105 kU/L have more chance of responding to folate receptor targeted immunotherapy. This might be explained by decreased immunogenicity of ovarian cancer cells with elevated expression of CA125. Preclinical research has also shown that thrombocytosis reduces the efficacy of taxane-based chemotherapy, which might explain the success of combination therapy of Paclitaxel-Carboplatin after treatment with Paclitaxel monotherapy. In short, these clinical variables could be easily applied to tailor therapy for ovarian cancer patients.

Keywords: CA125, Platelets, Neurophil Lymphocyte Ratio

A173 / Using single cell TCRseq to study tumor antigen-specific T cell responses in mice

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Following the success of checkpoint inhibitors and the realization that (neo-) antigen-specific T cells are crucial for cancer regression, renewed interests in adoptive T cell therapy and cancer vaccination approaches have arisen. Immunotherapeutic strategies targeting individual tumor antigens (TAs) either passively using high-affinity T cell receptors (TCRs) or actively using RNA- or peptide-based vaccines are being highly investigated in mice and humans. On the one hand, tumor models are crucial for the development of novel immuno-oncology therapies, particularly when providing insight into the mode of action of TA-specific T cells. On the other hand, vaccination of HLA-transgenic mice with human self-TAs facilitates the discovery of high-affinity TCRs, which are deleted in humans through central tolerance mechanisms, and also enables the identification of immunogenic HLA-restricted T cell epitopes. Such mouse studies have created a strong demand for accessible technologies that allow high-throughput sequencing of paired αβTCR genes from splenocytes or tumor infiltrating lymphocytes in mice. Here, we present the application of a newly developed single cell (sc) TCRseq platform to identify TA-specific TCRs from splenic T cells isolated from HLA-A*0201/DRB1*0101 transgenic mice after vaccination with TA-encoding mRNA.
ScTCRseq makes use of template switching technology to minimize amplification bias and introduces universal primer sequences to reduce PCR complexity. Barcoding permits early pooling of single-cell samples and multiplexing of 96-well plates allowing little hands-on work at a high-throughput. A data analysis pipeline retrieves the paired αβTCR information from the 2x300 bp MiSeq data. TCRs of interest are easily cloned for functional validation using the remaining 1st strand cDNA.

Using this platform, we could rapidly identify functional TA-specific αβTCRs. Sequences and frequencies were comparable to those obtained using TCR repertoire profiling indicating that a representative sample of the repertoire was analyzed. Additionally, the single cell data provided insight into the complex TCR repertoire including clonal dominance and the presence of TCR chains shared amongst various expanded clones. In addition to the mentioned properties and results, the use of standard lab equipment renders the platform economical, easily applicable, and thus highly beneficial for diverse pre-clinical studies.

Keywords: TCR seq single cell murine, Tumor-antigen -specific T cells, Immunmonitoring

A174 / The dynamics of neoepitope recognition as response to therapy in a patient with bladder cancer

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Recent data suggest that a substantial fraction of the T cell reactivity induced during checkpoint inhibition is directed towards neoepitopes that are generated through genetic alterations. Specific forms of chemotherapy may also boost immune recognition. Here we evaluate the dynamics of neoepitope-specific T cell reactivity in one patient with bladder cancer who have received six consecutive treatments with cisplatin and gemcitabine chemotherapy and one cycle of PD-L1 checkpoint inhibition therapy.

We apply peptide-MHC multimers labeled with DNA barcodes for large-scale detection of patient specific neoantigens. We have analyzed peripheral blood drawn before initiating treatment and prior to the start of each cycle of treatment. Based on the patient's specific mutations and tissue type we have screened for antigenic recognition of a total of 268 potential neoantigens, restricted to HLA-A0201, A2601, B0702, B4402 and C0702. The DNA-barcode labeled MHC multimer approach allowed us to screen for responsiveness to all of the potential neoepitopes in parallel, which was essential in the limited material available. Additionally, we have monitored the dynamics of the immune systems responsiveness in terms of Granzyme B (GzmB), PD-1, ICOS, CTLA-1 and 4-1BB expression in $CD8^+$ T cells, $CD4^+$ effector cells and T regulatory cells. Finally, we have applied the DNA-barcode based approach in combination with staining of surface markers, enabling simultaneous assessment of target recognition and activation-status of the cancer-responsive T cells.

We observed a dramatic increase in $GzmB^+$, $PD\text{-}1^+$, $ICOS^+$ $CD8^+$ T cells after the 4^{th} cycle of chemotherapy, indicating an ongoing immune response. Moreover, we detected a total of 9 different neo-epitope responses over the course of therapy. Among these the majority of responses were detected after the 4^{th} cycle of chemotherapy and thus co-occurring with the overall change in the $CD8^+$ T cell phenotype. Among these neoepitope responses, a preferable recognition of clonally-derived antigens (7 of 230 possible) was observed over subclonally-derived antigens (2 of 38 possible), in line with observations made in earlier studies.

This case-study suggests that chemotherapy may indeed induce neoepitope recognition and potentially facilitate T cell mediated tumor cell killing when combined with checkpoint inhibition.

Keywords: Response to treatment, DNA-barcode labeled MHC multimers, neoepitope recognition

A175 / Comprehensive analysis of immune cell profiles in chronic myeloid leukemia (CML) and novel risk stratification model

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The immunologic landscape of the CML bone marrow (BM) remains unknown, although increasing evidence suggest the immune system to affect treatment response.

We constructed tissue microarrays (TMA) from BM biopsy samples of CML patients (n=56) and non-leukemic controls (n=14). Using a novel multiplex immunohistochemistry (mIHC) method, we stained TMA slides with up to six markers simultaneously from a total of 30 lymphoid, myeloid, and immune checkpoint markers. IHC was supplemented with machine-learning based image analysis. Immune cell subsets were compared using Mann-Whitney U test and complemented with Benjamini&Hochberg's correction (q-values). We developed a risk stratification model predicting the current treatment goal of tyrosine kinase inhibitor (TKI) therapy, molecular remission

4.0 (MR4.0), with an L1-penalized elastic net regression method. Findings were validated with flow cytometry (FC) of BM aspirates of CML patients (n=47).

Study patients were representative in terms of age, gender and Sokal, Hasford, and EUTOS risk scores when compared to the ELN/EUTOS CML registry ***'Out-study cohort'.*** We observed lower amount of CD3+CD4+ (2.3% vs. 7.9% of all BM cells, q< 0.001) and CD3+CD8+ (1.8% vs. 3.7% of all BM cells, q< 0.001) T cells in CML vs. control BM. CML patients' CD4+ and CD8+ T cells expressed higher levels of putative exhaustion markers PD1+ (11.5% vs. 1.7% and 9.6% vs. 2.0%, q< 0.001), TIM3+ (6.9% vs. 0.8% and 5.4% vs. 0.9%, q< 0.001), and CTLA4+ (45.0% vs. 0.9% and 48.8% vs. 1.2%, q< 0.001) when compared to non-leukemic BM. In addition, M2-macrophages (11.8% vs. 1.8%, q< 0.001) and myeloid-derived suppressor cells (3.4% vs. 0.037% of all cells, q< 0.001) were more numerous in CML vs. control BM. We noted lower total HLA-ABC expression (91.8% vs. 100.0%, q< 0.001), but slightly higher PD-L1 (2.6% vs. 1.7%, q=0.02) and PD-L2 (5.0% vs. 1.5%, q< 0.001) expression in CML vs. control BM. Lower CD4+ T cell count, higher PD1+TIM3- proportion of CD8+ T cells, and higher peripheral blood (PB) neutrophil count were associated with lower MR4.0 frequency after categorizing into three groups and adjusting with TKI therapy (intermediate-risk HR 2.5, 95%CI [1.1-5.4] and high-risk HR 10.6, 95%CI [4.3-25.8]). We observed a R2 of 0.42 (p< 0.001) and AUC of 0.85 (p< 0.001). In the validation cohort, high CD4+ T cell in the BM aspirate and low PB neutrophil count predicted MR4.0 (HR 1.8, 95%CI [0.97-3.5] and HR 0.50, 95%CI [0.26-0.96]).

The combination of mIHC and machine learning -based image analysis enables fast and objective tissue phenotyping. The CML BM is characterized with myeloid and lymphoid lineage immune suppression. The novel risk model based on immune cell profiles performed superiorly to clinically used risk scores in predicting MR4.0, but results remain to be validated in a larger cohort.

Keywords: Multiplexed immunohistochemistry, Immune checkpoint, CML

A176 / CIP NK proficiency panel 2017: Inter-lab variation in NK activation and functional markers is markedly reduced by the use of harmonised stimulation protocols, panels and gating strategies

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Phase I of an NK harmonisation proficiency panel, organised in association with the CIMT Immunoguiding Program (CIP), was run in 2014 to assess inter-lab variations when phenotyping human NK cells by flow cytometry. The 21 participants quantified predefined NK cell phenotypes but chose their own staining panel configuration and stimulation protocols. The findings of phase one were that overall there was low inter-lab variation with NK phenotypic markers CD56, CD16, NKp46 , but high variation with activation markers CD69+ and NKG2D and functional markers IFNγ and CD107a. These variations increased further following stimulation. Based on these findings, a second phase of the NK harmonisation panel has been run 2016 - 2017. Phase 2 used harmonised pre-defined stimulation protocols, and an harmonised common staining panel and gating strategy to aim to reduce inter-lab variation when phenotyping these activation and functional markers.

10 laboratories across Europe participated in phase 2 of the NK panel harmonisation initiative. Matched PBMC samples obtained from buffy coats of 3 UK national blood service donors were sent out to each laboratory, with identical batches of reagents for stimulation and staining (i.e. identical lots of fluorochrome-antibody conjugates). Activation and functional activity was measured, using 4 different stimulation conditions: Phytohaemagglutinin (10ug/mL PHA), activation induced by target cell K562 (PBMC:K562 cell ratio 10:1 with 1ng/mL IL15), activation driven by cytokine stimulation (1ug/mL IL2 & 10ng/mL IL15), and TLR 7/8 ligand (10ug/mL R848). 2 control conditions were performed, unstimulated cells and cells with addition of 1ng/mL IL-15 only. Stimulation, antibody staining, local analysis and manual gating were performed using an harmonized pre-defined protocol and gating strategy by each participating laboratory. Participants also uploaded their fcs data to 'ReFlow', an automated cluster analysis software, which has been developed in association with CIP, for data sharing and to compare cluster analysis against the local manual analysis.

The collated data has been analysed centrally in Southampton. Inter-laboratory variation in the activation and functional markers has been substantially reduced by the use of an harmonised protocol, and gating strategy. The differential effects of the different stimulation protocols on the phenotypic and functional markers is also presented.

Keywords: NK cell phenotype and function, Harmonisation, flow cytometry

A177 / DC cell activation by immunogenic nanoparticles monitored using Raman spectroscopy

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Immune cell activation is the key mechanism in regulating the overall immune response by converting the immune cells to mature cells mediated by costimulatory molecules or pathogens thereby bringing cascade of intra and inter-cellular events to create the desired immune response. Understanding the status of cellular transformation of immune cells can contribute significantly to the recent day immunotherapeutic research. Currently, such information are gained using fluorescence activated cell sorter (FACS), magnetic activated cell sorting (MACS) and immuno-labelling of cells, in which the process is relative expensive, laborious and time consuming. In this study, we report a label free detection of DC activation in live cells using Raman spectroscopy. Human DCs derived from PBMC were activated using lipopolysaccharide (LPS -10 µg/ml) by 24 hrs incubation and the activated cell phenotype was confirmed using FACS analysis by monitoring increase in the expression levels of CD 80, CD 86 and MHC I/II. For Raman analysis, cells were cultured in CaF_2 substrate and taken in PBS solution for live cell measurements. Raman spectra in the range of 500 -1800 cm^{-1} were measured from activated and naïve cells (n=75) and the spectrum obtained were classified using principal component analysis and discriminant analysis (PCA-DA). Although, the overall spectral variation was less prominent, intensity variations in selective regions were observed which includes amide vibration regions (1250, 1660 cm^{-1}), CH_2 deformations (1450 cm^{-1}), C-N stretch (1215 cm^{-1}), C-C stretch (1120 cm^{-1}) and finger print regions between 750-1000 cm^{-1}. PCA-DA of the spectrum obtained from activated and naïve DC could give a classification accuracy of nearly 86%. Further, the method was used to study the activation efficiency by immunogenic NP and NP loaded with disease specific antigen on DC cells for an incubation period of 24 hrs. Using the spectral database generated, PCA-DA could classify the NP activated DC spectrums with an accuracy of nearly 90%, which was further verified with FACS analysis. Thus, we demonstrate that Raman spectroscopy can be a relatively simple and efficient tool for monitoring the immunogenic response at cellular level.

Keywords: Raman Spectroscopy, Dendritic cells, Immune cell activation

A178 / New biomarkers in the diagnosis of ovarian pathology

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Background: Ovarian cancer is a silent killer, metastasizing throughout the abdomen before causing symptoms. The majority will die of the disease. Survival can be ameliorated by screening, improving diagnosis or treatment. Research so far has mainly invested in optimizing treatment. Screening has been proven not to be beneficial. Optimizing the diagnosis has always been the lowest priority in literature. This research project focusses on three pillars in the search for new diagnostic biomarkers: proteins, ctDNA (cellfree/circulating tumor DNA resulting from apoptotic tumor cells) and immune profiles.
Materials and methods: Serum, plasma and peripheral blood mononuclear cells (PBMC) were collected, so far of 269 people. In 157 of them (68 with benign cysts, 13 with borderline tumor, 43 with invasive ovarian cancer, 33 healthy controls) an immune profile analyses based on the presence of CD4, CD8, MDSC (myeloid derived suppressor cells) and Treg (regulatory T cells) was done at the PBMC level by FACS (Fluorescence Activated Cell Sorting). In 39 invasive ovarian cancer patients, immune profile was correlated with survival.
Results: Most striking were the differences in monocytic MDSC (mMDSC). Their presence was statistically different between the patient groups ($p < 0.0001$). The proportion of mMDSC doubled in the presence of ovarian pathology and within this group increased with growing malignancy potential (benign ***vs*** borderline ***vs*** invasive). This graded difference remained ($p\ 0.006$), but rendered the invasive cancers more pronounced as a separate group, if mMDSC who were positive for PDL1 (programmed death ligand 1) expression were considered. Moreover, overall survival in these invasive ovarian cancer patients was significantly reduced in case of increasing amount of mMDSC positive for PDL1 ($p = 0.02$).
Conclusion: This project is an ongoing international study that will finally include 1000 patients. Although these results are preliminary and analyses are still ongoing, immune profiling in blood for ovarian pathology holds much promise. At this moment, all data point towards a main role for mMDSC.

Keywords: ovarian cancer, biomarker, MDSC

A179 / Characterization of peptide exchange in major histocompatibility complex class I molecules by mass spectrometry

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Recombinantly expressed major histocompatibility complex class I (MHC I) molecules loaded with known peptide antigens have emerged as a powerful tool to detect antigen specific T cells by flow cytometry. However, generation of these reagents requires a complex protein refolding process in the presence of specific antigen peptides followed by extensive purification. Methods have been developed to overcome these drawbacks and enable high throughput production of peptide-MHC I complexes by generating MHC I molecules loaded with a photolabile peptide that can then be degraded, allowing for a peptide of interest to exchange into the complex. Existing analytical techniques to evaluate this peptide exchange are mainly enzyme-linked immunosorbent assay (ELISA) based and provide limited information as to the degree of exchange and the quality of the resultant peptide-MHC I molecules. Here we present real-time characterization of peptide exchange in MHC I molecules with peptide antigens over a range of affinities using mass spectrometry. We developed methods using two-dimensional liquid chromatography mass spectrometry (MS) and native MS that enable the characterization of these non-covalent protein-ligand complexes. These complimentary techniques isolate the MHC I complex from unincorporated peptide during the exchange reaction and directly determine the peptide present in the complex. We examined the rate of exchange for peptides of known affinities to define the relationship between affinity and exchange. Our results suggest that established exchange times of 30 min to 1 hour are insufficient, and that high affinity peptides require upwards of 4 hours to reach completion. These data define exchange parameters to maximize complex recovery and impart confidence in the downstream utilization of these reagents for immune monitoring.

Keywords: Immune monitoring, MHC, Peptide

A180 / CD39 and CD103 identify highly tumor-reactive CD8 T cells in human solid tumors

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Tissue-resident memory (T_{RM}) cells, expressing the integrin CD103, play a crucial role in protecting epithelial tissues against viral infections. Previous reports have shown that CD103+ CD8 T cells are present in some human solid malignancies. Based on these observations, we identified a subset of CD103+ CD8 T cells that co-expresses the ectonucleotidase CD39. These CD39+CD103+ T cells are enriched in primary tumors and metastatic lymph nodes, but absent in the blood and normal lymph nodes of head and neck squamous cell carcinoma (HNSCC) patients. Comparison of different tumor types revealed high frequencies of these cells in HNSCC, ovarian, lung and rectal cancers, whereas the cells were absent in primary colon cancer and colorectal liver metastasis (CRLM). Gene expression analysis of CD103+ CD39+ (DP) CD8 T cells revealed a gene signature reminiscent of T_{RM} cells ($CCR7^{low}$, L-selectin^{lo}, $S1PR1^{lo}$ and $CD69^{hi}$), and an activated phenotype (PD-1^{hi}, Ki-67^{hi}, Granzyme B^{hi}). TCR repertoire analysis showed high clonality in this subset and little overlap of the CDR3 sequences with other CD8 T cell subsets present in the tumor. Based on this phenotype, gene signature, circulation pattern and clonality, we believe that DP CD8 T cells are being chronically stimulated within the tumor microenvironment. In support of this finding, ***in vitro*** data suggests that CD39 expression is induced as a result of strong, sustained TCR stimulation in naïve CD8 T cells. Finally, using expanded CD8 T cell subsets and a tumor cell line from the same patient, we were able to demonstrate that only CD39+CD103+ tumor-reactive CD8 T cells can kill autologous tumor cells. Taken together our findings suggest that targeting and/or expanding tumor-resident DP CD8 T cells may be a promising approach to boost immune-mediated tumor regression.

Keywords: tumor-reactive T cells, human, cytotoxic T cells

A181 / Immune monitoring of dendritic cell (DC) vaccines: Examining vaccine potential from the DC side

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In the past years, many trials using dendritic cell vaccines have been reported and immune monitoring data has been presented. Unfortunately, and incomprehensibly, the immune monitoring has concentrated only on the side of responding T cells whereas the DC side of immune response has mostly been overlooked. This may be due in part to difficulties in determining relevant DC functions for analysis. To activate T cells optimally in a cancer immunotherapy setting, three signals must be delivered by the DCs. First, the right antigen must be presented in adequate amounts by MHC complexes to trigger TCRs (Signal 1); second, activating costimulatory molecules like CD80/CD86 must dominate over negative regulatory molecules on the DC surface (Signal 2) to provide positive co-stimulation to the T cells and third, the bioactive form of the cytokine IL12 should be secreted in the absence of IL10 to polarize T-cells in a Th1/Tc1 direction (Signal

3). Signal 1 and Signal 2 can be monitored using flow cytometry. Signal 3 can be measured by mimicking the DC-T cell interaction via CD40/CD40L binding. One approach here is to co-culture DCs with irradiated cells of the hCD40L-transfected mouse fibroblast cell line L929, followed by detection of secreted IL10 and IL12 in the supernatant medium with an ELISA. The major drawback of this method is the need to cultivate the CD40L-transfected L929 cells, making the assay less flexible, laborious and difficult to utilize in a GMP facility. Additionally, it requires access to a radiation unit not present in every institute. This assay delivers only limited information on the cytokine profile of DCs since it is impossible to determine how many cells produce cytokines and whether cells can secrete both IL-10 and IL12. To overcome these deficiencies, we developed a protocol using soluble CD40L and the TLR 7/8 antagonist R848 to stimulate DCs and utilize a dual-color IL10/IL12 ELISPOT assay as the readout method. This approach bypasses the need for cell culture and irradiation and provides exact information about the number of cells secreting a given cytokine, while the spot size indicates strength of secretion. Applying this assay to our in-house dendritic cell vaccines, we could observe that the immature DCs of healthy donors predominantly produce IL10 while, dependent upon the maturation cocktail, mature DC of the same donors predominantly produce IL12. These data correspond with the presence of activating markers on the surface of the mature DC and with detection of intracellular IL12 in the mature DC. Furthermore, these DCs could polarize T cells in a Th1/Tc1 direction, inducing the types of T cells associated with optimal anti-tumor immune responses. In the future, this assay will be used to monitor DCs originating from patients included in our clinical trials.

Keywords: Dendritic cell vaccines, Immune monitoring, dual color IL10/IL12 ELISPOT

A182 / Circulating fresh human whole blood with intact cascade systems as a tool to predict first-infusion reactions and mechanism-of-action of immunotherapeutics

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Systemic administration of therapeutic antibodies can result in severe infusion reactions and cytokine release syndrome (CRS). Despite the wide variety of mechanisms and pathways that can take part in CRS development, many preclinical screening assays are devoid of at least one of the blood components that might be necessary for these reactions to occur. Here, we propose a modified chandler loop, herein called a whole blood loop assay (WBLA), as a tool to predict infusion reactions and study mechanism of action of immunotherapeutics. Fresh blood from healthy donors along with therapeutic antibodies are added to PVC tubings with an immobilized heparin conjugate in the inner surface. The tubings are connected at the ends with a special metal connector to form a loop. The loops are kept in circulatory motion by attaching them to a rotatory wheel, and they are incubated for 4-6 hours at 37 ºC before the blood is collected for cytokine or cellular analysis. The circulatory motion and the surface-heparinization minimize the amount of soluble heparin needed and therefore maintaining the integrity of the complement and coagulation cascades. Using WBLA we could detect rapid increase in plasma TNF-a, IFN-g, IL-2 and IL-6 in response to ANC28.1, OKT3, alemtuzumab and TGN1412 in a 4-hour assay. We further identified memory CD4+ and CD8+ T cells as the major source of these cytokines using intracellular flow cytometry. When compared to the whole blood assay in a plate, WBLA had a lower background cytokine release in the negative controls. Additionally, the non-agonistic antibody natalizumab did not induce cytokine release in WBLA consistent with its clinical response. Both LPS and alemtuzumab induce complement activation in WBLA, which cannot be observed in plate assays. Hence, the biological effects of alemtuzumab, which is killing of CD52+ lymphocytes through complement activation could be observed in WBLA but not in a plate assay. We could furthermore block alemtuzumab action and rescue lymphocytes with compstatin and eculizumab, which block complement components C3 and C5 respectively. Surprisingly, a C1q blocker did not rescue cells from death, suggesting involvement of the alternative or lectin pathways, rather than the classical pathway. Interestingly a CD16 blocker, which blocks ADCC, selectively abolish alemtuzumab action on B cells but not T cells; suggestion a role of ADCC in alemtuzumab induced B cell killing. In conclusion, the intact cascade systems along with the blood cellular and antibody components makes WBLA a powerful tool to predict infusion reaction and to study mechanism of action of immunotherapeutics. The system can be used in combination with other assays or as a stand-alone tool to investigate drug/blood interactions and for safety screening prior a first-in-man clinical trial.

Keywords: immunotherapeutics, Cytokines, Complement

A183 / A simple and integrated workflow for deep proteomic and transcriptomic analysis of sorted cell populations

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In addition to the long standing efforts to understand and manipulate the immune system in the treatment of autoimmune and other immune-related diseases, the immune system is increasingly becoming a direct target for cancer therapeutics. The

immense heterogeneity in immune cell phenotype and function and its corresponding role in both health and disease requires increasingly sophisticated analytical approaches. Recent advances in flow and mass cytometry have greatly expanded the number of immune cell parameters that can be interrogated resulting in an improved understanding of the immune system heterogeneity. These technologies, however, remain limited in the number and types of analytes that can be examined in a single clinical sample. Expanding these parameters could lead to the discovery of new therapeutics and biomarker signatures.
The NanoString nCounter® platform enables the highly multiplexed digital analysis of both RNA (1) and protein (2) from a single biological specimen for multiple research applications. We have recently developed the nCounter® Vantage 3D™ RNA:Protein Immune Cell Profiling Assay for research applications, which interrogates 30 cell surface proteins and 770 immune-related RNA starting with cells in suspension (3). Expanding on this, we demonstrate the development of a streamlined workflow that seamlessly integrates standard immune cell sorting with downstream nCounter® analysis. By co-staining PBMCs with both fluorescently-labeled and DNA barcoded antibodies, CD8+ and CD4+ T cells and CD19+ B cells were isolated followed by analysis of dozens of additional proteins and 770 RNA from each sorted population. Demonstrating the value of this workflow in analyzing potentially rare cell populations, the number of target cells were titrated to determine the sensitivity of the workflow. Without the requirement for additional molecular biology methods, such as RNA purification or sequencing library construction, this method is ideally suited for incorporation into any cell sorting workflow.

Keywords: T Cell Therapy, Signature Development, Low Input Assay

A184 / Antibody and auto-antibody serum profiling using high-density and combinatorial peptide array approaches to generate biomarker signatures

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Cancer is a result of a number of genetic alterations that disturb normal, controlled cell growth and differentiation. Mutational events leading to the activation of oncogenes or the inactivation of tumor-suppressor genes have been linked causally to the formation of tumors. p53 is one of the most important regulators of transcription, cellular cycle, DNA repair and apoptosis detected to date. Anti-p53 antibodies have been detected in the serum of cancer patients. This immune response is due to a self-immunization process linked to the strong immunogenicity of the p53 protein, and is associated predominantly with p53 missense mutation and p53 accumulation in the tumor. Auto-antibodies have also been proposed as potential diagnostic biomarkers for early stage diagnosis of cancers, since an increase in serum levels of specific auto-antibodies has been shown to precede the development of disease symptoms and correlate with cancer incidence for various cancers including breast and lung cancer. Here we systematically evaluate reactivity of antibodies in p53 positive serum samples and identify reactive epitopes to normal and mutant peptides using a high-density peptide microarray. We then propose a working framework on how to deploy peptide arrays in order to empirically identify and optimize peptides to which the serum antibodies / auto-antibodies bind in order to generate signatures that are predictive or discriminatory between patient populations.

Keywords: Biomarkers, Diagnostics, Technology

A185 / Characterizing the immune cell landscape of tumor microenvironment using 7 color multiplex IHC in melanoma patients

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Immune checkpoint therapy has shown impressive responses in various cancer types such as melanoma. However, long lasting (> 2 years) responses remain limited to about 20-40%. Many patients might not benefit, but are exposed to expensive non-effective treatment with possible severe/lethal side effects. Therefore, there is a stringent need to identify biomarkers that predict efficacy of immunotherapy. In our previous study we determined that the density and distribution of CD3+ T cells within primary cutaneous melanoma correlates with survival of metastatic melanoma patients after dendritic cell (DC) based immunotherapy.
In the present study we want to further automate and extend this analysis of tumor microenvironment (TME) to distinguish different immune cell phenotypes and their checkpoint expression. Here we study melanoma tumors of patients that have been treated with Ipilimumab.
Multispectral fluorescent immunohistochemistry (IHC) using the Opal tyramide signal amplification was exploited to develop and

optimize immune cell cell panel to analyze the TME of melanoma patients. Next to quantification of different cell subsets, their spatial distribution will also be thoroughly analyzed.
Panels visualizing cytotoxic and regulatory T cells, M1 and M2 macrophages, M- and G-MDSCs, and checkpoint expression such as PD-1, PD-L1 and CTLA-4 expression have been optimized. We developed computational tools to visualize data and subsequently improve analysis algorithms. Besides that, we developed procedures to quantify microscopy data in a user friendly manner. Quantity and localization of different subsets vary between tissue compartments (tumor vs invasive margin). Updated results will be presented.
We here show that handling multiplex IHC is challenging and require specialized tools for visualization and analysis. Interpretation of the data should be performed carefully.

Keywords: tumor microenvironment, immune checkpoint inhibitors, multiplex IHC

A186 / The CIMT Immunoguiding Program: objectives and ongoing activities

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Immunomonitoring has evolved as an essential tool for anti-tumor immunotherapies. The international workgroup CIP (CIMT Immunoguiding Program) has pioneered the concept of immune assay harmonization and of immunoguiding. Proficiency panels and wet workshops are organized regularly with the objective to support inter-laboratory harmonization of the ***in vitro*** assays used to assess the frequency and function of immune cells in cancer patients. Such activities strengthen exchanges between participating centers, allow identification of technical parameters involved in inter-laboratory variability and, for individual users, propose an optimal platform to control assay performance over time. Harmonization of the IFNgamma-ELISpot and HLA-multimer assays for assessment of antigen-specific T cells has been reached. CIP is currently working on the measurement of other immune cell subsets, i.e. regulatory T cells, myeloid-derived suppressor cells and natural killer cells. In addition, the workgroup has established cellular reference samples for quality control of ***in vitro*** assays over time and is also cooperating on the automated processing of flow cytometry data. Finally, CIP supports the transparent reporting of immunomonitoring results through its participation in the development of the MIATA reporting frame. A summary of the objectives and recent activities of the group will be presented.

Keywords: immunomonitoring, cellular biomarkers, interlaboratory harmonization

A187 / MHC Multimer Proficiency Panel 2017: Evaluating routine MHC multimer assays

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Monitoring antigen-specific T cell responses are becoming increasingly important in Immunotherapeutic research and development. Thanks to the harmonization efforts by the CIC and CIMT over many years, and the development of better reagents and protocols, MHC multimer assays are now reliable, accurate assays for monitoring antigen-specific T cell immunity.
Supported by CIMT and CIC, Immudex has conducted a MHC Multimer proficiency panel in the spring 2017 to evaluate assay performance in immune monitoring laboratories routinely using MHC multimer assays.
Each of the 20 worldwide participants received cell samples, representing low, medium and high responses for three predefined MHC multimer specificities.
Participants were asked to determine the number of CD8+, antigen-specific T cells corresponding to each of the three multimer specificities. Any MHC Multimer could be used; Dextramers were offered free of charge.
It was recommended to use additional antibody marker(s) for exclusion or inclusion of specific cell populations (e.g. anti-CD4 antibody, anti-CD3 antibody), and dead-cell dyes during data analysis.
After performing the MHC Multimer assay, the participants reported back their results, as percentage of MHC multimer-positive T cells of total CD8-positive T cells.
The data set will be analyzed, and each participating laboratory will receive a report detailing the individual laboratory's performance (in an anonymized format). The anonymized report will become publicly available after presenting the data at the CRI-CIMT-EATI-AACR conference in Main, September 2017.

Keywords: Immune monitoring, antigen-specific T cells, Standardization

A188 / Persistence of expanded TCRβ clonotypes is associated with clinical activity of ADXS-PSA immunotherapy in metastatic castration-resistant prostate cancer

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Active immunotherapies, such as ADXS-PSA, are designed to help a patient's immune system recognize and respond to tumor-associated antigens. ADXS-PSA is a bioengineered, highly attenuated strain of ***Listeria monocytogenes*** that secretes a fusion protein consisting of a truncated fragment of listeriolysin O, which has adjuvant properties, and the tumor-associated target antigen, prostate-specific antigen (PSA). Because αβ T cell effectors are an essential component of the antitumor immune response elicited by active immunotherapies, we sequenced the complementarity-determining region 3 (CDR3) region of rearranged TCRβ genes to monitor and characterize the peripheral αβ T cell responses of patients with metastatic castration-resistant prostate cancer (mCRPC) treated with ADXS-PSA. Sequencing was performed on genomic DNA from peripheral blood isolated at multiple time points from mCRPC patients participating in the ADXS-PSA dose-determining stage of the phase 1/2 trial evaluating the safety and tolerability of ADXS-PSA as a monotherapy and in combination with Keytruda®. Because 4 of the 12 patients in the dosing cohort had stable disease (SD) as per RECIST criteria, the pre- and post-treatment TCR repertoires of SD and progressive disease (PD) patients were compared. All analyses were performed using Adaptive Biotechnologies' immunoSEQ Analyzer.

We first quantified the diversity of TCRβ sequences in the peripheral blood of mCRPC patients at baseline and after treatment with ADXS-PSA. At baseline, a wide range of TCR diversity was observed in both SD and PD patients. After ADXS-PSA treatment, changes in TCR diversity were noted in all patients, but these changes were not significantly different between SD and PD patients. We next examined the dynamics of the peripheral αβ T cell response by tracking the abundance of the top 100 TCRβ rearrangements over a 9-week time course, which included 3 ADXS-PSA treatments. Expansions of new and pre-existing TCRβ clonotypes were observed in the peripheral blood of all patients after ADXS-PSA treatment. In the SD patients, the clonal expansions occurred primarily after the first ADXS-PSA treatment and were stable over the entire time course, consistent with the report that improved survival in Yervoy®-treated mCRPC patients is associated with the maintenance of TCRβ clonotypes. By contrast, the clonal expansions in PD patients were asynchronous, occurring after each of the 3 ADXS-PSA treatments, and were not sustained, as evidenced by the subsequent loss of many of the newly expanded TCRβ clones. In summary, these results indicate that the stability of clonal expansions during ADXS-PSA-treatment distinguishes SD from PD and suggest that stable clonal expansions may be used to identify clinical responders to ADXS-PSA treatment.

Keywords: TCRβ, PSA, metastatic castration-resistant prostate cancer

A189 / Gene expression profiles associated with stable disease in metastatic castration-resistant prostate cancer patients treated with ADXS-PSA immunotherapy

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ADXS-PSA, a ***Listeria monocytogenes (Lm)***-based immunotherapy that expresses the tumor-associated antigen prostate-specific antigen (PSA), is designed to stimulate an antitumor response by directly targeting and engaging the immune system. However, tumors, as well as chemotherapy and radiation therapy, are known to suppress the immune system. For these reasons, we examined the immune status of metastatic castration-resistant prostate cancer (mCRPC) patients, pre-and post-treatment with ADXS-PSA, by profiling immune-related gene expression in peripheral blood mononuclear cells (PBMCs). Total RNA was extracted from PBMCs isolated at multiple time points from mCRPC patients participating in the ADXS-PSA dose-determining stage of the phase 1/2 trial evaluating the safety and tolerability of ADXS-PSA as a monotherapy and in combination with Keytruda®. The NanoString nCounter® PanCancer Immune Profiling Panel was used to quantitate gene expression levels. Normalized NanoString gene-level counts were compared against established immune cell-specific gene signatures. Four of the 12 patients (33%) in the ADXS-PSA monotherapy cohort had stable disease (SD) according to RECIST criteria, thereby allowing us to compare the gene expression profiles of SD and progressive disease (PD) patients.

When the kinetics of expression of genes associated with T cell activation were analyzed, SD patients were found to have significantly higher levels of these genes post- ADXS-PSA-treatment than PD patients. In addition, SD patients had significantly higher levels of genes expressed by mature antigen-presenting cells and by activated $CD4^+$ T cell effectors than PD patients, which is consistent with findings observed in mouse tumor models treated with ***Lm***-based immunotherapies. By contrast, PD patients had significantly higher levels of genes associated with immunosuppression than SD patients. Collectively, these analyses not only provide insight into the mechanisms of action of ADXS-PSA, but also identify potential pharmacodynamic biomarkers of clinical response in mCRPC patients treated with ADXS-PSA monotherapy.

Keywords: PSA, biomarker, metastatic castration-resistant prostate cancer

A190 / Tumor-infiltrating lymphocytes in biopsied pancreatic cancer tissues

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Tumor-infiltrating immune cells represent host antitumor immune responses. We previously reported that tumor-infiltrating $CD4^+$ T cells or $CD8^+$ T cells, and tumor-infiltrating M2 macrophages, neutrophils, or the ratio of tumor-infiltrating $FOXP3^+$ Tregs to $CD4^+$ T cells were favorable and unfavorable prognosticators in patients with pancreatic ductal adenocarcinoma (PDAC), respectively. The methods to evaluate such tumor-infiltrating immune cells were carefully made, since these cells are distributed very heterogeneously within cancer tissues and they also infiltrate due to reasons other than cancer, i.e. ulceration, necrosis, etc. Therefore, we used surgically resected cancer tissues in these studies. While, the small biopsied tissue is usually only available cancer tissue obtained from patients with unresectable advanced PDACs, although it is unknown whether tumor-infiltrating lymphocytes (TILs) examined in small biopsied tissue represent tumor immune microenvironment.
Here we investigated the reliability of TILs data obtained from small biopsied tissues. At first we examined if expression of genes ***CD3, CD4, CD8,*** or ***FOXP3*** in PDAC tissue represent immune microenvironment and are prognosticators using 241 cases of PDAC. As a model study, we took twenty biopsy tissues (fine-needle aspiration biopsy) from a fresh surgical specimen resected for PDAC (n=17). Expressions of such genes were analyzed by quantitative RT-PCR using ten biopsied samples per case. TILs in the biopsied tissues were examined by immunohistochemistry using another ten biopsied samples per case. Intra-rater reliability was statistically tested by using the intraclass correlation coefficient (ICC). Survival analyses revealed that PDAC patients with higher expression of genes ***CD3, CD4, or CD8*** showed significantly longer survival compared with those with lower expression of the genes both in overall survival and disease-free survival. Expression of ***FOXP3*** gene and the ratio of ***FOXP3/CD4*** were not prognostic. ICC (ρ) was 0.58 (95% CI: 0.40 to 0.77, P< 0.001) for ***CD3*** gene expression, 0.61 (95% CI: 0.44 to 0.79, ***P***< 0.001) for ***CD4***, 0.46 (95% CI: 0.29 to 0.68, ***P***< 0.001) for ***CD8,*** 0.41 (95% CI: 0.24 to 0.64, ***P***< 0.001) for ***FOXP3***, and 0.42 (95% CI: 0.25 to 0.65, ***P***< 0.001) for ***FOXP3/CD4,*** they were "moderate" to "substantial" levels. If they reach to "almost perfect" level (ρ=0.8), at least 3, 3, 5, and 6 times biopsies are necessary for ***CD3, CD4, CD8,*** and ***FOXP3***. While ICC values of immune-labeled TILs were low (0.02-0.22) and ≥6 times biopsies are necessary to reach to "moderate" level (ρ=0.4).
It is suggested that reliability increases in TILs findings obtained from small biopsied PDAC tissues, if TILs are estimated by expression of their related genes and if PDAC tissues are biopsied repeatedly. Immunohistochemical analyses of TILs with reliability are necessary to perform repeated biopsies at least several times.

Keywords: Pancreatic cancer, tumor-infiltrating lymphocytes, intraclass correlation coefficient

A191 / A novel, highly standardized, peptide-based ex-vivo stimulation assay for whole blood

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The goal of monitoring the immune system following an immunotherapeutic intervention is the identification of intervention-induced changes, for example, an increase or decrease of the frequency of antigen-specific T-cells or cytokine production. A major problem of immune monitoring in multi-center studies, or when comparing the results between different studies, is standardization. Major issues affecting standardization are related to sample handling, transport and storage. However, additional variation occurs as a result of variable interpretation of and adherence to experimental protocols, and, last but not least, the inherent variability of the methods used to read samples, e.g. flow-cytometry (intracellular-cytokine staining, ICS) or Elispot. Multiple efforts have been made to standardize immune monitoring over the last two decades, but some main sources of variation have remained.
We have now developed a simple, peptide-based whole-blood stimulation protocol that provides a high level of standardization. Peptides used to stimulate whole blood are synthesized in a highly

standardized fashion and aliquoted into reaction tubes, one test per tube. Anticoagulated whole blood is then added to these tubes, which are subsequently sealed and incubated in a standard, programmable heat-block. Following the desired incubation time of (for example 16 hours or as required), cells are spun down in a micro-centrifuge, supernatants are aspirated and immediately frozen at -20°C until analysis. Our initial experiments used peptide pools from a variety of human viruses and bacteria for in vitro stimulation. SEB was used as positive control, peptide solvent alone (DMSO) as negative control. The Meso Quickplex SQ120 platform (Mesoscale Discovery) was utilized to measure, IL-1beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-12p70, IFN-gamma, and TNF-alpha in the supernatants (V-Plex Proinflammatory Panel 1 Human).
Our pilot data show that peptide pools are highly suitable antigens for inducing cytokine secretion from both T-cells and non-T-cells in whole blood. Optimum peptide concentrations were similar to the ones typically used in Elispot and ICS assays. Different levels/profiles of cytokines were observed, depending on the blood donor and the origin of the peptides.
Our new assay has potential to be used in immune monitoring after/during tumor immunotherapy or other clinical settings. Standardization is considerably higher for currently available monitoring tools and only simple, portable, and inexpensive equipment is required for sample processing, increasing its potential for multi-center as well as remote use. We believe that thanks to existing high quality, multiplex assay platforms for secreted mediators, our assay will provide useful information above and beyond T-cell responsiveness alone. It might, however, initially be used alongside existing monitoring tools to provide more insight into its potential as a monitoring tool.

Keywords: peptides, immune monitoring, secreted mediators

A192 / Glucose metabolism and O-linked GlcNAcylation in the tissue repair function of Treg cells

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Regulatory T (Treg) cells dampen immune responses to avoid excessive, deleterious inflammation in the context of autoimmunity, allergy, and infection. Treg cells also impede anti-tumor immunity. Therefore, modulation of Treg cell activity is of great therapeutic potential for treating many debilitating inflammatory diseases and cancer. Recent studies have suggested that in addition to their prominent immunosuppressive function, Treg cells have a distinct and non-redundant role in tissue maintenance and remodeling. These Treg cells exhibit a unique transcriptional signature represented by high expression of the EGF family member amphiregulin (Areg). Importantly, tumor infiltrating Treg cells highly express Areg. Since Areg is known to exert pleiotropic oncogenic activities, Areg producing Treg cells may be crucial for establishing the tumor microenvironment. It has been increasingly appreciated that lymphocyte activation and differentiation are coupled to specific metabolic pathways. Metabolic reprogramming is dictated by cytokine and growth factor signals, as well as the availability of nutrients. In addition, metabolic products provide substrates that can alter the functional status of a cell through posttranslational modifications (PTMs). Among the various nutrients, glucose is a key metabolic substrate for T cells. Aerobic glycolysis in activated T cells is required for the acquisition of full effector function. Treg cells in the secondary lymphoid organs and the tissue have distinct glucose availability and metabolic needs. We thus hypothesized that glucose sensing and glucose metabolism play essential roles in shaping the unique phenotype and function of tissue resident Treg cells. O-linked GlcNAcylation is a PTM dynamically regulated by glucose levels and controls the function of many proteins, including transcription factors that play key roles in T cell activation such as NF-κB, CREB, and NFAT. To understand the mechanism by which glucose metabolism controls the function of tissue-resident Treg cells, we began to characterize protein O-GlcNAcylation in Treg cells, and found that both human and mouse Foxp3, the master regulator transcription factor of Treg cells, undergoes O-GlcNAcylation. We are in the process of mapping the site of O-GlcNAcylation of Foxp3, and understanding the physiological significance of this PTM.

Keywords: Regulatory T cells, Foxp3, O-GlcNAcylation

A193 / PD-1 is a better prognostic biomarker than PD-L1 in triple negative breast cancers

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Antibodies blocking PD-1/PD-L1 co-inhibitory pathway molecules have shown promising results in advanced stage triple negative breast cancers (TNBC) clinical trials. Hence, the knowledge of the expression and relative contribution of PD-L1 and PD-1 in the tumor microenvironment is critical for identifying a predictive as well as prognostic signature. In this study, we investigate the role of PD-1 and its ligands PD-L1/2 as biomarkers for predicting clinical outcome in an Asian TNBC cohort.
TNBCs diagnosed between 2003 and 2013 in Singapore General Hospital were stained with anti-PD-L1 antibody (n=176). The same cohort was subjected to quantitative, digital gene expression NanoString assay to measure expression of a panel of 499 immune response genes. The cohort was divided into “PD-L1-positive” and “PD-L1-negative” based on PD-L1 protein expression.

Clinicopathological parameters were correlated with protein and mRNA expression. Positive PD-L1 expression was defined as >1%. Disease free survival (DFS) and overall survival (OS) were correlated with biomarkers. A p value of < 0.05 defined statistical significance in this study. Changes in the likelihood ratio (LR) values (ΔLRχ2) were used to quantitatively measure relative amount of prognostic information of one model compared with another.
Tumoral expression of PD-L1 protein was 17.6% in our TNBC cohort and patients with PD-L1-positive tumors experienced significantly longer DFS (p=0.03) but not OS compared to patients bearing PD-L1- negative tumors. Multivariate analysis further confirmed the findings (HR-0.28, p=0.002). PD-L1 expression on tumor infiltrating lymphocytes (TILs) also showed similar trend. However, PD-1 expression was a better indicator of DFS and OS in TNBC with statistically significant p values in both univariate and multivariate analyses. Continuous PD-1 scores added significant prognostic information for DFS (ΔLRχ2 = 4.83; P < 0.028) beyond that provided by standard clinicopathological variables. ΔLRχ2 values were lower and non-significant for PD-L1. At the transcription level, patients with high tumor CD274 mRNA expression showed non-statistically significant association with better OS (p=0.06) and DFS (p=0.07). However, statistically significant results were observed when CD274 was combined with PD-L2 (PDCDLG2) and PD-1 (PDCD1) mRNA expression profile (OS, p< 0.001 and DFS, p< 0.001). These results were validated in a published TNBC gene expression database (METABRIC). A three-gene signature of CD274, PDCD1LG2 and PDCD1 mRNA expression demonstrated potential as a better prognostic indicator of OS and DFS.
PD-1 expression status (mRNA and protein) or a combined tri-gene mRNA panel may be a better indicator of prognosis compared to PD-L1 in TNBC. Our sample size and heterogeneity of protein expression may be a limiting factor. Therefore, further studies on a larger TNBC cohort as well as other immune checkpoint molecules in PD-L1-negative tumors are also warranted.

Keywords: PD-L1, PD-1, CD274

A194 / Biomarkers for response of melanoma patients to immune checkpoint inhibitors; a systematic review

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Immune Checkpoint Inhibitors (ICI), targeting CTLA-4 or PD-1 molecules, have shown impressive therapeutic results. However, only 20%-40% of advanced melanoma patients have durable responses to ICI, and these positive effects must be balanced against severe off-target immune toxicities and high costs. This urges the development of predictive biomarkers for ICI response to select patients who are likely to benefit from treatment. Although many candidate biomarkers exist, a systematic overview of biomarkers and their usefulness is lacking. To identify predictive biomarkers for clinical response or survival to ICI therapy in melanoma patients, we systematically searched OVID MEDLINE and retrieved 472 publications, of which 67 were included in this review. Blood and genomic biomarkers were mainly studied for CTLA-4 ICI, while tumor tissue markers were analyzed for both ICI. Blood cytology and soluble factors were more frequently correlated to overall survival (OS) than to response, indicating their prognostic rather than predictive nature. Systemic T-cell response and regulation markers correlated to response, but progression-free survival (PFS) or OS were not analyzed. Tumor tissue analyses revealed correlations to response for mutational load, neoantigen load, immune-related gene expression and CD8+ T-cell infiltration at the invasive margin. The predictive value of PD-L1 varied between studies, possibly due to the influence of T-cell infiltration on tumor PD-L1 expression. Genomic biomarker studies addressed CTLA-4 and other immune-related genes. Variations in outcome parameters, statistical power and analyses hampered summary of the results. Further investigation of biomarkers in larger patient cohorts using standardized objectives and outcome measures is recommended.

Keywords: Immune checkpoint inhibitors, predictive biomarkers, melanoma patients

A195 / Comprehensive characterization of the immune landscape in non-small cell lung cancer

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The success of immune checkpoint inhibitors (ICI) for non- small cell lung cancer (NSCLC) has galvanized the field. Unfortunately, just ~20% of patients benefit from novel therapies and underlying mechanisms for treatment failure are mostly unknown. ICI therapy likely fails for one of two reasons: (1) an antigen-driven immune response is not present or (2) an antigen-driven immune response is present, but immune suppressive factors reside within the tumor microenvironment (TME) that derail an otherwise effective immune response.
Lung cancer, the leading cause of cancer deaths, is a heterogeneous disease classified by histologic subtypes, with adenocarcinoma (ADCA) and squamous cell carcinoma (SCCA) representing the majority of NSCLC. Just as the anatomical location and mutational signature of the NSCLC subtypes differ, one would expect that the immune cell composition and function would

also differ. A strong foundational knowledge of the immune cell composition in NSCLC, will likely prove prerequisite to realizing the full potential of such reagents. To evaluate the complexity of the immune landscape in NSCLC we examined tumor and non-adjacent lung tissue in a cohort of 73 patients. We used flow cytometry, fluorescent multiplex immunohistochemistry and gene expression analysis to comprehensively profile the immune cell content and function in attempts to identify the dominant immune suppressive factors. Further, we performed T cell receptor sequencing to delineate the frequency with which antigen-driven immune responses exist. The additional analysis of somatic mutations, gene fusions and signaling pathways of the same cohort allowed the comprehensive characterization of NSCLC.

We could show that many immune cell types are significantly increased in tumor, when compared to non-adjacent lung. Cluster analysis identified patient groups with immune inactive and exhausted adaptive immunity and immune cell compositions are unique for NSCLC subtypes. In SCCA we observed a neutrophil predominant signature with additional increased infiltration of Treg when compared to ADCA. SCCA displays immune suppressive cell content, indicated by high expression of exhaustion markers on T-cells (PD-1, TIM3). Further, a more clonal TCRβ repertoire was observed in SCCA, indicating the presence of an antigen-driven immune response. Additionally, we identified that neutrophils constrain adaptive immune responses and neutrophils were the most abundant immune cell type in NSCLC specimens. A strong negative correlation between neutrophil and $CD8^+$ cellular content was observed. Notably, this association did not exist in non-adjacent lung tissue, strongly suggesting that this is a tumor-specific phenomenon.

This multidimensional dataset provides evidence that the immune landscape present within different NSCLC subtypes displays unique phenotypes and identifying the immune suppressive factors in different subsets will be important for successful immune-based therapy.

Keywords: lung cancer, neutrophils, immune checkpoint inhibitor therapy

A196 / Immune analysis of fibrolamellar hepatocellular carcinoma

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Background: Fibrolamellar hepatocellular carcinoma (FLHCC) is a rare variant of hepatocellular carcinoma (HCC). Although FLHCC develops in the absence of cirrhosis, the surrounding hepatic parenchyma may have mononuclear cell and lymphocyte infiltrates. We aimed to analyze tumor microenvironment by immunohistochemistry and flow cytometry.

Methods: Surgical samples of two FLHCC cases, 23 year old woman (case 1) and 25 year old man (case 2) who underwent neoadjuvant chemotherapy were analyzed. Expression of CD4, CD8, programmed death 1 (PD-1), programmed death ligand 1(PD-L1), CD45RO, CD57, CD68, OX-40, inducible costimulator (ICOS), FoxP3 and granzyme B (Gr-B) was evaluated by immunohistochemistry. Flow cytometry analysis of costimulatory molecules (4-1BB; CTLA-4; GITR; ICOS; LAG-3; OX40; PD-1 and TIM-3) in CD8, CD4 effector (Teff) and regulatory (Treg) T cells were performed.

Results: By IHC quantification, both cases showed the presence of immune cell infiltration confirmed with various T-cell marker subsets such as CD4, CD8, CD45RO. Expression of costimulatory molecules like PD1, PD-L1, OX-40, ICOS and pan-macrophage marker CD68 was also shown. By flow cytometry T cell subsets (CD8 T cells, CD4 T effector cells and T regulatory cells) were shown to express several costimulatory molecules, including PD1, CTLA-4 and ICOS.

Conclusion: Both cases expressed moderate to high infiltration of effectors T-Cells and co-stimulatory molecules. The presence of such immune subsets in the tumor microenvironment opens the possibility of the use of immuno-modulatory agents as a potential therapy for FLHCC given the current lack of any standard of care therapy.

Keywords: fibrolamellar, HCC, immune analysis

A197 / Identification of an intratumoral immune gene signature associated with tumor regression in an *Axalimogene filolisbac*-treated murine HPV^+ tumor model

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Axalimogene filolisbac (AXAL), a live attenuated *Listeria monocytogenes (Lm)*-based immunotherapy that expresses and secretes the E7 protein of human papillomavirus (HPV) 16, is currently being evaluated in clinical trials as a treatment for patients with HPV-associated cancer. Advaxis´ *Lm*-based immunotherapies act by stimulating innate immunity through multiple mechanisms including the STING pathway, inducing the generation of tumor antigen-specific T cells that infiltrate and destroy the tumor and by reducing the numbers and functions of immunosuppressive cells in the tumor microenvironment. To gain a better understanding of the molecular mechanisms of action of AXAL and to identify immune gene signatures that associate with AXAL-mediated tumor regression, we performed immune-related gene expression profiling of tumors in an AXAL-treated

murine HPV$^+$ tumor model. Total RNA was extracted from intact tumors harvested on day 19 post tumor implantation, at the first appearance of tumor control, from HPV$^+$ TC-1 tumor-bearing mice treated with two doses of AXAL, XFL7 (parental strain of AXAL that lacks HPV-E7), or PBS. Gene expression levels were measured using the NanoString nCounter® PanCancer Immune Profiling Panel and were analyzed using the nSolver™ Software. Mice from any treatment group whose tumors exhibited a ≥50% increase in size between days 15 and 19 were classified as progressors (n=12), while mice whose tumors exhibited a ≥10% decrease in size between days 15 and 19, all of whom were in the AXAL treatment group, were classified as regressors (n=7). Statistically significant differences in gene expression levels were noted between regressors and progressors. First, high expression of gene signatures indicative of CD4$^+$ T cells, CD8$^+$ T cells, cytotoxic cells, and NK cells were observed in the regressors but not in the progressors. The high expression of these effector cell-specific gene signatures is consistent with our previous flow cytometric analyses of tumor-infiltrating lymphocytes isolated from regressing tumors in AXAL-treated mice. Next, significantly higher expression levels of 59 genes were detected in regressors compared to progressors. This 59-gene signature contains genes involved in T cell and NK cell cytotoxicity, in antigen processing and presentation, and in cytokine, chemokine, and interferon signaling. This study has identified an intratumoral immune gene signature that highlights the importance of effector lymphocytes, mature antigen presenting cells, and cellular communication in AXAL-mediated tumor regression. This intratumoral immune gene signature may serve as a guide to identify molecular biomarkers associated with clinical outcome in patients with HPV-associated cancer receiving AXAL immunotherapy.

Keywords: Biomarkers, Immunotherapy, Gene signature

A198 / Establishment and immunological evaluation of an NGS-based method for mouse T cell receptor (TCR) profiling

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Identification of biomarkers is pivotal towards the proper development of cancer immunotherapies, in particular for designing the most effective regimens and identifying patient subgroups most likely to benefit from a given treatment. T cell receptor (TCR) repertoire emerged as a promising biomarker in this respect, as it also allows measurement of the dynamics of the T cell response both in time and in the different compartments. In this work, we developed and evaluated an NGS-based mouse TCR profiling method (TCR alpha and TCR beta) for discovery and validation of biomarkers related to the quantity and quality of the anti-tumor T-cell response in the preclinical setting.

For this purpose, different primers for each PCR step/ mouse TCR chain (alpha and beta) were designed and a two-step PCR reaction protocol was developed. This protocol was then tested on different tissues (blood, spleen, splenocytes and T cell-infiltrated tumors) using different RNA input amounts (from 100 ng to 2 µg RNA). Up to 250 ng RNA could be used as input into the protocol without the need to deplete the sample from ribosomal RNA (rRNA). For higher RNA input amounts rRNA depletion prior to cDNA synthesis proved to be necessary. The reproducibility of the final protocol was tested on two tissue types (blood and tumor) and similar performance values were obtained between replicates well as independent experiments with two different operators.

To test if the method can recover a defined cell clone in expected proportions, OVA-specific OT-I CD8 T cells were spiked into mouse blood in different concentrations (serial 10-fold dilution from 10% to 0.0001% OT-I mouse blood in a wild type blood background). The method could detect t OT-I sequences in a linear fashion down to a frequency of 0,001% OT-1 cells for both TCR alpha and TCR beta chains. Moreover, the relative frequency of the OT-I clone detected in B16-OVA melanoma tumors upon adoptive transfer of OT-I cells by TCR sequencing correlated well with the degree of OT-I infiltration to the same tumors assessed by flow cytometry.

Comparative analysis of TCR repertoire in different compartments upon different immunotherapeutic interventions (vaccination, check-point blockade) in murine models of melanoma using this established TCR profiling method is under evaluation.

Keywords: T cell repertoire, NGS, TCR

A199 / Mismatch repair protein deficiency is a independent risk for the aberrant expression of HLA class I molecules: a putative "adaptive immune escape"

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Accumulating evidence has shown that immune-checkpoint inhibition mediated cancer immunotherapies provided excellent prognosis in the certain types of cancer. Currently, it is becoming a standard therapy as well as surgery, radiotherapy and chemotherapy. One of the urgent challenges in cancer immunotherapy is the investment of relevant biomarker that can distinguish the efficacy, since the cost for antibody drug is becoming a socioeconomic burden in many countries. Recent studies reported that colorectal adenocarcinoma with hereditary or sporadic deficiency of mismatch repair (MMR) proteins revealed high antigenicity, and thereby detection of these proteins have been postulated as promising candidate for estimating clinical response. In this study, we investigated the correlation between deficiency of MMR proteins and expression of HLA class I molecules, a prerequisite of cytotoxic T cell based immunotherapy, in 135 cases of colorectal cancer by means of immunohistochemistry. Interestingly, MMR protein deficiency was an independent risk factor (OR, 10.44, 95% CI, 3.15-34.62, p< 0.001) of the impaired expression of HLA class I molecules, suggesting an entity of putative "adaptive immune escape". Moreover, our result might provide a potential novel biomarker for refining the cases to cancer immunotherapies. At the same time, the result would propose that we have to overcome the impairment of the expression of HLA class I molecules for further development of the curative cancer immunotherapy.

Keywords: HLA Class I, mismatch repair protein, colon carcinoma

A200 / Circulating levels of PD-L1 and Galectin-9 are associated with patient survival in Hepatocellular Carcinoma independent of their intra-tumoral expression levels

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Tumor expression of co-inhibitory ligands, such as PD-L1 and Galectin-9, has prognostic value in Hepatocellular Carcinoma (HCC) and other types of cancer. In addition, intra-tumoral PD-L1 expression seems predictive for the therapeutic effect of anti-PD-1 antibodies in some types of cancer. Determination of tumor tissue expression of these molecules requires tumor biopsy or surgical intervention. To facilitate prognostication, less invasive prognostic biomarkers, such as circulating PD-L1 or Galectin-9, would be preferable. Therefore, the aims of the study were to assess the prognostic significance of circulating levels of PD-L1 and Galectin-9 in HCC patients and to compare their prognostic significance to the intra-tumoral expression of these molecules.
Archived tissues and stored peripheral blood samples from 81 patients who underwent HCC resection or liver transplantation, with curative intent, were used. Immunohistochemistry was performed to determine intra-tumoral expression of PD-L1 and Galectin-9, while ELISA was used to quantify their circulating levels. Median circulating PD-L1 concentration was 383 pg/ml (IQR 206-774 pg/ml), and median circulating Gal-9 concentration was 21 pg/ml (IQR 3-44 pg/ml). With optimal cutoffs of 700 pg/ml and 42 pg/ml, respectively, high circulating levels of PD-L1 (HR 0.12, 95%CI 0.16-0.86, p=.011) and Galectin-9 (HR 0.11, 95%CI 0.15-0.85, p=.010) were both associated with improved HCC-specific survival and reduced cancer recurrence. Surprisingly, there was no correlation between circulating levels of PD-L1 and Galectin-9 and their expression levels on tumor cells. In fact, circulating levels of PD-L1 and Galectin-9 were predictive of HCC-specific survival independently of intra-tumoral levels and baseline clinicopathologic characteristics. Combined analysis of circulating levels and intra-tumoral expression of PD-L1 (HR 0.33, 95%CI 0.16-0.68, p=.002) and Galectin-9 (HR 0.27, 95%CI 0.13-0.57, p=.001) resulted in more confident prediction of survival.
In conclusion, circulating PD-L1 and Galectin-9 levels prognostically differentiate resected HCC patients, independently of their intra-tumoral expression. Combining circulating and intra-tumoral expression levels of PD-L1 or Galectin-9 further improves the prognostic values of these immune biomarkers.

Keywords: Co-inhibition, PD-L1, Galectin-9

A201 / Cancer-specific antibodies to self-antigens in head and neck squamous cell carcinoma before curative treatment and in the course of the disease

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Background: Human Papilloma Virus (HPV) negative Head and neck squamous cell carcinoma (HNSCC) is associated with poor prognosis. We have previously shown that expression of different cancer-testis antigens is associated with shorter survival. We hypothesized that immune reactions to such antigens may be insufficient to clear the tumor. Here we present serologic immune-monitoring data for cancer-specific auto-antibodies before treatment, during treatment and follow-up.

Material/ Methods: The Immune Response Evaluation to Curative Conventional Therapy (IRECT) trial is a single institution non-interventional, prospective clinical trial (IRECT trial, NCT03053661). 21 HNSCC patients were immune-monitored over the course of standard-of-care treatment with curative intent. Serum was collected at defined time points before / during treatment and at every follow-up for the first 12 months. Additionally, a cohort of 86 HNSCC patients for which serum or plasma samples collected at the time of initial treatment were available from the biobank of the Head & Neck Cancer Center Ulm was analyzed to determine the prevalence of auto-antibody responses.

Samples were analyzed by multiplex serology for antibodies to 20 shared tumor specific self-antigens, 9 oncogenes, to HPV-16 E6 as marker for HPV transformation and to polyomavirus BKV and JCV VP1 capsid protein as positive control antigens. Antigens were expressed in E. coli as GST-fusion proteins and bound to glutathione-derivatized fluorescent microbeads. Median fluorescence intensity (MFI) of human bead-bound immunoglobulins was determined using a Luminex platform. MFI cut-offs were determined graphically.

Results: Among the 107 HNSCC patients 23 patients (21.5%) had serological evidence for HPV transformation. In 32 (29.9%) patients reactivity to one and in 55 (51.4%) patients to >1 self-antigen (max. 7) was detected, whereas 20 patients (18.7%) showed no antibody responses to any self-antigen. The five most frequent antibodies detected were: MAGE-A3 (13, 12.2%), SSX2 and p53 (12 each, 11.2%) and Rhodopsin (11, 10.3%).

Among the 21 IRECT patients, at baseline 18 had auto-antibodies and 7 to HPV. Among patients free of recurrent disease during follow-up, 13/16 at baseline, 9/13 at the end of treatment and 8/11 one year after end of treatment had detectable auto-antibodies. Among 13 patients with a sample at the time of disease recurrence (5 patients from the IRECT trial and 8 patients from the biobank cohort) 9 patients had antibodies against any of the selected antigens.

Conclusion: 81.3 % of patients had antibody reactivity to ≥1 of the selected auto-antigens at baseline. Individual antigen reactivity to the tested shared antigens was quite diverse. Data analysis is ongoing and more correlations with clinicopathologic characteristics and the course of disease will be available at the meeting.

Keywords: cancer-specific antibodies, HNSCC, cancer-specific self-antigens

A202 / Soluble and monocyte bound CD163 are differentially expressed in healthy controls and patients with metastatic renal cell carcinoma

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CD163 is a monocyte/macrophage specific receptor found in both a membrane- (mCD163) and a soluble (sCD163) form. The levels of mCD163 and sCD163 are suggested to be indicators of macrophage activation and state of differentiation and might be used as biomarkers in cancer. Thus, we aimed to examine the levels of mCD163 on circulating monocytes and sCD163 in serum of patients with metastatic renal cell carcinoma (mRCC).

Peripheral blood mononuclear cells (PBMCs) and serum samples from 89 patients enrolled in a randomized clinical trial investigating the therapeutic efficacy of bevacizumab (anti-VEGF) as well as 20 age- and gender-matched healthy controls (HCs) were analyzed. Patients were stratified by the Motzer scoring system and only patients with a favorable prognosis (FP) or an intermediate prognosis (IP) were included in the study. The expression of CD163 on classical ($CD14^{++}CD16^{-}$, Mϕ-CL), intermediate ($CD14^{+}CD16^{+}$, Mϕ-IM) and non-classical ($CD14^{-}CD16^{+}$, Mϕ-NC) monocytes was assessed by multi-parameter flow cytometry on PBMC samples from mRCC patients collected at baseline. Serum levels of sCD163 was measured by an in-house ELISA.

A differentiated expression of CD163 was seen on monocyte-subsets in HCs with CD163 PE MFI-values: Mϕ-CL; 26,062 (95% CI: 22,737-29,386), Mϕ-IM; 14,609 (95% CI: 12,442-16,777) and Mϕ-NC; 201 (95% CI: 159-252). In contrast, the CD163 PE MFI in mRCC patients were: Mϕ-CL; 14,405 (95% CI: 12,012-16,797), Mϕ-IM; 11,989 (95% CI: 10,135-13,843) and Mϕ-NC; 259 (95% CI: 225-301).

When analysing the total monocyte population, the expression of mCD163 was significantly lower in mRCC patients with a difference in MFI of 9,574 ($P < 0.0001$). Among the monocyte subsets, only the Mϕ-CL subset showed significantly lower mCD163 expression in the mRCC patients.

As for the sCD163, mRCC patients showed a 1.4 (95% CI: 1.11-1.75) fold higher concentration of sCD163 compared to HCs. The mean concentration of sCD163 in HCs was 1.7 mg/L (95% CI: 1.45-2.00). Further, a 1.23 (95% CI: 1.01-1.50) fold higher concentration of sCD163 was observed in the IP group compared to the FP group. There was a trend towards an inverse correlation between the MFI of CD163 on circulating monocytes and the sCD163 concentration ($R= -0.2$, $P = 0.06$) when analyzing all patients.

In conclusion, in patients with mRCC we found lower expression of CD163 on classical monocytes. Furthermore, these patients showed a higher concentration of sCD163, and there was a borderline negative correlation between sCD163 and mCD163, implying an increased shedding that may be caused by cancer-related inflammation. Associations between macrophage-related biomarkers and clinical endpoints need further investigation.

Keywords: monocytes, CD163, metastatic renal cell carcinoma

A203 / Identification of parameters to harmonize the phenotype of human myeloid-derived suppressor cells

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The Cancer Immunoguiding Program (CIP) is coordinating a proficiency panel program that aims at harmonizing myeloid-derived suppressor cells (MDSC) phenotyping. MDSCs are a heterogeneous group of myeloid cells at different stages of differentiation, able to interfere with T cell-mediated responses, and often expanded in cancer patients. In recent years, recognition of the clinical relevance of MDSCs has steadily increased and there is a growing interest for monitoring circulating MDSCs in cancer patients. An intriguing aspect of MDSC biology is their appearance in different phenotypic subsets, both of monocytic and PMN type, probably because of the reaction to a conditional environment induced by the tumor. This characteristic aspect of MDSC biology, however, challenges at present their phenotypic identification, and a large variance is suspected among different laboratories in their identification.

To understand the extent of this problem, and to overcome this obstacle, the CIP has organized a proficiency panel aimed at harmonizing MDSC phenotyping with a two-step approach. In the first step, an international consortium of 23 laboratories immune-phenotyped 10 putative MDSC subsets on pre-tested, peripheral blood mononuclear cells of healthy donors, to assess the level of concordance and to define robust marker combinations for the identification of circulating MDSCs. At this stage, no mandatory requirements to standardize either reagents or protocols were introduced.

Results from the first step showed a small intra-laboratory, but very high inter-laboratory variance for all MDSC subsets, especially for the granulocytic subsets. In particular, the use of a dead-cell marker altered significantly the reported percentage of granulocytic MDSCs, confirming that these cells are especially sensitive to cryopreservation and/or thawing. Moreover, from the analysis of the results we hypothesized that the gating strategy was a major parameter causing the large variations in the frequency of the 10 potential MDSC subsets. To test this hypothesis, we have prepared an additional step, consisting of an ***in-silico*** gating panel to study how the gating strategies affect the variation in the reported results. We thus invited previous participants to take part in this additional gating panel, in which reference flow cytometry files (FCS) were sent out so that all other sources of variation were removed and only the gating aspect was addressed. In the first step, participants were asked to analyse the provided FCS files by using their own gating strategy, while in the second step, organizers provided a gating strategy guideline to apply on the same FCS files. From the results obtained by the new analyses of the files, we will propose further strategies to harmonize gating parameters and to define strategies for a robust and consistent characterization of MDSC subsets.

Keywords: MDSC, Innate immunity, multicolor flow cytometry

A204 / Digital spatial profiling platform allows for spatially-resolved, multiplexed measurement of protein distribution and abundance in FFPE tissue sections

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Characterization of the spatial distribution and abundance of proteins within tissues enables a better understanding of biological systems in many research areas, including immunology and oncology. However, it has proven difficult to perform such studies in a highly multiplexed manner. To address this unmet need, we have developed a novel optical-barcode based microscope and tissue-sampling platform designed to simultaneously analyze hundreds of proteins on a single FFPE section (Digital Spatial Profiling, DSP). DSP probes are not multiplex-limited by spectral resolution. Instead, “colors” are determined using barcode indexing oligos that are conjugated to antibodies. These indexing oligos are conjugated via UV-cleavable linkers and, following UV light exposure, are released and siphoned off the tissue surface via a microcapillary tip. This UV-cleavage is precisely controlled by a digital micromirror device that can illuminate discrete regions ranging from entire tissue microenvironments to single-cells. For quantification of signal, the photocleaved oligos are hybridized to NanoString barcodes, providing digital counts of the protein in each region-of-interest using NanoString nCounter instruments. Using this novel approach, we spatially resolve protein expression over 30 immune targets, simultaneously, on melanoma and colorectal cancer, from whole tumor biopsies and TMAs. We demonstrate multiplexed detection from discrete regions within a tumor (tumor center and immune invasive margin), enabling systematic interrogation of immune activity in clinical FFPE samples. Finally, we validate that indexing oligo conjugation and high multiplexing of antibodies do not interfere with specificity. The simplicity of the DSP platform allows high-resolution, high-multiplexed, spatially-resolved protein characterization in any lab capable of performing immunohistochemistry procedures, providing a potential method that can bridge the gap between translational research discovery and clinical applications. Continued work on the platform will expand the library of protein targets accessible for profiling and future assay development will demonstrate multiplexing up to 800 targets.

Keywords: spatial protein profiling, FFPE, biomarkers

A205 / An oligoclonal gp100-specific TCR repertoire of high avid, polyfunctional and tumor-reactive CD8+ T-cell clones, identified in melanoma patients treated with chemoimmunotherapy

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We have recently reported that combined chemoimmunotherapy improves the anti-tumor response of CD8+ Melan-A-specific T cells and significantly impacts the overall survival of melanoma patients. In particular, dacarbazine (DTIC) injected one day before peptide (Melan-A/ gp100)-vaccination plus IFN-alpha improves the anti-tumor lytic activity and enlarges the repertoire of Melan-A-specific T-cell clones, as compared with vaccination alone. This functional advantage has been shown to be not impaired by the presence of high level of PD-1 in the Melan-A-specific CD8+ T cells.
To identify functional differences in the response against the two different Ags employed in the vaccination protocol, we have analyzed a panel of gp100-specific CD8+T-cell clones isolated from patients treated with peptide-vaccination alone (Arm1) or DTIC plus vaccination (Arm2). We have analyzed the treatment-induced response in terms of TCR-beta sequencing, differentiation phenotype, inhibitory receptor profile, polyfunctionality and anti-tumor lytic capability. CD8+ gp100-specific T cells isolated from patients treated with vaccination alone showed an early differentiated phenotype, while those isolated from patients treated with DTIC plus vaccination show a late differentiated profile, as defined by the expression of CD28 and/or CD27 molecules. Analysis of the TCR Vβ chains of 28 sequences with in frame rearrangements of TRBV, TRBD, TRBJ and TRBC segments showed that, irrespective of the vaccination protocol, treatment-driven TCR profile of gp100-specific CD8+ T-cell clones expressed an oligoclonal repertoire. High anti-tumor lytic activity and polyfunctionality (in terms of TNF-alpha, IFN-alpha and Granzime B production), accompanied by low PD-1 expression, were observed in gp100+ CD8+ T-cell clones isolated after chemoimmunotherapy. Differently, clones isolated after peptide vaccination alone expressed high level of PD-1 inhibitory molecule, either alone or along with LAG-3 and TIM-3, and were neither tumor-reactive nor polyfunctional. The anti-PD-1 mAb blockade was able to increase the low anti-tumor polyfunctionality of these T cells.
These findings allowed us to identify anti-gp100-specific clonotypes from high avid, polyfunctional and tumor-reactive CD8+ T-cell clones. Of relevance we found that PD-1 was directly involved in the low anti-tumor functionality of gp100 specific CD8+ T-cells, differently from what observed for Melan-A specific CD8+ T cells. Importantly, we have demonstrated that the beneficial

anti-melanoma T-cell functional advantage induced by combined chemoimmunotherapy consists of CD8+ T-cell population with different molecular and phenotype profile, depending on gp100 or Melan-A Ag specificity.

Keywords: chemo-immunotherapy, melanoma, TCR repertoire

A206 / Personalized therapy of pediatric B-cell acute lymphoblastic leukemia - molecular determinants of CD19 splicing variants as a biomarker for the resistance to CART-19-therapy

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B-cell acute lymphoblastic leukemia (B-ALL) is the most common childhood cancer and the prognosis of children with relapsed or therapy refractory disease remains a challenge. Over the past years, a form of immunotherapy that includes utilizing modified T-cells expressing chimeric antigen receptors (CAR-therapy) against CD19 (CART-19) has been presented as a promising approach towards improving therapy for refractory and relapsed leukemia. Due to its specificity to B-cells, CD19 has been established as a promising target for CAR-therapy. However, 10 to 20% of the patients suffer another relapse and 30-50% of these relapses are caused by the loss of detectable CD19 which leads to the question about alternative mechanisms tumor cells use to become invisible to treatment. Especially, epitope-loss under therapy pressure has been suggested as such a mechanism of tumor cells to escape the recognition from CART-19 therapy. We recently published that an alternatively spliced ***CD19*** mRNA isoform lacking exon 2, and therefore the CART-19 epitope, is expressed at different levels in leukemic blasts at diagnosis in children and in the bone marrow of non-leukemia-pediatric donors. These results prove that some of the CD19 isoforms contributing to CART-19 escape already pre-exist at diagnosis and could evolve as a dominant clone during CART-19 therapy. In this project, we propose to dissect the molecular determinants of ***CD19*** exon 2 splicing. We will analyze the genomic sequence of B-ALL patients for mutations and SNPs (Single Nucleotide Polymorphisms) at exon 2 and in splice factor genes and also screen for dysregulated splice factor levels. To explain the impact of patient-specific alterations, we will utilize high-throughput random mutagenesis to identify all possible mutations and SNPs that can affect exon 2 skipping, and link these to the splice factors. Together, these approaches will help us to explain the effect of B-ALL-associated mutations and SNPs as well as changes in splice factor abundance. This may help in the early identification of patients who will relapse under CAR 19-therapy.

Keywords: B-ALL, CAR-therapy, CD19

A207 / T cell recognition of breast cancer antigens

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Despite originally considered an immunologically silent malignancy, recent studies are encouraging research of breast cancer immunogenicity to evaluate the applicability of immunotherapy as a treatment strategy. The epitope landscape in breast cancer is minimally described. Consequently, this project investigates four proteins commonly upregulated in breast cancer and thus probable tumor associated antigens (TAAs). Aromatase, prolactin, never in mitosis a related kinase 3 (NEK3), and protein inhibitor of activated STAT3 (PIAS3) contribute to increase growth, survival, and motility of malignant cells.

Aspiring to uncover novel epitopes for cytotoxic T cells, a reverse immunology approach is applied. ***In silico*** screening via NetMHC is used to predict peptides within the full length of each of the four proteins that bind to HLA-A*0201 and HLA-B*0702. An MHC ELISA is then applied to experimentally confirm which of the peptides are indeed HLA-A*0201 and HLA-B*0702 binders. Hereafter, a novel method for high throughout detection of antigen specific T cells is applied. Via DNA barcode labeled MHC multimer technology parallel screening for T cell recognition of all MHC binding peptides is performed. A cohort of breast cancer patient samples and healthy donor samples are included and compared.

Via in silico screening of the protein sequences, 415 peptides were predicted as HLA-A*0201 and HLA-B*0702 binders. Subsequent in vitro binding analysis in a MHC ELISA platform confirmed binding for 147 of the 415 predicted binders. The 147 peptides were evaluated for T cell recognition utilizing DNA barcode labeled MHC multimers to screen peripheral blood lymphocytes from breast cancer patients and healthy donor samples. Significantly more TAA specific T cell responses were detected in breast cancer patients than healthy donors for both HLA-A*0201 ($p=0.0039$) and HLA-B*0702 ($p< 0.001$) restricted peptides. Importantly, several of the identified responses were directed towards peptides that were predicted as poor or intermediate affinity binders. This is indicative of the importance of inclusion of these in the search for epitopes within shared TAAs.

Thus, the inspected proteins aromatase, prolactin, NEK3 and PIAS3, indeed contain targets for T cell reactivity. Further research will include functional testing of peptide specific T cell cultures to validate the peptides as true T cell epitopes through demonstration of intracellular processing and presentation at the cell surface.

Keywords: Tumor associated antigens, Epitope mapping, Antigen specific T cells

A208 / A novel method to capture the pharmacological activity of bispecific antibodies in patients with hematological malignancies

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A significant increase number of bispecific antibodies (BsAbs) leading to T-cell activation and serial lysis of tumor cells are currently in different clinical stages. However, no methods to stratify patients with remarkable antibody-mediated cytotoxicity are available to select patients with higher therapeutic potential in vivo for these constructions. The aim of the present study is to develop and ***in vitro*** assay to better quantify the activity of BsAbs and capture the interpatient variability. Fresh whole Bone Marrow (BM) or Peripheral Blood (PB) were tested with their corresponding BsAbs at 8 different concentrations in different time points (24h-144h). We tested 31 AML BM samples with the CD123xCD3 BsAb and 7 CLL and 3 B-ALL samples with Blinatumumab. When appropriate, basal quantification of TAA was performed by flow cytometry (FCM). The PharmaFlow platform efficiently count by FCM how many tumor cells are killed by every activated T-cells, here called effective E:T ratio. Eight-colour FCM staining was performed to simultaneously analyze the leukemic population, activated CD4 and CD8 T-cells and the residual normal cells. EC_{50} or E_{max} was calculated to evaluate potency or efficacy. Kinetics of activity was measured repeating the dose response curves in 3 different days. Most of the samples present both T-cell activation (CD25+) and an effective lysis of tumor cells after BsAbs exposure in a time and dose dependent manner, even starting with low basal E:T ratios (< 1:100). By contrast, differences in T-cell cytotoxicity or leukemic immunoresistance were observed between samples in terms of EC_{50} or E_{max}, even more marked between CLL samples. The integration of effective E:T ratios, EC_{50}, E_{max}, and kinetics allow us to generate an in vitro response model and select those samples with higher T-cell cytotoxicity after the different BsAbs exposure. Interestingly, many of the samples for all the BsAbs leave a significant proportion of live cells, even at the higher BsAb concentrations or with a remarkable expansion of activated T-cells that suggest the use of immunecheckpoint to unblock this immnunoresistant status. We have developed an automated FCM assay for BsAbs screening that keep intact both basal effector to target (E:T) ratios and native environment using whole PB or BM samples. The PharmaFlow platform selects different in vitro T-cytotoxicity effects across patients identifying best patient candidates for adoptive antitumor immunotherapy with BsAbs. The integration of Effective E:T ratios and pharmacological parameters better predict the ***in vitro*** response of BsAbs. Because of the high capacity of the PharmaFlow platform, additional antibodies constructions alone or in combinations with immunomodulatory agents could be tested to identify the better agents or immunotherapeutics combinations in hematological diseases.

Keywords: Bispecific Antibodies, Hematological Malignancies, Immuno-Oncology

A209 / Noninvasive imaging of tumor microenvironment as a predictive tool

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Immunotherapy has yielded durable remissions across a spectrum of malignancies. While encouraging, a significant fraction of patients fail to respond to therapy and may suffer serious side effects. Predicting and monitoring therapeutic efficacy remains an important challenge. Techniques to achieve these goals without multiple biopsies or other invasive methods will be critical. Rarely does a patient present with a single metastasis: usually there are several lesions. It is not possible to do biopsies, immunohistochemistry and immunostaining on all lesions. Some lesions are not even accessible for biopsies. Moreover, many cancers evolve quickly and different lesions may differ substantially from each other. Immuno-PET can provide a whole body scan and provide information for all lesions. The efficacy of the various forms of immunotherapy correlates with changes evoked in the tumor microenvironment (TME). Non-invasive monitoring of events occurring in the TME can be used to tackle this issue.
The presence of CD8 T cells in the tumor microenvironment correlates with a favorable response to immunotherapy. However, their dynamics in the course of a response are not known. We used

immunoPET using an anti-CD8 single domain antibody (VHH) to noninvasively monitor intratumoral CD8 T cells. When injected into mice, the radiolabeled anti-CD8 VHH showed lymphoid structures with great clarity and specificity. We showed immunoPET can detect tumors (B16 melanoma and panc02) by virtue of the presence of intratumoral CD8 cells with excellent (~1mm) spatial resolution. We were able to distinguish infiltration into a tumor from a distribution more peripheral to an island of neoplastic cells. Next we tracked intratumoral CD8 T cells in the immunotherapy-susceptible B16 melanoma model in response to checkpoint blockade. We imaged tumor-bearing mice longitudinally over the course of treatment and analyzed dynamics of the CD8 T cell response. Animals that responded to CTLA-4 therapy showed homogeneous distribution of the anti-CD8 PET signal throughout the tumor, whereas more heterogeneous infiltration of CD8 T cells correlated with faster tumor growth and worse responses to checkpoint blockade. The critical parameter relevant for prognosis is the **distribution** of CD8 T cells, independent of PET signal strength (p value=0.035). To support the validity of these observations, we used two different transplantable breast cancer models, yielding results that conformed with predictions based on the anti-melanoma response. Conventional immunohistochemistry performed at necropsy confirmed the distribution of CD8 T cells as observed by immunoPET. It may thus be possible to use immuno-PET and monitor antitumor immune responses as a prognostic tool to predict patient responses to checkpoint therapies.

Keywords: CD8 T cells, ImmunoPET, Prognosis of immunotherapy

A210 / High throughput single T cell TCR and RNA sequencing in tumor infiltrating T cells

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Recognition of cancer cell antigens by the T-cell receptor (TCR) is essential for an effective T-cell response against tumors. Upon recognition and activation, tumor-reactive T cells undergo clonal expansion within the tumor mass and draining lymph nodes. Studying the TCR repertoire of tumor infiltrating T-cells (TILs) allows to evaluate tumor's immunogenicity and identify tumor-reactive T cells, hence providing prognostic and therapeutic opportunities. We have developed a novel high throughput protocol to characterize the sequence of TCR alpha and beta chains from single T cells coupled with whole cell's transcriptomic profiling. Thousands of T cells isolated from blood and tumors of ovarian cancer patients were sorted into 384-well plates and expression of relevant T cell markers, such as PD1 and TIM3, was recorded for each T cell, during sorting. Full-length RNA was reversed transcribed and alpha and beta TCR chains were amplified and sequenced. The TCR was successfully identified in 68-80% of sorted T cells. The ability to characterize the TCR sequence in individual T cells in a high throughput assay combined with single T cell RNA sequencing is providing us with the opportunity to interrogate the transcriptional program of tumor expanded T cell clones and compare their frequency and phenotype in blood and tumors.

Keywords: Tumour-infiltrating T cells, Single-cell RNA seq, TCR seq

A211 / Expansion of adaptive NK cells in HBV-associated HCC

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Hepatocellular carcinoma (HCC) is one of the most frequent cancers in both incidence and mortality worldwide. Since treatment options are limited new treatment strategies for HCC are urgently needed. NK cells offer a potential target for HCC treatment as they have antitumor activity and represent up to 40% of all liver immune cells. Importantly, the human NK-cell repertoire is highly diverse and subsets differ in their phenotype and effector function. Therefore, a better understanding of NK-cell subsets including their appearance and antitumor activity is needed for the design of effective NK-cell involving immunotherapeutic approaches in HCC. The deepest knowledge of NK-cell diversification has been gained in the context of cytomegalovirus (CMV) infection. Indeed, CMV-associated heterogeneous NK-cell subsets have been described including the so called adaptive NK cells that are characterized by high expression of the maturity marker CD57, downregulation of the transcription factors PLZF, Helios and the signaling molecule FcεRIγ. Compared to conventional NK cells, adaptive NK cells exhibit increased antibody-dependent cellular cytotoxicity but decreased cytokine responsiveness. Yet, only little is known about antitumor activity of adaptive NK cells and frequencies in solid tumors like HCC.
To address phenotypic and functional NK-cell diversification in HCC, we compared circulating NK cells derived from 53 HCC patients and 28 healthy donors (HD) taking HCC etiology and CMV serostatus into account. Our data revealed that the presence of adaptive

FcεRIγ- NK cells in HCC patients depended on CMV seropositivity. Interestingly, compared to HD, a significant expansion of adaptive NK cells could only be observed in HCC patients with underlying cHBV infection, but not with underlying hepatitis C, alcoholic liver disease or non-alcoholic fatty liver disease. Of note, adaptive NK cells of HD and HCC patients regardless of etiology displayed a similar phenotype by displaying a significant downregulation of PLZF and a significant upregulation of CD57. To test antitumor activity of adaptive NK cells we performed co-culture experiments with the hepatoma cell lines HepG2 and HuH7 cells and assessed degranulation and cytokine production. In preliminary experiments, we could observe that adaptive NK cells had impaired antitumor activity compared to conventional NK cells suggesting functional relevance of adaptive NK cells in HCC.

In sum, our data demonstrate that the NK-cell repertoire is heterogeneous in HCC including the presence of adaptive NK-cell subsets clearly depending on CMV seropositivity. Importantly, the occurrence of CMV-associated NK-cell diversification is linked to etiology in HCC. This may have implications for antitumor activity that is potentially reduced in adaptive compared to conventional NK cells and should therefore be considered in the design of NK-cell involving immunotherapeutic approaches.

Keywords: Adaptive NK cells, Hepatocellular carcinoma (HCC), Antitumor activity

A212 / Detection and functional assessment of regulatory T cells in clinical samples

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Regulatory T cell (Treg)-mediated immunosuppression is considered a major obstacle for successful cancer immunotherapy. Given their profound effect on the outcome of immunotherapy trials, Tregs are being studied extensively in clinical trials. However, the multitude of Treg definitions in the reported studies makes correct interpretation of data and comparisons between studies difficult. Also, unambiguous enumeration of Tregs by flow cytometry is hampered by the absence of an exclusive, highly specific marker and the inability to directly measure their function in immunomonitoring of clinical trials. The CIP reached consensus with leading experts in the field concerning the use of an essential marker set comprising antibodies to CD3, CD4, CD25, CD127, Foxp3, Ki67 and CD45RA to define human Tregs. The use of markers was validated in a series of PBMC from healthy donors and cancer patients, as well as in tumor-draining lymph nodes (TDLN) and freshly isolated tumors. Also a robust gating strategy for the flow cytometric analysis of Tregs in human samples was determined.

To prove that the cells identified through this marker set are indeed Tregs with suppressor function, we have developed an assay to assess Treg functionality in the setting of (limited) clinical trial samples. This assay, which was adapted from a protocol described by Tree ***et al.*** (Diabetes 64(11):3891-902, 2015), is a highly efficient (using only 10,000 cells) protocol based on measuring Treg suppressive function through a 3H-thymidine-based approach. Analysis through this assay revealed that CD4+ Tregs that were isolated from peripheral blood of CxCa patients via $CD25^{pos}CD127^{low}$-guided flow sorting indeed exhibited suppressive capacity. Also, this 3H-based approach was much more sensitive than flow cytometry-based assays relying on measuring inhibition of responder cell proliferation or activation.

In summary, CIP has set up a guideline for phenotyping Tregs by flow cytometry. In addition, a highly efficient and sensitive 3H-thymidine-based protocol for measuring Tregs suppressive function in limited cell samples was established. Moreover, Tregs identified and isolated through CD25, CD127 and expressing Foxp3 exhibit suppressive capacity and represent bonafide Tregs.

Keywords: CIP, Tregs, monitoring

A213 / Development of IFN- γ /IL-2 FluoroSpot assay for monitoring HPV L1-specific immune responses in HPV vaccinees

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Objective: Human Papillomavirus (HPV) vaccines are adopted worldwide. To monitor T cellular immune responses after vaccination, a quick and simple assay is needed. IFN-γ and IL-2 are robust sentinel cytokines secreted by antigen-specific T cells. The objective was to evaluate the performance of a multiplexed fluorogenic ELISpot assay (FluoroSpot), based on IFN-γ/IL-2 double staining, to detect T cell responses after HPV vaccination.

Methods: Six healthy women (age 20-30) were enrolled and vaccinated with 3 doses of HPV vaccine according to the recommended schedule. Blood samples were drawn before 1st dose, and 1-2 month after 2nd and 3rd dose application. For comparison a cytometric ***ex-vivo stimulation*** assay and the new fluorogenic ELISpot assay were performed in parallel. PBMC were stimulated with HPV16 or 18 L1 synthetic 30mer peptide pools. Intracellular IFN-γ/IL-2 expression was assessed by four color flow cytometry (FACS) in antigen-specific (CD154+) CD4+ T cells 24 h

after stimulation. In parallel PBMC were subjected to FluoroSpot by seeding 2x10^5 PBMC directly into the wells and stimulation with L1 synthetic peptides for 24 h. Secreted cytokines were visualized by fluorochrome-labeled mABs (hence termed FluoroSpot assay). In addition, PBMCs of a Cervarix™ vaccinee 1 year after the 3rd dose were stimulated with virus-like particles (VLPs) of HPV16/18L1 (Serum Institute of India) for a comparison to synthetic peptide pools. A concentration gradient and dialyzed vs non-dialyzed group was tested. Spot numbers were readout using AID *i*Spot Elispot Reader (AID, Strassberg, Germany).

Results: After HPV vaccination, IFN-γ and IL-2 expressing T cells were significantly increased in all samples after 2nd and 3rd dose by FACS measurement (3-41fold for IFN-γ, 7-60 fold for IL-2, depending on the vaccinee). Similar results were found by IFN-γ/IL-2 double-stained FluoroSpot assay (4-60 fold by IFN-γ, 3-43 fold by IL-2). The results assessed by FluoroSpot assay and FACS measurement correlated excellently. IFN-γ and IL-2 responses did not show significant differences with titration of dialyzed VLPs from 1 mg/ml to 9 mg/ml (315-445 spots/10^6 for IFN-γ, 850-1000 spots/10^6 for IL-2), similar to the stimulation by peptide pools (330 spots/10^6 for IFN-γ, 780 spots/10^6 for IL-2). No significant difference was found in stimulation with dialyzed/non-dialyzed VLPs (330 vs 310 spots /10^6 for IFN-γ, 915 vs 825 spots /10^6 for IL-2, respectively).

Conclusion: FluoroSpot assay demonstrated good analytical performance and showed excellent concordance with the ex vivo IC-FACS. VLPs showed similar stimulation as compared to synthetic peptide pools. Dialysis of VLPs is obsolete. The L1 FluoroSpot is much more easy and robust to perform and may be an attractive option to monitor the cellular immune responses after HPV vaccination. It will be further developed to a 3-color assay, accompanied by a L1 B-cell FluoroSpot assay for comprehensive readout.

Keywords: HPV vaccine, T cell FluoroSpot, IFN- γ/IL-2

A214 / Index ATAC-seq reveals single-cell T cell receptor identity and chromatin accessibility for precision immune profiling

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T cells create vast amounts of diversity in their T cell receptor (TCR) genes, enabling individual clones to recognize particular peptide-MHC ligands. Here we combine transposase accessible chromatin analysis and TCR sequencing (iATAC-seq) at the single cell level. Using this approach, we identify epigenomic signatures in immortalized leukemic T cells, primary human T cells from healthy volunteers, and primary leukemic T cells from clinical patient samples. In healthy peripheral blood CD4$^+$ T cells, we identify ***cis*** and ***trans*** regulators of naïve and memory T cell states and find significant heterogeneity in previously defined populations. In patients with cutaneous T cell lymphoma, we identify leukemic and non-leukemic regulatory pathways in cells from the same individual, which has not been possible previously. Thus iATAC-seq is a new tool enabling analysis of epigenomic landscapes in clonal T cells and should be valuable for studies of T cell malignancy, immunity, and immunotherapy.

Keywords: Epigenomics, Single cell profiling, T cell receptor

A215 / Treatment monitoring by ^{18}F-FDG-PET/MRI in the spleens of melanoma bearing mice demonstrates the induction of a peripheral immune response during a new combined immunotherapy

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Immune checkpoint (ICP) modulation is a promising but expensive therapeutic strategy for cancer patients. PD-1 blockade protects from the exhaustion of effector T cells but is only effective in < 40% of melanoma patients, which makes an early identification of non-responders essential. Molecular imaging may provide an important contribution to monitor these treatments and to identify non-responders at an early time point. Aim of our study was to analyze the response to a new immunotherapeutic combination of local tumor irradiation (LIR) followed by PD-L1 and LAG-3 (PDL1/LAG3) blockade. Thus, we measured the glucose metabolism in the spleens as surrogate for the peripheral T cell pool of melanoma-bearing mice using ***in vivo*** imaging.

We treated luciferase (luc) transgenic B16-F10 melanoma bearing mice with a single LIR (10 Gy) of the tumors followed by four injections of PDL1/LAG3 mAbs. To examine the glucose metabolism in tumors and spleens during the treatment, we performed three ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET/MRI scans: 6 days post

tumor inoculation (dpi; 1 day before LIR, baseline), 13 dpi (LIR + 2x PDL1/LAG3) and 20 dpi (LIR + 4x PDL1/LAG3). Tumor growth was determined by volumetric measurements and bioluminescence imaging (BLI) to quantify the luc-derived tumor signal; spleen volume was determined by MRI. Mice were sacrificed 21 dpi, organs were collected for histopathology. Animals receiving isotype mAbs combined with LIR (LIR+ISO, n=5) were used as control.
We successfully established a new therapeutic approach combining LIR+PDL1/LAG3 mAbs: 5/10 mice exhibited significantly reduced tumor growth (responders) (82±25 vs. 490±112 mm^3; $p< 0.01$) with constant stable BLI signal intensities compared to LIR+ISO treated mice, whereas 20% of the LIR+PDL1/LAG3 treated mice showed clearly progressive disease. Nevertheless, we observed an increase in ^{18}F-FDG-tumor uptake in all groups over time, suggesting a non-effective therapy. Next, we examined the effects on the spleen volume and its ^{18}F-FDG uptake by the 'total lesion glycolysis' (TLG, uptake*spleen volume). The spleen volume of LIR+ISO treated mice decreased significantly to 84±5% at 20 dpi compared to baseline ($p< 0.05$), while the spleens of the LIR+PDL1/LAG3 group were poorly affected. In contrast, we observed an increase in TLG exclusively in the spleens of LIR+PDL1/LAG3-treated mice at 20 dpi compared to baseline (responders: +40±6%; $p< 0.001$; non-responders: +70±23%) while the spleens of LIR+ISO treated mice were not affected.
In summary, we showed that the combination of LIR+PDL1/LAG3 mAbs is highly effective in the B16-F10 melanoma model. Moreover, we demonstrated that the glucose metabolism in the spleens of responders and non-responders is indicative. Considering the increased tumor-derived ^{18}F-FDG PET signal in responding mice, most probably mimicking progressive disease generated by T cell infiltration, spleen metabolism may be a new approach to detect responders to ICP blockade.

Keywords: Local Tumor Radiation, Immune Checkpoint blockade, Molecular imaging

A216 / *In vivo* imaging of splenic glucose metabolism and volume alterations induced by checkpoint inhibitor therapy in patients with malignant melanoma

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Immune-checkpointinhibitor therapy (ICIT) represent the most powerful treatment tool for patients with metastatic melanomas. Approximately 20% of anti cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibody and 40% of anti programmed cell death protein 1 (PD-1) antibody treated patients yield impressive anti-tumoral effects whereas 60-80% of patients do not respond to ICIT. The impact on the tumor and its microenvironment is studied intensively, whereas it is unclear where the induction of the anti tumor immune response takes place. Effects induced by ICIT on the immune system e.g. in secondary lymphoid organs in vivo remain elusive. Here we analyzed differences of the splenic glucose metabolism and volume under ICIT in patients with malignant melanoma by positron emission tomography using ^{18}F-fluorodeoxyglucose with simultaneous computed tomography (FDG-PET/CT).
FDG-PET/CT-data of 17 patients (baseline and post-therapy) were analyzed retrospectively. All patients suffered from multiple metastasis and received CTLA-4 (ipilimumab) and/or PD-1 (pembrolizumab or nivolumab) antibody treatment as third line therapy. Among them, 11 responded to checkpoint inhibitor therapy (3x nivolumab; 7x pembrolizumab; 1 x ipilimumab/pembrolizumab) whereas 6 did not respond (5 x pembrolizumab; 1 x ipilimumab). Patients with metastasis in the spleen were excluded from the study. Splenic volume was determined in CT, which was performed as a full diagnostic contrast enhanced CT, if contrast agent was not contraindicated. Regions of interest of the whole spleen were defined using the CT data and copied to the coregistered PET, which was analyzed semiquantively using the Hermes software. Total lesion glycolysis (TLG) was calculated by multiplication of the spleen volume and the SUVmean.
Therapy responder presented with a tendency towards smaller spleen volume (222±27,6 cm^3 vs.266 ±34,5 cm^3) in the baseline scan before ICIT, while SUV mean (1,7±0,06vs.1,8±0,07) and SUV peak (2,5±0,1vs. 2,7±0,1) were slightly higher. Additionally, in the responders we could observe a trend towards lower TLG (412±57 vs. 453±40).
ICIT therapy was associated with a spleen volume rising in both patient groups (responder +23,3±21,0; non-responder +28,9±20,4). Interestingly, the FDG uptake, as measured by SUVmean and SUVpeak increased in non- responders (SUVmean +0,14±0,1; SUVpeak +0,14±0,15) and decreased in responders (SUVmean -0,06±0,07; SUVpeak -0,1 ±0,09). In line with these findings, also the TLG increased in patients which are not responding to ICIT (+83,8±57,8).
These observations give insights into the effects of ICIT on the volume and glucose metabolism of the spleen. As it is unclear how the spleen contributes to the anti-tumoral immune response further preclinical studies are needed to elucidate the mechanisms of ICIT.

Furthermore we aim to evaluate on a larger patient cohort, if this is maybe useful to identify patients, which are more prone to respond to ICIT.

Keywords: immune checkpoint inhibitor, imaging, therapy monitoring

A217 / Interleukin 8 activity influences the efficacy of adenoviral immunotherapy in cancer patients

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Interleukin 8 (IL-8) production is upregulated in many cancers and it also connects to several pathways that have been shown to impair the efficacy of adenoviral immunotherapy. We studied the role of IL-8 in 103 cancer patients treated with oncolytic adenoviruses. High baseline serum IL-8 concentration was found to be independently associated with poor prognosis ($p< 0.001$). Intriguingly, a decrease in IL-8 concentration after treatment with oncolytic adenovirus also predicted better overall survival ($p< 0.001$). Moreover, normal baseline IL-8 was associated with improved prognostic potential of anti-tumor T cell activity and neutrophil-to-lymphocyte ratio. Fresh patient-derived ovarian tumor samples were used to study the combination of adenovirus and IL-8 neutralizing antibody ***ex vivo.*** We observed that IL-8 blockade i) augmented the cytolytic activity of oncolytic adenovirus and ii) influenced tumor-infiltrating lymphocyte (TIL) activation when co-cultured with tumor-associated neutrophils (TAN) and adenovirus. Together, these results indicate a role for IL-8 as a biomarker in oncolytic virotherapy, but additionally provide a rationale for targeting IL-8 to improve the efficacy of oncolytic adenovirus treatment. In conclusion, reduction of IL-8 activity in the tumor microenvironment could improve anti-tumor immune response resulting in enhanced efficacy of adenoviral immunotherapy of cancer.

Keywords: Interleukin 8, oncolytic adenovirus, biomarker

A218 / Spatial co-expression of immunomarkers at the cellular level in sequential sections of hepatic colorectal metastases

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Spatial profiling using immunohistochemistry is the current state-of-the-art, although the simultaneous visualization and quantification of multiple immunomarkers is not trivial. In simultaneous immunohistochemistry, multiple antibodies are used to label multiple antigens in one staining process. Effectively, multiplex-staining technologies are limited by the availability of primary antibodies from different species; directly labelled antibodies require an exclusive tissue preparation procedure. In contrast, sequential immunohistochemistry is more standardized and involves several iterations of labeling single antigens, each with a different label or secondary antibody type until all desired antigens are visualized. Hence, to open-up more opportunities in characterizing the immune contexture and tumor microenvironment, a multistage image analysis workflow in serial tissue sections, stained for specific immunomarkers and then computationally rendered into a single image, is developed. The approach is validated with the characterization of T cell function, exhaustion, and cell death in hepatic colorectal metastases using stains for CD3, CC3, CTLA-4, PD-1, and LCK on FFPE serial tissue sections. Extremely thin sections (approx. 1 µm thick) are used, in order to maximize the likelihood that all five consecutive stainings performed are within the average thickness of a single T cell. Automated immunohistochemistry is employed using prolonged incubation times to ensure sufficient staining on the thin sections. Image acquisition is carried out on a whole-slide imaging system which automatically detects the regions that contain the stained tissue and also determines a valid focal plane for scanning. Glass slides are imaged at high resolution in brightfield mode at a 40-fold magnification. The computational workflow is based on linear and nonlinear image registration, biomarker segmentation, and post-processing operations. Nonlinear registration is based on elastic deformations represented by B-splines; this involves the minimization of an energy functional that includes the dissimilarity between the source and target histological images, a regularization term, and an energy term that accounts for the geometrical consistency between elastic deformations. Biomarker segmentation on the aligned imagery is based on the supervised training of a random forest classifier using color, edge, and textural features. The resulting binary images can be further integrated into data

processing pipelines, e.g. for assessing overlapping cells, proximity to local cell neighborhoods, immune cell interactions, etc., or merged into a virtual section where immune cells are visualized with a customized additive color model. This in silico approach demonstrates that using standard immunohistochemistry methods, the distribution and visualization of multiple immunomarkers can be realized in a single virtual section.

Keywords: multiple immunomarkers, spatial profiling, computational workflow

A219 / Digital gene expression from low sample input: Highly multiplexed and robust profiling of formalin-fixed paraffin-embedded (FFPE) and fresh frozen samples from as little as 1 ng of RNA using the nCounter® platform

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Gene expression analysis from tissue biopsies and formalin-fixed paraffin-embedded (FFPE) samples are an industry standard and invaluable to scientists who seek to answer biological questions in the fields of translational and biomarker research. However, one of the major challenges faced by scientists and clinicians is one of sample conservation. Researchers with samples of low RNA content, biopsies with limited tissue amounts or working with rare cell types are faced with the difficult choice of how much (or little) of their irreplaceable samples to process, while ensuring sufficient sample remains for other analytical testing necessary to obtain the most biologically meaningful insights possible. Furthermore, samples such as FFPE are often degraded or crosslinked due to fixatives used in the tissue archival process. For this reason, it is important for molecular technologies to not only be generate quality data but to do so consuming the least amount of sample possible. NanoString's nCounter System is an automated platform that using a digital molecular barcoding technology, enables counting of unique transcripts within a sample. The standard nCounter protocol recommends RNA inputs of 100 ng for successful expression profiling. Here we demonstrate a method using a pre-amplification approach upfront of the nCounter workflow that significantly reduces RNA input requirements 10 to 100-fold depending on sample type. RNA extraction was performed on a sample set containing both F/F and FFPE samples from spleen, brain, lymph node and tumor tissues. Samples were profiled on the nCounter across NanoString gene expression panels using both the standard workflow and 100 ng of un-amplified input RNA, and either 1 ng (F/F) or 10 ng (FFPE) RNA with Low RNA Input workflow. Profiles were generated from all samples and the overall number of genes detected showed a high concordance (r>0.85) between both standard (amplified) and Low RNA Input (un-amplified) prepared samples. This indicates that the pre-amplification-based approach in the Low RNA Input method allows a significant reduction in the amount of sample needed to generate a robust expression data without a resulting negative impact in sample data quality. The data we present demonstrates successful validation of a Low RNA Input workflow for the nCounter platform enabling a robust measurement of expression levels for while increasing the sensitivity of detection of low expressing genes.

Keywords: Gene Expression Profiling, Low Input, FFPE; fresh/frozen

A220 / A fast, flexible and low cost process for neo-epitope based immune monitoring

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Advances in genomic technologies have paved the way to developing personalized cancer vaccines targeting neo-antigens. Patient-specific vaccines, targeting several neo-antigens in one go have already entered the clinic. Such personalized, multi-target approach poses a challenge for immune monitoring of vaccine-specific T-cell responses in a fast, flexible and cost-effective fashion. Most immune monitoring protocols use 9-15-mer synthetic peptides originating from the vaccine target antigen. In contrast to detection of shared antigen-specific T-cells, for which the same peptide batch can be used for several patients, immune monitoring of individual neo-antigen-specific T-cell responses requires small amounts of large numbers of peptides (e.g. 40 x 15-mer peptides for 10 neo-epitopes of 27 amino-acids), which can only be used for one single patient. Therefor, standard peptide synthesis approaches applying commercial peptide synthesizers not only lack required throughput and speed but also generate peptides at prohibitive costs.
Here, we present data demonstrating the flexible application of our high-throughput, low cost FastTrack peptide synthesis approach in comparison with different specifications of standard peptides in ex-vivo ELISPOT to monitor neo-antigen specific immune responses in patients participating in the IVAC MUTANOME Phase I clinical trial (NCT02035956).
Application of the new peptide synthesis method enables the assembly of up to 1200 peptides in less than 3 weeks at appr. 20% of standard synthesis costs.

Keywords: Immune Monitoring, Neo Epitopes, Peptides

A221 / Ubiquilin1 is necessary for antigen-receptor mediated proliferation of B cells by eliminating mislocalized mitochondrial proteins

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Proteins that have reached the end of their lifespan in the cell must be eliminated, and the proteasome is a major pathway by which cells degrade protein. Some cancers, such as multiple myeloma, are particularly susceptible to proteasomal inhibition, and the proteasome is the target of several FDA-approve anti-cancer drugs. However, proteasomal inhibition also targets healthy cells and can have potentially severe side-effects. Furthermore, resistance to these inhibitors typically evolves in the course of treatment. Ubiquilins (Ubqlns) are a family of ubiquitin receptors that promote the delivery of specific subsets of ubiquitinated proteins to the proteasome for degradation. UBQLN1, which is expressed in most cell types, has been shown to facilitate the degradation of mislocalized mitochondrial proteins. We found that ***Ubqln1***$^{-/-}$ knockout mice have a specific defect in peritoneal B1a cells, a subset of B cells that require tonic BCR signaling for their self-renewal. ***Ubqln1***$^{-/-}$ B cells also had defective responses to ***in vitro*** BCR stimulation, which causes inhibition of mitochondrial protein import due to a stimulation-dependent loss of mitochondrial polarity. Following BCR stimulation, ***Ubqln1***$^{-/-}$ B cells fail to upregulate protein synthesis to WT levels and therefore do not accumulate the proteins necessary for cell cycle entry. Diffuse Large B Cell Lymphomas (DLBCLs), a heterogeneous family of B cell cancers, can be categorized based on their expression profiles into three clusters, one of which is defined by overexpression of mitochondrial genes and referred to as the 'oxphos' cluster. Curated DLBCL cell lines can be similarly categorized. We found that unlike HeLa cells, BJAB cells that are of the 'oxphos' subtype of DLBCL are particularly sensitive to the loss of UBQLN1. We observed a rapid loss in proliferation and decreased protein synthesis in BJAB cells depleted of UBQLN1 by a doxycycline-inducible shRNA (compared to a non-targeting construct). The loss in proliferation was associated with an accumulation of mislocalized mitochondrial proteins as identified by quantitative global mass spectrometry of isolated cytosol from UBQLN1-depleted cells. We hypothesize that the accumulation of mislocalized mitochondrial proteins, either through overexpression of mitochondrial genes or depolarization of mitochondria upon cellular stimulation, renders UBQLN1 necessary for continued protein synthesis.

Keywords: B cell, proteasome, mitochondria

A222 / High-yield large scale generation of major histocompatibility class I (MHCI) complexes for immune monitoring

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The efficacy of cancer immunotherapy treatments, specifically checkpoint inhibitors, are dependent on the presence of neoantigen-specific T-cells, and these cell types can be important biomarkers of efficacy. Here, we sought to generate high-quality reagents to monitor the CD8+ T-cell response to neoantigen therapies at the patient-specific level. However, patient-specific immune monitoring requires the generation of 100s-1000s of unique recombinant major histocompatibility complex class I (MHCI) complexes, which is highly challenging given the elaborate, low-yield refolding protocol required to recombinantly generate UV cleavable peptide-loaded MHCI complexes. To address this limitation, we adopted the conditional UV peptide approach, which allows for the generation of large quantities of a single MHCI complex that could be subsequently utilized for high-throughput generation of 100-1000s of unique MHCI complexes through simple peptide exchange. Despite the potential of this approach for high-throughput generation of MHCI complexes, the refolding methods used to generate the UV cleavable peptide-loaded MHCI complexes have very low yield and have only been scaled to the 1-2 liter level. Therefore, we optimized refolding time and starting material ratios of UV MHCI complexes to improve the yield and quality of material generated, and the re-folded material was

analyzed by LCMS, SEC/MALS, and Native MS. These techniques allowed us to measure the ratio of components relative to each other, the presence of the stability-conferring UV peptide, and the amount of aggregated protein present in the sample. Additionally, we scaled up the refold volume from 1L to 5 and 15 L by removing a dialysis step present in some published refolding protocols. We found that a 1:1:100 molar ratio of human leukocyte antigen (HLA):beta-2 microglobulin (B2M):peptide and refolding over four days maximizes the yield of the exchange and minimizes the amount of aggregate produced. Refold volumes of 1L, 5L, and 15L produced yields of 4%, 10%, and 16%, respectively, and the 15L refold volume produced 95 mg of refolded material. Here, we developed and optimized methods to generate UV-cleavable peptide MHC complexes that can readily undergo peptide exchange following exposure to UV light. These scaled-up methods allow us to generate sufficient MHCI material for 950,000 neoantigens in a single week. These methods hold great promise in providing the tools needed to better understand the relationship between predicted neoantigens and the immune response in the context of cancer immunotherapy treatment.

Keywords: Major histocompatibility complex class I, MHC multimers, T-cell receptors

A223 / Tumor cell-intrinsic and immune cell expression of immune checkpoint molecules revealed by RNA in situ hybridization

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Cancer immunotherapy targeting the PD-1/PD-L1 pathway has been established as a new paradigm in cancer treatment with long lasting clinical benefits in multiple types of cancers. Despite the clinical efficacy observed in some patients, the majority of cancer patients do not respond to PD-1/PD-L1 blockades and the development of predictive biomarkers is in critical need to effectively stratify patients for PD-1 blockades. In addition to primary resistance, some responders relapse after a period of response and understanding the mechanism behind both types of resistance are critical to overcome current immunotherapy challenges.
In this study, we evaluated in situ single cell expression profiles of therapeutic checkpoint targets in the tumor microenvironment (TME) of archived FFPE tissues from two cancer types; 30 cases of non-small cell lung cancer (NSCLC) and 30 cases of ovarian cancer. Applying RNAscope® in-situ hybridization (ISH) assays, specific checkpoint target molecules are visualized in highly specific and sensitive manner in individual cells within tissue morphological context. We report that, in addition to PD-L1, multiple other immune checkpoint molecules, including TIM3, LAG3, PD-L2, and GITR (TNFRSF18) are expressed in the tumor cells in subsets of tumors from both NSCLC and ovarian cancer. Furthermore, we observed PD1 is often co-expressed with other therapeutic checkpoint targets, including LAG3, TIM3, and TIGIT, in the same immune cells infiltrated into the microenvironment. These findings demonstrate the utility of the RNAscope® ISH platform in helping to understanding cancer's mechanism to evade host immune surveillance and ultimately developing resistance against checkpoint blockades.

Keywords: Cancer immunotherapy, Checkpoint markers, Tumor microenvironment

A225 / CD103+ T cells are intraepithelial, activated cytotoxic T cells of interest as a target for immunotherapy in gynecological malignancies

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Cytotoxic T cells (CTL) are associated with an improved prognosis in a wide variety of epithelial cancers. However, CTL depend on close proximity to cancer cells to induce apoptosis. We have recently published that predominantly intraepithelial CD8+ T cells contribute to the prognostic effect of CTL infiltration in all major gynecological malignancies. Furthermore, we reported on the αE subunit (CD103) of the αEβ7 integrin as a marker for these intraepithelial CTL in endometrial, ovarian and cervical cancer. Here, we report on our extensive ***in vitro*** and ***in vivo*** characterization of these cells. First, flow cytometry analyses showed CD103+ cells to be mainly cytotoxic T cells with a CD3+TCRαβ+CD8αβ+CD4- phenotype and a heterogeneous differentiation status. Additionally, CD103+ CTL were found to dominantly co-express checkpoint molecules PD-1 and CD27. Multicolor immunofluorescence (IF) for CD3, CD8, CD103,

fibronectin, pSMAD2/3 and CD137 showed CD8+CD103+ CTL were indeed localized in close contact with the epithelium. They were characterized by nuclear pSMAD2/3 expression, a hallmark of TGF β signaling, and CD137 expression, indicating T cell receptor (TCR) signaling. ***In vitro*** modeling indicated that CD103 could easily be induced on peripheral blood CD8+ T cells upon contact with cancer epithelium when cancer-derived TGFβ was present. CD103+ and CD103- CD3+TCRαβ+CD8αβ+CD4- T cells were sorted from human tumor digests by fluorescence-activated cell sorting. Single cell RNA sequencing of the sorted cells revealed CD103+, but not CD103-, CTL highly express activation and exhaustion maker genes such as ***PDCD1*** and a novel set of potential immune checkpoints currently undergoing validation. ***In vivo***, human papilloma virus (HPV) E6/E7- targeted therapeutic vaccination combined with radiotherapy in a mouse model of cervical cancer increased intratumoral CD103+CD8+ CTL numbers, and CD103+ CTL infiltration was negatively correlated with tumor weight. Remarkably, HPV E7-specificity was largely restricted to CD103+CD8+ T cells, suggesting this subset might indeed be a population with great efficacy in eradicating tumors. Finally, CD103 immunohistochemistry of human tumors and TCGA analyses indicated CD103+ CTL are strongly associated with an improved prognosis in all three malignancies. Taken together, we have established that CD103+ CTL are intraepithelial T cells of a mainly exhausted cytotoxic phenotype that are highly effective in infiltrating and targeting tumors and are of great interest as a target for immunotherapy.

Keywords: CD103, Tumor infiltrating lymphocytes, Gynecological malignancies

A226 / Automated analysis of flow cytometry data to reduce inter-lab variation in the detection of MHC multimer binding T cells

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Manual analysis of flow cytometry data and subjective gate-border decisions taken by individuals continue to be a source of variation in the assessment of antigen-specific T cells when comparing data across laboratories, and also over time in individual labs. Therefore, strategies to provide automated analysis of MHC multimer-binding T cells represent an attractive solution to decrease subjectivity and technical variation. The challenge of using an automated analysis approach is that MHC multimer-binding T cell populations are often rare and therefore difficult to detect. We used a highly heterogeneous dataset from a recent MHC multimer proficiency panel to assess if MHC multimer-binding $CD8^+$ T cells could be analyzed with computational solutions currently available, and if such analyses would reduce the technical variation across different laboratories. We used three different methods, FLOCK, SWIFT, and ReFlow to analyze flow cytometry data files from 28 laboratories. Each laboratory screened for antigen-responsive T cell populations with frequency ranging from 0.01-1.5% of lymphocytes within samples from two donors. Experience from this analysis shows that all three programs can be used for the identification of high to intermediate frequency of MHC multimer-binding T cell populations, with results very similar to that of manual gating. For the less frequent populations (< 0.1% of live, single lymphocytes), SWIFT outperformed the other tools. As used in this study, none of the algorithms offered a completely automated pipeline for identification of MHC multimer populations, as varying degrees of human interventions were needed to complete the analysis. In this study, we demonstrate the feasibility of using automated analysis pipelines for assessing and identifying even rare populations of antigen responsive T cells and discuss the main properties, differences and advantages of the different methods tested.

Keywords: MHC multimers, Automated gating, Computational analysis

A227 / *Infino:* Bayesian inference to distinguish immune cell expression phenotypes and estimate immune infiltration into tumor microenvironment

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By developing Bayesian generative modeling techniques for RNA-seq expression mixtures of immune cells in the tumor microenvironment, we improve expression mixture deconvolution methods to investigate the differential effectiveness of immunotherapy. Several new cancer immunotherapies perform remarkably well for a fraction of patients, but responses are not universal. Robust infiltrate quantification may help clarify why certain patients benefit from treatment. A number of recent publications have demonstrated associations between the immune

composition in the tumor microenvironment with response to immunotherapy and with prognosis. Furthermore, there is mounting evidence that new exhausted immune cell phenotypes may play a role in the response to checkpoint blockade. However, accurate characterization of the immune cells that surround a tumor by cell surface markers is slow and expensive to ascertain. Several methods instead estimate the relative abundances of the immune cell types around a tumor by deconvolving gene expression in the tumor microenvironment region ***in silico***. First, we review the existing immune infiltrate quantification methods and their performance on synthetic mixtures of known composition. Our analysis suggests that existing techniques confuse similar cell types and fail to accurately characterize the rate of error in the deconvolution results. We then motivate and develop ***infino,*** a Bayesian hierarchical mixture deconvolution method tailored to RNA-seq data. Critically, we use information from the hierarchy of immune cell types to improve our deconvolution results. Our method produces a probability distribution of immune infiltration in RNA-seq mixtures for each cell type, with robust estimates of uncertainty at each level of the immune cell type hierarchy. While for many mixtures ***infino*** performs comparably to the state-of-the-art method CIBERSORT, we find that estimation at all levels of the immune cell type hierarchy enables more precise examination of complex tumor microenvironments.

Keywords: Immune infiltrate quantification, Tumor microenvironment mixture deconvolution, Immune cell expression phenotypes

CANCER-MEDIATED IMMUNOSUPPRESSION

A228 / Plasmodium infection inhibits the recruitment and activation of MDSCs and Tregs in the tumor microenvironment in a murine Lewis lung cancer model

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A major challenge in the development of an effective cancer immunotherapy is the ability of tumor and its microenvironment to suppress immune cells through a variety of mechanisms mediated by the release of cytokines, growth factors, exosomes, and the recruitment and activation of immunosuppressive cells. Myeloid -Derived suppressor cells (MDSCs) and regulatory Tcells (Tregs) are the major immunosuppressive cells recruited to the tumor microenvironment and are known to markedly inhibit Tcell antitumor response. Over expression of these immunosuppressive cells in cancer have been reported in several papers and are related to poor prognosis and diminished survival. We previously demonstrated that malaria infection promotes innate and adaptive immunity against cancer in a murine Lewis lung cancer (LLC) model. However, it is yet to be known if Plasmodium infection could inhibit the recruitment and activation of immunosuppressive cells in the tumor microenvironment. 6-8 weeks old female C57BL6 mice were randomized into four groups (n=5mice/group). Mice subcutaneously injected with $5X10^5$cells (LLC), tumor bearing mice injected with $5X10^5$ Plasmodium yoelli (LLC+Py), mice injected with Plasmodium yoelli (Py), and mice with neither LLC nor Py (Naïve). Spleens and tumor tissues were harvested 17 days post tumor implantation and the proportions of MDSCs were evaluated using flow cytometry. Total RNA from tumor tissues was extracted, reverse-transcribed and complementary DNA was generated. Cytokines analysis was then carried out using RT-QPCR. Immunohistochemical staining was used to analyze the tumor content of cancer associated fibroblasts and Treg. We found a significant reduction of MDSCs and Tregs in the tumor tissues of plasmodium treated tumor-bearing mice compared to that of the untreated group. RT-QPCR results show a significant inhibition of GM-CSF, M-CSF, G-CSF, VEGF, IL-6,IL-4, IL-1ß, COX-2, PGE2, and S100A9 proteins. Furthermore, Immunohistochemical results reveal a significantly reduced expression of cancer associated fibroblasts in the plasmodium treated tumor mice compared to the untreated group. More importantly, MDSC from the treated group show significant reduction in STAT3 protein expression compared

to those from the untreated group. These results reveal for the first time, the effect of plasmodium infection on the recruitment and activation of immunosuppressive cells within the tumor microenvironment and holds great promise for the development of effective immunotherapeutic strategies.

Keywords: MDSCs, Tumor microenvironment, Immunosuppression

A229 / Pharmacological activation of the HIF1-E2F7 complex may block the tumor infiltration capacity of immune suppressive cells through inhibition of NRP1 expression

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Tumor-induced immune suppression presents a major hurdle for Immunotherapy in which tumor infiltrating regulatory T cells and tumor associated macrophages dampen the anti-tumor immune response. These cells are therefore considered attractive therapeutic targets, of which the inhibition may improve the observed partial response rates to cancer immunotherapy.
Tumor hypoxia (intratumoral oxygen deprivation) is a hallmark of solid tumor development and an adverse prognostic factor as it promotes an immune suppressive tumor microenvironment. Namely, hypoxic tumor cells dampen the anti-tumor immune response by stimulating the secretion of the chemo attractants VEGFA and SEMA3A that respectively attract regulatory T cells and macrophages towards the tumor. Notably, two recent studies demonstrate that neuropilin (NRP1), a receptor protein expressed by regulatory T cells and macrophages, is essential for their tumor infiltration along these VEGFA or SEMA3A gradients. These data suggest that targeting of NRP1 may enhance the anti-tumor response by blocking the migration of regulatory T cells and macrophages towards the tumor.
Intriguingly, our data provides a novel opportunity to target NRP1 in these immune suppressive cells. Although NRP1 expression is known to be repressed by hypoxia, our recent data for the first time provide a detailed molecular mechanism for hypoxic NRP1 repression (***de Bruin A, et al., Nucleic Acids Res. 2016 May 5;44(8):3549-66***). By performing ChIP-seq and microarray analysis we identified NRP1 as a direct and repressed target of the novel, hypoxia-induced HIF1-E2F7 transcription complex, which represses ***NRP1*** expression through a E2F-binding site hub in the proximal promoter region. In addition, we confirmed ***in vivo*** repression of ***NRP1*** expression by the HIF1-E2F7 complex. Zebrafish embryos raised in hypoxia showed significantly reduced ***nrp1a*** and ***nrp1b*** expression, which could be rescued by injecting established morpholinos directed against hif1a and e2f7/8. In addition, our data revealed that morpholino-inhibition of the HIF1-E2F7 complex also derepresses NRP1 expressioin under physiological conditions. Based on these results we hypothesize that pharmacological stabilization and activation of the HIF1-E2F7 complex (using the FDA-approved DFO or PHD2 inhibitors such as DMOG), could result in inhibition of NRP1 expression. Our preliminary data indeed demonstrate that NRP1 expression can be repressed in human and mouse macrophages by DFO. We are currently testing this in regulatory T cells. Next we will analyze if pharmacological HIF1-E2F7 activation inhibits the tumor infiltration capacity of macrophages and regulatory T cells through repression of NRP1 expression in these cells, and if this enhances the anti-tumor response, using the B16 melanoma mouse model.

Keywords: NRP1, HIF, VEGFA

A230 / Role of HMGB1 in breast cancer resistance to chemotherapy

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Among other forms of breast cancer, triple negative breast cancer (TNBC) is considered a challenge to treat in today's clinical practice. While refractory to hormone therapies, currently cytotoxic chemotherapy is the only systemic treatment option for these patients. Recent data suggest that chemotherapy, which acts by determining the destruction of tumor cells, also trigger immune responses, which are relevant for the clinical outcome of the therapy. In fact, immune response plays a fundamental role in the rejection of cancer, but can also exert regulatory effects promoting tumor growth. HMGB1 is an important molecule that can regulate the function of numerous immune cell types and is therefore poised on the cusp of cancer promotion versus prevention.
The aim of my project is to establish the role of HMGB1, a multifaceted protein that has the ability to modulate the inflammatory response, in the onset, progression and response to chemotherapy of triple negative breast cancers (TNBC).
The strategy comprises the use of a well-established spontaneous mammary tumor model driven by the loss of p53, a tumor suppressor often lost or mutated in TNBC, in the context of altered HMGB1 functionality. In addition to the evaluation of the role of HMGB1 on the development of the mouse mammary tumours, its impact on the response to conventional chemotherapy will be studied.

Keywords: breast cancer, immune response, HMGB1

A231 / Discovery and distribution of BTN2A1 in solid tumours and its potential as target for immunotherapy

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Cancer immunotherapies target immune-regulatory signal molecules such as the programmed cell death receptor PD-1 and cytotoxic T lymphocyte antigen CTLA4. Clinical trials in a variety of cancers have shown encouraging outcomes including durable responses which were first seen in melanoma. With the success of these therapies and the deeper understanding of the immune-system and its regulation in the context of cancer, many potential immune-regulatory molecules are being discovered and examined. Using an unbiased discovery-strategy utilizing phage display on fresh melanoma samples, we recently found high expression of BTN2A1 on the surface of melanoma cells but which was virtually absent in normal cells including fresh PBMCs and melanocytes. BTN2A1 shares sequence and structure similarity to the B7 family of immune-regulatory molecules, which include Btnl2 and Skint1 in mice. BTN2A1 has been suggested as an immune-regulatory molecule alongside other members of the same super-family. Consequently, we developed multiple antibodies (n>25) targeting human BTN2A1 for additional studies.
By flow-cytometry we tested panels of cell lines derived from multiple tumor types, including melanoma, breast, lung and prostate with these antibodies. All tested cancer cell lines were found to be positive for BTN2A1 expression and antibody-specificities were confirmed using CRISPR/Cas9 mediated knockout. Using multiplex fluorescent immunohistochemical staining (VECTRA) BTN2A1 was found to be highly membrane-expressed in 8/10 melanomas tested and multiple solid malignancies. Although minor staining could be observed in some normal tissues, BTN2A1 was generally more highly expressed in cancer cells, with the exception of strong staining observed in pancreatic islets. Additional functional testing revealed that expression of butyrophilins can be induced on CD4+ and CD8+ T cells after activation and cytokine treatment. These data warrant further investigations into the use and mechanism of BTN2A1 in the context of cancer-immunotherapy.

Keywords: Melanoma, Butyrophilin, immune checkpoint

A232 / The transcriptome of lung tumor-infiltrating dendritic cells reveals a tumor-supporting phenotype and a micro-RNA signature with negative impact on clinical outcome

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Targeting immunomodulatory pathways has ushered a new era in lung cancer therapy. Further progress requires deeper insights into the biology of immune cells in the lung cancer micro-environment. Dendritic cells (DCs) represent a heterogeneous and highly plastic immune cell system with a central role in controlling immune responses. The intratumoral infiltration and activation status of DCs are emerging as clinically relevant parameters in lung cancer.
In this study we used an orthotopic preclinical model of lung cancer to dissect how the lung tumor micro-environment affects tissue-resident DCs and extract novel biologically and clinically relevant information.
Lung tumor-infiltrating leukocytes expressing generic DC markers were found to predominantly consist of CD11b+ cells which, compared to peritumoral lung DC counterparts, strongly over-express the T- cell inhibitory molecule PD-L1 and acquire classical surface markers of tumor-associated macrophages (TAMs). Transcriptome analysis of these CD11b+ tumor-infiltrating DCs (TIDCs) indicates impaired anti-tumoral immunogenicity, confirms the skewing towards TAM-related features, and indicates exposure to a hypoxic environment. In parallel, TIDCs display a specific microRNA signature dominated by the prototypical lung cancer oncomir miR-31. ***In vitro,*** hypoxia drives intrinsic miR-31 expression in CD11b+DCs. Conditioned medium of miR-31-overexpressing CD11b+DCs induces pro-invasive lung cancer cell shape changes and is enriched with pro-metastatic soluble factors. Finally, analysis of TCGA datasets reveals that the TIDC-associated microRNA (miRNA) signature has a negative prognostic impact in non-small cell lung cancer.
Together, these data suggest a novel mechanism through which the lung cancer micro-environment exploits the plasticity of the DC system to support tumoral progression.

Keywords: dendritic cell, lung cancer, micro-RNA

A233 / Resistance to interferon-gamma is a common immune escape mechanism in colorectal carcinoma

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In colorectal carcinoma (CRC), the presence of a Th1 immune response has been associated with improved clinical outcome. This effect has been notably attributed to the direct and indirect anti-tumorigenic properties of IFN-gamma, the Th1 activator and effector cytokine. However, clinically detectable tumors are entities which have overcome immunosurveillance and the eliminatory pressure of the immune system. Indeed, we observed a discrepancy in the ***in situ*** expression of guanylate-binding-1 (GBP-1) - the most abundant IFN-gamma stimulated protein and a marker of IFN activity - in CRC between stromal cells and tumor cells, where it was lost in almost half of the cases. This suggested that CRC tumor cells become resistant to IFN-gamma in the course of tumorigenesis. Indeed, we found that several colorectal carcinoma cell lines failed to express interferon-stimulated genes after treatment with IFN-gamma, and were resistant to IFN-gamma-induced apoptosis or proliferation inhibition. In these cell lines, the loss of IFN-gamma responsiveness correlated either with the down-regulation of the IFN-gamma receptor alpha chain (IFNgRa) or with the presence of a mis-glycosylated form of IFNgRa, which displayed an aberrant intracellular localization. We further validated these findings at the clinical level by investigating the expression of IFNgRa in colorectal carcinoma at the RNA and at the protein level using a tissue microarray and observed a correlation between the down-regulation of IFNgRa expression and shorter disease-free survival together with a higher rate of distant metastasis. In addition, studies in conditional knock-out mice revealed that the absence of IFN-gamma receptor expression by intestinal epithelial cells fosters tumor growth in vivo. Altogether, our data suggest that the loss of IFN-gamma responsiveness is a common event in CRC and protects tumor cells against the anti-tumorigenic effects of IFN-gamma.

Keywords: colorectal carcinoma, interferon-gamma, immune evasion

A234 / CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity

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Cancer cells exploit the expression of the programmed death-1 (PD-1) ligand 1 (PD-L1) to subvert T-cell mediated immunosurveillance. Therapies that disrupt PD-L1 mediated tumour immune evasion have demonstrated considerable clinical success, highlighting the need to understand the molecular regulation of PD-L1 expression.
Using a genome-wide CRISPR/Cas9 loss of function screen we have identified the uncharacterized protein CMTM6 to be a key regulator of both constitutive and cytokine-induced PD-L1 expression in a broad range of cancer cells. CMTM6 is a previously uncharacterised, ubiquitously expressed protein that associates with PD-L1 in a membrane-anchored complex. CMTM6 is not required for trafficking of PD-L1 to the cell surface but is essential to maintain PD-L1 expression at the plasma membrane, and in its absence PD-L1 is targeted for lysosome-dependent degradation. Using a quantitative global proteomics approach to profile plasma membrane proteins regulated by CMTM6, we find that CMTM6 displays remarkable specificity for PD-L1. Importantly, loss of CMTM6 specifically decreases PD-L1 without compromising the cell surface expression or antigen presentation capacity of MHC class I. CMTM6 depletion in tumour cell lines alleviates PD-L1-mediated suppression of tumour specific cytotoxic T-cell activity both ***in vitro*** and ***in vivo,*** highlighting the functional importance of CMTM6 in maintaining the PD-L1/PD-1 immune checkpoint. Together these findings provide novel insights into the biology of PD-L1 regulation, identify a new master regulator of this critical immune checkpoint and reveal a potential therapeutic target to overcome immune evasion by cancer cells.

Keywords: PD-L1, Tumour immune evasion, CMTM6

A235 / Natural killer cell anergy in liver cancer patients and potential of adoptive cell transfer

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Introduction/Background: Primary liver cancer, hepatocellular carcinoma (HCC), accounts for over 700,000 deaths annually and is the second leading cause of cancer death. Its incidence is steadily increasing in Western countries. Treatment options for patients with advance disease are limited and systemic chemotherapies have dismal response rates. Better therapeutic options are warranted. Natural killer (NK) cells have been considered to be innate immune cells, independent on expression of tumor-

associated antigens and lyse tumor cells without prior sensitization. Therapeutic potential of NK cells has been revealed in seminal studies that haploidentical donor NK cells can prevent leukaemia relapse following haematopoietic stem cell transplantation in patients with acute myeloid leukaemia. Nonetheless, varying clinical responses have been reported with infusion of NK cells in patients with solid tumors and the major hurdles are the NK expansion scale and functionality of expanded population.
Aim: The current study aims to characterize NK cells isolated from liver cancer patients, expand and activate ***ex vivo,*** and examine their functional properties.
Methods and Results: In the pilot study, we have examined a panel of up-to-date protocols on ***ex vivo*** NK expansion and tailor-made one that induced an average of over 700-fold expansion after 21-days of culture in defined medium. Large scale NK preparation for therapeutic use will provide important information for adoptive transfer including autologous and allogenic NK infusion approaches. Furthermore, we showed that expanded NK cells, compared to baseline when cells were freshly isolated from HCC patients, were more active with increased expression of NK stimulatory receptors NKG2D and CD69. In addition, these expanded NK cells were more cytotoxic against HCC cells. Intriguingly, expanded NK cells when supplemented with autologous HCC serum, compared to healthy serum, showed decreased cell number, reduced expression of NK stimulatory receptors NKG2D and CD69, diminished production of secretory functional molecules perforin and IFNɣ, and decreased cytotoxicity against HCC target cells.
Summary/Conclusion: *Ex vivo* expansion and activation of NK cells isolated from liver cancer patients were demonstrated. These findings will have important biological and clinical implications to exploit the full potential of immunotherapy in cancer treatment.

Keywords: natural killer cells, liver cancer, anergy

A236 / Differential analysis of macrophages from tumors and adjacent tissue

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Treatment of heterogeneous tumors is clinically challenging, in part as multiple 'driver mutations' impair standardized therapies. Promising treatments lie in utilizing 'broader approaches' including epigenetics and immunotherapy. With a median survival of, for example, 14.6 months for glioblastoma brain tumors (GBM) new therapeutic strategies are wanting. Macrophages are innate immune cells that specifically eliminate tumor cells. However, once cancer cells escape this first step of immunoediting, macrophages can become re-educated, suppress the immune system and promote tumor growth. Pharmacological options to inhibit macrophages are available, but their broad inhibition commonly decreases treatment efficiencies. Less explored targets with broad application in various inflammatory contexts are the molecular mechanisms underlying macrophage re-education. Using GBM as focal point, we are characterizing heterogeneity in identity of macrophages (microglia) and infiltrating monocytes. Comparative analysis of populations derived from tumor and adjacent tissue indicates distinct signatures and we now aim to decode the signals underlying this shift in macrophage identity.

Keywords: Macrophage, Tumor-associated Macrophages, re-education

A237 / Targeting latency-associated peptide promotes anti-tumor immunity

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Regulatory T cells (Tregs) promote cancer by suppressing anti-tumor immune responses. Latency-associated peptide positive (LAP+) Tregs have been implicated in human cancer pathogenesis. In this study, we hypothesized that targeting LAP+ Tregs with anti-LAP antibody which binds to the LAP/TGF-beta complex on Tregs will reduce cancer induced immunosuppression and promote anti-tumor immunity and tested this hypothesis in cancer models. We found that anti-LAP enhances anti-tumor immune responses and reduces tumor growth in murine models of melanoma, colorectal carcinoma and glioblastoma. Based on gene expression analysis and flow cytometry, LAP+ CD4 T cells have an enhanced tolerogenic signature in tumor-bearing mice. Anti-LAP antibody decreases LAP+ Tregs, tolerogenic dendritic cells and TGF-beta secretion, and is associated with CD8+ T cell activation. Anti-LAP increases infiltration of tumors by cytotoxic CD8+ T cells and reduces CD103+ CD8 T cells in dLNs and spleen. We identified a new role for CD103+ CD8 T cells in cancer. Tumor-associated CD103+ CD8 T cells have a tolerogenic phenotype with increased expression of CTLA-4 and IL-10 and decreased expression of IFN-gamma, TNF-alpha, and granzymes. Adoptive transfer of CD103+ CD8 T cells promotes tumor growth whereas CD103 blockade limits tumorigenesis. Moreover, treatment with anti-CD103 antibody reduced tumor growth in cancer models. Finally, we found that anti-LAP treatment combined with tumor-

specific antigen dendritic cell vaccination enhances antitumor response and immune memory. Thus, anti-LAP targets multiple immunoregulatory pathways and represents a potential approach for cancer immunotherapy. Plans are underway to initiate human clinical trials.

Funding: Supported by the National Institute of Health (R21NS090163 to H. L. Weiner) and Innovation Discovery Grant (from BWH to H. L. Weiner).

A patent related to this study, titled "Treatment of cancer with anti-LAP monoclonal antibodies", has been filed (PCT/US16/13408).

Keywords: Treg, LAP, TGF-beta

A238 / TGF-β signaling suppresses tumor surveillance by converting tumor NK cells into type 1 innate lymphoid cells

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Avoiding destruction by immune cells is a hallmark of cancer, yet how tumours ultimately evade natural killer (NK) cell control remains incompletely defined. In this study we describe the TGF-β signalling-dependent conversion of NK cells (CD49a-CD49b+Eomes+) into intermediate type 1 innate lymphoid (intILC1) cell (CD49a+CD49b+Eomes+) and ILC1 (CD49a+CD49b-Eomes+/-) cell populations in the tumour microenvironment. Strikingly, intILC1s/ILC1s were unable to control local tumour growth and metastasis, whereas NK cells favoured tumour immune surveillance. Experiments using TNF-neutralizing antibodies suggested that innate immune escape was partially mediated by TNF-producing ILC1s. Together our data highlight the unexpected plasticity of group 1 ILCs in tumours and reveal a novel mechanism by which tumours escape innate immune surveillance.

Keywords: NK cells, Innate lymphoid cells, Immunesurveillance

A239 / Non-invasive *in vivo* imaging of ^{64}Cu-NOTA-αCD11b-labeled myeloid-derived suppressor cell homing to the primary and metastatic tumor microenvironment

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The expansion of immunosuppressive polymorphonuclear and monocytic myeloid-derived suppressor cells (PMN-/M-MDSCs) elicited by aberrant signaling is one of the hallmarks of cancer. MDSC subpopulations contribute to the formation of an immunocompromising microenvironment leading to tumor growth, pre-metastatic niche formation and, consequently, metastasis. Therefore, we aimed to visualize MDSC migration in mouse models for human cancer with varying aggressiveness by positron emission tomography/magnetic resonance (PET/MR) imaging.

MDSC subpopulations were differentiated ***in vitro*** from freshly prepared bone marrow-derived cells with GM-CSF and IL-6 over 6 days and isolated via magnetic cell sorting. PMN- and M-MDSCs were radiolabeled with a NOTA-bearing, ^{64}Cu-radiolabeled αCD11b monoclonal antibody (mAb). Effects of the radiolabel on viability and labeling stability were evaluated by flow cytometry, confocal microscopy and γ-counting. To study MDSC recruitment ***in vivo,*** PyMT breast cancer primary tumors and B16 melanomas were inoculated orthotopically while PyMT-metastases were induced by intracardiac injection. $2x10^6$ ^{64}Cu-NOTA-αCD11b-labeled PMN- or M-MDSCs were adoptively transferred into primary (n=6) or metastatic (n=5) breast cancer- and melanoma-bearing mice (n=5) via tail vein injection. Static PET and T2-weighted anatomical MR scans were acquired 3, 24 and 48 h post injection (p.i.) of radiolabeled MDSCs in all animal models.

As confirmed by confocal microscopy, the internalization of the αCD11b-mAb-integrin complex was completed within 3 h after labeling resulting in a reasonable efflux of radioactivity. Labeling of PMN-MDSCs and M-MDSCs with the αCD11b-mAb did not negatively influence MDSC functionality as confirmed in a T cell suppression assay. Internalization of the radiolabeled mAb did not have any major effects on cell viability. Both PMN- and M-MDSCs accumulated in PyMT tumors and B16 melanomas over the course of 48 h. B16 melanomas had significantly higher uptake values for PMN-MDSCs both 24 h p.i. (2.54±0.32 %ID/cc vs. 1.69±0.52 %ID/cc, $p< 0.01$) and 48 h p.i. (5.62±0.64 %ID/cc vs. 2.44±0.17 %ID/cc, $p< 0.05$) compared to PyMT tumors. Likewise, M-MDSC

uptake was higher in B16 melanomas than in PyMT tumors 24 h p.i. (3.14±0.013 %ID/cc vs. 1.73±0.26 %ID/cc, p< 0.01). PyMT lung metastatic lesions had significantly higher uptake values for both PMN-MDSCs (2.58±0.48 %ID/cc vs. 1.11±0.19 %ID/cc, p< 0.001) and M-MDSCs (3.87±1.13 %ID/cc vs. 1.66±0.023 %ID/cc, p< 0.001) compared to healthy lung tissue.

In conclusion, ***in vivo*** imaging of radiolabeled MDSCs revealed differences in the recruitment of PMN- and M-MDSCs as well as differences in overall MDSC migration to the breast cancer primary tumor microenvironment and established metastases. Moreover, a faster and stronger MDSC recruitment to melanomas was detected presumably due to increased cytokine and chemokine secretion.

Keywords: myeloid-derived suppressor cells, cell trafficking, breast cancer

A240 / Expression patterns of novel lncRNAs define virus-specific effector and memory CD8$^+$ T cells

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Upon repeated exposure to antigen, pathogen- or cancer-specific CD8$^+$ T cells enter a dysfunctional, exhausted state characterized by diminshed proliferative potential, poor target killing, and reduced cytokine secretion. This T cell exhaustion is a major barrier to clearance of cancer and persistent viral infection by the human immune system. Large transcriptional changes underlie CD8$^+$ T cell differentiation, including the naïve-to-effector, effector-to-memory, and effector-to-exhausted T cell transitions. To examine the role of long non-coding RNAs in these differentiation processes, we performed RNA-sequencing on antigen-specific CD8$^+$ T cells in acute and chronic infection in both humans and mice. We identify thousands of expressed lncRNAs in CD8$^+$ T cells during CD8$^+$ T cell differentiation in both humans and mice, many of which were previously unannotated. Strikingly, well-known CD8$^+$ T cell subsets can be define exclusively by their expression of novel lncRNAs identified in this study. Additionally, homologous lncRNAs in humans and mice show similar regulation during the course of CD8$^+$ T cell differentiation ***in vivo,*** indicating that some lncRNAs may be functionally conserved across mammals. Finally, exhausted CD8+ T cells express a unique set of lncRNAs, whose profile is altered with PD-1 pathway blockade and/or IL-2 treatment. Together, these results indicate that we have discovered important lncRNAs in CD8$^+$ T cell function, and that lncRNAs play important roles in T cell differentiation and exhaustion.

Keywords: lncRNA, CD8 T cells, T cell exhaustion

A241 / Exploring immunotherapy resistance in melanoma at the single-cell resolution

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Immunotherapy is expected to affect both the malignant and non-malignant cells in the tumor. Many of these affects remain poorly characterized, limiting our ability to predict and tackle immunotherapy resistance. Here, we leverage the power of single cell RNA-Seq (scRNA-Seq) to study immunotherapy resistance in 29 melanoma patients, consisting of treatment naïve and post-treatment patients who developed resistance to immune checkpoint inhibition. We analyzed over 7,000 cells, including malignant, immune, and stromal cells, and discovered substantial shifts in the transcriptional state of both malignant and immune cells post-immunotherapy. In the malignant compartment, we show that immunotherapy resistant (ITR) malignant cells can be characterized by a robust, and generalizable, gene signature. The ITR oncogenic signature manifests well-established immune evasion mechanisms, such as the repression of antigen presentation and interferon gamma responses, as well as transcriptional modules that have not been previously linked to cancer immunotherapy, including BAF and MYC targets, metallothioneins and TNF alpha signaling. Integrative computational analysis with bulk expression profiles of hundreds of melanoma tumors showed that the ITR state is strongly associated with poor prognosis and with the exclusion of T-cells from the tumor microenvironment. Furthermore, we found that the ITR state in pre-treatment tumors is predictive of lack of subsequent response to immunotherapy in mouse models and in patients. Among immune cells, we find key differences in T-cells post-immunotherapy compared to T-cells from treatment naïve tumors. Post-immunotherapy T-cells have unique transcriptional states and show massive clonal expansions, which are not observed in the treatment naïve populations. Lastly, we derive a genome-scale cell-cell interaction network that depicts the crosstalk between the different cells in the tumor ecosystem. The network reveals that the ITR oncogenic state substantially represses a host of communication routes between the malignant and immune cells. We are now testing these interactions mechanistically in malignant cells alone and in co-cultures of matched T-cell and malignant cells, which we derived from melanoma patients. We are applying Perturb-Seq to inhibit different ITR genes in a pool of malignant cells, and monitor the effects on both malignant cells and T-cells by subsequent scRNA-Seq. Overall, our study suggests that, even in patients with no objective clinical response, immunotherapies have activated the T-cells, but simultaneously induced or selected

for oncogenic cellular states, which permit immune evasion and exclusion. These states include both known and novel programs that could potentially be blocked to overcome immunotherapy resistance.

Keywords: Single-cell RNA-sequencing, Immunotherapy resistance, Melanoma

A242 / Identification of miRNAs affecting surface expression of CD73, resulting in altered susceptibility to recognition by cytotoxic CD8$^+$ T cells

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miRNAs can have tumor suppressive as well as oncogenic function. Several oncogenic miRNAs (oncomiRs) have been shown to promote tumor immune escape. However, little is known about the effects oncomiRs exert on the expression of inhibitory immune checkpoint molecules that impair tumor cell recognition by cytotoxic T cells (CTLs). Recently, the AMP hydrolyzing ectonucleotidase CD73 was described as a new inhibitory checkpoint molecule expressed by various tumor types.
The goal of this project is to identify miRNAs that modulate surface expression of CD73 on tumor cell lines of various entities, thereby altering their susceptibility to cognate CTL recognition.
First, various miRNA target prediction tools will be applied to determine miRNAs potentially regulating CD73 expression by direct binding. To confirm the *in silico* analysis as well as to find miRNAs indirectly regulating CD73 surface expression, a miRNA library screen will be conducted for one human cancer cell line, followed by direct measurement of CD73 surface expression by flow cytometry. As a result, miRNAs capable of up regulating or down regulating CD73 expression would be identified, both of which are of potential clinical interest. Subsequently, the most promising hits will be further validated by qPCR, WB and 3'UTR-reporter assays. Also, the impact of selected miRNAs on CD73 enzymatic function will be tested using AMP-Glo assay. Finally, the impact of the miRNA dependent alterations in CD73 expression on CTL-mediated killing of the cancer cell line will be experimentally verified.

Keywords: miRNAs, CD73, CTL recognition

A243 / Evaluation of the functional role of TAM kinases in tumour-driven immune suppression

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TAM kinases (**T**yro 3, **A**XL and **M**erTK) are reported to be expressed on a variety of tumour types and cells of the innate immune system. TAM kinases contribute to tumour survival, migration and metastasis. Upon binding to the ligands, MerTK regulates efferocytosis of apoptotic cells mediated by macrophages. Targeting TAM kinases *in vivo* leads to favourable anti-tumour immune responses and synergises with immune checkpoint blocking antibodies. However, the role of TAM kinases in anti-tumour effects of primary human immune subsets is yet to be fully investigated. Here, using publicly available datasets, we identified that *MerTK* mRNA is enriched in human macrophages but *AXL* and *TYRO3* are expressed in multiple subsets in the myeloid lineage. In accordance, we demonstrate that while MerTK is consistently induced by rhM-CSF on primary human monocytes, up-regulation of surface AXL requires addition of IFN-alpha. Importantly, MerTK is expressed at higher level when primary human monocytes are co-cultured with human tumour cells. Using CRISPR technology, we have successfully generated human tumour sublines that are deficient for either MerTK or AXL. The resulted sublines show comparable proliferation rate *in vitro* but exhibit altered surface expression of immune-related proteins. Functionally, MerTK- or AXL-deficient tumour CRISPR subline enahnces activation of CD8+ T cells, NK cells and CD4+ T cells in a tumour-immune co-culture system (TICS), to a similar extent as a PD-L1 blocking antibody. Altogether, these results suggest that TAM kinase family members MerTK and AXL are involved in regulating anti-tumour immune responses.

Keywords: MerTK, AXL, Macrophage

A244 / Systems level mass cytometric analysis of the effects of tumor-derived cytokines on myeloid cell development and function in a pre-clinical model of breast cancer

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Cancer immunotherapies have improved patient outcome and survival in a subset of human malignancies. However, particular types of tumors and a significant number of patients do not respond to current therapies, highlighting the need to better understand the interactions between tumors and the immune system. Phagocytes such as dendritic cells (DCs), macrophages and myeloid-derived suppressor cells (MDSCs) are critical in orchestrating or antagonizing innate and adaptive immune responses against tumors. In turn, phagocyte activity and development is heavily dependent on tumor-derived factors. We have identified a tumor-derived factor that alters phagocyte development and function. We hypothesize that this cytokine perturbs the myeloid compartment by impairing phagocyte

development and maturation, resulting in tumor-induced immunosuppression. Our results show that this tumor-derived factor impairs DC maturation and induces the expansion of immature myeloid cell progenitors and MDSCs. Interestingly, the cytokine is particularly powerful in terms of its ability to block the development of a subset of DCs ($XCR1^+CD11b^-$), which has been associated with improved cancer treatment outcome and strong $CD8^+$ cytotoxic T cell activation potential. Our ***in vivo*** data show that this tumor-secreted factor leads to anemia, leukocytosis, splenomegaly and enhances extramedullary hematopoiesis. Finally, we present cytometry by time-of-flight (CyTOF) data showing that tumor growth, and its associated cytokine expression, leads to a systemic accumulation of a complex mixture of immature myeloid cells, with associated perturbations in a wide variety of signalling pathways throughout the immune system. We believe that modulating this tumor-secreted factor could be key to improve immunotherapy efficacy.

Keywords: mass cytometry, phagocyte development, tumor-induced immunosuppression

A245 / Role of non-immune functions of regulatory T cells in tissue homeostasis and cancer progression

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Regulatory T cells (T_{reg}) are a T cell lineage vital to promoting tolerance to self antigens, commensal bacteria, and environmental antigens; as well as limiting responses to acute and chronic infections. Because of their critical role in immunosuppression in diverse biological settings -including the tumor microenvironment- therapeutic targeting of T_{reg} cells holds promise for cancer immunotherapy. In addition to dampening immune responses against tumor antigens and the associated inflammation, tissue-resident T_{reg} cells may directly support tumor growth, homeostasis, and metastasis. Hence understanding T_{reg} cell functions independent from immunomodulation may provide novel targets for cancer therapy without the adverse side effects associated with immune checkpoint blockade. Studies of T_{reg} cells found in non-lymphoid tissues have revealed transcriptional signatures and functions distinguishable from those found in T_{reg} cells residing in secondary lymphoid organs, suggesting that T_{reg} cells may perform a variety of specialized functions within non-lymphoid tissues. Through RNA-seq analysis we find that T_{reg} cells in the normal lung and in lung tumors have differential expression of genes involved in adhesion and migration compared to $CD4^+Foxp3^-$ T cells. Consistent with gene expression analysis, T_{reg} cells show distinct localization within the lung parenchyma compared to conventional T cells in homeostasis and inflammation, raising the possibility that they may differentially interact with stromal and tissue resident cells. In particular, we find frequent T_{reg} cell localization in close proximity to peripheral neurons compared to other T cells. Gene expression analysis has also revealed expression of neuromodulatory molecules by lung T_{reg} cells, further suggesting interplay between T_{reg} cells and peripheral neurons. Interactions between T_{reg} cells and the peripheral nervous system may play an important role in limiting immune responses and/or promoting tissue homeostasis. Understanding potential neuromodulatory activity of T_{reg} cells and its effect on inflammation, tissue homeostasis and cancer could reveal novel strategies for therapeutic possibilities.

Keywords: Regulatory T cells, Cell migration, Neuro immune interactions

A246 / Screening for melanoma-derived miRNAs affecting tumor cell recognition by antigen specific CD8+ T cells

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The regulatory effect of microRNAs (miRNAs) in cellular anti-tumor immune responses has been described with predominant focus on immune effector cells. However, much less is known about the effects of miRNAs on the susceptibility of target cells during T cell - target cell interaction. The goal of this project is to identify miRNAs which affect tumor cell recognition by cognate cytotoxic T cells (CTLs). Therefore, the murine melanoma cell line B16F10 will be transfected with a murine miRNA library, followed by co-cultivation with a stable CD8+ CTL line specific for the tumor antigen TRP-2. Individual miRNAs modulating the susceptibility to CTL recognition will be identified using a high throughput screening assay based on luciferase activity detection. After an optimization phase, the high throughput assay was established and through pre-screen assays, miRNAs that have an impact on CTL mediated killing as well as a miRNA that has no impact on CTL mediated cytotoxicity were selected as positive and negative controls respectively. The hits obtained on basis of the proposed screen will be preselected and their effects on tumor antigen expression, processing and T cell epitope presentation will be investigated, followed by microarray based transcriptome analysis, to identify and validate the target mRNAs of these miRNAs. The role of identified target genes in immune recognition by CTLs will be validated in further functional assays including IFNγ and granzyme B ELISpot-assays, ELISAs and the impedance based xCELLigence system optimized in our lab. As a result, miRNAs sensitizing tumor cells for CTL recognition, as well as miRNAs promoting resistance to CTL recognition will be identified. Both miRNA species are of potential clinical interest, as they could help to improve future cancer treatment approaches,

either based on the inclusion of sensitizing miRNAs or on the inhibition of attenuating miRNAs. The proposed interventions may help improve the efficacy of both, classical chemotherapy approaches and novel immunotherapeutic treatment strategies against cancer.

Keywords: microRNA, Cytotoxic T cells, Melanoma

A248 / Loss of skin dendritic cells in melanoma: apoptosis induction and decreased survival within the tumor microenvironment

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A decrease in the percentage of skin dendritic cells (DCs) has been reported for various types of skin cancers, including melanoma. In the tg(Grm1)EPv spontaneous melanoma mouse model, ectopic expression of the metabotropic glutamate receptor-1 in melanocytes leads to a highly proliferative and anti-apoptotic phenotype, resulting in melanoma formation within the dermis. The slow progression of these tumors allows for the in depth analysis of immune infiltrate in growing tumors. We here show that total DCs (CD11c+ cells) are gradually lost as the tumor progresses. Mainly the dermal DCs were affected, including mostly CD11b+ DCs, whereas epidermal Langerhans cells remained unchanged. DC numbers in the tumor draining lymph nodes were not increased, suggesting that DC loss from the skin is not a result of increased migration to the tumor draining lymph nodes. We hypothesized that extrinsic cell death of the DCs may be induced within the growing tumor. Indeed, the tumor tissue upregulated the expression of Fas ligand (FasL) that can induce extrinsic apoptosis in immature DCs that express Fas (CD95). Fas-mediated apoptosis depends on activation of caspase-8 and mice in which the autoproteolytic cleavage site D387 is mutated to alanine (casp8$^{D387A/D387A}$ mice) show strong resistance to Fas-mediated death. Lethally irradiated tg(Grm1)EPv mice reconstituted with casp8$^{D387A/D387A}$ bone marrow showed an increased percentage of DCs in skin than tg(Grm1)EPv mice reconstituted with casp8$^{WT/WT}$ bone marrow, indicating that loss of skin DCs in this spontaneous melanoma mouse model might be the result of decreased survival due to receptor-mediated apoptosis. In addition, tumor development and growth was accompanied by a decreased expression of Flt3L, suggesting that growth factors that are critical for the survival of DCs are being downregulated in the skin. Our data thus indicate that the loss of DCs in melanoma is not due to DC migration, but depends on induction of apoptosis and reduction of survival signals within the tumor. A deeper understanding of the causal relationship between the decrease of skin DCs and tumor growth could lead to novel immunotherapeutical strategies to combat skin tumors.

Keywords: Dendritic cells, Melanoma, Apoptosis

A249 / Persistent antigen presentation impairs CD4$^+$ T cell memory generation

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Persistent antigen presentation by tumor cells and in chronic infections functionally impairs T cells over time. While exhaustion has been characterized extensively for CD8$^+$ T cells, information on how CD4$^+$ T cells become exhausted by persistent antigen presentation is scarce. To address how different kinetics and dosage of antigen presentation affect CD4$^+$ T cells, we followed TCR transgenic T cells transferred into transgenic recipients with regulatable antigen presentation where the cells received transient or chronic TCR signals of varying strengths, in the absence of confounding pathologies of chronic infections. We show that CD4$^+$ T cells exposed to persisting antigen presentation at three different levels display phenotypes with varying kinetics and consequences. These CD4$^+$ T cells showed impaired cytokine production upon re-stimulation and, at the highest antigen dose, TCR downregulation and apoptosis. Their transcriptional profiles reflect the qualitative and quantitative changes in antigen presentation by dose-dependent upregulation of exhaustion-associated genes such as Lag-3 and TIGIT. We tested the Ca^{2+}, MAPK and Akt pathways for functionality and found Ca^{2+} fluxes to be rather robust against persistent antigen presentation while the MAPK and Akt pathways were more easily tuned by it. Consequently, nuclear translocation of the transcription factor NFATc1 and its degradation parallel the dose of persisting antigen. The expression of the anergy markers CD73 and FR4 was also correlated with antigen load but remained dynamic upon antigen removal. Our results demonstrate that dose and timing of antigen presentation determine the range of CD4$^+$ T cell dysfunctionalities in an otherwise sterile environment.

Keywords: T cells, exhaustion, antigen

A250 / GARP on endothelial cells contributes to the development of experimental tumors in mice

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Regulatory T cells (Treg) are key players in the tumor microenvironment and fulfill tumor promoting activities. Glycoprotein A repetitions predominant protein (GARP) is a membrane receptor for latent transforming growth factor beta (TGF-β) expressed on Tregs. Blocking human GARP and thus the release of TGF-β with anti-GARP monoclonal antibodies inhibited the immunosuppressive activity of human Tregs in a xenogeneic graft-versus-host disease model. Besides on Tregs, GARP is also expressed on platelets and endothelial cells and deletion of GARP in mouse platelets resulted in a delayed tumor development. In this study, we investigated the contribution of GARP expressed on mouse Tregs or endothelial cells in tumor development. Treg and endothelial conditional GARP knockout (KO) mice were generated using the Cre-LoxP recombination system with Cre expression driven by the Foxp3 or Tie2 promotor respectively. These mice were challenged either orthotopically with GL261 glioma cells or subcutaneously with MC38 colon carcinoma cells, and overall survival or tumor volumes were monitored, respectively. The suppressive function of Tregs lacking GARP was further investigated using a transfer homeostasis model in which Tregs from Foxp3 GARP KO mice or littermates (LM) were transferred to immunodeficient NSG hosts together with WT CD4 effector T cells. After one week, proliferation of the effector T cells was measured in the spleen using flow cytometry. SMAD2 phosphorylation on ex vivo stimulated CD25 cells isolated from Treg GARP KO mice and LMs was measured using quantitative western blot. Unexpectedly, GL261 or MC38 challenged Treg GARP KO mice did not show prolonged survival or delayed tumor growth compared to LMs. The suppressive function of KO Tregs was compared to LM Tregs in a transfer model in NSG mice. Effector T cells proliferated equally in mice injected with effector T cells and Tregs with or without GARP, indicating that mouse Tregs with a genetic deletion of GARP showed no functional impairment of T cell suppression in vivo compared to LM Tregs. Furthermore, SMAD2 phosphorylation of stimulated CD25+ splenocytes from Foxp3 GARP KO mice was similar compared to LM splenocytes demonstrating that the TGF-β receptor is similarly activated. Finally, endothelial GARP KO mice were subcutaneously challenged with MC38 cancer cells to further investigate the role of GARP in tumor development. Interestingly, mice lacking GARP on endothelial cells did show a decreased tumor burden (LM 3.7 ± 0.2 cm^3 (n=5) vs KO 2.0 ± 0.5 cm^3 (n=6) 17 days post injection; $p < 0,0001$) revealing that endothelial GARP is important in tumor development. This study indicates that blocking endothelial GARP but not Treg GARP has potential clinical prospects in tumor immunotherapy. Further studies to unravel the mechanism of endothelial GARP in experimental cancer models are in progress.

Keywords: GARP, Endothelial cells, Regulatory T cells

A251 / Resistance to cancer immunotherapy mediated by apoptosis of tumor-infiltrating lymphocytes

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Despite impressive clinical success, cancer immunotherapy remains ineffective in many patients due to tumoral resistance. Here we used the autochthonous TiRP melanoma model, which recapitulates the innate anti-PD1 resistance (IPRES) signature recently described in human melanomas. TiRP tumors were resistant to immunotherapy based on CTLA4 or PD1 blockade, cancer vaccines or adoptive CD8+ T cell therapy (ACT). TiRP tumors recruited and activated tumor-specific CD8+ T cells, but these T cells underwent apoptosis and disappeared. This did not occur with isogenic transplanted tumors, which were rejected after ACT. Apoptosis of tumor infiltrating lymphocytes (TIL) was prevented by interrupting the Fas/Fas-ligand axis, and appeared triggered by polymorphonuclear-myeloid-derived suppressor cells (PMN-MDSC), which expressed high levels of Fas-ligand and were more numerous in TiRP tumors. Blocking Fas-ligand increased the anti-tumor efficacy of ACT in TiRP tumors, and increased the efficacy of anti-PD1/anti-CTLA4 in transplanted tumors. Therefore, TIL apoptosis appears as a relevant mechanism of immunotherapy resistance, which could be blocked by interfering with the Fas/Fas-ligand pathway.

Keywords: PMN-MDSC, Fas Ligand, Apoptosis

POSTER SESSION B

CANCER VACCINES AND TARGETS

B001 / Cancer awareness and screening in rural India

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Breast cancer is the most common cancer of urban Indian women and the second most common in rural women. Owing to lack of awareness of the disease in India and in absence of breast cancer screening programs, majority of breast cancers are diagnosed at a relatively advanced stage. Government agencies, NGOs and charity organisations have put great emphasis on improved breast cancer awareness among masses for promotion of early detection, providing comprehensive treatment module, providing support for breast cancer management and for screening and rehabilitation. The efforts have resulted in an improved survival and quality of life of Indian breast cancer patients but the improvement is more pronounced in urban population. In rural areas, there is still a lack of good health care and awareness among masses regarding the importance of early breast cancer screening and thus cases of late diagnosis are more prevalent. In addition, there is still an identified lack of breast cancer screening programs in rural areas which further causes late diagnosis. The other common factors that lead to late diagnosis include delays on the part of womenfolk of rural areas to seek advice for a recognized health problem which is mainly due to financial reasons, social/cultural reasons such as general inhibition of women to see the doctor for breast ailments, general scare of people towards cancer like disorders and a general indifference of women towards their health. In rural areas Illiteracy is widespread and also people are inhibited and not motivated to come to the hospitals for screening/check up.
Considering various factors of cancer incidence rate, to address the most common barriers such as lower cancer literacy, lesser availability and accessibility of proper medical facilities, three Indian states were shortlisted to initiate the project “ECHO” by organizing Breast Cancer Awareness and Screening Programs for Rural and Semi-Urban Indian Population. In addition to being a CSR approach, Project ECHO also increased the cancer literacy amongst the rural population and emphasized on health education, early diagnosis of breast cancers and more public facilities for breast cancer treatments.

Keywords: Cancer prevention, Preventive oncology, Cancer Awareness

B002 / A dendritic cell targeting NY-ESO-1 vaccine significantly augments immune responses in melanoma patients pretreated with human Flt-3 ligand

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Background: Patients with high-risk melanoma, (AJCC TNM stage II and stage III disease), have a 20-60% recurrence rate with 5-year overall survival (OS) between 45% and 70%. The adjuvant setting is an opportunity to test prevention vaccines that may have efficacy against disease recurrence. Vaccine therapy with CDX-1401 (a fusion protein consisting of human monoclonal IgG1 antibody targeting the dendritic cell (DC) receptor DEC-205 linked to the NY-ESO-1 tumor antigen) can safely lead to humoral and cellular immunity in cancer patients with advanced malignancies that express NY-ESO-1, including melanoma. CDX-301, a recombinant human Flt3 ligand (Flt3L), safely produces increases in DC in humans and may enhance vaccine responses through increased DC number and activity. We evaluated CDX-301 and CDX-1401 combination treatment in a phase II, open-label, multicenter, randomized study of subjects with resected melanoma, to determine whether immune responses to NY-ESO-1 elicited by vaccination with CDX-1401 + poly-ICLC are substantially increased by prior expansion of circulating DC with Flt3L therapy. We also assessed immune correlates of optimal vaccine responses.
Methods: 60 patients with resected melanoma were randomized to two cohorts: Cohort 1 received CDX-301 (Flt3L) pretreatment (25 ug/kg SC x 10 days) in two of four monthly cycles of vaccination with CDX-1401 (1mg IC) + poly-ICLC (2mg SC, days 1 and 2). Cohort 2 received 4 monthly cycles of vaccine with CDX-1401 and poly-ICLC without prior Flt3L. Patients were enrolled independent of NY-ESO-1 status and NY-ESO-1 expression was determined retrospectively. We also assessed immunogenicity to other melanoma-associated antigens and memory viral responses, character of PBMC subsets, and safety, tolerability and clinical efficacy of the regimens.

Results: Both treatments were well tolerated with grade 1-2 AEs of chills, injection site erythema and pain, fever and myalgia most common. Six patients progressed prior to completing 4 cycles of treatment. A substantial increase of between ~15- to ~32-fold of innate immune cells (DC, monocytes and NK cells) was observed in subjects treated with CDX-301. Further, there was development of significantly higher anti-NY-ESO-1 antibody titers and NY-ESO-1 specific T cells in cohort 1 vs. cohort 2. Comparative immune cell gene expression profiling of PBMC are consistent with these differences.

Conclusions: DC mobilization with vaccines targeting DC is safe and significantly enhances vaccine responses to widely expressed tumor associated antigens.

Keywords: Dendritic Cells, Melanoma, Vaccine Therapy

B003 / Naturally presented CML-associated HLA ligands represent novel immunogenic T-cell epitopes for immunotherapeutic approaches

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Due to tyrosine kinase inhibitor (TKI) treatment, the prognosis of chronic myeloid leukemia (CML) has improved. However, the presence of minimal residual disease precludes therapy termination in many cases. T-cell based immunotherapy, such as peptide vaccination, may be a promising approach to achieve elimination of these residual leukemic cells. For this aim, an exact knowledge of CML-associated T-cell epitopes is necessary. In the present study, we utilized mass spectrometry for the identification of frequently and naturally presented CML-associated human leukocyte antigen (HLA) ligands. Comparative profiling of the HLA ligandome of primary CML samples (class I n=16, 8,291 HLA ligands, class II n=15, 2,822 ligands) and a database comprising 188 ligandomes of various normal tissues (***e.g.*** blood, bone marrow, spleen) revealed 38 frequently presented CML-associated HLA class I and 44 class II ligands represented in ≥ 25% of CML patients. In order to investigate immunogenicity of these targets, peripheral blood mononuclear cells (PBMCs) from CML patients and healthy volunteers (HV) were analyzed in IFNγ ELISpot assays. Specific T-cell responses targeting 4/4 (100%) HLA class II peptides were detected exclusively in CML patients, with recognition frequencies ranging from 17% (4/23) to 4% (1/23) of CML patients. The immune recognition was found to be strictly CML-associated with no T-cell responses detected in HV (0/10). For HLA class I, 1/9 (11%) peptides evoked immune recognition in CML patients with a frequency of 6% (1/18 CML samples). The low frequency of immune responses to HLA class I peptides might result from the TKI treatment, which reportedly causes inhibition of $CD8^+$ T-cell responses. We thus compared T-cell responses against a panel of viral epitopes in IFNγ ELISpot assays of PBMCs from CML patients under TKI treatment with that of HV and chronic lymphocytic leukemia (CLL) patients. We detected a significant reduction of T-cell response in CML patients (mean 74±16 spots, n=19) compared to HV (mean 241±24 spots, n=42, $p< 0.001$, two-tailed ***t***-test) or CLL (mean 218±16 spots, n=125, p=0.008) patients. We performed ***in vitro*** artificial antigen-presenting cell-based priming experiments with $CD8^+$ cells obtained from HV in order to nonetheless prove the immunogenicity of our HLA class I peptides. Formation of tetramer-positive populations with frequencies ranging from 0.1-30.4% of viable $CD8^+$ cells could be observed for 10/10 peptides. For seven of those peptides functional characterization was performed using ICS, which revealed TNFα and IFNγ production in 5/8 (63%) tetramer-positive populations of three analyzed HV. Moreover, priming experiments using T cells of CML patients and killing assays with peptide-specific T cells are ongoing.

Taken together, we took a first step towards validation and immunological characterization of a set of newly defined CML-associated antigens as targets for off-the-shelf peptide vaccination in CML patients.

Keywords: CML, immunotherapy, peptide vaccination

B004 / A novel bicyclic peptide, a small-molecular binder of a prostate cancer marker Glutamate carboxypeptidase II

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Novel binders of known cancer markers can be useful for functionalization of therapeutic nanoparticles. Here, we describe the discovery and evaluation of a bicyclic peptide binder of Glutamate carboxypeptidase II, a membrane metalloprotease overexpressed in prostate cancer.

Using an fd phage library displaying bicyclic peptides of variable lengths on its G3P protein, we performed a three-round selection for a phage binder of the extracellular part of Glutamate carboxypeptidase II and analyzed the resulting pool on next generation sequencing. The variable peptide candidate displayed on the leading phage has been synthesized and evaluated in biochemical assays to determine its binding properties. Alternative cyclization linkers have been tested to assess potential improvements in binding.
Phage display methods have long been a useful tool to scan through a large chemical space. Using a recently introduced approach of three-cystein peptide libraries with post-translational cyclization resulting in bicyclic peptide we found a novel binder for a prostate cancer marker and assessed its properties. We expect that compared to linear peptide binders a bicyclic peptide will achieve higher stability in plasma and improved binding affinity due to a more constrained structure. We hope to utilize this molecule for functionalization of nanoparticles binding to our target.

Keywords: Prostate cancer, Peptide, Glutamate carboxypeptidase II

B005 / Developing ovarian cancer xenograft in mouse model to see FSHR mediated anticancer effect of Moringa oleifera root extract

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Introduction: Sex hormones play important role in ovarian cancer as novel redirected T-cell-based immunotherapy targeting human follicle-stimulating hormone receptor (FSHR)is tried. Moringa olifera root extract has shown efficacy in cancer cell as well as in hormonal milleu of ovary and reproductive organ. Hence, the aim of the present study was to study the anticancer effect of Moringa oleifera on human OAW 42 & MCF-7 cell line through its putative FSHR antagonistic role.
Methods: The colorimetric MTT metabolic activity assay was used to see cell viability. Western blot analysis was performed to check its effect in apoptosis. Clonogenic assay was carried out to see the effect of Moringa root extract on cancer cell proliferation. Cell cycle analysis was done based on incorporation of Propidium Iodide into DNA (BD Cycletest™ Plus DNA Kit). Analyses of the samples were carried out flow cytometrically using BD LSRFortessaTM SORP (San Jose, CA) cell analyzer and BD FACSDiva v8.0.1 software.On day 35, mice were sacrificed & histopathological analysis of the ovaries were carried out following standard protocol followed by IHC for detection of FSHR
Result: The dose dependent analysis on viability of OAW-42 and MCF-7 cells by MTT assay showed 50% lethal toxicity (IC-50) of MRE (moringa root extract) to be 250µg/ml and 200µg/ml respectively with synergestic effect of moringa extract with both cisplatin and paclitaxol in OAW-42 cell line. Moringa extract at its IC-50 value showed visible effect in case of cleaved caspase-9. In clonogenic assay approximately 40% reduction of colony formation was observed Flow cytometric data showed that after 24 hours and 48 hours of treatment with MOL root extract at its IC-50 dose, DNA content progressively increased in the sub G0 phase. However approximately 3% upregulation and 11.4% downgulation of PI intensity at G0/G1 phase was also observed. Profuse expression of FSHR and its downstream target cMyc in the stroma of carcinoma control mice compared to control or Moringa treated mice.
Conclusion: We can conclude that MRE is a promising candidate for treatment of ovarian cancer by a FSHR mediated pathway either alone or in combination with traditional chemotherapeutic drugs like Cisplatin and Paclitaxol at a sublethal dose of their toxicity.

Keywords: moringa oleifera, ovarian cancer xenograft, FSHR

B006 / Reassessing an immunological dogma: CIITA-dependent MHC class II-expressing cancer cells act as professional antigen presenting, prime virgin CD4+ T cells and trigger a potent adaptive immune response against the tumor

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Our previous studies showed that non immunogenic H-2d tumor cells of distinct histotypes can become highly immunogenic, induce a protective CD4+ T cell response and vaccinate the animals against parental MHC-II-negative cells if they are rendered MHC class II-positive by stable transfection with the Activator of immune response gene 1 (Air-1)-encoded MHC-II transcriptional activator (CIITA) discovered in our laboratory.
However, these studies did not established whether our strategy was valid for tumors of different genetic background and, more importantly, whether tumor immunity was the consequence of a direct priming of naïve CD4+ T lymphocytes by CIITA-driven MHC-II-expressing tumor cells or by MHC-II-tumor antigen complexes engulphed by dendritic cells and exposed on the surface of these professional APC.
By using a transgenic H-2^b mouse model, the CD11c.DTR C57BL/6 mice, in which dendritic cells can be functionally deleted by administration of difteria toxin, we now show that CIITA-tumor cells of two distinct histotypes can be rejected or strongly retarded in their growth in dendritic cell-deleted mice. Moreover, CD4+ T cells from CIITA-tumor vaccinated mice could transfer protection to naïve mice when these were injected with parental CIITA-negative tumors. Finally, naïve animals deleted of CD4+ T cells by injection of anti-CD4 monoclonal antibodies could not reject or retard the growth of CIITA-tumor cells anymore.

To rule out that in absence of dendritic cells, other professional APC could prime naïve CD4+ T cells, we deleted the macrophages in CD11c.DTR C57BL/6 mice by administration of liposome clodronate and still obtained rejection or strong retardation in tumor growth of CIITA-tumor cells.
These results provide definitive evidence that CIITA-tumor cells are the crucial APC ***in vivo*** for CD4+ T cell priming and demonstrate that our vaccination strategy can be extended to animals of distinct genetic background.
Our results challenge the diffuse belief that non-professional APC cannot efficiently prime naïve T cells ***in vivo***. Moreover the demonstration of the general validity of our approach in different genetic backgrounds may open the way to new strategies of anti-tumor treatment in clinical setting.

Keywords: Tumor vaccines, CIITA, CD4+ T cells

B007 / Conjugation of peptide vaccine directly to TLR agonists generates enhanced immune responses and tumor therapy

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Post-translational modifications can result in changes to proteins which cause them to be recognised by the immune system. One such modification is citrullination where the positive charged arginine is modified to a neutral citrulline. Our previous study has demonstrated the stimulation of potent CD4 T cell responses to citrullinated vimentin which result in strong anti-tumour immunity (Brentville et al. 2016). In this report we show the efficient induction of Th1 responses by peptide vaccination with two citrullinated peptides from vimentin and one from alpha enolase. Responses induced are restricted through both HLA-DR4 and HLA-DP4 alleles and provide long lived tumour therapy in an aggressive mouse melanoma model. High frequency Th1 responses are induced when peptides are mixed with both TLR4/9 and TLR1/2 agonists at doses of 10nmol and 1nmol but are lost at a dose of 0.1nmol. However, linking the TLR1/2 or TLR9 agonists directly to the peptides allowed the dose to be reduced without loss of activity. High frequency responses are seen with a dose of 0.1nmol and lower responses at 0.01nmol dose. 0.1nmol doses of agonist linked peptides provide efficient tumor therapy in mouse models with survival of 70-100% (p=0.0002). In addition, responses induced by low dose linked peptide vaccination provide protection against tumor rechallenge resulting in long term survival of 80-100% (p< 0.0001). The generation of anti-tumor immune responses with low dose of TLR agonist linked peptides should enable more efficient scaling of dose into human studies. These studies suggest that intradermal injection of citrullinated peptide linked directly to a Th1 polarising adjuvant such as TLR1/2 or TLR9 agonist should be considered for translation into the clinic.

Reference:

Brentville et al., 2016. Citrullinated vimentin presented on MHC-II in tumor cells is a target for CD4+ T cell-mediated antitumor immunity. Cancer Research 2016 Feb 1;76(3):548-60.

Keywords: peptide vaccine, citrullination, TLR agonist

B008 / TLR9 mediated conditioning of the liver environment is essential for successful intrahepatic immunotherapy and effective memory recall

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Hepatotropic viruses such as HBV and HCV pose a major public health threat, as the intrahepatic cytotoxic CD8+ T cells that are crucial for effective immune response against these viruses often become dysfunctional and exhausted what leads in consequence to viral persistence, chronic infection and development of hepatocellular carcinoma. Therapeutic vaccination strategies focusing on reviving the immune response to combat these viruses and transformed cells provide an attractive option. To elucidate the mechanisms downregulating intrahepatic immunity we aimed to evaluate the role of intrahepatic antigen load on adaptive CD8+ T cell responses induced upon vaccination. To mimic the varying viral loads observed in patients, we employed a conditional mouse model OVA X AlbCreERT2 with hepatocyte-specific ovalbumin antigen expression. With this model, variable percentages of OVA-expressing hepatocytes can be adjusted by tamoxifen, leading to a mosaic antigen distribution. Here we report that irrespective of the type of therapeutic vaccination applied and independent of the type of intrahepatic antigen, (OVA or HBV surface antigen) peripherally activated endogenous CD8 T cells could eliminate low antigen loads in liver but were functionally impaired if confronted with elevated antigen burden. Strikingly, upon conditioning the liver environment with the TLR9 ligand (CpG-ODN oligodeoxynucleotide), successful immunization against high intrahepatic antigen density could be achieved. Importantly, immunomodulation with the TLR9 agonist also supported a functional memory recall upon subsequent high antigen challenge.

This study reveals that by fine tuning the liver environment with potent modulators one could override the liver specific regulatory cues, thereby facilitating elimination of immunologically defined targets.

Keywords: Therapeutic vaccination, Liver, CD8 T cells

B009 / Stress activated IRES-dependent translation of the long-non coding RNA meloe in melanoma cells produces the most immunogenic MELOE antigens

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The use of tumor-specific neoantigens as targets for immunotherapy has been widely promoted in the past years. However, their patient variability and the recently described dynamics of their expression hampers their clinical usefulness. We previously described two antigens, MELOE-1 and MELOE-2, involved in melanoma immunosurveillance that are translated from the polycistronic ***meloe*** lncRNA by IRES-dependent mechanisms. While ***meloe*** RNA was found in both melanoma cells and normal melanocytes, MELOE-1 or 2 specific T cell clones recognized only melanoma cells suggesting that IRES sequences are only activated in melanoma cells. Thus MELOE-1 and 2 are shared specific melanoma antigens.
Recently we documented the translation of another ***meloe*** ORF, coined MELOE-3 that is expressed in both melanocytes and melanoma cells. The translation of MELOE-3 is strictly cap-dependent in contrast with the IRES-dependent translation of MELOE-1 and 2. We compared the immunogenicity of MELOE-3 and MELOE-1 by stimulating PBMC from healthy donors with overlapping peptides from either MELOE-1 or 3 and found only very rare CD8 and CD4 T cell responses against MELOE-3, in marked contrast with the high frequencies of responses against MELOE-1 in all donors. This strongly suggested an immune tolerance towards this antigen that would be consistent with its expression in normal melanocytes.
We are now studying the IRES activation mechanisms since they will be the key to understand MELOE-1 tumor specificity. We stably transfected a melanoma cell line with ***meloe*** cDNA in which the ORF coding for MELOE-1 was replaced by the YFP gene and monitored its translation under various stress stimuli. Preliminary results showed that the expression of MELOE-1 is indeed enhanced under stress conditions, particularly in cells exposed to endoplasmic reticulum stress. In addition, by ligand fishing of the proteins bound to the IRES putative sequence using SPR (BIACore 3000) and mass spectrometry identification, we documented the association of hnRNP-A1 to MELOE-1 IRES. Multiple roles of this RNA-binding protein have been described such as splicing, nucleo-cytoplasmic shuttling of RNAs and more recently IRES trans acting factor (ITAF) activity. We are currently testing whether hnRNP-A1 can functionnally activate MELOE-1 IRES dependent translation.
In conclusion, this work reports that IRES-dependent translation of the lncRNA ***meloe*** can generate a new class of antigens that are both tumor and lineage specific and thus may represent the best targets for immunotherapy. In light of recent studies highlighting the implication of IRES-dependent translation in the development and progression of various cancers we are currently testing whether this concept of highly immunogenic IRES-dependent antigens could be extended to other tumor types.

Keywords: tumor specific antigens, IRES dependent translation, lncRNA

B010 / Oncolytic measles virus for tumor-specific vaccination

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Measles virus vaccines (MVs) are exceptionally suited to prime antigen-specific immune responses and are in clinical development as oncolytic therapeutics. MV oncolysis leads to immunogenic cell death and release of both viral and tumor-associated antigens (TAAs) in an inflammatory milieu, promoting induction of immunity. We hypothesized that by encoding TAAs within MV vectors, we could direct the immune response towards specific antigens, shifting the balance from anti-viral to anti-tumor immunity.
We generated Schwarz vaccine-derived MVs encoding TAAs. To further enhance immune stimulation by oncolytic MV vaccines, we generated MVs encoding only immunodominant epitopes of TAAs. Three different cassettes for TAA epitopes were evaluated, including variants with a secretion signal and targeting for proteasomal cleavage. Insertion of TAA cassettes did not imped viral replication or oncolysis compared to the parental virus. Transduction of B16-hCD46 cells with the generated viruses induced a strong virus-mediated expression of TAAs. In IFNγ Elispot and cytotoxicity assays we demonstrated TAA-specific T cell stimulation and tumor cell lysis. We selected the cassette which induces the strongest antigen-specific IFNγ response for further studies. To evaluate MV encoding TAAs ***in vivo,*** we used an MV-susceptible, immunocompetent mouse model of melanoma, B16-hCD46. Treatment of B16-hCD46 tumors

with TAA-encoding oncolytic MV vaccines confers strong anti-tumor immunity without augmenting anti-viral immunity, thus supporting the concept of our approach.
Further analysis of induced immune responses and therapeutic mechanisms will be instrumental to identify effective combinations of immunovirotherapy. With the increasing availability of tumor sequencing data, this study has direct implications for future clinical trials of personalized immunovirotherapy especially concerning neoantigen-specific vaccination.

Keywords: Oncolytic vaccine, RNA vaccine, preclinical model

B011 / Great Apes Adenovirus neoantigens-based vaccine synergizes with immunomodulators in mouse models of cancer

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Several line of evidences have convincingly shown that neo-antigens are an important class of immunogenic tumor antigens and represent a better target of effective anti-cancer T-cell immunity than overexpressed classical tumor antigens. Neo-antigen specific T cells are rescued by treatment with checkpoint inhibitors contributing to their anti-tumor efficacy. Indeed, we demonstrated that treatment with anti-PD-1 in the CT26 mouse model could rescue CD4 reactivity against one of the predicted neo-antigens. Here, we developed a new neo-antigen vaccine approach based on the use of viral vectors, Great Apes Adenovirus (GAd), encoding multiple neoantigens in tandem. Starting from the mutanome of murine CT26 cancer cell lines, we identified mutations and selected neo-antigens to be encoded in the vaccine by the use of prediction algorithms. 31 selected neo-antigens were inserted in GAd multiantigenic vaccine, the largest so far generated to our knowledge, and tested in vivo. A fraction of them was shown to be immunogenic, inducing strong CD8 and CD4 interferon-γ (IFN-γ) producing T cells. Immunization with GAd vaccine encoding neoantigens induced the very same hierarchy of T cell reactivities in naïve and tumor bearing mice. Vaccination with GAd vaccine was able to control CT26 tumor growth. Importantly, single injection of GAd vaccine synergizes with anti-PD1 and others immunomodulators activity in a therapeutic setting. GAd vaccine strengthens and broadens T cell immune response induced by anti-PD1, thus potentiating the antitumor T cell responses, which may be limited by the poor antigenicity of tumors cells in case of checkpoint blockade monotherapy.

Keywords: neoantigen, vaccine, immunomodulators

B012 / Characterization of an HLA-A*24:02 peptide exchange platform for assessing MHC peptide binding and generating new specificity HLA-A*24:02 tetramers

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There is currently a great interest in discovering peptide sequences from pathogens or tumors (neoantigens) that can be used for developing novel T cell based immunotherapies tailored to each patient's specific HLA haplotype. The combination of whole-exome and transcriptome sequencing analysis with mass spectrometry now permits rapid identification of a myriad of peptide candidates. As a first screening step, potential binders to MHC molecules can be determined using MHC prediction algorithms. However, these algorithms typically generate a large proportion of false binders, therefore requiring experimental validation. We have devised, for this purpose, assays that can both help determine binding of peptides to MHC class I molecules and generate MHC class I tetramers with these peptides for detection of peptide specific T cells. We previously developed HLA-A*02:01 and H-2 Kb assays. Here we report on an assay platform for exchanging peptides on an HLA-A*24:02 tetramer prepared with an exchangeable peptide. After a short incubation of new peptides with this HLA-A*24:02 tetramer in presence of a peptide exchange factor, the peptide exchange rate is assessed by a capture assay and quantitated by flow cytometry. Results indicate that new specificity HLA-A*24:02 tetramers can recognize CD8+ T cells that are specific for these peptides. Peptide exchange rates obtained with the HLA-A*24:02 assay correlate with theoretical binding affinities of known HLA-A*24:02 binders.

Keywords: Cancer Vaccine, Peptide Exchange, MHC Tetramer

B013 / A linear tetanus derived peptide improves dendritic cell activation, antigen loading capacity and CD8+ activation *in vitro* and *in vivo*

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With current immunotherapeutic peptide vaccination strategies optimal immunogenicity cannot be accomplished, as they fail

to deliver adjuvants and antigens to the same cell. This results in less potent dendritic cell (DC) activation and increased risk of tolerance induction. It is known that antigen-antibody immune complexes (ICs) can serve as adjuvants and improve antigen presentation by DCs compared to soluble antigens alone. We propose a novel strategy that combines antigen and adjuvant into one entity, ensuring that the cell receiving the activation signal also receives the antigen material. We have developed a strategy where synthetic long peptides (SLP) containing T cell epitopes are linked to several linear tetanus derived B cell epitopes named minimal tetanus toxin epitopes (MTTE), to form complexes using endogenous circulating IgG antibodies against MTTE. To test this we generated hybridomas that produce mouse anti-MTTE antibodies of IgG1 and IgG2a isotype and additionally used polyclonal rabbit IgG specific for the MTTE sequence. We assessed DC activation, antigen uptake and loading capacity qualities of different MTTE-SIINFEKL conjugates using murine immature myeloid dendritic D1 cells and B3Z hybridoma T cells activated upon MHC class I/SIINFEKL engagement of the T cell receptor. As monomeric IgG does not engage low affinity Fc receptors to the same extent as complexed IgG, it was crucial to test whether the MTTE-SIINFEKL conjugates could form complexes with anti-MTTE IgGs. We investigated the need for one, two or three MTTEs in a conjugate to induce efficient DC activation, antigen uptake and cross-presentation. ICs with one MTTE sequence failed to activate DCs and did not activate T cells more efficiently than the conjugate in the same doses alone, while conjugates containing two or three MTTEs complexed with mIgG1 or mIgG2a were more efficient in activating DCs and T cells following antigen uptake and cross-presentation than the sole conjugate. Accompanying the ***in vitro*** data, MTTE-hgp100 conjugates and MTTE-specific antibodies were injected into mice to assess CD8+ cell proliferation and activation ***in vivo***. Elevated levels of hgp100-specific CD8+ cells in draining popliteal lymph nodes were seen when conjugate was administered into the footpad with MTTE-specific, but not with irrelevant antibodies. Intraperitoneal injection of the same conjugate together with MTTE-specific antibodies led to increased CD8+ cells expansion in mesenteric lymph nodes compared to use of unspecific antibodies or conjugate alone. The findings presented show that ICs improve the antigen loading capacity of dendritic cells and MHC-mediated cross-presentation of incorporated T cell epitopes compared to soluble antigen alone. Furthermore, we could show that MTTE-specific ICs enhance T cell expansion and activation ***in vivo.***

Keywords: immune complexes, therapeutic vaccination, synthetic long peptides

B014 / Optimizing the route of administration for nanoparticle based iNKT cell vaccines

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Introduction: We previously reported enhanced anti-tumor T cell responses by iv vaccination of PLGA nanoparticles containing both an iNKT cell analog and a protein antigen. Here we sought to identify if this is also relevant for other routes of vaccination which are frequently used in the clinic. To our knowledge, this is the first study to directly compare multiple routes for iNKT cell analog vaccines employing various immune response readouts.
Methods: PLGA nanoparticles encapsulating an iNKT cell analog and ovalbumin were injected to wild type BL/6 mice through iv, subcutaneous, intramuscular, intranasal, and intranodal routes. Proliferation of OT-I cells, numbers of endogenously generated CD8 and CD4 T cells, in vivo cytotoxic potentials of CD8 T cells, activation status of iNKT cells and antibody responses against ovalbumin were evaluated weekly.
Results: T cell proliferation in lymphoid organs was greatly enhanced by incorporation of glycolipids in nanoparticles if its administered via iv route. As an indication of iNKT cell activation, 24h serum IFN-y was also highest for the iv route followed by inodal and im routes. Additionally, other indicators of iNKT activation such as, decrease of population and lower NK1.1 expressions were pronounced only for the iv route throughout the 4 weeks. Moreover, the fastest and highest T cell cytotoxicity was also achieved by iv route. On the other hand, intranodal vaccination yielded to highest numbers of antigen specific IFN-y secreting CD4 T cells. Regarding B cell responses, iv and intranodal routes induced the highest Anti -OVA IgM, IgG1 and IgG2c levels.
Conclusion: These results suggest that iNKT cells are most accessible via iv administration of nanoparticles leading to rapid CD8 T cell and B cell responses. Depending on the similarity of mouse and human iNKT cell distributions, it may not be favorable to perform these vaccinations through subcutaneous or intramuscular routes in a clinical setup. Moreover, intranodal administration can be promising in a booster vaccine regime due to its efficiency in antigen delivery and activation of CD4 T cells.

Keywords: iNKT cells, nanoparticles, vaccination route

B015 / Modi-1 a novel cancer vaccine targeting citrullinated vimentin and enolase

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Stressful conditions in the tumor microenvironment induce autophagy in cancer cells to promote their survival. However, autophagy also causes post-translational modification of proteins, in particular citrullination, which is recognized by the immune system. We have previously shown that killer CD4 T cells which are stimulated by citrullinated vimentin result in strong anti-tumor responses (Brentville et al. 2016). In this study we show that citrullinated vimentin 415-433, vimentin 28-49 and alpha enolase 241-260 peptides (Modi-1) in the presence of GMCSF, TLR1/2, TLR4, TLR7 and TLR9 ligands stimulates Th1 responses in both HLA-DR4 and DP4 transgenic mice. Similarly Modi-1 could stimulate CD4 proliferative responses in normal donors which were shown to be restricted by HLA-DR4 and HLA-DP4. In the presence of TLR4/9 adjuvants Modi-1 induced strong anti-tumor immunity against established B16-DR4 melanoma (60% survival, $p< 0.0001$), B16-DP4 melanoma (100% survival, $p< 0.0001$), Pan02-DR4 pancreatic tumors (50% survival, $p=0.0076$), LLC2-DR4 lung cancer (40% survival, $p< 0.0142$) and ID8-DP4 ovarian cancer (70% survival, $p< 0.0014$). Since most tumors do not constitutively express MHC class II molecules a model was engineered that expressed HLA-DR4 under the control of an IFNγ inducible promoter. Immunisation with Modi-1 resulted in 100% survival ($p< 0.0001$) against established B16 tumor expressing inducible HLA-DR4. In contrast, immunisation with Modi-1 in Incomplete Freund's adjuvant (IFA) stimulates a strong IL-10 response that could be detected 2 days post immunisation, suggesting that there was a pre-existing regulatory response to citrullinated enolase and vimentin. These responses failed to show anti-tumor immunity demonstrating the need for Modi-1 to include Th1 polarizing adjuvants. Enolase is an enzyme which catalyzes the penultimate step in glycolysis. Many tumors have elevated glycolysis known as the "Warburg effect" as they use glucose to generate ATP by pyruvate to lactic acid conversion even in the presence of normal oxygen. They therefore have elevated levels of enolase. Vimentin is one of the early proteins induced during epithelial-to-mesenchymal transition of metastasizing epithelial tumors. Of great interest is that 97% of ovarian and triple negative breast cancers (TNBC) express citrullinated vimentin although only 50% of ovarian and TNBC express vimentin, suggesting degradation and citrullination of vimentin is common during the EMT process. These studies suggest that intradermal injection of Modi-1 in combination with a Th1 polarising adjuvant should be rapidly translated to the clinic for the treatment of TNBC and ovarian cancer.

Brentville et al., 2016. Citrullinated vimentin presented on MHC-II in tumor cells is a target for CD4+ T cell-mediated antitumor immunity. Cancer Research 2016 Feb 1;76(3):548-60.

Keywords: citrulline, cancer vaccine, TLR agonist

B016 / Sub-optimal T cell activation drives a tumor cell mutator phenotype that promotes escape from therapy

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Anti-tumor T cell responses raised by frontline therapies such as chemotherapy, radiation, tumor cell vaccines, and viroimmunotherapy are generally likely to be weak both quantitatively (low frequency) and qualitatively (low affinity). We show here that T cells which recognize tumor-associated antigens (TAAs) on tumor cells can directly kill tumor cells if used at high effector to target ratios. However, when these tumor reactive T cells were present at suboptimal ratios, direct T cell mediated tumor cell killing was reduced, and the ability of tumor cells to survive, and evolve away from, a co-applied frontline therapy was increased. Tumor cell escape, enhanced by the presence of tumor reactive T cells, was associated with cytosine to thymine transition mutations characteristic of APOBEC-induced cytosine deaminase activity, and was induced through an MHC class I, TNFα and PKC dependent pathway. Short hairpin RNA inhibition of endogenous APOBEC3 returned the level of tumor escape to levels seen in the absence of anti-tumor T cell co-culture. Conversely, overexpression of human APOBEC3B in tumor cells led to significantly enhanced escape from suicide gene therapy and oncolytic virus therapy both ***in vitro*** and ***in vivo.*** Our data suggest that the generation of weak, sub-optimal T cell responses against TAAs may actively drive a mutator phenotype in tumor cells that promote their genomic plasticity and ability to evolve away from frontline therapies. Therefore, indirect (bystander effects of chemotherapy, virotherapy or radiation therapies) or direct (tumor vaccines, adoptive T cell transfer) immunotherapies may even act as tumor cell mutators in the same way as other mutagenic frontline therapies. We reason that immunotherapies need to be optimized as early as possible so that, if they do not kill the tumor completely, they do not make it stronger.

Keywords: vaccines, oncolytic viruses, tumor escape

B017 / Formation of immune-complexes via a defined linear tetanus toxin-derived peptide boosts human T cell responses

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Enhancing immune responses against both viral and tumor antigens requires efficient co-stimulation and directed delivery of antigens into APCs. As short peptides can lead to T cell tolerance and suffer from HLA restrictions, long peptides are considered favourable from a clinical perspective. However, long peptides are not actively targeted to and taken up by APCs, and the standard non-conjugated adjuvant-peptide mixtures do not ensure co-targeting of the two to the same APC. We have now used a unique linear tetanus-toxin derived B cell epitope to mediate the formation of immune-complexes with circulating antibodies. These complexes, improves both antigen uptake by APCs (blood monocytes and CD1c+ DCs) and thereby CD8+ T cell recall responses in a human ***ex-vivo*** blood loop system. The uptake of the peptide conjugate by blood monocytes is dependent on antibodies and the complement component C1q. The defined linear peptide limits the number of antibodies that can bind the sequence, and therefore the number of linear tetanus sequences per T cell epitope determines the outcome of the response. Additionally, by vaccinating healthy individuals and cancer patients with DTP vaccine IgG titres against the tetanus epitope are boosted and improve T cell responses to a model antigen. We envision that this strategy can be used to facilitate active uptake of antigens into antigen-presenting cells and we currently have an active preclinical development program with a prostate cancer candidate drug that we aim to take into clinical trial testing.

Keywords: immune-complexes, synthetic long peptides, Therapeutic vaccination

B018 / Mapping the HLA peptidome of primary *versus* recurrent disease in glioblastoma by mass spectrometry

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Glioblastoma is the most aggressive and most frequent primary tumor of the central nervous system. Despite extensive research efforts, there has only been limited translation of scientific knowledge into clinical benefit. Owing to deep infiltration into surrounding benign tissue, complete surgical resection is impossible and glioblastoma inevitably recurs. Recurrent disease is characterized by a lack of therapeutic options resulting in poor clinical outcome, underscoring the high unmet need for innovative therapeutic concepts. Novel promising approaches include antigen-specific immunotherapy as exemplified by recent reports of glioblastoma regression after chimeric antigen receptor (CAR) T cell therapy. Importantly, such immunotherapeutic intervention typically takes place after standard radiochemotherapy, which has been shown to contribute to clonal evolution and hypermutation in glioblastoma and may thereby drastically alter the antigenic landscape of the tumor.

Using immunoaffinity chromatography and tandem mass spectrometry, we profiled the HLA peptidome of primary ***versus*** recurrent disease in seven glioblastoma patients. A total number of six antigens frequently and exclusively presented at recurrence (43% to 57% positive samples) and nine with frequent and exclusive HLA-presentation on primary tumors (57% to 86% positive samples) were identified. For six patients, we performed label-free quantification delineating HLA class I ligands showing significant up- or down-modulation at recurrence. On average, 13% of the patients' total HLA class I peptidomes showed significant modulation upon recurrence. Three antigens were over- and three antigens were under-represented in the HLA peptidome of recurrent glioblastoma of at least half of the patients. Furthermore, we investigated the presentation of HLA ligands derived from previously published tumor-associated antigens. Since most

of them were also identified on a broad spectrum of benign tissue, we defined a novel set of twelve tumor-exclusive antigens with frequent representation in the HLA class I or HLA class II peptidomes of both primary and recurrent tumors.
The present work provides insight into the antigenic landscape of glioblastoma as well as its modulation in the context of clonal evolution during progression from primary to recurrent disease. Using this strategy, we aim to identify (novel) robustly presented antigens as potential targets for the immunotherapy of recurrent glioblastoma.

Keywords: Glioblastoma, Mass spectrometry, HLA

B019 / Vaccination-induced skin-resident memory and circulating CD8+ T cells collaborate to protect against cutaneous melanoma

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Long-lasting memory $CD8^+$ T cells have the potential to control primary and disseminated tumors. Resident-memory CD8+ T (Trm) cells are a new subset of memory T cells that stably reside in non-lymphoid tissues and mediate potent protective immunity. However, their specific contribution to antitumor immunity remains poorly understood. Moreover, vaccination strategies that efficiently generate Trm cell responses are expected to improve protection against tumors. Here, we demonstrated that intradermal vaccination with DNA-encoded or DC-targeted protein antigens efficiently induced antigen-specific Trm cells, which accumulated in vaccinated and distant non-vaccinated skin, and were resistant to in vivo antibody-dependent depletion. Intradermal but not intraperitoneal vaccination generated memory precursors expressing skin-homing molecules and Trm cell responses in skin that completely suppressed the growth of B16 melanoma tumors, independently of circulating memory CD8+ T cells. Interestingly, Trm-mediated rejection of B16 melanoma expressing a model neoantigen lead to the generation of secondary cytotoxic $CD8^+$ T cell responses against melanoma-derived self-antigen GP100, providing protection against re-challenge with B16F10 cells lacking neoantigen expression. This work highlights the therapeutic potential of vaccination-induced Trm cells against skin malignancies and suggests a cooperative role with circulating CD8+ T cells to further broaden antitumor immunity and control potential antigen escape mutants.
Acknowledgements: This work was funded by CONICYT PFB-16, FONDECYT-11171703, P09/016-F and CONICYT scholarships.

Keywords: Memory CD8 T cells, Intradermal vaccines, Melanoma

B020 / Analysis of differential antigen processing in cancers and prevalence of presentation of tumor exclusive antigens using targeted mass spectrometry

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In the last years, cancer immunotherapies such as immune checkpoint blockade strongly improved clinical outcome in many malignancies, however at the cost of considerable side effects due to the lack of specific targets. Hopes are that antigen-specific immunotherapy might further improve cancer therapy in the near future. An extensive range of attractive targets are naturally presented tumor-exclusive HLA ligands in the HLA ligandome of multiple tumor entities. We selected promising HLA ligands and are now in the process of validating their tumor-exclusive nature in different benign and malignant sources.
Previously, we identified several tumor-exclusive HLA ligands in large-scale screenings of the HLA-ligandomic landscape of different malignancies.
We are further assessing the distribution and frequency of 38 identified HLA ligands derived from known tumor-specific antigens (MUC16, MSLN, IDO1...) and cancer testis antigens (MAGEA, MAGEB, PRAME...).
Moreover, tumor-exclusive HLA ligands generated by differential antigen processing were detected. Although differential antigen processing in cancer is a well described process, there is a lack of specific examples for this phenomenon which cannot be explained by tumor-associated changes in gene expression. The phenomenon is investigated based on the two model proteins deubiquitinating protein VCIP135 and cytochrome c oxidase subunit 8A and their respective HLA ligands. These proteins are translated from a single mRNA transcript and are abundant in malignant and benign tissues, but give rise to exclusive HLA ligands for both tumor and benign tissues.
The selected HLA ligands are identified and quantified on different malignant and benign tissues using quantitative targeted mass spectrometry in combination with synthesized isotope-labelled peptides.
In conclusion, employing quantitative targeted mass spectrometry the prevalence and quantity of presentation of the selected HLA ligands within as well as between different malignancies can be determined accurately. Additionally, the tumor-exclusive nature of the selected peptides can be validated and the previously identified cases of differential antigen processing can be characterized. The project will provide tumor-exclusive antigens and enable novel tangible insights into the differential antigen processing in cancer.

Keywords: differential antigen processing, tumor-exclusive antigens, targeted mass spectrometry

B021 / An antibody derived from a cured AML patient identifies CD43s, a unique epitope on CD43, as a novel target for acute myeloid leukemia and myelodysplastic syndrome

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Immunotherapy for acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) is hampered by the lack of tumor-specific targets. We took advantage of the tumor-immunotherapeutic effect of allogeneic hematopoietic stem cell transplantation (HSCT) and searched the B cell repertoire of a patient with a lasting and potent graft versus AML response for AML-specific antibodies.
We identified a donor-derived B cell clone that produced an IgG1 antibody, AT1413, that specifically interacted with AML cell lines, with the patient's autologous AML blasts, but not with lymphocytes or with cells from liver, colon, skin and other tissues. AT1413 recognized a unique, not previously described, sialylated epitope on CD43 (CD43s). CD43s is overexpressed on all types of AML and MDS, as illustrated by its reactivity with freshly isolated blasts of each of more than 60 randomly selected AML and MDS patients in our clinic, representing all WHO 2008 AML and MDS classes.
AT1413 induced antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) of target cells in vitro. To investigate the effect of AT1413 ***in vivo*** we first generated mice populated with human effector cells (NK cells, CTL and myeloid cells) by injecting human hematopoietic stem cells into new born immunodeficient mice. After establishment of a human immune system in these mice we inoculated luciferase labeled AML cells via tail vein injection. Following engraftment of the tumor we dosed the mice biweekly with AT1413 or a control antibody. We observed strongly reduced numbers of AML cells in AT1413- but not in control antibody treated mice. Importantly, AT1413 treatment was tolerated well and did not affect numbers of non-malignant human myeloid cell in these mice.
In conclusion, we have obtained an antibody from a cured AML patient which recognizes a unique sialylated epitope on CD43 (CD43s) that is selectively over-expressed on all WHO 2008 types of AML and MDS. This antibody was able to eliminate AML cells ***in vivo*** and therefore has high therapeutic potential.

Keywords: Acute myeloid leukemia, antibody, therapeutic

B022 / Elucidating the control of germinal center B cell proliferation

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A majority of B cell lymphomas originate from germinal center (GC) B cells. While somatic hypermutation in these cells can increase antibody affinity in response to infection, it can also cause collateral damage to DNA giving rise to cancer in this highly proliferative cell type. Successfully mutated antibody with increased affinity leads to increased antigen capture and presentation and subsequently increased follicular T cell help. T cell help in the light zone of the germinal center initiates a program of GC B cell proliferation with increased T cell help controlling dwell time in the dark zone where GC B cells proliferate. However, the mechanism underlying the number of cell cycles a selected B cell performs before returning to the light zone is elusive. We hypothesize that the number of cell cycles a selected GC B cell goes through is metabolically controlled by a mechanism in which a GC B cell is metabolically charged to perform a given number of divisions based on the strength of the selecting T cell signal. However, it is currently unknown what cell intrinsic factors control the dose-dependent proliferation of GC B cells.
To identify major dose-dependent regulators of proliferation of GC B cells we employed a model in which a small subpopulation of GC B cells receives different doses of T cell help by loading them with different amounts of cognate antigen. We used the cell cycle indicator Fucci to subsequently sort cells that had recently received T cell help and had progressed into the cell cycle while still remaining in the light zone. We then performed RNA sequencing on these recently selected GC B cell that had received different doses of T cell help. We identified genes that were regulated in a dose-dependent manner with T cell help and among the genes found strong signatures for many pathways positively correlating with the amount of T cell help. Among these several have been previously implicated in GC maintenance and CD40 signaling including NFkB signaling, mTOR signaling and STAT signaling.
We also noted that several metabolic pathways were strongly upregulated and showed that GC B cells are more metabolically active than their naïve counterparts in terms of glucose uptake and mitochondrial activity.
We are currently constructing genetic tools to further explore the contribution of key regulators to GC B cell proliferation, such as for the glucose transporter Glut1. In addition, we are examining several signaling pathways, which could control the extent of GC B cell proliferation after T cell help.
Our studies will shed light on the regulation of proliferation of GC B cells, a major source of lymphoma and its dependency on

several metabolic and signaling pathways with implications for B cell lymphoma research and vaccination approaches both in tumor immunology and elsewhere.

Keywords: Lymphoma, Germinal center, B cells

B023 / Cytokine-armed oncolytic adenovirus enables system-wide antitumor efficacy - the abscopal effect - following local treatment

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A traditional treatment for metastatic cancer is most commonly administered systemically, leading to systemic side-effects. Immunotherapies provide interesting means to provoke system-wide responses, but toxicity remains a problem with some of these approaches. We aim to study whether local treatment with oncolytic adenovirus coding for human Tumor Necrosis Factor alpha (TNFa) and Interleukin-2 (IL-2), Ad5/3-E2F-d24-hTNFa-IRES-hIL2 (a.k.a. TILT-123), can provoke systemic antitumor effects, known as the abscopal effect. Treatment with this virus activates locally innate and adoptive immune cells and induces epitope spreading. In addition, the virus enables T cell therapies including checkpoint modulation in solid tumors. Here, we studied the systemic effects in two immunocompetent animal models, Syrian hamsters and mice, and combined virus treatment to adoptive T cell transfer. Hamsters provided us a platform to study the effect of oncolysis, whereas with mice we could dissect the effects of the cytokines with replication-incompetent viruses. With both models, we saw reduction in tumor growth in both treated and untreated tumors. Virus spread into untreated tumors was modest, and only seen in the replication permissive setting, pointing out the importance of the arming device. The cytokine-armed virus was able to induce positive changes in immune cell compartments in the tumor microenvironment. In addition, oncolytic virus was able to enhance tumor antigen presentation by upregulating MHC-I expression on the tumor cell surface. Interestingly, a SPECT-CT study of cell trafficking revealed increased presence and sustainability of the administered T cells in both treated and untreated tumors. In summary, local treatment with TILT-123 enhanced systemic antitumor efficacy by inducing immune cell infiltration and trafficking into both treated and untreated tumors. Moreover, the oncolytic adenovirus platform increases systemic effects over replication deficient vectors since the virus spreads into metastases subsequent to local injection.

Keywords: Oncolytic adenovirus, Abscopal effect, Adoptive cell therapy

B024 / Targeting B7-H3 with different ADCC-mediating monoclonal antibody constructs in neuroblastoma

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We evaluated the use of B7-H3 as alternative target antigen to disialoganglioside GD2 in neuroblastoma and investigated different anti-B7-H3 monoclonal antibody (mAb) constructs for their ability to elicit antibody-dependent cellular cytotoxicity (ADCC).

Introduction: B7-H3 (CD276) belongs to the B7-CD28 family. It is an immune checkpoint and the ligand for a yet unknown receptor, through which T and NK cell response is regulated. B7-H3 shows limited protein expression on healthy human tissue, but is highly overexpressed on many solid tumors. This makes B7-H3 an interesting target for cancer immunotherapy. In high-risk neuroblastoma patients, treatment with mAbs targeting GD2, significantly improves survival. However, GD2 expression is heterogeneous and B7-H3 might serve as an alternative or additional antigen to improve therapy.

Method: A B7-H3-specific mAb clone (HEK5-1B3) as well as modified and optimized versions of it were evaluated *in vitro* for their capability to mediate ADCC against neuroblastoma cell lines, which express high levels of B7-H3 but variable levels of GD2 (LAN-1, LS, Kelly, SH SY5Y). Four different versions in addition to the initial HEK5 clone were engineered: (1) HEK5 fused with human IL-2 and human IgG1 constant regions (cHEK5-IL2), (2) chimeric and Fc-optimized (SDIE) HEK5 w/o fusion (cHEK5opt), (3) cHEK5opt fused with human IL-2 (cHEK5opt-IL2) and (4) cHEK5opt fused with IL-15 (cHEK5opt-IL15). All IL-2 fusions were to the C-terminus of the light chain. The abilities of all five anti-B7-H3 mAb constructs and the GD2-specific mAb CH14.18 to mediate ADCC were compared in cytotoxicity assays using the RTCA xCELLigence system. Human expanded NK cells were used as effector cells.

Results: All anti-B7-H3 mAb constructs were able to elicit ADCC against the tested neuroblastoma cell lines. Of interest, even in cell lines expressing high amounts of GD2, target cell lysis mediated by the Fc-optimized cHEK5opt-IL2 fusion mAb was higher or at least comparable to that mediated by CH14.18. Specific lysis of LAN-1 - a cell line expressing high amounts of both GD2 and B7-H3 - was calculated (after 36 hrs.; in ascending

order): Targets + effectors w/o mAb (24 %), initial HEK5 clone (29 %), cHEK5opt (44 %), cHEK5-IL2 (76 %), cHEK5opt-IL15 (85 %), CH14.18 (90 %) and cHEK5opt-IL2 (97 %). Similar results were obtained using SH SY5Y as target cell line, which expresses high levels of B7-H3 but no GD2, making CH14.18 ineffective. Indeed, compared to targets + effectors w/o mAb, specific lysis was not enhanced by addition of CH14.18 (45 % and 40 %, respectively) but drastically increased using the cHEK5opt-IL2 instead (99%).
Conclusion: Beside GD2, B7-H3 has been demonstrated to be a suitable target antigen in neuroblastoma and an alternative when GD2 expression is low or absent. Fc-optimized mAbs and mAb cytokine fusion constructs targeting B7-H3 might increase the efficacy of immunotherapy in GD2 negative tumors and in combinatory approaches.

Keywords: Neuroblastoma, Immunotherapy, B7H3

B025 / Attenuated *Listeria monocytogenes* as a promising vaccine candidate in the settings of hepatocellular carcinoma and cholangiocarcinoma

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The purpose of a present research study is the development and validation of a vaccine strain in the settings of primary liver cancer, comprising hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA).
Primary liver cancer is the second leading cause of cancer-related deaths with a constantly growing incidence. Current treatment approaches are insufficient and the disease prognosis is dismal. Therefore, new therapies are urgently needed. Since CCA and HCC are highly immunogenic, immunotherapy represents a promising alternative strategy. In our studies we decided to concentrate on attenuated *Listeria monocytogenes* (Lm) strains that have been recently successfully tested in the treatment of various solid tumors and that are potent inducers of anti-tumor immune responses.
We took advantage of recently established transposon-based autochthonous mouse models of HCC and CCA, both expressing a model antigen Ovalbumin (OVA). For this purpose we have applied a hydrodynamic tail vein injection to stably integrate oncogenic ***Nras^{G12V}*** and ***OVA*** or ***Kras^{G12V}*** and ***OVA*** into murine hepatocytes of B6.129X1-Cdkn2a^{tm1Cjs} (p19$^{Arf-/-}$) mice giving rise to autochthonous HCC-Ova and CCA-Ova, respectively. In these tumor models we tested theprophylactic- as well as therapeutic vaccination potential of the attenuated strain Lm ΔactA/ΔinlB (LmAI) expressing the tumor model antigen OVA (designated LmAIO) (both strains kindly provided by D. A. Portnoy).
Prophylactic vaccination resulted in a full protection from HCC- as well as CCA development when two vaccination doses of LmAIO strain were applied. In our preliminary data even one prophylactic vaccination with LmAIO strain was sufficient to decrease the tumor burden and prolong the survival 60 % of mice in which highly aggressive HCCs have been induced 14 days post vaccination.
In a therapeutic approach one treatment The vaccination dose with LmAIO strainwas efficient in decreasing tumor burden and led to an increase of overall survival comprising 16- and 7 weeks, respectively, in early- and advanced HCCs.
In both prophylactic- and therapeutic approaches vaccination with LmAIO delivering tumor antigen OVA showed an induction of tumor antigen (OVA)-specific CD4+/CD8+ T cells which correlated with the observed protection against HCC developmennt. Interestingly, the induction of tumor-specific antibodies was not required for protection against liver cancer development.
In conclusion, the attenuated strain LmAI delivering tumor antigens represent a promising vaccine candidate for both HCC- and CCA-based malignancies. However, for the future perspectives LmAI strain expressing specific tumor associated antigens has to be developed and tested ***in vivo*** using several autochthonous murine models of HCC and CCA.

Keywords: Primary liver cancer, Cancer vaccines, Immunotherapy

B026 / CTL responses against a cancer-stem cell specific antigen, ASB4, effectively prevent colorectal cancer formation

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Cancer-stem cells (CSC) are a cellular subset that is observed in many types of malignant tumors. Although they are small in numbers, those dormant cells tend to be resistant to conventional chemo and radiotherapies, being responsible for relapse or metastasis. In this study, we identified a novel antigen, ASB4, that was widely expressed in colorectal cancers (CRC) including primary tissues from patients. HLA-ligandome analysis revealed the peptide repertoire of colon CSC and non-CSCs, and following analysis demonstrated that the cytotoxic-CD8$^+$ T cell (CTL) epitope of ASB4 was naturally presented by a CSC subset, but not by non-CSCs. The epitope was immunogenic to elicit CTL responses, which discriminated and exclusively responded to CSCs, accordingly. Most interestingly, the adoptive transfer of the CTLs effectively prevented tumor development in mice implanted with heterogeneous CRC cells that were composed of a small number of CSC and tons of non-CSC subsets. Our results suggest that CSCs can be a necessary and minimal target to prevent

tumor formation *in vivo* as well as CTL immune surveillance is capable of recognizing CSCs. Because CTL responses against the ASB4 antigen were readily detected in CRC patients, the use of CSC-specific antigens offers a new and practical strategy in CTL-based immunotherapy against CRCs.

Keywords: Cancer stem cell, Tumor antigen, Cancer vaccine

B027 / Enhanced antitumor immunity using tumor cell lysate-encapsulated CO_2 generating liposomal carrier system by photothermal irradiation

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Tumor cell lysate-encapsulated liposomes as a tumor-specific antigen carrier is a promising approach to enhance intracellular delivery efficiency of lysate to dendritic cells (DCs). Although liposomes provide a promising strategy to increase delivery efficiency, triggered release of payload from liposomes is needed to achieve optimum immune response. Therefore, we developed whole tumor cell lysate containing bubble-generating liposomes (TCL-BG-LPs), which are capable of triggered release of payload when stimulated by near-infrared (NIR) irradiation in the tumor microenvironment. In addition, we developed doxorubicin (DOX) containing bubble-generating liposomes (DOX-BG-LPs) to increase combination therapeutic efficacy. Mean particle size of TCL-BG-LPs and DOX-BG-LPs was 115.23 ± 6.78 nm and 134.45 ± 1.42 nm, and surface charge was -21 mV and -29 mV, respectively. Bubble generation into liposomes by NIR irradiation was monitored by using an ultrasound imaging system. The TCL-BG-LPs and DOX-BG-LPs enhanced burst release of payloads by bubble generation into liposomes stimulated with NIR irradiation compared to non-BG-LPs. Moreover, the releases of tumor cell lysate from TCL-BG-LPs promoted dendritic cell maturation, leading to emergence of antigen-specific cytotoxic CD8+ T cells. Combination therapy of TCL-BG-LPs and DOX-BG-LPs showed significantly greater antitumor efficacy in B16F10 tumor-bearing mice compared to control (p< 0.01). Taken together, we demonstrate that a novel liposomal delivery system combined with NIR irradiation leads to potent immunotherapy efficacy in tumor-bearing mice. This approach has broad utility for enhancing the therapeutic effects on cancer immunotherapy.

Keywords: Bubble generating liposome, Tumor cell lysate, CD8+ T cell

B028 / Enhanced anti-tumor effect by combining oral cancer vaccine using *Bifidobacterium* displaying WT1 protein with anti-PD-1 antibody therapy in mouse prostate cancer model

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Prostate cancer is the most common cancer in men, and is the sixth leading cause of cancer-related death worldwide. Recently, an autologous active cellular immunotherapy, sipuleucel-T®, has been approved by US FDA for the treatment of metastatic castration-resistant prostate cancer (mCRPC), however, it achieved only modest effectiveness despite high cost. Recently, we had constructed the recombinant *Bifidobacterium longum* displaying Wilms' tumor 1 (WT1) protein (B. longum 420) as oral cancer vaccine for treatment of prostate cancer, based on the findings that WT1 protein was highly expressed in metastatic prostate cancer tissues. In this study, we investigated the feasibility of combination immunotherapy of *B. longum* 420 and anti-programmed death 1 (PD-1) immune checkpoint inhibitor therapy by using mouse prostate cancer model. Firstly, 2×10^6 of murine prostate cancer cell line, TRAMP-C2, which expressed WT1 and PD-L1 induced by IFN-γ, were inoculated into mice. After tumor formation, 1×10^9 colony forming units of *B. longum* 420, *B. longum* 2012, which was mock control, and PBS were orally administered into mice 5 days a week over the following weeks. In addition, 100 μg anti-murine PD-1 antibody or IgG isotype control were intraperitoneally injected at 31, 34, 38, 41 and 45 days after tumor inoculation. To analyze the WT1-specific cellular immune responses induced by *B. longum* 420, mice splenocytes were isolated and their cytokine production, cytotoxic activities against TRAMP-C2 were determined. As the results, oral administrations of *B. longum* 420 and anti-PD-1 antibody therapy significantly inhibited the growth of TRAMP-C2 tumor compared with the group treated with *B. longum* 420 and IgG isotype control at day 70 (192.6±72.8 mm^3 vs 684.4±145.7 mm^3, p< 0.05). The combination therapy significantly improved the overall survival compared with other treatments (p< 0.05). Splenocytes obtained from *B. longum* 420 treatment group produced significantly higher IFN-γ, IL-2 and TNF-α compared to both *B. longum* 2012 and PBS group (p< 0.05). *B. longum* 420 also induced significantly higher population of $CD4^+$T and $CD8^+$T cells that produced IFN-γ, IL-2 and TNF-α (p< 0.05). MHC-tetramer assay using the representative WT1-$CD8^+$T epitope

showed that epitope-specific CTLs were induced by immunization with whole WT1 protein displayed on the ***B. longum*** 420. CTLs isolated from ***B. longum*** 420 group elicited significantly higher WT1-specific cytotoxicity against TRAMP-C2 in a effector:target ratio dependent manner ***in vitro***. In conclusion, we demonstrated that a combination immunotherapy using ***B. longum*** 420 and anti-PD-1 antibody could elicit the synergistic anti-tumor effect in mouse prostate cancer model. These findings suggested that the combination immunotherapy of oral WT1 cancer vaccine and anti-PD-1 antibody could be a promising candidate for treatment of mCRPC.

Keywords: Cancer vaccine, Combination therapy, Prostate cancer

B029 / Enhanced stimulation of anti-stomach cancer T cells responses by dendritic cells loaded with poly lactic-co-glycolic acid (PLGA) nanoparticle encapsulated tumor antigens

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Background: Developing safe and effective cancer vaccine formulations is a primary focus in the field of cancer immunotherapy. Dendritic cells (DC) are currently employed as cellular vaccine in clinical trials of tumor immunotherapy. Recognizing the critical role of DCs in initiating anti-tumor immunity has resulted in the development of several strategies that target vaccine antigens to DCs to trigger anti-tumor T cell responses. To increase the efficiency of antigen delivery systems for anti-tumor vaccines, encapsulation of tumor-associated antigens in polymer nanoparticles (NPs) has been established.
Methods: In this study, the effect of tumor lysate antigen obtained from three stage III stomach cancer tissues encapsulated within PLGA NPs to enhance the DC maturation was investigated. The T-cell immune response activation was then fallowed up. Fresh stomach tumors were initially used to generate tumor lysate antigens containing poly lactic-co-glycolic acid (PLGA) NP. The encapsulation efficiency and release kinetics were profiled. The efficiency of encapsulation was measured using Bradford protein assays measuring the dissolved NPs. To evaluate the hypothesis that NPs enhances antigen presentation, including soluble tumor lysate, tumor lysate containing NPs and control NPs the efficiency of NP-mediated tumor lysate delivery to DCs was evaluated by assessing CD3+ T-cell stimulation after T cell/and DCs co-culture.
Results: The rate of encapsulation was increased by enhancing the antigen concentration of tumor lysate. However, increasing the antigen concentration diminished the encapsulation efficiency. In addition, higher initial protein contenting NPs led to a greater cumulative release. All three patients released variable amounts of IFN-γ, IL-10, IL-12 and IL-4 in response to re-stimulation. T cells stimulated with lysate-pulsed DCs induced a substantial increase in IFN-γ and IL-12 production. We demonstrated that NPs containing tumor lysate can induce maturation and activation of DCs, as antigen alone does.
Conclusion: PLGA-NPs are attractive vehicles for protein antigen delivery which effectively induce stimulation and maturation of DCs, allowing not only an enhanced antigen processing and immunogenicity or improved antigen stability, but also the targeted delivery and slow release of antigens.

Keywords: Dendritic cells, Tumor associated antigen, Nanoparticles

B030 / Intracellular cleavable antigen-adjuvant conjugates improve immunotherapy

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Co-delivery of tumour antigen and vaccine adjuvant has been shown to enhance cellular immune responses compared to delivery of antigen/adjuvant mixtures. Recently we generated an intracellular cleavable vaccine conjugate of class B CpG linked to the model tumour antigen ovalbumin (OVA-HYN-SS). An ***in vivo*** therapeutic tumour trial demonstrated enhanced survival in mice vaccinated with the intracellular cleavable OVA-HYN-SS conjugate compared to vaccination with a stable conjugate and a mixture of CpG and ovalbumin. To translate the vaccination strategy into a clinical setting, the clinically relevant melanoma associated antigen gp100 peptide was chosen to be conjugated to either CpG class B and class C using the same intracellular triggerable linker technology. The two classes of CpG are used to target multiple antigen-presenting cells, with class B CpG activating dendritic cells, B-cells and natural killer cells and class C CpG additionally inducing a type I interferon response with associated enhanced plasmacytoid dendritic cell stimulation. The gp100 peptide was conjugated to CpG B using either stable (gp100-HYN) or intracellular cleavable disulphide (gp100-HYN-SS) bis-arylhydrazone bond linking chemistry. The conjugates were analysed by reverse-phase high performance liquid chromatography. A molar ratio of 1.9 CpG per gp100 peptide was determined by UV-absorption at 354 nm for the formation of the bis-arylhydrazone bond. Murine bone marrow derived dendritic cells (BMDC) were stimulated with either PBS, CpG/gp100 mixture or a CpG-gp100 conjugate (gp-100-HYN or gp100-HYN-SS) and activation was analysed by flow cytometry. Preliminary results show effective activation of dendritic cells by the upregulation of activation markers CD40 and

CD86 after stimulation with either stable conjugate, intracellular cleavable conjugate or the mixture of CpG and gp100. Effective activation of dendritic cells is a crucial first step to induce an efficient cellular anti-tumour immune response. This study indicates that conjugates of CpG and the clinically relevant antigen gp100 peptide may induce an effective anti-cancer immune response comparable to the intracellular cleavable OVA-HYN-SS conjugate previously tested. Ongoing studies test conjugates of class B and class C CpG-gp100 for stimulation of dendritic cells, subsequent natural killer and T-cell activation ***in vitro*** as well as induction of an anti-cancer immune response ***in vivo.*** The intracellular cleavable conjugation strategy represents a promising approach to improve cancer immunotherapy of soluble vaccines.

Keywords: Antigen-Adjuvant conjugate, Immunotherapy, CpG

B031 / Induction of HPV16 E7-specific CD8$^+$ T cells in MHChumanized A2.DR1 mice via minimal epitope vaccination

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Several studies have demonstrated anti-tumor efficacy of therapeutic vaccinations against the viral oncoprotein E7 of human papillomavirus (HPV)16 in a model of cervical cancer in C57BL/6 mice. However, this approach is limited to the study of murine epitopes. To overcome this challenge in translatability, we use mice that are humanized for MHC molecules, called A2.DR1. In this model, we aim at the development of a vaccine formulation that effectively induces high numbers of HPV16-specific CD8$^+$ T cells to mediate anti-tumor effects.

We make use of minimal epitopes to induce only immune responses against epitopes that have been proven by mass-spectrometry to be naturally presented on HPV16$^+$ human cancer cells. Our vaccination approaches include emulsion-based vaccines, RNA vaccines, constructs exhibiting amphiphilic properties, silica-nanoparticles and the use of various TLR-agonists together with the minimal epitope HPV16 $E7_{11-19}$.

Our results show that robust immune responses against this HLA-A2-restricted epitope can be induced in A2.DR1 mice. The numbers of $E7_{11-19}$-specific CD8$^+$ T cells generated by our different vaccination formulations vary greatly, with amphiphilic constructs showing the most promising responses to date.

Additionally, we develop an A2.DR1-compatible HPV16 E6/E7-expressing tumor model. We propose our preclinical vaccination experiments in this A2.DR1 model as a means to achieve improved translatability, due to the possibility to use the same epitopes for vaccination as would be used in humans.

Keywords: cancer vaccine, immunotherapy, MHC-humanized mice

B032 / Assessing a novel PAP-derived vaccine for the treatment of prostate cancer

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The aim of the study is to develop a new and more effective, Prostatic Acid Phosphatase (PAP)-based, vaccine for the treatment of advanced prostate cancer. Our laboratory has previously shown that a 15mer PAP-derived vaccine, when injected as a DNA vaccine, could induce PAP-specific T-cell responses and reduce tumor growth in a syngeneic heterotopic murine prostate cancer model. We have subsequently developed an elongated (42mer) PAP-derived peptide containing a changed amino acid ('mutated') form of the vaccine, and this is the focus of the study.

To assess the efficacy of the vaccine, two preclinical murine models were used: C57Bl/6 mice and HHDII/DR1 transgenic mice. In both models, the abilities of wild type and mutated 42mer PAP-peptide sequences administered using different delivery/adjuvants systems (CpG ***versus*** CAF09 and ImmunoBody® vaccine) to induce PAP-specific immune responses were assessed and compared. For this, IFNγ release from splenocytes isolated from immunized mice in response to stimulation with wild-type short (8 to 9 amino acid) and long (15 amino acid) vaccine-derived peptides was measured using an ELISpot assay. Immunophenotyping of splenic T cells was performed using flow cytometry, and the ability of splenocytes to kill relevant target cells was assessed using a ^{51}chromium release assay. The anti-tumor efficacy of the most promising vaccine strategy was assessed in heterotopic PAP+ tumor models in a prophylactic setting. Finally, the presence of CD8$^+$ PAP-specific T-cells in the peripheral blood of patients with prostate cancer following ***in vitro*** stimulation with PAP-derived peptides was assessed using multimer-based flow cytometry.

The mutated PAP 42-mer vaccine was the most immunogenic sequence in both murine models, as demonstrated by the higher number of IFNγ-releasing splenocytes following ***in vitro*** stimulation with shorter vaccine-derived peptides. The functional avidity towards short vaccine-derived peptides was also greater. CAF09 and ImmunoBody® were superior to CpG in inducing PAP-specific immune responses, with CAF09 eliciting strong immune responses

in both models, and ImmunoBody® eliciting potent immune responses in C57Bl/6 mice. The mutated 42-mer PAP-derived peptide administered with CAF09 adjuvant induced MHC class I specific killing of target cells in vitro and attenuated the growth of PAP+ tumors in the HHDII/DR1 model. PAP-42mer derived, specific $CD8^+$ T cells were detected in the periphery of patients with prostate cancer.
In summary, we have developed a unique vaccine strategy which induces robust anti-PAP immunity and attenuates tumor growth ***in vivo***. The presence of PAP-specific $CD8^+$ T cells in the periphery of patients with advanced prostate cancer suggests that these patients will benefit from this new approach.

Keywords: Prostate cancer, Cancer vaccine, Prostatic Acid Phosphatase

B033 / FLJ43879 is a cancer/testis antigen, shows cancer stem-like cell`s aspects in colorectal cancer

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Cancer testis antigens (CTA) are a category of tumor antigens expressed in human tumors of varying histological origin, but not in normal tissues except for testis and placenta. Identification of cancer/testis antigens with oncogenic properties is of high priority in tumor-specific immunotherapeutic approaches and vaccine therapy Melanoma associated antigen-1 (MAGE-1) was the first identified CT antigen which exhibited autologous T cell response in melanoma patients in 1991. Until now at least 70 families of cancer-testis antigen with 270 members have been attributed to this group and their expression has been studied in different types of tumors.
Colorectal cancer (CRC) ranks third among the estimated cancers and the leading cause of cancer-related death for both men and women in Western countries. Recently, it has become largely accepted that effective anticancer therapies should focus on functionally significant cells with high tumor-initiating capacity and resistance to therapies. Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are a small sub-population of cancer cells that have the properties of high tumor-initiating ability, self-renewal ability and differentiation ability. A subsequent study studied revealed CSCs/CICs exist in solid cancers. In previous studies, we screened out that LYPD3, LEMD1 and ST6GALNAC1 expressed in CR-CSCs/CICs from the primary colorectal cancer cell lines.
In the present study, we isolated CR-CSCs/CICs as sphere-cultured cells from primary colorectal cancer cells and found that a product derived from FLJ43879 is a cancer testis antigen, preferentially expressed in CSCs/CICs. Gene overexpression and gene knockdown experiments revealed that FLJ43879 has roles in promoting cell proliferation, migration and sphere formation via PI3K/pAKT and Wnt/β-catenin pathway in colorectal cancers. Thus, FLJ43879 may represent a new diagnosis target in cancer and immunotherapeutic target for treatment of CRCs.

Keywords: FLJ43879, Cancer testis antigen, Cancer stem cell

B034 / UBR5, a novel E3 ubiquitin ligase and target for immunotherapy of breast and ovarian cancers

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Patients with triple negative breast cancers (TNBC) are at high risks for recurrence and metastasis at an early time despite standard treatment, underscoring the need for novel therapeutic modalities. Our recent analysis of primary TNBC specimen by whole exon sequencing revealed strong amplifications and overexpression of the gene encoding a novel HECT-domain E3 protein ubiquitin ligase, UBR5. A recent TCGA study shows that ***UBR5*** gene amplifications occur in 18-22% of human breast and ovarian cancers. Moreover, breast cancer patients carrying genetic lesions in the ***UBR5*** gene have strongly reduced survival rates. Subsequently, our experimental work via CRISPR/Cas9-mediated annulment of the ***ubr5*** gene in a murine mammary tumor model of TNBC for the first time uncovered a profoundly driving role of UBR5 in the growth and metastasis of breast cancer. Further, tumor-derived UBR5 facilitated malignant growth in a manner completely dependent on paracrine interactions with immune cells in the microenvironment via secreting a factor that deviates dendritic cell differentiation, thereby inhibiting T cell-mediated anti-tumor immunity. In contrast, UBR5 promoted metastasis in a tumor cell autonomous fashion through Id1/Id3 and E-cadherin to induce epithelial to mesenchymal transition (EMT) and the reverse process (MET) for colonization in secondary organs. Experimental evidence also suggests that the diminished UBR5 expression in tumors may have caused the expression of “neoantigens” that triggered antigen-specific immune responses. UBR5’s vital role in promoting tumorigenesis via suppressing host immunity renders itself a potentially impactful immunotherapeutic target in breast and ovarian cancers and beyond.

Keywords: Ubiquitin ligase, dendritic cell differentiation, neoantigen

B035 / The immunopeptidome atlas of the human body: mapping the HLA ligandome of benign tissues

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Adjacent benign tissue is often used as a reference for normal tissue to define tumor-specific targets for cancer immunotherapy. However, even tumor-free adjacent benign tissue might be influenced by the tumor microenvironment. As it is difficult to predict which antigens are presented on HLA molecules solely by using RNA expression data and HLA binding prediction, it becomes increasingly important to define a healthy-state HLA ligandome across multiple HLA allotypes and organs. Therefore, we aim to develop a benign database comprising the HLA ligandome acquired from autopsy samples.
In order to isolate HLA-peptide complexes, we employ immunoaffinity chromatography from solid tissue lysates. Subsequently, purified peptides are identified using LC-MS/MS.
In order to validate the stability of HLA ligands under standard pathology storage conditions, biological replicates of a liver were snap-frozen in liquid nitrogen at 8 h, 16 h, 24 h, 48 h, and 72 h *post-mortem*. This time series experiment revealed no qualitative and semi-quantitative changes until 72 h *post-mortem* during storage at 4°C.
Up to 22 different tissue types from 14 donors were acquired. So far, 85 samples and 810 MS runs have been analyzed, yielding 68,845 unique class I peptides and 103,600 unique class II peptides. When employing unsupervised hierarchical clustering using the Jaccard distance on the identified peptides and their corresponding source proteins, it becomes evident that donors cluster together, rather than organs. The low similarity on both peptide and protein level within organs can be attributed to motif stringency of different HLA allotypes.
Taken together, these findings indicate that using autopsy tissue is a suitable approach to map the HLA ligandome of benign tissues. Increasing coverage of organs and HLA allotypes will allow a deeper insight into the inter- and intra-donor diversity on HLA ligandome level as well as HLA allotype specific comparative analyses. The data acquired within this project will be made available through a new web platform: The HLA Ligand Atlas.

Keywords: HLA, Immunopeptidome, Mass Spectrometry

B036 / First-in-man clinical trial of a multi-peptide-based vaccine adjuvanted with CV8102 (RNAdjuvant®) for HCC - HEPAVAC

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IMA970A is a therapeutic cancer vaccine for primary liver cancer developed by the FP7 EU-funded HEPAVAC Consortium. It is based on an off-the-shelf cocktail of 16 peptides, of which 7 are restricted to HLA-A*02; 5 to HLA-A*24 and 4 to HLA class II.
IMA970A, combined with the CV8102 adjuvant (RNAdjuvant®), is going to be evaluated in a single-arm, first-in-man trial entitled HepaVac-101 (EudraCT Number: 2015-003389-10), in patients with very early, early and intermediate stage of HCC. Enrollment is predicted to start in Q2 2017. The vaccine administration will follow a single pre-vaccination infusion of low-dose cyclophosphamide acting as an immunomodulator. The study drugs are applied without concomitant anti-tumor therapy with the intention to reduce risk of tumor recurrence/progression in patients who have received all indicated standard treatments. The primary endpoints are safety, tolerability, and immunogenicity. Secondary/exploratory endpoints are additional immunological parameters in blood (e.g. regulatory T-cells, myeloid-derived suppressor cells, impact of the standard therapy on the natural immune response), infiltrating T-lymphocytes in tumor tissue, biomarkers in blood and tissue, disease-free survival/progression-free survival and overall survival.
The HepaVac project started in September 2013 and is supported by the European Commission's 7th Framework Program under the Grant Agreement Nr. 602893 (www.hepavac.eu). The clinical trial HepaVac-101 will be conduct in 6 centers located in 5 European countries, i.e. Italy (Naples and Varese), Germany (Tübingen), UK (Birmingham), Spain (Pamplona) and Belgium (Antwerpen).

Keywords: liver cancer, cancer vaccine, HEPAVAC

B037 / Evaluation of novel drug delivery technology platforms to induce potent anti-tumor immunity

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Radiotherapy is a frequently used strategy to fight cancer - however, this therapy is seldom curative, and increasing evidence points to the great advantage of combining radiation therapy with immunotherapy to increase the curative potential. Immunotherapy has a great potential as cancer therapy, with the prospect of training our own immune cells to actively kill cancer cells, not only targeting primary tumors, but also metastasis. Within this platform, one induces not only a treatment for current cancers, but could potentially prevent recurrence in the future by the induction of an immune memory response.
I the current project (XVac) we are elucidating various strategies for combining radiation therapy and immune stimulatory compounds. The primary goal of the XVac project is the development of new drug delivery technology platforms, which can induce a potent anti-tumor immune response. Individual strategies are evaluated in syngeneic cancer mouse models.
We will investigate how the combination of radiation and immunotherapy engages the immune system. In pre-clinical tumor models we will evaluate 1) the infiltration of immune cells to the tumor lesions, 2) systemic alterations in immune reactivity and immune regulation, 3) the ability to induce T-cell recognition of cancer specific antigens, and finally 4) the tumor rejection capacity. Mutation-derived antigens (neoantigens) are of prime interest as these provide a set of tumor specific targets, and are truly foreign to the immune system. For the identification of the T-cell specific neoantigen landscaping pre- and post-immunization, a novel technology, based on multimerized peptide-MHC-I coupled with a specific barcode, will be applied. This technique gives the possibility of detecting >1000 antigen-specific T cell populations in one sample. This will allow us to gain insight to T cells that recognize tumor cells, and provide basis for rational design of personalized cancer vaccines.
Radiotherapy is effective, but does not effectively stimulate a specific immunogenic eradication of all cancer cells. With the combination of immunotherapy this could be overcome. The end goal of this project is to find effective and safe delivery mechanisms of immune stimulatory compounds that can provide sustained clinical efficacy in cancer patients. Preliminary result will be presented at the conference.

Keywords: Vaccine, Radiotherapy, Cancer

B038 / High-avidity T-cell receptors induced by alpha fetoprotein-derived peptides are associated with durable anti-tumor effects in patients with hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. There are no multiple therapeutic options for advanced HCC patients; sorafenib is the sole agent that has been proven to prolong survivals. Therefore, new approaches to advanced HCC treatment are required. Levels of alpha fetoprotein (AFP) are measured for surveillance and diagnosis of HCC. It has also been reported that AFP has an immunogenic potential in hepatocellular carcinoma patients. Here, we performed a phase 1 trial to evaluate the safety and efficacy of AFP-derived peptides as an anti-tumor vaccine for patients with advanced HCC. Moreover, we characterized the induced AFP-specific T-cell receptors (TCRs) in a relationship with their outcomes.
A prospective study of 15 patients with advanced HCC seen at Kanazawa University Hospital in Japan from March 2010 through March 2012 was conducted. Each patient was given a subcutaneous injection of 3 mg of AFP-derived peptides (AFP_{357} and AFP_{403}) in an emulsion with incomplete Freund's adjuvant every other week for at least 6 weeks. Patients were evaluated every 8 weeks by radiologic imaging using the response evaluation criteria in solid tumors criteria ver. 1.0. Adverse events and toxicities were categorized and graded using the common terminology criteria for adverse events. Peptide-specific TCRs were assessed using a rapid TCR cloning and evaluation system. We transduced the TCRs in primary T cells and estimated their ability to recognize the antigen. We also checked whether these TCRs could target AFP-producing cells and finally determine their functional avidities to compare with each other.
No serious adverse reactions to the peptides were observed. One of the 15 patients had a complete response and disease stabilized in 8 patients. In 4 of the 15 patients, we detected AFP_{357}-specific CD8 T-cells; we cloned 14 different TCRs with different avidities for the peptide. The TCR with the highest avidity against AFP-expressing cells was observed in the patient who achieved a complete response for more than 2 years. The second most potent TCR was identified in the patient who maintained stable disease for more than 2 years.
In conclusion, administration of AFP-derived peptides to 15 patients with HCC induced T-cells with receptors specific for the antigen peptide. It was suggested that the functional avidities of the induced TCRs were related to anti-tumor effects and outcomes of the patients.

Keywords: hepatocellular carcinoma, alpha fetoprotein, T cell receptor

B039 / Development of a therapeutic peptide vaccine for the treatment of acute myeloid leukemia (AML) based on naturally presented HLA ligands of CD34$^+$CD38$^-$ AML progenitor cells

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Acute myeloid leukemia (AML) is characterized by high relapse rates and a poor overall survival mainly due to the presence of minimal residual disease (MRD) associated with the persistence of chemotherapy-resistant leukemic progenitor cells (LPCs) after standard chemotherapy and allogeneic stem cell transplantation. Thus, to maintain long-lasting remissions, novel strategies including T-cell based immunotherapy are needed to eliminate MRD. The basis for clinically successful immunotherapy is the availability of suitable target antigens. In a recent study, we characterized the antigenic landscape of primary AML blasts by mass spectrometric analysis of naturally presented HLA ligands and identified AML-associated CD4$^+$ and CD8$^+$ Tcell epitopes (Berlin et al. ***Leukemia*** 2015). A potential avenue to further improve immunotherapy for AML patients is specifically targeting the highly chemotherapy-resistant LPCs. Here we present a mass spectrometry-based study, which - to our knowledge - for the first time analyzes the naturally presented HLA ligandome of AML progenitor cell enriched (LPC_{enr}) fractions of primary AML samples to identify novel LPC-associated antigens. Enrichment of LPCs was performed by magnetic or fluorescence-activated cell sorting for the lin$^-$CD34$^+$CD38- phenotype of PBMCs from eleven AML patients. LPC-containing populations of 1-3% within the PBMCs were enriched to > 80% with cell counts up to 200 x 10^6 per sample. The expression levels of HLA class I molecules on the cell surface of AML blasts and LPCs were found to be comparable with 145,000-175,000 molecules/cell. We identified more than 26,000 different naturally presented HLA class I ligands representing 9,150 source proteins on LPC_{enr} fractions and autologous blasts (n = 11). Furthermore, we were able to identify more than 8,000 different HLA class II ligands from 1,700 source proteins. To specify LPC-associated antigens, we performed comparative profiling of the HLA ligandomes of LPCs, AML blasts (n = 48) and a database comprising 230 ligandomes of various normal tissues (***e.g.*** blood, bone marrow, spleen, liver). We identified three groups of highly interesting LPC targets: 1. LPC-exclusive antigens as 6.4% (986/15,356) of the LPC-presented peptides were not found on non-LPC AML blasts or benign tissue. 2. LPC-associated antigens highly enriched on LPCs as identified by semi-quantitative comparison with autologous blasts. 3. Frequently presented AML-associated antigens expressed on both, AML blasts and LPCs, but not on benign tissues. The identified LPC-associated peptides are highly interesting targets for immunotherapeutic approaches in AML and will be further evaluated for their potential to elicit specific T-cell responses.

Keywords: HLA, mass spectrometry, AML progenitor cells

B040 / Mouse model for a proof of concept of preventive and therapeutic vaccines against microsatellite-unstable cancers

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Microsatellite-unstable (MSI) cancers occurring in the context of Lynch syndrome elicit pronounced tumor-specific immune responses. These immune responses are specifically directed against frameshift peptide (FSP) neoantigens, which result from mismatch repair (MMR) deficiency-induced insertion/deletion mutations in coding microsatellites (cMS). We have recently completed a clinical phase I/IIa trial that successfully demonstrated safety and immunogenicity of an FSP neoantigen-based vaccine in MSI colorectal cancer patients (Clinical trial number: NCT01461148). Previously, we also detected cMS frameshift mutations in MMR-deficient mouse intestinal tumors. To further develop a vaccine against MSI cancers in Lynch syndrome, we aimed to establish a preclinical mouse model. In order to identify potential mutational targets and derived FSP neoantigens in MMR-deficient mouse tumors, a systematic murine genome database search was performed. Subsequently, intestinal cancers obtained from Lynch syndrome mice (***Msh2$^{flox/flox}$ VpC$^{+/+}$***) were evaluated for mutations affecting these candidate microsatellites. Coding microsatellite mutation profiling of murine Lynch syndrome colorectal cancers revealed 13 candidate cMS showing a mutation frequency of 15% or higher. The cMS most frequently affected by frameshift mutations was located in the ***Nacad*** gene (75% of tested tumors). Ten FSP neoantigens were synthesized for the most frequently mutated cMS. Immunogenicity was evaluated after vaccination of C57BL/6 mice using IFN-gamma ELISpot to measure CD4/CD8 T cell responses and peptide ELISA to measure humoral immune responses. Four FSP neoantigens derived from

cMS mutations in the genes ***Nacad, Maz, Xirp1,*** and ***Senp6*** elicited strong antigen-specific cellular and humoral immune responses. Based on the cMS mutation data, a vaccine with these four FSP neoantigens is predicted to cover about 75% of cancers in Lynch mice. In summary, we have identified 4 immunogenic FSP neoantigens derived from commonly mutated cMS in murine Lynch syndrome colorectal cancers. These results provide the basis for evaluating the concept of cancer-preventive FSP vaccines in a mouse model of Lynch syndrome. This model allows longitudinal monitoring of immune responses and tumor development in different vaccination schemes, adjuvants and combination with chemoprevention.

Keywords: cancer vaccine, neoantigens, Lynch syndrome

B042 / iVacALL: A personalized peptide-vaccination design platform for pediatric acute lymphoblastic leukemia patients based on patient-individual tumor-specific variants

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We established a platform for the design of patient-individual peptide vaccination cocktails by combination of whole exome sequencing of tumor and normal tissue with ***in silico*** epitope prediction algorithms for individual patient HLA types.
Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. Standard chemotherapy is a successful treatment in 80% of patients, only about 20% develop a relapse, however these patients have a dismal prognosis. Prevention of relapse after first-line chemotherapy or stem cell transplantation (SCT) is therefore mandatory. Accumulation of somatic mutations is one characteristic feature of malignant cells. These single nucleotide variants (SNVs) can lead to altered amino acid sequences of the translated proteins, which in turn can be presented by malignant cells as antigenic peptides on HLA molecules. A peptide vaccination to induce a neoantigen-specific T cell response is therefore a promising approach to prevent relapse in high-risk patients. For this purpose we detect nonsynonymous mutations by whole exome and transcriptome sequencing of patient leukemic blasts and healthy reference tissue. HLA binding peptides harboring the altered amino acids are subsequently predicted ***in silico*** by algorithms SYFPEITHI, NetMHC and NetMHCpan for the patients' individual HLA type.
Whole exome sequencing was performed for 25 patients to identify ALL-specific SNVs using a comparative bioinformatics pipeline. We found an average of 39.2 mutations per patient on DNA level, with an average validation rate of 47% by RNA sequencing.
We applied the platform for 5 patients based on compassionate need and designed individual peptide vaccines. In all cases validated mutations could be identified and epitope prediction was performed for MHC I & II binders. The predicted peptides were synthesized and vaccination cocktails were formulated. The vaccination schedule provides 16 vaccinations over 33 weeks using GM-CSF and Imiquimod as adjuvant. Response to the vaccination was monitored by detection of T cells recognizing the vaccinated peptides occurring over time in peripheral blood of the patients. Monitoring was performed for each vaccination time point by prestimulation with the peptides and subsequent intracellular cytokine staining (ICS) of T cells and FACS analysis. In 4/5 patients a ***de novo*** induced CD4+ T cell response against the vaccinated mutated MHC II binding peptides was detectable.
Whole exome sequencing of pediatric ALL patients is feasible and yields a small amount of mutations per patients. However, these few mutations are sufficient to predict HLA-binding peptides that are immunogenic when vaccinated and elicit specific T cell responses in patients. The concept is now translated to clinical application in a phase I/II clinical trial, started in 2016.

Keywords: Peptide vaccination, Neoepitopes, Immune monitoring

B043 / Peripheral tolerance restricts immune responses against melanoma-associated self-antigens

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Immune tolerance limits the efficacy of tumor vaccination against self-antigens. To elucidate the mechanism underlying peripheral tolerance in melanoma, we used ***Dct***-deficient mice lacking the tyrosinase-related protein-2 (TRP-2), which is expressed in melanocytes but not in the thymus. To assess whether peripheral tolerance against this self-antigen can be overcome with a potent vaccine strategy, we used a recently developed viral vaccine vector expressing TRP-2. After immunization ***Dct***$^{-/-}$ mice showed

significantly increased frequencies and improved functionality of TRP-2-specific CD8$^+$ T cells compared to ***Dct***-proficient mice. In addition, therapeutic immunization of B16F10-tumor-bearing ***Dct***-deficient mice resulted in complete eradication of the tumors and long-term survival whereas wild-type mice only showed a delay in tumor growth. Therefore, our data suggest that peripheral tolerance is responsible for failures of vaccination therapy against melanoma-associated self-antigens.

Keywords: melanoma, peripheral tolerance, vaccination

B044 / Therapeutic DNA vaccination against colorectal cancer by targeting the Myb oncoprotein

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Colorectal cancer is the third most common and the third leading cause of cancer-related death worldwide. Historically, disease management has been mainly based on TNM staging. In 2009 Galon et al. introduced the Immunoscore to predict clinical outcome. Infiltrating CD8$^+$ cytotoxic T lymphocytes form the main component of the Immunoscore, predicting tumor recurrence and patient survival superior to TNM staging. Furthermore, patients with a low density of CD8$^+$ T cell infiltrate typically do not benefit from immune checkpoint inhibitors, as this treatment requires the existence of tumor-specific T cells. These patients are the main target group for colorectal cancer vaccines.
Vaccine antigens are optimally expressed and presented by the cancer and not by normal cells. However, the low immunogenicity of tumors of patients with low Immunoscore presents a big challenge. Therefore, we decided to trial a very unconventional route by designing a vaccine that breaks tolerance to the self-antigen Myb. Myb is a nuclear oncoprotein overexpressed in 80% of colorectal cancers, but also expressed in stem cells of the bone marrow and gastrointestinal tract. We decided to generate a DNA vaccine, which uses the FDA-approved pVAX1 vector backbone and inserted a fusion construct consisting of the universal tetanus toxin T cell epitopes P2 and P30 as well as mutated Myb. The P2-Myb-P30 DNA vaccine did not show any induction of autoimmunity in a mouse model, as analyzed by bone marrow stem cell transplantation, haematocrit, platelet, RBC and white blood cell analysis, as well as histological analysis of gastrointestinal crypts. Interestingly, however, an anti-tumor immune response dependent on CD8$^+$ and CD4$^+$ T cells was observed in the MC38 and CT26 animal models of colorectal cancer. Therapeutic vaccination commencing two days after tumor transplantation significantly increased survival, while therapeutic vaccination starting 5 days post tumor transplantation did not show any benefit. We postulated that the larger tumor burden on day 5 induced immunosuppression. By combining our P2-Myb-P30 DNA vaccine with the immune checkpoint inhibitor anti-PD1, we were able to restore vaccine function. Moreover, we were able to increase complete response rate from 33 to 50% and survival of non-complete responders from 24 to 33 days by changing the delivery from intramuscular to intradermal injection and by supplementing our vaccine with 5% DMSO to enhance vaccine uptake. Vaccinated mice surviving the first MC38 challenge were resistant to further challenges without the need of treatment, indicating the establishment of anti-tumor memory. These findings have led to a funded phase I/II clinical trial.

Keywords: Cancer vaccine, colorectal cancer, immune checkpoint inhibitor

B045 / Developing a novel virus-like particle based delivery system of long peptides for immunotherapeutic treatment of colorectal cancer

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Colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women, representing a significant health burden worldwide. Metastatic CRC has a poor prognosis with limited treatment options which highlights the need for more effective therapies to treat this disease. We have developed an immunotherapeutic cancer vaccine from virus-like particles (VLPs) derived from ***Rabbit hemorrhagic disease virus*** (RHDV) which acts as a highly immunogenic scaffold to deliver tumor-associated antigens (TAAs) to antigen presenting cells (APCs) of the immune system. Previous work has shown that delivering minimal MHC-I binding epitopes derived from TAAs (8-9 amino acids) incorporated into the VLP vector can elicit a potent, long-lasting anti-tumour response and trigger the formation of immunological memory against the epitope to prevent tumour recurrence in an MC38 mouse model of CRC. In order to translate this work into a clinically relevant context, this study has involved redesigning the RHDV VLP vector to allow for the incorporation of longer peptides. These long peptides contain several epitopes that can be presented on both MHC-I and MHC-II across multiple HLA haplotypes. Prediction tools were used to identify potentially immunogenic peptide sequences derived from survivin, mucin-1 and carcinoembryonic antigen. These peptides were validated as suitable vaccine candidates by screening them against cells isolated from patients with colorectal

cancer and analyzing the recall response induced in T-cells. The RHDV VLP capsid protein was truncated at two sites to facilitate the insertion of longer peptides without affecting particle formation. These truncated forms of the vector have been successfully synthesized in a baculovirus expression system and shown to be as immunogenic as the full-length form in mouse models. These results indicate that the RHDV VLP scaffold can be modified for use as a vaccine vector in a more clinically relevant setting. Future work will focus on evaluating the use of this vaccine to treat primary vs. secondary tumors, further enhancing the anti-tumor immune response by administering the vaccine in conjunction with other therapies such as chemotherapy and immune checkpoint inhibitors and determining a clinically relevant dosage schedule.

Keywords: Virus-like particle, therapeutic vaccination, colorectal cancer

B046 / Antigen spreading by targeted killing of tumor cells via chimeric antigen receptors

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Antigen presenting cells (APCs), especially macrophages, play an important role in the growth or regression of tumors. M2-type macrophages reside mainly in human cancers and produce tumor growth promoting factors. Tumors can be recognized by tumor specific T-cells and rejected by “cancer vaccines”, however tumor specific antigens recognized by the immune system in most human cancers are rare and difficult to identify. Thus, modulating APCs to specifically target and kill tumor cells as well as to stimulate T-cells by tumor specific antigen presentation might be a feasible alternative to boost an anticancer immune defense. We used the Orf-virus (ORFV) of the family of Poxviridae to establish a highly attenuated and apathogenic viral vector vaccine platform that allows the fast development of new recombinants and further the simultaneous expression of several target antigens in different expression levels as a promising candidate for virus-based vaccines. ORFV is taken up by APCs such as macrophages and dendritic cells and causes a massive stimulation of the cellular and humoral immune system, which provokes a long lasting and balanced immune response. Since no neutralizing antibodies against the virus could be detected, our platform technology allows repeated boost immunizations or immunizations against different target antigens. In this proof of principle study, ten ORFV recombinants were generated to incorporate different chimeric antigen receptors (CARs) on macrophages and dendritic cells directed against CD19 as a B cell antigen. Antigen binding via the scFv part was hypothesized to induce a crosslinking of the CAR eventually eliciting phagocytosis of the recognized cell. Using this innovative approach, a single tumor associated antigen may elicit antigen spreading to subsequently induce multispecific antitumoral responses.

Keywords: Chimeric antigen receptor, Antigen spreading, Viral vector vaccine

B047 / Antibody-dependent cell cytotoxicity induced by active immunotherapy in non-small cell lung cancer patients vaccinated with racotumomab

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The antitumor strategies based on positive modulation of immune system actually represent therapeutic options with prominent acceptance for cancer patients’ treatment due to its selectivity and higher tolerance compared to chemotherapy. Racotumomab is an anti-idiotype monoclonal antibody directed to NGlycolyl (NGc) gangliosides such as NGcGM3. Racotumomab has been approved in Latin American countries as an active immunotherapy for advanced non-small cell lung cancer (NSCLC) treatment. The aim of this work is to evaluate if vaccination with racotumomab induces antibody-dependent cellular cytotoxicity (ADCC) in NSCLC patients included in a phase III clinical trial. Development of anti-NGcGM3 antibodies in serum samples of immunized patients (3 and 6 months after treatment beginning) was first evaluated by FACS using the NGcGM3-expresing X63 murine myeloma cells. The comparison was made between pre and hyperimmune serum samples for each individual patient. The percentage of X63 cells stained with patients´ hyperimmune serum samples was significantly higher in comparison with patients´ pre-immune sera ($p< 0.0001$ ANOVA). We then explored the induction of ADCC against X63 cells using a lactate dehydrogenase release assay. Serum samples from 36 vaccinated patients and peripheral blood mononuclear cells from healthy donors were incubated with target cells, and the specific lysis was measure. We considered a positive ADCC activity when the specific X63 lysis obtained with hyperimmune sera had a 2-fold increase with respect to that obtained with preimmune sera. The results indicated that anti-NGcGM3 Abs in hyperimmune serum samples from 22 out of 36 vaccinated patients elicited a specific cytotoxic activity against X63 cells (61%). Serum samples from no treated patients (control group) were also analyzed. In this set of patients, specific lysis values showed no significant differences between samples

collected at the beginning of the protocol and 3 or 6 months after starting the treatment (p>0.05, Unpaired t-Test). When target cells were devoid of NGcGM3 by a glucosylceramide synthase inhibitor (PDMP) treatment, we observed a significant reduction of the ADCC activity using hyperimmune sera, suggesting a target specific response. (p< 0.01 two-way ANOVA followed mean CI comparison test). Our data strongly suggest that anti-NGcGM3 antibodies induced by racotumomab vaccination are able to mediate an antigen specific cellular immune response in NSCLC patients. This is the first report of ADCC induction in response to racotumomab treatment, demonstrating a cell-mediated cytotoxicity related to NGcGM3 expression.

Keywords: Active immunotherapy, ADCC response, NGcGM3 ganglioside

B048 / Enhancement of innate and adaptive antitumor immunity by lymph node targeting with synthetic nanoparticle vaccine

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In this work, we designed synthetic vaccine nanoparticles (SVNPs) that effectively targeted lymph nodes, where immune responses against foreign antigens are primed, were developed to enhance anti-tumor immunity. For effective loading and delivery of model tumor antigen (Ovalbumin) and toll like receptor 3 agonist (poly (I:C)) to immune cells in lymph nodes, we selected poly(γ-glutamic acid) based SVNPs, which size is 20~70nm, modified with amine group. When antigen presenting cells are treated by Ovalbumin loaded SVNP (SVNP-OVA) and poly (I:C) loaded SVNP (SVNP-IC), they showed not only higher level of inflammatory cytokines (TNF-α, IL-6) and type 1 interferon (IFN-α, IFN-β) secretion, but higher uptake of OVA and poly (I:C) than those treated with OVA and poly (I:C) alone. In vivo experiment, the mice treated with SVNP-IC showed higher levels of activation markers, inflammatory cytokines, and type I IFNs in the lymph nodes compared to those of mice in other groups. Moreover, natural killer cell expansion/ activation ($NK1.1^+$ cells) and $CD8^+$ T cell response ($CD8^+$ $INF\text{-}\gamma^+$ cells) in innate and adaptive immunity was also increased, respectively. In cancer model, vaccination of EG7-OVA tumor-bearing mice with SVNP-OVA and SVNP-IC simultaneously showed better antitumor immunity and inhibited tumor growth in both preventive and therapeutic model.

Keywords: Lymph node, Vaccine, Adjuvant

B049 / Phase 1/2 study of in situ vaccination with tremelimumab + intravenous durvalumab + polyICLC in patients with select relapsed, advanced cancers with measurable, biopsy-accessible tumors

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Immunotherapy has demonstrated promising antitumor activity in various advanced cancers. Combined tumor targeting from multiple drugs with unique mechanisms may provide further improved outcomes. Tremelimumab (TRE) is a cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) antibody and durvalumab (DUR) blocks programmed death-ligand 1(PD-L1). Poly-ICLC is a toll-like receptor 3 agonist. Intratumoral (intra-T) injection of polyICLC directly alters the tumor microenvironment (TME) and, by creating an in situ vaccination, may trigger a clinically effective systemic anti-tumor response when also combined with DUR and TRE. This is an ongoing Phase 1/2, open-label, multicenter study (NCT02643303). The study is designed to evaluate the safety, preliminary efficacy and immune activity of intra-T administration of TRE + intravenous (IV) DUR + intra-T and intramuscular (IM) poly-ICLC in patients with the following advanced, measurable, biopsy-accessible tumors: non-viral-associated head and neck squamous cell carcinoma, locally recurrent breast cancer, sarcoma, Merkel cell carcinoma, cutaneous T-cell lymphoma, melanoma after failure of available therapies, genitourinary cancer with accessible metastases, and other solid tumors with masses accessible without imaging. Up to 36 patients are anticipated for enrollment in Phase 1, which determines the recommended combination dosing (RCD) for the regimen, with dose deescalation based on dose-limiting toxicities (DLTs) and standard 3 + 3 rules. Starting doses are: DUR, 1500 mg IV; TRE, 75 mg IV; TRE, 10 mg intra-T; poly-ICLC, 1 mg intraT and IM. Possible dose de-escalation levels are DUR, 750 mg IV; TRE, 22.5 mg IV; and TRE, 3 mg intra-T. Phase 1 starts with Cohort 1A (DUR + poly-ICLC). Upon demonstration of tolerability, enrollment proceeds with Cohort 1B (DUR + IV TRE + poly-ICLC) and Cohort 1C (DUR + intra-T TRE + poly-ICLC). The RCD is the highest dose at which < 2/6 patients have DLTs. In Phase 2, up to 66 evaluable patients are treated using the RCD regimen, with enrollment of 6 patients per tumor type initially, and enrollment of 6 additional patients per 3 tumor types contingent upon at least 1 response among the initial 6 patients. Study endpoints are RCD determination and evaluation of safety according to the National Cancer Institute Common Terminology Criteria for Adverse Events, objective response rate per the

Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 and the immune-related RECIST, progression-free survival, and overall survival. Exploratory endpoints are biological activity, including effects on the TME and immunological responses. Enrollment opened on 28 Dec 2016.

Keywords: Tremelimumab, durvalumab, Poly-ICLC

B050 / Landscape of immunogenic tumor antigens in metastatic cervical carcinoma: mutated neoantigens and cancer-germline antigen KK-LC-1 as new T-cell targets

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Cancer immunotherapies such as adoptive cell therapy with tumor-infiltrating lymphocytes (TILs) and immune checkpoint blockade have demonstrated clinical activity in advanced cervical carcinoma, as well as in other human papillomavirus (HPV)-induced epithelial cancers. However, the tumor antigens targeted by T cells in these virally-induced malignancies remain poorly defined. The viral proteins expressed by HPV$^+$ cancers are considered the primary targets of T-cell based immune attack. Still, HPV$^+$ cancers also harbor somatic mutations and express cancer-germline antigens that may be immunogenic. We recently elucidated the landscape of the viral and non-viral tumor antigens targeted by T cells in two patients with metastatic HPV$^+$ cervical cancer who experienced complete tumor regression after adoptive transfer of TILs. Examination of T-cell reactivity in these TILs revealed predominant tumor antigen-specific T-cell populations targeting either unique mutated neoantigens or the cancer-germline antigen Kita-kyushu lung cancer antigen 1 (KK-LC-1), in addition to subdominant populations of HPV-specific T cells. These findings established that non-viral tumor antigens can be prominent targets of T cells in immunotherapy of HPV$^+$ cervical cancers.
We next focused our attention to the cancer-germline antigen KK-LC-1, because of its desirable attributes of shared expression in a variety of non-viral epithelial cancers, and absent expression in normal non-germline tissues. Due to this favorable expression pattern, KK-LC-1 is considered an attractive target antigen for T-cell based immunotherapies. To investigate its usefulness as a T-cell target in HPV$^+$ cervical cancers, we assessed the expression and immunogenicity of KK-LC-1 in these malignancies. Expression of KK-LC-1 encoding gene ***CT83*** was assessed by quantitative real-time polymerase chain reaction. KK-LC-1 was found to be expressed in 40% (12/30) of metastatic HPV$^+$ cervical cancers. To assess immunogenicity of KK-LC-1, TILs grown from metastatic HPV$^+$ cervical cancers were tested for reactivity against autologous dendritic cells electroporated with ***in vitro*** transcribed RNA encoding full-length KK-LC-1. T-cell reactivity against KK-LC-1 was detected in 33% (3/9) KK-LC-1$^+$ cervical cancers tested. These results suggest that KK-LC-1 is frequently expressed by metastatic HPV$^+$ cervical cancers and naturally immunogenic. Further studies are underway to characterize the KK-LC-1 specific T-cell reactivities in these TILs, as well as to assess the global immunogenicity of the viral and non-viral tumor antigens in HPV$^+$ cancers. Overall, our findings provide new T-cell targets for personalized cancer vaccines, adoptive T-cell therapies and immune-monitoring studies of various cancer immunotherapies for HPV$^+$ cancers.

Keywords: HPV+ cancer, Tumor antigens, Immunotherapy targets

B051 / Preclinical development of Agenus' next generation cancer vaccine

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Agenus is developing a broad immunotherapy portfolio comprising peptide-based vaccines and immune checkpoint modulators (CPMs) which are being deployed in combination to combat cancer and infectious disease. Upon transformation, tumor cells accumulate somatic mutations and abnormal post-translational modifications (PTMs), giving rise to immunogenic neoepitopes recognizable as non-self by T-cells. Similarly, virally infected cells can present immunogenic viral epitopes that can be recognized and targeted by the adaptive immune system. Next-generation sequencing, proteomics and bioinformatics combined with a better understanding of the immune synapse enable prediction of HLA-restricted immunogenic tumor neoantigens and viral epitopes directly from genomic, transcriptomic or proteomic analysis of patient samples and pathogens. The Agenus Rapid Response Vaccine Platform (ARRVP™) relies on a proprietary ***in-silico*** pipeline, Agenus Immunogenic Mutation (AIM™), to predict immunogenic peptides containing neoantigens or pathogen-associated antigens for incorporation into personalized anti-tumor therapeutic vaccines, AutoSynVax™ (ASV™), or anti-microbial vaccines. Additionally, using proteomic analysis of the HLA ligandome in tumor samples, AIM allows the identification of tumor-specific PTMs which form the basis of our off-the-shelf vaccines including PhosphoSynVax™ (PSV™). To optimize vaccinogenic immune responses, we utilize synthetic long peptides comprising predicted immunogenic epitopes and a chaperone-targeting

sequence complexed to recombinant heat shock proteins (HSPs) and administered with QS-21 Stimulon® adjuvant. This format is intended to optimize vaccine priming by facilitating processing and presentation of immunogenic epitopes to T-cells, resulting in robust immune responses. We have demonstrated immunogenicity and efficacy of our vaccine platforms in prophylactic and therapeutic murine tumor models and immunogenicity in a single patient in a compassionate use setting. To improve duration and potency of the vaccinogenic response, Agenus' vaccines are being tested in combination with agonistic and antagonistic monoclonal antibodies targeting checkpoint pathways. Preclinical evidence of enhanced HSP-based vaccine efficacy when combined with CPMs has been observed in both ASV and antiviral vaccine formats. A first-in-human Phase 1 clinical study of ASV has fully enrolled and combination studies with CPMs are planned.

Keywords: Personal Vaccination, Neo-Antigens, CPM Vaccine Combination

B052 / Testing potency of induced dendritic cells and chimeric antigen receptor T cells targeted against HCMV-gB in humanized mice for protection against of glioblastoma multiforme spread

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Introduction: After surgical removal of glioblastoma multiforme (GBM), the recurrent tumor rapidly spreads in the central nervous system and survival is dismal. Multiple human cytomegalovirus (HCMV) proteins are found in GBM. Although the mechanisms of HCMV in the immunosuppression and tumor recurrence are not fully understood, the HCMV glycoprotein B (gB) has been implicated in tumor invasiveness ***in vitro***, which can be blocked by neutralizing anti-gB antibodies. Immunologic recognition and killing of GBM expressing HCMV proteins by B and T cells opens the perspective of exploring these antigens for cancer immunotherapy. Recent phase I clinical trials have explored the HCMV pp65 antigen used to load dendritic cell vaccines in the form of peptides or RNA as an adjuvant therapy for glioma patients. Increased progression-free survival and overall survival were observed in the immunized patients compared with historical controls, which was also correlated with an increased T cell response against pp65 epitopes.
Experimental procedure: We hypothesize that a potent immunomodulatory vaccine blocking GBM spread through gB targeting could slow down or block tumor invasion. Thus, we propose to explore the expression of membrane-anchored HCMV-gB in dendritic cells to generate combined humoral and T cell responses.
Results: After co-transduction of human monocytes with lentiviral vectors expressing GM-CSF/IFN-a and HCMV-gB overnight, we demonstrated the self-differentiation of "induced dendritic cells" (iDCs) ***in vitro*** expressing the gB protein on the cell surface. We tested the effects of immunization/boost of cryopreserved/thawed iDCgB in Nod-Rag mice transplanted with human hematopoietic cells developing human T and B cells ("humanized mice"). After repeated s.c. administration of iDCgB into humanized mice, blood and lymphatic tissues (spleen, lymph nodes and bone marrow) were collected to analyze the immune effects. Immunization resulted into an increase in the absolute numbers of human effector memory $CD4^+$ and $CD8^+$ T cells ($CD45RA^-$/ $CD62L^-$) in spleen and peripheral lymph nodes. Immunizations also produced significant increases in the numbers of human memory and plasma B cells (IgM, IgA, and IgG) in spleen and elevated levels of human and IgG (IgG2 and IgG3) in serum. We have also generated T cells expressing chimeric antigens (CARs) targeted against gB, which are currently being tested ***in vivo.*** We have developed a HCMV (TB40-EI1-Gaussia Luciferase) challenge model in humanized mice demonstrating systemic viral infection in lymphatic tissues (by optical imaging and RT-q-PCR).
Conclusion: Ectopic and orthotopic PDX models of GBM combined with HCMV infection remains to be established to test the ***in vivo*** effects of iDCgB immunization and gB-CAR-T gainst gB^+GBM.

Keywords: HCMV, Dendritic cells, Glioblastoma

B053 / Defining superior migratory dendritic cells for cancer vaccination

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Migratory dendritic cells (migDCs) are pivotal antigen-presenting cells that are essential to transport antigens to draining lymph nodes (dLN) and regulate immune responses. Although, migDCs were effectively used to induce immune responses against cancers, it is still unclear which migDC subset to target in vivo or how in vitro generated DCs should be stimulated to increase the efficacy and clinical outcome. Recently, we discovered that the neuronal plasticity molecule activity-regulated cytoskeleton associated protein/activity-regulated gene 3.1 (Arc/Arg3.1) was exclusively expressed by migDCs within the immune system and drives fast inflammatory DC migration. However, it is unknown to what extend migDCs express Arc/Arg3.1 and how Arc/Arg3.1 expression is regulated within migDCs and their in vitro generated counterpart of granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated bone marrow-derived DCs (BMDCs). Here we show that Arc/Arg3.1 is only partly expressed by migDCs and BMDCs using transgenic ***Arc/Arg3.1***$^{eGFP/+}$ mice, mRNA expression analysis and Arc/Arg3.1-antibody staining. Further, Arc/Arg3.1 expression was

independent of ontogeny and transcription factors by analyzing naïve, irradiated and bone marrow transplanted transgenic ***Arc/Arg3.1***$^{eGFP/+}$ and naïve ***Irf4***$^{-/-}$ and ***Batf3***$^{-/-}$ mice in vivo, whereas Arc/Arg3.1^{+} BMDC depended on Irf4 and GM-CSF. Moreover, Arc/Arg3.1^{+} migDCs from all subsets showed a pivotal function in inflammatory antigen transport from skin to dLN and immune response to intradermal challenge with Staphylococcus aureus was diminished in ***Arc/Arg3.1***$^{-/-}$ mice. To uncover the mechanism of Arc/Arg3.1 controlled migration and to reveal surface proteins specifically expressed by Arc/Arg3.1^{+} migDCs, we sorted Arc/Arg3.1-eGFP^{+} and Arc/Arg3.1-eGFP^{-} migDCs and sequenced their mRNA. Biological processes associated with cytoskeletal shape were highly enriched in Arc/Arg3.1-eGFP^{+} migDCs, matching their pivotal migratory capacity. We found surface protein N-cadherin (Cdh2) enriched by Arc/Arg3.1-eGFP^{+} sorted migDCs that was also expressed on a comparable subset within GM-CSF derived BMDC. Together, Arc/Arg3.1^{+} migDCs and BMDC presented with superior migratory capacity independent of ontogeny and phenotype. Since Arc/Arg3.1 expression was highly specific to GM-CSF-derived BMDC that are usually used in vaccination experiments, we further expect an important role of Arc/Arg3.1^{+} migDCs and BMDCs in cancer vaccination. Thereby, Cdh2 might serve to target Arc/Arg3.1^{+} migDCs in vivo or enrich Arc/Arg3.1^{+} BMDC for vaccination in vitro.

Keywords: Migratory dendritic cells, Cancer vaccination, Superior migration

B054 / Identification of a novel human T cell population with the characteristics of stem-like chemo-resistance

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Understanding of the long-lasting immunological memory is important for the protection of pathogens and cancer in human. Generally, CD8+ T cells are divided into four subsets: naive (T_N), central memory (T_{CM}), effector memory (T_{EM}) and effector (T_{EFF}) T cells. Among memory T cells, T_{CM} cells have a longer lifespan and can differentiate into T_{EM} cells after antigenic challenge, whereas T_{EM} cells can immediately differentiate into T_{EFF} with the effector function after the second challenge with antigens. We found a novel human memory CD8+ T cells with the high activity of the aldehyde dehydrogenase 1 (ALDH^{high}). ALDH^{high} cells showed the ability of higher proliferation and chemo-resistance. Using gene profiling between ALDH^{high} and ALDH^{low} cells, CD73 was identified as the marker of ALDH^{high} cells and was highly expressed in CD45RA+CD62L+ population. Because CD73+ cells in CD45RA+CD62L+ contained both naive and memory cells, we divided CD73+CD45RA+CD62L fraction into CXCR3+ and CXCR3- and found that CD73+CXCR3+CD45RA+CD62L+ fraction is the novel memory T cells, "Young Memory T cells (T_{YM})", with the characteristics of higher proliferation, chemo-resistance and memory function directed at viral and tumor-associated antigens. Moreover, T_{YM} barely expressed the other memory markers including CD95. These suggested that T_{YM} might locate at closer to naive cells than stem cell memory T cell (T_{SCM}) defined by CD95. The regulation of T_{YM} is attractive for peptide-based immunotherapy and adoptive cell transfer therapy.

Keywords: memory T stem cell, peptide vaccination, ALDH

B055 / Identification of mutated neo-epitopes in the viral oncogene-driven tumor model TC1

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Mutated neo-epitope specific T cells are crucial effectors in cancer immunity. It has been repetitively shown that they significantly contribute to the clinical success of immunotherapies such as checkpoint blockade and adoptive cell therapy of tumor-infiltrating lymphocytes. Especially in mutation-rich tumor types the antigenome seems to be predominantly comprised of mutated neo-epitopes. However, even in virally induced cancer, as recently reported, mutated neo-antigens can constitute the targets of immunodominant T-cell reactivities. In this regard, we set out to analyze the mutanome of the widely used viral oncogene-driven mouse tumor model TC1.

Applying a set of ***in-silico*** prioritization steps on non-synonymous single nucleotide variations (nsSNVs) detected via exome and RNA sequencing followed by RNA-lipoplex vaccination we were able to identify multiple cancer-specific neo-antigens. From 78 expressed nsSNVs, 25 were selected for immunogenicity testing based on coinciding favorable MHC binding determined via two prediction algorithms. RNA lipoplex vaccination of mice against selected candidate neo-antigens induced strong T-cell responses against five MHC class I- and two MHC class II-restricted neo-epitopes. Importantly, induced CD8 T-cell responses were functionally relevant as they

specifically produced IFNγ upon recognition of TC1 tumor cells. These results confirm the presence of neo-epitopes in virally induced cancers and allow further analysis of the interplay between virus and mutation specific T cells in a common murine tumor model.

Keywords: Neoantigens, RNA vaccine, TC1

B056 / Responsive poly-antigen/adjuvant cancer nanovaccine for enhanced lymph node targeting and cross-presentation

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Cancer immunotherapy has made unprecedented progress in the last few decades. In particular, checkpoint blockade and adoptive T cell therapy have shown remarkable clinical results. However, the clinical efficacy of therapeutic cancer vaccine remains modest. In many phase I/II studies, cancer vaccines have shown clinical benefit, in particular extended overall survival, while objective durable regressions were rarely seen. Suboptimal vaccine design resulting in general weak and short-lived antigen-specific T cell responses is one of the root causes of the lack of cancer eradication in therapeutic vaccine treatment. The major challenges are the inefficient delivery of antigen/adjuvant to secondary lymphoid organs, where immune responses are initiated and orchestrated, as well as the lack of effective cross-presentation for cytotoxic T cell response. Here, we developed a novel responsive poly-antigen/adjuvant (PAA) nanovaccine to address these challenges and enhance cancer vaccines. The responsive PAA nanovaccines were prepared through reversible chemical crosslinking of antigen and adjuvant components together. Sizes of the nanovaccines were controlled by varying the concentration and linker-to-protein ratio, and optimized for lymph node (LN) targeting and persistence in mice. We found the PAA nanovaccine with size ≤100 nm showed highest LN targeting efficiency compared to larger nanovaccines. The PAA nanovaccine efficiently activated the bone marrow-derived dendritic cells (BMDCs) harvested from C57Bl/6 mice in vitro. Confocal microscopic studies revealed the intracellular co-delivery of antigen and adjuvants to DCs. The reversible chemical crosslinkers were cleavable in response to intracellular low redox potential, or acidic pH in endosomes, facilitating the endosomal escape and cytosol delivery of released antigens. The BMDCs treated with the responsive PAA nanovaccines cross-primed the CFSE-labeled OT-I CD8+ T cells in vitro and in vivo more effectively than the mixture of soluble antigen and adjuvant. Immunization of C57Bl/6 mice subcutaneously at tail base with PAA nanovaccines also elicited robust antigen-specific CD8+ T cells response. The responsive PAA nanovaccine provided a promising platform for the therapeutic cancer vaccine development with enhanced efficacy.

Keywords: cancer nanovaccine, lymph node targeting, cross-presentation

B057 / A live attenuated *Salmonella* Typhimurium oral T cell vaccine against PD-L1 protects 100% of animals from a leukemia challenge

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Immunotherapy is a revolution in cancer therapy, and this is being largely driven by advances in immuno-oncology, and the recent approval of novel treatment modalities like immune checkpoint inhibitors in various cancer indications. However, further solutions are necessary to overcome the peripheral tolerance and the immunosuppressive tumor microenvironment that prevent the eradication of cancer in most of patients. VAXIMM is developing a unique oral T-cell vaccination platform based on the FDA-approved live-attenuated ***Salmonella*** Typhi strain Ty21a vaccine Vivotif®. This study summarizes the immunogenicity and anti-cancer efficacy of ***Salmonella*** Typhimurium based vaccines VXM10m and VXM10ma, transformed with eukaryotic expression plasmids encoding the full-length murine programmed death-ligand 1 (PD-L1) protein and a truncated form of PD-L1, respectively.

The anti-tumor efficacy of VXM10m and VXM10ma was evaluated in the FBL-3 disseminated model of erythroleukemia. Multiple oral administrations of VXM10m and VXM10ma used at 10^{10} colony-forming units (CFU) were generally well tolerated, and neither sign of toxicity nor body weight loss were observed throughout the study. Moreover, oral administration of VXM10m and VXM10ma produced a strong anti-tumor effect in the FBL-3 leukemia model, with 100% of surviving animals 80 days after leukemia challenge in the highest dose groups. In contrast, administration of the empty vector control did not show any anti-cancer effect, with a median survival of 21 days and 0% of tumor regression. Importantly, 100% of long-term surviving mice resisted re-challenge with FBL-3 cells, demonstrating that vaccination with VXM10m and VXM10ma generated a potent memory T cell response against the leukemia. Immunogenicity and antibody response towards PD-L1 are being evaluated to dissect the exact mechanism of action of these novel vaccines.

Prophylactic vaccination with VXM10m and VXM10ma induced a strong and sustained anti-cancer activity in the FBL-3 model of leukemia. This study provides further evidence that VAXIMM's oral T-cell vaccination platform can be used to stimulate anti-tumor immunity, not only against proper tumor-associated antigens but also against PD-L1-expressing cells.

Keywords: PD-L1, FBL-3 leukemia, Salmonella oral T cell vaccine

B058 / Non-clinical safety, immunogenicity and antitumor efficacy of VXM06m, a live attenuated *Salmonella* Typhimurium oral T cell vaccine against WT-1

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Significant progresses have been achieved recently in cancer vaccines that aim at engaging or reengaging the tumor immunity. Yet novel immunization solutions to deliver efficiently tumor-associated antigens to professional antigen-presenting cells, and to overcome the peripheral tolerance and the immunosuppressive tumor microenvironment, are urgently needed. VAXIMM is developing a unique oral T-cell vaccination platform based on the FDA-approved live-attenuated ***Salmonella*** Typhi strain Ty21a vaccine Vivotif®. This study summarizes the preclinical toxicity, immunogenicity and anti-cancer efficacy of the ***Salmonella*** Typhimurium based vaccine VXM06m transformed with a eukaryotic expression plasmid encoding a murine Wilm´s tumor 1 (WT1) protein variant lacking the zinc fingers.
The non-clinical safety profile was assessed in C57BL/6J mice after repeated administrations by gavage with doses up to 10^8 colony-forming units (CFU) in a 13-week GLP-compliant toxicity study. The immunokinetic study was performed in healthy C57BL/6J mice treated with doses up to 10^{10} CFU, and antigen-specific T cells were detected in the spleen by flow cytometry using MHC class I pentamers. The anti-cancer activity was evaluated in the FBL-3 disseminated model of erythroleukemia expressing WT1. Multiple administrations of VXM06m were generally well tolerated, and extra-intestinal manifestations were restricted to the liver and the kidney, which were attributed to the bacterial vector. Moreover, vaccination with VXM06m induced a systemic antigen-specific immune response peaking 10 days after the last dose. Finally, oral administration of VXM06m generated a strong anti-tumor effect in the FBL-3 leukemia model, with 100% of surviving animals 80 days after leukemia challenge. In contrast, administration of the empty vector control did not show any anti-cancer effect. Importantly, 100% of surviving mice resisted re-challenge with FBL-3 cells, demonstrating that vaccination with VXM06m generated a potent memory T cell response against the leukemia.
VMX06m was well-tolerated, and generated substantial immunogenicity in healthy animals. Prophylactic vaccination with VXM06m induced a sustained anti-cancer activity in the FBL-3 model of leukemia. This study provides further evidence that VAXIMM's oral T-cell vaccination platform can be used to stimulate anti-tumor immunity against various tumor-associated antigens. Further studies of VAXIMM´s cancer vaccine candidates, as monotherapy and in combination with immune checkpoint blocking modalities, are warranted.

Keywords: WT1 Wilm´s tumor 1, FBL-3 leukemia, Salmonella oral T cell vaccine

B059 / Activation of endogenous anergic self-specific CD8$^+$ T cells by polymeric nanoparticles for enhanced cancer immunotherapy

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While the human immune system is often able to protect the body from infectious pathogens, it has multiple mechanisms to inhibit mounting an immune system against what it perceives as "self" and thus often fails to eliminate cancer cells since they seem to have the characteristics of "self". Recently, our lab found that self-peptide MHC-specific CD8$^+$ T cells in the blood of healthy humans were present in frequencies similar to those specific for non-self antigens, but these cells are resistant to activation and/or expansion, except with a very strong stimulus (anti-CD3 plus anti-CD28 antibodies). We hypothesized that tumor infiltrating T cells are often in an anergized state but that they could be activated with the appropriate innate immunity signals in addition to antigen-MHC exposure. To test this idea, we have screened a series of combinations of immunological stimulants. In order to activate these cells, however, a large dose of stimulating agents needs to be injected into the body. Those drugs often have life-threatening side effects. For example, one class of these drugs that has been tested in clinical trials is interleukins-naturally occurring chemicals that help promote T cell growth but have severe side effects, including heart and lung failure, when given in large doses. To address this issue, we have developed a poly(lactide-***co***-glycolide)

nanoparticle (PLGA NP) based immunostimulatory platform which is functionalized with monoclonal anti-CD28 antibody (Ab), and can sustainably release interleukin-2 (IL-2) and toll-like receptor-2 (TLR2) agonist, providing sustained immune stimulation in the tumor microenvironment to continuously activate anergic self-specific $CD8^+$ T cells. Compared to systemic administration of stimuli, the favorable release kinetics provided by PLGA NPs also allows for minimal systemic toxicity often associated with systemic immunostimulation. The result demonstrated that the mice bearing melanoma treated with the developed NPs have elicited potent immune response with markedly increased self-specific effector $CD8^+$ T cells in the tumor tissue, resulting in significantly delayed tumor growth. In addition, the developed NPs can also be combined with the current immunotherapeutic strategies such as checkpoint blocking antibodies to achieve the synergistic effect, significantly prolonged the overall mice survival. We also applied the single cell sequencing approach we recently developed to assess the TCR sequences and phenotypic characteristics of $CD8^+$ T cells harvested from tumor after NP stimulation. The results we obtained are crucial for identifying potentially important melanoma-associated TCRs and subsequent discovering their antigens.

Keywords: anergic self-specific CD8+ T cells, Single T cell sequencing, combination therapy

B060 / The leukaemia stem cell surfaceome in LMO2-dependent T-ALL reveals co-expression of GPR56 and CD25 as dual immuno-oncology targets

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The treatment of cancer can fail to eliminate the cancer stem cells or leukaemia stem cells (LSCs) in haematopoietic cancer. These cancer stem cells may reside as residual disease and propagate relapsing disease due to specific mechanisms of resistance against conventional therapies such as chemotherapy. Antibody-based therapeutics have increasing impact following a series of clinical applications of anti-cancer antibodies. Effective targeted therapy should eliminate LSCs while sparing normal counterparts to reduce side effects. Therefore, identification of specific and accessible antigens on the cell surface of LSCs is a prerequisite for successful antibody-based drug applications. The analysis of whole cellular transcriptomes by next-generation deep RNA sequencing (RNA-seq) provides a new method for novel therapeutic target discovery. Furthermore, a surface protein (surfaceome) database can be used to identify the candidates that are accessible to antibody binding. We have used a transgenic mouse model of LMO2-dependent T-cell acute leukaemia (T-ALL), in which the LSCs are presented as immature thymocytes, to identify cell surface proteins using RNA-seq and surfaceome database mining. By comparing the surfaceomes of transgenic LSCs with age-matched wild type counterpart thymocytes, we have identified several markers on the LSCs. These markers are potential therapeutic targets for LSCs and these candidates were evaluated on primary human T-ALL samples. In particular, GPR56 was found co-exist with CD25 on LSCs and these two cell surface markers together showed their potential as specific dual-target for treating leukaemia and LSCs. GPR56, a G-protein coupled receptor, internalizes when bound by bivalent antibody whereas CD25 does not internalize. Implementation of a tripartite green fluorescent protein complementation assay demonstrated that GPR56 could co-localise with CD25 at the cell surface and facilitate the internalization of CD25. Therefore GPR56 and CD25 represent combined targets on LSCs for immuno-oncology therapeutic approaches such as antibody-drug conjugates (ADCs) and antibody-dependent cell-mediated cytotoxicity (ADCC). In conclusion, we have identified surface markers on LMO2-dependent T-ALL leukaemia stem cells (LSCs). Two of these markers, GPR56 and CD25, can co-localise and internalize, providing a dual target specific to LSCs that should significantly reduce, or eliminate, side effects due to ADCs damaging normal cells.

Keywords: Leukaemia stem cells, chromosomal translocations, Antibody-drug conjugates (ADCs)

COMBINATION THERAPIES

B061 / Combination of oncolytic virotherapy and DC-based immunotherapy for the treatment of melanoma

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VSV-GP, a novel chimeric vesicular stomatitis virus (VSV) pseudotyped with the glycoprotein of the lymphocytic choriomeningitis virus represents a promising oncolytic virus (OV) that preferentially targets and kills cancer cells. Release of tumor antigens and activation of immune response by OV therapy might support dendritic cell (DC)-mediated anti-tumor immunity. Thus in our study we analyzed the efficacy and immune mechanisms of the combination of VSV-GP oncolytic virotherapy with DC-based immunotherapy. Combination of VSV-GP therapy and DC-based vaccination was investigated in the syngeneic subcutaneous B16-OVA melanoma model. SIINFEKL-loaded CpG-activated DCs (DCVacc) and VSV-GP were applied intra- and peritumorally and immune responses were analyzed in the spleen and tumor tissue. The DCVacc/VSV-GP combination therapy resulted in a significantly improved survival compared to single treatments. Surviving mice from the DCVacc/VSVGP treated group showed a long lasting anti-tumor immunity against B16-OVA and partial anti-tumor immunity against non-OVA B16 melanoma in rechallenge experiments. Analyzing specific cytotoxic T lymphocyte (CTL) responses induced by DCVacc and VSV-GP single and combination treatments we found that both DCVacc and DCVacc/VSV-GP induced comparable levels of OVA-specific CD8 T cell responses. In addition, a strong VSV N peptide-specific CD8 T cell response was detected upon VSV-GP and DCVacc/VSV-GP treatments. The improved therapeutic effect by the DCVacc/VSV-GP combination treatment correlated with increased numbers of tumor infiltrating lymphocytes (TIL) and elevated Tconv/Treg and CD8/Treg ratios seen also in non-treated collateral tumors. Furthermore, depletion of CD8 T cells but not NK cells abrogated the therapeutic effect of DCVacc/VSV-GP. Taken together, the combination of VSV-GP and DC-based immunotherapy might represent a promising therapeutic option for the treatment of melanoma.

Keywords: oncolytic virus, dendritic cell, melanoma

B062 / Systematic screening of small molecule inhibitors targeting oncogenic signaling pathways (MAPK, PI3K/AKT) for combinatorial immunotherapy with focus on agonist anti-CD40 antibody

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Combinations of immunostimulatory compounds and cytostatic agents are currently being extensively tested against different tumor types. Clearly synergistic anti-tumor responses are rare, however. Furthermore the impact of the compounds on the immune cells itself is insufficiently understood, as is the risk of unanticipated side effects of combination regimens. Therefore, our aim is to study combinatorial immunotherapeutic approaches in a systematic fashion by making use of robust in vitro and in vivo immune cell assays.
In order to select the most effective and immune response potentiating compounds, we are screening cytotoxic/cytostatic compounds, such as chemotherapeutic agents and small molecule inhibitors (SMi) against MAPK or PI3K/AKT signaling for their efficacy against human and murine tumors cell lines and their effects on T cell proliferation and effector functions.
In syngeneic tumor models combination of selected MEK inhibitors with an agonist CD40 antibody showed T-cell dependent anti-tumor effects. For one MEK inhibitor mechanistic studies revealed clear synergy with respect to blocking tumor growth even though this particular MEK inhibitor negatively affected T-cell priming/expansion and suppressed the expression of immune related genes in the tumor microenvironment (TME). Addition of agonist CD40 antibody rescued the immune suppressive properties of this MEK inhibitor resulting in a positive net anti-tumor effect also manifested by reduced expression of tumor growth related genes and induction of transcripts associated with cellular stress and autophagy. Preliminary experiments with other MEK inhibitors revealed that not all SMi have negative effects on immune cell function and synergize with agonist anti-CD40. Further characterization SMi with respect to their impact on all stages anti-tumor T-cell responses und effect on the TME will help to select the most suitable compounds for combinatorial immunotherapy. In more advanced models, such as the autochthonous BRaf-driven model for metastatic melanoma, the combinatorial effect of MEK inhibition and agonist CD40 was much less prominent, presumably the lack of mutated neoantigens. In order to optimize our models with respect to prediction of clinical outcome, we are developing electroporation-based autochthonous tumor models, which are equipped with immunogenic foreign antigens.

Keywords: CD40, Small molecule inhibitors, Mouse tumor models

B063 / Depletion of tumor-associated macrophages switches the epigenetic profile of pancreatic cancer infiltrating T cells and restores their anti-tumor phenotype

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Pancreatic Ductal Adenocarcinoma (PDA) is characterized by a complex tumor microenvironment that supports its progression, aggressiveness and resistance to therapies. The delicate interplay between cancer and immune cells creates the conditions for PDA development, particularly due to the functional suppression of T cell anti-tumor effector activity. However, some of the mechanisms involved in this process are still poorly understood. In this study, we analyze whether the functional and epigenetic profile of T cells that infiltrate PDA is modulated by the microenvironment, and in particular by tumor-associated macrophages (TAMs). CD4 and CD8 T cells obtained from mice orthotopically injected with syngeneic PDA cells, and untreated or treated with Trabectedin, a cytotoxic drug that specifically targets TAMs, were sorted and analyzed by flow cytometry and characterized for their epigenetic profile. Assessment of cytokine production and the epigenetic profile of genes coding for IL10, T-bet and PD1 revealed that T cells that infiltrated PDA displayed activated Il10 promoter and repressed T-bet activity, in agreement with their regulatory phenotype ($IL10^{high}$/$IFN\gamma^{low}$, $PD1^{high}$). By contrast, in Trabectedin-treated mice, PDA-infiltrating T cells displayed repressed Il10 and Pdcd1 and activated T-bet promoter activity, in accordance with their anti-tumor effector phenotype ($IL10^{low}$/$IFN\gamma^{high}$), indicating a key role of TAMs in orchestrating functions of PDA-infiltrating T cells by modulating their epigenetic profile towards a pro-tumoral phenotype. These results suggest the targeting of TAMs as an efficient strategy to obtain an appropriate T cell anti-tumor immune response and open new potential combinations for PDA treatment.

Keywords: pancreatic cancer, T cell epigenetics, Trabectedin

B064 / Systematic discovery of combination immunotherapy targets

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Checkpoint blockade (e.g. anti-CTLA-4 and anti-PD-1) induces durable responses in a subset of cancer patients. However, it is frequently reported that tumors are able to overcome - or are resistant to - immunotherapy treatments. This is most likely due to exploitation of additional, and unidentified, negative feedback loops.
Current approaches for discovery of combination therapies emphasize a 1 + 1 approach in which an active drug is tested in combination with an experimental agent. We hypothesize that many opportunities for synergistic combinations are missed with this strategy. We are therefore using CRISPR/Cas9 to systematically identify combination therapies in the tumor microenvironment. We have used both ***in vivo*** and ***in vitro*** screening techniques to rapidly identify key targets that may synergize with current checkpoint blockade therapies.
We show here that possible targets can be rapidly discovered ***in vivo*** from a library of genes that could be used in combination with anti-PD-1 treatment. We have also shown that ***in vitro*** assays can be utilized to identify novel targets that may overcome MDSC-mediated suppression within the tumor.

Keywords: CD8 T cells, Immunotherapy, CRISPR/Cas9

B065 / Combination of IL-2/anti-IL-2 antibody complexes and checkpoint blockade controls established transplanted and autochthonous tumors

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High dose IL-2 administration is one of the first treatments that demonstrate that manipulation of the immune system can induce durable cancer regression. Yet, IL-2 therapy can be very toxic and the reached efficacy is not optimal (5-20% of responders). Low efficacy is in part associated to the unwanted effect of IL-2 on Tregs, which are able to block the anti-tumor immune response. Unwanted activation of Tregs can be avoided by the use of IL-2/anti-IL-2 antibody (IL-2C) complexes, that re-direct IL-2 action to $CD8^+$ T and NK cells. Moreover, IL-2Cs have a longer half-life, accounting for a better pharmacodynamics and can be used at lower doses, reducing toxicity. In this study, we evaluated whether

combining IL-2C administration with the blockade of inhibitory immune pathways could result in an effective therapy in two experimental models: i) the B16-OVA, naturally resistant to anti-checkpoint inhibition and, ii) the ***K-ras***$^{LSL\text{-}G12D/+}$***;p53***$^{fl/fl}$ genetically-engineered mouse lung adenocarcinoma. We observed that in both models, combination of IL-2C with blockade of the PD-1 or CTLA-4 pathways showed enhanced effectiveness, leading to complete tumor regression in some mice. Mechanistic studies indicated that the combined treatment works by activating NK cells and/or re-invigorating exhausted CD8+ T cells infiltrating the tumor. Overall, our pre-clinical results indicate that combination of IL-2C with anti-checkpoint mAbs represent a valid therapeutic approach for fighting against cancer, worth being translated into the clinic.

Keywords: Immunotherapy, Cancer, Interleukine 2

B066 / Tumor-associated macrophages-like nanoparticles for brain tumor therapy

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Tumors contain many infiltrating cells, such as tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs). We have previously shown that these cells have the homing ability to irradiated tumors. This study reports a new therapeutic strategy by adapting characteristics of the tumor tropism of TAMs to modify drug-loaded nanoparticles as adjuvant for brain tumor therapy. In this study, the cellular membrane was extracted from TAMs and then coated on the drug-loaded nanoparticles. Results show that the cellular membranes of TAMs not only reduce the uptake of nanoparticle from the mononuclear phagocyte system, but also enhance the tumor targeting of the nanoparticles toward astrocytoma cell line, ALTS1C1. We also found that the tumor targeting effect of the TAM membrane-coated nanoparticles is diminished against SDF-1 knockdown tumor cells. These results indicate that the SDF-1/CXCR4 axis is contributed to the tumor-tropism of the TAM membrane-coated nanoparticles toward brain tumor. In conclusion, we established a TAMs-based drug delivery platform against brain tumor with the cellular membrane coating technology.

(This work was supported by the research grant of NHRI-EX106-10514BI from National Health Research Institutes).

Keywords: Brain tumor, Tumor-associated macrophages, Tumor microenvironment

B067 / Synergistic effect of mitochondrial activation and PD-1 blockade in boosting antitumor immunity

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PD-1, a surface receptor expressed mostly by activated T cells, acts as a negative regulator in the effector phase of the immune response. Compelling progress has been made in the clinical application of cancer immunotherapy by blocking the interaction between PD-1 and PD-L1 (ligand of PD-1, mainly expressed on tumors), which reactivates the immune system against cancers. However, a significant fraction of patients are still unresponsive to this therapy. One of the approaches to expand its success rate has been combination with existing cancer treatments. Although the preliminary results of combination therapy showed some improvement from PD-1 blockade therapy alone, significant synergistic effect is lacking. In order to develop effective combination therapy, we considered the factors that regulate killer T cell ($CD8^+$ T cell) differentiation. Based on recent literature, demonstrating the association of mitochondrial energy metabolism with killer T cell differentiation, we administered small molecules that perturb mitochondrial activity in combination with PD-1 blockade to tumor bearing mice. We have successfully demonstrated that perturbation of mitochondrial activity with small molecules such as ROS inducers or uncouplers, synergized with PD-1 blockade. Importantly we found that up-regulation of transcriptional coactivator PPAR-gamma coactivator 1α (PGC-1α), a key regulator of mitochondrial biogenesis, is an integral component of such robust tumor inhibition. PGC-1α is known to activate mitochondria through its partner transcription factors such as PPARs. Subsequently, we found that the activation of the PGC-1α/PPAR pathway showed impressive synergism with PD-1 blockade through enhancing effector/memory $CD8^+$ T cells, their expression of T-bet and their accumulation in the tumor site. The enhancement of antitumor immunity by activation of PGC-1α/PPAR in combination with PD-1 blockade was accompanied by increased oxygen consumption rate (OCR), which is an indicator of mitochondrial respiration. The combinatorial treatment strategy that has been established in this study should improve the response rate of cancer patients, particular those who are less sensitive to the PD-1 blockade therapy alone. Further research is necessary to integrate the mitochondrial activation and $CD8^+$ T cell function that lead to an effective anti-tumor immune response.

Keywords: PD-1, Immunotherapy, Mitochondria

B069 / Poly(I:C) drives primary human glioblastoma cells towards activating lymphocytes invigorated by PD-L1 blockade

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Prognosis of glioblastoma (GBM) remains dismal and underscores the need for novel therapies. Immunotherapy is generating promising results, but presumably requires combination strategies to unlock its full potential. We investigated the immunomodulatory profile of primary human GBM cells following poly(I:C) treatment for its immunomodulatory capacities and combinatorial potential to programmed death ligand 1 (PD-L1) blockade. Primary human GBM cells were cultured from tumor tissue obtained from standard surgery of GBM patients and stimulated with 10µg/ml poly(I:C). Cytokine analysis was performed by electrochemiluminescence, while PD-L1 and PD-L2 expression was evaluated using flow cytometry, quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC). Immune activation in cocultures with peripheral blood mononuclear cells (PBMC) was analyzed using flow cytometry (CD69, CD107a) and ELISA (interferon-γ, IFN-γ). Migration of PBMC to treated GBM cells was assessed using a transwell assay with flow cytometric detection. The value of blocking PD-L1 or PD-L2 was evaluated by IFN-γ ELISA. In our experiments, poly(I:C) stimulated a pro-inflammatory secretome by GBM cells, including type I IFN, interleukin 15 and reduced transforming growth factor β. Moreover, we observed an upregulation and induction of PD-L1 and PD-L2 on the membrane, which was partially due to secreted IFN-β. Furthermore, qRT-PCR and IHC analysis revealed ***de novo*** production of the PD-1 ligands rather than translocation from the cytoplasm. Upregulation of the adaptor molecule for Toll-like receptor (TLR) 3 indicated that poly(I:C) mediated its effect via the TLR3 axis. Notwithstanding expression of PD-1 ligands, cocultures of poly(I:C)-treated GBM cells with PBMC resulted in enhanced lymphocytic activation (CD69) and degranulation (CD107a), as well as overall immune activation (IFN-γ). Additional PD-L1 blockade further propagated immune activation, while this effect was not consistent for PD-L2. Next to activating immunity, poly(I:C)-treated GBM cells also

doubled the attraction of $CD8^+$ T cells, and to lesser extent $CD4^+$ T cells. Downregulation of CXCR3 and CCR5 on migrated lymphocytes suggested that ligands of these receptors are involved in the increased chemotaxis. Indeed, poly(I:C) induced secretion of CXCL9, CXCL10 and CCL4 by GBM cells. Our results indicate that by triggering GBM cells, poly(I:C) primes the tumor microenvironment for an immune response. Secreted cytokines allow for immune activation while chemokines attract $CD8^+$ T cells to the front, which have been postulated as a prerequisite for effective PD-1/PD-L1 blockade. In conclusion, our data proposes poly(I:C) treatment combined with PD-L1 blockade in order to invigorate the immune checkpoint inhibition response in GBM. We are also the first to show PD-L2 expression on human GBM cells.

Keywords: Poly(I:C), Glioblastoma, PD-L1

B070 / Improving efficacy of adoptive T cell therapy against cancer by combining targeted cytokines and inhibitory immune checkpoint blockers

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Crossing the abnormal vessel barrier and interstitium characterizing most solid tumors remains a major hurdle that either endogenous or adoptively transferred tumor-specific cytotoxic T lymphocytes (CTLs) must overcome to get in direct contact with their targets. This explains resistance of some solid tumors to blockade of inhibitory immune checkpoints (ICB), like programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). We previously demonstrated that NGR-TNF, a Cys-Asn-Gly-Arg-Cys-Gly-TNF fusion protein capable of targeting tumor vasculature and activating endothelial cells, substantially improves tumor infiltration by CTLs, and efficacy of adoptive cell therapy (ACT) with TCR-redirected T cells.
We tested the hypothesis that anti-tumor immunity could be ameliorated by combining ACT and antibodies blocking the PD-1 and CTLA-4 receptors with NGR-TNF, thus favoring intratumoral T cell infiltration and enhancing intratumoral effector functions.
This triple combination was tested in the autochthonous transgenic adenocarcinoma of the mouse prostate (TRAMP) model, and in C57BL/6 mice bearing established, orthotopic B16 melanoma, as both tumors are poorly sensitive to the single therapeutic agents, therefore mimicking human prostate cancer and ICB-resistant melanoma. The combination of anti-CTLA-4 and anti-PD-1 antibodies was chosen based on available clinical results. Disease progression and animal survival were analyzed in relation to tumor-specific T cells representation and function. We found that the triple combination of ACT, NGR-TNF and ICB was the most effective treatment in delaying disease progression, in improving overall mice survival and in promoting tumor infiltration by $granzymeB^+$/interferon-g^+ CTL. We also found Tregs to be less represented in the triple combined settings, thus resulting in most favorable T effector/Treg ratio.
Our findings thus indicate that the simultaneous targeting of tumor cells with ACT, of tumor endothelial cells with NGR-TNF, and of the immunosuppressive tumor microenvironment with checkpoint blockade results in increased T cell infiltration and immune responses, thus outperforming current strategies in the treatment of highly immunosuppressive tumors.

Keywords: vessel targeting, immune checkpoint blockade, resistance

B071 / Duokines made of the TNF superfamily ligands CD40L, 4-1BBL, OX40L and CD27L are novel bifunctional co-stimulatory fusion proteins that amplify T cell responses

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Efficient costimulation is crucial for antitumoral T cell responses. Modulating costimulatory signals via the receptors of the tumor necrosis factor receptor super family (TNFRSF) playing a central role in T cell costimulation has therefore emerged as promising strategy in cancer immunotherapy. By targeting the receptors with their natural ligands, T cell responses are supported without any supraagonistic activity that has been observed for agonistic antibodies. By establishing a novel class of bifunctional costimulatory fusion proteins consisting of two tumor necrosis factor superfamily (TNFSF) ligands, we boosted T cell activation by utilizing the spatial proximity of T cells and antigen-presenting cells (APCs) as well as the upregulation of TNFRSF receptors on T cells upon primary stimulation. The novel Duokine (neologism for dualacting cytokines) fusion proteins were created by connecting two different TNFSF ligands to form either homotrimeric molecules by inter-molecular homotrimerization or homotrimeric single-chain molecules by intra-molecular homotrimerization.
By connecting the TNFSF ligands 41BBL, OX40L and CD27L in all possible combinations, cisacting Duokines were generated that act on the same or adjacent T cells, while combining CD40L with 41BBL, OX40L and CD27L resulted in transacting Duokines simultaneously acting on APCs and T cells. All Duokines and

scDuokines stably assembled in the correct protein configuration and proved to activate their receptors on HT1080 reporter cells. In a heterogeneous population of peripheral blood mononuclear cells, cis-acting molecules preferably bound to T cells, while trans-acting molecules bound to APCs. ***In vitro*** co-stimulation of T cells was seen for cis- and transacting Duokines and scDuokines in an antigen-unspecific as well as antigen-specific setting. Trans-acting molecules furthermore activated B cells, which represent a class of APCs. The antitumoral potential of scDuokines was proven ***in vivo***, where scDuokines as costimulatory agent in combination with a primary T cell stimulating antibody successfully reduced the formation of lung tumors. In conclusion, Duokines have been established as novel bifunctional fusion proteins that act co-stimulatory and have the potential to enhance the antitumoral activity of other immunotherapeutic approaches. Thus, together with their highly modular molecular architecture, TNFSF ligand-based Duokines present themselves as promising molecules with high potential for further developments.

Keywords: TNFSF ligands, co-stimulation, cancer immunotherapy

B072 / Synergistic immunotherapeutic efficacy of RIG-I activation in combination with PD1-checkpoint blockade in a model of hepatocellular carcinoma

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Background: Hepatocellular carcinoma (HCC) it is one of the most common type of cancer worldwide, characterized by poor prognosis with a 5-year survival rate of solely 5-6%. The innate immune receptor RIG-I triggers a type I IFN-mediated immune response and an immunogenic form of cell death in tumor cells upon detection of triphosphate-RNA (3p-RNA). Here, we evaluate RIG-I activation as single immunotherapeutic agent and in combination with immune checkpoint inhibition in HCC.
Methods: RIG-I expression in HCC was assessed by immunohistochemistry analysis of tissue microarrays of human HCC biopsies. Furthermore, activation of the RIG-I pathway following 3p-RNA treatment was evaluated ***in vitro*** in human and murine HCC cell lines, whilst the immunotherapeutic efficacy of RIG-I activation was tested in an orthotopic syngeneic HCC mouse model.
Results: Transfection of 3p-RNA in both human and murine HCC cell lines leads to phosphorylation of IRF-3, production of IFN-b and CXCL10 and the induction of tumor cell death, confirming the activation and functional activity of RIG-I. Treatment with 3p-RNA significantly prolonged survival in orthotopic HCC mouse model and the treatment efficacy proved to be dependent on $CD8^+$- and $CD4^+$ T cells. While anti-PD1 checkpoint inhibition used a single agent did not show significant survival benefits, a combinatorial approach of anti-PD1 checkpoint inhibition and 3p-RNA dramatically enhanced therapeutic efficacy leading to significantly prolonged and increased overall survival.
Conclusions: In conclusion, RIG-I represents a promising target for HCC therapy, and the combination of RIG-I ligands and checkpoint inhibition shows prominent therapeutic benefits in HCC and possibly in other tumor models.

Keywords: Innate Immune receptor RIG-I, Combinatorial Therapy, Checkpoint Inhibition

B073 / Viro-immunotherapy with oncolytic parvovirus H-1 in combination with bevacicumab and PD-1 checkpoint blockade shows safety and objective anti-tumor response in patients with recurrent glioblastoma

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Viro-immunotherapy is an emerging concept to improve the cinical effects of current oncolytic virus based anti-cancer strategies. Based on results from a phase I/IIa trial (ParvOryx01) in which the oncolytic H-1 parvovirus (H-1PV) showed evidence for the induction of immune responses in patients with recurrent glioblastoma we aimed to enhanced H-1PV treatment with immune activators, in particular bevacicumab and checkpoint blockade.
8 patients with recurrent (n=6) or primary (n=2) glioblastoma received H-1PV based viro-immunotherapy on a compassionate use (CU) agreement. 7 of the patients were treated by intratumoral and intravenous injection of H-1PV and 1 patient by intravenous injection only, followed by bevacicumab and nivolumab or pembrolizumab. GMP-grade H-1 virus and all medication were provided by the sponsor of the ParvOryx01 trial (Oryx GmbH&Co KG, Baldham, Germany) on a humanitarian basis.
In all eight patients the combination treatment was safe. Side effects were attributed to checkpoint inhibitors (hepatitis, n=2 and thyroiditis (n=1) or bevacicumab (impaired wound healing, n=3). MRI showed marked and rapid tumor regression in all eight patients within four to eight weeks after virus injection. The objective radiological response according to RANO criteria was between 49% and 96%, accompanied by clinical improvement in all symptomatic patients.

Viro-immunotherapy based on H-1PV showed safety and consistent induction of clinical and radiological responses in all treated patients. Even though the observations were derived from non-trial patients the data gives strong support for this novel concept in malignant glioma therapy.

Keywords: Oncolytic Virus, Parvovirus H-1, Combination therapy

B074 / Design of multivalent liposomal therapeutic antibodies for treatment of neuroblastoma

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Monoclonal antibodies attached to liposomal formulations have typically been considered as targeting moiety selected to facilitate delivery of an encapsulated drug to target cell populations (e.g. cancer cells) ***in vivo***. Although this is interesting in concept there is little evidence to support the idea that liposomes with surface associated targeting moieties can distribute efficiently within heterogeneous tumors. Our interest is more focussed on the development and application of liposomes with multiple active agents. In this context, therapeutic antibodies can be attached to drug loaded liposomes to generate combination products where one of the active agents is the attached antibody. To test this concept we designed a multivalent liposomal rituximab formulation. The methodology allowed the production of liposomes with controlled valences ranging from 19 to 158. These formulations induced cancer cell apoptosis in a valence-dependent manner. The activity of these formulations was up to 14-fold greater than that achieved with bivalent rituximab. Other studies confirmed that there was no loss of antibody-dependent cell-mediated cytotoxicity and complement dependent cytotoxicity achieved when using multivalent liposomal rituximab. This data highlights the therapeutic potential of rituximab liposomes; even in the absence of an encapsulated drug. This concept is now being expanded to include the development of liposomal camptothecin formulations with surface associated dinutuximab; a humanized monoclonal antibody targeting the disialoganglioside GD2 recently approved for use in the treatment of neuroblastoma. We hypothesize that multivalent liposomal dinutuximab will exhibit improved therapeutic activity and concomitantly the side effects observed following administration of this antibody (mainly infusion reactions and neuropathy) will be reduced due to changes in dinutuximab pharmacokinetics and biodistribution. Multivalent liposomal dinutuximab will be characterized ***in vitro*** for binding and cytotoxicity against a number of neuroblastoma cell lines including LAN-1, IMR-32 and SK-N-AS. A murine model of systemic disease is being developed to assess the therapeutic activity of the resulting formulations. Liposomal formulations of topotecan and irinotecan, previously optimized in our lab, will be evaluated in combination with the bivalent antibody as well as the multivalent liposomal dinutuximab. The resulting combination product should provide significant benefits when used to treat neuroblastoma.

Keywords: Liposomes, Dinutuximab, Camptothecin

B075 / Activation of HGF/c-MET signaling in neutrophils limits cancer immunotherapy

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Oncogenic signal transduction inhibitors and T- cell immunotherapies are currently revolutionizing cancer treatment. The HGF/c-MET signaling pathway is dysregulated in many solid cancers, but the therapeutic efficacy of targeting oncogenic c-MET is limited to a subgroup of patients with certain cancer entities. In addition, HGF/c- MET signaling also participates in the regulation of immune responses.

Therefore, we hypothesized that c-MET inhibitors (METi) could have a broader applicability in combination with immunotherapies. To test this, we combined METi with immunotherapies in experimental mouse tumor models.

Here, we demonstrate that adjuvant c-MET inhibition increases the efficacy of T-cell immunotherapies in different cancer models regardless of tumor cells' dependence on c- Met signaling. We found out that in response to cancer immunotherapies HGF/c-MET signaling in neutrophils promoted their reactive mobilization from the bone marrow and their recruitment into T-cell inflamed lymph nodes and tumor tissues, where neutrophils acquired an immunosuppressive phenotype and thereby restrained anti-tumoral T-cell responses. Adjuvant METi treatment caused retention of c-MET$^+$ neutrophils in the bone marrow and inhibited their recruitment into T-cell inflamed tissues. Consequently, this promoted the expansion of anti-tumoral T cells and led to an enhanced tumor control.

In conclusion, we identified c-MET inhibition as an adjuvant treatment strategy to increase the efficacy of T-cell

immunotherapies in different cancer mouse models. As reactive neutrophil responses are also seen in patients undergoing immunotherapy, this strategy could have a broad clinical applicability and could offer therapeutic benefit in a variety of cancer types regardless of tumor cells dependence' on c-MET signaling.

Keywords: c-MET, neutrophils, immunotherapy

B076 / Oncolytic virotherapy of melanoma cells with the oncolytic parvovirus H-1 benefits from the combination with the checkpoint inhibitors Ipilimumab and Nivolumab

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In the last year the treatment of melanoma has undergone big changes. Immune-based therapies such as ipilimumab (ipi) and nivolumab (nivo) or oncolytic virus therapies have been developed which improve patient survival. Metastatic melanoma is an immunogenic cancer and is seen as a model to use to learn even more about immunotherapy and checkpoint inhibitors. Expression of checkpoints such as CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) and PD-L1 (programmed death receptor ligand 1) on tumors or its induction on the surface of T cells may lead to suppression of the human anti-tumor immune response. Here we analyze the effect of the oncolytic parvovirus H-1 (H-1PV) in combination with the checkpoint inhibitors ipilimumab and nivolumab to induce maturation of dendritic cells, inhibition of regulatory T cells (Tregs) and activation of cytotoxic T cells (CTL). A human ex vivo melanoma model, was used to increase our knowledge about oncolytic virotherapy with the H-1PV in combination with ipilimumab and nivolumab. To test this untreated and H-1PV infected melanoma cells were cocultured with dendritic cells in presence of ipi, nivo or both to induce their maturation which were determined by expression of CD80, CD83 and CD86. To analyze the effect on T cell activation, T lymphocytes were added one day after DC: tumor cell coculture. Expression of the activation marker CD69 and the Treg marker FoxP3 was measured after another 48 hours.
The benefit of such combinations on the differentiation and activation of human T lymphocytes was demonstrated. Expression of the three proteins CTLA-4, PD-1 and PD-L1 is upregulated in H-1PV-infected melanoma cells. Ipi and nivo were not able to induce DC maturation. While H-1PV induced maturation of DCs could not be further strengthen by ipi and nivo, combination of H-1PV with the checkpoint inhibitors ipi and nivo, increased the activity of T lymphocytes. We showed that nivo in combination with H-1PV induced stronger CTL activation than ipi. Conversely, ipi was able to reduce the number of Tregs and their activity. These data support the concept of a treatment benefit from combining oncolytic H-1PV with the checkpoint inhibitors ipilimumab and nivolumab, with nivolumab inducing stronger effects on cytotoxic T cells, and ipilimumab strengthening T lymphocyte activity.

Keywords: oncolytic parvovirus H-1, ipilimumab, nivolumab

B077 / *In situ* vaccination improves efficacy of PD-1 blockade in unresponsive lymphoma tumors through induction of a highly efficient cross-presenting dendritic cell subset expressing TLR3

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Purpose: Low-grade non-Hodgkin's B-cell lymphomas are generally incurable, with standard therapies inducing only temporary remissions; and preliminary results with anti-PD-1 therapy have yielded low response rates. It has been shown that tumoral DC infiltration correlates with efficacy of checkpoint blockade. Tumor-targeted vaccines represent promising, novel treatment strategies able to induce anti-tumor T cells. Therefore, we tested the combination of ***in situ*** vaccination with PD-1 blockade in a pre-clinical mouse model.
Methods: A20 lymphoma-bearing mice were treated with a PD-1 blocking antibody with or without an ***in situ*** vaccine consisting of intratumoral injections of FMS-like tyrosine kinase-3 ligand (Flt3L), local irradiation (XRT) of the tumor and intratumoral injections of the TLR3 agonist poly-ICLC (pIC).
Data: Untreated lymphoma tumors contained very low numbers of DC and treatment with anti-PD1 alone did not induce tumor regression or increase survival. After Flt3L treatment, flow cytometry analysis revealed a dramatic increase of IRF8+TLR3+ DC at the tumor site, the tumor-draining lymph node (TdLN) and the spleen. XRT of A20 cells induced activation of Flt3L-treated splenic DC in vitro and local XRT of the tumor ***in vivo*** induced expression of CD103 on infiltrating TLR3+ DC, indicating maturation of these cells. Local XRT also increased the amount of intratumoral DC taking up tumor-associated antigen (TAA), suggesting enhanced uptake of dying tumor cells. Interestingly, tumor antigens were taken up mainly by CD103+ DC and not CD103- subtypes. CD103+ expression distinguishes a subset of migratory DC that are very efficient at cross-presenting exogenous antigens to CD8+ T cells. Accordingly, CD103+ DC isolated from the tumor induced proliferation of tumor-specific CD8+ T cells more efficiently than CD103- subsets. The combination of Flt3L with XRT and pIC

induced tumor-reactive, Interferon g (IFN g)-producing T cells, but delayed tumor growth and improved survival only in 40% of mice. Importantly, ***in situ*** vaccination also increased expression of PD-L1 on tumor cells and tumor infiltrating DC. Consistent with these findings, combination of ***in situ*** vaccination with PD-1 blockade led to complete tumor regression and increased long-term survival in the majority of mice. Moreover, PD-1 blockade also increased the number of tumor-reactive T cells and depletion of CD8+ T cells abrogated the anti-tumor effect.

Conclusions: *In situ* vaccination combining intratumoral Flt3L injection with local XRT and poly-ICLC can improve efficacy of anti-PD-1 in checkpoint-unresponsive lymphoma tumors through induction of a highly efficient cross-presenting DC subset leading to long-term regression of established lymphoma tumors.

Keywords: In situ vaccination, TLR3, lymphoma

B078 / Intratumoral RNA therapy demonstrates durable anti-tumor efficacy

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Over the last decade new therapeutic strategies to treat cancer by breaking the cancer-induced immune tolerance rather than targeting the tumor itself have garnered considerable interest. These therapeutic strategies involve approaches such as activation of immune cells via cytokines, re-activating T cells via immune checkpoint blockade or influencing the tumor microenvironment towards immune activation. In spite of great successes in the field of cancer immune therapy, administration of such drugs is frequently accompanied by toxic side effects. We thus sought to leverage local drug administration as an alternative to avoid the dose-limiting toxicity associated with systemic applications. In particular, immune-modulating substances administered intratumorally offer an attractive option to induce a local pro-inflammatory tumor microenvironment and anti-tumoral effects. Here, we present a novel approach for the therapy of solid tumors by intratumoral treatment with RNAdjuvant®, which consists of a synthetic non-coding, immunostimulatory RNA and a polymeric carrier.

CT26 tumor-bearing mice were treated intratumorally with RNAdjuvant® with or without systemic PD1 checkpoint inhibition for three weeks. The median tumor growth and survival of mice was monitored over a time span of 15 weeks. In case of complete tumor eradication, re-challenge experiments were performed at least 100 days after the first tumor inoculation.

Intratumoral application of RNAdjuvant® substantially reduced tumor growth in CT26 tumor-bearing mice. The anti-tumoral effect of RNAdjuvant® was dose-dependent and induced complete remission in up to 50 % of treated mice. In combination with systemic PD1 immune checkpoint inhibition even low doses of RNAdjuvant® administered intratumorally resulted in complete tumor eradication in the majority of mice. Moreover, local treatment of tumor lesions with RNAdjuvant® in conjunction with PD1 blockade induced systemic anti-tumor memory responses protecting cured mice against a second challenge with CT26 tumor cells performed at least 100 days after the first tumor cell inoculation.

Our data suggest that intratumoral treatment with RNAdjuvant® in combination with PD1 checkpoint inhibition represents a promising therapeutic approach for the local treatment of solid tumors by enabling systemic anti-tumor immune responses without the drawback of inducing immune toxicity. Based on these promising preclinical data, a Phase I clinical trial is currently in preparation to assess the safety and tolerability of intratumoral application of RNAdjuvant® in combination with immune checkpoint blockade.

Keywords: RNA therapeutics, intratumoral therapy, immune checkpoint inhibitors

B079 / Standing in the GAP

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A new radioimmunotherapeutic regime GAP15R was formulated with the aim to (a) stimulate GITR(G) to overcome regulatory T suppression, (b) add IFNα(A) to promote inflammatory milieu, (c) block PD1(P) to disinhibit T effector cytotoxicity, (d) add IL-15(15) to enhance danger signals & T cell expansion, and (e) apply radiation(R) at critical time point to sustain localised inflammation. This was tested in a murine 4T1 metastatic mouse carcinoma model with treatment given over a period of 28 days and regular monitoring of tumor volume and survival of mice. It demonstrated long term complete remission up to 66% of treated mice, which is not associated with major treatment related complication in cases with specific tumor burden.

Keywords: Radioimmunotherapy, Breast carcinoma, GITR, TNFa, PD1, IL15

B080 / Enhancing the sensitivity of breast cancer cells to innate immune attack using low-dose chemotherapy treatment

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The administration of low non-toxic doses of chemotherapeutic agents is emerging as a promising modality that has been found to have anti-tumor immune modulatory effects. In this study, we determined the influence of low-dose chemotherapy treatment on the sensitivity of human breast cancer cells to natural killer (NK) cell-mediated cytotoxicity.
The influence of treatment on a panel of human breast cancer cell lines corresponding to different molecular subtypes (MCF7 (Luminal A, ER+), SKBR3 (Her2+), MDA-MB-468 and MDA-MB-231 (Triple-negative)) with low-dose doxorubicin on the expression of senescence and senescence-related markers and the cell cycle was determined using flow cytometry. The potential impact of treatment on the sensitivity of these cells to NK cell cytotoxicity was evaluated on the basis of its impact on the expression of NK cell ligands and their sensitivity to resting and cytokine-stimulated donor-derived primary NK cells and the NK-92 cell line, as measured using an ***in vitro,*** flow cytometry-based assay.
Treatment with non-toxic, low-doses of doxorubicin induced a "senescence-like" state which was best identified on the basis of cellular morphology and the expression of senescence-associated beta- galactosidase (SA-β-gal). Treated cells expressed higher levels of ligands for activatory NK cell receptors such as NKG2D and DNAM-1, and lower levels of ligands for inhibitory receptors such as KLRG-1 than their non-treated counterparts. Furthermore, cells that were responsive to the treatment were more sensitive to NK cell-mediated killing and clearance. This sensitivity was further enhanced with prior stimulation of NK cells with IL-2. Cells corresponding to the triple negative subtype of breast cancer (MDA-MB-468 and MDA-MB-231), which is associated with the most aggressive phenotype, showed the highest sensitivity amongst the panel.
This study shows that the treatment of breast cancer cells with low dose doxorubicin upregulates their expression of NK activatory ligands and renders them more sensitive to NK cell-mediated killing. Ongoing studies are assessing the influence of low-dose chemotherapy on the sensitivity of implanted human breast tumors to adoptively transferred human NK cell populations in murine xenograft models. This scenario provides a promising opportunity for combining low-dose chemotherapy with NK cell-based therapeutic strategies for the treatment of triple negative breast cancer.

Keywords: Low-dose chemotherapy, Natural killer (NK) cells, Breast cancer

B081 / Combination of cisplatin and anti-CD70 therapy in non-small cell lung cancer: an excellent match in the era of combination therapy

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In contrast to the negligible expression of the immunomodulating protein CD70 in normal tissue, we have demonstrated constitutive overexpression of CD70 on tumor cells in a subset of primary non-small cell lung cancer (NSCLC) biopsies. This can be exploited by CD70-targeting antibody-dependent cellular cytotoxicity (ADCC)-inducing antibodies, though this would only be applicable to a subset of patients with CD70-expressing tumor cells. Recently, the immunomodulatory properties of chemotherapy in combination with immunotherapy have been demonstrated. Although chemotherapy has long been considered immune suppressive, recent studies have shown that in addition to its direct cytotoxic effects on cancer cells, certain chemotherapeutics can elicit changes in the tumor microenvironment by triggering an immunogenic mode of tumor cell death.
In this study, we explored the potential of different chemotherapeutics (cisplatin, oxaliplatin and cyclophosphamide) to induce CD70 expression on NSCLC cells. Furthermore, using real-time measurement tools, we also assessed the efficacy of a combination regimen with chemotherapy and anti-CD70 therapy under normoxia and hypoxia. Finally, we examined the potential induction of immunogenic tumor cell death by these chemotherapeutic agents, measured by the membrane expression of calreticulin and the extracellular secretion of ATP.
We identified the highest induction of CD70 expression on lung cancer cells upon low doses of cisplatin, independent of oxygen levels. More importantly, the use of cisplatin resulted in an enhanced ADCC-effect of anti-CD70 therapy. As such, this combination regimen led to a significant decrease in lung cancer cell survival and broadens the applicability of CD70-targeting therapy. Finally, cisplatin induced calreticulin exposure and ATP secretion in NSCLC cell lines, signifying that this combination regimen might also stimulate immune reactions that ignite a strong anticancer immune response.
This is the first study that proves the potential of a combination therapy with cisplatin and CD70-targeting drugs in NSCLC. Based

on our data, we postulate that this combination strategy is an interesting approach to increase tumor-specific cytotoxicity and reduce drug-related side effects.

Keywords: non-small cell lung cancer, Cisplatin, CD70

B082 / The natural histone deacetylase inhibitor sulforaphane prevents resistance to everolimus in renal cell carcinoma in vitro

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Purpose: Our purpose was to evaluate whether the natural histone deacetylase (HDAC) inhibitor, sulforaphane (SFN), can prevent the development of resistance to everolimus in renal cell carcinoma (RCC).
Methods: The therapeutic efficacy of an SFN-everolimus-combination on RCC cell growth and proliferation was investigated in vitro. RCC cell lines A498, Caki-1, and KTCTL-26 were treated with everolimus [1 nM], SFN [5 µM] or an everolimus-SFN-combination for either 24 h (short-term) or 8 weeks (long-term). Cell growth, proliferation, and apoptosis were assessed, whereby the cell cycle phases and cell cycle regulating proteins cdk1, cdk2, cyclin A, and cyclin B were evaluated. The Akt-mTOR signaling axis with akt and raptor (both total and activated) was also analyzed.
Results: Exposure to everolimus or SFN for 24h significantly reduced RCC cell growth. Combining SFN and everolimus in short-term treatment induced an additive reduction on tumor growth and proliferation in the RCC cells. Responsiveness to everolimus alone was lost over time in Caki-1 cells, evidenced by elevated growth and proliferation. These functional effects on growth behavior in the everolimus resistant Caki-1 cells were associated with an increased percentage of G2/M (non-synchronized cell model) or S-phase (synchronized cell model) cells. Moreover, molecular analysis revealed up-regulation of the cdk1-cyclin B and cdk2-cyclin A axis and elevated phosphorylation of the mTOR sub-member, raptor. In contrast, no resistance development was observed during long-term treatment with the SFN-everolimus-combination. The combination resulted in suppressed Caki-1 growth and proliferation, and was associated with an increase in the G0/G1-phase cells. Furthermore, cdk1 and akt (both total and activated), cyclin B, and raptor expression diminished after long-term exposure to the SFN-everolimus-combination.
Conclusion: The natural HDAC inhibitor, SFN, delays resistance development to everolimus, which was observed with chronic everolimus monotherapy in RCC in vitro. Further investigations in vivo are necessary to verify the in vitro findings.

Keywords: renal cell carcinoma, everolimus, sulforaphane

B083 / In vivo siRNA knockdown of WNT1 rescues dendritic cells from b-catenin activation and sensitizes tumors to vaccination

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Aberrant WNT-b-catenin signalling is strongly associated with several types of cancers by increasing cancer cell proliferative, metastatic potential and stemness. Recent melanoma studies further suggest a profound immunosuppressive effect of intratumoral WNT-b-catenin pathway in dendritic cells (DCs), making its components particularly attractive immunotherapeutic targets. There are two important caveats in exploiting the WNT pathway: firstly, it plays a crucial role in homeostasis and regeneration and secondly, there are 19 human WNT ligands which are found differentially expressed in several human cancers. Targeting upstream the WNT signalling cascade the specific cancer cell-derived ligands that induce WNT pathway-dependent immunosuppression is expected to be a safer and more effective immunotherapeutic approach than targeting its intracellular counterparts.
We have set-up a combinatorial experimental approach that integrates primary human tumor analysis and syngeneic tumor models to identify cancer cell-derived WNTs that could become targets for a next-generation immunotherapeutic trials.
Firstly, we analysed the human Cancer Genome Atlas (TCGA) RNA-sequencing database and an in-house biobank of human lung adenocarcinoma to investigate correlations between WNT1-19 ligand expression and expression of immunosuppressive genes (RNA level) or numbers of T cytotoxic cells (IHC). WNT1 showed the strongest correlations. Silencing human WNT1 in dissociated human lung adenocarcinoma cultures confirmed its prominent immunosuppressive properties . We therefore created two Wnt1-overexpressing murine cancer cells lines, i.e. the Lewis-Lung-Carcinoma (LLC) and the B16-BL6 melanoma cell line and a WNT1-silenced LLC cell line, which we used in two syngeneic tumor transplantation models (one subcutaneous, one intrathoracic)

and one metastatic tumor model (intravenous administration). Murine lung adenocarcinoma and melanoma growth in immunocompetent, but not in immunodeficient mice, was strongly dependent on WNT-1. We observed strong tolerogenic effects of WNT1 on endogenous and adoptively transferred cancer antigen-specific T cells, such as low proliferation, activation, in vivo cytotoxicity and impaired immunological memory. Using Wnt pathway-reporter mice and an inducible DC-knock-out mouse model, we further show that WNT1-exposed DCs are sufficient and required for failure of adoptive T cell therapy. Pre-clinical trials showed that WNT1 siRNA-loaded nanoparticles rescued intratumoral DCs from b-catenin activation/immunosuppression and acted in synergy with DC-targeted vaccination against a model cancer-specific antigen, to induce regression of established tumors. Our studies unravel the crucial role of WNT1 in adaptive immune resistance of solid tumors and raise hopes that if in vivo RNA interference against WNT1 is safe in humans it could give excellent therapeutic responses, especially in rational combination immunotherapies.

Keywords: WNT pathway, dendritic cells, silencing nanoparticles

B085 / *In vivo* bioluminescence imaging of the sites of immune cell action and *ex vivo* characterization of the tumor microenvironment during a combined T cell, anti-PD-L1 and anti-LAG-3 based cancer immunotherapy

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Tumor antigen (TA)-specific IFN-γ producing $CD4^+$ T cells (Th1) are capable to inhibit tumor growth by induction of tumor senescence and activation of effector cells in mice and patients. In contrast TA-specific $CD8^+$ T (Tc1) cells directly kill tumors via MHCI. Expression of inhibitory immune checkpoints (ICP, e.g. PD-L1, PD-1, LAG-3) by tumor cells and immune cells inhibit TA-specific T cells. Consequently, ICP-blockade with specific antibodies is applicable to restore TA-specific T cell functions. The aim of our study was to establish a novel highly efficient OT-II-Th1/OT-I-Tc1 and ICP inhibitor-based combined immunotherapy (CIT) in the OVA-B16 melanoma model and to monitor the sides of T cell action using non-invasive i*n vivo* bioluminescence imaging (BLI) of NF-κB-luciferase-reporter (NF-κB-luc)- or wild type (wt)-mice. In addition, we aimed to monitore changes in the tumor microenvironment (TME) during CIT *ex vivo* by immunohistochemistry and gene expression analysis to reveal potential mechanisms of resistance to ICP inhibitor therapies.

OVA-B16 bearing NF-κB-luc- or wt-mice (n = 5-6) underwent a single 2 Gy whole body radiation (WBR) followed by either OT-II-Th1 or NF-κB-luc-OT-I-Tc1 cell treatment (***intraperitoneal injection***) and anti-PD-L1 and anti-LAG-3 mAbs (PD-L1+LAG-3) every second day. We measured the tumor volume, conducted in vivo as well as *ex vivo* BLI and gained the tumors for H&E histology, $CD3^+$-immunohistochemistry and qRT-PCR analysis.
Our CIT (OT-II-Th1+PD-L1+LAG-3) caused a reduction in tumor volume (184 ± 40 mm^3 vs. 256 ± 100 mm^3) and an increase in spleen weight compared to sham-treated mice. The efficacy of the CIT was WBR dependent. BLI of the sites of OT-II-Th1 cell action revealed a reduction in NF-κB activation in tumors followed by a strong reduction in the thymus and bone marrow when compared to sham treated mice. Activated NF-κB-luc-OT-I-Tc1 cells were detectable in OVA-B16 tumors. Histological evaluation ($CD3^+$) confirmed T-cell exclusion in tumors of sham-treated control mice and only a slight infiltrate in OT-II-Th1-treated mice. In contrast, a pronounced number of T cells were detected in tumors of OT-II-Th1+PD-L1+LAG-3-treated mice. In addition, we observed impressive molecular changes in the TME of OVA-B16 melanomas upon CIT (with OT-II-Th1 cells): An increase in HIF-1α and SDF1 mRNA expression indicating an increase in cancer associated fibroblasts. Furthermore, CIT with NF-κB-luc-OT-I-Tc1 cells caused an increase in SDF1, PD-L1 and HIF-1α expression in melanomas, which are discussed as prognostic biomarkers.
Our efficient combined immunotherapy suppressed NF-κB activation in melanomas, thymus and bone marrow and causes major changes in the TME. Thus, continuative studies are indispensable to uncover the temporal dynamics of the immune cell effector sites during T cell and ICP-based immunotherapies of cancer.

Keywords: Checkpoint Blockade, Tumor Microenvironment, Adoptive Cell Therapy

B087 / Rhabdoid tumors are highly infiltrated by lymphoid and myeloid cells, and sensitive to checkpoint blockade and TLR3 activation

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Rationale: Rhabdoid tumors (RTs) are highly aggressive cancers of infancy, arising from the central nervous system (Atypical Teratoid Rhabdoid Tumors, AT/RT) and other miscellaneous extra-cranial locations. RTs are mainly characterized by the biallelic inactivation of ***SMARCB1*** tumor suppressor gene and an otherwise highly stable genome. Prospective trials based on intensive multimodal therapies have led to few survivals, and the long-term side effects are a great matter of concern. Innovative therapies are therefore urgently warranted. In the prospect of new therapeutic strategies, we explored immune contexture of both human and mouse RT; then we explored in vivo targeting of immune infiltrate, in order to determine the best immunotherapy strategy adapted to the immune contexture.

Methods: At this aim, we first explored immune infiltrate of a cohort of human tumor samples, including both AT/RT and extracranial RT, by immunohistochemistry, expression profiling and flow cytometry. In parallel, we performed the same analyses on tumors obtained from the genetically engineered mouse model (GEMM) of RT Smarcb1$^{flox/flox}$;Rosa26-Cre^{ERT2} previously established in our lab, and syngeneic heterotopic engraftment models that we derived from the previous one.

Results: We found that both human and mouse RT are highly infiltrated by immune cells of both lymphoid and myeloid lineages; this consists equal repartition of CD4+ and CD8+ T cells with few regulatory T cells. We observed that T cells express PD-1 and TIM3 activation/exhaustion markers, while tumor and myeloid cells express their respective ligands, i.e. PD-L1 and Galectin 9. Consistently, we found that blockade of PD-1/PD-L1 pathway was able to impair tumor growth in GEMM and syngeneic models of RT and induced memory against second engraftment in syngeneic models. When tumors were less sensitive to PD-1 blockade, combination of anti-TIM3 and anti-PD1 shows a synergistic activity. In the context of RT syngeneic engraftment, we observed the expansion of aberrant myelopoiesis corresponding to suppressive myeloid cells. Myeloid transcriptomic signatures correlated with the expression of some chemokines known to recruit such myeloid cells, such as CCL2. We found that tumor infiltrating myeloid cells corresponded for a major part to macrophages of pro-tumoral phenotype, both in human and mouse RT. Targeting this myeloid infiltrate by TLR3 activation with poly(I:C) induced a potent anti-tumor effect.

Conclusion: We show here for the first time that rhabdoid tumors are highly infiltrated tumors susceptible to PD-1/PD-L1 and TIM3 blockade, and to reprogramming of myeloid suppressive cells. Immunogenicity of rhabdoid tumor cells is a surprising observation in the field of pediatric tumors; we hypothesize that rhabdoid tumor recognition by T cells is initiated by a ***SMARCB1*** dependent mechanism under investigation.

Keywords: SMARCB1, checkpoint blockade, TLR3

B088 / Codon-optimized P1A-encoding DNA vaccine and immune-checkpoint blockade combination: towards a therapeutic vaccination against P815 mastocytoma

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This study aims to find new strategies to improve the cancer DNA vaccine performance alone or in combination with immune-checkpoint inhibitors, inhibitory pathways crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses. Immune-checkpoints are now considered a major mechanism of immune resistance, particularly against T cells that are specific for tumor antigens. Cancer DNA vaccines are plasmids encoding tumor-associated antigens delivered in vivo to mount an immune response. DNA vaccine sequences can be modified to enhance the antigen-specific immune responses. To optimize the efficiency of a P815 mastocytoma DNA vaccine, the P1A gene sequence was modified by substituting specific codons with synonymous ones thus obtaining 3 codon-optimized P1A vaccines, with a different amount of CpG unmethylated motifs. Codon optimization can facilitate the translation to protein thus improving the antigen production and can notably improve the activation of the innate immune system due to an increased number of CpG motifs. In vitro, increased production of the P815A murine antigen was obtained with optimized plasmids. To evaluate the innate immune response to the vaccine, P1A plasmids were injected in the tibial muscle of DBA/2 mice. Six hours after the vaccine injection, a local increase in the expression level of cytokines involved in the TLR-9 response was measured when more unmethylated CpG motifs were present. In vivo, mice were vaccinated by administering 3 times the P1A vaccines at 2 weeks of interval. Mice immunized with the optimized vaccines showed higher levels of tumor infiltrating CD8+ in the P815 mastocytoma tumor. Consequently, tumor growth was delayed and mouse survival was improved after prophylactic vaccination. Therapeutic vaccination was effective when the DNA vaccine containing the codon-optimized P1A sequence with the highest number of CpG motifs was used. The best vaccine was selected and used in combination with immunocheckpoint blockade inhibitors, directed against CTLA-4 and PD-1 receptors on T cells. The effect of the combination further increased mice survival and significantly delayed the tumor growth, improving the efficacy of the therapeutic vaccination. This study demonstrates that the optimization of a DNA vaccine can improve its efficacy thanks to the increased innate immune stimulation and antigen production thus allowing its use for a therapeutic vaccination. Furthermore, expanded efficacy is achieved when the improved vaccine is combined with CTLA-4/PD1-pathway blockade, as the combination of these two strategies further potentiate the T cell-mediated immunity.

Keywords: Cancer DNA vaccines, Therapeutic vaccination, Immune-checkpoint blockade

B090 / Targeting the complement system to improve radiation treatment

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Radiotherapy is an effective treatment strategy for cancer, but a significant proportion of patients still experience radiation-induced toxicity due to damage to normal tissue in the irradiation field. Increasing the therapeutic window of radiotherapy may be achieved by using molecularly targeted therapies against pathways that are altered in cancer. The complement system is an important pathway in immunity composed of soluble and cell surface proteins. Several members of this pathway are upregulated in cancer and complement inhibition is under investigation as a therapeutic strategy, including in combination with radiotherapy. Interestingly, our data suggests that in response to radiotherapy, expression of complement regulators CD55 and CD59 is decreased in normal colon. Importantly, these expression changes correlate with an increase in the C5b-9 complex (thought to be responsible for cell lysis) in irradiated colon ***in vivo***. Furthermore, our results suggest that targeting the complement system (either genetically or pharmacologically) can result in increased survival of mice following radiotherapy, through protection of the gastrointestinal tract from radiation-induced toxicity. Together, these findings suggest that targeting the complement system could be a promising approach to reduced radiation-induced gastrointestinal toxicity thereby increasing the therapeutic window of radiotherapy.

Keywords: Radiation, Innate immunity, Complement system

B091 / Natural killer cells and cytotoxic T lymphocytes are required for successful combination immunotherapy in xenograft model of human lung adenocarcinoma

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A well-recognized mechanism for tumor evasion is the induction of immune checkpoint molecules (e.g. PD-1, CTLA4) which prevent efficient T cell-mediated tumor recognition, and render local immune responses to malignant cells ineffective. We developed a patient derived xenograft (PDX) model of human lung adenocarcinoma by engrafting freshly resected, treatment naive tumors, into lymphopenic Nod/Scid/IL2Rgc-deficient (NSG) mice. Tumors successfully engrafted, and tumor derived immune cells expanded in about 70% of recipient NSG mice to varying degree. Using this translational model, we

1) identified potential pathways of immune cell exhaustion in lung tumors;

2) pharmacologically reversed or blocked pathways of interest to evaluate their effects on tumor growth mediated by reconstituted tumor-derived immune cells;

3) demonstrated that tumor rejection in treated animals requires the presence of immune cells; and

4) using antibody-mediated depletion or functional blockade, we identified syngeneic immune cells that were rescued from tumor exhaustion by immunotherapy. Specifically, we demonstrate that Interleukin 15 (IL-15) mediated cytokine stimulation, in addition to PD-1 specific checkpoint blockade, can elicit potent anti-tumor activity in previously exhausted Natural Killer (NK) cells and Cytotoxic T Lymphocytes (CTL). Tumor eradication, or reduced growth in response to anti-PD-1 and IL-15 treatment required both NK and CTL. Interestingly, therapeutic treatments with trans-presented IL-15 alone also showed beneficial anti-tumor effects in the absence of checkpoint blockade. Our findings demonstrate the plasticity of exhausted NK and CTL in the tumor microenvironment that could be rescued and reactivated in vivo to reject solid tumors. We provide evidence that in vivo IL-15 stimulation is an important activating signal for tumor specific human immune cells, and that combination immunotherapy with cytokine and checkpoint blockade provides an effective anti-tumor therapy. In addition, the PDX model can be established in a way that allows successful evaluation of immunotherapies to reverse tumor immune exhaustion using reconstituted endogenous immune cells to successfully eradicate tumor.

We acknowledge the joint participation by Diana Helis Henry Medical Research Foundation through its direct engagement in the continuous active conduct of medical research in conjunction with Baylor College of Medicine and the Natural Killer Cell Immunotherapy to Cure Lung Cancer Program (to SP and FK), the Department of Pathology and Immunology and the Department of Medicine at Baylor College of Medicine, and the Center for Human Immunobiology and the Department of Pediatrics at Texas Children's Hospital.

Keywords: Patient Derived Xenograft, Natural Killer and Cytotoxic T Lymphocyte, Combination Immunotherapy

B093 / Antigens derived from human endogenous retroviruses as potential targets for immune recognition following epigenetic therapy for hematological malignancies

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There is increasing interests in understanding the effect of combination therapy in the field of checkpoint inhibition. More recently the combination of epigenetic modulation and checkpoint inhibition has been explored. Epigenetic modulation may open the epitope landscape by increased expression of antigens normally tightly regulated through epigenetic silencing. Among such antigens are human endogenous retroviruses (HERV) that comprises up to 8% of the human genome. Here we have studied 77 of these HERV that has been shown to be transcribed in human tissues Treatment with DNA methyltransferase inhibitors (DNMTi's), 5-azacytidine (5-aza-CR) and 5aza-2'-deoxycytidine (5-aza-CdR), for higher risk myelodysplastic syndromes (MDS) have shown clinical benefits. DNMTi's have been shown to up-regulate ERV transcription in vitro. Here we evaluate if treatment with DNMTi's would induce specific T cell recognition of ERV, and form potential valuable antigens for immunotherapy strategies. To identify ERV derived immune recognition, we generated a library of 1250 ERV

derived potential antigenic peptides restricted to most abundant MHC class I molecules in Caucasian population. Peripheral blood mononuclear cells (PBMCs) from a cohort of 27 patients treated with DNMTi's for different hematological malignancies (MDS, AML, and CMML) were used to detect $CD8^+$ T cells reactive to ERV-derived peptides. We employed our recently developed T cell detection technology, which utilizes DNA barcodes, capable of assessing more than 1000 specificities in a single sample. Preliminary data suggests HERV-derived peptides are indeed recognized by T cells, both in healthy and diseased individuals. Our data suggest an overall increase in $CD8^+$ T cells reactive to ERV derived epitopes post treatment with DNMTi's. This observation correlates with the post treatment transcriptional analysis of AML patients showing upregulation in transcription of HERVs specifically in malignant cells. We will present the detailed outcome of T cell recognition of HERV derived antigens and the effect of DNMTi treatment on such responses.
Since mechanism of action for DNMTi's has not been well characterized this study would provide an insight to the role of cytotoxic T cells, and possibly provide alternate opportunities to target HERV derived antigens for therapeutic applications.

Keywords: Human endogenous retroviruses, Epigenetic modulation, Immunotherapy

B094 / A patient derived antibody targeting the tetraspanin CD9 synergistically inhibits tumor growth with an anti PD1 antibody

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Immune reactions against cancer cells can be induced by immunotherapy. We investigated the possibility that an antibody response contributed to the success of the immunotherapy of a cancer patient.
A patient with metastatic melanoma was cured by adoptive transfer of ***ex vivo*** expanded autologous tumor reactive T cells. Peripheral blood memory B cells were immortalized by ectopic Bcl-6 and Bcl-xL expression and analyzed for the presence of tumor-reactive B cells.
We isolated a B cell clone that produced antibody AT1412 which recognized a novel cell surface epitope on the tetraspanin CD9. CD9 is broadly expressed and involved in multiple cellular activities including proliferation and adhesion. Cell surface staining showed enhanced binding of AT1412 to melanoma cells as compared to healthy melanocytes indicating that the epitope is overexpressed on tumor cells. The antibody also reacted strongly with other tumor types including colon and pancreas cancer. The stronger binding of AT1412 to tumor cells is probably caused by the fact that the antibody bound preferably to a clustered form of CD9 which is known to be dependent on palmitoylation and highly expressed on metastatic tumor cells.
Strikingly, AT1412 reduced growth of melanoma tumors and blocked metastasis in melanoma xenografted mice. A very strong synergistic inhibition of tumor growth was observed in mice that were treated with AT1412 in the presence of an anti-PD1 antibody that is currently being used in the clinic for treatment of melanoma. Taken together this data suggests that the anti CD9 antibody contributed to the success of the immunotherapy in this patient. This antibody could act together with tumor-reactive T cells in eradicating circulating tumor cells and preventing settlement of metastatic tumor cells.
We addressed the question whether safety issues could prevent therapeutic use of AT1412 because in the past antibodies targeting human CD9 were found to trigger aggregation of platelets and were therefore not suitable for use in patients. However, AT1412 which has a low affinity for CD9, does not induce platelet aggregation. Together with the observation that the patient from whom the antibody was derived did not show any adverse effects that could be ascribed to the antibody these data indicate that AT1412 is safe for therapeutic use.

Keywords: Patient derived antibody, Melanoma, Combination therapy

B097 / 2 Gy total body irradiation enables effective tumor-directed T helper 1 cell based immunotherapy by modifying the immune cell composition, inducing a proinflammatory environment, and prolonging adoptive cell persistence

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Adoptive cell therapy (ACT) using tumor-directed $CD4^+$ T cells induces potent antitumoral activity in several tumor entities. Clinical and preclinical studies revealed therapeutic benefit, when ACT is combined with preparative lymphodepleting regimes such as cyclophosphamide or total body irradiation (TBI). In the endogenous pancreatic islet tumor RIP1-Tag2 mouse model ACT with tumor antigen-specific Tag-TH1 cells is efficient exclusively after 2 Gy TBI. The impact of local irradiation on tumor environment and immune responses has been investigated extensively. Aim of our study was to uncover the systemic effects of preparative TBI on the host immune system as well as on the biodistribution patterns of adoptively transferred Tag-TH1 cells.
To investigate 2 Gy TBI-induced changes in myeloid and lymphoid cell populations, we analyzed blood, thymus, spleen, and lymph nodes by flow cytometry on different time points for up to 28 days in irradiated and non-irradiated C3H mice. PET/CT imaging using radiolabeled CD3 monoclonal antibodies (^{64}Cu-DOTA-CD3-mAbs) was performed for visualization of endogenous T cells non-invasively ***in vivo***. In addition, we followed homing patterns of adoptively transferred Cy5-labeled Tag-TH1 cells by optical imaging and conducted consecutive ***ex vivo*** flow cytometry as well as multiplex cytokine arrays of the sera.
2 Gy TBI induced profound lymphodepletion in line with weight reduction of spleens and lymph nodes by 50 %. Fractions of $CD4^+$ T cells increased in relation to $CD8^+$ T cells and B cells, whereas myeloid cells showed least radiosensitivity. Tag-TH1 to endogenous $CD4^+$ and regulatory T cell ratios increased significantly, although the absolute number of Tag-TH1 cells remained unchanged in blood and lymphatic organs. Biodistribution analysis of intraperitoneally injected Tag-TH1 cells 1 day after TBI yielded enhanced accumulation in the liver and lung as well as prolonged persistence in the peritoneal cavity. Proinflammatory and TH1-associated cytokines such as IL-1, IL12, IL-15, IL-17, TNF, G-CSF, and CXCL-11 increased after combination of ACT and TBI in a synergistical manner.
In summary, 2 Gy TBI induces profound changes in the immune cell composition impressively increasing the Tag-TH1 to endogenous $CD4^+$ cell ratios. Moreover, TBI provokes the persistence of adoptively transferred Tag-TH1 cells by enhanced migration into non-lymphoid tissue and promotes the release of proinflammatory cytokines. Thus, TBI generates an ideal milieu for effective antitumoral immune responses and might represent a beneficial tool for ACT in the clinical setup.

Keywords: Total body irradiation, T cell therapy, systemic immunity

B098 / Inhibition of tumor growth by cancer vaccine combined with metronomic chemotherapy and anti-PD-1 in a pre-clinical setting

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Tumor microenvironment (TME) is characterized by multiple immune suppressive mechanisms able to suppress anti-tumor effector cell immunity. Combinatorial strategies, including vaccine and immunomodulatory drugs, needs to be developed for improved immunotherapy efficacy.
A novel combinatorial approach was assessed in C57BL/6 mice injected with mouse melanoma B16F10 cells. A multi-peptide vaccine (PEPT) was combined with a low dose metronomic

chemotherapy (MCT) and an anti-PD-1 checkpoint inhibitor (CI). Statistical analysis were performed with the unpaired two-sided Student's ***t***-test and ANOVA.
Animals treated with the multi-peptide vaccine combined with MCT or CI showed remarkable delay in tumor growth and prolonged survival as compared to control groups. The multi-pronged combination including PEPT+weekly MCT+CI was able to prolong survival in all mice (100%) and inhibit tumor growth in 66.6% of mice. Re-challenged tumor-negative mice showed prolonged survival and tumor growth inhibition in 25% of mice. The anti-tumor effect was associated with strong T cell immune response to vaccine mutated peptides and significant reduction of regulatory T cells.
The combination of a vaccine with MCT and CI was highly efficient in potentiating the vaccine's anti-tumor effects. Overall, these results provide strong indications for the efficacy of combinatorial immunotherapy including a cancer vaccine, targeting mutated tumor-antigens, and immune modulatory treatments aiming at inhibiting immune suppressive cells (MCT) as well as blocking the immune checkpoints (CI). The observed results suggest that such combinatorial immunotherapy protocol can be predicted to provide an outscoring clinical outcome in early-diagnosed cancer patients and has gained momentum for translation into clinical evaluations.

Keywords: Combinatorial strategy, cancer vaccine, metronomic chemotherapy

B099 / Potent and selective C-C chemokine receptor (CCR4) antagonists potentiate anti-tumor immune responses by inhibiting regulatory T cells (Treg)-recruitment into the tumor

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Naturally suppressive $CD4^+$ $Foxp3^+$ regulatory T cells (T_{reg}) are essential for immune tolerance. Although T_{reg}-mediated suppression of effector cells is important to control inflammation and prevent autoimmune diseases, the presence of T_{reg} in the tumor microenvironment (TME) has been shown to dampen anti-tumor immune responses. Human T_{reg} express CCR4, the receptor for the chemokines CCL17 and CCL22. These chemokines are produced by tumor cells, tumor-associated macrophages and dendritic cells, as well as by effector T cells (T_{eff}). Preclinical and clinical data supports a role for CCR4-mediated recruitment and accumulation of T_{reg} in the TME which can be associated with poor prognosis. Further, recent longitudinal studies in patients receiving IO agents demonstrate an influx of T_{reg} in responding patients which may dampen optimal anti-tumor responses. Therefore, CCR4 is an ideal target to selectively block T_{reg} recruitment into the TME.
We have developed structurally unique series of small molecule antagonists of CCR4 with cellular potencies in multiple assays (e.g. chemotaxis of primary human T_{reg} in 100% serum) in the low double-digit nM range. Representative compounds are selective against other chemokine receptors, GPCRs and ion channels, including the hERG channel, and lack inhibition of common human CYP450 enzymes. Moreover, compounds have excellent in vitro and in vivo ADME properties, consistent with convenient oral dosing. In preclinical syngeneic tumor models, these CCR4 antagonists block T_{reg} migration and support expansion of activated T_{eff}. In contrast to the non-selective approach of depleting anti-CCR4 antibodies, our compounds reduce T_{reg} in the tumor, but not in peripheral tissues such as blood, spleen or skin. In preclinical efficacy studies, treatment with various checkpoint inhibitors and immune stimulators (e.g., anti-CTLA-4 or anti-CD137) induce the upregulation of CCR4 ligands. In these models, we observe enhanced tumor growth inhibition and increased tumor regressions when these agents are combined with CCR4 antagonists, without any gross toxicity. At cellular level, combination therapy with CCR4 antagonist and IO agents reduced T_{reg} number and increased number of activated and total T_{eff}, resulting in an increase in the intratumoral ratios of both, $CD4^+$ and $CD8^+$ T_{eff} to T_{reg}.
Combination therapy with CCR4 antagonist and IO agents overcome T_{reg}-mediated suppression in tumors and tip the balance toward tumor rejection.

Keywords: CCR4, Treg recruitment, Combination therapy

B100 / PRIMMO study: combining PD-1 blockade, radiation and immunomodulation to tackle cervical and uterine cancer

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Background: Immunotherapeutic approaches have revolutionized the field of cancer therapy but are less abundantly evaluated in gynecological malignancies.
For HPV-positive cervical cancer, HPV-targeting vaccines and adoptive T cell therapies are in development. Given the high expression of PD-L1, immune checkpoint blockade is also being evaluated for cervical cancer. The overall response rate to Pembrolizumab treatment in cervical cancer (Keynote-028) was however only 17%.

For endometrial cancer, the overall response rate to Pembrolizumab treatment was 13%, but recent data indicate that immune checkpoint inhibitors are particularly efficient in the MSI- and POLE-mutated subtypes, which is however a minority of advanced endometrial cancer patients.
Thus, although the results are promising, it is clear that, like in other tumor types, only a subset of patients responds to immunotherapy.
Besides, gynecological tumors are also known to exert immunosuppressive effects on the microenvironment, by inducing acidification (Warburg effect), expressing/secreting immunosuppressive molecules and attracting immunosuppressive cells, which might hamper the effectiveness of immunotherapy.
Therefore, we hypothesize that a combinatorial treatment approach to tackle the tumor and its microenvironment at different fronts is of utmost importance.
Study set-up: We designed this study in patients with recurrent or refractory cervical carcinoma, endometrial carcinoma or uterine sarcoma wherein will be combined:

1. A cocktail of repurposed drugs with immunomodulatory properties consisting of vitamin D, curcumin, aspirin, lansoprazole and low-dose cyclophosphamide to tackle immunosuppressive players in tumor-induced immunosuppression.

2. Radiotherapy to one lesion to induce immunogenic cell death, release of (neo)antigens and T cell priming, thereby acting as an ***in situ*** vaccine

3. PD-1 blockade to release the brakes of the immune system

We anticipate that this combinatorial treatment regimen will work synergistically to induce and sustain the anti-tumor immune response, resulting in tumor regression.
The main study endpoints are clinical response, progression-free and overall survival and quality of life. Besides these clinical endpoints, this study is accompanied by an extensive exploratory research program to evaluate the effects of the combinatorial treatment on the anti-tumor immune response (both in blood and tumor), the induction of immunogenic cell death and the secretion and nature of extracellular vesicles. We believe that these analyses will help to shed light on the mode of action of this innovative combination treatment and identify possible predictive or prognostic biomarkers.

Keywords: PD-1 blockade, Radiation, Immunomodulation

B101 / Cytotoxic T cell-mediated delivery of nanoparticles as a new approach to cancer therapy

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One of the hurdles in cancer immunotherapy is the existence of a suppressive tumor microenvironment which favors tumor growth. Many nanoparticle (NP) strategies are currently under investigation to address this issue. However, achieving favorable biodistribution resulting in sufficient NP infiltration in the tumor bed remains challenging. Using liposomes as a model nanoparticle, we demonstrated that NPs can be covalently coupled to the surface of T cells. By exploiting the intrinsic capability of activated T cells to infiltrate into the tumor mass, this strategy aims to enhance the accumulation of these hitchhiking NPs in the tumor bed, targeting pro-tumorigenic immune cells. Importantly, we showed that the attached NPs do not compromise important T cell functions such as proliferation, cytolytic activity and cytokine production ***in vitro***. In addition, by stimulating T cell proliferation with either IL-2 or IL-15, we could induce distinct T cell phenotypes, which affected T cell size and the absolute amount of NPs coupled. Following these results, we developed an siRNA-loaded NP that can be coupled to the surface of cytotoxic T cells. Furthermore, we successfully demonstrated the uptake of T cell-bound NPs by bone-marrow-derived dendritic cells ***in vitro*** using co-culture experiments. Based on these results, we aim to further optimize this strategy to achieve effective delivery of siRNA-NPs to modulate the suppressive tumor microenvironment.

Keywords: Adoptive T cell therapy, Nanoparticle, siRNA

B102 / Radiotherapy induces directional migration of Natural Killer cells via induction of CXCL8

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Adoptive Natural Killer (=NK) cell therapy has shown promising results in the treatment of leukemia. However, solid tumors frequently do not respond, an event attributed to their low permissiveness for NK cell infiltration. Radiotherapy (=RT) is an emerging combination partner for immunotherapy. While RT increases leukocyte infiltration in preclinical models, its effects on NK cell infiltration remain poorly understood. In this study we

evaluated RT as a potential means to increase NK cell infiltration. For this purpose we used Boyden chamber migration assays to analyze the effect of tumor cell irradiation on NK cell migration *in vitro* and analyzed chemokine expression by ELISA. We show that irradiation of human melanoma cells triggers the release of soluble factors, which lead to directional migration of NK cells *in vitro*. This effect was partially mediated by CXCL8, which was released by irradiated melanoma cells in a radiation-dose dependent manner. We show that radiation-induced CXCL8 specifically attracts the highly cytotoxic CD56dim subset of NK cells, which expresses the cognate CXCL8 receptors CXCR1 and CXCR2. Thus, we identified a novel mechanism by which radiotherapy can enhance migration of cytotoxic NK cells. Although CXCL8 is known to induce metastasis and invasiveness of melanoma cells, these adverse effects could be overcome when RT is combined with adoptive NK cell transfers. Tools for enhancing NK cell migration into solid tumors are desperately needed and radiotherapy-induced CXCL8 release presents an approach that warrants further investigation.

Keywords: Natural Killer, Radiation, IL-8

NEW AGENTS AND THEIR MODE OF ACTION

B103 / Self-recognition of Alu duplex RNAs is the basis for MDA5-mediated interferonopathies

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Melanoma **D**ifferentiation **A**ssociated **G**ene-5 (MDA5) is an innate immune receptor that senses viral long double stranded RNAs (dsRNAs) and initiates type I and III interferon signaling cascade thereby playing a vital role in antiviral immunity. Recently, a number of mutations were identified in MDA5 that lead to its aberrant activation and have been implicated in various autoinflammatory conditions including Aicardi-Goutières syndrome. The mechanistic basis of this constitutive MDA5 activation, however, has remained elusive. An understanding of the subtle balance of self vs. non-self discrimination by MDA5 is important especially in the context of recent reports demonstrating the targeted activation of MDA5 as a potential therapeutic strategy against diverse carcinoma. Our work revealed a hitherto unknown role played by the RNA-rich cellular environment in preventing aberrant MDA5 activation by imposing cooperative filament assembly on dsRNAs as a functional requirement for signal activation. We further show that the disease-causing gain-of-function (GOF) mutants of MDA5 can form signaling-competent filaments on endogenous RNA populations comprising mainly of Alu RNA duplexes. Strikingly, under physiological conditions, the wild type MDA5 is not activated by Alu RNAs because of its sensitivity to structural irregularities such as bulges and mismatches commonly occurring in Alu:Alu hybrids. The GOF mutants, on the other hand, show reduced sensitivity to disruptions in duplex RNA structures. In order to demonstrate the critical role played by Alu transcripts in MDA5-mediated inflammation, we employed a novel cell-free approach to show that specifically depleting Alu RNAs from the cellular pool remarkably compromised its ability to stimulate MDA5 GOF mutants. Overall, the work reveals the underlying mechanism behind MDA5-mediated inflammatory disorders. Moreover, it highlights the unique role played by Alu RNAs as an evolutionary tether on MDA5 keeping its affinity towards "self" ligands under check during the course of evolution.

Keywords: MDA5, innate immunity, Alu

B104 / AT1413 BAIT: A T-cell engaging antibody targeting a unique sialylated epitope on CD43 overexpressed in acute myeloid leukemia

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From the B cell repertoire of an acute myeloid leukemia (AML) patient in long-term remission, we recently identified the antibody AT1413. AT1413 binds CD43s, a unique sialylated epitope on CD43, which is overexpressed on AML cells. Besides its therapeutic potential as a naked antibody, AT1413 provides an interesting candidate for a bispecific T-cell engaging antibody.
Bispecific T-cell engaging antibodies have been clinically validated as a powerful tool for harnessing the cytotoxicity of polyclonal T cells against a hematologic cancer. Simultaneous binding to a cancer surface antigen and the T-cell surface protein CD3ε mediates cancer cell recognition independent of the T-cell receptor specificity. As a result, T-cell activation and cytotoxicity towards the cancer cell are triggered.
To investigate CD43s as a target for a T-cell engaging antibody, we generated AT1413 BAIT (**B**ivalent **A**ntibody **I**mmune **T**herapeutic). First, the antibody Fc-region was modified to abolish Fc-receptor interaction. Second, the bispecific was assembled by linking the modified AT1413 IgG to two single chain variable fragments (scFv) against CD3ε using a combination of site-specific enzymatic and chemical coupling. In a sortase-catalyzed transpeptidation reaction, complimentary click handles were introduced at the C-termini of the IgG heavy chains and the scFv and subsequently joined in a cycloaddition reaction. With this approach, the stability and bivalency of the native IgG was maintained, molecular engineering was minimized and a format readily accessible for further antibody candidates was created.
AT1413 BAIT was confirmed to bind both AML cells and CD3ε-expressing Jurkat cells. *In vitro*, AT1413 BAIT successfully induced T-cell mediated cytotoxicity against different CD43s expressing AML cell lines. Endothelial cells that have a detectable but considerably lower binding capacity for AT1413 remained unaffected. Besides cytotoxicity, T-cell activation, as indicated by upregulation of activation markers CD69 and CD25, release of IFN-γ and T-cell proliferation were observed in the presence of AML cells. AT1413 BAIT was tested *in vivo* in a mouse model with human immune cells (human immunesystem (HIS) mice). Newborn NSG mice were injected with human hematopoietic stem cells and, after establishment of a human hematopoietic system, inoculated with AML cells. Upon tumor cell engraftment, the mice were dosed with AT1413 BAIT or control BAIT at 2 mg/kg and showed, in case of AT1413 BAIT, a very strong inhibition of tumor growth but no effects on normal human hematopoietic cells.
Taken together, our results indicate that CD43s is a potential new target for T-cell engaging antibodies. AT1413, consequently, does not only provide a promising new antibody candidate against AML, but also holds therapeutic potential in a bispecific, T-cell engaging format.

Keywords: T-cell engagement, Acute myeloid leukemia, Bispecific antibodies

B105 / Oxidants inactivate murine melanoma and increase immunogenicity

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Metastatic melanoma is an aggressive type of skin cancer. Palliation often involves electro chemotherapy and recently advances have been made with photodynamic therapy, both involving oxidants. We here extend on this approach by utilizing cold physical plasma, an ionized gas that expels a multitude of reactive oxygen and nitrogen species.
Murine metastatic B16F10 melanoma cells were treated with plasma in vitro. Viability was investigated using metabolic activity testing. Cell viability, motility, expression of the junctional protein zonula occludens 1, cell cycle, and growth kinetics were monitored using high content imaging. Biomechanical properties were assessed with real-time deformability cytometry. Expression of MHCI, calreticulin, and MC1R were followed with flow cytometry. Plasma oxidized melanoma cells and decreased their metabolic activity. Cell death was seen within an hour after plasma treatment in a caspase-independent fashion. Accordingly, cell growth was impeded and nuclear inflation was observed. Cell motility was decreased upon plasma treatment, which was accompanied by an increase of cytosolic zonula occludens 1 protein and an increase in cellular stiffness. Finally, plasma treatment upregulated ecto-calreticulin on the cell surface and increase expression of membrane-bound MHCI molecules.
These results suggest that treatment with cold physical plasma can kill melanoma cells and increase their immunogenicity with potential relevance for tumor immunology. In vivo studies are underway to refine relevance and mechanisms of this new technology.

Keywords: Melanoma, Calreticulin, Cold physical plasma

B106 / Bi-specific TCR-based reagents and their detailed *in vitro* preclinical package

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A novel class of bi-functional protein therapeutics has been engineered by Immunocore to overcome the poor affinity of T cell receptors (TCRs) towards tumour self-antigens, and the extremely low surface epitope densities on cancer cells. These soluble mTCR are known as ImmTACTM molecules (immune mobilizing monoclonal T cell receptors against cancer), as they are able to circumvent the limitations of the natural TCR repertoire and break tolerance in the tumour microenvironment.
ImmTAC molecules are formed of a monoclonal TCR which recognises HLA-restricted tumour-associated antigens on cancer cells with picomolar affinity, and an anti-CD3-specific scFv which redirects the host's polyclonal T cells towards these antigens, resulting in a potent anti-tumour response.
As both the TCR and the anti-CD3 portions are entirely human specific, the assessment of safety and potency of these drug candidates is based on a broad range of molecular and cellular in ***vitro*** assays. Our preclinical package is designed to characterize ImmTAC molecules' efficacy and ascertain their specificity. To test ImmTAC potency we use HLA-appropriate antigen-positive tumour cells, whilst to characterize their safety profile we use HLA appropriate normal cell lines that represent a wide range of human tissues. In addition, we use extensive molecular analysis tools to predict unwarranted off-target effects. IMCgp100 is now in phase II clinical trial for patients with advanced malignant melanoma (cutaneous and uveal). We describe here the assays used in our extended preclinical package.

Keywords: ImmTAC, immunotherapy, soluble TCR

B107 / Blocking IL-2 signal with an IL-2 mutant reduces tumour growth through the control of regulatory T cell

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Interleukin 2 (IL2) is critical for peripheral tolerance mediated by the Regulatory T cells, which represent an obstacle for effective cancer immunotherapy. It has been long hypothesized that therapies blocking IL-2 signals will weaken Tregs activity promoting immune responses, although IL-2 is also important for effector T cell function. The latter has been partially tested using anti-IL-2 or anti-IL-2R mAbs, and showing some antitumoral effects, which cannot be exclusively attributed to the block of IL2 signal in vivo. In this work we pursue an alternative strategy. We design antagonist IL2 mutants, which conserve the capacity to bind to the α and β chains of the IL-2R but not to the γc chain having therefore drastically affected its signaling capacity. These mutants inhibit IL2-dependent T cells proliferation and Treg differentiation in vitro. Moreover in vivo treatment of mice leads to a slower tumor growth and to less accumulation of Tregs. A mathematical model is used to better understand the effect of these mutants and to suggest suitable strategies to improve their design as potential drugs. Overall our results shows that it is enough to inhibit transiently IL2 signaling to bias helper and regulatory T cells balance in vivo towards immunity. The obtained IL-2 antagonist or an improved version could be useful for cancer inmunetherapy based on Regulatory T cell inhibition.

Keywords: Regulatory T cells, Interleukin 2 mutant, cancer therapy

B108 / Dendritic cell-targeted AcTaferon: generic and safe type I interferon-based cancer immunotherapy, synergizing with immune checkpoint blockade and yielding anti-tumor immunity

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An ideal cancer immunotherapy should mobilize the immune system to destroy tumor cells selectively, without harming healthy cells, and remain active in case of tumor recurrence. Furthermore, it should be readily at hand off-the-shelf and preferably not rely on tumor-specific markers, as these are only available in a limited set of malignancies. Immunotherapeutics include antibodies, cellular therapies and cytokines. Despite approval for the treatment of various cancers, clinical application of cytokines is severely impeded by their systemic toxic side effects. Type I interferon (IFN), for instance, is approved for the treatment of several hematological and solid cancers. However, best results are generally obtained with the highest doses, bordering on the maximally tolerated dose. To curtail cytokine toxicity, we are developing "AcTakines" (**A**ctivity-on-**T**arget cyto**kines**), which are optimized (mutated) immunocytokines, with drastically reduced affinity for their cognate ubiquitous receptors when not targeted, but up to 1000-fold more potent on targeted cells than on non-targeted cells.
As conventional Dendritic Cells (cDC) are essential for IFN antitumor efficacy, we targeted type I IFN-derived "AcTaferon" to Clec9A^{+} cDC. Clec9A-AcTaferon therapy displayed strong antitumor activity in murine melanoma (B16), breast carcinoma (4T1) and lymphoma models (A20), as well as against human RL

lymphoma in immunodeficient NSG mice reconstituted with a human immune system. In sharp contrast to WT mIFN therapy, the antitumor efficacy of Clec9A-AcTaferon was not accompanied by any toxicity, assessed via body weight and several hematological parameters. Clec9A-AcTaferon effects were lost in CD8-depleted or Batf3-/- mice, and depended on IFN signaling in cDCs but not in T lymphocytes, evaluated using CD11c- or CD4-IFNAR-/-, respectively. Combined with a-PDL1 immune checkpoint blockade, Treg-depleting a-CTLA4 + a-OX40 therapy, immunogenic chemotherapy, or low-dose TNF, complete tumor regressions and long-lasting tumor immunity (memory) were obtained, still without any adverse effects. Our findings thus indicate that DC-targeted AcTaferons provide a novel, highly efficient and safe cancer (immuno)therapy, with no need for tumor-specific markers or ***ex vivo*** manipulations, and possible application in a broad range of malignancies.

Keywords: Interferon, dendritic cell, combination therapies

B109 / Study of B-cell antigen receptor trafficking in primary cells

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B cells display B cell antigen receptor (BCR) on their surface to detect environmental antigens and, upon binding, initiate immune responses that produce protective antibodies. Steps that link BCR behavior to humoral immune responses are important to achieve protective and long-lasting immunity. Alternatively, dysregulation of BCR operation can contribute to development and propagation of B cell cancers. Historically, the properties of the BCR in primary B cells have been studied mostly for cells that produce immunoglobulin-M (IgM), while little is known about the behavior of BCRs on primary B cells of other Ig isotypes, such as IgG1. Fundamental questions of relevance to B cell cancer biology, such as the extent to which BCR is spontaneously internalized in primary B cells and whether BCR recycles to the cell surface following internalization remain incompletely characterized. The role of the unfolded protein response (UPR) in regulating trafficking of Ig isotypes other than IgM is similarly under studied. In this work I use transnuclear mouse models to provide primary B cells of a defined isotype and antigen specificity, such as B cells that start out expressing BCR of the IgG1 isotype that bind ovalbumin (OB-1 mice). I subsequently crossed these mice with a mouse line harboring a B-cell-specific knockout of the transcription factor Xbp-1 (Xbp^{KO}), which is essential for execution of the UPR in B cells. Preliminary analysis of the impact of Xbp-1 deficiency in OB-1 B cells indicates that, in contrast to IgM producing B cells, there is only a minor attenuation in IgG1 production and secretion. This finding is of critical importance for current efforts to identify agents that inhibit Xbp-1 to treat multiple myeloma or other cancers. I have also made use of a mouse model derived from the OB-1 line (OB-1 κ mice), in which BCRs amenable to site-specific modification via sortase-catalyzed ligation were created using Cas9-CRISPR. Using this method BCR can be labeled without use of antigen or crosslinking Ig reagents, thus avoiding activation, which is induced by most conventional approaches for labeling BCR. I have used B cells from the OB-1 κ model to track unactivated, cell surface IgG1 BCR in live cells labeled with a self-quenched, protease-activated fluorescent probe in real-time. Preliminary findings indicate that the internalization of BCR in the presence or absence of antigen is observable in real time by fluorescence microscopy. A comparison of the internalization kinetics and endosomal routing for BCR on primary B cells in the presence or absence of various stimuli and for different Ig isotypes will provide a useful starting point for understanding the role of BCR internalization in B cell cancers.

Keywords: B cell receptor, Unfolded protein response, Immunoglobulin isotype

B110 / The OX40-CTLA-4 bispecific antibody, ATOR-1015, induces immune activation and anti-tumor effect

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ATOR-1015 is an OX40-CTLA-4 bispecific immune activating antibody, developed for tumor-directed immunotherapy. The compound was generated by fusing a high affinity CTLA-4 binder, derived by FIND® optimization of CD86, to an agonistic OX40 antibody derived from the human antibody library, ALLIGATOR-GOLD®.
ATOR-1015 binds both targets simultaneously resulting in cell-cell interactions, which is expected to enhance the immuno-stimulating effect of the compound. The mode of action of ATOR-1015 is thought to be a combination of effector T cell activation and regulatory T cell (Treg) depletion, which is supported by cell-based ***in vitro*** studies. The efficacy and potency of the bispecific compound demonstrate superiority compared to the monospecific counterparts, either alone or in combination, in an ADCC reporter assay.
In vivo, treatment with ATOR-1015 reduces tumor growth and prolongs survival in syngeneic tumor models in human OX40 transgenic mice. Further, the ***in vivo*** data supports the tumor-directed mode of action of ATOR-1015, demonstrating an increase in the intratumoral effector T cell/Treg ratio without affecting systemic T cells. The compound has excellent developability properties and is currently in the second phase of production, process development, and is planned to enter clinical trials in 2018.

Keywords: OX40, CTLA-4, Bispecific

B111 / Introducing ImmTAC™ Reagents: IMCgp100, a novel TCR-based bi-specific agent targeting malignant melanoma

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Immunotherapeutic strategies that activate T cells to attack cancer cells have the potential to eradicate tumors. Efficient T cell-mediated clearing of cancer cells is limited by the ability of T cells to recognize tumor-associated antigens (TAAs). The expression of TAAs is not limited to tumor tissue and as T cells that express T cell receptors (TCRs) with a high affinity to self-antigens are removed during immune system development, self-tolerance to TAAs is common. T cell recognition of cancer cells is further compromised by downregulation of HLA (human leukocyte antigens) on the cancer cell surface, and establishment of an immunosuppressive environment. To overcome these limitations and harness the power of T cells in cancer therapeutics, Immunocore Ltd has developed ImmTAC reagents (immune-mobilizing monoclonal TCRs against cancer); a new class of soluble bi-specifics with the ability to re-direct the immune system to destroy tumor cells in a potent and specific manner. ImmTAC reagents comprise an affinity enhanced TCR, recognizing cancer peptide-HLA, fused to an anti-CD3 effector domain that engages and activates a T cell killing response.
The process of generating ImmTAC reagents is complex but can be described in a few essential steps. Firstly, T cell clones recognizing validated cancer antigens are isolated from donor blood and the TCR-encoding sequences identified using a single cell sequencing approach. To confirm antigen binding, TCR α and β chains are expressed in ***E. Coli***, co-refolded ***in vitro***, and their binding to the target peptide:HLA complex tested by Surface Plasmon Resonance. The affinity of the TCR is enhanced up to a million-fold by mutagenesis of the complementarity determining regions using phage display. The TCR is then fused to an anti-CD3 domain and an additional di-sulfide bond is introduced to ensure stability of the molecule in solution. Finally, the ImmTAC molecule undergoes a range of biochemical and cellular assays to confirm its potency and specificity. Our lead ImmTAC, IMCgp100, has been engineered to possess a picomolar affinity for the peptide $gp100_{280-288}$, a well-characterized melanoma-specific TAA, in complex with HLA-A2. Extensive pre-clinical ***in vitro*** studies have shown that IMCgp100 specifically redirects T cells against melanoma targets expressing the $gp100_{280-288}$/HLA-A2 complex. A first in human clinical study with IMCgp100 has demonstrated a favorable safety profile for the therapeutic. These clinical data also show evidence of T cell mobilization, cytokine release and durable tumor responses in both cutaneous and uveal melanoma patients. The current clinical data suggests that the ImmTAC platform is a promising new technology in the cancer immunotherapy field.

Keywords: TCR-based bi-specific, Redirected T-cell killing, Malignant melanoma

B112 / Inhibition of immune suppressive CD39 by 3rd generation antisense oligonucleotides in the tumor microenvironment

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During the last decades it became obvious that the immune system can be utilized to evoke effective anti-tumor responses. However, cancer cells develop mechanisms to circumvent this. The two ectonucleotidases CD39 and CD73 are promising drug targets, as they act in concert to convert extracellular immune-stimulating ATP to immunosuppressive adenosine. CD39 is expressed on different immune cells as well as on a range of cancer cells and the latter are recognized to co-opt CD39 for circumventing anti-tumor immune responses. Accordingly, in order to enhance immunity against tumors it would be favorable to increase extracellular ATP- and to simultaneously reduce adenosine concentrations in the tumor microenvironment. Therefore, as therapeutic strategy we designed Antisense Oligonucleotides (ASOs) to target CD39 mRNA expression in tumor- and immune cells.
The application of CD39 ASOs led to a successful knockdown of CD39 mRNA and protein expression in different cancer cell lines and in primary human $CD4^+$ and $CD8^+$ T cells without the need of any transfection reagent. Furthermore, degradation of extracellular ATP was significantly blocked by CD39-ASO on these cells when compared to treatment with control ASO. Supplementation of cell culture medium with extracellular ATP impaired proliferation and viability of CD39 expressing $CD8^+$ T cells. Strikingly, CD39-knockdown by ASO treatment -but not treatment with unspecific control ASO- successfully reversed the inhibitory effects of ATP on cell proliferation and viability.
Treatment of mice with mouse specific CD39-ASOs yielded a successful knockdown of CD39 mRNA expression in spleen and lymph nodes ***in vivo***. Moreover, CD39-ASO treatment of tumor-bearing mice significantly reduced CD39-protein expression on tumor infiltrating macrophages and T_{regs}.
The impact of CD39-ASO ***in vivo*** as monotherapy and in combination with PD-1 blockade on immune cell infiltration and tumor rejection is currently being investigated.

In summary, our data indicate that dysfunction of primary human $CD8^+CD39^+$T cells in the presence of extracellular ATP was successfully reversed by CD39-ASO treatment ***in vitro***. Strikingly, treatment of MC-38 tumor bearing mice with CD39-ASO significantly reduced CD39 protein expression on tumor infiltrated immune cells.
Altogether, our data reveal that targeting CD39 by ASOs represents a very promising state-of-the art therapeutic approach to improve anti-tumor immune responses and has great potential to ameliorate current established immunotherapies for the treatment of cancers.

Keywords: CD39 ectonucleotidase, Antisense Oligonucleotides (ASOs), Anti-tumor immunity

B113 / Inhibition of CD73 by 3rd generation antisense oligonucleotides to improve immunity against tumors

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During the last decades it became obvious that the immune system can be utilized to evoke effective anti-tumor responses. However, cancer cells develop mechanisms to circumvent this. The two ectonucleotidases CD39 and CD73 are promising drug targets, as they act in concert to convert extracellular immune-stimulating ATP to immunosuppressive Adenosine. CD73 is expressed on different types of immune cells as well as on cancer cells. Accordingly, in order to enhance immunity against tumors it would be favorable to reduce Adenosine concentrations in the tumor microenvironment. Therefore, as therapeutic strategy we designed Antisense Oligonucleotides (ASOs) to target CD73 mRNA expression in tumor- and immune cells.
Our experiments revealed a specific knockdown of CD73 mRNA and protein levels in cancer cell lines and in primary human $CD4^+$ T cells without the need of any transfection reagent. Moreover, mass spectrometry analysis of supernatants from ovarian cancer cell lines supplemented with extracellular AMP revealed a significant reduction of Adenosine concentrations by CD73 ASO treated cells, when compared to control ASO treated cells. Supplementation of cell culture media with extracellular AMP significantly impaired proliferation and viability of $CD73^+CD4^+$ T cells. Strikingly, CD73-ASO treatment reversed inhibition of proliferation and strongly improved viability of $CD4^+$ T cells. Our initial ***in vivo*** studies in mice using human/mouse cross-reactive CD73-ASOs demonstrated a successful knockdown of CD73 expression ***in vivo*** upon systemic administration with unformulated ASO. The impact of CD73-ASO knockdown ***in vivo*** on T cell mediated anti-tumor immune responses, tumor growth and survival will be subsequently analyzed in forthcoming studies.
Altogether, our data reveal that targeting CD73 by ASOs represents a very promising state-of-the art therapeutic approach to improve anti-tumor immune responses and has great potential to ameliorate current established immunotherapies for the treatment of cancers.

Keywords: CD73 Ectonucleotidase, Antisense Oligonucleotides (ASOs), Anti-tumor immunity

B114 / CIS checkpoint inhibition synergizes with activating receptors and impairs inhibitory/suppressive pathways in NK cells resulting in superior tumor immunity

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Immune "checkpoint" inhibitor antibodies have revolutionized cancer therapy and function by reactivating tumor-resident cytotoxic lymphocytes. Checkpoint inhibitors primarily block inhibitory pathways in tumor-resident T cells, however interest in other effector populations, such as natural killer (NK) cells is growing. NK cells possess an innate ability to detect cellular transformation and are key to cancer immunosurveillance, particularly in settings of metastasis or hematological cancers. NK cells are dependent on the cytokine interleukin (IL)-15 for their development and function but how this cytokine is regulated remains enigmatic. In a screen to identify suppressors of NK cell function, we discovered that the SOCS protein CIS (encoded by ***Cish***; cytokine-induced SH2 domain), is induced by IL-15 and acts as a potent intracellular NK cell checkpoint by suppressing IL-15 receptor signaling. ***Cish***-deficient NK cells are hyper-responsive to IL-15, with dramatically enhanced proliferation, survival, pro-inflammatory cytokine production and cytotoxicity ***in vitro***. Loss of CIS function ***in vivo*** results in a pool of differentiated, cycling NK cells that are pre-primed. ***Cish***-/- NK cells have a lower threshold for activation via stimulatory ligands even in the absence of IL-15 and are largely refractory to inhibition via MHC-I and suppressive cytokines. Interestingly, NK cell suppression in the tumor microenvironment was associated with NK cell differentiation towards a tissue resident phenotype. As a result ***Cish***-deficient mice display superior immune control of tumors expressing ligands to activating NK cell receptors and are largely resistant to tumour initiation and metastasis in multiple models where MHC-I expression is high and suppressive cytokines dictate tumor outcomes.

Keywords: Natural Killer cells, Cytokines, immune suppression

B115 / Mechanism of action of 4-substituted phenols to induce vitiligo and their potential as anti-melanoma agents

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Monobenzone is a 4-substituted phenol that interacts with the enzyme tyrosinase in pigmented cells and can induce vitiligo and antimelanoma immunity. 4-methoxyphenol and 4-tertiarybutylphenol have skin depigmenting effects but their ability to induce antimelanoma immunity is unknown. We here investigated a series of nine structurally-related 4-substituted phenols in vitro for their depigmenting action and evaluated their potential as melanoma immunotherapy drugs. The phenols were tested for tyrosinase inhibition, toxicity against pigmented cells or keratinocytes, quinone formation and immunizing ability. Tyrosinase inhibition was analyzed by spectrometry using the substrate L-DOPA or 3-methylbenzothiazolinone (MBTH) that eliminates false positive absorbance from converted phenols. Depletion of glutathione (GSH) was used as surrogate marker for reactive quinone formation and binding to protein thiol groups, which can cause immunogenic hapten formation. Immunization was tested by stimulating T-cells with dendritic cells (DC) loaded with melanoma cells that were pre-exposed to phenol or not, and analyzing T-cell activation by flow cytometry. Most phenols displayed both tyrosinase inhibition and quinone formation, although the levels did not correlate. Phenols with most immunizing ability also demonstrated tyrosinase inhibition and quinone formation, whereas their toxicity did not correlate to immunizing potential. Most phenols specifically induced CD8+ T-cell responses against pigmented cells, and not against keratinocytes, except for 4-tertiarybutylphenol also inducing aspecific immunity. This indicates that the induced T-cell response is melanocyte/melanoma antigen specific. T-cells stimulated with DC loaded with phenol-exposed melanoma cells, also targeted unexposed melanoma cells. This suggests that the T-cell response may act both locally against exposed skin melanocytes or melanoma cells and against melanocytes or melanoma cells at distant sites that were not exposed, e.g. systemic spread of depigmentation or melanoma regression. In conclusion, we identified the biochemical and immunologic mechanism of action of 4-substituted phenols underlying their depigmenting and vitiligo-inducing effects, which may be applicable for melanoma immunotherapy.

Keywords: immune activation, vitiligo, skin-bleaching phenols

B117 / Tumor-specific carbohydrate antigens as preferable targets for novel T cell engaging bispecific antibody constructs to reduce on-target/off-tumor toxicities

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T cell redirecting bi-specific antibodies like blinatumomab are a potent approach to treat hematological malignancies. Excellent results in the treatment of relapsed/refractory B cell acute lymphocytic leukemia led to the approval of blinatumomab by the U.S. Food and Drug Administration in 2014 and the clinical development of several other T cell redirecting bi-specifics to treat hematological malignancies.
However, positive results in the treatment of solid tumors by T cell bi-specifics (TCBs) are rare and only minor progress has been made in the last years. One of the main obstacles to the successful utilization of TCBs for treatment of solid malignancies is the availability of suitable tumor antigens. Most antigens used today are overexpressed on tumor cells but are also present at lower levels on normal healthy tissues. Therefore, clinical use of TCBs is often accompanied by strong on-target/off-tumor toxicities limiting their broad applicability and safety. To reduce effects on healthy cells, antigens that are exclusively expressed on tumor cells are of highest interest. One promising tumor antigen is TA-MUC1. TA-MUC1 is a highly tumor-specific carbohydrate / protein mixed epitope on the tumor marker MUC1 that is virtually absent from normal cells. TA-MUC1 shows a broad distribution among epithelial cancers of different origin and is also present on metastases and cancer stem cells underpinning its broad therapeutic potential. However, it was shown for different antigens that the potency of TCBs strongly depends on the distance between T cell and tumor cell. To achieve a close proximity of T cell and target cell, small antigens and membrane proximal antigen binding are highly favored for TCBs. In contrast, TA-MUC1 is present on the extracellular domain of MUC1 that extends up to 200-500 nm from the cell surface and therefore represents a quite challenging antigen for TCBs.
To address this challenge we developed a platform approach consisting of 24 different TA-MUC1 targeting TCB constructs that differ in CD3 binding affinity, CD3 and TA-MUC1 binding valencies, serum half-life, molecule size and Fc-functionality and produced them in our human expression platform GlycoExpress. By using a broad screening approach consisting of antigen binding, T cell activation, proliferation and cytotoxicity, we were able to identify a suitable TCB construct mediating strong and specific anti-tumor effects. Surprisingly, the identified construct did not mediate closest proximity of T cell and tumor cell, probably accounting for the large size of the N-terminal domain of MUC1 carrying

the TA-MUC1 epitope. These results emphasize the influence of the particular tumor antigen on TCB efficacy and highlight the importance of careful construct selection during the development of T cell recruiting bispecifics.

Keywords: T cell bispecific antibody, Tumor-specific carbohydrate antigen TA-MUC1, Construct selection

B118 / Targeting hIDO1 with 3rd generation antisense oligonucleotides for modulation of the tumor microenvironment

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Targeting the immunosuppressive shield of tumors has emerged as a promising treatment option for oncologic indications in the last years. Especially the success of antibodies targeting CTLA-4 and PD-1 in patients with different malignancies paved the way for immuno-oncology in daily clinical practice. However, despite long lasting remissions in a small subset of tumor patients the majority of patients does not respond to the currently available immunotherapies. One possible reason is the fact that tumor cells and suppressive immune cells can express a plethora of immunosuppressive factors leading to immune evasion of the tumor. One of those factors is indoleamin-2,3-dioxygenase 1 (IDO1) which is a critical enzyme in the metabolism of tryptophan. The degradation of tryptophan in the tumor microenvironment leads on the one hand to tryptophan starvation and on the other hand to the generation of kynurenines both resulting in a suppression of immune effector cells.

We designed antisense oligonucleotides (ASOs) with specificity for human IDO1 (hIDO1). ASOs were synthesized as GapmeRs with flanking locked nucleic acids to increase stability and affinity to the target RNA. The knockdown efficacy of ASOs on the mRNA and protein level was investigated in cancer cells and human immune cells without addition of a transfection reagent. The effect of hIDO1 knockdown in cancer cells on the production of L-Kynurenine and the proliferation of cocultured T cells was investigated.

We furthermore developed ASOs with specificity for murine IDO1 (mIDO1) to investigate the efficacy of ASO-mediated IDO1 knockdown in syngeneic tumor models.

We identified a subset of ASOs that resulted in a hIDO1 mRNA knockdown of >90% in cancer cell lines. Two highly potent ASOs with IC50 values in the low nanomolar range were selected for further experiments. The treatment of cancer cells as well as human immune cells resulted in reduction of IDO protein levels by >85%. Importantly, we observed a complete block in the production of immunosuppressive L-Kynurenine in ASO treated cells and IC50 values in the low nanomolar range. In line with those results, we observed a strongly increased proliferation of T cells when hIDO1 was knocked down in cocultured tumor cells. Preliminary ***in vivo*** experiments suggest that treatment of tumor-bearing mice with mIDO1-specific ASOs results in the knockdown of IDO1 in tumor cells as well as tumor infiltrating myeloid cells. In conclusion, we selected highly potent hIDO1 ASOs that efficiently knock down hIDO1 mRNA and protein in cancer cells as well as primary human cells and potently reduce the immunosuppressive capacity of cancer cells. Potent mouse specific IDO1 ASOs have been identified and will be used for ***in vivo*** efficacy studies in tumor-bearing mice. Taken together, we developed an innovative immunotherapeutic tool to block the expression of hIDO1 that will potentially improve treatment options for cancer patients in the future.

Keywords: IDO1 (Indoleamine-2,3-dioxygenase 1), Antisense Oligonucleotides (ASOs), Anti-tumor immunity

B119 / Multimeric soluble 4-1BB ligand (CD137 ligand) as a co-stimulator of T cells and NK cells for adoptive immunotherapy

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Introduction: A many-trimer soluble form of 4-1BBL/CD137L was constructed that supports the activation and proliferation of T cells and NK cells.

Background and procedures: Activated dendritic cells express 4-1BBL as a trimeric membrane protein organized in patches on the membrane. This is effectively an array of ligand molecules that, after the formation of an immunological synapse, clusters and thereby activates 4-1BB/CD137 receptors on contacting T cells. In contrast, a single-trimer soluble form of 4-1BBL is essentially inactive for this purpose. In order to make an activating soluble, many trimer form of 4-1BBL, we fused its extracellular domain to a multimerization scaffold taken from surfactant protein D (SPD). The resulting soluble protein, SPD-4-1BBL (Ultra4-1BBL^{tm}) was produced in CHO cells or 293 cells in serum-free media and concentrated by ultrafiltration.

Results: Expression of the 4-1BB/CD137 receptor was induced on CD4+ and CD8+ T cells by plate-bound anti-CD3 (Signal 1). As a Signal 2, the addition of murine SPD-4-1BBL induced IL-2 production by CD4+ T cells and IFN-gamma production by CD8+ T cells. Likewise, CD3-activated human CD8+ T cells were stimulated by the human form of SPD-4-1BBL. This protein was also active on human NK cells and promoted their proliferation following stimulation by K562 cells and co-culture with IL-2 and IL-15.

Conclusions: SPD-4-1BBL (Ultra4-1BBL^{tm}) is a soluble protein that can stimulate T cells and NK cells for adoptive cell therapy. As a cell culture media additive, it may have advantages over using irradiated 4-1BBL-expressing K562 cells to provide 4-1BB/CD137 stimulation.

Keywords: CD137, 4-1BB, 4-1BBL

B120 / Evolution of a designed nucleocapsid for non-viral targeted delivery

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Nucleic acid encapsulation is a fundamental requirement of life, elegantly solving the challenges of evolution in a complex biochemical environment by coupling genotype to phenotype and protecting genetic material. In the simplest examples, viruses use capsids to surround their genomes. While these naturally occurring systems show considerable promise for drug delivery, their evolution in complex environments to simultaneously optimize genome packaging, surviving the environment, targeting host cells, and hijacking host cells' translation machinery have resulted in delicate systems that are difficult to re-engineer for therapeutic applications. Based on simple first principles, we have created synthetic nucleocapsids (synNCs) capable of encapsulating their own RNA genome by (1) self-assembling into icosahedral capsids and (2) electrostatically packaging their genomes with a positively-charged interior surface. This synthetic system provides a "blank slate" to evolve desired properties for drug delivery, while avoiding the safety risks and engineering challenges associated with viruses. We explored the ability of synNCs to evolve by generating diversified populations in ***Escherichia coli*** and selecting for resistance to ribonuclease A and whole murine blood. The genomes of synNCs that retained the ability to encapsulate and protect their mRNAs under these conditions were recovered by reverse transcription PCR and reintroduced into ***E. coli*** to produce a new population for continued evolution. Using a combination of design and evolution, we generated synNCs that packaged >500-fold more mRNA than the starting computational design with one full-length mRNA for every 4 icosahedral assemblies, better than the best recombinant adeno-associated virus (AAV) vectors. Evolution also improved RNA recovery after a four-hour challenge in whole blood from below 1.3% to 88%, demonstrating that completely synthetic protein-RNA systems can evolve in complex environments. Most recently, we have evolved the exterior nucleocapsid surface to increase serum half-life from < 5 minutes to approximately 3 hours in living mice. We envision applications including display of multivalent vaccine epitopes and grafting targeting domains capable of delivering synNCs to specific target cells. Considerable effort has been directed at "top-down" modification of viruses to be safe and effective for delivery and vaccine applications; the ability to computationally design synthetic nanomaterials and to optimize them through evolution now enables a complementary "bottom-up" approach with considerable advantages in programmability and control.

Keywords: Delivery, Non-viral, Evolution

B122 / Transcriptional programs linking IRF7 signaling to enhanced cytolytic activity are modulated by DNA-demethylating agents in effector CD8+ T lymphocytes

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We recently showed that DNA methyltransferase inhibitors (DNMTi), such as 5-AZA-CdR, can induce IRF7 activation and Type I/III interferon signalling through double-stranded-RNA (dsRNA) mediated viral mimicry in cancer cells. For this present work, we performed a large pan-cancer analysis using TCGA data, and showed that IRF7 activation is associated with increased CD8+ T infiltration and higher cytolytic activity across multiple cancer types. Further analysis identifies the immune compartment as the main source of IRF7 signalling. Remarkably, in single-cell RNA-seq analysis from melanomas, we also find that higher IRF7-expression in CD8+ T cells is associated with higher cytolytic activity. Consequently, we used DNMTis as pharmacological modulators of IRF7 signalling in CD8+ T cells. We hypothesize that modulation of IRF7 would increase activity of T cells and thereby, increase antitumor activity. Indeed, ***ex vivo*** expansion of human CD8+ T cells in the presence of low dose 5-AZA-CdR resulted in enhanced functional potential of T cells. Remarkably, 5-AZA-CdR-treated CD8+ T cells have higher expression and production of the effector cytokines IFN-gamma and TNF-alpha, and the Granzyme B cytotoxic granules. Furthermore, ***in vitro*** co-culture of 5-AZA-CdR-treated CD8+ T cells with HER2+ target cells resulted in increased depletion of such target cells when engaging with HER2/CD3-Bispecific antibodies. Altogether, these results suggest that DNMTi could specifically modulate effector activity in CD8+ T cells. Moreover, in a syngeneic CT26 colorectal tumor model, mice that received low doses of 5-AZA-CdR developed smaller tumors and had remarkably higher CD8+ T cell tumor infiltration confirmed by immunohistochemistry. Importantly, these effects were abrogated when CD8+ T cells were selectively depleted after administering a neutralizing antibody. Altogether, modulation of the IRF7 pathway in CD8+ T cells using 5-AZA-CdR results in higher production of functional proteins, and consequently, in enhanced anti-tumor potential both in vitro and ***in vivo***.

Finally, we show that DNMTi triggers a process marked by the induction of viral mimicry in human CD8+ T cells, which also involves the activation of the mitochondrial protein MAVS. Indeed, we observed increased expression levels of endogenous retroviruses repeats in CD8+ T cells upon 5-AZA-CdR treatment. Strikingly, we found stronger induction of MAVS activation and higher IRF7 protein expression in 5-AZA-CdR treated CD8+ T cells measured by western blots. These results implicate the IRF7-associated transcriptional programme we previously discovered in tumors, is also a key mechanism by which DNMTi regulates CD8+ T cell effector function. Our collective findings highlight the potential for pharmacologic DNMTi to boost cancer immunotherapy by modulating cells within the immune compartment.

Keywords: DNA-demethylating agents, CD8+ T cell modulation, cytolytic activity

B123 / HERA-GITRL: a novel hexavalent human GITR agonist promoting anti-cancer immunity by enhancing T cell activation

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Glucocorticoid-induced TNFR-related protein (GITR, TNFRSF18, CD357), a member of the tumor necrosis factor receptor superfamily (TNFR-SF), is a co-stimulatory receptor that increases anti-tumor T cell activation. Based on the Apogenix HERA (hexavalent TNFR-SF agonist) technology platform, we created three variants of fully human hexavalent GITR ligand fusion proteins (HERA-GITRL) intended for T cell costimulatory approaches in cancer immunotherapy. Here we report ***in vitro*** and ***in vivo*** properties of our novel HERA-GITRL constructs.

HERA-GITRL proteins were expressed in CHO-S cells and purified by a two-column based lab-scale chromatographic process resulting in pure non-aggregating protein lots. Pharmacokinetic (PK) parameters were determined by ELISA of serum samples from CD1-mice and cynomolgus monkeys treated with HERA-GITRL. For functional characterization of HERA-GITRL ***in vitro***, human immune cells isolated from healthy-donor blood were profiled by multicolor flow cytometry for changes in activation marker expression, proliferation rate (CFSE) and cytokine production. Signaling efficacy of HERA-GITRL was assessed by a reporter gene assay. For the evaluation of ***in vivo*** anti-tumor effects, a mouse surrogate molecule, mmHERA-GITRL, was tested in mice.

Structural modifications led to three fully human HERA-GITRL drug candidates with unique PK properties. Terminal half-life was between 62 and 201 hours in mice and 24 and 38 hours in cynomolgus monkeys as a relevant species. HERA-GITRL was well tolerated in a pilot tolerability study in cynomolgus monkeys. In a direct ***in vitro*** comparison of trivalent GITRL and hexavalent HERA-GITRL, only HERA-GITRL showed full biological activity without additional crosslinking. Stimulation of human total T cells or purified naïve CD4+ T cells by anti-CD3 antibody was further augmented by HERA-GITRL. This effect was accompanied by increased proliferation and differentiation. Furthermore, we observed elevated levels of intracellular TNF-α and IFN-γ in naïve CD4+ T cells pre-activated with anti-CD3 that could be further increased by the addition of HERAGITRL. In line with these findings, the murine surrogate mmHERA-GITRL enhanced antigen-specific clonal expansion of CD4+ (OT-II) and CD8+ T (OT-I) cells ***in vivo***. Importantly, mmHERA-GITRL showed ***in vivo*** anti-tumor efficacy as a single agent in a subcutaneous syngeneic colon cancer model in mice.

The immuno-oncology field is currently dominated by bivalent antibodies, however, natural TNFR-SF signaling is activated upon binding of trimeric ligands followed by clustering of multiple receptors. The hexavalent GITR agonist HERA-GITRL is a member of a novel class of TNFR-SF agonists which show biological activity without the need for Fc receptor-mediated crosslinking and excellent ***in vivo*** stability while combining efficient exposure with fast-in/fast-out dynamics.

Keywords: GITR agonist for cancer immunotherapy, hexavalent TNF superfamily receptor agonist, HERA technology

B124 / Remediation of the p53/Arf and Interferon-β pathways as a novel cancer immunotherapy strategy: a gene transfer approach

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Previously we demonstrated that adenovirus meditated gene transfer of p19Arf (tumor suppressor and p53 functional partner) in combination with Interferon-β (IFNβ, pleiotropic immunomodulatory cytokine) drastically enhances death of tumor cells that harbor wild type p53. Here, in B16 (mouse melanoma) tumor vaccine models as well as in an LLC (mouse lung carcinoma) model of ***in situ*** gene therapy, we investigate the molecular mechanisms of cell death in response to p19Arf+IFNβ's cooperation and explore potential applications of this combination as a cancer immunotherapy strategy. First, applying dying B16 cells as the vaccine agent, p19Arf+IFNβ treatment up regulates IL-15, ULBP1 ligand, FAS/APO1, KILLER/DR5 death receptors and, consequently, tumor formation is strongly abrogated in hosts that present functional NK cells. Upon prophylactic vaccination, contralateral tumor challenge shows a remarkable reduction in tumor progression that is dependent on both CD4+ and CD8+ T cells and augments production of Th1 related cytokines. Strikingly, this antitumor protection was observed even when challenge was performed 73 days after vaccination as well as in a therapeutic vaccine setting. Secondly, ***in situ*** gene transfer of LLC tumors conferred significant protection against a secondary tumor challenge only when the primary tumor was treated with the p19Arf+IFNβ combination. Transcriptome analysis of these treated tumors indicated a chemotaxic signature of neutrophils and CD8+ T cells with the involvement of CCL3, CXCL3, IL-1α and IL-1β. Indeed, accumulation of CD11b+Ly6G+ neutrophils was confirmed exclusively in the p19Arf+IFNβ group and granulocyte depletion greatly impaired therapeutic efficacy.

Next, aiming to reveal the molecular mechanism of cell death, use of pharmacological mimetics could only induce cell death in the presence of an irrelevant adenovirus vector, indicating the importance of an endogenous antiviral response. Furthermore, detection of apoptosis markers were more closely correlated with p19Arf single treatment, as the addition of IFNβ activated an alternative pathway, evidenced by the detection of necroptosis mediators, Rip3 and Tnfrsf, as well as all three markers of immunogenc cell death, calreticulin, ATP and HMGB1. Lastly, seeking to potentiate the therapeutic efficacy of our strategy, we investigated association with current cancer treatments, revealing that association with cisplatin provided full tumor regression in the LLC model, CTLA-4 checkpoint blockade improved therapeutic vaccine control and remarkably, pre-treatment with p19Arf+IFNβ immunotherapy restored therapeutic efficacy of sub-therapeutic dose of doxorubicin while preserving cardiac function. In sum, we have gathered critical evidence to characterize the combined gene transfer of p19Arf+IFNβ as a promising and novel cancer immunotherapy strategy.

Keywords: Interferon-beta, immunogenic cell death, vaccine

B125 / HERA-CD137L: a novel hexavalent human CD137/4-1BB agonist promoting anti-cancer immunity by activation of CD8 T cells and induction of memory formation

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The TNF receptor superfamily (TNFR-SF) member CD137/4-1BB is an inducible costimulatory receptor mainly expressed on immune cells following activation. Binding of the cognate ligand CD137L/4-1BBL leads to receptor trimerization and activation of signaling cascades involved in expansion and survival of T cells and myeloid cells as well as memory formation and protection against autoimmunity. Recent evidence suggests the therapeutic potential of CD137 agonists for cancer therapy. The HERA technology platform developed by Apogenix is based on trivalent but single-chain molecular mimics of the TNF-SF Receptor binding domains (scTNF-SF-RBDs) fused to a silenced human IgG1-Fc-domain serving exclusively as a dimerization scaffold. This protein-design concept generates hexavalent agonists that replicate the natural receptor binding mode on a structural level. Due to their inherent high clustering capacity for their cognate receptor, the HERA compounds are true agonists and their biological activity is, in contrast to bivalent anti-TNFR-SF antibodies, independent of FcR-mediated crosslinking events. Here we report *in vitro* and *in viv*o properties of the novel HERA-CD137L.

HERA-CD137L was produced in CHO-S cells and purified by a two-step lab-scale purification process resulting in highly pure non-aggregating protein lots. PK studies in mice demonstrated a terminal half-life of approximately 75 hours indicating an excellent *in vivo* stability of the fusion protein. To study the effects on immune cells *in vitro*, T cells were isolated from healthy-donor buffy coats and stimulated with anti-CD3 antibody alone or in combination with HERA-CD137L. Using multicolor flow cytometry analysis, we confirmed that expression of CD137 increased following stimulation with anti-CD3 antibody on $CD8^+$ T cells. In accord with upregulation of the activation markers CD25, CD69 and CD45RO as marker for memory formation, treatment with HERA-CD137L enhanced proliferation of both CD4+ and CD8+ T cells, as determined by CFSE analysis. Intracellular accumulation of IFN-γ, TNF-α, Granzyme B and Perforin upon CD137 ligation was observed in CD8+ but not CD4+ T cells. HERA-CD137L treatment of THP-1 monocytes co-cultured with primary T cells also induced cytotoxic activity against multiple tumor cell lines, including colorectal and mammary, as shown in a real-time live cell analysis assay. Primary human monocytes express low levels of CD137 and differentiation to macrophages *in vitro* did not increase expression. However, pro-inflammatory cytokines such as TNF-a and the chemokine CCL4 were secreted after stimulation with HERA-CD137L.
The hexavalent CD137 agonist HERA-CD137L produced by the Apogenix HERA technology platform shows biological activity *in vitro* and triggers CD137 signaling in T cells and monocytes. Based on the *in vitro* data presented, HERA-CD137L is a promising candidate to promote anti-tumor immune responses either as single agent or in combination with other IO-compounds.

Keywords: CD137/4-1BB, HERA technology, TNF receptor superfamily

B126 / HERA-CD40L: a novel hexavalent human CD40 agonist promoting anti-cancer immunity by enhancing activation of B lymphocytes and antigen presenting cells

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CD40 is a member of the TNF superfamily of costimulatory receptors (TNFRSF), which are mainly expressed on cells of the immune system, but also by tumor cells. TNFRSF signaling complexes induced by binding of the cognate TNFSF ligands consist of clusters of trimers. The HERA technology platform developed by Apogenix generates hexavalent fusion proteins targeting the TNF-receptor superfamily with high clustering capacity for the cognate receptor independent of Fc-receptor-mediated crosslinking, which

is a major advantage over bivalent antibody-based drugs. The efficacy of different CD40 agonist formats, including the novel hexavalent HERA-CD40L, trimeric CD40L and multiple "agonistic" antibodies, and the functional consequences of differential receptor clustering was compared.
Primary human B cells and monocytes were isolated from buffy coats and expression of activation markers upon CD40 ligation was analyzed by multicolor flow cytometry. In all ***in vitro*** assays, direct comparison of bivalent anti-CD40 antibodies with a trivalent CD40L and the hexavalent HERA-CD40L demonstrated the superiority of the hexavalent compound. CD40-activated B cells showed upregulation of CCR7, CD86 and CD95, while primary monocytes acquired an M1-like phenotype in the presence of HERA-CD40L. Treatment of previously ***in vitro*** generated M2-macrophages with HERA-CD40L led to re-programming to M1-like macrophages, which stimulated enhanced proliferation of naïve T cells in direct allogenic co-cultures. HERA-CD40L was also the most potent inducer of IκBα degradation and NF-κB activation in Ramos B cells. Real-time cell analysis (xCELLigence) demonstrated that T cells acquired cytotoxic activity in direct co-culture, dependent on cell-cell contact, with Ramos B cells in the presence of HERA-CD40L.
A murine surrogate HERA-CD40L molecule was tested in a mouse OT-I assay and the syngeneic MC38-CEA mouse tumor model. The data showed that murine HERA-CD40L enhanced the ovalbumin-antigen-specific OT-I CD8+ T cell response without affecting endogenous T cells. Additionally, single agent activity of murine HERA-CD40L reduced the growth of MC38-CEA tumors, indicating a functional anti-cancer immune response in this model.
The hexavalent CD40 agonist HERA-CD40L produced by the Apogenix HERA technology platform triggers CD40 signaling in B cells and in cells of the myeloid lineage and shifts the M1/M2 balance towards proinflammatory conditions. The CD40-induced activation of B cells and macrophages further promotes proliferation and activation of T cells which results in an efficient anti-cancer immune response. Unlike bivalent anti-CD40 antibodies or trivalent CD40L-based agonists, the hexavalent HERA-CD40L generates highly clustered signaling complexes and thus exhibits superior biological activity over other agonistic formats without the need for Fc-receptor-mediated crosslinking.

Keywords: CD40 agonist, HERA technology, Hexavalent TNF superfamily receptor agonist

B127 / Activin-A: a novel regulator of anti-tumor immunity in lung cancer

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Activin-A is a cytokine that exerts both beneficial and detrimental effects on immune responses depending on the spatiotemporal context. Activin-A is over-expressed in patients with lung cancer and associated with metastasis; still, its role in the development of T cell-mediated anti-tumor responses remains unexplored.
Our findings demonstrate a significant increase in activin-A serum levels in lung tumor bearing mice, accompanied by enhanced expression by bronchial epithelial cells at the tumor site. Notably, therapeutic administration of activin-A in distinct mouse models of lung metastasis, induced a marked regression in cancer progression, evidenced by macroscopic, PET/CT imaging and histological studies, concomitant with a greatly extended overall survival. Activin-A anti-tumor effects were associated with enhanced infiltration of IFN-γ, TNF-α and IL-17 secreting $CD4^+$ T effector cells in lung tumors and decreased frequencies of $Foxp3^+$ Treg and myeloid derived suppressor cells. Moreover, activin-A treatment significantly decreased the expression of the immune checkpoint inhibitors, PD-1, CTLA-4, Lag3 and Tim3, among lung TILs. Mechanistic studies demonstrated that T cells were essential in mediating activin-A anti-cancer effects, as administration of activin-A in lung tumor bearing ***Rag-1***$^{-/-}$ mice, abolished tumor regression. Finally, combination therapy of activin-A along with sub-optimal concentrations of immunogenic drugs, enhanced mouse survival and potentiated $CD8^+$ T effector functions, as reflected by heightened expression of granzyme B and perforin, accompanied by significantly increased cytotoxic functions against lung tumor cells ex vivo. Collectively, our studies reveal a novel role for activin-A in boosting anti-tumor T cell-mediated responses that may be beneficial for the combat of lung cancer progression.

Keywords: activin-A, anti-tumor immunity, lung cancer

B128 / Functional evaluation of SF-25 IgE and IgG1 isotypes as novel candidates for cancer immunotherapy

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Background: Monoclonal antibody therapeutics for cancer are designed with Fc regions of only one antibody class, namely IgG. Building upon previous and on-going studies, we hypothesize that the unique properties of the IgE class antibodies: a) the high affinity to their receptors and b) their natural retention in tissues will result in more effective antibodies for the treatment of solid tumours. SF-25 IgE, an engineered chimeric antibody, recognizes a tumour antigen expressed on the surface of tumour cells with low or no expression on non-malignant tissues.
Methods: SF-25 IgE antibody was initially produced from the Sp2/0 hybridoma cell line and its ability to trigger *in vitro* degranulation of basophils was measured via β-hexosaminidase release. SF-25 IgE direct effects on tumour cells were tested via colony formation and MTS assays.
SF-25 IgG1 and IgE were then cloned in pVitro1, expressed in Expi293™ cells and purified through Protein A and K-select columns, respectively. Functional assays to investigate SF-25 IgE/IgG1 ability to a) bind to IgE and IgG1 receptors, b) bind to SF-25 antigen on target cells and c) induce antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (ADCP) were analysed by flow cytometry.
Results: SF-25 antigen expression was detected on a broad panel of tumour cell lines, including high expression on melanoma, colorectal, pancreatic and ovarian cancer cell lines. Low or no expression was detected on non-tumour cells. The direct effects of SF-25 IgE were tested on multiple target cell lines, showing that it does not affect proliferation nor their clonogenic ability. Functional studies demonstrated that SF-25 IgE was able to trigger basophil degranulation when cross-linked via anti-IgE antibodies or by tumour cells expressing the antigen. Once we unravelled SF-25 IgE sequence, the IgE and IgG1 versions were successfully cloned, expressed and purified. SF-25 IgE and IgG1 showed the same affinity to SF-25 antigen and they both bound to FcεRs and FcγRs, respectively, on immune primary cells and monocytic cell lines. Among the three versions of SF-25 antibody (SF-25 IgE produced in Sp2/0 hybridoma, SF-25 IgE and IgG1 cloned in pVitro1 vector), SF-25 IgE pVitro1 has the highest effect in mediating ADCC and ADCP *in vitro*. The IgE versions of SF-25 antibody resulted to be anyway superior to the IgG1 counterpart in mediating ADCC *in vitro.*
Conclusions: Functional comparison between different versions of SF-25 IgE and the IgG1 counterpart showed the former to be more efficient in mediating ADCC *in vitro*. We therefore present the SF-25 IgE clone as a good candidate to investigate IgE antibodies role in the tumour microenvironment. Nevertheless, through more *in vitro* and *in vivo* on-going studies, it could represent a novel therapeutic tool for solid tumour malignancies.

Keywords: IgE, Solid tumours, Allergo-oncology

B129 / Systemic application of a TLR7 agonist proves efficient and safe for anti-cancer therapy

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Toll-like receptor (TLR) engagement activates the immune system and plays an important role in antiviral and antitumoral immunity. Among the various TLR ligands, so far only imiquimod, a TLR7 agonist has been approved for topical application. When TLR agonists were delivered systemically, disadvantageous toxicities were described in clinical studies. Here, we report a novel small molecule TLR7 agonist, SC1.2, which displays a favourable safety profile after systemic application.
Here we describe a preclinical study using SC1, a novel small molecule TLR7 agonist, against several murine tumor models. Using a therapeutic setting in the CT26 colon carcinomas model, SC1 conferred a strong anti-tumoral effect, prolonging survival and increasing the anti-tumoral immune response. We found that SC1 therapy increased frequency of tumor antigen specific (gp70) CD8 T cells detectable in blood, spleen and tumors with potent effector function, which are the major effector cells mediating tumor therapy. SC1 anti-tumoral treatment activated splenic pDC, cDC, macrophages as well as monocytes and augmented the effector immune response by increasing CD8/regulatory T cell ratio. In addition, SC1 induced activation and polarization of macrophages towards an M1 phenotype in spleen as well as in tumor by inducing an overall a change of the tumor environment favoring the recruitment of immune cells and promoting Th1 immune responses. Interestingly, a combination of SC1 treatment with anti-PDL1 antibody revealed a synergistically improved synergize improving anti-tumoral therapeutic effects, indicating promising implications of SC1 in oncological treatments.
In conclusion, these novel TLR7 agonist SC1 and SC1.2, in a non-toxic systemic regimen act as strong anti-tumoral mono-therapeutic agents, potentiating anti-tumoral immune response via CD8 T cells, indicating promising implications for treatment of several cancers entities.

Keywords: TLR, systemic application, anti-tumoral

B130 / Establishment of human monoclonal anti tumor antibodies with high affinity to CD9 using an in vitro B cell affinity maturation platform

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Introduction: Several attempts to develop an anti-CD9 antibody for multiple cancer types has failed in pre-clinical phase due to the life-threatening side effect of platelet aggregation. Here, we describe the development of an anti-CD9 antibody (AT1412) derived from a cured melanoma patient (see abstract Schotte et al.). This patient, who suffered a stage IV melanoma with brain metastases, was treated with autologous ex vivo expanded T cells and is still in remission 10 years after treatment. The antibody derived from the patient´s B cells targets a unique new epitope on CD9 with low affinity, mediates ADCC against melanoma cells in the presence of NK cells *in vitro*, affects growth of tumor cells in mouse models but in contrast to other anti-CD9 antibodies does not aggregate platelets. To investigate whether we can improve the anti tumor efficacy of the antibody that still lacks the capacity to induce platelet aggregation we generated variants using a novel affinity maturation platform.
Methods: We developed an in vitro selection technique that mimics the natural process of affinity maturation of antigen specific B cells. Primary human B cells immortalized by forced expression of Bcl-6 and Bcl-xL are B-cell receptor positive, antibody-producing germinal center like B cells. Low frequencies of AID induced somatic hypermutations in B cell clones are detected, which allows for the selection of sub clones with altered antibody binding characteristics. Sub clones of a B cell clone producing anti-CD9 antibody AT1412 were seeded and maintained for 3 weeks. Antibody containing supernatants were collected and tested for binding to CD9 by Surface plasmon resonance (SPR).
Results: Out of 800 B cell sub clones tested, nine produced an antibody with a higher affinity to CD9 as compared to the parental clone. The clones could be divided into two groups based on binding characteristics; (1) faster association and slower dissociation (5 clones) and (2) faster association and faster dissociation (4 clones). Purified recombinant antibodies were generated to confirm enhanced binding to CD9 expressing cells by flow cytometry and SPR. One antibody version combining two mutations reached similar affinity levels as commercial anti-CD9 mabs. Extensive epitope mapping of the high affinity antibody suggest that all variants recognize the same epitope as the parental AT1412 antibody. These high affinity antibodies mediate much stronger ADCC against melanoma cells than the wild type antibody but, importantly, still do not aggregate platelets in contrast to commercial anti-CD9 mabs.
Conclusion: We describe a highly efficient technology that allows for rapid generation of antibodies with modified affinities in both the heavy and light chains within 10 weeks. This method allows for establishment of more efficacious anti tumor antibodies.

Keywords: Antibody affinity maturation, Antibody screening, Tumor efficacy

B131 / Agonistic hexavalent CD27 ligand, HERA-CD27L, enhances T cell activation and induces potent anti-tumor immunity

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Key immune regulators belong to the tumor necrosis factor receptor superfamily (TNFRSF). Apogenix has developed a proprietary technology platform to develop novel hexavalent TNFRSF agonists (HERA) for cancer therapy. HERA proteins are capable of clustering six receptor chains in a spatially well-defined manner thereby inducing potent agonistic activity. In contrast, “agonistic” antibodies achieve their minimal effect through secondary crosslinking. In addition, the short half-life of HERA proteins, relative to antibodies, facilitates the fast-in/fast-out dynamic essential for combination therapy and reducing serious side effects associated with immune system overstimulation. The HERA concept has been successfully translated to HERA-TRAIL (now in Phase I), -GITRL, -CD40L, -OX40L, -4-1BBL, -LIGHT and -CD27L. HERA-CD27L was produced in CHO-S cells and purified by a two-step lab-scale purification process resulting in highly pure and non-aggregating protein lots. Human HERA-CD27L binds to purified human, mouse and cynomolgus monkey CD27 as well as primary human T cells. HERA-CD27L increased T cell activation and proliferation following all stimulatory conditions tested *in vitro*.
In addition, treated cells also upregulated expression of effector molecules such as IL-2, TNF-α and IFN-γ. Interestingly, treatment with HERA-CD27L also increased the expression of other TNFRSF members, e.g., GITR, OX40 and 4-1BB, suggesting that combination of HERA compounds might be a promising approach to further boost immune responses and anti-tumor efficacy.
The effect of HERA-CD27L treatment on antigen-specific T cell responses was assessed using the OVA-specific CD8+ “OT-I” T cell adoptive transfer model. Following a single dose of HERA-CD27L and OVA protein, serial blood samples showed a significant and HERA-CD27L dose-dependent clonal expansion of OT-I T cells. OT-I T cells expressed high levels of activation/differentiation markers, e.g., CD69 and CD44, while the endogenous T cells failed to show any response to HERA-CD27L treatment.
HERACD27L treatment produced dose-dependent tumor-growth

inhibition (TGI) in two syngeneic mouse models, MC38-CEA and CT26wt. For example, 10 mg/kg HERA-CD27 achieved 48% TGI in the MC38-CEA model. In the CT26wt model, a lower dose of HERA-CD27L (1 mg/kg) alone showed 43% TGI compared to anti-PD-1 (10 mg/kg) alone (29% TGI). Interestingly, combination therapy increased TGI to 66% highlighting the importance of both T cell activation and checkpoint inhibition in anti-cancer immunity. Finally, pilot studies in mice and cynomolgus monkeys demonstrated that HERA-CD27L was well tolerated. In addition, treatment with the murine surrogate HERA-CD27L did not result in anti-drug antibody development in mice even after three weeks of twice weekly administration.
Altogether, HERA-CD27L shows potent anti-tumor efficacy, is well tolerated by multiple relevant species and the lead candidate is currently being prepared for GMP cell line development.

Keywords: CD27, HERA technology, TNF receptor superfamily

B132 / Programming antigen-presenting dendritic cells from fibroblasts

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Cell fate reprogramming of adult cells towards pluripotency or unrelated somatic cell-types has been demonstrated and explored in the context of regenerative medicine and cell replacement therapy. Dendritic cells (DCs) are professional antigen presenting cells (APCs) specialized in the recognition, processing and presentation of antigens to T-cells, inducing adaptive immunity. We hypothesized that the unique properties of DCs could be induced by cell reprogramming to allow the direct control of immune responses.
Here, the requirements to induce antigen-presenting DCs were investigated using combinatorial overexpression of Transcription Factors (TFs) in Clec9a-tdTomato mouse fibroblasts. This reporter system specifically marks the conventional DC lineage. We have identified a pool of three TFs sufficient to induce reporter activation, establish DC morphology and activate a DC transcriptional program. The activation of the reporter occurs within 48 hours without cell division and its efficiency is increased by including additional TFs. Induced DCs (iDCs) express MHC-II and co-stimulatory molecules CD40, CD80 and CD86 at the cell surface, essential for antigen presentation. iDCs engulf particles, proteins and dead cells and upon stimulation of toll-like receptors, secrete inflammatory cytokines. iDCs capture, process and present antigens to CD4+ T-cells, inducing their proliferation and activation. Remarkably, iDCs have established the competence for cross-presentation, a hallmark of DCs, eliciting antigen-specific CD8+ T-cell responses. Finally, transduced human dermal fibroblasts also acquire DC morphology, expression of human DC surface markers and competence to engulf beads, proteins and dead cells.
Hence, we provide evidence that antigen presentation can be programmed in unrelated cell-types by a small combination of TFs. Collectively, our results provide insights into DC specification and cellular identity. The generation of APCs by direct reprogramming opens avenues for inducing immune responses with autologous-engineered cells. This strategy may give rise to the development of powerful cancer immunotherapies.

Keywords: Antigen-presenting cell, Dendritic cell, Cellular reprogramming

B133 / Cancer immunotherapy using glycolipid-modified *Listeria monocytogenes* in a partially humanized mouse model

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Listeria monocytogenes (Lm) is a gram positive bacterium associated most often with gastrointestinal infections caused by the consumption of contaminated food. ***Lm*** initially infects epithelial cells in the small intestine, and then migrates to other organs such as the spleen and the liver, where it is taken up mainly by phagocytic cells. Once phagocytized, ***Lm may then be processed in the phagosome to generate peptides presented on MHC Class II for activation of Lm***-specific $CD4^+$ T cell responses. ***Lm*** can also escape the phagosome, leading to secretion of protein antigens into the cytosol. These are processed and presented by MHC Class I molecules to $CD8^+$ T cells, generating strong protective immunity that can clear infection and prevent subsequent re-infection. These features of Lm have been exploited for its use as a vaccine vector, particularly for the delivery of tumor-associated antigens. Our previous work showed that the efficacy of Lm as an anti-tumor therapeutic vaccine could be significantly improved by incorporation of synthetic glycolipid activators of CD1d-restricted invariant Natural Killer T cells (iNKT cells) into the bacteria. Using an attenuated Lm strain expressing the tumor-associated antigen Mage-B, this approach gave improvements in prevention of metastasis in the 4T1 breast carcinoma model in BALB/c mice, and also gave modest reductions in the size of primary tumors. Currently, our efforts are directed at further improving this approach to more effectively target primary tumors, and also at validating the effects in a more humanized experimental model. To this end, we are using a human CD1d knock-in mouse line

in which the mouse CD1d protein has been replaced by human CD1d. These mice have diminished percentages of iNKT cells closely resembling what is seen in most humans, and thus mount iNKT cell responses that are comparable to those seen in humans. We found that incorporation of iNKT cell activating glycolipids into Lm leads to improved iNKT cell-dependent immune responses in hCD1d knock-in mice, and induces synergistic anti-tumor immune mechanisms against implanted primary syngeneic tumors. In addition, administration of iNKT cell activating glycolipids in association with Lm did not induce anergy or exhaustion of the responding cells, thus enabling repeated treatment to boost anti-tumor immune responses

Keywords: Listeria monocytogenes, iNKT cells, Antigen specific responses

B134 / Novel small-molecule inhibitors of ecto-nucleotidase CD73 promote activation of human CD4$^+$ and CD8$^+$ T cells and have profound effects in experimental tumor models

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Extracellular adenosine (ADO) is a potent inhibitor of T cell activation, able to induce an immunosuppressed phenotype. The intra-tumoral generation of ADO depends on the coordinated and sequential cleavage of extracellular ATP by the two ecto-nucleotidases CD39 and CD73. Various anti-CD73 antibodies are being studied clinically but only a limited number of potent and selective small-molecule CD73 inhibitors, such as those presented here, have been described.

Methods: CD73 activity was assessed using a malachite green or AMP-glo assay. The ability of CD73 inhibitors to reverse AMP-mediated immune suppression was determined by adding exogenous AMP during CD8$^+$ T cell activation. Proliferation, CD25 and IFN-g expression were determined. DC/T cell mixed-lymphocyte-reaction (MLR) assays were conducted by culturing differentiated monocyte-derived DC with CD4$^+$ T cells; proliferation and CD25 expression were assessed. Two-way total PBMC MLR were performed by co-culturing PBMC from different donors. The ability of CD73 inhibitors to limit AMP hydrolysis *in vivo* was determined by quantifying plasma AMP:ADO ratios from mice dosed with various dose levels of CD73 inhibitor. Levels of CD73 inhibitor, AMP and ADO were determined by mass spectrometry. A syngeneic CT26 mouse tumor model was used to assess the effects of CD73 inhibitor.

Results: A001421 is a first-in-class, highly selective, reversible small-molecule inhibitor of human (IC_{50} = 40 pM) and mouse (IC_{50} = 1 nM) CD73. CD73 was expressed on human CD8$^+$ T cells (naïve and memory), memory CD4$^+$ T cells, B cells, DC and a very small fraction of circulating T_{reg}. AMP:ADO conversion by CD4$^+$ and CD8$^+$ T cells was inhibited by A001421 with IC_{50} values of 6 and 5 pM, respectively. Addition of AMP or ADO strongly suppressed IFN-g production and CD25 expression by activated CD8$^+$ cells *in vitro.* Furthermore, AMP also inhibited proliferation of CD8$^+$ and CD4$^+$ T cells. Addition of A001421 potently rescued the AMP-induced inhibition for both cell types. AMP is also able to inhibit cytokine production in an allogeneic CD4$^+$ T cell activation setting. A001421 rescued the AMP-induced inhibition of IL-2 production in a DC/T cell MLR and effectively reversed the AMP-mediated immune suppression seen in a two-way PBMC MLR. Administration of A001421 to mice resulted in elevated AMP:ADO ratios that paralleled plasma concentrations of drug. Therapeutic dosing of mice with CD73 inhibitor in combination with anti-PD1 antibody produced robust tumor growth inhibition and resulted in a profound shift of the TIL towards greater inflammatory phenotype.

Conclusions: A001421 is representative of a novel class of selective, potent, and reversible small-molecule CD73 inhibitors that effectively block the generation of ADO from extracellular ATP, reverse the ADO-driven inhibition of human and mouse T cell activation, and display promising anti-tumor activity when dosed in combination with PD-1 blockade.

Keywords: ATP:Adenosine axis, CD73, immune suppression

B135 / AB928, a potent, dual $A_{2a}R$ / $A_{2b}R$ small molecule antagonist for the treatment of cancer

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In the tumor micro-environment, extracellular adenosine (ADO) is abundant, as a result of extracellular ATP/NAD hydrolysis. The potent immunosuppressive effects of ADO are mediated by 2 receptors: $A_{2a}R$, which leads to impaired activation/proliferation of T and NK cells, and $A_{2b}R$, which induces a suppressive phenotype in myeloid cells such as dendritic cells (DC) and macrophages. Thus, pharmacological inhibition of ADO's effects has recently generated much interest in immuno-oncology, although, thus far, only repurposed $A_{2a}R$ antagonists, initially developed for CNS indications, have been studied in clinical oncology. AB928 is a selective, CNS-excluded and highly potent small molecule inhibitor of both $A_{2a}R$ and $A_{2b}R$, which is slated to enter the clinic in 2017.

Methods: The potency of compounds against human $A_{2a}R$ and $A_{2b}R$ was quantified by compound-dependent decreases in cAMP levels induced by NECA, using CHO cells stably over-expressing either of

these G_s-coupled receptors. DC/T cell mixed-lymphocyte-reaction (MLR) tests were conducted by culturing differentiated monocyte-derived DC with $CD4^+$ T cells. Cytokine release, proliferation and CD25 expression were assessed after 4 days. The ability of AB928 to reverse ADO-mediated suppression of human $CD8^+$ T cells (proliferation, CD25 expression and cytokine production) was determined following anti-CD2/CD3/CD28 activation. Inhibition of $A_{2a}R/A_{2b}R$ activation in monocytic cell populations was assessed in human whole blood by quantifying the extent of NECA-driven CREB phosphorylation by flow cytometry.

Results: AB928 inhibited NECA-mediated $A_{2a}R$ and $A_{2b}R$ activation with a potency (K_B) of 1.4 nM and 2.0 nM, respectively, and it maintained potency in the presence of human serum. AB928 fully reversed the ability of 12 µM ADO to suppress $CD8^+$ T cell activation as indicated by proliferation, CD25 and cytokine expression. DC generated in the presence of ADO exhibited a significantly reduced capacity to stimulate $CD4^+$ T cells in a DC/MLR assay; the inhibitory effect was restored by AB928. In a whole blood assay AB928 inhibited ADO receptor activation and resulting CREB phosphorylation with a potency (IC_{50}) of 66 nM. AB928 retains its potency against mouse $A_{2a}R/A_{2b}R$ and has demonstrated anti-tumor activity in syngeneic mouse tumor models. AB928 displays excellent oral bioavailability in multiple species, with characteristics suitable for human dosing that will allow 90% target inhibition between dose intervals. A receptor coverage assay was developed to assess target engagement for clinical applications.

Conclusion: AB928 is a potent and selective antagonist of the $A_{2a}R$ and $A_{2b}R$ receptors with the ability to block the various immunosuppressive effects of high concentrations of ADO on both receptors. AB928 differs from other known $A_{2a}R$ antagonists based on its dual mode of action, its minimal loss of potency due to non-specific binding to plasma proteins, and its lack of brain penetrance.

Keywords: Adenosine receptor A2aR/A2bR, immune modulation, Dual receptor antagonist

B136 / Immunologic reshaping and therapy of cancer by stimulation of innate nucleic acid sensor RIG-I

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We describe a novel immunotherapy approach in which the viral defense system is harnessed to stimulate anti-tumor immunity. Cancer immunotherapy has revolutionized oncology in recent years, yet many tumors become resistant or do not respond to current treatments such as checkpoint inhibitors. Stimulation of the innate immune system opens a new therapeutic strategy that could be combined effectively with other immunotherapeutic regimens. The ubiquitously expressed cytosolic RNA receptor retinoic acid inducible gene I (RIG-I) recognizes double-stranded RNA bearing a 5´-triphosphate. Its activation induces apoptosis preferentially in tumor cells and simultaneously activates the innate immune system via type I interferon (IFN) signaling. We developed an optimized, fully synthetic oligonucleotide, designated RGT100, which is a RIG-I selective ligand. RGT100 activates the RIG-I pathway leading to the induction of Th1-dominated cytokines, including IFN-a and IFN-b. The treatment of tumor-bearing mice with RGT100 encapsulated in a delivery device demonstrated potent anti-tumor activity in a variety of tumor models. Histological and flow cytometric analysis of the tumors revealed infiltration and activation of immune cells after RGT100 treatment. Treatment of tumors by intratumoral injection led to efficacy of both the treated tumors as well as untreated contralateral tumors. Furthermore, systemic delivery of RGT100 was efficacious against both local subcutaneous B16 melanoma as well as its lung metastases, as well as a variety of other tumors. Data support both natural killer (NK) cell-mediated and T cell-mediated anti-tumor activities, and resistance to tumor re-challenge has been demonstrated. Immunologic characterization of the tumors and draining lymph nodes after therapy reveals expression of a type-I IFN signature and parameters of immunogenic cell death as well as markers of local activation and cytotoxic activity of NK- and T-cells. In summary, Rigontec's RIG-I-selective ligand RGT100 shows strong anti-tumor activity in several clinically relevant mouse tumor models and routes, while bearing an advantageous safety profile. RGT100 has entered clinical evaluation in advanced cancer patients in Q1 2017, and will be clinically evaluated for single-agent activity as well as in combination with checkpoint inhibitors.

Keywords: Nucleic Acid-based Immune Therapy, Innate immune receptor RIG-I, Combination Therapy

B137 / Novel hexavalent OX40 agonist HERA-OX40L promotes T cell activation and expansion

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The tumor necrosis factor receptor superfamily (TNFRSF) member OX40 (CD134, TNFRSF4) plays an important role during T cell activation by delivering co-stimulatory signals. Stimulation of OX40 by its natural ligand OX40L (CD252) has been shown to support T cell survival as well as memory formation. Additional data suggest that OX40 engagement expands tumor-reactive T cells and hampers regulatory T cell activity within the tumor tissue, thereby promoting anti-tumor immunity.

The HERA technology platform developed by Apogenix generates fully human hexavalent TNFSF fusion proteins that mimic the natural receptor binding mode in order to co-stimulate T cells. HERA ligands are pure agonists whose signaling capacity does not rely on secondary Fc-receptor crosslinking. Here we report for the first time the ***in vitro*** and ***in vivo*** properties of a novel HERA-OX40L construct.

Similar to all HERA fusion proteins, HERA-OX40L has been engineered as a perfect molecular mimic of the natural ligand with high clustering capacity for the cognate receptor. The core unit consists of a single chain polypeptide comprising the three minimal OX40L-subsequences necessary for folding into a functional trivalent receptor binding domain (RBD). Fusing a silenced IgG1 Fc-domain as a dimerization scaffold to the C-terminus of the RBD resulted in a fully human, hexavalent fusion protein termed HERA-OX40L.

HERA-OX40L was expressed in CHO suspension cells followed by a lab-scale purification process including affinity chromatography and SEC-based polishing, resulting in homogenous, aggregate-free protein lots. HERA-OX40L was proven to bind the human but not the murine receptor. As assessed by means of a specific ELISA assay using serum samples from a PK study in CD1-mice, the terminal half-life of the compound was 93.4 hours, indicating good ***in vivo*** stability.

In order to test biological activity, T cells were isolated by magnetic sorting from human PBMCs and treated with HERA-OX40L ***in vitro***. Flow cytometric analysis revealed that HERA-OX40L significantly enhanced proliferation of naïve $CD4^+$ T cells following stimulation with anti-CD3 antibody, as determined by CFSE dilution, while showing weaker effects on $CD8^+$ T cells. In line with these results, $CD4^+$ T cells exhibited a more activated and differentiated phenotype ($CD25^+$, $CD45RA^-$, $CD45RO^+$). Moreover, $CD4^+$ T cells revealed strikingly enhanced production of IL-2 and the effector cytokines IFN-γ and TNF-α following stimulation in the presence of HERA-OX40L.

In summary, the unique hexavalent design of HERA-OX40L enabled the compound to enhance activation and expansion of human $CD4^+$ T cells as well as to boost production of effector cytokines independent of secondary crosslinking events. Being a true OX40 agonist differentiates HERA-OX40L from current antibody-based concepts and renders it an attractive candidate for cancer immunotherapy.

Keywords: TNF receptor superfamily agonist, HERA technology, OX40

B138 / Biomaterial scaffolds for recapitulating niche interactions in T-cell development

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The profound long-term deficiency in adaptive immune cells after hematopoietic stem cell transplantation (HSCT) limits its applicability as a treatment for life-threatening blood disorders. One major reason for post-HSCT T-cell deficiency is a lack of available T-competent cells, which are generated in the bone marrow. We hypothesized that the development of T-cells can be promoted by using a biomaterial-based scaffold that which specifies the T-cell lineage program in progenitor hematopoietic cells through the Notch-Delta pathway. We designed an injectable biomaterial hydrogel scaffold comprised of alginate with tethered notch ligand, which was capable of self-assembling into a bony nodule. In vitro, we observed that the generation of common lymphoid progenitors (CLPs) from hematopoietic stem cells was enhanced by the scaffold, in which the generation of CLPs was dependent on the degree of DLL-4 functionalization. Mouse and humanized mouse models were used to evaluate the biomaterial scaffold in vivo. Histology, flow cytometry and RNA sequencing were used to assess T-cell reconstitution. The hydrogel concentrated transplanted cells and induced a bony nodule . Within the hydrogel, CLPs derived from transplanted cells selectively expanded. The hydrogel accelerated reconstitution of T-cells, increased thymic output, and diversified the T-cell receptor, relative to animal that received just the transplant. In humanized mice, the hydrogel significantly accelerated human T-cell reconstitution, slowed the onset of graft-versus-host disease and prolonged survival. The results indicate that a programmable biomaterial scaffold provided cues for T-cell lymphopoiesis, accelerated reconstitution of T-cells after HSCT. Such an approach could potentially reduce HSCT associated immunological complications, and broaden its applicability.

Keywords: Biomaterials, T-cell development, Hematopoietic stem cell transplant

B139 / Cell penetrating peptide restoring p53 activity for cancer therapy

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Tumor suppressor p53 plays pivotal roles in many physiological processes and its inactivation is closely related to many types of cancer. Reactivation of p53 has been a great interest in cancer therapeutics. Several different strategies have been developed to restore p53 activity, among those synthetic peptides are promising therapeutic agents because of their high selectivity,

efficacy, and safety. A hydrocarbon-stapled α-helical peptide has been developed to restore p53 activity by inhibiting the interaction between p53 and its negative regulator MDM2. However, insufficient cell penetration limits its efficacy ***in vivo.*** Recently, we developed a novel technology to modify this peptide to increase its stability and cell penetration efficiency. We implanted human multiple myeloma (MM.1S) and colon cancer (HCT116) cell lines in mice, and treated tumors with the modified peptide ***in vivo***. Both local and systemic treatments of the modified peptide significantly inhibit tumor growth compared to vehicle or unmodified peptide treatments ***in vivo***. Cell proliferation and MDM2 activation (p-MDM2 level) in the tumors were significantly decreased after the modified peptide treatment. Our results demonstrate that the modification of peptide increases its stability and cell penetration efficiency. Therefore, the modified peptide shows high potency to suppress the growth of human xenograft tumors. Our results show that this modification of peptide has a great potential to improve the intracellular delivery of current therapeutic peptides for treatment of human diseases.

Keywords: Cell penetrating peptide, cancer therapeutics, p53

B140 / Advanced tumors in mice are eliminated by mRNA-encoded bispecific antibodies

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The potential of bispecific T-cell-engaging antibodies is well-known and has been clinically proven by blinatumomab, a CD19xCD3 bispecific antibody (bsAb), for the treatment of relapsed/refractory acute lymphoblastic leukemia (ALL). The mechanism of action behind bispecific T-cell-engaging antibodies is to recruit cytotoxic T cells to tumor cells and induce target-dependent polyclonal T-cell activation and tumor cell lysis. A plethora of different formats has been created in the last years mostly with the goal to solve technical problems of protein engineering. Even though a number of protein-derived problems could be solved, the success of most bsAbs is still hindered by manufacturing challenges, poor solubility and short serum half-life. We hypothesized that these limitations can be circumvented by an mRNA-based approach. Therefore, we used ***in vitro*** transcribed, pharmacologically optimized and nucleoside-modified mRNA encoding for single-chain bi-$(scFv)_2$ bsAbs.

The mRNA-encoded bsAbs, so-called RiboMABs, elicited highly potent and target-specific T-cell activation and tumor target cell lysis in ***in vitro*** assays. Furthermore, by administering polymer-lipid complexed mRNA intravenously into mice we were able to ensure a sustained supply of endogenously synthesized and functional antibody at therapeutic dose levels ***in vivo***. In a therapeutic setting, advanced subcutaneous xenograft tumors established in humanized mice were as effectively eliminated by endogenously translated RiboMABs targeting different tumor-associated antigens (Claudin 6 & EpCAM) as with the corresponding recombinant bsAbs. No evidence of liver toxicity or release of proinflammatory cytokines neither by the RiboMAB nor by the modified mRNA were detected. Besides the proof for the feasibility of a treatment with mRNA encoding for bi-$(scFv)_2$, we also showed that Fab-$(scFv)_2$ encoded by two individual mRNAs results in correctly assembled and functional antibody ***in vitro.***

In summary, our data show the feasibility to generate pharmacologically active and sustained levels of RiboMAB through intravenous mRNA administration into mice. The mRNA-based approach to produce bsAbs in the patient may facilitate the development of protein-based drugs substantially.

(authors H.B., R.A.S. & R.R. contributed equally)

Keywords: bispecific antibodies, RNA-based cancer immunotherapy, drug delivery

B141 / *Ex-vivo* multicolor flow cytometry analysis to understand immune cell composition and modulation in syngeneic mouse tumor models

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Many cancer patients do not respond to immunotherapy alone, pointing out the urgent need for more effective and/or combination therapies. To get a better understanding of the mechanisms leading to full tumor regressions, we analyzed the immune cell composition in tumors and draining lymph nodes before and under treatment conditions in different syngeneic mouse tumor models. Using 17-color flow cytometry, we were able to detect up to 19 cell populations simultaneously and to define the activation status of selected cell types. Detailed immune landscaping showed, that immune cell composition varies significantly between different syngeneic mouse tumor models. This information can be used to guide the selection of models for the testing of novel cancer therapies. As an example of a mode of action analysis we show the treatment of the EMT-6 mouse tumor model with a SMAC mimetics (BI 891065) in

combination with a mouse tool anti-PD-1 antibody, which leads to tumor regressions (effective therapy). BI 891065 treatment alone, resulted in significant changes of the immune cell composition within the tumor microenvironment and the draining lymph nodes, but also in an upregulation of PD-1 on tumor infiltrating CD8+ T cells, which leads to tumor outgrowth. Additional blockade of PD-1 likely leads to reactivation of T cells and to potent and long term tumor eradication. We show that detailed multicolor flow cytometry can be used for classification of mouse tumor models and to delineate the mode of action of anti-cancer therapeutics, which in turn can guide the selection of effective combination therapies.

Keywords: Multicolor flow cytometry, syngeneic mouse tumor models, SMAC mimetics

B142 / *In vitro* characterization of immunogenic cell death induced by BI 891065, a novel SMAC mimetic

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Emerging evidence demonstrates that IAPs are critical components of immune-modulatory pathways that control innate and adaptive immunity. SMAC mimetics appear to function by inducing rapid degradation of cIAP1 (by activating the auto ubiquitin ligase activity and targeting the protein for proteasomal degradation), which results in altered immune signaling and sensitizes tumor cells to cell death by extrinsic death ligands from the immune system such as TNF-alpha, TRAIL and FasL. Accordingly, SMAC mimetics hold the promise of both facilitating tumor cell killing and stimulating the immune system to recognize and eliminate dying tumor cells. Here we report the characterization of the effect of BI 891065 on dendritic cell activation as a single agent or in combination with TNF-alpha. Also, effects on dendritic cell activation were evaluated in cultures of dendritic cells with tumors that had been pre-treated with BI 891065 or BI 891065 plus TNF-alpha. Known mediators of immunogenic cell death were analyzed in this experimental setup. In vitro treatment of dendritic cells with BI 891065 leads to dendritic cell activation, measured as increased surface levels of costimulatory and antigen-presenting molecules. As expected, expression of these markers is reduced in the presence of tumor cells. Pre-treatment of tumor cells with BI 891065 or BI 891065 plus TNF-alpha leads to an increased dendritic cell activation, only when the tumor cells are sensitive to BI 891065. When the SMAC mimetic is administered in vivo in syngeneic mouse tumor models it shows remarkable tumor regressions in combination with an anti-PD-1 antibody. We show that our SMAC mimetic leads to a potent induction of immunogenic cell death and sets up a "virtuous cycle" by potentiating dendritic cell and T-cell mediated immune responses that further promote induction of cell death. These effects are potentiated by checkpoint inhibitors, leading to long term tumor control.

Keywords: SMAC, immunogenic cell death, dendritic cells

B143 / Coalescence of nanoscale cytoplasmic signalosomes contributes to T cell receptor signaling to NF-κB

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T cell receptor (TCR) activation of the transcription factor NF-κB is a crucial determinant of effector T lymphocyte function. However, characterization of the signaling events which are further removed in space and time from initial TCR activation remains limited. We have previously demonstrated that following activation of the TCR and initial signaling events at the immunological synapse, the proteins p62, Bcl10, and Malt1 rapidly combine to form a cytoplasmic filamentous signalosome called POLKADOTS, which recruits further signaling proteins and initiates the terminal steps in activation of NF-κB. Here, we examine the fate of POLKADOTS signalosomes following initial activation of NF-κB. We demonstrate via super-resolution and confocal microscopy techniques that POLKADOTS filaments converge on the microtubule-organizing center via microtubule transport, aggregating in a peri-nuclear structure. We establish that this structure exhibits all the characteristics of a classic aggresome, including its location near the microtubule-organizing center, a "dented" nucleus, and a surrounding cage of intermediate vimentin filaments. Aggresomes are poorly understood structures thought to be depots of misfolded protein destined for degradation; however, our data suggest that the aggresomal accumulations of POLKADOTS continue to promote T cell activation. We show that the formation and maintenance of this aggresome corresponds with a secondary increase in NF-κB translocation. Additionally, this aggresomal structure prolongs both IKK phosphorylation and colocalization with Malt1, implying that the aggresome promotes IKK-mediated signaling processes. Furthermore, the POLKADOTS aggresome promotes Malt1-mediated proteolytic cleavage of NF-κB inhibitors such

as CYLD and roquin, which further promotes NF-κB activation. Together, these results demonstrate that TCR signaling pathways to NF-κB utilize the orchestrated assembly, transport and stable coalescence of nanoscale cytoplasmic signaling complexes into a classic aggresome. More broadly, our findings provide evidence that aggresomes can serve as stable platforms of signal transduction, thereby proposing a new role for these little understood structures.

Keywords: T cell receptor signaling, NF-κB, Aggresome

B144 / OX40 as a model target to investigate the importance of isotype selection in agonist antibodies

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OX40 is a member of the Tumour Necrosis Factor Receptor (TNFR) superfamily and a co-stimulatory molecule whose signalling has been shown to inhibit Treg function. OX40 has therefore attracted attention as a possible target for cancer immunotherapy: with agonist monoclonal antibodies (mAb) having the potential to both attenuate regulatory T cell (Treg) function and boost cytotoxic T cell activities. However, OX40 agonist antibodies have generally shown limited efficacy as monotherapy and it remains unclear what is the optimal antibody isotype. Here we used an agonistic mAb to human OX40 to explore the role of isotype in driving human T cell proliferation ***in vitro***, and created a novel human OX40 knock-in mouse model to address the role of IgG isotype ***in vivo***. In a human PBMC assay system, the human IgG2 isotype was capable of driving costimulation of CD4+ and CD8+ T cells. However, anti-human OX40 hIgG1 resulted in reduced proliferation of CD4+ and CD8+ T cells compared with an isotype control antibody. This reduction was dependent on Fc:FcR interactions but independent of NK cells. The OX40 hIgG1 also showed a small effect on reducing Treg numbers and proliferation which was also Fc-dependent but NK cell-independent. Adoptive transfer experiments using OT-I cells expressing human OX40 into OX40 KI mice revealed that the anti-human OX40 mAb was costimulatory as both mIgG1 and mIgG2a but only the latter showed a reduction in Tregs thus confirming that the two isotypes were capable of immune-stimulatory activity through different means. Furthermore, when human isotypes were studied in the OX40 knock-in mouse, a hierarchy of hIgG1>>hIgG2>hIgG4 was revealed in their ability to expand antigen specific CD8+ T cells and induce memory, with only hIgG1 and hIgG4 reducing Treg numbers. These studies provide insight into the mechanisms of action through which anti-OX40 agonist mAb induce their effects and the role of antibody isotype in these mechanisms.

Keywords: antibody isotype, Human OX40 knock-in model, Treg depletion

B145 / Conversion from human IgG1 to IgG2 isotype can reverse anti-CD40 antibody antagonistic activity to deliver powerful FcγR-independent agonism through ligand-like CD40 binding profiles

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Agonistic human (h) CD40 monoclonal antibodies (mAb) exert their clinical efficacy through the activation of CD40 on antigen presenting cells, a process initially reported to be dependent upon further receptor clustering provided by the co-engagement of the inhibitory FcγR2b. Subsequent studies demonstrated that a more complex interplay between CD40 epitope and IgG isotype dictates the threshold and level of agonistic activity, which presents opportunities for agonistic fine-tuning. As such, mAb targeting the membrane-distal CRD1 domain of CD40 exhibit greater agonistic activity than those targeting membrane-proximal CRD2-4 domains. Moreover, the hIgG2 isotype, through its distinct disulfide bond architecture within the CH1 and hinge regions, has been shown to impart super-agonistic activity both in vivo and in vitro. However, the precise molecular mechanism of hIgG2-mediated super-agonism remains unclear.

In this study, we used a clinically-relevant antagonistic anti-CD40 mAb, 341G2, currently in clinical development for transplant rejection, to demonstrate that isotype-switching from its current hIgG1 to hIgG2 delivers a fully FcγR-independent super-agonist that exhibits highly potent therapeutic efficacy; above that seen with CP-870,893, the most agonistic anti-CD40 mAb seen to date in clinical trials. To dissect the molecular mechanism of FcγR-independence, we employed a series of biophysical and imaging techniques. Using analytical ultracentrifugation we showed that both hIgG1 and IgG2 bound soluble CD40 at the same molar ratio, displaying identical molecular masses for the complex; but that the agonistic hIgG2 formed a more hydrodynamically favorable, compact complex with CD40, suggesting that conformational differences conferred by the Fc domain account for the functional divergence. This conformational difference is further manifested in the distinct patterns of CD40 binding observed with confocal imaging, with hIgG2 engaging distinctive CD40 clusters in both

primary and transfected cells, akin to those seen with hCD40 ligand. Thus, our study highlights the importance of engaging cell surface CD40 clusters for optimal CD40-mediated immune activation, and point to the selective targeting of these clusters for therapeutic applications.

Keywords: CD40, FcγR, agonistic antibody

CLINICAL TRIALS OF CANCER IMMUNOTHERAPIES

B146 / Profiling mutational landscape and immune cell infiltrates of follicular lymphoma successfully treated with *in situ* immunotherapy

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Mutational load and tumor T-cell infiltration correlate positively with response to immunotherapy in cancers with a high mutational burden, such as melanoma and non-small cell lung cancer. However, it is unclear whether this relation holds for cancers with lower mutation frequencies. In the present study, we aimed to correlate the mutational landscape and immune cell profiles in pre-treatment tumors to clinical responses to immunotherapy in follicular lymphoma, representing a tumor with intermediate/low mutational burden.

Whole exome and RNA sequencing was performed on paired follicular lymphoma/normal peripheral blood mononuclear cell samples from patients treated with ***in situ*** immunotherapy (LYMVAC-1, n=14, Kolstad ***et al., Blood 2015***). Briefly, patients with stage III/IV disease were treated with local radiotherapy combined with intra-nodal injections of low-dose rituximab and immature autologous dendritic cells. Thirty-six percent of the patients achieved a complete or partial response along with high frequencies of peripheral blood tumor-reactive CD8 T cells, and these frequencies correlated closely with degree of tumor reduction.

Tumor sequencing data revealed that both clinical responder and non-responder patients had similar numbers of nonsynonymous mutations in their tumor samples. However, a trend towards increased Bcl-2 gene expression was found in non-responders. We speculate that this could result in immunotherapy escape due to increased resistance to apoptosis. Immunohistochemistry of tumor sections was performed to correlate T-cell, macrophage and dendritic cell infiltration to clinical response. The data showed that clinical responders and non-responders have a similar wide range of T cell infiltrates.

Although the number of patients was limited, these data suggest that neo-antigenic load and T-cell infiltration are not important indicators of clinical response to immunotherapy in follicular lymphoma. A more detailed qualitative analysis on mutational signatures and gene expression as well as immune cell infiltration is underway.

Trial Registration NCT01926639

Keywords: Neo-antigens, Immunotherapy, Follicular lymphoma

B147 / Delivery of a dendritic cell vaccine as part of standard of care chemotherapy for patients with liver cancer - the ImmunoTACE trial

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Introduction: As a consequence of the increased prevalence of cirrhosis the incidence of hepatocellular carcinoma (HCC) is rising. We have previously demonstrated tumour-pulsed dendritic cells (DC) can induce effective T cell responses, are well tolerated and can be localized to the liver by intra-arterial infusion. Here, we describe a clinical trial delivering DC vaccination alongside standard of care chemotherapy.
Methods: Peripheral blood monocytes (Mo) are selected from apheresis products by semi-automated magnetic bead selection (Miltenyi Biotec, CliniMACS Prodigy®) before differentiation into immature Mo-DC in medium (DendriMACS™, Miltenyi Biotec) supplemented with GM-CSF and IL-4. After 5 days in culture, cells are antigen loaded with a lysate from the HepG2 cell line and matured by the addition of monophosphoryl lipid A, a potent Toll Like Receptor-4 agonist. Additionally, keyhole limpet hemocyanin is added to facilitate demonstration of functional antigen presentation. After a further 40 hours in culture, the mature Mo-DC are harvested and cryopreserved in CryoStor® CS10 (BioLife solutions). Eligible patients are pre-conditioned on day 1 and day 29 with low-dose cyclophosphamide (250 mg/m^2) to deplete regulatory T-cells before receiving DC vaccination on day 31. The initial vaccination is given directly into the hepatic artery at the time of Trans-Arterial-Chemo-Embolisation (TACE). Follow up vaccinations are given monthly for the next 3 months via a peripheral vein.
Results: To date, seven patients have received a total of 28 vaccinations without adverse event. Immunophenotypic analysis of peripheral blood samples reveals reduction in Treg frequency following cyclophosphamide induction with additional variations after TACE. Increase in tumour associated antigen-specific T-cell responses can be detected after treatment.
Conclusion: The ImmunoTACE clinical trial demonstrates the feasibility of delivering bespoke cellular therapy vaccines alongside standard of care treatment. Lessons learnt from these early studies will streamline the integration of cell-based immunotherapy into mainstream cancer treatment.

Keywords: Dendritic cell, Hepatocellular carcinoma, ImmunoTACE

B148 / IMA950 multipeptide vaccine adjuvanted with Poly-ICLC in combination with standard therapy in newly diagnosed HLA-A2 glioblastoma patients

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Immunotherapy is a promising alternative strategy for patients with glioblastoma (GBM). Here, we conducted a phase I/II trial to address the safety and immunogenicity of a multipeptide therapeutic vaccine (IMA950) adjuvanted with Poly-ICLC in patients with newly diagnosed high-grade glioma. Sixteen HLA-A2$^+$ GBM patients were included, as well as three HLA-A2$^+$ patients with anaplastic (grade III) astrocytoma. Patients received the standard of care treatment including surgery, 6-week chemo-radiation therapy with temozolomide, and 6-12 adjuvant cycles of temozolomide. Vaccines (composed of nine glioma-associated CD8 peptides and two tumor-associated CD4 peptides) were injected starting one week after the end of chemo-radiation therapy. Primary endpoints were safety and immunogenicity; secondary endpoints were OS and PFS at six and nine months. In the first six patients, peptides were injected intradermally (i.d.) and with Hiltonol (Poly-ICLC) intramuscularly (i.m.) at the peptide injection site. Low levels of vaccine-induced CD8 T cell responses were detected following this initial vaccination schedule, leading us to modify the vaccination protocol. In the modified schedule, peptides and adjuvant were mixed before injection, which was given i.m. for six patients and subcutaneously (s.c.) for seven patients, in an effort to determine the optimal injection route. Clinically, the IMA950/Poly-ICLC vaccine was well tolerated, the most common side effect being local inflammation at the injection site with mild fever. A few patients experienced cerebral edema, which was manageable with steroids. Analysis of vaccine-induced T cell responses revealed that 63% of patients responded to one CD8 peptide and 37% responded to two or more CD8 peptides. In addition, modifying the vaccination protocol resulted in detection of multipeptide responses in 46% of patients, as compared with 17% in the initial protocol. Vaccine-induced CD4 T cell responses were detected in the majority of patients and were

of Th1 phenotype. Median OS of the 16 vaccinated patients was 22.2 months (range 10 to 37 months). Overall, the IMA950/Poly-ICLC is safe, immunogenic and preliminary median OS is encouraging. Definitive clinical results and correlation with immunological data will indicate the most efficacious route of vaccination for use in further trials in glioma patients.

Keywords: therapeutic vaccine, glioma, T cell responses

B149 / Checkpoint blockade for inhibition of relapsed mesothelioma: CONFIRM: A phase III trial to evaluate the efficacy of nivolumab in relapsed mesothelioma

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Mesothelioma is an incurable, apoptosis-resistant cancer caused in most cases by previous exposure to asbestos and is increasing in incidence in the UK and beyond. The majority of patients with mesothelioma present with advanced disease and prognosis is poor. Mesothelioma therefore represents a growing health burden, but it remains under-researched and treatment options are limited. Despite a significant number of clinical studies in the second line setting, no randomized study has been positive.

To date there have been no placebo controlled randomized trials for mesothelioma using PD-L1 or PD-1 checkpoint inhibition. Early promising signals of activity relating to both PD-L1 and PD-1 targeted treatment in mesothelioma implicate a dependency of mesothelioma on this immune checkpoint, and support the development of a randomized phase III trial to evaluate the efficacy of nivolumab. CONFIRM is the first ever placebo controlled, randomized phase III trial of a PD-1/PD-L1 immune checkpoint inhibitor.

The primary objective is to determine whether nivolumab increases overall survival in relapsed mesothelioma. Secondary objectives are to determine whether nivolumab

a) increases progression-free survival,
b) increases response rate,
c) has good safety/tolerability, and
d) results in acceptable patient quality of life and cost per quality adjusted life year.

A translational study will be undertaken to determine the correlation between overall survival and
i) PD-L1 expression,
ii) mutational burden (estimated by genome-wide analysis of copy number alterations),
iii) immunotranscriptomic profile.

Co-ordinated by the CR UK Southampton Clinical Trials Unit within the Centre of Cancer Immunotherapy the trial will recruit 336 patients with pleural or peritoneal mesothelioma who have received at least two prior lines of therapy from UK sites, between March 2017 and 2021. We are also investigating opening recruitment to international sites. Patients will be randomized 2:1 (treatment: control), stratified according to epithelioid vs. non-epithelioid and center, to receive 240 mg nivolumab (anti PD-1 antibody) monotherapy or saline placebo as a 30 minute intravenous infusion. Allocation will be double blind. Treatment will be on day one of each 14-day cycle, until disease progression for a maximum of 12 months. Trial follow up will continue for 6 months after the last participant has progressed, or completed or discontinued treatment. The trial is powered (80%, with 2-sided 4% significant) to detect a hazard ratio of 0.7 using an adjusted Cox regression model (time to event) and will be analyzed using intention to treat.

This trial is funded by CRUK (C16728/A21400) and Bristol Myers Squibb (CA 209-841). Trial registrations: NCT03063450 and ISRCTN79814141.

Keywords: mesothelioma, PD-1; PD-L1, checkpoint blockade

B150 / Mutanome Engineered RNA Immuno-Therapy (MERIT) for patients with TNBC- first insights into phase I trial

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Tumor-cell-selective treatment of triple negative breast cancer (TNBC) is hampered by the lack of validated therapeutic targets like hormonal or growth factor receptors. Chemotherapy and radiotherapy is currently the standard of care and survival rates in TNBC remain poor. Approaches tailored to the patient's individual tumor signature may bring improvement. The **Mutanome Engineered RNA Immuno-Therapy (MERIT)** consortium is validating an innovative, individualized mRNA-based vaccine for the treatment of TNBC.
MERIT is a collaboration project of five European partners from academia and industry dedicated to realize a personalized approach to treatment of TNBC. The consortium has set up a clinical workflow and trial design, which covers the drug development cycle from target discovery, validation to GMP manufacturing and drug release for each individual patient. Moreover, the consortium has generated a pre-synthesized mRNA vaccine warehouse containing the most frequent shared tumor antigens in TNBC for drug supply.
The **first-in-human phase I trial** planned at sites in Germany, Belgium, Sweden and France assesses the feasibility, safety and biological efficacy of this approach. TNBC patients (pT1cN0M0 - T_xN_xM0) after surgery and adjuvant chemotherapy are allocated to one of two study arms. All patients receive eight vaccination cycles with up to three shared tumor antigens from the pre-synthesized vaccine warehouse which are expressed in their personal tumor as well as the universal tumor-associated antigen p53. Additionally, patients in ARM2 are subsequently treated with eight cycles of an on-demand manufactured MUTANOME vaccine encoding unique mutation signature of the individual patient identified by next generation sequencing. The mRNAs are administered intravenously as a nanoparticulate lipoplex formulation, which protects RNA from degradation, enhances effective uptake by antigen-presenting cells and subsequently activates innate immunity and induces highly potent antigen-specific T-cell responses. Four clinical sites are open for recruitment; >10 patients have been screened for ARM1 and the vaccinations with pre-manufactured warehouse RNAs have started. Here, we give insights into peculiarities of the established process and present first preliminary data on safety and tolerability of the vaccine and immune responses of the first treated patients. With this innovative trial concept we seek to overcome the current limitations of fixed, off-the-shelf therapeutics aiming to increase the clinical benefit for TNBC patients.

This project is funded by the European Commission's FP7 and led by BioNTech AG.

Keywords: personalized immunotherapy, mRNA vaccine, TNBC

B151 / Phase 1/2 study to evaluate safety and efficacy of an ALK inhibitor, ensartinib, and a PD-L1 antibody, durvalumab, in patients with ALK-rearranged non-small cell lung cancer (NSCLC)

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Abnormal expression of anaplastic lymphoma kinase (ALK) in malignant tissues carrying an ALK gene rearrangement is a key driver of certain types of NSCLC. ALK tyrosine kinase inhibitors have been approved for ALKrearranged (ALK-pos) NSCLC, but the development of resistance over time is universal. Several programmed cell death-1 (PD1) and programmed death-ligand 1 (PD-L1) blocking antibodies are also approved in NSCLC, but response rates are lower in non-smokers, a group which includes most ALKpos NSCLC. PDL1 is expressed in 25-80% of NSCLC tumors, both on tumor cells and within the tumor microenvironment (TME). Combining targeted therapy with a PD-1/PD-L1 checkpoint inhibitor may be important, as the effective tumor shrinkage seen with targeted therapy combined with the durable responses seen with PD-1/PD-L1 checkpoint inhibitors may result in lasting tumor control. Thus, the combination of ensartinib (ENS), a next generation ALK inhibitor, and durvalumab (DUR), a PDL1 antibody, is a promising regimen for ALK-pos NSCLC. This ongoing Phase 1/2, open-label, multicenter study (NCT02898116) is designed to evaluate the safety and preliminary efficacy of ENS + DUR in patients with histologically confirmed metastatic ALK-pos NSCLC; patients may have had prior ALK inhibitor therapy. Up to 32 patients are anticipated for enrollment. The study includes a pre-immunotherapy Run-in prior to starting the combination drug. During the Run-in, patients receive ENS monotherapy (oral, daily) for two 28-day cycles to evaluate safety; those patients with rash < Grade 2 and/or no dose reduction continue ENS therapy with the addition of intravenous DUR. In Phase 1, dose-escalation of ENS, using a standard 3 + 3 design, determines the recommended combination dosing (RCD) of ENS (starting at 200 mg, with potential escalation to 225 mg or de-escalation to 150 mg) + DUR (1500 mg every 4 weeks). Cohort initiation depends on observation of < 2/6 patients experiencing dose-limiting toxicities in the previous cohort. In Phase 2, the RCD cohort is expanded to 20 patients. The primary endpoints of the study are determination of RCD and evaluation of safety according to the National Cancer Institute Common Terminology Criteria for Adverse Events. Secondary endpoints are objective response rate at 8 and 24 weeks per RECIST 1.1 and immune-related RECIST, progression-free survival at 8 and 24 weeks, as well as overall best response, disease control rate, duration of response, and overall survival. Exploratory

endpoints include effects of initial priming with ENS on the TME and immunological responses, which are evaluated in biopsies taken prior to initiation of combination therapy. Enrollment opened 01 March 2017. As of 07 June 2017, there are 2 patients enrolled; enrollment is ongoing.

Keywords: durvalumab, ensartinib, NSCLC

B152 / E6/E7 RNA$_{(LIP)}$: A novel RNA cancer vaccine for treatment of patients with HPV16-positive malignancies

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Human papillomavirus (HPV) has emerged as a major risk factor for Head Neck squamous cell carcinoma (HNSCC) and the incidence of HPV-positive HNSCC continues to rise. Standard treatment of HNSCC given with curative intent causes substantial and long-term physical and functional impairments, but nonetheless, ~50% of patients die of their disease. Alternative treatments are urgently needed that improve survival but also to reduce treatment-associated morbidity. Both $CD8^+$ and $CD4^+$ T cells are important for viral clearance and regression of HPV-positive premalignant lesions; density of tumor-infiltrating lymphocytes (TILs) is a strong predictor of the outcome of HPV-positive oropharyngeal cancers. Consequently, therapeutic cancer vaccines hold great promise to achieve long-term disease control in HPV-positive malignancies if they generate, tumor antigen-specific and durable anti-cancer immunity that can recognize viral antigen at the tumor site. Recent advances in the systemic delivery of antigen-encoding lipid-complexed RNA (RNA$_{(LIP)}$) allow efficient targeting and body-wide RNA delivery to secondary lymphoid organs with early data supporting substantial immunological and clinical effects. With E6/E7 RNA$_{(LIP)}$, BioNTech has developed a promising immunotherapeutic approach to address a high unmet medical need. E6/E7 RNA$_{(LIP)}$ is the first systemically administrable therapeutic cancer vaccine based on two lipid-complexed RNA drug products targeting the well-characterized HPV16-derived viral neoantigens E6 and E7. Preclinical studies with E6/E7 RNA$_{(LIP)}$ demonstrate a high degree of immunogenicity with induction of antigen-specific $CD8^+$ and $CD4^+$ T cell responses in human HLA-transgenic mice. Moreover, in two independent syngeneic mouse tumor models vaccine treatment significantly increased the frequency of TILs, leading to strong tumor microenvironment polarization and consistent regression of large established tumors. Importantly, preliminary data from another currently ongoing clinical trial with a tetravalent RNA$_{(LIP)}$ cancer vaccine in patients with advanced melanoma (NCT02410733) confirm the safety and tolerability of the RNA$_{(LIP)}$ immunotherapy platform with patients experiencing mostly mild to moderate adverse drug reactions typically associated with immune activation.
Building on the preclinical data E6/E7 RNA$_{(LIP)}$ is currently evaluated in a multi-center, first-in-human phase I/II trial in patients with HPV16-positive HNSCC and other HPV16-positive cancers (Sponsor: University Hospital of Southampton NHS Foundation Trust; EudraCT No.: 2014-002061-30). Objectives of the trial are to study safety, tolerability, and immunogenicity of the novel immunotherapeutic approach. Where measurable tumor is present clinical response rate and expansion of HPV-specific TIL will be evaluated. Here we present the preclinical development of E6/E7 RNA$_{(LIP)}$, as well as the study design, recruitment plan, and treatment status of the ongoing clinical trial.

Keywords: Therapeutic cancer vaccine, HPV16-positive malignancies, E6/E7 RNA(LIP)

B153 / Intratumoral IL-15-immunotherapy to target distant tumor burden

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In transit metastatic melanoma is effectively treated with intratumoral (i.t.) IL-2-immunotherapy. However, like most local therapies, targeting multiple metastatic sites is often problematic. Another common γ chain cytokine, IL-15, facilitates dendritic cell maturation, the formation of memory T cells and imprints naive CD8+ T cells with surface molecules that heighten their ability to traffic to areas of inflammation and secondary lymphoid organs (SLOs) for primary immune activation. We asked whether i.t. IL-15 might enhance systemic antitumor immunity by increasing antigen (Ag)-experienced $CD8^+$ T cell numbers. To do this, we used the murine bilateral B16F10-melanoma implantation model, high-dimensional single cell immune profiling, and ***in vitro*** coculture assays. Strikingly, i.t. IL-15 (I) is superior to IL-2 at

controlling treated and distant non-treated tumor growth and is well-tolerated; (II) orchestrates an influx of Ag-experienced $CD8^+$ T cells to treated-tumors and distant SLOs, which were mostly of the effector and effector memory phenotypes; and (III) enhances the functional quality and reactivity of systemic $CD8^+$ T cells to tumor-associated immunogens; while (IV) beneficially having limited influence on regulatory T cell numbers. Collectively, these data demonstrate that i.t. IL-15 is superior to IL-2 at controlling the growth of established tumors, treated and distant non-treated, by increasing immune surveillance of peripheral tissues mediated in part by IL-15-programmed $CD8^+$ T cells.

Keywords: Immunotherapy, Cytokines, IL-15

B154 / A first-in-human phase I/II clinical trial assessing novel mRNA-lipoplex nanoparticles encoding shared tumor antigens for immunotherapy of malignant melanoma

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Therapeutic vaccination with tumor antigen-encoding RNAs by local administration is currently successfully employed in various clinical trials. Advancing from local to more efficient systemic targeting of antigen-presenting cells (APCs), we have developed a novel class of RNA-lipoplex ($RNA_{(LIP)}$) immunotherapeutics for intravenous application. $RNA(_{LIP})$ is a nanoparticulate formulation of lipid-complexed RNA which was engineered to selectively deliver the antigen-encoding RNA to APCs in lymphoid compartments body-wide. Efficient RNA uptake and expression of the encoded antigen by the APCs results in the synchronized induction of both, potent adaptive as well as type-I-IFN-mediated innate immune responses.

The first-in-human phase I/II dose escalation Lipo-MERIT trial (NCT02410733) conducted in four German study centers assesses the safety, tolerability, and biological efficacy of $RNA_{(LIP)}$ immunotherapy in patients with stage IIIB/C and IV melanoma. This is the first example of an intravenously administered, clinically applicable RNA-based cancer vaccine. Following selective antigen stratification on routinely collected tumor samples, eligible patients are treated with increasing doses of the tetravalent Lipo-MERIT vaccine composed of $RNA_{(LIP)}$ products encoding the shared melanoma-associated antigens NY-ESO-1, tyrosinase, MAGE-A3, and TPTE, that are administered successively within each treatment cycle.

Preliminary data obtained from >20 patients of this ongoing trial confirm the safety and tolerability of the universally applicable $RNA_{(LIP)}$ immunotherapy platform. Patients experience mostly mild to moderate adverse drug reactions typically associated with immune activation. Preliminary results from immunological assessments show a high rate of vaccine-induced immunity and indicate that multiple applications of the Lipo-MERIT vaccine lead to the ***de novo*** induction of antigen-specific immune responses and potent expansion of pre-existing immunity.

Keywords: RNA immunotherapy, malignant melanoma, liposome

B155 / Turning cold into hot - reversing non-inflamed tumor entities through therapeutic vaccination: results of a phase I/II trial in AML using a dendritic cell vaccine

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Innovative treatment options for postremission therapy of AML are needed to successfully eliminate minimal residual disease. Therapeutic vaccination with autologous dendritic cells (DCs) is a promising strategy to induce immune responses against leukemia-associated antigens (LAAs).

We have conducted a phase I/II proof-of-concept study using monocyte-derived next-generation DCs as postremission therapy of AML patients with a non-favorable risk profile in CR/CRi after intensive induction therapy (***NCT01734304***). These DCs were generated within 3 days using a TLR7/8 agonist, loaded with RNA encoding the LAAs WT1 and PRAME as well as CMVpp65 and applied intradermally up to 10 times within 26 weeks. After evaluating the safety profile within phase I (n=6), the cohort was expanded to 13 patients. DCs of sufficient number and quality were generated from leukapheresis in 12/13 cases. DCs showed high costimulatory molecule expression, IL-12p70 secretion, migration towards a chemokine gradient and antigen processing and presentation. In 10/10 vaccinated patients we observed delayed-type hypersensitivity (DTH) responses at the vaccination site, but no grade III/IV toxicities. $CD4^+$ as well as $CD8^+$ T cells accumulated at DTH sites, and TCR repertoire analysis revealed clonotype enrichment. Vaccination-specific T cell responses were detected by multimer staining and ELISPOT analysis: 7/7 patients showed responses to CMVpp65, including boosting of pre-existing T cells in CMV^+ patients and induction of a primary T cell response in CMV^- patients. 2/7 patients exhibited responses to PRAME and WT each. Interestingly, we also observed the induction of CMV-specific antibody responses in CMV seronegative patients. 7/10 vaccinated patients are still alive, and 5/10 are in complete response, with an observation period of up to 976 days.

Various strategies to further optimize immunological and clinical responses are being evaluated. In an individual treatment attempt, continuation of DC vaccinations in combination with 5-azacytidine resulted in MRD conversion. ***In vitro*** data demonstrates enhancement of IFN-γ secretion and proliferation of T cells by blockade of PD-1 and LAG-3. Inhibition of these checkpoints might therefore be a promising combination partner for further clinical studies based on DC vaccination.

Keywords: Dendritic cells, Cancer vaccines, AML

B157 / Phase 1/2 study to evaluate durvalumab ± tremelimumab + chemo(radio)therapy in patients with esophageal cancer (EC)

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Checkpoint blockade immunotherapy (IMT) has demonstrated considerable antitumor activity and is an approved therapy for some solid tumors. PDL1 expression in up to nearly half of EC suggests amenability to IMT. Durvalumab (DUR), a PDL1 antibody, and tremelimumab (TRE), a CTLA-4 antibody, block distinct inhibitory pathways in T cells, thus providing enhanced synergistic activity. Chemo(radio)therapy (C[R]T)-mediated immune priming of the tumor microenvironment, either in combination or in sequence with anti-PD-L1/CTLA-4 therapy, offers a novel therapeutic strategy in EC. This ongoing Phase 1/2, open-label study (NCT02735239) is designed to evaluate the safety and preliminary efficacy of DUR ± TRE in combination with chemotherapy (CT) in metastatic/locally advanced EC (Cohorts [COHs] A1, A2, and B) and DUR in combination with neoadjuvant C[R]T before surgery in operable EC (COHs C and D). The study consists of a safety run-in Phase 1 (COHs A1 [n=6-9] and A2 [n=6-12]) and an expansion Phase 2 (COHs B [n=14], C [n=20], and D [n=20]). Patients in all COHs have a 4week period of IMT pre-C[R]T: COHs A1, C, and D receive DUR 750 mg intravenous (IV) every 2 weeks (Q2W); COH A2 receives TRE (37.5 mg IV once on Day 1, with possible dose escalation to 75 mg) + DUR Q2W; and COH B receives IMT at recommended combination dose (RCD) from COH A1 or A2. C[R]T follows the 4-week IMT period. Phase 1 starts with COH A1 where the safety of DUR is evaluated for 4 weeks followed by 6 x 3week cycles of standard oxaliplatin (OX, 130 mg/m2 IV every 21 days) / capecitabine (CAP, 1250 mg/m^2/day, orally) with continuation of DUR during CT. After COH A1 is complete, Phase 2 expansion begins with COH C (4 weeks of DUR + 6 weeks of neoadjuvant OX/CAP and continuing DUR during CT) and COH D (4 weeks of DUR + 4 weekly IV doses of neoadjuvant paclitaxel [PAC, 50 mg/m2] / carboplatin [CAR, AUC 2] + concurrent radiotherapy [RT, 41.4 Gy in 23 fractions; 5 weeks], but no DUR during CRT). COH C and D patients undergo surgery 6-8 weeks after C[R]T; those who adequately recover from surgery within 12 weeks post-surgery may resume DUR (up to 12 doses). Concurrent with expansion of COHs C/D, a safety review determines whether to enroll COH A2, in which patients receive TRE on Day 1 in addition to the COH A1 treatments. After COH A2 is complete, a safety review determines which RCD, A1 or A2, is used for expansion of COH B (DUR ± TRE + OX/CAP). The primary objective is to assess the safety of DUR ± TRE + CT in metastatic/locally advanced EC and DUR + neoadjuvant C[R]T before surgery in operable EC. Secondary endpoints are tumor response by immune-related RECIST, progression-free survival and overall survival. Exploratory endpoints are immunological effects and genomic response correlations. As of 30 May 2017, 10 patients have been enrolled. Enrollment is ongoing.

Keywords: durvalumab, chemoradiotherapy, esophageal cancer

B158 / „INSIGHT" - An explorative, single center, open-labeled, phase I study to evaluate the feasibility and safety of intra-tumoral, intra-peritoneal, and subcutaneous injections with IMP321 (LAG-3Ig fusion protein) for advanced stage solid tumor entities

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The INSIGHT study focuses on evaluation of the feasibility and safety of intratumoral and intraperitoneal injections of IMP321 (mono-agent) for the treatment of advanced stage solid tumors as well as to generate first efficacy data on such treatment. This proof-of-concept (POC) data could build the basis for further clinical studies exploring the therapeutic potential of active immunotherapy with IMP321 by direct injection into the tumor mass or the peritoneal space.
IMP321 is a soluble form of the LAG-3 T cell surface receptor and represents a highly potent activator of antigen presenting cells (APC). It is a member of a new class of drugs known as "APC activators" (primary mode of action).
Initial studies showed that IMP321 is able to induce strong immune and clinical responses if administered by sub cutaneous (s.c.) injection as a mono-therapy or together with chemotherapy. Together with the favorable safety profile of IMP321, we were encouraged to conduct a phase I trial investigating a potential enhancement of IMP321 immune-activating effects by new routes of administration. It is well known that inadequate drug delivery to solid tumors is a major cause for treatment failure. This seems also true for the new class of immunotherapeutic anticancer agents for which - despite great advancements in specific constellations - the results of several clinical trials have been disappointing.
Up to now, IMP321 has been solely administered by s.c. injection. Here, we investigate whether a direct injection of IMP321 into the tumor tissue will be a useful option to improve anti-tumor immune response by placing the immune-activating agent in direct vicinity of immune infiltrates in the tumor bed. In contrast to s.c. injection, the resulting locally and temporarily high IMP321 concentration could additionally allow for the efficient utilization of the second part of the dual mode of action of IMP321 (prevention of exhaustion of activated T-cells). In cases of peritoneal metastases, we will furthermore explore if an intra-peritoneal therapy represents a feasible alternative by means of delivering high drug concentrations directly to tumors located in the peritoneal cavity. Of note, peritoneal carcinomatosis is still a major area of unmet need in oncology.
If patients treated in course of this study display immune and clinical responses, this POC data will build the basis to evaluate the safety and efficacy of IMP321 direct injection for treatment of the respective tumor entities in larger sets of patients.

Keywords: Cancer, Immunotherapy, intratumoral injection

B159 / Enabling reproducible biomarker discovery: a case study in immune checkpoint blockade

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Checkpoint blockade biomarker discovery requires a large number of manual tasks, including clinical data management, genomic data processing, statistical analyses, and final figure construction. In addition, data and methods are often updated during the course of an analysis. Given that analysis and manuscript drafting are commonly iterative processes, manual tasks must be repeated often to account for these updates, prompting the need for an automated way to check for differences between data and methods revisions and to regenerate an entire publication's content and figures. Further, readers should be able to interrogate a publication's data and review its analyses on-demand. We introduce a suite of publicly available*, open source tools for the reproducible execution of checkpoint blockade biomarker analyses and demonstrate their use in a recent publication (Snyder et al. 2017). All analysis code for this publication is publicly available and executable by any reader, and each result in the manuscript directly links to its supporting analysis code. Our suite of tools for biomarker discovery includes: Cohorts, Discohorts, SurvivalStan, and Hyper. The analysis is orchestrated by Cohorts, a tool for uniformly specifying, plotting and statistically analyzing both clinical and genomic patient data. For example, in a single one-line command, a Cohorts user may calculate all missense single nucleotide variants (SNVs) or neoantigens per patient, create a figure comparing durable clinical benefit (DCB) and no durable benefit (NDB) patient SNV counts, and run a Mann-Whitney U test for this comparison. In another one-line command, a user may make use of SurvivalStan, our software that runs Bayesian survival models and stores model fitting results reproducibly. Cohorts keeps track of the software and data versions that it uses to generate results, thereby ensuring reproducibility of those results. Cohorts also makes use of other tools that we have developed for genomic data analysis, such as Varcode for variant effect prediction and Topiary for tumor neoantigen prediction. Our Discohorts tool allows Cohorts users to remotely execute longer tasks and pipelines for all patients in a cohort, such as those involving genetic sequence alignment, genetic variant calling, generative model fitting, and transcript abundance quantification. These pipelines make use of our workflow management software (Ketrew) and bioinformatics pipeline

software (Biokepi), which keep track of all intermediate data and parameters used. Finally, Hyper is a Google Docs add-on that synchronizes numbers and figures in a publication with updated analysis results and optionally alerts the user of any text differences. Together, our open source tools for biomarker discovery have allowed us to reproducibly turn raw clinical and sequencing data into checkpoint blockade biomarker discovery publications.

* Software described is open source and publicly available at: https://github.com/hammerlab

Keywords: Reproducibility, Biomarkers, Checkpoint blockade

B160 / Phase I clinical study for validation of photochemical internalisation (fimaVacc) - a novel technology for enhancing cellular immune responses important for therapeutic effect of peptide- and protein based vaccines

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The aim of this clinical study is to investigate the safety and immune response of fimaVacc-based peptide- and protein antigen vaccination in healthy volunteers.

On the basis of promising preclinical results, a phase I clinical study with fimaVACC has been started in healthy volunteers. FimaVacc is a technology that in pre-clinical studies has been shown to increase MHC class I antigen presentation, leading to strongly enhanced cytotoxic- and helper T-cell responses to various types of vaccines. FimaVacc involves formulating the vaccine with a photosensitising compound (fimaporfin) and a toll-like receptor (TLR) agonist. The vaccine will be given as intradermal injections followed by illumination of the vaccination site. A phase I clinical study in healthy volunteers is on-going in the United Kingdom. The subjects will be vaccinated with models for peptide- and protein-based vaccines; HPV16 E7 peptide antigens and Keyhole Limpet Hemocyanin. Both antigens will be formulated with the TLR3 agonist poly-ICLC (Hiltonol), and up to three vaccinations will be given. Local and systemic adverse effects will be assessed, and cellular and humoral immune responses will be analyzed by ELISPOT and ELISA assays, respectively.

The principle of the fimaVacc technology will be presented, together with preclinical results showing that fimaVacc strongly enhances both cellular and humoral immune responses and improves anti-tumour effects in different clinically relevant mouse models. The design of the clinical study, and initial clinical results will be presented. Early results show that intradermal treatment with fimaVacc is tolerated. Control Group (without **fima*Vacc***) is completed with detection of immune responses measured as IFN-g ELISPOT which makes a reference for testing **fima*Vacc*** study groups.

The fimaVacc technology strongly enhances the effect of therapeutic cancer vaccines in pre-clinical models. A phase I study in healthy volunteers is currently on-going in the UK to assess the PCI technology for vaccination in humans.

Keywords: cancer vaccine, immunotherapy, Phase I study

B161 / LTX-315: A first-in-class oncolytic peptide that reshapes the tumor microenvironment - Results from an ongoing clinical phase I study

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Background: Intratumoral LTX-315 disintegrates cytoplasmic organelles with release of tumor antigens in preclinical models accompanied by increase in tumor-infiltrating lymphocytes (TILs). LTX-315 induces complete regression in several rodent models, with systemic immune responses. LTX-315 is strongly synergistic preclinically with immune checkpoint inhibitors (ICI). We are conducting a phase 1 trial to evaluate LTX-315 in combination therapy.

Methods: Patients with advanced metastatic solid tumors received injections of LTX-315 into a single accessible tumor over 6 weeks. Additional injections could be administered thereafter every 2 weeks. Biopsies of injected lesions were taken at baseline, and after LTX-315 treatment. Immunoprofiling was assessed by immunohistochemistry and Nanostring analysis.

Results: 28 patients have been enrolled. LTX-315 monotherapy was administered at doses of 2-7mg for a median of 9 weeks (range 1-33). In 50% (14/28) patients all LTX-315-related adverse events were CTC grade 1 or 2, including local erythema, flushing, pruritis and hypotension (asymptomatic). Best response in 44 injected lesions in 20 evaluable patients included: 2 complete responses, 5 partial responses (> 50% reduction), and 20 stable disease (< 50% reduction or < 25% increase)). Significant increases in CD8+ TILs occurred in 67% (14 of 21) of patients with evaluable biopsies. The

HalioDx Immune Gene Signature analysis of LTX-315 treated tumors showed clear effect on key genes (effector T cell, Th1 orientation, chemokines, and cytokines) involved in immune-mediated tumor regression. Regression in injected and non-injected lesions has been observed. Stable disease ((SD) median duration 11 weeks) in non-injected tumor lesions (CT scan (irRC criteria)) was observed in 8 of 15 evaluable patients (53%) (melanoma (4), sarcoma (3), breast (1)). There is a clear correlation of LTX-315 treatment resulting in TIL infiltration, cold to hot tumor transition as assessed by Immunosign® gene signature and clinical benefit (achievement of SD on CT scan (irRC criteria).

Conclusions: This phase 1 study demonstrates that intratumoural LTX-315 has a generally safe and tolerable safety profile resulting in increase in TILs after LTX-315 treatment. LTX-315 converts cold tumors to hot, as evidenced by Immunosign® gene expression analysis. Partial and complete regression was observed in some injected tumours and regression in non-injected tumours. Evaluation of LTX-315 in combination with ICIs in breast and melanoma is ongoing.

Clinical trial information: NCT01986426

Keywords: Oncolytic peptide, tumor microenvironment, immunotherapy

B162 / IVAC MUTANOME - A first-in-human phase I clinical trial targeting individual mutant neo-antigens for the treatment of melanoma

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The genome of cancer cells is inherently instable promoting multiple genomic alterations and epigenetic changes. This often stochastic process leads to a unique molecular profile of every given tumor. Recently, a series of independent reports revealed that neo-antigen specific T-cell responses are seminal for the clinical efficacy of immune checkpoint inhibitors. However, less than 1% of mutations appear to raise spontaneously occurring T-cell responses in the tumor-bearing patient. Accordingly, only patients with a high burden of mutations profit from currently approved therapies.

To overcome this restriction, the IVAC® MUTANOME, a highly potent personalized neo-antigen-encoding RNA vaccine approach, harnesses the individual patient's mutation profile. To this aim, the individual mutation repertoire is identified by next-generation-sequencing and 10 potentially immunogenic mutated sequences per patient are selected. These are incorporated into a poly-epitopic RNA vaccine for intra-lymph node injection that is tailored to activate and expand the individual patient's $CD4^+$ and $CD8^+$ T cells against the respective patient`s unique cancer mutanome.

A phase I first-in-human trial has been initiated in 2013 in patients with stage III and IV malignant melanoma (NCT02035956) to test this fully personalized RNA vaccine concept. The objective of this clinical trial is to study the feasibility, safety, tolerability, immunogenicity and the potential anti-tumoral activity of the IVAC® MUTANOME approach.

Notably, in each and every patient (n=13) a strong poly-neo-epitopic immune response against vaccine antigens was detected. Overall, 60% of the selected neo-epitopes elicited a T-cell response. Simultaneously, no severe adverse drug reactions were reported and indications for clinical activity were observed.

In this contribution, the multi-step manufacturing process triggered by a patient's enrollment, the study design and comprehensive immunological and clinical data from the treated patients will be presented.

Keywords: Personalized cancer vaccine, First-in-human trial, Neo-epitope encoding RNA vaccines

B163 / Immunostimulatory AdCD40L gene therapy combined with low-dose cyclophosphamide ± radiotherapy in metastatic melanoma patients

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Malignant melanoma is an aggressive tumor sensitive for immunotherapy such as checkpoint blockade antibodies. Still, most patients do not respond, and the side effects can be severe underlining the need for better treatments. CD40 is an important co-stimulatory molecule. Stimulation of the CD40 pathway to initiate anti-tumor immunity is a promising alternative. Herein, we demonstrate toxicity, tumor response and immune profiling data from melanoma patients treated with an adenovirus-based CD40 ligand gene therapy (AdCD40L).

In a phase I/IIa study, twenty-four patients with advanced melanoma who had failed standard treatments were enrolled to receive AdCD40L injections intratumorally. They were treated with AdCD40L monotherapy (cohort I, n=6) or combined with low dose cyclophosphamide conditioning to reduce systemic immunosuppressive cells and their mediators (cohort II, n=9) or low dose cyclophosphamide and single fraction radiotherapy (cohort III, n=9). The patients were monitored for 10 weeks using immunological and radiological evaluations and thereafter for survival. Cells from patients in cohort I and II were analyzed by flow cytometry while plasma samples from all the patients were analyzed by a multi-array proteomics. Radiology was performed at enrolment and repeated twice after the treatment.

Treatment with AdCD40L was safe with mild transient reactions. No objective responses were recorded by MRI, however, local and distant responses were seen on FDG-PET. In cohort II and III where conditioning with cyclophosphamide was given, a significantly better 6 month overall survival (OS) was observed compared to cohort I in which patients received no conditioning. All patients from cohort I and II had an increased Teffector/Tregulatory cell ratio post therapy. At the same time, the death receptors TNFR1 and TRAIL-R2 were significantly up-regulated post treatment. Stem cell factor, E-selectin, and CD6 correlated to enhanced OS while the levels of granulocytic myeloid-derived suppressor cells, IL8, IL10, TGFb1, CCL4, PlGF and Fl3t ligand were highest in patients with short survival. In all three cohorts, high initial plasma levels of IL12 and MIP3b correlated to OS, whereas IL8 responses post-treatment correlated negatively with survival. Interestingly, antibody reactions to the virus correlated negatively with post IL6 and pre IL1b levels in blood.

We demonstrated that AdCD40L intratumoral injections induced desirable systemic immune effects that correlated to prolonged survival in patients with advanced malignant melanoma. AdCD40L was safely administered to patients and the effect was improved by cyclophosphamide but not by radiotherapy. Immune activation profile at baseline may predict responders better than shortly after treatment. Further studies using CD40 stimulation in malignant melanoma are warranted.

Keywords: Gene therapy, Malignant melanoma, AdCD40L

B164 / INSPIRE: A multicenter randomized trial of neoadjuvant and adjuvant therapy with the IRX-2 regimen in patients with newly diagnosed stage II, III, or IVa squamous cell carcinoma of the oral cavity

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The IRX-2 biologic is an injectable cancer immunotherapy composed of physiologic levels of multiple cytokines. It activates T cells and natural killer cells and differentiates immature dendritic cells into mature antigen-presenting cells. The IRX-2 biologic is provided as part of the IRX-2 regimen, which contains cyclophosphamide, indomethacin, and zinc to support an anticancer immune response. In a phase 2a clinical trial in 27 patients with therapy-naïve head and neck squamous cell carcinoma (HNSCC), treatment with the IRX-2 regimen raised no significant safety concerns and was associated with antitumor signals thought to be immune mediated. The new multicenter randomized IRX-2 Neoadjuvant Therapy in Head and Neck SCC to Provide Immune Response Enhancement (INSPIRE) trial (NCT02609386) is designed to evaluate the safety and effectiveness of combined neoadjuvant and adjuvant therapy with the IRX-2 regimen in patients with oral cavity HNSCC, a disease known to have a disordered immune function. In the INSPIRE trial, 200 patients with stage II, III, or IVa oral cavity HNSCC will be randomized 2:1 to either the IRX-2 biologic regimen arm or the IRX-2 regimen control arm (the IRX regimen minus the IRX-2 biologic). The neoadjuvant stage of INSPIRE begins 21 days before resection, when patients in both treatment arms will receive the IRX-2 regimen each day until resection. During this period, patients in the IRX-2 biologic regimen arm will also receive 10 days of the IRX-2 biologic subcutaneously injected into their bilateral sternocleidomastoid insertion regions. After resection, patients will receive standard-of-care adjuvant radiation or chemoradiation therapy followed by adjuvant IRX-2 booster regimens at 3, 6, 9, and 12 months. During each 10-day booster

period, patients in the IRX-2 biologic regimen arm will also receive, for 5 consecutive days, the IRX-2 biologic subcutaneously injected into their bilateral deltoid regions. The primary endpoint of INSPIRE is event-free survival (EFS). Secondary endpoints include overall survival (OS) and safety as assessed by the incidence and severity of adverse events. Exploratory endpoints include changes in tumor size; histologic differences between pre- and post-treatment specimens in lymphocytic infiltration assessed by multiplex immunohistochemistry and immune cell gene signature analysis; changes in the peripheral T cell receptor profile pre- and post-treatment; and human papilloma virus status assessed by p16 protein expression. With an enrollment of 200 patients, the INSPIRE trial will evaluate the ability of the IRX-2 regimen to improve EFS and OS by inhibiting tumor-mediated immunosuppression. Analysis of the exploratory endpoints will help elucidate the biological-activity profile of the IRX-2 regimen

Keywords: Head and neck squamous cell carcinoma, Tumor-mediated immunosuppression, Cytokines

TUMOR MICROENVIRONMENT AND ITS MODULATION

B165 / Sialic acid mimetics synergize with cancer immunotherapy

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Introduction: Altered glycosylation is a hallmark of tumor cells. One of the most remarkable changes in tumor glycosylation is the upregulation of sialic acid-carrying glycans (sialoglycans) upon malignant transformation. A dense layer of sialoglycans confers resistance to apoptosis, promotes tumor cell adhesion and migration and mediates therapy resistance. More recent insights demonstrate a role for sialoglycans as potent immune modulators contributing to the immunosuppressive microenvironment and tumor immune evasion. Therapeutic strategies directed against tumor sialic acids are, however, very limited and unexplored. We set out to investigate the Sialic Acid/Siglec axis in cancer immunotherapy, applying Sialic Acid Blockade with novel rationally designed glycomimetics.
Results and Experimental procedure: Using three murine tumor models, our data show for the first time that sialic acid blockade using an inhibitory sialic acid mimetic called $Ac_5 3F_a$xNeu5Ac is feasible ***in vivo***, potently reduces tumor growth and can even result in regression of established tumors and inhibition of tumor metastasis. Our data show that blocking sialic acid expression in the tumor microenvironment leads to a dominant decrease in sialic acids on tumor cells, increased infiltration of effector immune cells and decreased numbers of immunosuppressive regulatory cells. Mechanistic studies on the mode of action of Sialic Acid Blockade confirmed a minor role for NK cells and highlight a dominant role for CD8+ cytotoxic T cells. Moreover, sialic acid blockade synergized with adoptive transfer of tumor-specific $CD8^+$ T cells and enhanced CpG immune adjuvant therapy. Results will be presented that are consistent with a dual effect of Sialic Acid Blockade on reprogramming the immunosuppressive tumor microenvironment ***and*** on the activation of antigen presenting dendritic cells and induction of potent CD8+ T cell responses.
Conclusions: Collectively, these data emphasize the crucial role of sialic acids in tumor immune evasion and show the therapeutic effects of sialic acid blockade. Furthermore, these findings identify $Ac_5 3F_{ax}$Neu5Ac as potent therapeutic prototype molecule able to create an immune-permissive tumor microenvironment for $CD8^+$ T cell-mediated tumor immunity either as monotherapy or in combination with other immune based intervention strategies.

Keywords: Tumor microenvironment, Sialic Acid Blockade, Immuno combination therapies

B166 / Hypoxia reduction sensitizes solid tumors to T cell checkpoint immunotherapy

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Tumor hypoxia predicts poor outcomes across all cancers and has long been recognized as a critical source of resistance to both chemotherapy and radiotherapy. Despite the success of T cell immune checkpoint blockade in treating melanoma, many solid tumors such as prostate and pancreatic cancer are largely resistant to αCTLA-4 and αPD-1 antibody therapy in the mouse and in man. We find that hypoxic zones of these tumors resist infiltration by T cells even in the context of robust infiltration of normoxic areas of the same tumor (e.g. in the context of T cell checkpoint blockade). Beyond this lack of accessibility to tumor-specific T cells, hypoxia drives the establishment of a highly interdependent network of immunosuppressive stromal cells. Among these, we find myeloid-derived suppressor cells, tumor-associated macrophages and myofibroblasts to be critical populations which act to both exclude and suppress T cells, thereby mediating immunotherapy resistance. Using the hypoxia-specific prodrug TH-302, we show that disruption of hypoxia in both transplantable and genetically-engineered murine models of prostate cancer sensitizes them to antibody blockade of the T cell immune checkpoint receptors CTLA-4 and PD-1. Loss of immune resistance is a consequence of re-oxygenation of hypoxic zones which results in loss of myeloid suppressor cells coupled with a reduced capacity to suppressively polarize new myeloid immigrants. This combination of hypoxia disruption and T cell checkpoint blockade has the potential to render some of the most therapeutically resistant cancers sensitive to immunotherapy.
We are currently evaluating the combination of hypoxia-reduction with TH-302 and CTLA-4 blockade in a Phase I clinical trial for prostate, pancreatic, head and neck, and melanoma patients.
In addition, we are evaluating multiple approaches to hypoxia ablation in both prostate and pancreatic cancer to assess their impact on immune infiltration, myeloid stromal composition, and sensitivity to T cell checkpoint modulation.

Keywords: Hypoxia, MDSC, Checkpoint Blockade

B167 / Hodgkin lymphoma cells trigger *in vitro* differentiation of human monocytes into macrophages of the M2-subtype

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Interactions between tumor cells and microenvironment crucially regulate disease progression, including tumor cell survival, metastasis and therapy resistance. A paradigmatic microenvironment-controlled cancer entity is classical Hodgkin lymphoma (HL). The malignant Hodgkin-Reed-Sternberg (HRS) cells account for less than 1 % of the disease-related cells. HRS cells highly depend on signaling cross-talk with non-transformed neighboring cells, and thus HL perfectly serves as a model to analyze tumor-stroma interactions. The role of macrophages in lymphoma development and dissemination is currently under intensive debate. Notably, several studies reported on more rapid disease progression and even treatment failure in patients with high numbers of macrophages in the lesions.
Tumor-associated macrophages (TAMs) have often been assigned as tumor-supportive microenvironmental cells, able to sustain proliferation and angiogenesis and to suppress anti-tumor immune responses. Therefore, the aim of our study is the characterization of mutual interactions between HRS cells and monocyte-derived macrophages. Primary human monocytes isolated from peripheral blood were differentiated ***in vitro*** in the presence of HL-conditioned supernatant in comparison to colony-stimulating factor 1 (CSF1/M-CSF). Gene expression and cell surface protein expression were measured using qRT-PCR and flow cytometry. HL-derived factors strongly support monocyte differentiation into macrophages, specifically the M2-related subtype. These macrophages are characterized by strong expression of CD40, CD68, CD163, CD206, and significant expression of adhesion molecules. Functional analyses revealed high endocytic activity of the HL-conditioned macrophages. In addition, we used chick chorioallantoic membrane (CAM) assays to study the role of the M2-related macrophages for lymphoma behavior ***in vivo***. We found that the presence of macrophages increases the probability of lymphogenic dissemination of HRS cells.
Our observations support a model in which HRS cells are able to induce monocyte differentiation into M2-related macrophages to rebuild the lymphoma microenvironment. Further molecular and functional studies are in progress to characterize mutual interactions between TAMs and HRS cells more deeply.

Keywords: Hodgkin lymphoma, Macrophages, Tumor microenvironment

B168 / Immunological response and anti-tumor effects in mouse tumor model

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Introduction: Local tumor ablation has been shown to induce immunologically mediated anti-tumor effects in various pre-clinical models as well as in the clinical setting. Several lines of evidence indicate that more powerful effects are evoked following ablation at lower temperatures than higher temperatures. This prompted development of a laser system capable of irreversible tissue destruction while keeping the temperature of the perimeter of the ablated volume constant at 46°C. This treatment protocol has been described previously including data demonstrating abscopal effects in a twin tumor rat model. This poster shows the first preliminary treatment results of the newly developed laser system adapted for being used in ablation of tumors in mice.

Methods: Mice were subcutaneously inoculated with tumor cells on both sides of the flank. The animals were randomized into two groups, one of which received active treatment and a non-treated control group. Interstitial laser thermotherapy was performed in one of the tumors in the treatment group and the growth of both tumors was recorded.

Results: Preliminary data show that interstitial laser thermotherapy can create local tumor control and prohibit tumor re-growth during the time studied. A tendency toward reduced growth of the contralateral tumor compared to control suggest the treatment was also able to induce immunologically mediated anti-tumor effects.

Conclusion: This preliminary data indicate that low temperature interstitial laser thermotherapy is capable of inducing abscopal effects in mice as a monotherapy, although confirmation in a larger study population is needed. Ongoing studies are aiming at doing this. Previous studies in the field suggest interstitial thermotherapy to have a mechanism of action different from many immunomodulatory pharmaceutical anti-cancer agents, making it a suitable candidate for future treatment combinations.

Keywords: Laser interstitial thermotherapy, Abscopal effect, Twin tumor mouse model

B169 / Searching for synergy between standard chemotherapy and immunotherapy in an ovarian cancer mouse model

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Introduction: Up till now, results from trials with checkpoint inhibitors in ovarian cancer (OC) have yielded response rates ranging from 10-15%, possibly due to severe (innate) immunosuppression. The combination of standard chemotherapy with immunotherapy could overcome this immunosuppressive hurdle. To forge synergistic combinations we need more information on the immunological effects of chemotherapy for OC.

Material and method: Tumorbearing ID8-fLuc-C57BL/6 mice received a single dose of Carboplatin (100 mg/kg), Gemcitabine (500mg/kg), Pegylated Liposomal Doxorubicin (PLD) (6 mg/kg), Paclitaxel (10 mg/kg) or the combination of Carboplatin (100 mg/kg) and Paclitaxel (TC) (10 mg/kg) or Gemcitabine (CG) (2 mg/kg). Immune-monitoring on tumor tissue was performed by fluorescent activated cell sorting (FACS). Regulatory T cells were defined as $CD3^+CD4^+FoxP3^+$ cells. Granulocytic myeloid derived suppressor cells (gMDSC) were determined as $CD11b^+Ly6C^-Ly6G^+F4/80^-$ cells and monocytic MDSC (mMDSC) were defined as CD11b+Ly6Chi cells. Total macrophages were selected as $CD11b^+F4/80^+$ cells and the M2 subtype was defined by CD206 positivity.

Results: Significant differences in immune cell infiltration between chemotherapy-treated mice and untreated controls were observed for several commonly used chemotherapies in OC. After treatment with Carboplatin, an increase in gMDSC (p=0,071) and a reduction in CD8 (p=0,034) and M2 infiltration (p=0,018) was observed. This last one was also observed after treatment with Gemcitabine (p=0,075). Administration of TC reduced CD4 infiltration (p=0,045) and CG decreased mMDSC (p=0,032) but increased gMDSC (p=0,008) and total macrophages (p=0,004). No significant differences or trends were observed after treatment with PLD or Paclitaxel.

Conclusion: We demonstrated that the use of Carboplatin reduced M2 macrophages. Combinatorial chemotherapy included Carboplatin (CG) however, did not, due to an increase in total macrophages, but reduced mMDSC. M2 and mMDSC are major players of innate immunosuppression. Further research is needed to investigate the synergistic effect of chemotherapy in combination with immunotherapy.

Keywords: Chemotherapie, tumor microenvironment, Ovarian cancer

B170 / Myeloid derived suppressor cells (MDSC) determine outcome in ovarian cancer

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Introduction: Immunotherapy trials in ovarian cancer have so far yielded poor results, possibly due to severe immunosuppression. Macrophages are abundant in ovarian cancer (OC) and a predominance of M2 macrophages is correlated with poor survival. Clodronate Liposomes (CL) are a commonly used drug, which effectively depletes macrophages.

Material and method: The murine experiments were performed in C57BL/6 mice (n=50) and Rag1^{tm1Mom} mice (n=12), inoculated intraperitoneal with 5 x 10^6 ID8-fLuc cells. Mice received ip injections with phosphate buffered saline or CL twice weekly starting from tumorinoculation. Six mice per group were evaluated for survival analysis. Immune-monitoring on ascites was performed by fluorescent activated cell sorting (FACS). Next, peripheral blood mononuclear cells were collected prospectively in 39 patients at diagnosis of ovarian cancer.

Results and discussion: Mice treated with CL died significantly faster compared to immunocompetent mice (p=0.004), whereas the survival was not altered in mice lacking T- and B-cells (Rag1^{tm1Mom}). After treatment with CL, macrophages were nearly absent. There were no significant changes in T- and B-cell populations. After treatment with CL, monocytic myeloid derived suppressor cells (mMDSC) (CD11b$^+$Ly6C^{Hi}) increased significantly (p=0,004), whereas granulocytic MDSC (CD11b$^+$Ly6C$^-$ Ly6G^{Hi}) decreased. In patient samples, we observed a decrease in progression free survival in patients with a high number of mMDSC (CD11b$^+$HLA-DR$^-$CD14$^+$) (17.5 ***vs*** 20 months).

Conclusion: In OC mice the absence of adaptive immune system did not influence survival. Depletion of macrophages by CL significantly reduced survival in tumorbearing mice. This could be explained by a significant rise in mMDSC in mice. The increase was also observed in patients with OC. Our research demonstrates for the first time a prominent role for mMDSC as the driver of immunosuppression in OC, which is a new concept in the field of OC.

Keywords: myeloid derived suppressor cells, ovarian cancer, clodronate liposomes

B171 / The interplay between CLL cells and the dendritic cells in their bone marrow microenvironment

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Chronic lymphocytic leukemia (CLL) is a disease of mature resting B lymphocytes that evolves from a clonal B-cell population. The lymphocytes lose their natural life homeostasis due to a defective apoptosis mechanism and start to accumulate in the blood, lymphoid organs and in the final stages of the disease also in the bone marrow (BM). CLL cell survival is dependent on external signals, originating from different cellular elements of their microenvironment including soluble and surface-bound factors. Recently, there has been increased focus on exploring the myeloid components of this lymphocytic disease, including dendritic cells (DCs) that play an important part in mediating the innate to adaptive immunity. DCs have been previously demonstrated to play an important role in tumor microenvironment, but their role in the CLL BM niche has not been studied. To follow DCs function in CLL, we have followed their formation and function in adoptive transfer and chimeric TCL-1 mice models. Our results show that CLL induced expansion of inflammatory derived CD11b+ BM DCs population. This population shared similarities with other myeloid cells in the BM, but was different from BM monocytes/macrophages on a transcriptomic level and expressed tolarogenic markers. Our previous studies have shown that CD84, a cell surface molecule belonging to the Signaling Lymphocyte Activating Molecule (SLAM) family of immunoreceptors, mediates the interactions between CLL and its microenvironment, inducing cell survival. We therefore hypothesized that CD84 can bridge between BM DCs and CLL. Our results show that DCs ablated mice or mice expressing CD84-/-DCs reduced disease progression. In conclusion, better understanding of the CLL BM niche microenvironment will lead to more effective and knowledgeable development of therapeutic modalities to target the niche for CLL and other malignancies.

Keywords: Chronic lymphocytic leukemia, Dendritic cells, Bone marrow

B172 / Metabolic barriers to T cell function and immunotherapy in renal cell carcinoma

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Cancer cells can inhibit effector T cells through both immunomodulatory receptors and alteration of the tumor microenvironment as a result of cancer metabolism. A majority of patients treated with immune checkpoint inhibitors recently approved by the FDA fail to exhibit a clinical response. The extent to which metabolic conditions within the tumor impede T cell activation and anti-tumor effector function in RCC are unknown. To study the tumor microenvironment of RCC two models a mouse xenograft and patient samples were used. Under the IACUC protocol M1600005-00, BALB/c or Rag mice were subcutaneously injected with RenCa cells obtained from ATCC and growth was monitored by caliper measurements every 3 days. ***In vivo*** PD-1 blockade was performed by 200 mcg/i.p. injection every 3 days using purified mPD-1 (BioXcell, J43). Deidentified tissue donations from patients with RCC were collected under the IRB protocol #151549 and processed into single cell suspensions following mechanical dissociation for the functional assays indicated below. Through work with Rag deficient mice lacking functional B and T cells, we have established that RenCa tumor growth is regulated in a T cell dependent manner as evidenced by earlier formation and faster tumor growth. In a syngeneic mouse model of RCC (RenCa), we find that inhibition of PD-1 delays tumor growth and size. Tumor infiltrating lymphocytes (TILs) isolated from xenograft exhibit markers of activation and chronic stimulation with high PD-1. ***Ex vivo*** analysis of CD8 TIL suggested differences in metabolic dependency compared to control CD8 from splenocytes. Patient samples were analyzed and found to be phenotypically distinct and are impaired both functionally and metabolically from healthy surveilling CD8 control. At a global level, based on RNA-seq data, CD8 from TIL rely on distinct metabolic pathways compared to control. Instead of efficient use of aerobic glycolysis, TILs fail to increase glucose metabolism, and instead display increased reactive oxygen species (ROS) and mitochondrial dysfunction. CD8 effector cells found in tumors have notable differences in mitochondrial morphology compared to healthy control CD8 T cells by electron microscopy and immunofluorescence where CD8 TIL are punctate and dispersed throughout the cell while healthy control CD8 mitochondria are fused in networks. Bypassing metabolic defects partially restore markers of TIL activation and effector function. Preclinical data suggests that improved understanding of metabolic dysfunction in TIL of RCC may allow for combined therapies to improve response rates of checkpoint inhibition in this disease.

Keywords: metabolism, renal cell carcinoma, T cell

B173 / HHLA2 (B7H7) is highly expressed in human hepatocellular carcinoma cells and is associated with better patient survival

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Introduction: HHLA2 is a recently discovered member of the B7-family and is thought to function predominantly as a T-cell co-inhibitory molecule. Its expression on cancer cells was found amongst others in osteosarcoma, breast cancer and lung cancer. In this study, we assess the expression patterns of HHLA2 in hepatocellular carcinoma (HCC) and determine its relation to patient survival.
Method: Tumors and tumor-free liver (TFL) tissues collected from HCC patients during tumor resection were used to construct Tissue-microarrays (TMA) containing 3 cores per tumor and 2 cores per TFL tissue. TMA were immunohistochemically stained with a validated monoclonal antibody against HHLA2 (clone: 566.1) and scored as either negative or weak, intermediate, or strong expression. FACS-analysis of single cells isolated from freshly isolated tumors was used to further characterize HHLA2 expression in HCC.
Results: TFL tissues showed in 9.7% of patients no HHLA2 expression, in 20.1% weak expression, in 55.2% intermediate expression, and in 14.9% strong expression. There was no association between HHLA2 expression in TFL tissues (n=154) and patient survival. In 27.8% of patients HHLA2-expression was absent on tumor cells, while 15.5% had weak expression, 37.1% had intermediate expression, and 19.6% had strong expression on tumor cells (n=194). Absent or weak tumor expression of HHLA2 was associated with poorer HCC-specific survival (average

72 months, p=0.002) compared with intermediate or strong HHLA2 expression (average 94.7 months). HHLA2 expression was predictive of HCC-specific survival independent of baseline clinicopathologic characteristics, like liver cirrhosis, alfa-feto protein serum level, tumor size, and number of lesions (HR 0.43; P=0.004). Expression of HHLA2 correlated positively with expression of PDL1 (r=0.448; P< 0.0001) and galectin-9 (r=0.411; P< 0.0001), suggesting that HHLA2 expression in HCC is induced in response to inflammation. FACS analysis confirmed that HHLA2 was expressed on $CD45^-$ tumor cells in HCC, while in $CD45^+$ cells only $CD19^-$ $BDCA1^+$ myeloid dendritic cells and $CD14^+$ cells showed expression.

Conclusion: Tumor cell expression of HHLA2 was observed in the majority of HCC patients and is associated with better HCC-specific survival and PDL1 expression. HHLA2 expression in tumors may be induced in response to immunologic pressure, which may explain the positive association with prolonged survival.

Keywords: Hepatocellular carcinoma, HHLA2, PDL1

B174 / NK cells control the recruitment of cDC1 into the tumor microenvironment promoting cancer immune control

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Conventional type 1 dendritic cells (cDC1) are critical for antitumor immunity and the abundance of cDC1 within tumors determines T cell-mediated anti-tumor immunity and the success of cancer immunotherapy. Here, we investigated the mechanisms underlying the accumulation and positioning of intratumoral cDC1. We show that cDC1 accumulation in cyclooxygenase (COX)-deficient $BRAF^{V600E}$ tumors, which cannot produce prostaglandin E2 (PGE_2) and are therefore susceptible to immune control, critically depends on NK cells. Intratumoral NK cells produce the chemokines CCL5 and XCL1 to recruit cDC1 and overexpression of XCL1 and CCL5 in COX-deficient mouse tumors augments cDC1 accumulation resulting in enhanced immune-mediated tumor rejection. cDC1 recruitment by NK cells is prevented by PGE_2 produced by tumors that evade anti-tumor immunity through a combination of inhibition of chemokine production by NK cells and downregulation of the relevant chemokine receptors in cDC1. In human cancer patient data CCL5, XCL1 and XCL2 transcripts closely correlate with gene signatures for both NK cells and cDC1, implying a similar involvement of NK cells for cDC1 recruitment into human tumors. Notably, NK cell, chemokine and cDC1 signatures associated with patient overall survival in melanoma, head and neck cancer, lung adenocarcinoma and triple-negative breast cancer. Our findings reveal a cellular and molecular pathway for cDC1 recruitment into tumors, which is associated with survival and could be exploited for cancer therapy.

Keywords: NK cells, Dendritic cells, Immune evasion

B175 / The activation status of STAT3/IL-6/AKT signaling pathways in the microenvironment of human prostate pathologies

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IL-6 is a pro inflammatory cytokine with multifunction in human prostate cells. The major signaling transduction of IL-6 is through the transcription factor STAT3. However, PI3-K/AKT signaling pathway can also activated by IL-6 under prostate pathological conditions. The aim of this study is to evaluate the status of STAT3/IL-6/ AKT axis signaling in the microenvironment of prostate tissues and prostate epithelial cells from patients with Benign Prostatic Hyperplasia (BPH) and Prostate Cancer (PC). Then we analyzed the AKT/IL-6/STAT3 axis signaling expression according to sera Prostate Specific Antigen tumoral marker (PSA) and the correlation of AKT activation with activated of IL-6 and STAT3 expression in BPH and PC. Immunohistochemical and Western blot analyses for IL-6, Gp130, pSTAT3(Tyr705) and pAKT (Ser473) were carried out in 25 samples of BPH, 16 samples of PC. Serum levels of PSA have been assayed by Immulite autoanalyser. Immunoreactivity to IL-6 was consistently observed in stroma compartment of BPH and cytoplasmic epithelial cell in PC samples. pAKT was mainly expressed in membrane and the cytoplasm in PC compared to BPH.). Immunoreactivity for pSTAT3(Tyr705) was found in the stroma and the nucleus of epithelial and tumoral cells. The analysis of the expression status of the STA3/IL-6/AKT pathways in BPH and PC revealed that positive profile (IL-6+, pSTAT3+) was correlated with expression of pAKT (Ser473) whereas patients with the positive profile (IL-6+, pAKT +) were also positive for Gp130 and pSTAT3(Tyr705). This suggests that IL-6/AKT axis could be one of mechanism to activate STAT3 by facilitating inflammatory cell migration in the microenvironnment induced chronic inflammation and cancer progression by promoting cell growth in PC.

Keywords: microrenvironnemnt, prostate epithelial cell, STAT3/IL6/ AKT

B176 / The novel histone modifier and tumor suppressor gene *MLL3* prevents T cell dysfunction and Ly6C^{int-hi} myeloid cell differentiation *in vivo*

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Breast cancer accounts for most cancer mortality in women (571,000 deaths in 2015) with ~2 million new cases diagnosed every year. Various therapeutic approaches combining surgery, chemotherapy and hormonal therapy have been developed to improve outcomes, however, their success still remains limited. Among these cancers, invasive breast cancer is the most common and aggressive as a result of multiple mutations and epigenetic alterations. Forty percent of breast cancers overexpress the gene encoding for the Phosphoinositide 3-kinase (PI3K) which is involved in cancer cell survival and growth. Recently, a gene encoding for the histone methyltransferase ***MLL3*** was identified as a novel tumor suppressor gene. MLL3 gene inactivation is detected in several cancers beyond breast cancers and is reported in 8-27% of human breast cancers, and ***MLL3***$^{-/-}$ breast cancers have significantly poorer outcomes compared to wild type counterparts (***MLL3***^{WT}). ***MLL3*** is also found frequently co-mutated with constitutively active PI3K (PI3KCA^{+}). However, the role of MLL3 in breast cancer development remains unknown.
Here, we have investigated how MLL3 contributes to the establishment of efficient antitumoral response against breast tumors. We hypothesize that ***MLL3*** prevents the development of breast tumors by modulating both the proportion and the functions of tumor-infiltrating lymphocytes (TILs) and myeloid cells. Using a novel method, we have developed, and which relies on the "custom genome editing" of mouse mammary stem cells (MaSCs) with the CRISPR/Cas9 system, we generated genetically engineered mammary glands containing the clinically relevant ***MLL3*** loss of function mutation in mice. With this system, we established that inactivation of ***MLL3*** coupled to PI3KCA, promoted MaSCs to form accelerated mammary tumors compared to non-mutated counterparts (***MLL3***^{WT}, PI3KCA^{+}). Analysis of the TILs in ***MLL3***$^{-/-}$ tumors revealed that less than 10% expressed the cytolytic marker granzyme B and secreted IFNg, while in ***MLL3***^{WT} tumors, more than 50% of the TILs expressed these effector molecules as well as the PD-1 inhibitory receptor. In addition, TILs from ***MLL3***$^{-/-}$ tumors were actively proliferating (Ki67^{+}) and differentiated into effector cells (KLRG1^{+}). Phenotyping of myeloid cells revealed that ***MLL3***$^{-/-}$ tumors exhibited a majority of CD11b^{int}Ly6C^{low} F4/80^{+} MHC-II^{+} cells while multiple populations of CD11b^{int} F4/80^{+} MHC-II^{+} cells expressing varying levels of the Ly6C marker (Ly6C$^{lo/int/hi}$) were found in ***MLL3***^{WT} tumors. Analysis of the tumor cells further showed that non-mutated tumors expressed high level of the T cell co-inhibitory receptors B7x and PD-L1. Altogether, our data suggest that TILs in ***MLL3***$^{-/-}$ tumors are largely dysfunctional, most likely through a mechanism that does not involve B7x or PD-L1 and which remains to be elucidated. Our results on myeloid cells also suggest that loss of MLL3 may be associated with impaired Ly6C^{hi} myeloid cell differentiation in these tumors.

Keywords: MLL3 breast cancer mutation, T cell activation, Tumor microenvironment

B177 / Interrogating evolving tumor-immune interaction through combination of genome perturbation and single-cell genomics

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Advances in genome sequencing and related technology have led to unprecedented pace at which we can identify genomic and epigenetic changes associated with human diseases. This enabled wide range of application, particularly single-cell genomics to interrogate cellular gene function at unprecedented resolution. At the same time, to match the power of single-cell genomics, genome editing tools such as CRISPR-Cas system enabled the perturbation of human genome at massive scale. Here we employed a combination of single-cell genomics and genome engineering technology to probe the evolving interaction between malignant and immune cells in a range of in vivo cancer models, while taking into account human patient data. We demonstrate these technologies can be deployed as versatile discovery tool for dissecting T-cell regulation during tumor growth, particularly with genetically-engineered non-small-cell lung cancer (NSCLC) model. We uncover intriguing heterogeneity and potential antigen-specific transcriptional regulation within the tumor-infiltrating T-cells, focusing on CD8 and Treg sub-types across multiple time points in our longitudinal study. We could employ genetic perturbation to validate molecular regulators of this process based on our findings. Overall, we highlight the emerging trend on how single-cell analysis could be integrated with perturbation tools to transform our ability to leverage tumor immunology insight to target key regulatory nodes as novel therapeutic approaches.

Keywords: single-cell RNA-seq, CRISPR, tumor immunology

B178 / Re-polarization of tumor macrophages following anti-PD-L1 treatment

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Tumor-associated macrophages play a critical role in shaping the tumor microenvironment and contribute to the cancer-immune set point. Immunotherapies such as anti-PD1/anti-PD-L1 have demonstrated remarkable therapeutic efficacy in a subset of patients, partly through reinvigoration of CD8+ T cell responses. However, the impact of these T cell dependent therapies on macrophages remains largely unknown. Here, by using the anti-PD-L1 responsive murine MC38 colorectal cancer model, we show that anti-PD-L1 treatment altered the balance of tumor associated myeloid cells by significantly decreasing the levels of Arginase-I (ARG1), a suppressive effector gene expressed by M2 cells, while increasing the levels of iNOS, on macrophages and their precursor, monocytes. Additionally, anti-PD-L1 treatment enhanced MHC-II expression on macrophages, a characteristic M1-associated marker. We next sought to investigate the mediators of anti-PD-L1 treatment responsible for these phenotypic changes on macrophages. We identified IFN-g as a major cytokine produced by CD8+ T cells, which was significantly increased following anti-PD-L1 treatment and which was associated with macrophage re-polarization into a less suppressive and more M1-like phenotype. Additionally, blocking IFN-g levels in vivo abrogated these phenotypic changes on macrophages and monocytes. Finally, we examined the effect of anti-PD-L1 treatment on additional tumor models and observed a similar phenotypic change in the anti-PDL1 responsive model (EMT6), but not in a non-responsive model (JC1). We conclude that the phenotype of tumor associated macrophages can be modified from an M2-like to a M1-like phenotype following anti-PD-L1 treatment, affecting the tumor immune set point by reducing the suppressive characteristics of tumor-associated macrophages and providing a more pro-inflammatory microenvironment. This effect was mediated by enhanced IFNg production from responding CD8+ T cells. Given that these phenotypic changes on macrophages were only observed in responding tumor models, strategies to independently promote macrophage re-polarization could help augment responses to T cell dependent immunotherapies by lowering the immune set point and allowing patients with reduced CD8 T cells responses to overcome the threshold for generating anti-tumor activity. Combining agents that modulate the monocyte/macrophage phenotype could therefore help expand the proportion of patients responding to CIT agents.

Keywords: anti-PD-L1, macrophages, polarization

B179 / IL-17 affects pancreatic cancer progression by modulating stromal cells

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Pancreatic ductal adenocarcinoma (PDA) is a fatal medical condition with few advances in therapy and patient survival. The role of IL17 and Th17 in cancer progression and anti-tumour immunity is still controversial. In this study we have investigated the role of IL17 in PDA carcinogenesis by crossing genetically engineered mice (GEM) that spontaneously develop PDA with IL-17 KO mice (GEM/IL17KO). Survival was analysed by Kaplan-Meier, and pancreata and organs from GEM and GEM/IL17KO were analysed by immunohistochemistry and second harmonic generation to evaluate collagen into tumor stroma. Isolated fibroblasts from GEM and GEM/IL17KO mice were analysed by RNA expression and cytokine arrays. GEM/IL17 KO mice survived significantly more than GEM mice, even if there were no changes in the tumor onset. In addition, we observed an increased fibrotic reaction in GEM/IL17 KO compared to GEM mice starting from early stage of disease with the formation of compact nets of collagen fibers. Fibroblasts isolated from GEM/IL17KO mice unveiled a wound-healing profile more than a pro-tumoral phenotype. Lastly, IL17 directly increased PDA cell migration and invasion but not proliferation. Overall these data suggest that the absence of IL17 does not affect the tumor onset but the progression, likely affecting the fibrotic and immune responses, which seem to be able to better counteract tumor growth.

Keywords: Pancreatic cancer, IL 17, Cancer associated fibroblast

B180 / Effect of high fat diet in different types of liver cancer- how diet can affect innate immune system and liver cancer progression

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Currently there is little information about the differences between conventional hepatocellular carcinoma (cHCC) and fibrolamellar hepatocellular carcinoma (FL-HCC). Therapies used in cHCC are not successful in FL-HCC. The lack of accurate animal models to study in vivo cellular and molecular mechanisms involved in the progression of FL-HCC are hampering our knowledge. The recruitment of innate immune cells to tumor niche can be a powerful drug target to modulate liver cancer progression and

invasion. Nothing is known about the innate immune cells role in FL-HCC, although their presence at the tumor microenvironment can either enhance or deter cancer progression. Non-alcoholic fatty liver disease (NAFLD) has been associated with increased incidence of liver cancer. Our new food habits and sedentary life lead to increased accumulation of lipids in hepatocytes and further chronic inflammation of the liver (NASH). In order to develop more powerful tools able to treat cHCC and FL-HCC it is crucial to understand how our diet is affecting not just liver cancer progression but also the innate immune system. For such, in this project we aim to combine a high fat diet protocol with cHCC and with our new FL-HCC zebrafish model.
We have established a protocol to induce NASH by supplementing our normal larvae diet for 10 days with 10%cholesterol. NASH was confirmed by Oil Red staining, bright-field imaging of lipid droplets, increase of liver size and ballooning effect, and increased leukocyte recruitment lo the liver. Next, isolating RNA from hepatocyte using TRAP we observed an upregulation of lipogenic (pparg and srebp1), oxidative stress (gpx) and inflammatory (il1b and il8) genes. Same approach with neutrophils and macrophages also showed distinct genetic profile of these cells suggesting that HFD is able to prime innate immune system. To address the effect of HFD over leukocyte response to an injury we used the tail-fin transection model. Massive leukocyte recruitment was observed to wounds in larvae fed with HFD. Additionally, after photo-conversion of neutrophils/macrophages at liver area, we observed that more than 50% of leukocytes being recruited in HFD to wounds are coming from the liver. Combining our HFD with cHCC model (SH-cHCC), an increase of hepatocyte mobility was observed as well as the release of small vesicles. Not surprisingly SH-cHCC larvae display bigger and non-organized livers with “fat” hepatocytes and big nuclei, and massive leukocyte recruitment. Importantly, leukocytes from SH-cHCC larvae showed reduced recruitment to wounds compared to HFD or cHCC conditions alone indicating that pro-inflammatory signals in livers from SH-cHCC larvae are extremely high, able to retain these primed cells and inhibit their massive recruitment to wounds. These results suggest that the increased recruitment, as well as persistence of leukocytes at the liver observed in SH-cHCC larvae might be associated with increased liver cancer progression.

Keywords: Non-alcoholic fatty liver disease, Conventional Hepatocellular Carcinoma, Fibrolamellar Hepatocellular Carcinoma

B181 / Engineering cell sensing and responses using GPCR-coupled CRISPR system

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We describe a new three-component molecular device called CRISPR ChaCha, a new synthetic device that couples programmable genome regulation via CRISPR-dCas9 to the ligand-sensing ability of the highly diverse G-protein coupled receptor (GPCR) family. CRISPR ChaCha displayed superior performance over the previously reported Tango assay. Its superior ability to recruit and activate multiple Cas9 molecules per ligand-activated GPCR is explained in a parsimonious model. We systematically characterized various design parameters to optimize device performance. The new device is expanded to diverse GPCRs that can sense both synthetic and natural signals such as chemokines, mitogens, and fatty acids, and convert those signals to customizable genetic programs such as reporter and endogenous genes in mammalian cells. This novel GPCR-CRISPR technology opens up novel cell biological programs in response to diverse extracellular ligands and can be utilized to interrogate or manipulate existing signaling pathways. This emerging technology can be combined with adoptive cell transfer therapy to modulate the response of immune cells to the soluble milieu of tumor microenvironments.

Keywords: CRISPR-Cas9, Genome Engineering, G-protein coupled receptor

B182 / Antigen-specific interaction with activated CD4 T cells induces M1 associated gene expression in M2 polarized macrophages

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The immunosuppressive tumor micro milieu established by inflammatory immune cells such as myeloid derived suppressor cells (MDSCs), regulatory T cells (Tregs) and tumor associated macrophages (TAMs) still forms a major obstacle for successful immunotherapies. TAMs can be attracted from the circulation by chemokines secreted by the growing tumor. Depending on the activating stimuli, these cells can develop into different subsets including classically (M1-like) or alternatively (M2-like) activated macrophages with distinct gene and protein expression patterns. Here, we used ***in vitro*** polarized peritoneal exudate cells (PECs) to establish a panel of surface markers to distinguish between the two major subtypes of macrophages, M1 and M2, by flow cytometry.

In addition, 18 different genes, nine for each macrophage subset were selected to distinguish the two subtypes by quantitative real-time PCR. Next, we showed that immunosuppressive M2-like macrophages can be repolarized into pro-inflammatory M1-like macrophages when co-cultured with purified ovalbumin specific $CD4^+$ T helper cells obtained from immunized C57BL/6 mice. Peptide loaded PECs co-cultured with $CD4^+$ T helper cells for 24h showed a strong upregulation of M1-like markers IA^b and iNOS. The gene expression analysis revealed an increased expression of M1-like associated genes and a decreased expression of M2-like associated genes. The recognition of PECs by $CD4^+$ T helper cells became weaker the longer they were polarized into M2. Taken together, our results show that M2-like polarized macrophages can be repolarized into M1-like macrophages through direct interaction with $CD4^+$ T helper cells. In ongoing ***in vivo*** experiments we are using the murine B16F10-OVA tumor model to study the effects of adoptively transferred ovalbumin specific $CD4^+$ T cells on the polarization of TAMs. Tumor antigen specific $CD4^+$ T cells might polarize TAMs into immunostimulatory M1, thereby neutralizing the immunosuppressive micro-milieu. In a final step of the project, tumor antigen specific $CD4^+$ T cells will be co-administered with $CD8^+$ T cells isolated from OT-I mice to investigate whether the induced changes in the tumor microenvironment would facilitate CTL mediated tumor rejection.

Keywords: Macrophage polarization, Tumor Microenvironment, Adoptive T cell transfer

B183 / Analysis of tumor-infiltrating T cells made easy - complete workflows improve the isolation and analysis of tumor-infiltrating T cells

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Question: Immunotherapy has proven clinical efficacy and tremendous potential in multiple cancers. Syngeneic mouse tumor models represent the gold standard to analyze effects of immunotherapy, as they possess a fully competent immune repertoire. However, the amount and composition of tumor-infiltrating leukocytes (TILs) is highly variable, complicating the analysis of individual subpopulations. In particular, small subpopulations might escape analysis as they could get lost in the background noise. When working with large cohort sizes, even immunophenotyping of TILs by flow cytometry is time consuming and data processing highly work intensive. Therefore, pre-enrichment of TILs is highly desirable to increase the sensitivity of analysis and save time and effort during flow cytometry.

Methods: We have established workflows combining tissue dissociation with specific T cell isolation and phenotyping. Tumor dissociation was automated using the gentleMACS™ Octo Dissociator and optimized for epitope preservation. Moreover, isolation of tumor infiltrating T cells was improved by developing new CD4-, CD8- and Pan-T cell specific enrichment reagent for magnetic cell sorting, based on MACS® Technology, directly from dissociated tumor tissue. Finally, we used optimized panels of recombinant REAfinity fluorescently-labelled antibodies to phenotypically characterize tumor infiltrating T cells.
Results: Our workflows were validated in different mouse tumor models. Tumor-infiltrating T cells were isolated to purities above 80% and yields ranging from 60-95%. Time of downstream analysis was reduced up to 50-fold while enhancing the detection and phenotypic characterization of T cell subpopulations within the tumor.
Conclusions: Standardized processing of tumor samples and magnetic isolation of tumor infiltrating T cells greatly reduces time and cost of downstream analysis while significantly increasing reproducibility and the quality of data obtained from TIL analysis.

Keywords: T cells, Tumor, TIL

B184 / Anti-C5aR antibodies inhibit both neutrophils and myeloid cells and overcome PD-1 checkpoint resistance in vivo

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The generation of an inflammatory microenvironment promotes primary tumor growth and metastasis. Pro-tumoral inflammation is largely mediated by myeloid cells and neutrophils which accumulate within the tumor microenvironment and secrete pro-angiogenic factors and inflammatory cytokines. At the same time, these cells suppress anti-tumor immunity by expressing surface molecules and secreting cytokines that inhibit NK and T cells. These cells are also associated with poor prognosis in many cancer types as well as resistance to checkpoint blockade. From a therapy perspective, it seemed attractive to target both suppressive myeloid cells and neutrophils and to inhibit both their pro-tumor and immunosuppressive functions. C5aR, the receptor for complement factor C5a, appeared to fit this profile. C5a is a strong pro-inflammatory mediator and a potent chemoattractant and activator of myeloid cells and neutrophils. In C5aR KO mice, tumors are infiltrated by markedly fewer MDSCs, which produce less immuno-suppressive mediators, and are functionally unable to suppress T cells. IPH5401 is a novel, first-in-class fully human anti-C5aR therapeutic antibody candidate. To evaluate the therapeutic potential of this antibody in cancer, we first tested its activity ***in vitro*** with human cells. We confirmed the selective expression profile of C5aR on circulating human neutrophils and myeloid

cell subsets. C5aR expression was higher on ***in vitro*** derived suppressive M2 macrophages compared to the M1 subset, and interestingly, C5a preferentially induced migration of M2 cells in chemotaxis assays. In line with the suppressive capacity of M2 but not M1 cells, the C5aR^{hi} M2 subset specifically inhibited NK and T cell cytotoxicity ***in vitro***. Importantly, blockade of C5aR ***in vitro*** with IPH5401 effectively inhibited the C5a-mediated effects on M2 chemotaxis and neutrophil activation. Recent reports have associated resistance to anti-PD1 therapy with an increased infiltration of myeloid cells in patients with melanoma or HNSCC. To test whether C5aR blockade may overcome checkpoint blockade resistance, the efficacy of a surrogate anti-C5aR blocking antibody was assessed in the B16F10 melanoma model, which exhibits poor T cell infiltration and is resistant to anti-PD-1 therapy. In established tumors in this model, the combined administration of anti-C5aR with anti-PD1 synergistically and statistically reduced tumor growth (tumor growth inhibition was achieved in over 50% of the mice treated with the combination compared to less than 10% with either agent alone). Taken together, these data suggest that C5aR blockade may result in a more permissive environment for immune-mediated tumor rejection. IPH5401 thus represents an opportunity to reverse the tumor immunosuppressive microenvironment, and potentially overcome the checkpoint resistance seen in many patients.

Keywords: MDSC, Neutrophils, Checkpoint

B185 / Macrophage regulation of tissue homeostasis

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The ability of organisms to maintain homeostasis is fundamental for their survival and is controlled at the cellular, tissue, and organismal levels. While maintenance mechanisms at the cellular and systemic levels are well characterized, the relevant cell types and signaling components controlling homeostasis in tissue compartments are undefined. Macrophages are found in almost all mammalian tissues and are known to orchestrate responses to damage and infection. Furthermore, these innate immune cells may perceive more subtle changes within the surrounding environment and participate in steady state maintenance of tissues. In fact, we recently found that macrophages are engaged in a growth factor-dependent circuit with fibroblasts to maintain stable cell ratios. In addition to this circuit, macrophages likely communicate with neighboring cells, to not only maintain appropriate cell composition, but to promote tissue function. Perturbations in homeostasis activate stress response pathways and result in both cell-intrinsic and cell-extrinsic responses, the latter of which can result in tissue level adaptations. We propose that macrophages regulate tissue level changes to maintain homeostasis and that this feature may become dysregulated during chronic inflammatory conditions, including cancer. In order to model disrupted homeostasis, we applied diverse stressors to bone marrow-derived macrophages (BMDMs) ***in vitro***: endoplasmic reticulum (ER) stress, osmotic stress, amino acid deprivation, glucose deprivation, hypoxia, and heat shock. Next, we performed RNA sequencing to compare gene expression patterns between different stress conditions. We found that each stressor induces a unique transcriptional program in BMDMs, as well as a shared program, common between multiple stress conditions. To identify potential paracrine signals originating from macrophages and acting on surrounding tissues, we focused on genes encoding secreted proteins, upregulated in multiple conditions, and containing known stress-associated motifs in their promoters. Many of these genes encode extracellular matrix components and chemokines/cytokines and are associated with tissue remodeling, angiogenesis, cell adhesion and cell migration. We have identified a number of proteins that may be involved in this cell-extrinsic stress response in macrophages and are currently generating mice deficient in these factors to investigate their roles in settings of tissue stress.

Keywords: macrophages, stress responses, homeostasis

B186 / Atypical chemokine receptor 4 restrains anti-tumor CD8$^+$ T cell recruitment into solid tumors

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Current immunotherapies directed at enhancing the quality of CD8$^+$ T cell responses show remarkable promise, but their efficacy in patients with solid tumors is limited. This is mediated in part by tumor-specific T cells being inefficiently recruited or actively excluded from the tumor parenchyma. Thus, understanding the chemotactic cues that dictate T cell trafficking into solid tumors is critical in developing strategies to enhance clinical responses. The atypical chemokine receptor 4 (ACKR4) is expressed in the stromal compartment of a wide range of tissues and has been reported to scavenge the T cell-attracting chemokines CCL19, CCL21 and CCL25, thus regulating their bioavailability. Here, we have identified a novel role for ACKR4 in repressing anti-tumor immunity, using the E0771 and transgenic MMTV-PyMT models of breast cancer. ACKR4 expression in the tumor microenvironment is upregulated over the course of tumor progression. In the absence of ACKR4, increased levels of intratumoral CCL21 are associated with enhanced recruitment of IFNγ$^+$ CD8$^+$ T cells to mammary tumors, with unaffected recruitment of Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells. This leads to a significant attenuation in tumor

growth that is dependent upon cytotoxic T cells, as depletion of $CD8^+$ T cells restores growth to wildtype levels. Furthermore, deletion of ACKR4 also strongly suppresses tumor growth in orthotopic and chemically-induced models of skin cancer. Together, these data support the notion that ACKR4 is an important regulator of the tumor microenvironment and prevents recruitment of tumor-specific T cells through scavenging of CCL21.

Keywords: CD8+ T cells, cell migration, breast cancer

B187 / The effect of vitamin E on the function and frequency of myeloid derived suppressor cells in an experimental breast cancer model

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Background: Vitamin E has been shown to have strong anti-carcinogenic properties, including antioxidant and apoptotic characteristics, making it appealing candidate for cancer therapy. On the other hand, among the tumor immunosuppressive components, it has been shown myeloid derived suppressor cells (MDSCs) have remarkable ability to suppress anti-tumor immunity through multiple mechanisms. The aim of current study was to assess whether the alpha-tocopherol succinate can alleviate MDSCs-mediated immunosuppression in vitro and in an experimental breast cancer model.
Material and methods: After assessing the effect of α-tocopherol succinate on MDSC viability and gene expression in vitro, mice were challenged with $7x10^5$ 4T1 murine breast adenocarcinoma cell line. After 5 days, tumor-bearing mice were intraperitoneally injected with vitamin E (5mg/kg) or DMSO/Tween (vitamin E solvent) at one day interval for a total of five times. After isolation of MDSCs from the spleen and tumor tissue, MDSCs frequency, nitric oxide (NO) production and gene expression analysis were performed by flow cytometry and quantitative RT PCR respectively.
Results: Based on our experiments, vitamin E diminished tumor growth rate in tumor bearing mice but it had no effect on the percentage of CD11b+ Gr-1+ MDSCs in the spleen and tumor tissues in tumor-bearing mice. Q-PCR showed that α-tocopherol succinate reduced iNOS, Arginase and indoleamine-pyrrole 2,3-dioxygenase (IDO) gene expression in isolated MDSCs.
Conclusion: According to our data, vitamin E has not effect on MDSCs frequency in tumor bearing mice however it may modulate their functions through decreasing MDSC expression of immunosuppression-related genes ***in vitro***.

Keywords: Alpha-tocopherol succinate, Myeloid-derived tumor suppressor cells,, Experimental breast cancer model

B188 / Spatial distributions of CD8 T cells are prognostic in Triple Negative Breast Cancer and reflect distinct immunogenotypes

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Tumor infiltrating lymphocytes (TILs) are frequently present in Triple Negative Breast Cancer (TNBC) and, although representing a prognostic factor, only a minority of patients respond to immune therapies. In order to investigate immune evasive mechanisms of TNBC, we addressed the composition of TILs, their spatial distributions and evaluated potential immune evasive pathways in TNBC using gene expression arrays and in situ stainings. We performed immune stainings on 153 whole tissue slices in a unique cohort of lymph node negative, primary TNBC patients, who have not received adjuvant therapy. Immune stainings of CD8 T cells revealed three distinct distribution patterns: homogenous distribution (inflamed; 48%); predominant distribution at border and not in center (excluded; 28%); not/negligible presence of CD8 T cells (ignored; 24%). Importantly, these patterns were significantly correlated with clinical outcome, with inflamed tumors having the best prognosis, while ignored tumors had the worst prognosis (DSF, MFS and OS, $p< 0.001$). Other lymphocytes, such as B cells (CD20) showed similar distribution patterns while tumor associated macrophages (CD163) do not. Notably, tumors with different lymphocyte distributions are characterized by unique immune profiles. We observed that inflamed tumors showed up-regulated gene expression of checkpoint molecules and CD8 T cells showed low expression of activation markers; excluded tumors showed up-regulated expression of immunosuppressive extracellular matrix components; and ignored tumors showed down-regulated expression of MHC molecules and chemoattractants. Taken together, TNBC can be subgrouped according to distinct spatial distributions of lymphocytes, and these subgroups may have differential treatment requirements to sensitize tumors towards immune therapies.

Keywords: T cell exclusion, Triple negative breast cancer, immune evasive machanisms

B189 / Subtype-specific prognostic impact of different immune signatures in node-negative breast cancer

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Background: The role of different subtypes of immune cells is still a matter of debate.

Methods: We compared the prognostic relevance for metastasis-free survival (MFS) of a B-cell signature (BS), a T-cell signature (TS) and an immune check-point signature (CPS) in node-negative breast cancer (BC) using mRNA expression. Microarray based gene-expression data were analyzed in six previously published cohorts of node-negative breast cancer patients not treated with adjuvant therapy (n=824). The prognostic relevance of the individual immune markers was assessed using univariate analysis. The amount of independent prognostic information provided by each immune signature was then compared using a likelihood ratio statistic in the whole cohort as well as in different molecular subtypes.

Results: Univariate Cox regression in the whole cohort revealed prognostic significance of CD4 (HR 0.66, CI 0.50-0.87, p=0.004), CXCL13 (HR 0.86, CI 0.81-0.92, p< 0.001), CD20 (HR 0.76, CI 0.64-0.89, p=0.001), IgκC (HR 0.81, CI 0.75-0.88, p< 0.001) and CTLA-4 (HR 0.67, CI 0.46-0.97, p=0.032). Multivariate analyses of the immune signatures showed that both TS (p< 0.001) and BS (p< 0.001) showed a significant prognostic information in the whole cohort. After accounting for clinical-pathological variables TS (p< 0.001), BS (p< 0.05) and CPS (p< 0.05) had an independent effect for MFS. In subgroup analyses, the prognostic effect of immune cells was most pronounced in HER2+ BC: BS as well as TS showed a strong association with MFS when included first in the model (p< 0.001).

Conclusion: Immune signatures provide subtype-specific additional prognostic information over clinical-pathological variables in node negative breast cancer.

Keywords: TILs, antitumor immunity, immune signatures

B190 / The effects of a TNFα and IL-2 armed oncolytic adenovirus on pathogen associated molecular pattern signaling

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Adenoviral-mediated gene therapy is a promising new treatment for malignant tumors with poor responses to traditional cancer treatments. In this study, we investigate signaling responses to a chimeric, cytokine armed oncolytic adenovirus (Ad5/3-E2F-d24-hTNFα-IRES-IL-2). Previous studies has shown that this virus provides potent anti-tumor effects in association with induction of immune responses. However, more in-depth information on the molecular mechanism behind these phenomena are needed. Pattern recognition receptors, such as Toll-like receptors (TLR) are responsible for the recognition of damage and pathogen-associated molecular patterns (DAMP and PAMP), which are present during cellular stress and (adenovirus) infections. Binding of adenovirus DNA to TLR9 seems to result in the activation of a signaling cascade consisting of a plethora of proteins, finally culminating in the activation of nuclear transcription factors, e.g. the nuclear factor-κB (NFκB). This starts the production and release of several cytokines, leading to both cellular activation and extracellular signaling, consequently affecting neighboring cells as well. The produced cytokines, such as TNFα and IL-2, might enhance vascular leakage, thus limiting blood flow and lowering nutrient and oxygen levels in tumors. Also, the leaky and unorganized tumor vasculature, seems to cause local enhanced cancer cell death and retained drug delivery. In this study several methods such as Western blot and flow cytometry were used to study the tumor microenvironment and DAMP and PAMP signaling *in vitro*. Key results were confirmed *in vivo*, using tumor bearing mice and Syrian hamsters as animal models. These methods were utilized to study Ad5/3 induced, TLR-dependent, cytokine production and its consequent effect on tumor development and tumor microenvironment. The knowledge gained about the signaling pathways in this study provides new insight into cell signaling, which can be used to further develop the tumor immunotherapy and to provide a new method of treating poorly responsive tumors.

Keywords: Oncolytic adenovirus, PAMP and DAMP signaling, Tumor microenvironment

B191 / Expression and function of tryptophan 2,3-dioxygenase as an immunoregulatory enzyme in tumor-associated vessels

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The enzyme tryptophan 2,3-dioxygenase 2 (TDO2), ensuring the degradation of the essential amino acid tryptophan, causes T cell inhibition by depleting tryptophan in the cell environment and increasing the concentration of its metabolites. This inhibition mechanism has been shown to induce immune tolerance in pre-clinical murine tumor models, but little is known about the expression of TDO2 in human tumors.

Thanks to a homemade antibody, we could reveal by immunohistochemistry (IHC) that TDO2 is expressed by vascular smooth muscle cells (vSMCs) in grade 4 glioblastomas, breast cancers, melanomas and their lymph node metastases, but not in the healthy surrounding tissue. We showed that those TDO2-positive vSMCs generally surround immature vessel, as TDO2 co-localizes with the immaturity marker Regulator of G-protein signalling 5 (RGS5). Those vSMCs also surround morphologically abnormal vessels, which are either large vessels with a single, irregular layer of vSMCs or an accumulation of many small vessels surrounded by several layers of vSMCs. Taken together, those data suggest that TDO2-positive vessels are probably poorly functional vessels. To complete this part of the project, we will stain other tumor types (bladder, liver, kidney, colorectal, head and neck, lung, stomach, pancreas, uterus and ovary cancer) to confirm that our observations are not restricted to glioblastomas, melanomas and breast cancers. This IHC analysis will be compared to the corresponding clinical data of the patients to reveal potential correlations between the expression of TDO2 and some characteristics like disease progression, metastases formation or treatments (radio- or chemotherapy).

After the characterization of TDO2-positive vessels, we wondered whether those vessels degraded tryptophan sufficiently to cause immunosuppression. We aimed to reveal tryptophan-depleted regions of the tumors by IHC. As tryptophan itself cannot be revealed by IHC, we stained tryptophanyl-tRNA synthetase (WARS), a protein that is up-regulated in the absence of tryptophan. Unfortunately, WARS was not up-regulated in TDO2-positive tumors, indicating that tryptophan was not depleted. However, this does not exclude a role for TDO2 in immunosuppression because T cell inhibition can be mediated by tryptophan metabolites. To evaluate the impact of TDO2 on the immune system, we will quantify the immune cell infiltration in human tumors by IHC. More precisely, we will quantify the number of infiltrating immune cells (lymphocytes, macrophages, dendritic cells) in TDO2-positive and -negative tumors as well as their distance towards TDO2-positive and -negative vessels.

Keywords: TDO2, Tumor-associated vessels, Immunosuppression

B192 / CD70^{+} cancer associated fibroblasts as an adverse prognostic factor and a new immunotherapeutic target for colorectal cancer

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Numerous studies have reported that tumor progression and invasiveness are determined not only by the malignant cancer cells themselves but also by the surrounding tumor microenvironment, including cancer-associated fibroblasts (CAFs). Although CAFs are implicated in tumor progression, their total depletion has been demonstrated to induce more aggressive tumors, indicating that different CAF subpopulations have opposing tumor-promoting or tumor-inhibitory roles. Unfortunately, specific markers to target the tumor-promoting CAFs are lacking. Expression of the immune checkpoint CD70 is normally tightly regulated and limited to cells of the lymphoid lineage only. Instead, tumors hijack CD70 to facilitate immune evasion by increasing the amount of suppressive regulatory T cells (Tregs), inducing T cell apoptosis and skewing T cells towards T cell exhaustion. Over the past years, a lot of clinical successes have been generated by the blockade of immune checkpoints. However, in colorectal cancer (CRC) the efficacy remains limited to a small subset of patients with mismatch repair-deficient (MSI) tumors which might be caused by the intense dialogue between stroma and malignant cells. Therefore, in this study we have explored the expression patterns of the immune checkpoint molecule CD70 in CRC, with a particular focus on CAFs.

In the study, the expression of CD70 was examined by immunohistochemistry in 51 CRC specimens. In addition, the prognostic value of CD70, and the relationship with Tregs and MSI was explored. To elucidate the pro-tumorigenic role of CD70-positive CAFs in more detail, the CT5.3 hTERT myofibroblast cell line was sorted for the membrane expression of CD70. CD70^{high}

and CD70^{low}myofibroblasts were used to study the effects of CD70 on migration (IncuCyte™ Live-Cell Imaging System, Essen BioScience) and Treg survival (FACS Aria II, Becton Dickinson).
We are the first to demonstrate the expression of CD70, not just on some malignant cells but also on the majority of CAFs in invasive CRC specimens. Thereby, CD70-expression on CAFs was significantly associated with negative clinicopathological parameters such as metastasis and advanced stage. Moreover, CD70-positive CAFs served as an independent prognostic marker in CRC. Functionally, we reveal increased migratory capacities of CD70-positive CAFs and their role in immune escape by the accumulation of naturally occurring Tregs.
We have identified a new targetable CAF subpopulation, marked by the expression of CD70 and equipped with strong tumor-promoting properties. Thereby, we have found evidence of a cross talk between CD70^{+} CAFs and Treg, paving the way towards immune escape. The lack of association between CD70 expression and MSI-status highlights the potential of this target in CRC subsets that do not benefit from immune checkpoint blockade. We believe that targeting CD70 holds great potential in CRC, especially in light of the limited immunotherapeutic options available in CRC.

Keywords: CD70, Cancer associated fibroblasts, regulatory T cells

B193 / PD-L1 expression is not a predominant characteristic of Ewing sarcomas

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Programmed cell death-1 receptor (PD-1) engagement by its ligand, PD-L1, can inhibit the function of tumor antigen-specific T cells and contribute to immune evasion of many cancers. Disruption of the PD-1/PD-L1 interaction, e.g. by specific antibody blockade, was effective to unleash antitumor T cell responses in several malignancies. PD-L1 expression in tumor biopsies has emerged as a relevant factor to predict successful intervention with PD-L1/PD-1 checkpoint inhibitors. We hypothesized that PD-L1/PD-1 may contribute to immune evasion of Ewing sarcoma (EwS), a highly aggressive cancer of bone and soft tissue with an urgent need for optional therapeutic approaches.
We characterized the expression of the immune checkpoint proteins PD-1 and PD-L1 on pretherapeutic EwS tumor biopsies via immunohistochemistry. In addition, PD-L1 expression on GD2-expressing EwS xenografts after treatment with GD2-specific, chimeric antigen receptor (CAR) expressing, T cells was evaluated on archived specimens. Flow cytometry analysis was employed to assess the ability of EwS cell lines to express PD-L1 after IFN-γ stimulation. Of 60 EwS patient biopsies, including 12 relapse samples, none expressed PD-L1 on tumor cells, while the ligand was detectable on single macrophages in 8 specimens and on infiltrating T cells in one individual case. Evaluation of CAR T cell treated xenografts revealed lack of PD-L1 expression on EwS cells despite T cell infiltration in tumor tissues. PD-L1 was expressed on the cell surface of all of 13 tested EwS cells lines in response to stimulation with IFN-γ. Staining with the PD-1 specific mAb NAT105 detected PD-1+ infiltrating T cells in 4 of the 60 biopsies. In addition, in a noticeable proportion of EwS patient biopsies (35/60), the tumor cells were reactive with this antibody. Reactivity of tumor cells with PD-1 specific mAb was also found with an additional PD-1 specific mAb clone (4H4D1) on 28 of 29 EwS samples. In CAR T cell treated EwS xenografts (VH-64 and TC-32), PD-1 expression was limited to infiltrating T cells.
We conclude that PD-L1 expression by EwS cells is rare in their natural microenvironment, while in vitro results demonstrate that EwS cells are generally able to express PD-L1 under inflammatory conditions. Our finding that EwS are reactive with PD-1 specific mAbs deserves further exploration to characterize possible biologic effects of PD-1 expression of tumor cells. These findings discourage the use of PD-L1/PD-1 immune checkpoint blockade as a single-agent therapeutic option for this cancer.

Keywords: PD-L1, PD-1, Ewing Sarcoma

B194 / Effect of Ta99 antibody on myeloid-derived suppressor cells in mice bearing B16 melanoma

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Myeloid-derived suppressor cells (MDSCs) are immature immune cells that have been shown to promote tumor progression, correlate with poor clinical outcome and decrease the efficacy of immunotherapies. Tumor antigen-specific monoclonal antibodies (mAbs) are used to treat hematological malignancies and solid tumors. They induce tumor cell killing through mechanisms involving antibody dependent cell-mediated cytotoxicity (ADCC). In addition to that mechanism, the Fc portion of therapeutic mAb might interact with Fc-gamma receptors (FcγR) on MDSCs and modulate their suppressive activity. Nevertheless, little is known about this alternative mechanism. In an attempt to identify the effect of mAbs in modulating MDSCs through FcγR-dependent mechanisms, we evaluated the effect of a mAb, Ta99, in regulating the expansion of MDSCs and their expression of FcγR in blood, tumor, and spleen of mice bearing B16 melanoma.

Ta99 treatment decreased the expansion of MDSCs and down-regulated the expression of FcγRI on the surface of those cells. These findings suggest that Ta99 mainly interacts with FcγRI on MDSCs and this might represent an alternative mechanism to inhibit the expansion of these suppressor cells.

Keywords: Myeloid-derived suppressor cells, Ta99, Fc-gamma receptors

B195 / Negative feedback of SL-15-cholesterol impairs colon cancer progression

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Summary: Increased cholesterol content in cancer cells is well documented. SL-15, involved in cholesterol biosynthesis, is reported as a proto-oncogene. Here we unexpectedly show that SL-15 depletion caused by cholesterol accumulated over a threshold point profoundly potentiates colon cancer progression. Intriguingly, the SL-15 reduction by intracellular accumulated cholesterol simultaneously triggers p53 degradation and GSK3b inhibition, together inducing epithelial-mesenchymal transition, anoikis resistance and oncogene-induced senescence bypass. A high-cholesterol regimen further confirms that SL-15 depletion by accumulated cholesterol confers aggressive capacities through the production of migratory cancer stem cells. Furthermore, the strong relationship between the dissociation of SL-15 and p53 and the inhibition of GSK3b in human advanced colon cancers suggests SL-15 as a key regulator in cholesterol-initiated colon cancer progression.

Significance: Despite recent advances in therapy, the incidence of colon cancer is increasing, primarily due to increased dietary cholesterol intake. Moreover, metastasis is responsible for most colon cancer deaths. Here, we exploit colon cancer progression caused by accumulated cholesterol and show a novel pathway in which excess cholesterol facilitates malignant conversion and metastatic dissemination of cancer cells via SL-15 degradation. Thus, this finding suggests SL-15 as a pharmacologically viable target to suppress colon cancer progression, and possibly colon cancer metastases.

Keywords: colorectal cancer metastasis, cholesterol, a novel target

B196 / The immune microenvironment in an orthotoptically growing tumor is distinct from a subcutaneous tumor when studying the bladder cancer MB49 model system

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Herein we examined the differences of the immune cell infiltration profiles of orthotopic and subcutaneous (heterotopic) MB49 tumors, a syngeneic murine bladder cancer model. Previous studies have shown that orthotopic and subcutaneous MB49 tumors respond well to immunotherapies such as CpG oligodeoxynucleotides (ODNs) or CD40 stimulating therapies. Here, we studied the immune cells' frequency in the tumors in an early time point. On day 11 after the initial tumor inoculation, CpG ODN 1668 or dilution buffer, as a control, were administered locally. CpG ODN 1668 is a type B CpG and a strong immunostimulatory agent. In the MB49 model this agent can cure mice from tumors and induce tumor-specific immunity. 24 hours post drug exposure, the animals were sacrificed and the tumors were immediately frozen. Immunofluorescence staining for CD4 and CD8 was performed on 7um thick cryosections. The infiltration of $CD4^+$ and $CD8^+$ cells in MB49 tumors alone or in the bladders including MB49 tumors was quantified in ImageJ and expressed as positive cells' area per tissue section area. Interestingly, we observed that the frequency of $CD4^+$ and $CD8^+$ cells increased inside the orthotopic tumors 24 hours post CpG ODN 1668 administration, while it appeared to remain stable when the quantification was performed on tumors along with the bladder walls surrounding them. In the subcutaneous tumors the frequency of $CD4^+$ and $CD8^+$ cells was lower. Our data suggest that the bladder of orthotopic tumor bearing mice hosts a reservoir of $CD4^+$ and $CD8^+$ cells in proximity to the tumor lesion, where they migrate rapidly upon immune adjuvant stimulation. On the other hand, the infiltration of subcutaneous tumors probably requires prolonged stimulation or later analysis time points. This difference might be due to the lack of an accessible immune microenvironment provided by the surrounding organ, which can mediate the homing of cells on site even before stimulation. It seems that the orthotopic tumor growth may be beneficial for studying rapid immune cell infiltration events. Therefore, we propose this as an insightful model to study the impact of immunotherapy for bladder cancer on the tumor microenvironment (TME) composition in early time points of therapy. Nevertheless, the functionality of the immune cell infiltrate and the impact of those findings on the efficacy of cancer immunotherapy on each variant of the models have to be assessed further.

Keywords: Orthotopic tumors, MB49 model, Bladder cancer

B197 / STING agonist modulates tumor immune microenvironment of non-inflamed lung cancer to potentiate the efficacy of immune checkpoint blockade

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Cancer immunotherapy targeting immune checkpoints are now emerging as a promising therapeutic strategy in various tumors. However, the treatment of T-cell non-inflamed tumor which lacks intratumoral T cell infiltrates are still major clinical hurdle. To overcome non-inflamed tumor microenvironment, we used cyclic dinucleotides, which is an agonist of stimulator of interferon genes (STING) in mouse model of non-inflamed lung cancer. Localized intratumoral injection of STING agonist remodels tumor microenvironment in lung cancer, leading to increased intratumoral T cell infiltration, decreased tumor angiogenesis, and augmented anti-cancer immune response. The localized STING therapy activates systemic and tumor-specific immune response. Combination therapy of STING agonist and immune checkpoint inhibitors (ICI) targeting PD-1 effectively suppresses the growth of lung cancer which was resistant to ICI monotherapy. Moreover, optimal sequence and route of administration was critical for synergistic effects of this combination. Collectively, our findings demonstrate that localized STING therapy effectively sensitizes non-inflamed lung cancer to systemic ICI treatment and induce a maximal anti-cancer immune response.

Keywords: STING agonist, Tumor microenvironment, Immune checkpoint inhibitor

B198 / Soft tissue sarcoma subtypes: differential quantities and qualities of tumor infiltrating lymphocytes

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Soft tissue sarcomas (STS) are a heterogeneous group of malignant tumors with over 50 subtypes, differing in tumor biology and clinical outcome. To explore whether STS would be sensitive to immune therapies, and since current data on tumor immunology in STS subtypes is scarce, we have assessed quantity and quality of Tumor Infiltrating Lymphocytes (TILs) in 6 prevalent STS subtypes. Tumors were freshly obtained after surgery and dissociated into single cell suspensions. Lipoma and melanoma were taken along as controls, representing a benign soft tissue tumor and immunogenic tumor, respectively. T cells were counted per mg wet tumor tissue, and phenotypically analyzed by flow cytometry, with emphasis on CD8 T cell maturation (assessed via CD45RA and CCR7) and T cell co-inhibition (PD1, LAG3, TIM3, BTLA). STS subtypes demonstrated a range of T cell numbers and fractions of T cells expressing the co-inhibitory receptors PD1, LAG3 and TIM3. Interestingly, for most STS subtypes these parameters varied concordantly. Leimyosarcoma and myxofibrosarcoma have high numbers of T cells; the highest fractions of effector memory CD8 T cells; and the highest fractions of CD8 T cells expressing co-inhibitory receptors (IRs). In contrast, these parameters varied in a non-concordant manner for GIST, a subtype that showed high numbers of T cells, but low fractions of CD8 T cells that were differentiated or expressed IRs. STS subtypes clearly show differential quantities and qualities of TILs. Remarkably, despite the high number of TILs, GIST has relatively low fractions of T cells showing the characteristics of an antigen-specific encounter. Myxofibrosarcoma is high in absolute numbers of TILs, as well as fractions of CD8 T cells that have differentiated and express IRs, and may represent a subtype amenable for treatment with checkpoint inhibitors.

Keywords: Soft Tissue Sarcoma, Tumor infiltrating lymphocytes, Tumor microenvironment

B199 / Modulation of cancer cell proliferation, growth and microenvironmental factors in viable human tissue co-cultured with patient-derived or commercial cancer cells

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With over 90% of cancer-associated deaths, the formation of metastasis, characterized by high cellular heterogeneity and genetic disparity in regards to the primary tumor, is listed as the main cause of death in most cancers. Currently, this complex process can only partly be reflected in ***in vitro*** models due to lacking human microenvironment.
Efficacy of the anti-tumor drugs cisplatin, bevacizumab and vemurafenib on tumor growth reduction and modulation within its natural microenvironment was determined by preparing fresh human tumor tissue slices to reflect the response of solid tumors. Co-cultures of fresh human lung tissue slices with either allogenic GFP-labeled MDA-MB-231 or patient-derived (lymph nodes), disseminated and GFP-labeled melanoma cells were treated analogously to the tumor slices to gain insight into first steps in the metastatic process.
Cancer cells integrate into the healthy lung tissue and proliferate resulting in 6fold increase of cancer cells within the first 24 hours of co-culture. Biomarkers for neoangiogenesis, such as extrinsic

VEGF-levels, were elevated 3.5fold in lung tissue co-cultures with MDA-MB-231 cells and 5.4fold in lung tumor tissue slices after 48h. Treatment with bevacizumab [200 µg/mL], an angiogenesis inhibitor, suppressed VEGF-release up to 24fold in the co-cultures and up to 25fold in lung tumor tissue slices after 48h. Supernatants of these treatments also showed impaired migration of endothelial cells by up to 81% in co-cultures and up to 83% in tumor slices. Other tumors biomarkers like extrinsic GM-CSF were also highly elevated [13fold after 24h] in co-cultures compared to healthy tissue controls. Cisplatin treatment [50µM] led to a decline of viability and reduced cancer cell number in co-cultures by up to 37.5% and in tumor slices up to 48.7% after 72h. MDA-MB-231-invaded lung tissue and tumor slices were less sensitive in regards to their respective anti-cancer drug efficacy than the 2D culture. To address patient-specific genetic disparities, co-cultures of lung tissue and disseminated melanoma cells were performed with both patient-derived cells that carried the driver mutation V600E of the BRAF gene and non-mutated melanoma cells. Treatment with vemurafenib [50µM], which interrupts the B-Raf/MEK/ERK pathway in V600E mutants resulting in programmed cell death, led to a 71% decrease of V600E cancer cells after 48 hours whereas non-mutated cells showed no significant cancer cell decrease.
Here we modulate cancer cell proliferation, growth and mediator concentrations in human lung tissue and show that tumor cells in solid tumors ***ex vivo*** and freshly seeded into healthy lung tissue ex vivo are sensitive to tumor treatments. This work aims to translate cancer drug efficacy data from animals towards humans to optimize clinical trial outcome.

Keywords: Tumor microenvironment, Human ex vivo lung tissue, Metastasis

B201 / Reviving chemotherapy sensitivity after anti-CCR4 mAb (mogamulizumab) treatment in lung cancer patients

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Patients with advanced lung cancer have poor survival, although they have received multidisciplinary therapy. Therefore, the novel effective therapy is needed. In various malignancies, tumor cells escape the host immune defenses, in which regulatory T cells (Tregs) play an important role. Tregs, maintaining self-tolerance and homeostasis in the immune system, suppress antitumor immune responses in cancer patients. Thus, Tregs are crucial in controlling antitumor immune responses. Several clinical studies show that a number of Tregs at tumor site was correlated with poor prognosis and Tregs suppress the antigen-specific T-cell induction in immunotherapy. Therefore, controlling Treg functions is probably promising immunotherapy.
The study of adult T-cell leukemia-lymphoma (ATL) revealed that Tregs strongly express CCR4 molecule of a CC chemokine receptor on their surface. The humanized anti-human-CCR4 monoclonal antibody (mogamulizumab) recognizes CCR4 molecule and shows a robust ADCC activity against CCR4-positive cells such as Tregs. Thus, Tregs depletion by mogamulizumab probably enhances the host immune response against the tumor. We recently finished the clinical trial of mogamulizumab treatment in advanced solid cancer, and the monitoring of $FoxP3^+$ Tregs in the peripheral blood mononuclear cells during treatment indicated efficient depletion of those cells, even at the lowest dose of 0.1 mg/kg used.
In this study, we analyzed the response against chemotherapy before and after mogamulizumab treatment in 6 advanced lung cancer patients who were enrolled in the clinical trial. Although the patients finished standard chemotherapy and therefore were to be refractory, 4 of 6 patients showed the partial response (PR) in chemotherapy after mogamulizumab treatment. While 2 of 6 patients showed PR in chemotherapy before mogamulizumab. In 3 of those patients, we analyzed the number of immune cells (CD3 T cells and $CCR4^{+/-}$ $FoxP3^+$Tregs) and expression of PD-L1 (SP142) on tumor cells in lung cancer tissues by immunohistochemistry at diagnosis and after mogamulizumab treatment. We observed efficient depletion of $CCR4^+FoxP3^+$Tregs after mogamulizumab treatment in all patients, while $CCR4^-FoxP3^+$Tregs were detected in lung cancer tissues. In 2 PR patients in chemotherapy after mogamulizumab treatment, we observed increased number of CD3 and $PD\text{-}1^+$cells. In one patient, increased PD-L1 expression on tumor cells was observed. On the other hand, in one SD patient in chemotherapy after mogamulizumab, the number of CD3, $PD\text{-}1^+$ cells, and expression of PD-L1 on tumor cells were decreased. Treg depletion by mogamulizumab may induce inflamed tumor microenvironment in some lung cancer patients, and result in reviving chemotherapy sensitivity.

Keywords: anti-CCR4 mAb, Treg, inflamed tumor

B202 / Characterization of tumor-associated B-cell subpopulations and humoral immune response in head and neck squamous cell carcinoma (HNSCC)

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The immune system is important in both preventing and promoting malignancies. While there is increasing knowledge about T-cellular functions in this context, little is known about the role of B cells in cancer pathophysiology. The B-cell compartment consists of subpopulations with various distinct functions. They can exert antitumorigenic effects, for example by antigen-presentation or production of tumor-targeting antibodies. However, protumorigenic regulatory B cells can inhibit adequate immune responses against malignancies. The purpose of this study is therefore to provide a comprehensive analysis of tumor-associated B-cell subsets in head and neck squamous cell carcinoma (HNSCC) and to analyze humoral immune responses against various tumor-associated antigens in these patients.
10-colour flow cytometry was performed on single cell suspensions derived from treatment-naïve HNSCC (n = 36) and non-cancerous mucosa (n = 7), peripheral blood mononuclear cells (PBMC) of HNSCC patients (n = 36) and healthy controls (n = 20). Tumor-infiltrating lymphocytes were detected in all analyzed HNSCC and a substantial B-cell infiltration in the majority of tumors. Analysis of B-cell subsets showed increased percentages of activated B cells ($CD86^+$), antigen-presenting B cells ($CD86^+CD21^-$), plasmablasts ($CD38^{high}$), plasmacells ($CD38^{high}CD138^{high}$) and memory B cells (IgD^-CD27^+) compared to PBMCs of cancer patients and healthy controls. Different regulatory B-cell phenotypes did not show a significant increase in the tumor microenvironment. Although cancer associated with human papillomavirus (HPV) showed higher numbers of B cells within the tumor, the composition of B-cell subsets did not show major differences compared to HPV-negative tumors. LUMINEX bead assay revealed the presence of tumor-specific antibodies against at least one of the analyzed 36 tumor-associated antigens in serum samples of 32/36 patients.
We could detect a marked B-cell infiltration in the majority of HNSCC and increased numbers of B cells in HPV-positive HNSCC. The results are indicative of an active, antigen-recognizing B-cell phenotype. Our study provides a first comprehensive analysis of tumor-infiltrating B-cell subsets in HNSCC. In-depth knowledge of tumor-associated immune cells will be crucial for the understanding of mechanisms underlying immunotherapeutic approaches.

Keywords: B cells, head and neck squamous cell carcinoma, microenvironment

B203 / Multiplex three-dimensional optical mapping of tumor immune microenvironment

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Untangling the complexity of programmed death-ligand 1 (PD-L1) expression within a heterogeneous tumor microenvironment is an urgent challenge in PD-1/PD-L1 immune checkpoint blockade therapy. Here, we address this challenge with a method, termed transparent tissue tomography (T3), facilitating three-dimensional (3D) visualization and spatial analysis of distributions of multiple biomarkers regarding to cancer cells, vasculature, and immune cells in context in the tumor microenvironment. With T3 analysis of transgenic mouse mammary tumors immunostained against Her2, CD45, Ki-67, CD31, and PD-L1, we reveal that PD-L1 expression within the tumor microenvironment is highly adaptable for efficiently preventing immune cell infiltration into the tumor. Stronger correlation of Her2 and PD-L1 expression in the tumor periphery where has a high $CD45^+$ immune infiltrate density is determined by tumor-wide analysis. Also, tomographic analysis shows blood vessels expressing PD-L1 in the tumor core, where PD-L1 expression is lower. Furthermore, high-resolution T3 image localizes PD-L1 expression to a region between the endothelium and the surrounding smooth muscle cells in blood vessels. We investigate spatial pharmacokinetics of anti-PD-L1 antibody in the whole mouse mammary tumor in the context of hypoxia, $CD31^+$ blood vessels, and target $PD\text{-}L1^+$ cells. We also evaluate anti-tumor immune responses after PD-L1 blockade therapy using T3. We observe broad distribution of tumor infiltrating $CD3^+CD8^+$ cytotoxic T cells in 3D tumor section following combination therapy of radiation and anti-PD-L1 antibody compared to PBS, anti-PD-L1 antibody alone, or radiation alone treatment group. Moreover, we apply T3 for immunoanalysis of whole core needle biopsies of mouse mammary and human head and neck tumors. We spatially map PD-L1 expression and granzymeB producing immune cells including $CD3^+CD8^+$ cytotoxic T cells in pre-treatment and in-treatment core needle biopsies at cellular resolution and in three dimensions. Meanwhile, T3 analysis is nondestructive, allowing

secondary analysis by conventional immunohistochemistry (IHC). We anticipate that T3 can be applied broadly to facilitate preclinical studies of immunotherapy and also find use in spatial, multiparameter analysis of patient biopsies, particularly to improve predictive testing and analysis of immune responses to tumor immunotherapy.

Keywords: Tumor microenvironment, Multiplex 3D tumor imaging, PD-L1

B204 / Frequency of tumor infiltrating lymphocytes and the potential of immunogenic lipids in pediatric solid tumor entities

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In Germany, we have an incidence rate for all pediatric malignancies of approximately 2000 children per year and a 15-year-survival rate of 81 %. Nevertheless, the outcome of patients with relapsed or refractory cancer remains poor. Moreover, there are several entities, e.g. intrinsic childhood brain tumors, which pose a considerable challenge for treatment options. Accordingly, the need of new therapeutic strategies remains a matter of utmost urgency. Latest research suggests that tumor cells can be recognized and eliminated by nonconventional T cells such as iNKT and γδ T cells. Unlike conventional αβ T cells, antigen recognition is independent of HLA haplotype in iNKT and γδ T cells and offers new options for off-the-shelf, pan-population cancer immunotherapies. iNKT and Vδ1 T cells recognize lipids via MHC class-1 related CD1d molecules, but so far, the nature of those lipids is largely unknown. The glycolipid ligand, α-Galactosylceramide (α-GalCer) is known as a synthetic model antigen for iNKT cells. Several combinatory immunotherapeutic approaches of iNKT cells and α-GalCer have been investigated, but with dose-limiting toxicities. Therefore, we aimed for the analysis of the infiltration of nonconventional and conventional T cells and the identification of endogenous lipid ligands by utilizing mass spectrometry and cellular assays.
First, we isolated tumor infiltrating lymphocytes (TILs) from seven pediatric brain tumor samples (pilocytic astrocytoma, CNS HGNET-BCOR and ependymoma) and nine other pediatric solid tumor samples (teratoma, AT/RT, neuroblastoma, giant cell tumor, benign soft tissue tumor, LCH, undifferentiated sarcoma, papillary renal cell carcinoma and papillary thyroid carcinoma) by percoll density centrifugation and analyzed the TILs via flow cytometry. In all entities described above, we identified infiltrations of γδ T cells and conventional CD4 and CD8 positive T cells. Notably, samples of papillary renal cell carcinoma and papillary thyroid carcinoma were infiltrated with Vδ1, Vδ2 and iNKT cells. Furthermore, we fractionated tumor lipids and tested the fractions for their ability to induce the proliferation of iNKT cells. According to our first analysis of seven polar and seven nonpolar lipid fractions, we found one nonpolar fraction with the potential to activate iNKT cells. Further characterization of this fraction by LC/MS is ongoing.
Our project is expected to provide new insights into the therapeutic utility of iNKT cell therapy in pediatric solid tumors.

Keywords: Tumor infiltrating lymphocytes, invariant NKT cells, pediatric cancer

B205 / Robust digital dissection of immune cell types in fresh and cultivated human bladder cancer samples

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We have developed a combinatorial workflow employing deconvolution based and signature based digital dissection of immune cell types in nearly all kind of tissue samples. During the last years the immune microenvironment of solid tumors turns into focus of both, survival prognosis and patient stratification for immunotherapy. To that extend standardized quantification and qualification of tumor immune cell infiltrates in a broad range of either fresh or cultivated tissue samples is highly recommended. In addition to histological attempts based on immunostaining of discriminating surface markers, such as CD3 and CD8 in the case of the well established „Immunoscore" there are now additional approaches coming up with the comprehensive use of high throughput RNA sequencing (RNASeq). Deconvolution based tools with their most prominent representative CIBERSORT calculate the relative proportion of different cell types within one sample whereas signature based ones calculate the enrichment (-scores) for each cell type in a minimum of two samples. Quality and/or granularity of digital immune cell typing heavily depends on the set of marker genes used in combination with the associated algorithm and therefore a meaningfull combination of those tools might be more helpfull than the use of a single one. To that extend we have developed a combinatorial procedure consisting of CIBERSORT, ImmQuant and xCell as a representative of signature

based tools for high quality digital immune cell typing. In addition some of the tools were used with different reference data sets to achieve maximal granularity in cell subtyping. For proof-of-quality, RNAseq based results are further evaluated independently by subsequent multiplex marker staining in histological analysis (Abstract Miegel et al. Ex vivo human bladder cancer tissue system for comprehensive application and monitoring of immune microenvironment therapies). The entire setting was used in two types of studies encompassing i) cocultivation of A549 cells together with healthy donor PBMCs and ii) ex vivo cultured primary human bladder cancer samples. In both cases changes in immune cell composition were monitored after treatment with a recombinant adenovirus (Im02) expressing human IL-2, single-chain IL-12 and the costimulator 4-1BBL in one single construct. Time course experiments revealed Im02 specific upregulation of M1 macrophages, dendritic cells, CD4+ as well as CD8+ T-cells at different time points up to 96h underpinning both, anti-tumor immune stimulation by Im02 and usability of digital immune cell dissection for monitoring the Im02 effects.

Keywords: immune microenvironment, expression-based deconvolution, bioinformatics

B207 / Blockade of FOXP3 transcription factor dimerization and FOXP3/ AML1 interaction inhibits T regulatory cell activity: sequence optimization of a peptide inhibitor

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Although T regulatory cells (Treg) are essential for the prevention of autoimmune diseases, their immunoregulatory function restrains the induction of immune responses against cancer. Thus, development of inhibitors of FOXP3, a key transcription factor for the immunosuppressive activity of Treg, might give new therapeutic opportunities. In a previous work we identified a peptide (named P60) able to enter into the cells, bind to FOXP3, and impair Treg activity *in vitro* and *in vivo*. Here we show that P60 binds to the intermediate region of FOXP3 and inhibits its homodimerization as well as its interaction with the transcription factor AML1. Alanine-scanning of P60 revealed the relevance of each position on FOXP3 binding, homodimerization, association with AML1 and inhibition of Treg activity. Introduction of alanine at positions 2, 5 and 11 improved the activity of the original P60, whereas alanine mutations at positions 1, 7, 8, 9, 10 and 12 were detrimental. Multiple mutation experiments allowed us to identify peptides with higher FOXP3 binding affinity and stronger biological activity than the original P60. Head to tail macrocyclization of peptide P60-D2A-S5A improved Treg inhibition and enhanced anti-tumor activity of anti-PD1 antibodies in a model of hepatocellular carcinoma. Introduction of a D-aminoacid at position 2 augmented significantly microsomal stability while maintained FOXP3 binding capacity and Treg inhibition *in vitro*. In vivo, when combined with the cytotoxic T-cell epitope AH1, it induced protection against CT26 tumor implantation. This study provides important structure-function relationships essential for further drug design to inhibit Treg cells in cancer.

Keywords: T regulatory cells, Foxp3, Inhibitory peptide

B208 / Polymerase epsilon proofreading mutations are associated with tertiary lymphoid structure formation in Endometrial and Colorectal Cancer

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Endometrial cancer (EC) is the most common gynecological malignancy and new therapeutic options are needed. Highly mutated ECs, due to microsatellite instability (MSI) or a mutation in the exonuclease domain of polymerase epsilon (***POLE***), have previously been linked to increased immune infiltration and a better prognosis. In patients with ***POLE*** mutant cancers in particular, a so-called ultramutation phenotype is observed, which is thought to result in an increase of antigenic neo-epitopes. This may explain the enhanced cytotoxic T cell response and improved prognosis of POLE patients. Although CD8+ T cell infiltration is associated with an improved prognosis in a wide range of tumors, there are also other factors involved in the anti-tumor immune response. Several studies indicate that tertiary lymphoid structures (TLS) might have an important function in orchestrating T and B cell responses. To further understand the role of TLS in anti-tumor immunity, we assessed the presence of TLS and their relation to tumor infiltrating CTLs in ***POLE*** mutated, MSI and microsatellite stable (MSS) subtypes of EC by immunohistochemistry, immunofluorescence (IF) and analysis of RNA sequencing data. We report for the first time the observation of higher numbers of TLS in ***POLE***-mutant cancers compared to endometrial tumors with other molecular subtypes. CD20+ B cell follicles were scored

in FFPE slides from 109 EC patients. Interestingly, we noticed a striking increase in B cell follicles in ***POLE*** mutated tumors compared to MSS and (to a lesser extend) to MSI cases. We used multicolor IF of sequential slides to produce a detailed overlay image of a B cell follicle in ***POLE*** mutated EC. These follicles contain the cell types typically associated with TLS, including B cells, plasma cells, T cells, dendritic cells and high endothelial venules, indicating they are indeed TLS. We then turned to publically available endometrial TCGA RNA sequencing. Gene expression of ***MS4A1*** (CD20) and ***CD8A*** (CD8) is increased in ***POLE*** and MSI cases compared to MSS tumors. Furthermore, using a list of 71 genes associated with TLS formation we found an extensive mutual correlation pattern in POLE tumors, especially compared to MSS cases. Since POLE mutations have been described in 1-2% of colorectal cancers (CRC), we have validated these findings in FFPE slides and TCGA RNAseq data from ***POLE*** mutant, MSI and MSS CRC.

Our observations indicate an association between genomic instability (or neo-epitope presentation) and TLS presence. We suggest that the anti-tumor immunity of T cells instigated by the abundant neo-epitope presentation of ***POLE*** tumors could enhance the B cell response and formation of TLS. Subsequently, the TLS may play a role in coordinating and thereby enhancing the local anti-tumor immune response.

Keywords: POLE mutant cancer, Genomic instability, Tertiary lymphoid structure

B209 / Differentiating the role of host IDO from tumor IDO in regulating anti-tumor immunity

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Indoleamine 2,3-dioxygenase (IDO) catalyses the first and the rate-limiting step in tryptophan metabolism in the kynurenine pathway. IDO-mediated tryptophan depletion induces T cell anergy and apoptosis. IDO expressed by tumors in response to interferon-gamma produced by immune cells mediates adaptive resistance to immunotherapy. In fact, IDO is shown to induce resistance to ipilimumab. IDO produced by tumor cells, or by non-tumor cells in the tumor microenvironment, could explain the lack of response to some checkpoint inhibitors in cancer patients. We have previously shown that, through production of nicotinamide adenine dinucleotide (NAD^+), IDO confers resistance to chemotherapy and radiation therapy independent of its role in regulating the immune system. We have also shown that targeting IDO in melanoma cells restored anti-tumor T cell response and inhibited tumor growth. Moreover, silencing IDO in dendritic cells enhanced anti-tumor T cell function. However, there is little known about the differential role of host IDO versus tumor IDO in suppressing anti-tumor immunity. Normal, non-tumor cells of tissues including lung, placenta, and endothelium produce IDO. Moreover, in the presence of a tumor, dendritic cells, myeloid-derived suppressor cells (MDSCs), macrophages, and other myeloid cells can produce IDO and help further suppress immunosurveillance of cancer. Here we set out to determine which source of IDO, tumor or immune cells, is most important in suppressing anti-tumor T cells and cancer immunotherapy. Since not all patient tumors express IDO despite IDO expression by host cells, it is critical to determine whether non-tumor IDO is as important as tumor IDO in protecting cancer cells from the immune system.

We used CRISPR/CAS9 to permanently knockout (KO) IDO in B16-F10 melanoma cells. IDO wild type (WT) and KO melanoma cells are being grown in IDO WT and KO C57BL/6 mice to determine the role of host IDO vs tumor IDO ***in vivo***. We will compare tumor growth in syngeneic mice and tumor-specific T cell responses including pro- and anti-inflammatory cytokine production (IFN-γ, TNF-α, and IL-10). We will also examine T cell cytotoxic function to determine the effect of IDO expressed in tumor vs host on suppressing adaptive anti-tumor immunity. We will further study the presence of various macrophage subtypes (M1/M2) in tumors to compare the role of tumor IDO to host IDO in establishing pro-tumor macrophage subtypes in the tumor microenvironment. This study will determine mechanistically, and for the first time, the relative importance of the source of IDO (tumor or non-tumor) for suppression of tumor-reactive T cells and anti-tumor immunity and establishment of tumor-associated macrophages in the tumor microenvironment. Therefore, it will help us determine whether tumor IDO or host IDO should be the primary target of IDO inhibitors in the clinic.

Keywords: IDO, tumor, T cell

B210 / Immune cell composition and checkpoint expression in mesothelioma patient samples

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Till today, human malignant pleural mesothelioma (MPM) remains an aggressive cancer with a poor prognosis due to the limited impact on overall survival of the current treatments. Preliminary data from clinical trials show promising results with immune checkpoint blockade in mesothelioma patients. In order to develop an efficient immunotherapy, it would be of great interest to gain more insight in the immunological aspects of the tumor microenvironment (TME) and to investigate its immune checkpoint expression profile. Therefore, we identified different subsets of immune cells and looked at the expression of TIM-3, LAG-3, PD-1 and its ligand PD-L1 in formalin fixed paraffin embedded (FFPE) tissue and fluid samples from MPM patients.

Immunohistochemistry (IHC) was used to investigate tissue samples of 54 MPM patients (40 at diagnosis, 14 treated with chemotherapy), while multicolor flow cytometry was used to examine pleural (n=6) and ascites (n=5) fluid samples collected from 11 different chemotherapy-treated MPM patients via thoracocentesis (pleura) or paracentesis (ascites).

CD8+ tumor infiltrating lymphocytes (TILs), CD68+ histiocytes and macrophages and CD45RO+ memory T cells were present in all tissue samples, with CD8+ TILs as the predominant cell type. CD4+ TILs were present in the stroma of more than half of all samples. Expression of PD-1 and PD-L1 was seen on TILs in the untreated and treated tissue samples, while the expression on tumor cells was only noted in a few untreated samples. TIM-3 was expressed on tumor cells, TILs and plasma cells, less often in pretreated samples. CD45RO expression in the stroma was found to be a negative predictive factor for response to chemotherapy and expression of CD4 and TIM-3 in lymphoid aggregates were good prognostic factors.

In the fluid samples, CD4+ and CD8+ T cells, B cells, macrophages, natural killer (NK) cells, dendritic cells (DCs) and podoplanin+ (PDPN) tumor cells were found. CD4+ T cells, macrophages and DCs were the predominant cell types. Though LAG-3 expression was absent in our tissue samples it was expressed in 64% of the fluid samples on T cells and NK cells. PD-1 and TIM-3 expression was also found on these cells. PD-L1 was expressed on DCs, B cells, macrophages and PDPN+ MPM tumor cells in all the ascites samples, while less pleural samples were found to be positive. In both fluid types highest PD-L1 expression was found on the PDPN+ tumor cells. The percentage of PD-L1+PDPN+ tumor cells was a bad prognostic factor and CD4+ T cells were significantly correlated with better response to chemotherapy.

We demonstrated a patient-dependent variation in immune cell composition and immune checkpoint expression in MPM tissue and fluid samples. Our data point to TIM-3 and LAG-3 as new targets in mesothelioma.

Keywords: Mesothelioma, Immune checkpoints, Tumor microenvironment

B212 / The role of hMENA-related splicing in the cross/talk between cancer cells and CAFs in PDAC and NSCLC

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The dynamic interactions between tumor and cancer associated fibroblasts (CAFs), abundant cells in the tumor stroma, play a complex role in supporting carcinogenesis, immunosuppression and drug resistance.
Pancreatic ductal adenocarcinoma (PDAC) and non-small cell lung cancer (NSCLC) are tumors with a fibrotic stroma compartment, and we have demonstrated that the tissue-specific alternative splicing of hMENA, an actin regulator protein, generates hMENA11a and hMENAΔv6 isoforms that represent powerful diagnostic and prognostic factors in early stage NSCLC and PDAC. The overexpression of hMENA/hMENAΔv6, in the absence of hMENA11a, is crucial in cancer cell invasiveness and in TGF-β1 mediated Epithelial Mesenchymal Transition (EMT), but as yet no data are available on the role of hMENA/hMENAΔv6 in CAF biology. The aim of this study is to investigate whether hMENA/hMENAΔv6 play a role in CAF functions and in the regulation of cancer cells-CAFs crosstalk.
Herein, we have analyzed the expression of hMENA isoforms in organ-specific fibroblasts, freshly isolated from pancreatic and lung cancer tissues, by WB analysis. CAFs express hMENA and hMENAΔv6 isoforms but not hMENA^{11a}. hMENAΔv6 is overexpressed in CAFs compared to normal pancreatic fibroblasts (NFs) and lung fibroblasts isolated from tissue distant from the tumor (DFs). We have demonstrated, by gain and loss of function experiments, that hMENA/hMENAΔv6 are required for CAF activity. We found that the downregulation of hMENA/hMENAΔv6 isoforms in CAFs, by siRNA, reduced the contractile activity of CAFs in Col-1 gel and MMP2 activity. Accordingly, hMENAΔv6 overexpression in CAFs promoted their ability to invade into matrigel, to activate the MMP2 and to increase CAF-mediated cancer cell invasiveness. Reciprocally, co-culture experiments demonstrated that signals derived from tumor cells over-expressing hMENA/hMENAΔv6 are required for hMENAΔv6 overexpression and CAF activation.
Since conditioned medium (CM) of CAFs with high hMenaΔv6 expression correlates with their ability to increase cancer cell invasiveness, we performed a comparative secretome analysis of NFs vs CAFs with high and low hMENAΔv6 expression, by mass spectrometry-based proteomics analysis (LC-MS/MS). We identified an hMENAΔv6 related-signature including Gas6, the ligand of AXL, crucial receptor in EMT and drug resistance, and demonstrated that hMENAΔv6-dependent Gas6 secretion triggers cancer cell invasion.
In addition hMENA/hMENAΔv6 in cancer cells sustain Gas6/AXL axis activation leading to cancer cell invasion and survival.
These results demonstrated a new role for hMENA/hMENAΔv6 in orchestrating the cross-talk between cancer cells and CAFs ***via*** the regulation of Gas6/AXL axis. We suggest that in a subgroup of PDAC and NSCLC patients, the pattern of hMENA isoform expression in both tumor cells and CAFs may serve as a biomarker for patient stratification also for AXL-targeting agents in clinical practice.

Supported by AIRC

Keywords: PDAC and NSCLC, Cancer associated fibroblasts, Gas6-AXL axis

B213 / Targeting autophagy inhibits melanoma tumor growth by enhancing Natural Killer cells infiltration in a CCL5-dependent manner

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The failure in achieving a sustained and durable clinical anticancer immune response likely depends on the establishment of an immunosuppressive microenvironment by the tumor cells, which limits the infiltration of cytotoxic immune cells. Therefore, the key issue to achieve successful tumor immune response is to harness strategies allowing the redirection of immune effector cells to the tumor bed. It is now well established that targeting autophagy blocks the tumor growth of several cancers, however, its role on the infiltration of Natural Killer (NK) cells into tumors remains unknown. Here, we investigated the impact of targeting autophagy gene ***BECN1*** on the infiltration of Natural Killer (NK) cells into

melanomas. We showed that, in addition to inhibiting tumor growth, targeting ***BECN1*** increased the infiltration of functional NK cells into melanoma tumors. We provided evidence that driving NK cells to the tumor bed relied on the ability of autophagy-defective tumors to transcriptionally overexpress chemokine gene ***CCL5***. Such infiltration and tumor regression were abrogated by silencing CCL5 in BECN1-defective tumors. Mechanistically, we showed that the upregulated expression of CCL5 occurred through the activation of its transcription factor c-Jun by a mechanism involving the impairment of phosphatase PP2A catalytic activity and the subsequent activation of JNK. Similar to ***BECN1***, targeting other autophagy genes such as ***ATG5, p62/SQSTM1***, or inhibiting autophagy pharmacologically by chloroquine, also induced the expression of ***CCL5*** in melanoma cells. Clinically, a positive correlation between CCL5 and NK cell marker NKp46 expression was found in melanoma patients and high expression level of CCL5 was correlated with a significant improvement of melanoma patients' survival. We believe that this is the first study highlighting the impact of targeting autophagy on the tumor infiltration by NK cells and its benefit as a novel therapeutic approach to improve NK-based immunotherapy.

Keywords: natural killer, autophagy, melanoma

B214 / *Ex vivo* human bladder cancer tissue system for comprehensive application and monitoring of immune microenvironment therapies

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The composition and activity of the tumor microenvironment plays a decisive role to achieve therapeutic success in all current immunotherapeutic strategies, from checkpoint modulation to cell-based immunotherapies. Therapeutic approaches are required to modulate the immune microenvironment of cancers towards a responsive and defensive state. So far, rodent models are the mainstay of preclinical cancer immunotherapy development, however, most transplanted syngeneic models do not provide a suppressive immune microenvironment and as such lack predictability for human tumors.
Human tumor biopsies may be regarded as the best system for tumor microenvironment studies as they display all cellular relationships in their three-dimensional arrangement. However whole specimen tissue culture is challenging.
We introduce here a comprehensive test platform for human tumor samples based on biopsy-derived tumor specimens, ***ex vivo*** tissue culture, and analytic methods for all major gene and cell regulation levels. Basic and immunotherapy-induced effects are studied by (i) mRNA-expression bioinformatics of whole transcriptome sequencing results using bioinformatics, pathway-analyses, immune-cell infiltrate deconvolution, (ii) histologic cell profiling using multi-color immunofluorescence and electron microscopy (in collaboration with Vironova AB, Stockholm), (iii) immunologic cell profiling after tissue dissociation, and (iv) monitoring cytokine/chemokine profiles in tissue culture media using multiplex protein assays.
Employing this system for evaluation of the adenoviral immunotherapeutic Im02, a multivalent vector expressing the combination of 4-1BBL, IL-2, and scIL-12, we could show efficient and rapid re-programming of the tumor microenvironment in bladder carcinoma tissues. Within 6 days of culture after transduction with Im02, mRNA-profiles revealed specific induction of an activated Th1/M1/NK milieu. Cellular changes were investigated by multi-color immune cell profiles and cytokine/chemokine alterations confirmed the mRNA-profiles by induction of Th1 pronounced mediators and effectors.

Keywords: bladder carcinoma, multivalent adenoviral immunotherapy, immune microenvironment activation

B215 / Disrupting signaling between ectopic osteosarcoma-associated VCAM-1 and α4β1 integrin in macrophages ameliorates pulmonary metastasis

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Osteosarcoma (OS) is the most prevalent aggressive malignant bone cancer, affecting children and young adults with a predilection in boys and African American descent. Of the 400-600 OS diagnosed each year, 20% initially presents with pulmonary metastasis (pOS), while ~30% of patients with local disease develop pulmonary metastasis after initiation of multimodal therapy. Outcome for metastatic disease remains dismal (< 30%) over the last 3 decades, which accounts for almost all of OS-related mortality. OS contains complex genetic alterations, making molecular targeted therapy challenging. Recent exciting scientific development implicates the immune system as a potential important new armamentarium as a novel approach to control or reduce pOS. Breast cancer literature and our ongoing research have implicated over-expression of tumor-expressed Vascular Cell Adhesion Molecule-1 (tVCAM-1) as a critical step in establishing a

metastatic tumor niche in the lung tissue through its interaction with α4β1 (VLA4) integrin on lung macrophages (MACs). Using a syngeneic OS implantation model derived from Balb/c mice, we interrogated the functional role of this interaction axis in pOS. Compared to non-metastatic parental OS tumor (K7), pOS cells (K7M2) express high surface VCAM-1, and absence of either tVCAM-1 by genetic deletions or MACs by intranasal liposomal clodronate administration prevents the initial development as well as abrogated established pOS in 50-60% of mice. Mechanistically, surface-bound or soluble tVCAM-1 can drive macrophage differentiation towards $CD206^{+}$, $Arg\text{-}1^{+}$ M2 subset ***in vitro*** and ***in vivo***. Importantly, weekly treatment of established pOS with intranasal administration of either anti-CD49d (anti-α4) antibody or a novel anti-VCAM-1 antagonist peptide identified through phage-display was effective in rendering tumor-free survival in 80% and 60% of the mice, respectively. These observations make VCAM-1/α4β1 potentially a set of high-impact targets for treating pOS. Our data identified VCAM-1 as among the first surface markers in pOS that can be targeted in future clinical translational immunotherapy. Additionally, our observation affords the opportunity to target pulmonary MACs through direct pulmonary administration of anti-α4 blocking antibody in pOS. Finally, our data support further investigation into the molecular and signaling mechanisms between integrin signaling and macrophage functional differentiation.

Keywords: osteosarcoma metastasis, macrophages, integrin signaling

B216 / Spatial T cell dysfunction between tumor and draining lymph nodes of breast cancer patients

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Lymph node (LN) invasion by tumor cells, which can induce immune suppression by multiple mechanisms, is a critical negative prognosis factor. We compared the phenotype and function of T cells from non-invaded (NI), invaded (I) LNs, and tumor samples from untreated luminal breast cancer patients, to understand whether tumor-invaded LNs represent or not tolerogenic sites for T cells. As expected, T cells from the corresponding primary tumors failed to proliferate and produce effector cytokines upon stimulation ex-vivo. Unexpectedly, T cells in I TDLNs showed evidence of activation and differentiation, produced high levels of IFN-g and responded to recall antigens. From the physiopathology standpoint, our findings indicate that while the tumor represents an immunosuppressive environment for T cells, tumor invasion of draining LNs is associated with a functional local ongoing immune response. From a clinical perspective, these results let us hypothesize that patients with LN involvement could be susceptible to immune-modulation. Moreover, our findings that CD8+, CD4+, and regulatory T lymphocytes show different expression patterns of targetable immunecheckpoint molecules may guide the rationalized use of selective agonistic or antagonistic antibodies directed to these molecules in view of personalized immunotherapies. Our results indicate that while the tumor represents an immunosuppressive environment for T cells, solid tumor invasion of draining lymph nodes is a critical step in the onset of both effector and regulatory immune responses, predicting that patients with LN involvement are good candidates to benefit from immunotherapies.

Keywords: Invaded Lymph node, Breast Cancer, Tumor

B217 / Targeting immune cells of the tumor microenvironment with antisense oligonucleotides to increase anti-tumor immunity

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Tumor microenvironment (TME) includes blood vessels, immune and non-immune cells, signaling molecules, and the extracellular matrix (ECM) and has profound contribution toward tumor heterogeneity, growth, drug resistance, immune evasion, metastasis and other properties. Immunotherapeutic treatment, a type of cancer treatment aimed to help the immune system fight cancer is an attractive area of drug discovery for various platforms. Antisense oligonucleotides (ASOs) can be designed to target and degrade any type of RNAs, including non-coding RNA thus offering broader therapeutic opportunities compared to other treatment modalities.
Here we show that ASOs can accumulate and produce pharmacological activity in a variety of cells within the TME including immune cells. Mouse ASOs targeting signal transducer and activator of transcript 3 (STAT3) produce anti-tumor activity in a variety of mouse syngeneic models by altering the immune cell composition of the TME and exerting a strong interferon response (reference). In the clinic, human ASO STAT3rx/AZD9150 was shown to be active in clinical samples obtained from treated humans clearly demonstrating that antisense technology is a promising platform for the discovery (reference).

Th2/M2 responses are associated with anti-inflammatory microenvironment and involved in tumor progression. In the TME, interleukins (IL) such as IL4 and IL13, IL21 and IL33 are known to induce a Th2/M2 response. We hypothesized that targeting STAT6, a major transcriptional factor controlling IL-4/IL-13 signaling, would result in an immune shift from a Th2/M2 towards Th1/M1 response thus skewing the environment from a pro- to anti-tumor phenotype. We demonstrate that mouse STAT6 ASOs lead to a dose-dependent reduction of mouse STAT6 in murine bone-marrow derived macrophages (BMDM) and prevented IL4 and IL13 mediated M2-polarization in a dose-dependent manner. M2 polarization prevention was measured by monitoring M2 markers such as ***Arg1***, ***Mrc1*** and ***Tmem26***. ***In vivo***, tumor growth inhibition was observed in various syngeneic models such as colorectal cancer model CT26.WT, diffuse large B cell lymphoma model A20 and ovarian cancer model ID8-VEFF. In addition, we observed significant increase in immune cell infiltrate (CD45+, CD11b+, NK, CD8+ cells), increase in IFN response (IFNg and type I IFNs) and in cytotoxic T cell responses (increase in CD8 infiltrate, as well as granzyme B and perforin expression) in all syngeneic models. Finally, combination treatment with PD-L1 antibody, resulted in an increase of antitumor activity in A20 syngeneic model. Altogether, our results demonstrate that ASOs targeting immunosuppressive mechanisms of the TME (such as STAT3 and STAT6) hold great therapeutic promise.

Keywords: Antisense oligonucleotides, Tumor Microenvironment, Tumor associated macrophages

B218 / Quantification of tumor microenvironment's immune and stromal populations from transcriptomic data: the MCP-counter method and its application to human cancers and preclinical mouse models

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It is now established that components of the tumor microenvironment (TME) sharply influence tumor growth, invasion and therefore patient's survival. The TME is complex, with many different cells of the immune and inflammatory systems, as well as endothelial and fibroblasts components. It is characterized by the heterogeneity of the cellular orientation, and the cytokines and chemokines produced, which dynamically govern the dialectical interactions between tumor cells and the host. Moreover, complexity is increased by the differential impact of various cellular elements, such as lymphocytes, macrophages, endothelial cells or fibroblasts, on patient's clinical outcome in different cancers. To understand how the TME can exert opposite influences on cancer evolution, it is essential to be able to comprehensively and robustly assess the composition of the TME. Transcriptomic data offer both the advantage of enabling the analysis of a large number of markers and the availability of large public datasets (TCGA, GEO, ArrayExpress).
We designed a method, called "MCP-counter" (http://cit.ligue-cancer.net/language/en/mcp-counter/), based on transcriptomic markers allowing us to accurately quantify 8 immune populations, endothelial cells and fibroblasts in transcriptomes of human heterogeneous samples. It was applied to a wide range of bulk tumor data to assess the profiles of the tumor microenvironment, and their association with prognosis.
Since mouse models are particularly relevant to cancer immunity research, as they allow a deeper understanding of underlying mechanisms, we are adapting our MCP-counter method to mouse models to enable precise quantification of murine immune and stromal cell populations in the transcriptome of heterogeneous samples, using publicly available data from the Immune Genome Project.
These methods, when applied to tumor samples, can provide meaningful insights regarding the relationships between the malignant cells and their microenvironment, allowing for intra-malignancies as well as pan-cancer approaches.

Keywords: Tumor microenvironment, Cell population quantification, Transcriptome

B219 / BRAF inhibitor alters the melanoma antigen profile dependent on the duration of treatment

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Melanoma therapy has been dramatically improved with the implementation of inhibitors, such as vemurafenib, targeting oncogenic BRAF-V600E and immunotherapies blocking immune checkpoints. BRAF inhibitor (BRAFi) therapy can induce regression of bulky tumor masses, however; its clinical responses are frequently transient whereas immunotherapy can induce long-lasting clinical benefit but only in a subgroup of patients. In order to exploit the advantages of both therapies, the combination of

BRAFi and checkpoint blockers is currently tested in clinical trials. Previous studies demonstrated that BRAFi treatment can enhance $CD8^+$ T cell infiltration in melanoma in the early phase ***in vivo***. Furthermore, short-term BRAFi treated melanoma cells showed enhanced sensitivity towards differentiation antigen-specific $CD8^+$ T cells. However, the impact of prolonged BRAFi treatment on melanoma T cell recognition is poorly defined. To understand this, we treated BRAF-V600E mutant melanoma cells with vemurafenib, a BRAFi, for 3, 7, 14 and 21 days and investigated the expression of antigen processing/presentation components in vital melanoma cells as well as their capacity to activate autologous $CD8^+$ T cell clones. In the presence of the inhibitor, alive melanoma cells developed a senescence-like phenotype. There was no negative effect of vemurafenib on the expression of antigen processing and presentation components over time. However, differences in T cell responsiveness towards short-term (3-7 d) and long-term (14-21 d) BRAFi-treated melanoma cells were observed. Autologous differentiation antigen-specific $CD8^+$ T cells showed enhanced recognition of short-term BRAFi-treated melanoma cells but strongly decreased responsiveness towards long-term treated tumor cells. Notably, RNA and protein expression analyses revealed that altered T cell activation was due to changes in the target antigen level under BRAFi treatment. Similar results were obtained for autologous T cells being specific for another shared antigen and two neoantigens. Taken together, our data indicate that BRAFi strongly impacts on the antigen expression profile of melanoma cells which might also influence the outcome of combined immunotherapy and targeted therapy.

Keywords: BRAF inhibitor, melanoma immunogenicity, T-cell resistance

B220 / Tumour microenvironment tunes the ability of neuroblastoma cells to protrude, migrate and colonize through activation of the Wnt/ β-catenin pathway

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The main challenge in treating high-risk neuroblastoma is to combat tumour metastasis and development of resistance to multiple chemotherapeutic drugs. At the time of diagnosis 50% of primary tumours have already metastasised or spread to other parts of the body (i.e. stage 4). Despite major advances in available therapies, children with drug resistant and/or recurrent neuroblastoma have a dismal outlook with 5 year survival rates of less than 20%. In the native tissue, cancer cells are surrounded by a three-dimensional (3D) microenvironment which provides biological and physical support and determines disease initiation, progression, patient prognosis and response to treatment. An ability to distinguish the relative contributions of specific genetic, epigenetic and microenvironmental changes to the migration and local dissemination of neuroblastoma cells is paramount in our understudying of disease pathogenesis and development of new targeted therapeutics.
Six mice xenograft neuroblastomas derived from cisplatin sensitive Kelly and resistant KellyCis83 cell lines, along with their corresponding tissue engineered 3D ***in vitro*** models were analysed by western blot and ICH using multiple markers from the Wnt/ β-catenin pathway (c-jun, TCF1, LEF1, Met, CyclinD1, β-catenin, CD44, Dvl2/3, LRP6, Wnt5a/b, Axin1) and specific matrix metalloproteinases (MMP-3, MMP-7, MMP-9 and TIMP-1). Neuroblastoma cells KellyCis83 demonstrated no significant changes in its invasion capability using BD BioCoat GFR Matrigel assay. However, the same cell line behaved differently on RGF BME R1 gel forming protrudes and disseminations. Kelly and KellyCis83 cell lines grown on two different collagen-based scaffolds demonstrated both scaffold- and cell type dependent growth patterns suggesting altered adhesion and migration potential of the cells. c-Jun, TCF1, LEF1, β-catenin, Dvl2/3, LRP6, Axin1 were detected at significantly higher levels in 3D ***in vitro*** and ***in vivo*** KellyCis83 when compared to their 2D cultures and the same of parental Kelly cells.
The results demonstrated that 3D ***in vitro*** cell lines mimic native neuroblastoma cell growth and microenvironment ***in vivo***. Increased expression of key players in Wnt/β-catenin pathway can be potentially activated by tumour microenvironment in cisplatin resistant neuroblastoma cell line KellyCis83 contributing to the ability of these neuroblastoma cells to protrude, migrate and colonize the matrix.

Keywords: Wnt/ β-catenin pathway, neuroblastoma, drug resistance

B221 / Neutrophil chemotaxis to oncogene-transformed cells in vivo

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Neutrophils are the first responder cells to sites of infection and tissue damage. Evidence from patient tumors and animal models of cancer also suggest that they are highly recruited to the tumor microenvironment, though their role there is still poorly understood. The larval zebrafish is an excellent model to study

neutrophil migration and chemotaxis in vivo and is an emerging model for cancer development and progression. Here, we use the zebrafish to model Kras-driven oncogenesis in epithelial cells and glia. Beginning at 3 days post fertilization (dpf), we observe invasive EMT-like cell shape changes in oncogenic mutant Kras ($Kras^{G12V}$) expressing cells as well as enhanced cell proliferation as marked by phospho-histone H3. Using fluorescently labeled transgenic lines, we demonstrate that neutrophils are recruited to transformed cells and that blocking this recruitment results in decreased proliferation of transformed cells, suggesting that neutrophils may play a tumor-promotional role at early stages of oncogenesis in our model. To determine the method of neutrophil chemotaxis to transformed cells, we used Transcription Activator-like Effector Nuclease (TALEN) mutagenesis to generate zebrafish mutants for the chemokine receptor CXCR1, which is known to regulate neutrophil chemotaxis to sterile wounds. We demonstrate that CXCR1 is required for neutrophil chemotaxis to Kras-transformed cells in zebrafish. Together these data suggest that blocking CXCR1 activity may reduce neutrophilic inflammation in the tumor microenvironment which may in turn reduce tumor cell proliferation and slow tumor progression.

Keywords: neutrophils, chemotaxis, CXCR1

B222 / Innate lymphoid cells in human colorectal cancer

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Colorectal cancer (CRC) is the second most common cancer in Europe, both in terms of incidence and mortality. Lymphocyte infiltration of the tumor is associated with better prognosis, suggesting that understanding of immunological processes in the tumor microenvironment could enable the development of new successful CRC therapies. Innate lymphoid cells (ILCs) are a recently identified type of immune cells that facilitate gut barrier function while playing a central role in regulating intestinal immune responses. Studies performed in several mouse models indicate that ILCs participate in tumor development in the colon. Nonetheless, the role for ILCs in human CRC remains unknown. In this project we address the role of ILCs with regard to human CRC initiation and/or progression. As part of a productive collaboration with the gastroenterology unit at the Karolinska University Hospital, Stockholm, we obtain paired peripheral blood and resected tissue from patients undergoing CRC surgery. Pertaining to the resected material, we receive tissue from the non-affected, tumor border and central tumor areas. Phenotypic and functional profiles of distinct ILC subsets are assessed using a combination of multi-color flow cytometry and cell sorting, as well as a range of *in vitro* cell culture techniques. To date, data obtained from the CRC patient samples show that there is a gradually increasing switch in ILC phenotype stretching from non-affected into the cancerous tissue areas. Specifically, we observe an accumulation of putatively IFN-γ producing ILC1, and a decrease in IL-22 producing ILC3 in colon cancer compared to non-affected tissue, implying an existing crosstalk between ILCs and the tumor microenvironment in CRC. To determine how ILCs behave in the complex intratumoral cellular network, we are investigating the crosstalk between ILCs and tumor cells, with the intention of dissecting factors driving ILC plasticity and understanding the possible impact on oncogenesis and/or tumor progression. Moreover, we observed increased HLA-DR expression on ILCs in the tumor, suggesting that ILCs may present cancer antigens to T cells and thus, regulate cellular immune responses in the tumor microenvironment. In this regard, we have demonstrated the ability of class 3 ILCs to take up and process exogenous proteins, hinting at their potential as antigen-presenting cells. In summary, we have observed a switch in ILC plasticity in terms of composition and phenotype in human CRC compared to non-affected autologous tissue, which may be influenced by the tumor microenvironment itself. Additionally, the increased expression of HLA-DR on intratumoral ILCs implies their involvement in regulating adaptive immune responses in CRC, with potential clinical relevance.

Keywords: Colorectal cancer, Innate Lymphoid Cells, Tumor microenvironment

B223 / Expeditious detection of hypoxic tumor microenvironment by fluorophore loaded enzyme-responsive nanoparticles

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Hypoxia, a decreased level of available oxygen in tumor tissues, shows great importance in tumor microenvironment including conventional therapy resistance, angiogenesis, tyrosine hydroxylase synthesis and increase of cancer stem cells. Besides, the degree of hypoxia shows positive correlation with tumor progression, and has a negative effect in tumor prognosis. Among all the representative reductive enzymes in hypoxic tissue, the concentration of nitroreductase (NTR) has a close relationship with the degree of hypoxia. Up to now, rapid and precise detection of hypoxic tumor microenvironment remains challenging. We synthesized an amphiphilic polymer by grafting NTR-responsive moiety (nitro-benzyl derivative) to hydrophilic chitosan backbone, followed by encapsulating hydrophobic dye with the help of

hydrophobic interaction to fabricate the NTR-responsive polymeric nanoparticles. With the enzymatic activity of NTR (overexpressed in hypoxic tissues), nitro-benzyl structure was reduced to amino-benzyl derivative, which broke the balance of hydrophobic interaction between hydrophobic dye and nitro-benzyl substrate on amphiphilic polymer. Therefore, the encapsulated fluorescent dye could be released within hypoxic regions. The introduction of 6-hydroxyhexanoic acid as spacer in the enzyme-responsive moiety accelerate the 1,6-elimination process, which speeds up the release of fluorescent payload. In the meantime, the reductive agents globally existed in the biological system could not trigger the release of dye. Further, the fabricated nanoparticles could be successfully applied to determine the hypoxic status of A549 cells with about 3-fold fluorescence intensity enhancement in the cytosol. The detection process could be done within 30 mins, while several hours were required in conventional assays. Collectively, these findings support the expeditious diagnostic method of hypoxic tumor microenvironment with relative short time. Ongoing work seeks to further apply the platform to the controlled drug release system for target therapy of hypoxia-related diseases.

Keywords: Hypoxia, Fluorescence detection, Tumor microenvironment

B224 / Stroma-derived cytokines mediate dedifferentiation of bronchial epithelial cancer cells

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Cancer cells are able to hijack the machinery responsible for the intercellular communication network, thus modulating the behavior of the neighboring cells and consequently, establishing the tumor microenvironment. Therefore, through the extensive crosstalk mediated by cytokines and chemokines, cancer cells can overcome the restrains of the invasion-metastasis cascade, allowing the development of the ill-fated metastatic disease. Cancer stem cells (CSCs) have recently been implicated in major steps of the tumorigenic process, namely in tumor initiation, metastasis formation and tumor relapse following therapy. Thus far, despite their relevance, the mechanisms underlying their formation are still unclear but, a pivotal role has already been ascribed to the tumor microenvironment. Accordingly, noticing that the malignant human bronchial epithelial RenG2 cells acquired a CSCs phenotype following culture in the subcutaneous mouse lumbar region, co-cultures of isolated mice lumbar stromal cells or human bronchial fibroblasts (HBF) with RenG2 cells were established and the conditioned media screened for cytokines implicated in the dedifferentiation process. Consequently, fibroblasts-derived Interleukin-6 (IL-6), Granulocyte colony-stimulating factor (G-CSF) and Activin-A were identified as the paracrine mediators of the intercellular communication process. Aiming to understand the intercellular communication featuring RenG2 cells' dedifferentiation, the same co-cultures were repeated in the presence of specific cytokine-neutralizing antibodies, used either individually or in combinations of up to three, and an exosome uptake inhibitor. Finally, exosomes were also isolated from the co-cultures' conditioned media and their content screened for the target cytokines by ELISA. The cytokine-blocking experiments revealed that the cytokines play distinct roles in the dedifferentiation process, more precisely only IL-6 and Activin-A have the potential to orchestrate dedifferentiation, as a stem cell-like pool only arises inside RenG2 cells when at least one of these cytokines was present. G-CSF, in turn, appeared to be critical in maintaining the undifferentiated phenotype, as a larger stem cell-like pool was attained when this cytokine was present alongside with either IL-6 or Activin-A. Lastly, ELISA results established that fibroblasts-derived exosomes were the vehicle for the paracrine transport of the aforementioned cytokines since they were present inside exosomes. Additionally, supporting this, whenever exosomes' uptake was blocked, dedifferentiation was abrogated. Following studies are essential to access the use of these cytokines as therapeutic targets, thus empowering and improving the strategies against tumors, and consequently, patients' outcome and welfare.

Work sponsored by FEDER, POFC-COMPETE and the FCT grants PTDC/BBBBQB/
2450/2012 and SFRH/BD/33884/2009.

Keywords: Cancer stem cells (CSCs), Dedifferentiation, Intercellular communication

B225 / Tumor cells undergoing ER stress produce a novel lipid that drives cell extrinsic myeloid immune dysfunction

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The tumor microenvironment harbors a variety of insults that privilege tumor cells and enable their ability to co-opt host immunity. Many of these conditions create endoplasmic reticulum (ER) stress for resident tumor cells, which is mediated by the unfolded protein response (UPR). We and others showed that ER stress affects infiltrating myeloid cells, promoting a complex immune dysfunction that favors tumor growth. Central to this immune dysfunction is the polarization of myeloid cells, macrophages and dendritic cells, to a mixed pro-inflammatory/immune suppressive phenotype. Our in vitro studies demonstrated that this effect can be mediated by factors derived from tumor cells undergoing ER stress and act cell-nonautonomously. Because this phenomenon is hallmarked by the transmission of ER stress from tumor cells to target cells, we termed it transmissible ER stress (TERS).
Here, we sought to identify if TERS related genes that were differentially expressed in vitro were similarly expressed in vivo. Tumor infiltrating myeloid isolated from orthotopic murine tumors underwent substantial ER stress relative to those harvested from distant secondary lymphoid sites, the spleen and bone marrow, as well as displayed increased transcription for both pro-inflammatory and immune-suppressive genes. Concordantly, this same profile existed in myeloid cells isolated from the less aggressive ***adenomatous polyposis coli (APC)*** model. TLR4 signaling partially mediated this profile as tumor infiltrating $TLR4^{-/-}$ myeloid cells showed no signs of ER stress, were not inflammatory, but still were immune suppressive. In $TLR4^{-/-}$ hosts, tumors grew at slower rates than in WT mice.
To identify which arm of the UPR is responsible for TERS polarization in macrophages, we used chemical and genetic models. We found that the IRE1α axis was responsible for the TERS driven phenotype, while ATF6 and PERK appeared to be dispensable. The inhibition of IRE1α kinase and endonuclease activity led to a dramatic reduction in TERS mediated inflammation, immune-suppression, and angiogenesis. Moreover, inhibiting IRE1α signaling during TERS treatment prevented the surface expression of a variety of TERS related targets. Macrophage polarization induced by other molecules also appeared to be IRE1α dependent.
Through a variety of biochemical methods we characterized the molecule responsible for TERS. TERS effects were not mediated by proteins, DNA, or extracellular vesicles. Activity was assignable to a stress-dependent lipid compartment. Through classical chromatographic isolation as well as lipidomic techniques, we found the activity to be consistent with a novel signaling lipid molecule.
Our findings provide elucidation of the novel mechanism and factors released by tumor cells undergoing ER stress able to promote immune evasion and successful tumor escape. They reveal potential novel biomarkers of the tumor microenvironment and novel targets to bolster antitumor immunity.

Keywords: UPR, Myeloid, Tumor microenvironment

B226 / Repurposing virus-specific immunity to fight tumors

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The immunosuppressive tumor microenvironment is a major hurdle to overcome in the development of successful cancer therapies. Newly discovered tissue resident memory CD8+ T cells (T_{RM}) function to create a potent immunostimulatory environment to protect against local reinfection. As T_{RM} are present in abundance in nearly every tissue and can be triggered by cognate peptide alone, without adjuvant, we tested whether we could hijack infection-specific CD8+ T cells in tumors to reverse the immunosuppressive tumor microenvironment and enhance existing immunotherapies. Mice with established vesicular stomatitis virus (VSV)-specific CD8+ T cells were challenged with the transplantable B16 melanoma cell line. We find VSV-specific CD8+ T cells within established tumors and within 12 hours of cognate peptide delivery, these cells upregulate IFNγ, CD25, and granzyme B. This led to 1) tumor NK cell and CD8+ T cell activation through granzyme B upregulation, 2) increased numbers of NK cells in the tumor, 3) bystander memory CD8+ T cell recruitment to the tumor and 4) DC activation in the tumor and tumor draining lymph nodes through CCR7 and CD86 upregulation and 5) recruitment of transferred CAR T cells. Finally, we demonstrate that tumor T cell reactivation can mediate regression in B16 growth and confers a significant increase in survival. Ongoing work includes testing the ability of virus-specific T cell reactivation to synergize with checkpoint blockade and adoptive cell therapies in mouse models, and interrogating the location and functionality of virus-specific T cells within human tumors.

Keywords: CD8 T cells, melanoma, virus

B227 / Deciphering local immunity with CyTOF in HPV16- and HPV16+ oropharyngeal tumors

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer. While the number of HNSCC cases is decreasing, the number of oropharyngeal SCC (OPSCC) is rising. OPSCC are increasingly (45-90%) caused by human papillomavirus type 16 (HPV16). Intriguingly, patients with HPV-induced OPSCC (HPV16+) respond better to therapy than HPV-negative (HPV16-) OPSCC. This is independent of nodal status, age, stage, tumor differentiation or gender. We hypothesize that tumors of HPV16+ OPSCC patients display a different immune contexture that contributes to a better response of these tumors to standard therapy.
To test our hypothesis, an exploratory in-depth analysis of immune infiltrates in the tumor microenvironment (TME) of 13 HPV16- and HPV16+ OPSCC patients was performed using 36-parameter mass cytometry (CyTOF) analysis. Tumor cell suspensions were prepared by mechanistic dissociation using Gentlemacs and cryopreserved until CyTOF analysis. HPV16 status of the tumors was determined by GP5+/6+ PCR and p16 immunohistochemistry staining.
HPV16 E6/E7-specific T cell reactivity within HPV16+ tumors was determined by proliferation assay on directly ex vivo tumor samples and/or IL-2-expanded tumor infiltrating lymphocyte (TIL) batches. In-depth tumor immune infiltrate analysis was performed by CyTOF technology on ex vivo tumor samples from 13 OPSCC patients, of which four were found to be HPV16-, four HPV16+ but HPV16 immune response-negative (HPV16+ IR-) and five HPV16+ immune response-positive (HPV16+ IR+).
Analysis of the TME through 36-parameter CyTOF revealed clear phenotypic differences between immune cells infiltrating the TME of HPV16 IR+, HPV16+ IR- and HPV16- tumors. Whereas HPV16+ IR- tumors were strongly infiltrated with B cells, HPV16+ IR+ tumors were strongly infiltrated with effector memory CD4+ and CD8+ T cells with a highly activated, i.e. CD38+, HLA-DR+ and/or PD-1+ phenotype. Interestingly, subsequent unsupervised hierarchical clustering through the CITRUS algorithm led to the identification of two distinctive populations of activated CD4+ T cells, separated on basis of CD161 expression, and one population of CD103-expressing tissue-resident effector memory CD8+ T cells that were present at significantly higher levels in HPV16+ IR+ patients.
Subsequently, we analyzed the survival of 75 patients with HPV16+ OPSCC in the publicly available TCGA database focusing on the expression of CD4, CD8, CD103 and CD161. A high expression of CD4, CD8 or CD161 was associated with better overall survival but this was not the case for CD103 expression.
In conclusion, our data revealed that distinct immune cell populations infiltrate the TME of HPV16+ IR+ tumors that may contribute to a better response of these tumors to standard therapy.

Keywords: tumor microenvironment, mass cytometry, T cell infiltrate

B228 / Role of TLR-2 alarmin, matrix metalloproteinase-2, in inflammasome driven dendritic cell modulation and pro-tumorigenic T cell differentiation in the tumor microenvironment

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Extracellular proteinases, such as matrix metalloproteinases (MMPs), support tumor progression through modulation of the tumor microenvironment (TME). MMP-2, in particular, is over-expressed in several cancers and high MMP-2 levels are associated with advanced tumor stages, increased dissemination and poorer survival/prognosis. Our lab has previously demonstrated that upon antigenic stimulation, MMP-2-specific CD4+ T cells, derived from patients with melanoma, secrete inflammatory T_H2 cytokines. We subsequently showed that active MMP-2 drives the differentiation of T_H2 responses by inhibiting IL-12 production and up-regulating OX40L expression on dendritic cells (DCs). We published our novel discovery identifying MMP-2 as a ligand for TLR-2 and showed that MMP-2 mediated TLR-2 stimulation lead to up-regulation OX40L on DCs (Cell Reports, 2014). This is particularly interesting as TLR-2 stimulating adjuvants are being tested for immunotherapy. However, the full spectrum of how TLR-2 activation affects tumor cells or immune cells remains unclear.
The main purpose of this study is to characterize the influence of tumor cell secreted-MMP-2 on the tumor infiltrating immune cells, especially through TLR-2 activation, so as to develop effective cancer therapies.
Towards this end we performed RNA sequencing to identify genes induced in human DCs upon MMP-2 stimulation. One of these targets is an atypical member of the canonical NFκB family, IkappaBzeta (***NFKBIZ*** or IκBζ). We show that MMP-2 secreted by melanoma cells up regulates IκBζ in DCs through TLR-2. Moreover, initial studies suggest a novel role for MMP-2 in inflammasome dependent T cell priming in DCs. We hypothesize that MMP-2-TLR-2 driven induction of IκBζ in DCs within the

TME drives the T cells towards a T_H17 phenotype thus enhancing tumor progression. We have screened several human melanoma cell lines for high and low MMP-2 and TLR-2 expression. Currently we are employing CRISPR/Cas9 technology to stably knock down TLR-2 and MMP-2 in tumor cell lines. These modified cells lines are being used to investigate the effect of MMP-2 and TLR-2 on DCs, T cells, NK cells and B cells and on tumor cell invasiveness and proliferation.
In summary, we have identified a novel role for MMP-2 as a TLR-2 alarmin with particular emphasis on induction of atypical signaling modulator IκBζ and have uncovered a new role for MMP-2 in inflammasome activation. Taken together our previous research and current data indicates that MMP-2 acts simultaneously as an endogenous T cell differentiation "conditioner" and a tumor-associated antigen. Therefore delving into MMP-2 signaling mechanisms in the TME holds a strong potential for discovering novel therapeutic options for treating melanoma.

Keywords: Matrix Metalloproteinase-2 (MMP-2), Dendritic Cells and melanoma, Toll like receptor-2

B229 / Tumor-associated B cells and humoral immune response in esophago-gastric adenocarcinoma

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Purpose: Tumor-infiltrating lymphocytes play a crucial role in several kinds of cancer. Whereas tumor-infiltrating T cells have been extensively studied, the role of tumor-associated B cells (TABs) is widely unknown. The B cell compartment consists of specific subpopulations with distinct functions. B cells stimulate immune responses as antigen-presenting cells or by production of tumor-specific antibodies. On the other hand regulatory B cell subsets can inhibit immune responses, especially in the context of autoimmune disease. This is a comprehensive analysis of TABs in gastric adenocarcinoma including complementary analyses of major T cell subsets.
Methods: Tumor samples of 80 patients with a pathological diagnosis of gastro-esophageal adenocarcinoma were collected. Single-cell suspensions were obtained using enzymatic digestion on a GentleMacs dissociator. PBMCs of cancer patients and 20 healthy controls were isolated using density-gradient centrifugation. Tumor-associated B cells in tumor sections, normal mucosa, lymph nodes and peripheral blood were studied by 10-colour flow cytometry. LUMINEX bead assay, intracellular cytokine staining and mixed lymphocyte reactions were used for functional analyses. Spatial distribution of tumor-associated B cells was analyzed by 4-color confocal immunofluorescence-microscopy.
Results: $CD45^+$ tumor-infiltrating lymphocytes (TIL) could be detected in all analyzed tumor samples, but samples of patients who previously received neoadjuvant chemoradiotherapy contained significantly less TIL. B cells (defined as $CD19^+/CD20^+$ in % of $CD45^+$ lymphocytes) were elevated in tumor samples compared to PBMCs of gastric cancer patients or normal mucosa samples. Subset-analyses of TAB revealed a significant elevation of antigen-presenting B cells, plasmablasts and plasmacells in tumor samples compared to normal mucosa or PBMC. TABs were predominantly of an activated ($CD86^+$) and memory phenotype (IgD^-CD27^+). TABs with a regulatory phenotype were also detectable and this finding was confirmed by detection of IL-10 and GrB production of tumor-associated B cells. Confocal microscopy revealed that TABs were mainly located in tertiary lymphoid structures at the invasive tumor margin. Tumor-specific antibodies against on or more of the analyzed 36 cancer testis antigens were detectable in serum samples of 25/31 patients with primary resections.
Conclusion: B cells make up a relevant part of tumor-infiltrating lymphocytes in esophago-gastric adenocarcinoma. These results are of important translational relevance to emerging immunotherapies.

Keywords: B cells, tumor-specific antibody, Gastric Cancer

B230 / Regulatory T cells infiltrating gastrointestinal stromal tumor display distinct phenotypic and transcriptional features

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Regulatory T (Treg) cells are an influential component of the immunosuppressive tumor microenvironment. In gastrointestinal stromal tumor (GIST), imatinib therapy induces Treg cell apoptosis selectively within the tumor through inhibition of indoleamine 2,3-dioxygenase. The frequency of Treg cells correlates with

response to imatinib therapy in human GIST. The phenotypic and transcriptional features of Treg cells infiltrating GIST and their response to oncogene inhibition, however, remain largely unknown. In this study, systemic Treg cell depletion through diphtheria toxin injection in *Kit*$^{V558\Delta/+}$;*Foxp3*^{DTR} mice resulted in a significant reduction of tumor growth, but did not further improve the efficacy of imatinib in combination therapy. Flow cytometric analysis of Treg cells from *Kit*$^{V558\Delta/+}$;*Foxp3*^{GFP} mice revealed a highly activated phenotype in the tumor compared to the tumor draining lymph node, with upregulation of CD44, CD69 and Granzyme B. Moreover, RNA-sequencing on sorted Treg cells demonstrated distinct transcriptional features and differential expression of several genes responsible for cell adhesion and immune response. Notably, the Treg signature remained largely unaffected by imatinib therapy. These data suggest that Treg cells have a major tumor-promoting effect in GIST and are a promising target for immunotherapy, especially in the setting of imatinib resistance.

Keywords: Regulatory T cells, immunotherapy, gastrointestinal stromal tumor

B231 / Corticosteroids impair the development of tertiary lymphoid structures in lung squamous cell carcinoma

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Tertiary lymphoid structures (TLS) form in chronically inflamed tissues including cancer and resemble follicles of secondary lymphoid organs both by structure and function. TLS activate adaptive immune cells in autoimmunity and infection and correlate with improved survival in various cancer types. This suggests that TLS might contribute to anti-tumor immunity and that TLS induction in cancer could be a novel immunotherapeutic approach. However, not much is known about TLS formation and function in the context of human cancer or the impact of cancer therapies. To pursue these questions we performed a comprehensive histological, gene expression and quantitative pathology analysis of lung squamous cell carcinoma (LSCC) patients (n=138) and animal models of TLS induction.
We demonstrate that TLS develop in the periphery of most tumors following sequential stages of maturation that culminate in germinal center (GC) reaction. Peritumoral TLS density (TLS number per mm2) in untreated patients was the most significant prognostic marker independent from TNM staging and other clinical parameters (multivariate Cox regression p=0.000, hazard ratio=0.29, CI 95% 0.16-0.55). Patients with low TLS density showed a significantly hampered TLS maturation towards GCs and a decreased expression of adaptive immune response-related genes. Furthermore, GCs were significantly reduced in patients after neoadjuvant chemotherapy and the prognostic relevance of TLS density was lost in these patients. The cytotoxic effects of chemotherapy might negatively impact the proliferating cells within GCs but these patients were also simultaneously treated with corticosteroids to manage side effects. We thus hypothesized that anti-inflammatory properties of steroids might hamper TLS development. To test this we analyzed TLS formation in chemotherapy-naïve patients with no history of steroid treatment and compared it to patients that received steroids to treat other cancer non-related comorbidities. Both, the density of TLS and GC formation, were significantly reduced in steroid treated patients. To validate this observation experimentally we administered alum particulates and ovalbumin intranasally, which induces robust TLS formation in the lungs of mice, in combination with low-dose systemic dexamethasone treatment. We observed similar total numbers of TLS in mouse lungs after dexamethasone treatment but GC formation was significantly reduced supporting our observations in patients.
In conclusion, we suggest that GC formation in tumor-associated TLS indicates to a favorable immune environment with considerable prognostic value in untreated LSCC patients. Further, corticosteroids impair TLS development independently of chemotherapy that might impair potential TLS-mediated anti-tumor effects.

Keywords: tertiary lymphoid structures, lung cancer, corticosteroids

B232 / Function of miR-146a-5p as a regulatory switch between tumor cell death and angiogenesis: macrophage therapy revisited

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Tumors survive and progress by evading the immune system´s killing mechanisms, and by generating a tumor microenvironment that reprograms macrophages ***in situ*** to produce factors that support tumor growth, angiogenesis and metastasis. We have previously shown that by blocking the translation of iNOS, miR-146a-5p inhibits NO production in a mouse renal carcinoma cell line (RENCA), thereby endowing RENCA cells with resistance to macrophage-induced cell death. We expand these findings to demonstrate that neutralizing miR-146a-5p´s activity, by transfecting both RENCA and mouse colon carcinoma CT26 cells with its antagomir, restored iNOS expression and NO production

and enhanced susceptibility to macrophage-induced cell death (by 48% and 25%, respectively, p< 0.001). Simultaneously, miR-146a-5p suppression inhibited the expression of the pro-angiogenic EMMPRIN (3 folds, p< 0.001), leading to reduced MMP-9 and VEGF secretion (2- and 3-folds, respectively, p< 0.05), and reduced angiogenesis, as estimated by ***in vitro*** tube formation and scratch assays. When we injected tumors with pro-inflammatory-stimulated RAW 264.7 macrophages together with ***i.v.*** injection of the miR-146a-5p antagomir, we found inhibited tumor growth (6-folds, p< 0.001) and angiogenesis (2-folds, p< 0.01), and increased apoptosis (2-folds, p< 0.01). This combination increased nitrites and reduced TGFβ concentrations in tumor lysates, alleviated immune suppression and allowed enhanced infiltration of cytotoxic $CD8^+$ T cells. Thus, the role of miR-146a-5p as a control switch between angiogenesis and cell death can be therapeutically utilized in combination with macrophage therapy to induce the immune system to attack the tumor. This should be further explored as a new therapy for the treatment of cancer.

Keywords: miR-146a-5p, iNOS, EMMPRIN

B233 / Targetable immune checkpoint molecule expression in the primary, untreated breast tumor microenvironment

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There is an exponentially growing interest in identifying novel immunotherapy targets for breast cancer (BC), particularly in the triple negative (TN) subtype where there are unmet treatment needs. This study examined primary BC for expression of targetable immune checkpoint molecules with known regulatory roles. First, we analyzed the publically available METABRIC microarray dataset to determine ***PDCD1*** (PD-1), ***PDCDLG1*** (PD-L1), ***PDCDLG2*** (PD-L2), ***CTLA4, LAG3*** and ***HAVCR2*** (TIM-3) gene expression levels in primary, untreated BC. Second, we used flow cytometry to quantify protein levels for the above markers on tumor infiltrating lymphocyte (TIL) subpopulations [$CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD45^+$ (total TIL)] in fresh, untreated primary BC tissues. TILs were analyzed the day of surgery in a cohort (n=95) that included all four molecular subtypes: luminal A (34%), luminal B (33%), HER2+ (20%) and TN (13%). Third, formalin-fixed paraffin embedded blocks from the same tumors were stained using immunohistochemistry (IHC) and/or immunofluorescence (IF) for CD3, CD20, PD-1, PD-L1, PD-L2, LAG-3 and TIM-3. Gene expression analyses show that ***PDCD1*** (PD-1), ***CTLA4***, ***LAG3*** and ***HAVCR2*** (TIM3-) are expressed at higher levels in TNBC compared to luminal A BC. Surface PD-1, LAG-3 and TIM-3 are detected on $CD4^+$ and $CD8^+$ T cell TIL, intracellular CTLA-4 is predominantly found in $CD4^+$ TILs and surface PD-L1/PD-L2 on $CD19^+$ B cell TILs and $CD8^+$ and $CD4^+$ TILs. The proportion of $PD\text{-}1^+$ TILs was significantly higher than $PD\text{-}L1^+$, $PD\text{-}L2^+$, $LAG\text{-}3^+$ or $TIM\text{-}3^+$ TILs; however, some markers were frequently detected in the same tumor, particularly in TNBC. IHC and IF staining identified TILs expressing PD-1, PD-L1, LAG-3 and TIM-3 in peri-tumoral tertiary lymphoid structures (TLS). PD-1 is most frequently found on TLS-associated TILs, while PD-L1, LAG-3 and TIM-3 were detected both on TILs and stromal immune cells. Overall, we found heterogeneous expression of these immune checkpoint molecules between the four BC molecular subtypes with their predominance in TN. We further found that their expression is also observed in TLS suggesting that these organized immune structures house important immune regulatory activities that control immune responses directed against the tumor.

Keywords: Immune checkpoint, Breast cancer, Tumor-infiltrating lymphocytes

B234 / HMGB1 regulates CXCL12-dependent recruitment of MDSCs to tumors

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During pathologic stress conditions such as infection, injury and inflammation, the high mobility group box 1 protein (HMGB1) is actively released by damaged cells and activated immune cells. Secreted HMGB1 represents a potent alarmin that modulates immune responses through several mechanisms. In particular, HMGB1 forms a heterocomplex with CXCL12 to potentiate CXCR4-dependent cell migration. Since overexpression of both CXCL12 and HMGB1 is frequently observed within tumors, these molecules may synergize to impact the recruitment of $CXCR4^+$ cells such as B and T lymphocytes, monocytes, macrophages and granulocytes. Here we aimed to determine the contribution of HMGB1 to the composition of immune cell infiltrates in cancer and its impact on anticancer immunity. We generated B16 and CT26 cancer cell lines where HMGB1 production is limited by shRNA-mediated downregulation and we observed that although $HMGB1^{high}$ and $HMGB1^{low}$ cell lines divide at the same rate in culture, they show different growing patterns ***in vivo***. Subcutaneous tumors induced from $HMGB1^{low}$ cells are smaller than tumors that express high levels of HMGB1. Importantly, $HMGB1^{low}$ tumors showed decreased infiltration of myeloid-

derived suppressor cells (MDSCs), in contrast to other subtypes of leukocytes that infiltrate HMGB1^{high} and HMGB1^{low} tumors equally. MDSCs are a heterogeneous population of immature myeloid cells that dampen the antitumor activity of T cells and NK cells. The presence of MDSCs in cancer patients correlates with immunosuppression and poor prognosis. Interestingly we observed that the effect of HMGB1 expression on potentiating MDSCs recruitment is limited to the tumor environment, as we found similar numbers of MDSCs in spleens and bone marrow of mice bearing HMGB1^{high} or HMGB1^{low} tumors. We hypothesize that in our model MDSCs migrate toward CXCL12 in an HMGB1-sensitive manner, resulting in reduced numbers of MDSCs among the tumor-infiltrating cells. Our results suggest that inhibition of HMGB1 may constitute a new promising strategy for limiting MDSC migration to tumors and reducing their immunosuppressive function.

Keywords: HMGB1, MDSCs, CXCR4

B235 / Tumor microenvironment-dependent inhibition of leukocyte infiltration to tumors: insights from a model of gastric cancer

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Gastric cancer is one of the most frequent cancers worldwide. Patients affected by advanced stage gastric cancer have a poor prognosis, and the development of new and effective therapeutic strategies is essential to improve outcome in this disease. In principle, cancer immunotherapy may be applied to many types of cancer, but the lack of appropriate models has impeded studies on the application of immunotherapy to stomach tumors. We investigate anticancer immunity in CEA424-SV40 TAg transgenic mice, which develop spontaneous tumors in the pyloric region of the stomach. We have previously shown that adoptive transfer of tumor-specific T cells fails to target these autochthonous tumors, suggesting that factors dependent on the tumor microenvironment limit T cell migration. Similarly, sequential stimulation of Rig-I-like receptor and Toll-like receptor pathways, alone or in combination with checkpoint blockade therapy, did not increase the number of T cells infiltrating the autochthonous tumors. Indeed, immunostaining of the gastric tumors revealed that leukocytes, including CD8$^+$ and CD4$^+$ T cells, preferentially localized in the tumor-surrounding stroma. F4/80$^+$ and CD11b$^+$ myeloid cells likewise accumulated in the same region. Moreover, cells expressing CD11c were found around tumor plaques. These observations suggest that unidentified factors in the tumor microenvironment restrain entry of T cells and other leukocytes into the tumors. Uncovering the molecular mechanisms involved in the inhibition of T-cell recruitment may lead to improved efficacy of cancer immunotherapy.

Keywords: tumor microenvironment, gastric cancer, lymphocyte infiltration

B236 / CCL5 expression and omental fat in ovarian cancer: impact on the anti-tumor immune response

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The expression of CCL5 in our cohort of 60 ovarian cancers has shown high variability. Interestingly, tissues displaying high CCL5 expression are characterized by the presence of big fatty patches inside or in close vicinity to the tumor. The presence of fat in epithelial ovarian carcinoma originates from the omentum, a privileged site for metastasis. In histological sections of fat-containing tumor tissue, the expression of CCL5 was exclusively derived from T cells.
Trying to understand the impact of the presence of fatty patches, and of CCL5 expression, on the anti-tumor immune response, we observed an association between the presence of fat in ovarian tumors and massive tumor infiltration by T cells. However, this massive T cell influx seems to fail in its anti-tumor activity since
(1) tumor-infiltrating T cells seem to be hijacked away from tumor cells and accumulate around fatty areas and
(2) tumor-infiltrating T cells display an exhausted phenotype.
In order to decipher the impact of fat on effector functions of T cells, we have cultured T cells, isolated from ascites, with supernatant from adipocyte cultures, in the presence of a CCR5 inhibitor. We have also developed a new model of tissue culture of explants from primary human tumors that are treated with a CCR5 inhibitor in an attempt to circumvent this fat-derived immune exhaustion and restore an efficient anti-tumor immune response.

Keywords: CCL5, Ovarian carcinoma, fat

B237 / LTX-315: A first-in-class oncolytic peptide that reshapes the tumor microenvironment

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The oncolytic peptide LTX-315, which has been de novo designed based on structure-activity relationship studies of host-defense peptides, has the ability to kill human cancer cells and induce long-lasting anticancer immune response when injected locally into tumors established in immunocompetent murine models. The oncolytic effect of LTX-315 involves perturbation of the plasma membrane and the mitochondria with subsequent release of danger-associated molecular pattern molecules (DAMPs) such as ATP, Cytochrome C and HMGB1. Furthermore, LTX-315 effectively disintegrates the cellular compartments with subsequent release of tumor antigens as demonstrated by a greater T-cell infiltration (TILs) , TILs clonality and the number of clones with greater abundance in the tumor microenvironment. In experimental tumor models, LTX-315 exerts abscopal effects and reshapes the tumor microenvironment by decreasing the local abundance of immunosuppressive cells and by increasing the frequency of effector T-cells. LTX-315´s ability to convert immunogenically "cold" tumors to "hot" makes it ideal combination partner with other immunotherapies as confirmed in experimental tumors combining LTX-315 with immune checkpoint inhibitors and immunochemotherapy.

Keywords: oncolytic peptide, immunotherapy, DAMP

B238 / The role of the centrosome in cytotoxic T cell function

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Cytotoxic T lymphocytes (CTLs) are highly effective killers capable of destroying virally infected and cancerous cells. Cytotoxic responses are induced by the formation of the immune synapse after the recognition of the peptide-Major Histocompatibility Complex by the T Cell Receptor. The synapse plays an important role by focusing signaling, secretion, and endocytosis at the point of contact between effector and antigen-presenting cells. How this delivery process precisely occurs remains unclear, but is thought to be is ensured by a series of actin and microtubules remodeling events leading to the positioning of the centrosome to a position just beneath the center of the contact site. Lytic granules, cluster around the centrosome in activated CTLs, and it has been proposed that polarization toward the synapse promotes the selective fusion of these granules with the synaptic membrane, leading to directional release of their contents toward the target cell.

Although the idea that the centrosome guides directional secretion is visually appealing, it is almost entirely based on correlative imaging experiments. In order to study precisely how the centrosome influences secretory responses and cytotoxic function, we have developed a system using OT-I mice bearing a conditional allele of both p53 ($p53^{flox}$) and SAS4 ($SAS4^{flox}$), a scaffolding protein that is absolutely required for the formation and maintenance of the centrosome.

In order to obtain $SAS4^{-/-}p53^{-/-}$ CTLs, $SAS4^{fl/fl}p53^{fl/fl}$ cells are transduced with Cre expressing retroviruses. SAS4 deletion in this manner effectively eliminates interphase centrosomes, as evidenced by the complete absence of g-tubulin^{+} and pericentrin^{+} puncta in the transduced CTLs. The microtubule cytoskeleton, which typically radiates from the centrosome, adopts an alternative configuration in the absence of the centrosome. Importantly, these cellular changes do not appear to compromise TCR signaling. Indeed, $SAS4^{-/-}p53^{-/-}$ CTLs exhibit robust activation of MAP kinase, PI 3-kinase, and NF-kB signaling is also normal. Consistent with these results, TCR-induced IFN-g production and secretion is unaffected by loss of SAS4.

Interestingly, $SAS4^{-/-}$ $p53^{-/-}$ CTLs exhibit a marked defect in target cell killing after 24h, implying a crucial role for the centrosome in cytotoxic responses. This killing defect is accompanied by a decrease in both granzyme B and perforin production. In addition, $SAS4^{-/-}$ $p53^{-/-}$ CTLs display altered synapse architecture as evidenced by a defect in F-actin clearance.

Taken together, these data indicate that centrosome deletion impairs target cell killing without affecting the overall magnitude of CTL effector responses. Given the importance of the centrosome as an organizing structure for lytic granules, it is tempting to speculate that the ablation of this organelle selectively alters cytotoxic efficiency by disrupting lytic granules maintenance and synaptic secretion.

Keywords: Synapse, Centrosome, Cytotoxic T Lymphocyte

B239 / Dissecting immune cell heterogeneity in human cancer by single-cell RNA-sequencing

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Checkpoint blockade therapies that aim to reactivate anti-tumor immune responses have revolutionized cancer treatment, resulting in durable responses in a significant proportion of patients with advanced disease. Nevertheless, many patients fail to reach long-term clinical benefit, and therefore, a better insight in the mechanisms underlying response to immunotherapy is required to enhance its success rates. Recent data suggest that immune cell infiltrates in human cancer can be highly heterogeneous, potentially contributing to differential therapy outcomes. However, immune cell states have thus far been studied mainly in patients with end-stage melanoma and diverse treatment histories, whereas patients with early-stage metastatic disease are generally treatment naïve, allowing assessment of naturally evolved T cell functionality and immune cell composition. To characterize the heterogeneity in the tumor immune environment within and between patients, we profiled the tumor microenvironment of six stage III melanoma patients by single-cell RNA sequencing, generating an unbiased map of the expression signatures of immune cells as well as tumor and stromal cells. In parallel, we derived the T cell receptor sequences from single T cells, and used them to determine intratumoral T cell clonality and infer the functionality of clonally expanded T cell populations in the tumor. A series of different cell types and cellular states that have been described previously in end-stage melanoma was also observed in this early-stage metastatic disease, including cytotoxic, exhausted, and regulatory T cells, as well as various myeloid subsets. Notably, despite identical disease stage and treatment background, the composition of the immune cell populations differed considerably between patients. In addition, while analysis of T cell states showed high variability between and within patients, clonally expanded T cells identified within patients predominantly adapted similar cell profiles. Currently we are assessing whether the occurrence of distinct T cell states can be coupled to other aspects of the immune infiltrate. In addition to describing T cell heterogeneity, our data suggest the existence of novel immune cell subsets within the tumor microenvironment. Our findings demonstrate that, using our single-cell RNA-sequencing approach, we can determine the immunological make-up in melanoma during early stages of metastatic disease, in an unbiased manner. Collectively, these experiments should be helpful in determining the mechanisms underlying the development and effectiveness of tumor-specific immune responses in metastatic melanoma.

Keywords: Tumor immune environment, Melanoma, Single-cell RNA-sequencing

B240 / Intratumoral IMO-2125 treatment in combination with anti-CTLA4 mAb induces durable anti-tumor responses associated with tumor-specific memory in preclinical studies

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IMO-2125 is a novel TLR9 agonist in clinical development in combination with ipilimumab in anti-PD-1 refractory melanoma patients. The hypothesis underlying intratumoral (i.t.) administration of IMO-2125 is that the compound modulates the tumor microenvironment (TME) by increasing tumor infiltrating lymphocytes (TILs). These TILs then allow the conversion of tumors from non-immunogenic to immunogenic, and thereby potentiate anti-tumor responses when combined with ipilimumab and other checkpoint inhibitors.

This study of i.t. IMO-2125 in combination with anti-CTLA4 mAb (clone 9H10) was conducted to further evaluate the combination's anti-tumor activity, associated immunological changes, and the impact on long term survival. Murine syngeneic colon carcinoma CT26.CL25, a subclone of CT26 expressing a model antigen b-galactosidase (b-gal), was employed in the study. Six to seven week-old BALB/c mice were subcutaneously implanted with 5 x 106 CT26.CL25 cells, on both flanks. Mice with dual solid tumors were randomized into four groups based on tumor size (150-300 mm3): placebo, IMO-2125, anti-CTLA4 mAb or a combination of IMO-2125 and anti-CTLA4 mAb. Treated mice received 100 µL volume of placebo, 50 µg of IMO-2125 (2.5 mg/kg) in 100 µL volume in the right tumor, or 200 µg (10 mg/kg) anti-CTLA4 mAb by intraperitoneal (i.p.) administration, or both IMO-2125 and anti-CTLA4 mAb. IMO-2125 was administered q4d x 5 and anti-CTLA4 mAb was administered q2d x 10. Mice were observed for safety, immune response parameters, and anti-tumor activity.

The treatment was well tolerated. By day 28, tumors on both flanks in the placebo group were 1.5 g or over. In comparison, in i.t IMO-2125, anti-CTLA4 mAb and combination groups, tumor growth inhibition in the treated tumors was 94%, 50% and 98%, respectively, and 52%, 63% and 98%, respectively in the distant untreated tumors. By day 185, 10% of mice in the IMO-2125

monotherapy group, 50% of the anti-CTLA4 mAb monotherapy group, and 100% of the combination group remained tumor-free.
In a separate study, combination treatment with i.t. IMO-2125 and i.t anti-CTLA4 mAb resulted in long term tumor free survival (384 days) in 7 out of 10 mice. Analyses of spleen cells from these surviving mice showed robust memory T cell responses against tumor-associated antigens (AH1 and beta-gal). Furthermore, these mice rejected tumor rechallenge 334 days post last treatment, indicating a durable tumor-specific immune response.
In conclusion, IMO-2125 i.t and anti-CTLA4 mAb combination therapy induced tumor-specific memory T cells, leading to long-lasting durable tumor regression and preventing relapse. A phase 1/2 clinical trial of IMO-2125 in combination with ipilimumab in patients with metastatic melanoma is ongoing (NCT Identifier: NCT02644967).

Keywords: Tumor microenvironment, TLR9 agonist, Combination therapy

B241 / The expression and function of IL-36g in colorectal cancer

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IL-36g is a member of the IL-1 superfamily of cytokines and a mediator of Type 1 immunity. This cytokine was recently reported to be a crucial mediator of tertiary lymphoid organ (TLO) formation in a murine model of colorectal carcinoma. Specifically, when IL-36g was introduced therapeutically into the tumor microenvironment of MC38-bearing mice, the treated tumors exhibited delayed progression in correlation with TLO formation, neither of which was observed in control-treated mice. In that system, CD11c+ dendritic cells (DC) were observed to be a major source of IL-36g in the tumor microenvironment (TME), and signaling through IL-36R+ host cells (such as those of the stroma and vasculature) was necessary for the therapeutic efficacy of IL-36g-based treatments. In the present study, we sought to identify a role for IL-36g in the ***de novo*** formation of TLO in a cohort of 33 patients with primary colorectal cancer. TLO have been reported in multiple studies to be a positive prognostic marker in both primary and metastatic colorectal cancer. Here, we investigated which cell types in the TME produce IL-36g, using immunohistochemical and immunofluorescent imaging. Results indicate that IL-36g can be expressed by the smooth muscle cells/myofibroblasts surrounding the vasculature, supporting existing data indicating that IL-1b signaling can induce IL-36g expression by colonic myofibroblasts. In our studies, expression of IL-36g by the tumor vasculature correlates with CD20+ B cell density in TLO, but not the number of TLO observed in the tumor. Interestingly, some of the IL-36g+ vessels appear to be high endothelial venules (HEV; co-expressing CD31 and PNAd), specialized vessels that aid in the recruitment of L-selectin/CD62L+ lymphocytes to sites of inflammation. Additionally, we identified that within the immune compartment, CD8+ T cells and CD68+ macrophages are major sources of IL-36g in the TME, while DC-LAMP+ DC are less so and CD20+ B cells do not appear to produce the cytokine at all. Together, these data suggest that the pro-inflammatory conditions of colorectal cancer promote a Type 1-polarized immune microenvironment; that IL-36g (a known alarmin and Type 1 cytokine) is secreted by a large number of cells within the TME; and that IL-36g expression may play a role in the maintenance of TLO and in B cell recruitment to these structures, within the immune contexture of colorectal cancer.

Keywords: Tertiary lymphoid organ, IL-36g, colorectal cancer

B242 / Intratumoral human papillomavirus (HPV) directed immunity as predictor of response to therapy

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Patients with HPV+ oropharyngeal squamous cell cancer (OPSCC) respond better to chemo)radiotherapy than HPV- OPSCC patients. This better prognosis might be related to the presence of virally-derived E6 and E7 antigens expressed on HPV+ OPSCC, making them visible to the immune system. To understand the constitution of such a better antitumor response in HPV+ OPSCC we performed an in-depth analysis of the immune contexture of OPSCC and evaluated the relation between immune infiltration composition and function with the clinical outcome.
In our cohort of 97 HPV16+ and HPV- OPSCC patients tumor infiltrated lymphocytes (TILs) were obtained from fresh tumor tissue taken prior to therapy and analyzed phenotypically and functionally (proliferation, cytokine production) upon stimulation

with HPV16 E6 and E7 antigens or PHA. The effect of the TIL-produced cytokines on growth of five different OPSCC cell lines was tested in presence or absence of cisplatin, the commonly used chemotherapeutic agent. Archived tumor tissue was used for HPV DNA typing, $p16^{INK4a}$ staining and quantification of T-cell infiltration. Using the RNA sequencing data of a cohort of 75 HPV16+ OPSCCs in the publicly available cancer genomic atlas (TCGA) findings were validated and tumor milieu analysis was done.
The majority of HPV16+ OPSCC patients in our cohort displayed a HPV16-specific CD4+ and CD8+ T-cell response. These patients had a significant better overall survival (OS) than HPV16+ patients without a HPV-specific immune response or HPV- OPSCC patients. The HPV-reactive T cells produced IFNg, TNFa and IL-17, a T-helper type 1 (Th1)/Th17 profile. The presence of significant more intratumoral T cells expressing the, with IFNg-production associated, transcription factor Tbet in immune responders was confirmed by immunofluorescence in archived tumor tissue. Culture of OPSCC tumor cell lines in the presence of IFNg and TNFa lowered their proliferation, while the combination with cisplatin resulted in increased apoptosis of the tumor cells. Validation of our results in the TCGA database confirmed that a high expression of CD4, CD8 and IFNg correlated with better OS in OPSCC. Furthermore, it suggested that HPV16+ OPSCC with many CD4+ T cells (enriched for activated effector memory T cells), dendritic cells (DCs) and DC-like macrophages. Indeed, mass and flow cytometric analysis of the freshly dispersed OPSCC in our cohort confirmed these findings.
In conclusion, the presence of activated Th1-oriented HPV16-specific T cells present in pre-treated HPV16+ OPSCC improves clinical outcome by making the tumor more susceptible for cisplatin and driving a type 1 oriented tumor immune microenvironment. Therefore, these HPV16-specific TILs can be used as predictor of clinical outcome upon therapy and might be boosted by vaccination.

Keywords: oropharyngeal cancer, human papillomavirus, tumor microenvironment

B243 / Evaluation of lymphoid and myeloid immune cells and secreted factors in the tumor microenvironment by RNA in situ hybridization

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The interactions between tumor cells and surrounding immune cells in the tumor microenvironment play a key role in tumor progression and treatment response. Accumulating evidence revealed crucial role by tumor infiltrating immune cells. Infiltration of T cells has been correlated with improved clinical outcome. However, T cells are ineffective in eradicating tumors due to inhibition of tumor infiltrated lymphocytes by various immune checkpoint molecules. In addition, immunosuppressive factors in the local tumor microenvironment contribute to tumor evasion by recruiting and modulating regulatory T cells and myeloid derived cells.
In this study, we evaluated immune infiltration of lymphoid and myeloid cells in the tumor microenvironment of archived FFPE tissues from two cancer types; 30 cases of non-small cell lung cancer (NSCLC) and 30 cases of ovarian cancer. Applying RNAscope® in-situ hybridization assays, expression of several key functional markers such as FOXP3, IFNg, CXCL10, and CCL22 are evaluated in addition to key cell lineage markers (CD4, CD8, CD68, and CD163). We observed abundant infiltration of both lymphoid and myeloid cells in most of tissues from both lung and ovarian tumors. Recruitment of CXCL10 expressing immune cells in local tumor region is clearly visualized. The presence and abundance of regulatory T cells (FOXP3+CD4+) are evaluated in relation to CCL22 expression in immunosuppressive local environment. Multiple immune checkpoint molecules, including PD1, PD-L1, TIM3, and CTLA-4, are detected in the same immune environment, especially highly inflamed tumors. Among tumor samples evaluated in this study, IFNg positive CD8 cells are rarely detected.
In summary, we present a valuable approach to visualize and evaluate states of lymphoid and myeloid cells present in the local tumor environment by detecting key functional molecules, such as cytokines and chemokines, in addition to immune co-inhibitory and lineage molecules. Considering highly plastic immune environment with a spectrum of maturation and polarization, characterizing immunomodulatory phenotype by RNAscope® ISH may bring insights to understand key contributing factors in immunosuppressive environment and facilitate intervention through cancer immunotherapy.

Keywords: Tumor microenvironment, Immune infiltration, Immune checkpoint molecules

B244 / Immune barriers in stromal regions of ovarian cancer: a new world of heterogeneity to explore

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Variable immune profiles can be distinguished amongst tumors of high-grade serous ovarian cancer (HGSC) patients. While the subgroup of patients that has a high infiltrate of tumor-infiltrating lymphocytes (TIL) in tumor epithelium is associated with a higher survival rate and likely responds to current immunotherapies (eg checkpoint blockade), most patients lack such major infiltration.

Instead, many patients present with TIL in stromal regions of the tumor that fail to confer prognostic benefit. To date, the role the stroma plays in immune evasion remains largely unknown. To address this issue, we aimed to determine the relationship between stromal composition and immune infiltration. Multicolor immunohistochemistry and multiplex immunofluorescence were used for comprehensive phenotyping and colocalization studies. We assessed markers of TIL, macrophages, PD-L1, cancer-associated fibroblasts, mesenchymal cells, and epithelial-mesenchymal transition (EMT) states. These were profiled in a prospective cohort with well-annotated clinical history and matching Nanostring gene expression profiles.
We identified multiple populations of mesenchymal cells and fibroblasts in the microenvironment of HGSC based on the expression of fibroblast-associated protein, vimentin, desmin, platelet-derived growth factor receptor-β, fibroblast-specific protein-1 (S100A4), and α-smooth muscle actin. Distinctive spatial relationships of these cells with immune cells were found with high variability between patients. While TIL infiltration was a relative good marker for differentiating gene expression-defined molecular subtypes, stromal patterns do not appear to align with these molecular subtypes. The presence of cancer-associated fibroblasts with variable phenotypes potentially further defines the tumor microenvironment and may have specific immune barrier functions, either mechanistically or chemically. The vast majority of tumors studied appear to at least in part have undergone EMT, with a medium to high expression of N-cadherin and downregulation of E-cadherin on epithelial cells. PD-L1 expression on tumor cells is generally low in HGSC, and therefore no direct link between EMT and PD-L1 could be distinguished. Other potential immune evasive mechanisms related to EMT and stromal components are under active investigation.
Even though molecular subtypes of HGSC are associated with specific immune profiles, no clear survival differences were found between patients with tumors of these various subtypes. Since stromal composition does not align directly with the subtypes, stratification on basis of fibroblast and mesenchymal cell phenotypes combined with immune composition may help define prognostically relevant subtypes. This could pave way for exploration of new combinatory treatment strategies to release TIL from suppressive effects of the tumor microenvironment to improve the success of immunotherapy in a larger subgroup of patients.

Keywords: High-grade serous ovarian cancer, stroma, immune evasion

B245 / Enhancing infiltration and function of effector T-cells in solid tumor through tumor-specific immunomodulator delivery

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In the past decades, breakthroughs in cancer immunotherapy have led to unprecedented clinical responses in patients with advanced-stage cancers that would otherwise be fatal. However, treatment of many solid organ malignancies with highly immunosuppressive local microenvironment remains challenging. Tumors develop a number of mechanisms to prevent effector T-cells from reaching tumors and to suppress their function. T-cell infiltration into solid tumor, a key limiting factor for efficacious cancer immunotherapy, has been demonstrated to be associated with good clinical outcomes in various cancer immunotherapies. Safe and effective strategies to improve T-cell infiltration and augment T-cell expansion and function in solid tumors are highly desired to enhance the clinical response rate of cancer immunotherapies in the treatment of solid tumors. Disruption of chemotaxis of lymphocytes is likely a main contributing factor for effector T-cell exclusion; chemokines are known to play a critical role in orchestrating T-cell trafficking and tumor infiltration. Here, we developed a tumor microenvironment-responsive nanogel (NG) based on a novel carrier-free delivery strategy to effectively and specifically co-deliver T-cell recruiting chemokines, such as CCL5, and immunostimulatory cytokines, such as interleukin-2 (IL-2), in order to enhance infiltration and function of effector T-cells in solid tumors. A half-life prolongated IL-2-Fc fusion cytokine and CCL5 were chemically co-crosslinked with a responsive linker to form the binary protein nanogels (BNG). These BNGs had exceptionally high loading capacity of cytokines and chemokines (~84% and 4% respectively) and uniform size (78.6 ± 8.2 nm). BNGs released native cytokine/chemokine proteins in response to increased reduction or decreased pH which are characteristics of tumor microenvironment, and thus enabled tumor-specific delivery of the incorporated cytokines and chemokines. In vitro chemotaxis assays showed that the released chemokines from BNG possessed the similar chemotactic ability in recruiting activated T-cells. Intratumorally or intravenously administered BNGs showed markedly enhanced T-cell infiltration and expansion in subcutaneous B16F10 mouse melanoma in C57BL/6 mice compared to free cytokines or chemokines at equivalent doses. The same dose of free cytokine administered systemically as a bolus or repeated lower-dose injections were highly toxic in mice, eliciting systemic cytokine syndrome. We demonstrated that the

responsive BNGs effectively enhance the T-cell infiltration in solid tumor and safely provide potent adjuvant immunostimulants to tumor infiltration T-cells.

Keywords: T-cell infiltration, Immunomodulator delivery, Cytokine/chemokine nanogel

MICROBIOTA

B247 / RANKL controls effector cytokine production in group 3 innate lymphoid cells

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Group 3 innate lymphoid cells (ILC3s) are specialized innate lymphocytes that secrete the cytokines IL-22 and IL-17A. In the intestine, these cells contribute to maintenance of the gut epithelial barrier and the host response to extracellular microbes. While soluble mediators that activate ILC3s have been described, the signals in tissues that negatively regulate these cells remain poorly understood. Here, we demonstrate that the tumor necrosis factor (TNF) family member receptor activator of nuclear factor kappa-B ligand (RANKL) provides signals that constitutively suppress ILC3 activity. RANKL controls intestinal ILC3 abundance and regulates production of IL-17A and IL-22 in response to IL-23 and during infection with the enteric murine pathogen ***Citrobacter rodentium.*** Suppression of ILC3 activity occurs in gut tissue microenvironments that are enriched for RANKL. Thus, RANKL is a novel regulator of ILC3 abundance and activation.

Keywords: Intestine, Innate lymphoid cells, RANKL

B248 / Microbiota-driven interleukin-17-producing cells and eosinophils synergize to accelerate multiple myeloma progression

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The gut microbiota has been causally linked to cancer, yet the mechanisms by which intestinal microbes influence progression of extramucosal tumors are poorly understood. Here we show that the commensal microbiota promotes the differentiation of Th17 cells colonizing the Peyer's patches and migrating to the bone marrow (BM) of transgenic Vk*MYC mice, where they favor progression of multiple myeloma (MM). Lack of IL-17 in Vk*MYC mice, or disturbance of their microbiome imposed either by sterility of the housing conditions or by antibiotics led to decreased BM levels of IL-17 and delayed disease appearance. Similarly, in patients with smoldering MM a higher level of BM IL-17 predicted faster disease progression. Mechanistically, IL-17 induced STAT3 phosphorylation in plasma cells, and activated eosinophils. Indeed, treatment of Vk*MYC mice with antibodies blocking IL-17, IL-17RA and IL-5, the latter activating and recruiting eosinophils, reduced BM accumulation of Th17 cells and eosinophils and delayed disease progression. Thus, in Vk*MYC mice, the gut microbiota precociously unleashes a paracrine signaling network between adaptive and innate immunity that accelerates progression to MM, and can be targeted by already available therapies.

Keywords: multiple myeloma, microbiota, cytokines

B249 / Mucosal-associated invariant T cells respond to cutaneous microbiota

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Infectious agents are estimated to cause approximately 18% of cancers worldwide, supporting a link between microbial products and carcinogenesis. The diverse array of commensal microorganisms that reside at barrier sites of the human body, collectively termed the microbiome, provide the primary source of constitutive microbial stimulation and promote immune homeostasis through their release of microbial products. Mucosal-associated invariant T (MAIT) cells are unconventional T cells that recognize microbial-derived metabolites and provide an initial defense to pathogens through their rapid production of Th1- or Th17-associated cytokines. While MAIT cells have been shown to infiltrate colorectal tumors, it remains to be determined how MAIT cells contribute to tumorigenesis and whether commensals regulate MAIT cell function. Here we show that MAIT cells are present in murine skin at a high frequency and their homeostasis requires the microbiota, as these lymphocytes are nearly absent in germ-free animals. Furthermore, application of distinct human commensal bacteria to the skin of mice induces the proliferation of IL-17A-producing MAIT cells that exhibit a unique transcriptional profile. The induction of these cells occurs in a manner that is partially dependent on both antigen presentation and IL-23 signaling. This work identifies MAIT cells as the only cell population that is entirely dependent on the microbiota and reveals the mechanism by which these cells respond to the commensal microbial community. Since IL-17A promotes tumor vascularization and neutrophil recruitment, the high frequency of MAIT cells in human skin suggests that modulation of the skin-resident bacteria may have clinical applications for the progression of melanoma and current studies are exploring this possibility using the B16 melanoma model in mice.

This work was supported by the NIH (ZIAAI001115 and ZIAAI001132 to YB). MGC is a Cancer Research Institute Irvington Fellow supported by the Cancer Research Institute.

Keywords: MAIT, microbiota, melanoma

B251 / Identifying novel effectors of the gut microbiota that modulate cancer cell killing by CTLs using functional metagenomics

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The immune system, which is profoundly affected by the microbiota composition and activity, plays an anti-tumorigenic role by surveying for and killing cancer cells. Indeed, recent studies found the microbiota to be essential for the success of cancer immunotherapy treatments in mice, while the bacterial effectors remain unknown. We developed an innovative high throughput screening system to isolate microbiota-derived effectors that modulate the ability of immune cells to kill cancer cells. We utilize the mouse CT26 colon carcinoma cell line as target cells and the GSW11 specific CCD2Z T cell hybridoma cell line as the effector cells. Cytotoxicity and T cell activation assays, measured by LDH release and b-gal expression, respectively, were calibrated to both positive and negative controls and in response to an array of perturbations. Bacterial DNA extracted from fecal contents of mice bearing colorectal tumors served for the generation of the metagenomics libraries. We are now screening ~ 15,000 clones and already have preliminary results that several clones indeed modulate T cell activation. The next step will be to characterize the cellular and immune responses of identified effectors both in tissue cultures and in animals. Finally, we hope that our future results could be taken to the clinic and serve for diagnosis, prognosis and therapeutics.

Keywords: Microbiota, T cell, Metagenomics