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Complex interactions between the microbiome and cancer immune therapy

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ABSTRACT

Immuno-oncology has rapidly grown in the last thirty years, and immunotherapeutic agents are now approved to treat many disparate cancers. Immune checkpoint inhibitors (ICIs) are employed to augment cytotoxic anti-cancer activity by inhibiting negative regulatory elements of the immune system. Modulating the immune system to target neoplasms has improved survivability of numerous cancers in many individuals, but forecasting outcomes post therapy is difficult due to insufficient predictive biomarkers. Recently, the tumor and gastrointestinal microbiome and immune milieu have been investigated as predictors and influencers of cancer immune therapy. In this review, we discuss: (1) ways to measure the microbiome including relevant bioinformatic analyses, (2) recent developments in animal studies and human clinical trials utilizing gut microbial composition and function as biomarkers of cancer immune therapy response and toxicity, and (3) using prebiotics, probiotics, postbiotics, antibiotics, and fecal microbiota transplant (FMT) to modulate immune therapy. We discuss the respective benefits of 16S ribosomal RNA (rRNA) gene and shotgun metagenomic sequencing including important considerations in obtaining samples and in designing and interpreting human and animal microbiome studies. We then focus on studies discussing the differences in response to ICIs in relation to the microbiome and inflammatory mediators. ICIs cause colitis in up to 25% of individuals, and colitis is often refractory to common immunosuppressive medications. Researchers have measured microbiota composition prior to ICI therapy and correlated baseline microbiota composition with efficacy and colitis. Certain bacterial taxa that appear to enhance therapeutic benefit are also implicated in increased susceptibility to colitis, alluding to a delicate balance between pro-inflammatory tumor killing and anti-inflammatory protection from colitis. Pre-clinical and clinical models have trialed probiotic administration, e.g. *Bifidobacterium spp.* or FMT, to treat colitis when immune suppressive agents fail. We are excited about the future of modulating the microbiome to predict and influence cancer outcomes. Furthermore, novel therapies employed for other illnesses including bacteriophage and genetically-engineered microbes can be adapted in the future to promote increased advancements in cancer treatment and side effect management.

Abbreviations: ASVs: amplicon sequence variants; CD: cluster of differentiation; CTLA-4: cytotoxic T lymphocyte antigen 4; EBV: Epstein-Barr virus; ETBF: enterotoxigenic *Bacteroides fragilis*; FMT: fecal microbiota transplant; HPV: human papilloma viruses; HSCT: hematopoietic stem cell transplantation; ICI: immune checkpoint inhibitor; IL: interleukin; OTUs: operational taxonomic units; PCA: principal component analysis; PCoA: principal coordinate analysis; PD-1: programmed cell death protein 1; PD-L1: programmed death ligand 1; PSA: polysaccharide A; rRNA: ribosomal RNA; SCFAs: short-chain fatty acids; SNPs: single nucleotide polymorphisms; TLR: toll-like receptor; TNF- α : tumor necrosis factor alpha; Tregs: regulatory T cells

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Introduction

Patient survival for most cancers has increased dramatically with earlier diagnosis, improved treatments, and faster and more effective management of side effects [1]. Novel cancer therapies include medications that augment elements of the immune response to directly

target and kill cancer cells or to replace and boost immune system functions; these collectively are referred to as cancer immunotherapy [2]. Commensurate with increased survival from new standards of care are increased efforts to understand and predict each individual's response to and toxicity from

these therapies [2]. For reasons not fully understood, variability exists in both efficacy and toxicity-related complications among patients receiving immune therapies [3]. Recently, gut microbiome and immune profiles have been identified as contributing factors to this inter-individual variability (reviewed in [4–7]).

Microorganisms inhabit our skin and respiratory and gastrointestinal tracts and comprise a large portion of our overall cellular, metabolic, and genetic mass [8]. The organisms that comprise this community are collectively referred to as the microbiota while their collective genetic material is termed the microbiome [9]. The intimate relationship among microbiota composition, metabolic function, and the development and regulation of the immune system has been correlated with entities as disparate as inflammatory bowel disease, autism, asthma, and obesity [10–14]. Several microbes directly inflame tissues, and certain viruses and bacteria have been well described as direct causes of cancer, for example, Epstein-Barr virus (EBV) with Burkitt's and Hodgkin's lymphoma, human papilloma viruses (HPV) with cervical, head, neck, and anal cancers, and *Helicobacter pylori* with gastric cancers, among others [15]. Given the connection between gut microbiota and inflammatory diseases, researchers are exploring whether differences in the human microbiome have the potential to impact individual responses to cancer immune therapies (additionally reviewed in [4–7]). Further, recent advances in sequencing technology have provided fine granular detail of a potential tumor microbiota in certain cancers [16–18]. With decreasing metagenomic sequencing costs, large scale observational studies and placebo-controlled trials can now be analyzed for longitudinal changes in microbial structure and gene content [19]. These techniques have revealed correlations between the microbiome and cancer immunotherapy outcomes in pre-clinical animal models and human clinical trials [20].

In this review, we first discuss methodologies for characterizing the microbiome, including technical limitations and challenges. Next, we describe ways in which the commensal gastrointestinal microbiota has been shown to interact directly with the immune system and how these interactions influence oncogenesis. We then focus on interactions among cancer immunotherapies and the microbiome and ways in which the microbiome can be modulated using prebiotics, probiotics, postbiotics, antibiotics and fecal microbiota transplantation (FMT) to improve survival and limit toxicity.

Measuring the microbiome

Traditionally, clinical assessment of bacterial populations is achieved by sampling the site of interest and

culturing organisms in broth and agar plates, followed by phenotypic and molecular identification [21]. Although we have made significant advances in our ability to culture various organisms, a large proportion of human-associated microbes are time- and labor-intensive to grow [22]. Additionally, bacterial enrichment through culture can obscure abundance differences in the original sample because of differential and exclusionary growth [23]. Cell-free methods to measure bacterial presence and activity have become more popular and have allowed measurement of microbial populations at previously impossible scales, including in diagnostic microbiology [24]. Nucleic-acid based methods for detecting viruses and bacteria initially focused exclusively on their presence or absence [25], but recent advances have incorporated relative abundance measures to determine bacterial presence in clinical samples quantitatively to better understand the burden of disease [26].

The first step in determining microbial colonization of a body site by sequencing is proper sampling with sterile technique and preservation from nucleases [27]. Once a sample is obtained, genomic DNA is extracted using phenol-chloroform or commercially-available kits with yield and purity dependent on sample type [28,29] (Figure 1(A)). The 16S ribosomal RNA (rRNA) gene is highly conserved and is nearly universally present in bacteria, and its sequence can be used to distinguish bacterial genera or species [30]. 16S rRNA gene amplicon sequencing is a cheap, quick, and reliable method for determining bacterial taxonomy after computational processing with publicly-available tools such as QIIME 2 or DADA2 [31,32] (Figure 1(B)). Although 16S rRNA gene sequencing is a useful tool, it has several limitations. Precision is often limited to the genus level, especially when assigning operational taxonomic units (OTUs) in lieu of amplicon sequence variants (ASVs), which are preferred due to enhanced specificity [33,34]. OTU-picking methods cluster reads that differ by less than 3% from a standard, but often group several species into a common genus. For example, OTU methods would assign the genus *Neisseria* to both pathogenic (e.g. *Neisseria gonorrhoea* and *N. meningitidis*) and commensal *Neisseria spp.*, which is clearly an important clinical distinction depending on the sample type [34]. Therefore, methods that assign ASVs to each sequence are preferred, thereby allowing resolution of single nucleotide polymorphisms (SNPs) and strain-level differences [34]. Further, 16S rRNA gene sequencing is limited to genomic DNA, does not account for variable copy number within bacterial genera, which could artificially increase relative abundance [35], and ignores

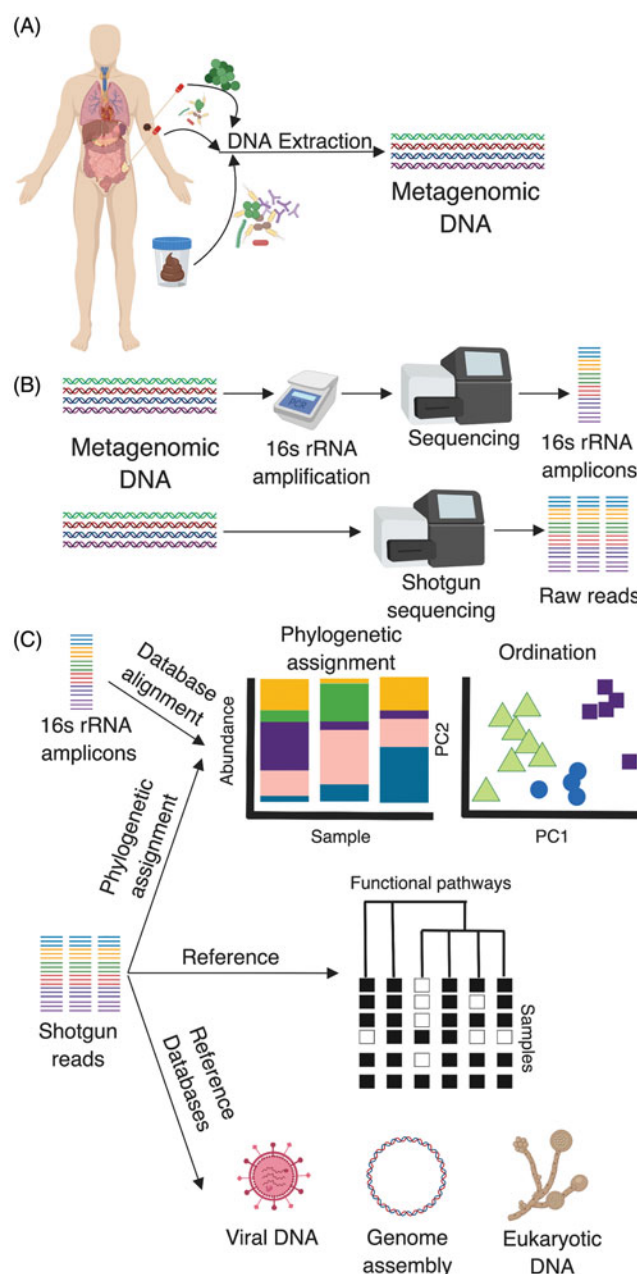


Figure 1. Schematic for metagenomic analysis. (A) The first step in microbiome analysis is selecting the sample, obtaining it with sterile technique, and preserving it with consideration of DNases and RNases. Samples can be obtained directly from tumors, skin, or fecal samples. DNA extraction via phenol chloroform or commercially available kits yields metagenomic DNA. (B) The 16S rRNA gene can be amplified and sequenced. Alternatively, metagenomic DNA can be shotgun sequenced to yield DNA fragments of varying sizes. (C) 16S rRNA reads can be computationally processed with publicly available pipelines yielding relative abundance of taxa per sample. Ordination methods such as PCA and PCoA are utilized to identify similarities and differences in diversity metrics (alpha, beta, gamma) between samples or between groups. Reads from metagenomic shotgun sequencing can be processed to yield the same information. In addition to taxonomy, shotgun reads can be processed and mapped to databases to determine functional pathways, assemble bacterial genomes, and understand the presence of viral or eukaryotic DNA depending on appropriate sequencing depth. Created with BioRender.

plasmids and genomic DNA that encode antibiotic resistance, toxin production, and other virulence factors of clinical importance.

Alternatively, DNA from all organisms in the sample, collectively referred to as the metagenome, can be shotgun sequenced using commercial technology, and

the taxonomy can be obtained using clade-specific markers with bioinformatic tools such as Kraken or MetaPhlan2 [36,37,38] (Figure 1(C)). Additionally, shotgun metagenomic sequencing can be used to infer whole community microbial functional information and annotate genes that confer antibiotic resistance,

produce toxins, or provide metabolic pathway information via comparison to known databases, assuming appropriate sequencing depth [27,39,40,41] (Figure 1(C)). Metagenomic sequencing can also identify non-bacterial components of the microbiota, such as fungi, viruses, and bacteriophages, which are gaining attention for their roles in the microbiome and human health [42,43]. Therefore, desired analyses necessarily determine whether 16S rRNA or metagenomic shotgun sequencing is appropriate to profile the microbiota or microbiome, respectively.

After the sequencing data is obtained, the first question asked is ‘who is there?’ Both 16S rRNA and shotgun metagenomic sequencing can answer this question (Figure 1(C)). Data from sequencing analysis can be visualized in terms of relative abundance of taxa within a sample or as a similarity or dissimilarity ordination plot based on measures of alpha and beta diversity (Figure 1(C)). Alpha diversity measurements describe intra-sample diversity: measurements include Shannon diversity and species richness. Beta diversity measures are pairwise comparisons of dissimilarity between all sample pairs and are typically viewed as an ordination plot with principal coordinate analysis (PCoA). Measures of beta diversity include Bray-Curtis, binary-Jaccard, and weighted UniFrac (Figure 1(C)) (reviewed extensively in [44]). In addition to overall assessments of similarity and dissimilarity between samples, these same measures can be used to examine differences in microbial gene content (e.g. functional pathways and resistance genes) from metagenomic shotgun sequencing (Figure 1(C)) (reviewed in [27]). Although shotgun metagenomic sequencing is more expensive than 16S rRNA gene sequencing per sample when performed at a depth of 2.5 – 5 million reads per sample, the validation of shallow shotgun sequencing with as few as 500,000 reads per sample increases the affordability of this method for large datasets [33]. Thus, in designing any study interrogating the microbiome, it is vital to determine the desired downstream analyses so that sampling schema and sequencing depth can be planned appropriately.

After sequencing and characterizing a microbial community, correlations can be discerned by connecting individual taxa, groups of taxa, or overall trends in community composition (e.g. diversity) to host metadata. However, this observation-driven strategy is frequently insufficient to draw concrete conclusions or to elucidate underlying mechanisms in the absence of manipulation or recapitulation in animal models. To achieve mechanistic understanding, microbiota-humanized mice are produced by gavaging germ-free or

antibiotic-treated, conventional mice with stool samples from healthy humans, patient cohorts, or pre-defined consortia [45,46]. Different microbes colonize mice with varying success, necessitating quality checks to ensure the input is comparable to the output community with at least 65% uptake at the phylum level, though the definition of “successful” microbiota-colonization depends on the richness and taxonomic abundance of the input community [45–47].

Designing *in vivo* microbiome-related experiments is accompanied by its own set of challenges and considerations. If humanized mice are used, will they be derived from germ-free mice or antibiotic-treated mice? Germ-free mice are free of all currently detectable microorganisms and allow complete recolonization, but they are expensive to maintain, have severely underdeveloped immune systems that are not always restorable, and must be individually derived for unique genotypes [48,49]. If antibiotic-treated mice are used, which antibiotic(s) will be used to deplete the conventional mouse microbiota, and what confounding effects may they produce? Individual antibiotics and antibiotic cocktails affect different microbial populations and have varying efficacy and tolerability in mice [48]. Even seemingly arbitrary decisions require due diligence. Considering the appropriate vendor and housing conditions for isogenic conventional mice is vital because microbiota compositions differ among mice that are obtained from different suppliers and that are housed in different facilities [50]. Once mice are obtained and appropriately humanized, it is important to ensure they are consistently colonized and that their microbiota are stably established over time with minimal strain dropout [45–47]. For short experiments, this can be done by cohousing animals, wherein they will engage in coprophagy and exchange microbiota content [51]. For longer studies, littermate crossing can aid in microbiome stability between cages [51,52]. All these factors must be taken into consideration not only when designing experiments, but also when interpreting results, reading the literature, and extrapolating conclusions from animal studies about the role of the microbiota in human health.

An additional concern, especially when considering environments previously presumed to be sterile or when tissue is isolated, is to include proper negative controls. Sequencing contamination can strongly influence results; therefore, researchers should include the extraction kit alone with no sample added or a skin swab control obtained at the same time and processed in the same way as the tumor sample. With proper controls, several bioinformatic tools exist to minimize the

impact of contamination [53,54]. Because of the advances in sequencing and computational techniques, it is vital to consider contamination when designing experiments to examine relationships between cancer and the local or gut microbiota. Indeed, Robinson *et al.* analyzed a large public repository, Cancer Genome Atlas, for microbial DNA and identified numerous skin contaminant taxa such as *Staphylococcus epidermidis* and *Cutibacterium acnes*. They also identified sequencing center-specific contaminants, reinforcing the importance of including appropriate negative controls [16]. To uncover associations between microbes and cancer, consideration of experimental design, including negative controls and sterile sample collection, is therefore vital.

Local tumor microbiota

Up to 15% of cancer malignancies worldwide can be attributed to infectious diseases [55]. Oncogenic viruses such as EBV and HPV carry genes that directly interfere with cell cycle checkpoints and inhibit the activity of tumor suppressors, and thus can be considered initiators of cancer [56]. In contrast, bacteria tend to be considered cancer promoters; they contribute to oncogenesis by inducing inflammation and recruiting immune cells, thus increasing the production of reactive oxygen species [56]. However, this paradigm is shifting, and recent studies show that bacteria can directly interact with host genes that regulate the cell cycle, apoptosis, and other mechanisms of tumor suppression [57].

Helicobacter pylori-associated gastric cancer is a classic example of inflammation-induced carcinogenesis. Prolonged or dysregulated inflammation can lead to uncontrolled cellular proliferation and subsequent tumorigenesis [56]. Several groups have found that *H. pylori*-associated gastritis and peptic ulcer disease, a common precursor to stomach cancer, are often accompanied by upregulated Th17 activity [58,59]. Further, *H. pylori*-infected tissues and associated gastric tumor microenvironments exhibit similarly upregulated Th17 activity and an increase in interleukin (IL)-17 production [60] (Figure 2(A)). Other bacterial taxa have been associated with increased tumorigenesis accompanied by upregulation of Th17 cells. Enterotoxigenic *Bacteroides fragilis* (ETBF) enhances colonic tumor formation in a mouse model in an enterotoxin and Stat3/Th17-dependent manner [61] (Figure 2(A)). This increase of pro-inflammatory Th17 cells in both *H. pylori*-associated gastric cancer and ETBF-associated colon cancer

suggests that certain bacterial taxa have oncogenic potential.

Inflammation is not the only mechanism by which bacteria can contribute to tumorigenesis. *Fusobacterium nucleatum* has been shown to induce inflammation and upregulate host oncogenes through interactions between its virulence factor FadA and E-cadherin expressed on epithelial cells, including colorectal cancer cells [62]. This binding triggers clathrin-mediated endocytosis of the bacterium, leading to phosphorylation of β -catenin and subsequent activation of NF- κ B, which upregulates inflammatory cytokine production. Downstream activation of WNT, Myc, and CyclinD1 promote cellular proliferation and override cell cycle checkpoints (Figure 2(A)). Inhibition of clathrin prevented internalization and NF- κ B activation, but the oncogenes were still upregulated [62]. This illustrates that bacteria can promote carcinogenesis simultaneously in multiple ways; inhibition of a single mechanism may be insufficient to treat or prevent disease.

Enrichment of certain bacteria has been identified in tumors without elucidated oncogenic mechanisms. For example, bacteria from the *Enterobacteriaceae* family were found by 16S rRNA gene sequencing and fluorescence *in situ* hybridization in 76% (86/113) of pancreatic adenocarcinoma tumor samples relative to 15% (3/20) of normal pancreas control samples [18]. In a mouse model of subcutaneous colon carcinoma, bacterial presence within tumors led to increased tumor size, and antibiotic treatment enhanced the efficacy of the chemotherapy drug, gemcitabine [18] (Figure 2(A)). It is unclear whether bacteria are present prior to cancer development or whether they colonize opportunistically, but local bacterial presence is an important consideration in cancer diagnosis and treatment. These data suggest the existence of a local tumor microbiota in certain cancers that can modulate therapy and also that the bacteria-immune interaction may be adjusted to fight cancer.

In addition to the presence of specific bacteria leading to immunological consequences connected to cancer, large scale case-control studies have demonstrated an association with frequent antibiotic courses in the ten-year interval prior to certain cancer diagnoses [63,64]. Because cancer can affect the immune system for an interval prior to diagnosis, these authors excluded all antibiotics administered in the year prior to diagnosis, again suggesting that long-term bacterial dysbiosis could affect cancer predisposition [63]. More mechanistic studies are required to determine whether this association is driven by gastrointestinal dysbiosis or local, tumor-promoting communities and/or whether it is a direct oncogenic effect of antibiotics in humans. Animal studies have

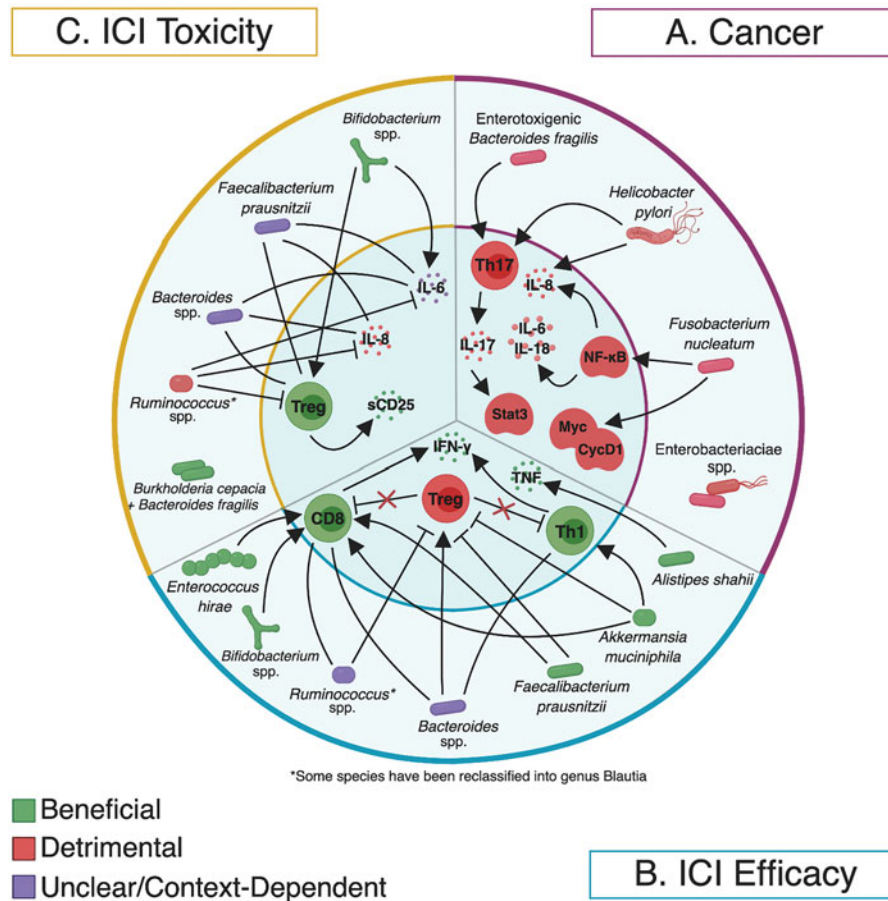


Figure 2. Interconnected relationships of the microbiota and immune system to affect cancer (A), immune checkpoint inhibitor efficacy (B), and colitis (C). A line with an arrowhead indicates a positive association. A line with a perpendicular end indicates a negative association. A straight line indicates context- or species-specific association. An organism with no line indicates mechanism not yet elucidated. (A) Enterotoxigenic *Bacteroides fragilis* (ETBF), *Helicobacter pylori*, and *Fusobacterium nucleatum* have been shown to increase expression of inflammatory cytokines and/or host oncogenes resulting in cancer promotion [58,62,139]. Bacteria from the family Enterobacteriaceae have been detected in the pancreatic adenocarcinoma microenvironment [18]. (B) *Alistipes shahii*, *Akkermansia muciniphila*, *Enterococcus hirae*, *Faecalibacterium prausnitzii*, and *Bifidobacterium* spp., are associated with beneficial immunophenotypes in the context of immune checkpoint inhibitor therapy. *Bacteroides* spp. have mixed effects; they have been cited as detrimental, different strains enriched in both responders or nonresponders depending on the species, or beneficial. *Ruminococcus* spp. are associated with beneficial immunophenotypes in the context of therapy. *Blautia obeum* is associated with a detrimental immunophenotype [20,83–85,88–90]. (C) *Bacteroides fragilis*, *Burkholderia cepacia*, and *Bifidobacterium* spp. may be protective against colitis [20,104,133]. *Blautia obeum* is associated with an increased risk of developing colitis. Different OTUs identified as *Faecalibacterium prausnitzii* and *Bacteroides* spp. were enriched in patients who did and did not develop ICI-associated colitis [85,94]. Created with BioRender.

demonstrated that certain antibiotics enrich for pathogens capable of stimulating the immune system, leading to bacterial translocation into the mesenteric lymph nodes and disruption of the local inflammatory balance [65–67]. Certain antibiotics have also been shown to directly inhibit immune cells [67], but the mechanistic interplay among specific antibiotics, the immune system, and the microbiota is not fully elucidated.

Cancer immune therapy

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies that block inhibition of T cell function. ICIs

have been developed to block programmed cell death protein 1 (PD-1; nivolumab, pembrolizumab), programmed death ligand 1 (PD-L1; atezolizumab, avelumab, durvalumab), or cytotoxic T lymphocyte antigen 4 (CTLA-4; ipilimumab). The inhibition of these proteins, which negatively regulate immune function, enhances the cytotoxic activity of T cells to increase anti-tumor immunity [61]. The proteins natively suppress T cells at different steps; therefore, the inhibitors each have a unique mechanism of action. CTLA-4 is an inhibitory receptor found on T cells that competes with cluster of differentiation (CD)28, a constitutively expressed receptor critical for T cell activation [61]. CTLA-4 is not

constitutively expressed on resting T cells but is upregulated following T cell activation [68,69]. Therefore, CTLA-4 plays an important role as an “off” switch to suppress immune activity, but it can detrimentally affect control of cancer growth by inhibiting cytotoxic CD8⁺ T cells. Antibody blockade of CTLA-4 prevents this inhibition, enabling anti-tumor cytotoxic lymphocyte activity to resume. Similarly, PD-1 plays an important role in immune suppression. It is expressed on activated T cells, and upon binding to PD-L1 or PD-L2 on the surface of various immune cells, it inhibits T cell activity [70]. Like CTLA-4, it is upregulated upon T cell activation, particularly in regulatory T cells (Tregs) [61]. The PD-1 signaling pathway is essential for preventing autoimmune disease by limiting peripheral T cell activity [70]. Although these two pathways vary somewhat in the mechanisms by which they inhibit T cell activation, both separately and combined, they have been demonstrated to be valuable targets for ICIs in the control of cancer progression [71–74].

Fundamentally, all ICIs upregulate immune activity that may have been suppressed by Tregs and other immunomodulatory cells. Tregs inhibit immune activity, which can be particularly detrimental in the tumor microenvironment where they have been implicated in the suppression of the anti-tumor inflammatory response that leads to immune tolerance of cancer [61,75]. However, Treg activity can be beneficial in mouse models of microbially-induced cancer, suggesting that context and the local microbiome-immune milieu is crucial [76–79]. Gastrointestinal microbiota is essential for the induction of Tregs that tolerate commensal organisms. In germ-free mouse models, induction of Tregs is significantly impaired, but it can be restored by colonization with a defined, nonpathogenic microbial consortium [80]. Furthermore, short-chain fatty acids (SCFAs), abundant metabolites produced by intestinal microbiota, have been demonstrated to be important drivers in the induction and activity of Tregs [81]. Overall, this evidence establishes the intimate relationship shared between commensal microbiota and the immunomodulatory activity of Tregs. This implies that the microbiota has the potential to act as a complicating factor in the ongoing battle among cancer, the immune system, and ICIs by acting on the same immune cells synergistically or antagonistically.

Microbiota and immune checkpoint inhibitor response

Recently, several groups have begun to characterize the role of microbiota in cancer immunotherapy treatment

outcomes to better understand the dichotomy between responders and non-responders (Figure 2(B)). Anti-IL-10R/CpG therapy, which works synergistically with PD-1 inhibitors [82] to allow proliferation of tumor-specific CD8⁺ T cells, is rendered less effective in the presence of antibiotics, thereby providing evidence that the microbiota plays a crucial role in modulating immune therapy efficacy in a mouse model [83]. Conventional mice treated with vancomycin, imipenem, and neomycin in drinking water for three weeks before tumor injection showed decreased tumor necrosis factor alpha (TNF- α) secretion and increased tumor volume [83]. Further suggesting a role of the microbiota, administration of probiotic *Alistipes shahii* via oral gavage restored TNF- α production in conventional mice, a promising proof-of-concept supporting the therapeutic potential of probiotics (Figure 2(B)) [83]. TNF- α was similarly low in tumor-bearing germ-free mice, again implicating the commensal microbiota in the anti-cancer immune response. Germ-free mice were not treated with antibiotics, so the additional effect of antibiotics in the absence of the microbiota is not clear. It is also unknown whether the replenishment of TNF- α levels was sufficient to restore the efficacy of the immune therapy to reduce tumor volume and increase survival. TNF- α secretion was not restored until four weeks after antibiotic therapy, suggesting there is an important pre-chemotherapy window in which to consider microbial composition during which antibiotics might be particularly detrimental.

ICI efficacy can be manipulated by altering microbiota composition [20,84]. Anti-PD-L1 efficacy differed in C57BL/6 mice from two different vendors; although these mice were considered to be genetically identical, they have unique microbiota compositions, which the authors hypothesized explained the differential efficacy that was observed [50,84]. Further implicating the microbiota, co-housing mice or orally gavaging feces between groups to obtain similar microbiota compositions reduced the differential treatment response between mice [84]. Alternatively, a transferred, soluble metabolite could manipulate gastrointestinal microbiota composition indirectly through an altered immune milieu. Further evidence supporting the microbiota as a factor mediating tumor regression was the correlation with *Bifidobacterium spp.* abundance determined by 16s rRNA sequencing and increased tumor infiltration by CD8⁺ T cells, a hallmark of anticancer activity. Administration of a *Bifidobacterium spp.* cocktail was sufficient to significantly increase the anti-PD-L1 efficacy in the non-responder group of mice [84] (Figure 2(B)). In a similar study, mice were colonized via

FMT from patient samples, followed by tumor inoculation, and then treated with anti-CTLA-4 therapy [20]. They found that donor stool could be clustered according to taxonomy using principal component analysis (PCA), and the cluster correlating with highest relative abundance, *Bacteroides spp.* was most predictive of tumor decrease after anti-CTLA-4 treatment. Although *Bacteroides spp.* abundance correlated with response, a causal relationship could have been solidified following probiotic administration of a bacterial consortium. The correlation between microbial abundance and ICI response provides exciting avenues of research interrogation and suggests a possible intervention to adjust the patient microbiome to influence cancer therapy outcomes.

These preclinical studies invoked closer examination of human cohorts and led to a series of prospective studies in search of microbial and immune signatures that could accurately predict patient response to treatment. Patients with the longest overall survival (>18 months) after ipilimumab treatment for metastatic melanoma had gut microbiota strongly enriched for *Faecalibacterium prausnitzii*, *Gemmiger formicilis*, and *butyrate-producing bacterium* SS2-1 [85] (Figure 2(B)). They found that certain baseline immune signatures, particularly lower frequencies of Tregs and $\alpha 4^+ \beta 7^+$ -expressing T cells (an integrin which directs T cells to the intestine during periods of inflammation), are correlated with long term benefit (Figure 2(B)) [85,86]. It is plausible that the baseline decrease in Tregs systemically could imply a local tumor-microenvironment with fewer Tregs that could more easily be inhibited by ipilimumab [87]. Finally, in a PCA identifying different microbial compositions between responders and non-responders, the authors found significantly increased inducible T cell activation in response to CTLA-4 blockade [85]. This study was the first in-depth analysis of the relationship among the microbiota, immunotherapy efficacy and toxicity (discussed in the following section), but the cohort size was small, only one type of cancer and drug therapy combination was examined, and the study was strictly observational without any recapitulation in an animal model. It is thus promising to link microbiota composition to ICI response (Figure 2(B)), and several clinical trials are currently investigating this relationship (Table 1).

Less than a year later, three large scale studies characterized the relationship between the gut microbiome and immune efficacy [88–90]. These three landmark papers have been the subject of several reviews by the authors as well as by third parties [4,6,7,91,92]. Importantly, all three studies identified key bacterial

species associated with responders and non-responders (Figure 2(B)) and immunophenotypes associated with responders, and recapitulated these findings in a mouse model.

Routy et al. evaluated the microbiome and outcomes of a cohort of 100 patients diagnosed with either non-small cell lung cancer or renal cell carcinoma being treated with PD-1 blockade [88]. They discovered that responders had increased microbiota diversity that was enriched for *Akkermansia muciniphila* and *Enterococcus hirae*, among other species within the Firmicutes phylum (Figure 2(B)). Upon FMT of responder and non-responder stool samples into mice, they recapitulated clinical outcomes following tumor inoculation and anti-PD-1 treatment. To illustrate the role of the microbiota, mice that received a non-responder microbiota were supplemented with *A. muciniphila* and *E. hirae*, resulting in a favorable anticancer immune phenotype characterized by an increased CD4⁺ T cell: Treg ratio (Figure 2(B)) [88]. This study illustrated the correlation among microbiota, cancer immune therapy efficacy, and local immune response, and demonstrated the possibility of microbial supplementation to augment ICI activity. Gopalakrishnan et al. conducted a similar study in a cohort of 43 patients being treated with PD-1 blockade for metastatic melanoma. They too found that responders had increased microbiota diversity compared to non-responders [89]. However, rather than identify species-specific enrichments between the strata, they characterized responders to have higher proportions of taxa belonging to the order Clostridiales (Phylum Firmicutes) compared to non-responders, which had gut microbiota dominated by Bacteroidales (phylum Bacteroides) (Figure 2(B)). Mice colonized with responder fecal microbiota had significantly lower tumor growth accompanied by higher CD8⁺ tumor infiltration, an immune phenotype consistent with responder patients (Figure 2(B)) [89]. Matson et al. identified distinct microbiota compositions driven by increased *Bifidobacterium longum* proportions in responders within their cohort of 42 patients receiving anti-PD-1 or anti-CTLA-4 treatments for metastatic melanoma (Figure 2(B)) [90]. In agreement with Routy et al., *A. muciniphila* was detected in four patients who were all classified as responders, but it was undetected in the other survivors and therefore it was not considered a significant influencer of response in this cohort [88]. Matson et al. also found unique microbial signatures associated with modulating the speed of tumor growth upon introduction into a mouse model in the absence of ICI treatment but did not describe which key taxa were associated with fast or slow tumor growth. Mice

Table 1. Clinical trials currently recruiting to study microbiota with cancer immune therapy.

National clinical trial	Number enrolled	Malignancy	Intervention	Objective	Outcomes	Analysis
03196609	55 (single group)	NSCLC, hepatocellular, colorectal, breast, prostate, glioblastoma	ICI	Catalog of microbial genes in patient treated with immune checkpoint inhibitors	Microbial changes after one month of ICI	Stool sample prior to therapy; NSCLC subset will have 1 month post-treatment sample
03819296	800	Cutaneous melanoma, genitourinary cancer	ICI, infliximab, vedolizumab	Role of gut microbiota on immune checkpoint toxicity; effectiveness of FMT to treat colitis; Incidence of adverse events and toxicity	Adverse events and toxicities	Microbiome and immune comparison between before and after colitis and between those with toxicity versus those without
03445858	15	Pediatric/adult solid tumor, lymphoma	Pembrolizumab, decitabine, and radiotherapy	Feasibility and response of combination therapy	Toxicity, tolerability, and anti-tumor activity	Immune response kinetics and stool microbiota diversity
03331562	24	Pancreas	PD1 inhibitor pembrolizumab with or without vitamin D	Microbiota differences between groups	Disease progression	16S rRNA sequencing with alpha and beta diversity metrics
03341143	20	PD-1 resistant melanoma	Responder- FMT prior to start of pembrolizumab	Response rates to therapy	Compare gut microbiota between responders and non-responders and before and after treatment	OTU and bacterial diversity metrics
03353402	40	PD-1 resistant melanoma	Responder-FMT after PD-1 blockade failure	Response rate to therapy	Adverse events and FMT engraftment; immune cell composition	Stool OTU abundance; immune cell proportions and cytokines
03358511	20	Breast adenocarcinoma	Probiotics	Increase CD8+ T cell abundance	Number of cytotoxic CD8+ T cells	Flow cytometry for CD8+ T cells
03290651	40	Breast cancer predisposition	Lactobacillus probiotics	Change local breast microbiota	Breast microbiota diversity	Inflammatory cytokine analysis

NSCLC: non-small cell lung cancer; FMT: fecal microbiota transplant; OTU: operational taxonomic unit.

transplanted with responder microbiota had significantly smaller tumors than mice transplanted with non-responder microbiota even in the absence of PD-1 blockade. This finding implies an anti-cancer effect of the specific bacteria-immune interaction. Mice colonized with responder microbiota had increased relative abundance of *Collinsella aerofaciens*, *B. longum*, *Lactobacillus* spp., *Klebsiella pneumoniae*, and *Parabacterioides merdae*, and their tumors were characterized by increased CD8⁺ T cells and IFN γ production (Figure 2(B)) [90].

Each of these studies identified different microbial signatures, with some overlap between them, that were correlated with ICI efficacy (Figure 2(B)). This may be explained by differences in cohorts (e.g. cancer diagnosis, co-morbidities, prior or concurrent exposure to antibiotics) or experimental design (e.g. microbiota analysis pipeline, production of humanized mice, *in vivo* cancer model). Alternatively, these findings could imply that different bacterial species or genera are accomplishing the same metabolic role or that some unifying feature (e.g. membrane composition, lipopolysaccharide, or other molecular signal) is being recognized by the gut mucosal immune system. These data encourage the use of deeper sequencing methods, such as shotgun metagenomic sequencing, as was done by some of the studies described above, instead of 16S rRNA sequencing, and they highlight the utility of performing metatranscriptomics, metaproteomics, and metabolic pathway analysis (Figure 1) to identify unifying signatures indicative of protective and detrimental microbiota.

Microbiome and immune checkpoint inhibitor toxicity

By removing inhibitory checkpoints on immune cell activity, ICIs activate effector T cells to target tumors but have adverse effects against numerous other tissue types [93]. The most common sites of adverse effects (gut and skin) are characterized by rapid cellular turnover and have close association with bacteria, suggesting a role of the microbiota in toxicity. The relationship between the microbiota and host biology is particularly complex in the context of inflammation. Microbes can modulate inflammation, which can in turn shape the microbiota, leading to a positive feedback loop characterized by dysbiosis and disease. Although there is strong evidence that both the microbiota and the immune system play critical roles, it is unclear which is the driving force. It is likely a combination of both, with dynamics that vary with host background, microbial

composition and function, and cancer diagnosis and treatments.

Up to 25% of patients treated with ipilimumab (CTLA-4 inhibitor) experience colitis, so clinicians and researchers have undertaken studies to identify associations between the microbiota and toxicity [94]. In their 2015 mouse experiments, Vétizou et al. showed that toll-like receptor (TLR) agonists (i.e. the microbiota), intraepithelial lymphocytes, and ICI treatment were all required to induce apoptosis and ICI-associated colitis [20]. Further, they demonstrated that a prophylactic gavage of *Burkholderia cepacia* and *Bacteroides fragilis* provided protection against colitis during anti-CTLA-4 treatment (Figure 2(C)). To the best of our knowledge, this was the first demonstration, not only that ICI-associated colitis was microbiota-dependent, but also that prophylactic probiotic treatment could protect against colitis in mice [20].

Human cohorts have also been studied to determine whether gut microbiota composition could predict or modulate colitis in addition to ICI efficacy. In a prospective study, Dubin et al. obtained fecal samples at the start of ipilimumab therapy for metastatic melanoma and stratified patients who did or did not develop colitis thereafter [94]. The authors used both 16S rRNA and shotgun sequencing to identify an increased relative abundance of Bacteroidetes in patients who did not develop colitis (Figure 2(C)). Similarly, they identified functional pathways using HUMAnN to show that the polyamine transport and riboflavin, pantothenate, thiamin, and biotin biosynthetic pathways were enriched in those protected from colitis [39,94]. It is unclear whether these pathways are simply *Bacteroides*-associated functional pathways, directly inhibit the cytotoxic activity of T cells, or otherwise provide local immunomodulatory effects, but it is intriguing to be able to utilize patient stool at therapy onset to predict toxicity.

In a similar cohort, increased abundance of Bacteroidetes relative to Firmicutes correlated with a longer colitis-free therapy period [85] (Figure 2(C)). However, the observed decreased incidence of colitis was offset by decreased survival. The authors initially show that ipilimumab, a CTLA-4 antibody, did not affect microbiota composition at the phylum level during treatment in the absence of colitis. They also conclude that ipilimumab did not decrease Shannon or Simpson diversity throughout treatment, but because of the small numbers of patients (only four patients sampled at the end of therapy), the null hypothesis of no difference between timepoints could be secondary to high variance in microbial composition. During colitis,

however, they found decreased Shannon diversity as well as specific reductions in members of the Firmicutes. These authors proceeded to show that in addition to better clinical response, increased relative abundance of Firmicutes at baseline correlated with colitis. In a linear discriminant analysis to identify taxa associated with colitis, two OTUs designated as *Faecalibacterium prausnitzii* were strongly correlated with differential response (Figure 2(C)). This discrepancy, which may be artifactual based on OTU picking or may represent different metabolic contributions, highlights the importance of identifying strain level differences using ASV-based methods for 16S rRNA sequencing [31,34] or with shotgun metagenomic sequencing [41,95]. To explain the balance between ICI response and toxicity, they performed flow cytometry on whole blood at baseline and correlated immune populations with microbiota composition. They found that patients who ultimately developed colitis had decreased baseline levels of blood IL-6, IL-8, and sCD-25 relative to those who did not develop colitis [85] (Figure 2(C)). IL-6 and IL-8 are pro-inflammatory cytokines and sCD-25 is important for IL-2 function in promoting Treg development [96]. Paradoxically, patients with a pro-inflammatory state at baseline had a lower incidence of developing colitis during treatment. Although preliminary and requiring replication, it is interesting that potentially microbial and immune biomarkers exist that could stratify patients at the start of treatment such that probiotics or fecal microbiota transplant could alleviate or lessen toxicity while maintaining appropriate therapeutic efficacy.

Modulation of the microbiota to improve therapeutic outcomes

Antibiotics

Antibiotic treatment of patients before and during ICI therapy is associated with worse outcomes in the majority of studies [88,97–99]. Antibiotic prophylaxis is common with many chemotherapeutic regimens, and antibiotics are given during periods of febrile neutropenia, which is accompanied by bacteremia in up to 25% of episodes [100,101]. An important consideration in interpreting the findings discussed in the section above and incorporating changes into clinical practice is the spectrum of antibiotic activity. Gram-positive anaerobes are responsible for many effects discussed previously, but not all antibiotics have anti-anaerobic activity. Effect depends not only on antibiotic activity and duration, but also on whether they are delivered during a critical developmental period [102,103]. Most

literature on antibiotic administration in the context of cancer immunotherapy is binary, that is, it indicates only treatment or lack thereof. When the antibiotic spectrum was accounted for, only broad-spectrum antibiotics, defined as active against gram-negative, gram-positive, and anaerobic bacteria, were associated with the greatest relative risk of ICI non-response in a multivariate analysis of a small cohort [97]. Oral vancomycin, which has minimal systemic absorption and targets only gram-positive bacteria, enhanced anti-CTLA-4 anti-tumor activity potentially by increasing Bacteroidales and local gut inflammation in mice [20]. The observed ~25% decrease in tumor size was offset by increased colitis, which implied a delicate balance between pro-inflammatory and anti-cancer effects. The increase in colitis observed with anti-CTLA-4 antibodies and vancomycin has since been replicated in a dextran sulfate sodium mouse model with added rescue by a *Bifidobacterium* consortium administration [104]. Antibiotic prescribing practices are at the discretion of the clinician; antibiotics with differential spectrum of activity and bioavailability could be theoretically administered for the same disease [98,105,106]. Antibiotic resistant infections are common in patients with cancer, with both antibiotic administration and chemotherapeutics being likely contributing factors [107]. It is therefore crucial to consider antimicrobial stewardship to obtain the best therapeutic response from ICIs while maintaining a low incidence of antibiotic-resistant infections that complicate cancer therapy [108].

Prebiotics

Prebiotics are various fibers that are digestible by individual commensal species, promoting expansion of bacteria that can metabolize them and produce secondary metabolites such as SCFAs. Preclinical studies employing prebiotics to alter the microbiota to inhibit oncogenesis have shown promising results. In 2015, Hu et al. tested the effects of a diet enriched with breakdown-resistant starch in a colitis-associated colorectal cancer model in rats. They found that rats fed the prebiotic-supplemented food had increased relative abundances of *Ruminococcus spp.* and *Bifidobacterium spp.*, which correlated with significantly decreased tumorigenesis, increased cecal and fecal SCFA concentrations, and decreased expression of the inflammatory markers, NF- κ B and TNF- α [109].

While numerous studies have linked high fiber diets to decreased cancer incidence [110–112], most failed to characterize the corresponding change in microbiota or posit an underlying mechanism. A high-fiber diet in

xenograft mice led to an increase in serum butyrate, a microbially-derived SCFA, and a decrease in tumor volume, an effect that was lost with antibiotic treatment [112]. They also observed increased expression of Fas, a host-derived apoptosis-inducing receptor, as well as increased mRNA expression of tumor suppressor genes *CDKN1A* (p21) and *CDKN1B* (p27), but did not propose any bacterial taxa that could be regulating these changes [112]. A retrospective study of healthcare professionals discovered that a prudent (whole grain and dietary fiber rich) diet was associated with a lower risk of *F. nucleatum*-positive cancers compared to a Western (red and processed meat, refined grains, and sugar-rich) diet [111]. Further mechanistic studies need to be performed to fully elucidate these relationships and the dynamics among diet, microbiota, and cancer. If certain taxa are identified and validated as biomarkers predictive of patient response to immunotherapy, patient diet could be adjusted to encourage the flourishing of responder-associated taxa and the reduction of non-responder taxa. Prebiotics are generally accepted as a safe, noninvasive method to modulate the microbiota.

Probiotics

Probiotics are an increasingly promising tool to manipulate the gut microbiota to fight disease. The ingestion of live bacterial strains can help correct microbial dysbiosis, exclude pathogenic bacteria, and modulate the host immune status by directly interacting with immune cells or through the production of secondary metabolites [113]. Unlike prebiotics, which require bacteria of interest to exist already in the microbiota, probiotics can introduce exogenous bacteria and establish either transient or stable colonization. In addition to the aforementioned experiments demonstrating the potential for administration of probiotic bacteria to differentially affect ICI efficacy and toxicity, research is ongoing to find specific taxa that predictably modulate the immune system and shape the microbiota so as to preclude cancer development.

Dissecting complex interactions from prospective studies in which patients have distinct microbiome compositions (during a disease state and at baseline), co-morbidities, and treatments is a challenging endeavor. In 2017, Geva-Zatorsky et al. performed an ambitious screen of the human microbiota for immunomodulatory bacteria. They monocolonized germfree mice with 53 different bacterial species that broadly represented the genetic diversity of the human gut microbiota and quantified their ability to influence immune cell populations in the colon [114]. In

concordance with other literature, segmented filamentous bacteria induced an increase in Th17 cells [115]. Interestingly, they also demonstrated that *Bifidobacterium longum* increased Th1 cells, further contributing to an already incongruous body of evidence describing the immunomodulatory potential of *B. longum*, which has previously been described as an inducer of Th2 response [116]. This further emphasizes the importance of considering strain level differences, which could be an underlying factor in these discrepant findings [117].

The use of probiotics to improve cancer outcomes and ICI efficacy and toxicity is still in experimental stages and is being evaluated in ongoing clinical trials (Table 1). One recent study characterized the effects of probiotic supplementation of *Bifidobacterium lactis* Bl-04 and *Lactobacillus acidophilus* NCFM on the gut microbiota in a small cohort of patients with colon cancer [118]. They began by comparing the microbiota of mucosal biopsies from healthy controls and mucosal and tumor biopsies from patients with colon cancer. They observed several taxa, including *Fusobacterium spp.*, with significantly different relative abundances in the mucosa and tumor biopsies of cancer patients compared to healthy controls, demonstrating that the microbiota within the tumor microenvironment could be contributing to oncogenesis. Intriguingly, they showed that the relative abundances of several taxa were substantially affected by the probiotic intervention. Bacteria belonging to the genus *Faecalibacterium* expanded, whereas bacteria belonging to the Clostridiales order and *Fusobacterium* genus decreased in relative abundance [118]. Although these results did not reach statistical significance following false discovery rate correction, the small sample size ($N=15$ colon cancer patients) and range of probiotic treatment length (8–78 days) could be contributing to excessive variation. These results demonstrate an important proof of concept for the ability of orally administered probiotics to alter the tumor microenvironment. It remains to be seen whether this change in the microbiota contributes to an altered immune phenotype or improved patient outcomes.

In addition to modulating the gut microbiota to increase therapy responsiveness, researchers have trialed direct instillation of bacteria to promote tumor killing. Uropathogenic *E. coli* strain CP1, a chronic prostatitis isolate, was instilled into the urethra of mice with prostate cancer [119]. Mice treated with anti-PD-1 inhibitor and CP1 had increased survival over either treatment alone. The immunomodulatory effect of CP1 related to its local increase in cytotoxic T cells and

decrease of Tregs. Given the interconnectivity of the gut microbiota and urogenital tract and the systemic immune system in the context of Uropathogenic *E. coli* infection, future experiments should consider the potential change in the gut microbiota and immune response resultant from bacteria instillation into the urinary tract [120–122]. Instillation of microbes to elicit anti-cancer activity has been demonstrated by Bacillus Calmette-Guerin treatment for bladder cancer, but engineering tumor-homing microbes that persist therein has the potential added benefit of preventing recurrence by maintaining an anti-cancer environment [119]. Long-term effects of this increased inflammatory state and the safety of these treatments needs to be evaluated before widespread use given possible side effects of local or systemic infection or secondary cancer development [123].

Postbiotics

In addition to prebiotics and probiotics, postbiotics are yet another approach to modulating the microbiome and immune milieu using microbial metabolites. Rather than relying on bacteria supported by prebiotics or introduced through probiotics, postbiotics surpasses the bacteria entirely by providing the microbial product directly [124]. Although they have distinct advantages over prebiotics and probiotics by not being dependent on the cultivation of specific microbiota compositions with complex community dynamics, they require elucidation of a clear mechanism of action, which can be challenging. Once effector metabolites are identified, their increased shelf life, dosing reliability, safety profile, and decreased biological regulations compared to probiotics make postbiotics an appealing avenue for developing microbiota-based therapies [124].

One example of an effector molecule that can induce an immune phenotype in the absence of the viable microorganism is polysaccharide A (PSA) derived from *B. fragilis*. Round & Mazmanian showed that PSA administration alone was sufficient to induce expansion of Tregs and increase production of anti-inflammatory IL-10 in mice via TLR2 activation. Furthermore, PSA treatment in a 2,4,6-trinitrobenzene sulfonic acid model of colitis in mice was both protective and curative. PSA treatment one day post-induction of colitis resulted in decreased weight loss and improved colitis scores comparable to prophylactic treatment [125]. Although microbial antigens are often considered to act as adjuvants to stimulate an immune response, this study provides evidence that the immune cells affected by these antigens can promote immune suppression as well.

Propionate, acetate, butyrate, and succinate, collectively known as SCFAs, represent a class of metabolites produced by several bacteria in the gut [124,126]. Because of their ubiquitous presence despite vast differences in gut microbiota composition from person to person, SCFAs are being investigated for their potential as universal metabolic regulators of the immune system. Smith et al. demonstrated their ability to induce colonic Treg proliferation in germfree mice in a manner dependent on free fatty acid receptor 2 [81]. Another study by Arpaia et al. showed that butyrate similarly induced peripheral Treg proliferation [127]. These experiments are critical demonstrations that even in the absence of bacteria, exogenous bacterial metabolites can be utilized to influence immune activity.

Fecal microbiota transplantation

FMT is a process whereby healthy donor stools from another individual (allo-) or the same individual (auto-) are delivered via oral gavage or direct delivery into the gastrointestinal tract to balance or restore gut microbial composition. The largest use of FMT has been in the treatment of *Clostridioides difficile* colitis, and it has recently been employed for immunocompromised individuals, including in patients after hematopoietic stem cell transplantation (HSCT) [128–130]. Induction prior to HSCT is accompanied by broad-spectrum antibiotic treatment for prophylaxis and to treat infections. Mortality is increased in patients with decreased gut microbial diversity after HSCT relative to those with more diverse microbiota [130]. 16S rRNA sequencing of pre-allo-HSCT fecal samples and division of an 80-patient cohort into 3 groups based on inverse Simpson index showed that patients in the lowest diversity group had 36% survival at 3 years whereas the highest diversity group had 67% survival [130]. The lower diversity group had received significantly more courses of vancomycin, metronidazole, and β -lactam antibiotics and had 10-fold higher rate of *C. difficile* infection. Therefore, the negative correlation between gut microbial diversity at the time of allo-HSCT and 3-year mortality may result from a loss of colonization resistance to pathobionts. Patients who died during the observation period were more likely to die from graft versus host disease or infection, both linked to decreased microbial diversity [130,131]. Taur et al. additionally identified *Enterobacteriaceae* and *Actinomycetaceae* as biomarkers for death and survival, respectively [130]. Members of the *Enterobacteriaceae* such as *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii*, and *Enterobacter cloacae* are common causes of bacteremia

in this population, and gram-negative bacteremia was associated with 45% mortality in a multi-center prospective cohort of patients after HSCT [132]. Thus, low diversity gut microbiota enriched with *Enterobacteriaceae*, which are often antibiotic resistant, may predispose patients to subsequent infection. It is therefore reasonable to hypothesize that increasing microbial diversity after HSCT or immunotherapy-induced dysbiosis via FMT could protect recipients from microbiota- and immune-related complications.

FMT was recently employed for the first time to treat two patients with steroid- and immune-suppressive refractory ICI-induced colitis [133]. After FMT, both patients had decreased systemic inflammatory cytotoxic T cells. Using 16S rRNA sequencing, the authors demonstrated differential microbial changes between the patients despite both becoming colitis-free. One patient had an expansion of *Bifidobacterium* in her gut after FMT, which is intriguing given a recent demonstration of its effect in abrogating colitis in mice [104,133]. Although preliminary, these successes are promising and need to be confirmed with larger cohorts replete with frequent microbiome sampling and analysis to determine the protective species and functional pathways indicative of a therapeutic response.

Future directions

Baseline immunologic, gut microbial taxonomic, and functional pathway abundance could be used to develop personalized cancer immunotherapy treatments to maximize progression-free survival while minimizing toxicity. In the near future, fecal samples could be sequenced by clinical and molecular microbiology labs and directed microbiome adjustments could proceed simultaneously with or prior to initiating ICI treatment to augment response and limit toxicity. It is therefore vital to maximally replicate the findings described above and elsewhere in the literature in different cohorts with numerous analytical techniques (Figure 1). Studies are currently ongoing to identify microbial and immune differences correlating with toxicity and response to ICI (Table 1). Research studies with apparently conflicting taxonomic correlations discussed above could be related to insufficient resolution or different metabolic contributions of strain-level differences among the same species [134,135]. We encourage researchers to incorporate functional metabolic profiling and strain tracking into longitudinal analysis to more precisely define the changes accompanying ICI therapy, toxicity, and response to microbial intervention.

In addition to potential microbiome adjustments with prebiotics, probiotics, postbiotics, antibiotics, and FMT, ample novel therapeutic strategies are emerging. An exciting technique would be to selectively kill pro-oncogenic or pathogenic bacteria with specific bacteriophages as has been recently achieved in other immunocompromised populations [136]. Genetically engineered microbes can also be used *in vivo* to diagnose and treat cancers [137]. It is compelling to consider other non-bacterial members of the microbiota including viruses and fungi and their role in modulating microbial composition and immune function as well as producing anti-cancer metabolites [138]. The era of personalized medicine is fast approaching due to the decreased cost of next-generation sequencing and the increased understanding researchers and clinicians share regarding the importance of the gut microbiome in regulating the immune system and disease.

Conclusions

Understanding microbiome compositions, manipulations, and interventions is now vital to understanding cancer progression and immunotherapy-based treatments. The details underlying the intricate relationship among the host immune system, cancer progression, the commensal and tumor microbiota, and therapeutic interventions continue to emerge. In addition, the tools and techniques used to characterize the microbiome are continually evolving to provide more data at lower costs. Although we have collectively learned much about commensal microorganisms and the modulatory role in local and systemic homeostasis, novel cancer immunotherapies have brought microbiome correlations and manipulations to the forefront. To progress beyond mere association in order to elucidate causation, we need to characterize precise molecular mechanisms by which the microbiota mediates cancer immunotherapy response and toxicity. Only after these molecular mechanisms are uncovered can interventions to modulate the microbiome to affect response and toxicity be widely employed. Continued partnerships among clinical scientists, laboratory physicians, and other translational researchers will help to usher in the era of personalized medicine with improved outcomes of many diseases while limiting often intolerable side effects.

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