T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition

Enfu Hui,1* Jeanne Cheung,2 Jing Zhu,2 Xiaolei Su,1 Marcus J. Taylor,1 Heidi A. Wallweber,2 Dibyendu K. Sasmal,3 Jun Huang,3 Jeong M. Kim,2 Ira Mellman,2† Ronald D. Vale†

1Department of Cellular and Molecular Pharmacology and the Howard Hughes Medical Institute, University of California, San Francisco, CA 94158, USA. 2Department of Cancer Immunology, Genentech, South San Francisco, CA 94080, USA. 3Institute for Molecular Engineering, University of Chicago, IL 60637, USA.

*Present address: Section of Cell and Developmental Biology, University of California, San Diego, CA 92093, USA.
†Corresponding author. Email: ron.vale@ucsf.edu (R.D.V.); mellman.ira@gene.com (I.M.)

Programmed death-1 (PD-1) is a co-inhibitory receptor that suppresses T cell activation and is an important cancer immunotherapy target. Upon activation by its ligand PD-L1, PD-1 is thought to suppress signaling through the T cell receptor (TCR). Here, by titrating PD-1 signaling in a biochemical reconstitution system, we demonstrate that the co-receptor CD28 is strongly preferred over the TCR as a target for dephosphorylation by PD-1-recruited Shp2 phosphatase. We also show that CD28, but not the TCR, is preferentially dephosphorylated in response to PD-1 activation by PD-L1 in an intact cell system. These results reveal that PD-1 suppresses T cell function primarily by inactivating CD28 signaling, suggesting that co-stimulatory pathways play key roles in regulating effector T cell function and therapeutic responses to anti-PD-L1/PD-1.

T cells become activated through a combination of antigen-specific signals from the T cell receptor (TCR) along with antigen-independent signals from co-signaling receptors. Two sets of co-signaling receptors are expressed on the T cell surface: costimulatory receptors, which deliver positive signals that are essential for full activation of naive T cells, and co-inhibitory receptors, which decrease the strength of T cell signaling (1). The co-inhibitory receptors serve as “checkpoints” against unrestrained T cell activation and play an important role in maintaining peripheral tolerance as well as immune homeostasis during infection (2). One such receptor is PD-1, which binds to two ligands, PD-L1 and PD-L2, expressed by a variety of immune and non-immune cells (3–5). The expression of PD-L1 is often induced by interferon-γ, and thus is indirectly controlled by T cells that secrete this cytokine upon activation (4, 6). In addition, T cell activation increases the expression of PD-1 on the T cell itself (3). Thus, during chronic viral infection, T cells become progressively “exhausted,” in part reflecting a homeostatic negative feedback loop due to increased expression of PD-1 and PD-L1 (7–9). The interaction between PD-1 and its ligands also has been shown to restrain effector T cell activity against human cancers (10–14). Antibodies that block the PD-L1/PD-1 axis have exhibited durable clinical benefit in a variety of cancer indications, especially in patients exhibiting evidence of pre-existing anti-cancer immunity by expression of PD-L1 (15–19). Interestingly, benefit often correlates with PD-L1 expression by tumor infiltrating immune cells rather than by the tumor cells themselves.

Despite its demonstrated importance in the treatment of human cancer, the mechanism of PD-1-mediated inhibition of T cell function remains poorly understood. Early work demonstrated that binding of PD-1 to PD-L1 causes the phosphorylation of two tyrosines in the PD-1 cytoplasmic domain. Co-immunoprecipitation (co-IP) and co-localization studies in transfected cells suggested that phosphorylated PD-1 then recruits, directly or indirectly, the cytosolic tyrosine phosphatases Shp2, Shp1, the TCR phosphorylating kinase Lck, and the inhibitory tyrosine kinase Csk (20, 27). Defining the direct targets of inhibitory effectors will be critical for understanding the mechanism of anti-PD-L1/PD-1 immunotherapy. However, the downstream targets of PD-1-bound effectors remain poorly understood. Current studies have suggested that PD-1 activation suppresses T cell receptor (TCR) signaling (21–23), CD28 co-stimulatory signaling (24), ICOS co-stimulatory signaling (25), or a combination of pathways. Decreased phosphorylation of various signaling molecules, such as Erk, Vav, PLCγ and PI3 kinase (PI3K), has been reported (21, 24), but these molecules are common effectors shared by both the TCR and co-stimulatory pathways and also may not be direct targets of PD-1. Here, we have sought to identify the immediate targets of PD-1 bound phosphatase(s) through a combination of in vitro biochemical reconstitution and cell-based experiments.

To gain insight into potential signaling pathways affected by activation of PD-1, we turned to a cell-free reconstitution system in which the cytoplasmic domain of PD-1 was bound to the surface of large unilamellar vesicles (LUVs)
that mimic the plasma membrane of T cells (Fig. 1A). We first determined which kinase(s) phosphorylate PD-1 by comparing the catalytic activities of Lck and Csk, the two kinases that were found to co-IP with PD-1 in cell lysates (20). Using a Fluorescence Resonance Energy Transfer (FRET)-based assay (Fig. 1A), we found that Lck, but not Csk, efficiently phosphorylates PD-1 in vitro. Although Csk can weakly phosphorylate PD-1 on its own, it slows down PD-1 phosphorylation in the presence of Lck (Fig. S1), likely due to its ability to inhibit Lck. This finding, together with previous co-IP results (20), suggests that Lck is the major PD-1 kinase. We then asked which SH2 domain-containing proteins bind directly to phosphorylated PD-1. In addition to Lck and Csk, PD-1 also was shown to co-IP with tyrosine phosphatases Shp2 and Shp1 (20) and contains a structural motif that might recruit the lipid phosphatase SHIP-1 (26). The biochemical FRET-based assay (Fig. 1A) demonstrated that phosphorylated PD-1 directly binds Shp2 but not Shp1, Csk, SHIP-1, or other SH2 proteins tested (Fig. 1B). A full titration experiment revealed a 29-fold selectivity of PD-1 toward full length Shp2 over Shp1 (fig. S2A), in agreement with qualitative cellular studies (21). Surprisingly, however, the tandem SH2 domains (tSH2) of Shp1 and Shp2 bind phosphorylated PD-1 with indistinguishable affinities (fig. S2B). Taken together, these data are consistent with a tighter auto-inhibited conformation for Shp1 than Shp2 (27), which may decrease Shp1’s affinity for PD-1. Mutation of either tyrosine (Y224 and Y248) in the cytosolic tail of PD-1 led to a partial defect in Shp2 binding and mutation of both tyrosines eliminated binding (Fig. 1C and fig. S3). Although Y224 was reported to be dispensable for the ability of PD-1 to co-immunoprecipitate with Shp2 (28, 29), our quantitative, direct binding assay shows that both tyrosines in the PD-1 cytosolic domain contribute to Shp2 binding. Collectively, these data suggest that Shp2 is the major effector of PD-1 and that Lck-mediated dual phosphorylation of PD-1 is needed for optimal Shp2 recruitment.

Using this reconstituted system, we next asked if other signaling receptors besides PD-1 (CD3ζ, CD3ε, CD28, ICOS, DAP10, CD225, CD96, TIGIT, and CTLA4) could recruit Shp2 (Fig. 1D). Remarkably, recruitment of Shp2 was not observed for any of these receptors, including two other co-inhibitory molecules TIGIT and CTLA4 (Fig. 1E). CTLA4 was reported to co-immunoprecipitate with Shp2 (30) and is widely believed to suppress T cell signaling at least partly through Shp2 (37). Our data suggest that Shp2 does not directly bind CTLA4, and that other proteins are likely required to bridge these two proteins. Overall, our results reveal an unexpected binding specificity of Shp2 for phosphorylated PD-1.

Recruitment of Shp2 to PD-1 raises the question of whether Shp2 might directly dephosphorylate PD-1 itself and cause the disassembly of the PD-1–Shp2 complex. To test this idea, we determined the stability of the PD-1–Shp2 complex using a full length Shp2 in the FRET assay (Fig. 1F). ATP-triggered phosphorylation of PD-1 caused the rapid recruitment of Shp2 (Fig. 1G) and activation of its phosphatase activity (fig. S4). Termination of the Lck activity by rapid ATP depletion caused a complete dissociation of Shp2 (Fig. 1G). This result indicates that the Shp2 dephosphorylates PD-1 to destabilize the PD-1–Shp2 complex and that continuous Lck kinase activity is required to activate and sustain PD-1–Shp2 mediated inhibitory signaling. Interestingly, a slow spontaneous disassembly of PD-1–Shp2 complex was observed even before the termination of Lck activity (Fig. 1G), and was not due to depletion of ATP as the dissociation continued even after further ATP addition (Fig. 1H). This result suggests that the activation of Shp2 upon binding to PD-1 allows Shp2 to override Lck, causing a gradual net dephosphorylation of PD-1. This positive-negative feedback loop of the Lck/PD-1/Shp2 network would allow the system to quickly reset in the absence of PD-1 ligation or Lck activation.

Having established a highly specific recruitment of Shp2 by PD-1, we turned to identify substrates for dephosphorylation by the PD-1–Shp2 complex. We utilized a titration system that can provide insight into how the T cell network responds to gradual upregulation of PD-1 during T cell development (32), activation (33), and exhaustion (e.g., in tumors or chronic viral infection) (7). To this end, we reconstituted a diverse set of components involved in the T cell signaling network including: (i) the cytosolic domains of various receptors (PD-1, TCR, CD28, and ICOS (another co-stimulatory receptor (34)) on the liposomes; (ii) the tyrosine kinases Lck, ZAP70 (a key cytosolic tyrosine kinase that binds to phosphorylated CD3 subunits to propagate the TCR signal (35)), and in some experiments the inhibitory kinase Csk (36); and (iii) the downstream adapter proteins LAT, Gads, and SLP76 (37) as well as the regulatory subunit of Type 1 PI3K (p85α), which is known to be recruited by phosphorylated co-stimulatory receptors (fig. S5) (38, 39). All protein components were reconstituted onto LUVs or added in solution at close to their physiological levels (fig. S6 and table S1). A reaction cascade consisting of phosphorylation, dephosphorylation, and protein–protein interactions at the membrane surface was triggered by ATP addition. To test the sensitivity of components in this biochemical network to PD-1, we systematically titrated the levels of PD-1 on the LUVs and measured the susceptibility to dephosphorylation of each component by phosphotyrosine (PY) Western blots (Fig. 2A).

Strikingly, CD28 but not the TCR or its associated components was found to be the most sensitive target of PD-1–Shp2. As shown in Fig. 2, B and C (Left), CD28 was very effi-
ciently dephosphorylated, with a 50% inhibitory concentration (IC$_{50}$) of ~96 PD-1 molecules per $\mu$m$^2$ (table S2). In contrast, PD-1–Shp2 dephosphorylated the TCR signaling components only to a minor extent, including the TCR intrinsic signaling subunit CD3$\zeta$, the associated kinase ZAP70, as well as its downstream adaptors LAT and SLP76, whose 50% dephosphorylation occurred at substantially higher PD-1 concentrations (>1000 molecules per $\mu$m$^2$, table S2). Lck, the kinase that phosphorylates TCR, CD28, and PD-1, was the second best target for PD-1–bound Shp2 in the reconstitution system. Both the activating (Y394) and inhibitory (Y505) tyrosines were ~50% dephosphorylated at similar levels of PD-1 (400 - 600 molecules per $\mu$m$^2$). This result, however, suggests a net positive effect of PD-1 on Lck activity, owing to the stronger regulatory effect of the inhibitory tyrosine (40). The addition of the Lck inhibiting kinase Skn rendered CD28 as well as TCR signaling components more sensitive to PD-1–Shp2, although CD28 clearly remained the most sensitive PD-1 target (fig. S7 and table S2). The strong preferential dephosphorylation of CD28 was observed also at later time points in the in vitro reaction (fig. S8). In contrast to the strong CD28 preference of PD-1–Shp2, the transmembrane phosphatase CD45 efficiently dephosphorylated all of the signaling components tested (fig. B, B and C, Right), with only 3-4 fold selectivity on CD28 over CD3$\zeta$ and ZAP70 (table S2).

To better understand the basis of the PD-1–Shp2 sensitivity to CD28, we deconstructed the reconstitution system into its individual modules (fig. S9). These experiments revealed that Shp2 alone dephosphorylates CD3$\zeta$, and CD28 with similar activities (fig. S9C), but that Lck has a six-fold higher $k_{cat}$ for CD3$\zeta$ over CD28 for phosphorylation (fig. S9, D and E). Thus, CD28 is a weaker kinase substrate, which in effect renders it more sensitive to PD-1–Shp2 inhibition in a kinase-phosphatase network. In summary, by reconstitution of components at physiological concentrations, CD28, and to a lesser extent Lck, are the major substrates for PD-1–Shp2 mediated dephosphorylation.

Having established that CD28 is highly sensitive to dephosphorylation by PD-1–Shp2 in vitro, we next sought to examine whether these two co-receptors co-localize in living cells and whether CD28 is indeed dephosphorylated in a PD-L1-dependent manner. Using TIRF microscopy and a supported lipid bilayer functionalized with an ovalbumin peptide-MHC class I complex (pMHC; TCR ligand) and B7.1 (CD28 ligand), we found that PD-1 strongly co-localized with the co-stimulatory receptor CD28 in plasma membrane microclusters (Fig. 3 and movie S1). Previous work reported the co-localization of TCR and CD28 into submicron-size clusters after binding their ligands (44); however, we found significantly less ($p < 0.0001$) overlap between PD-1 and TCR (Pearson correlation coefficient (PCC) of 0.69 ± 0.09 (mean ± S.D.; $n = 17$ cells)) compared to PD-1 and CD28 (PCC of 0.89 ± 0.05) (Fig. 3). Interestingly, although not itself a PD-1 substrate (Fig. 2, B and C), the ICOS co-receptor also more strongly co-localized with PD-1 than the TCR (fig. S10). Strong co-localization of PD-1 and CD28 began from the time of initial cell–bilayer contact (0 s, Fig. 3B) and was sustained until the T cells fully spread (30 s, Fig. 3B). The molecules moved centripetally and eventually became segregated into a canonical bull’s eye pattern with a center island surrounded by CD28 and PD-1, with the latter partially excluded from the TCR rich zone (145 s, Fig. 3B). Because of their rapid co-localization and actin-driven flow, the clusters of PD-1 and CD28 are most likely forming on the plasma membrane and are not extracellular microvesicles secreted by T cells (42). Some degree of CD28 / PD-1 co-clustering also was detected in the absence of pMHC, though the two co-receptors remain largely diffusive without TCR activation (fig. S11). As shown previously (21), PD-1 clusters also represented sites of Shp2 recruitment to the membrane (fig. S12). In the absence of PD-L1 on the bilayer, but with pMHC and B7.1 ligands, PD-1 remained diffusely localized (fig. S13 and movie S2), indicating that PD-L1 is required to bring PD-1 and co-stimulatory receptors into close proximity. Overall, these findings indicate that CD28 and PD-1 strongly co-cluster with PD-1 in the same plasma membrane microdomains in stimulated CD8+ T cells.

We next tested whether CD28 is the preferential target of PD-1 in intact T cells. For these studies, we used Jurkat T cells together with the Raji B cell line as an antigen-presenting cell (APC), as this system has been widely used for studying TCR and CD28 signaling (43). Because these cells lack PD-1 and PD-L1, we lentivirally transduced PD-1 and PD-L1 into Jurkat and Raji respectively, obtaining PD-1+ Jurkat T cells that express ~40 PD-1 molecules per $\mu$m$^2$ (table S1) and Raji B cells that express ~86 PD-L1 molecules per $\mu$m$^2$ (designated as PD-L1$^{High}$, fig. 4A). PD-1+ Jurkat cells stimulated by antigen-loaded PD-L1$^{High}$ Raji secrete significantly less interleukin-2 (IL-2) than those stimulated with antigen-loaded PD-L1$^{Negative}$ parental Raji B (63% decrease measured at 24 hours; Fig. 4B), indicating an inhibitory activity of PD-1 signaling in this cell system. We next tested how PD-L1 binding to PD-1 affects phosphorylation at the receptor level. To titrate the strength of PD-L1–PD-1 signaling, the PD-1–expressing Jurkat T cells were incubated with different ratios of PD-L1$^{High}$/PD-L1$^{Negative}$ Raji B cells; since a T cell can interact with multiple APCs, this mixture of APCs might be expected to modulate the PD-1 response. Two min after APC and T cell contact, CD28 phosphorylation decreased as a function of the percentage of PD-L1$^{High}$ cells (Fig. 4, C and D). In contrast, either no or substantially less dephosphorylation was observed for ZAP70 and CD3$\zeta$, respectively. Interestingly, the PD-L1–PD-1 inhibitory effect
on phosphorylation was transient, with far less dephosphorylation detected at 10 min (Fig. 4, C and D, t = 10 min), perhaps reflecting the feedback loop described in vitro (Fig. 1, G and H) that enables recruited Shp2 to dephosphorylate PD-1 and thereby repressing the inhibitory signal. We next tested these results using a Raji B cell line that expresses lower levels of PD-L1 (~16 molecules per μm², designated PD-L1low, fig. S14A), a density similar to that found in tumor infiltrating macrophages and tumor cells (table S3). Using this lower-expressing APC line alone, we still detected a transient dephosphorylation of CD28 with little to no effect on TCR signaling components (fig. S14, B and C, t = 2 min).

Taken together, results obtained from both membrane reconstitution and intact cell assays demonstrate that PD1–Shp2 strongly favors dephosphorylation of the co-stimulatory receptor CD28 over TCR (fig. S15). At high PD-L1 levels, we also observed some dephosphorylation of TCR components such as SLP76 and ZAP70 in agreement with previous reports (20–22). However, by performing direct and quantitative comparison, we find that the degree of TCR dephosphorylation was consistently much weaker than for CD28. The unexpected preference for inhibition of co-stimulatory receptor signaling may have interesting implications for cancer immunology and immunotherapy. Although co-stimulation via CD28 is most often associated with the priming of naïve T cells, there is increasing evidence that it may play a role at later stages of T cell immunity in cancer and in chronic viral infection. Indeed, recent studies have demonstrated that the ability of anti-PD-L1/PD-1 to rescue anti-viral (LCMV) T cell responses, as well as anti-tumor responses, depends on CD28 expression by T cells (44). Blockade of B7.1/B7.2 binding to CD28 also completely eliminated the ability of anti-PD-L1/PD-1 to rescue or prevent, T cell exhaustion (44). These in vivo observations are consistent with expectations from our results, namely that PD-1 exerts its primary effect by regulating CD28 signaling.

Our work and that of Kamphorst et al. (44) also have important implications for cancer immunotherapy. In at least a subset of human cancer patients, inhibition of T cell immunity is associated with the upregulation of PD-L1 in the tumor bed in response to the release of IFNγ (2, 6, 15, 16). However, expression of PD-L1 by tumor infiltrating immune cells can be independently and in some types of cancer even more predictive of clinical response than PD-L1 expression by tumor cells (45). Infiltrating cells including lymphocytes, monocyteic cells, and dendritic cells, all express CD28 ligands while generally tumor cells do not. If the primary target of PD-1 signaling regulation is through CD28 or another co-stimulatory molecule, then the therapeutic effect is likely to reflect re-activation of co-stimulatory molecule signaling on T effector cells rather than (or at least in addition to) TCR signaling. Conceivably, co-stimulation is required to expand tumor antigen-specific early memory T cells, a process controlled intratumorally by B7.1+ APCs. Indeed, recent LCMV experiments have implicated an early memory population as being the targets for expansion by anti-PD-L1/PD-1 (46, 47). These findings strongly suggest the need for broadly considering the roles of co-stimulatory molecules in addition to CD28 in anti-tumor immunity.

REFERENCES AND NOTES


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SUPPLEMENTARY MATERIALS
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Fig. 1. Lck sustains the formation of a highly specific PD-1-Shp2 complex. (A) Cartoon depicting a FRET assay for measuring the interaction between a SH2-containing protein and membrane-bound PD-1. Rhod-PE (energy acceptor) bearing LUVs were reconstituted with purified Lck kinase and the cytosolic domain of PD-1, as described in Methods. The SNAP-tag fused SH2 protein of interest was labeled with SNAP-cell-505 (energy acceptor) and presented in the solution. Addition of ATP triggered Lck-catalyzed phosphorylation of PD-1 and caused the recruitment of certain SH2 proteins to the LUV surface, leading to FRET. (B) A comparison of PD-1 binding activities of a panel of SH2-domain-containing proteins, using the FRET assay as described in (A). Shown are representative time courses of SNAP-cell-505 fluorescence before and after the addition of 1 mM ATP. Concentrations of components: 300 nM PD-1, 7.2 nM Lck and 100 nM labeled SH2 protein. (C) A comparison of the relative contribution of the two tyrosines of PD-1 in recruiting Shp2. Shown is the degree of Shp2 recruitment against the concentration of LUV-bound PD-1 WT or tyrosine mutant, measured by the FRET assay described in (A). See fig. S3 for raw data. (D) Cartoon depicting a FRET assay for measuring the ability of a membrane-bound receptor to recruit Shp2. The experiment set up was the same as in (A), except that PD-1 was replaced with another receptor of interest, using the tandem SH2 domains of Shp2 (Shp2SH2) as a fixed donor bearer. (E) A comparison of the Shp2 binding activities of the designated LUV-bound receptors, using the FRET assay shown in (D). Concentrations: 300 nM receptor, 7.2 nM Lck and 100 nM labeled Shp2SH2. (F) Cartoon showing a FRET assay for measuring the localization dynamics of full length Shp2 (Shp2FL). Rhodamine-PE (energy acceptor) bearing LUVs were reconstituted with purified Lck kinase and the cytosolic domain of PD-1, as described in Methods. SNAP-tag fused Shp2FL was labeled with SNAP-cell-505 (energy acceptor) and presented in the extravesicular solution. (G) Time course of the fluorescence of Shp2FL in response to sequential addition of ATP (2 mM) and the ATP scavenger apyrase (80 µg/ml) to the reaction shown in (F). Concentrations of components: 300 nM PD-1, 10 nM Lck and 50 nM Shp2FL. (H) Time course of the Shp2FL fluorescence showing the dynamics of Shp2 at indicated Lck concentrations. Assay was set up as in (F) and 2 mM ATP was added twice at 0 min and 30 min, respectively.
Fig. 2. CD28 is uniquely sensitive to PD-1 bound Shp2. (A) Cartoon depicting a LUV reconstitution system for assaying the sensitivities of different targets to PD-1:Shp2 or CD45. Purified cytosolic domains of plasma membrane bound receptors (CD3ζ, CD28, PD-1), the adaptor LAT, and the kinase Lck were reconstituted onto LUVs at their physiological molecular densities (table S1). Cytosolic factors (ZAP70, p85α, Gads, SLP76 and Shp2) were presented in the extravesicular solution at their physiological concentrations (table S1). In a parallel experiment, PD-1 and Shp2 were replaced with liposome-attached cytoplasmic portion of CD45. Addition of ATP triggered a cascade of enzymatic reactions and protein-protein interactions. (B) Shp2-containing reactions with increasing [PD-1] or CD45-containing reaction with increasing [CD45] terminated at 30 min, and subjected to SDS-PAGE and phosphotyrosine Western blots (WB), as described in Methods. (C) The optical density of each band in (B) was quantified by ImageJ. The 50% inhibitory concentrations (IC50) of PD-1 and CD45 on different targets were determined by fitting the dose response data in (B) using Graphpad Prism 5.0. or estimated based on the dose response plots if the inhibition was incomplete even at the highest PD-1 or CD45 concentration (summarized in table S2). Error bars: S.D. from three independent experiments.
Fig. 3. PD-1 co-clusters with co-stimulatory receptor CD28 but partially segregates with TCR. (A) (Left) Representative TIRF images of PD-1, CD28 and TCR of an OT-I CD8+ T cell 10 s after landing onto a supported lipid bilayer functionalized with recombinant ligands (100 – 250 molecules per µm²), which included peptide-loaded MHC-I (H2Kb), B7.1 (CD28 ligand), and ICAM-1 (integrin LF1 ligand). Cells were retrovirally transduced with PD-1-mCherry and CD28-mGFP and the TCR was labeled with an Alexa Fluor647-conjugated anti-TCR antibody (see Methods). Scale bars: 5 µm. Experiment is representative of five independent experiments. (Middle) Intensities were calculated from the raw fluorescence intensities along the two diagonal lines in the overlaid images (see Methods). PD-1: red; CD28 or TCR: black. (Right) Column scattered plot summarizing the Pearson’s correlation coefficient (PCC) values for PD-1/CD28 overlay (0.89 ± 0.05, mean ± S.D) and for PD-1/TCR overlay (0.69 ± 0.09) of 17 fully spread cells, with each dot representing a unique cell. Statistical significance was evaluated by two-tailed Student’s t test, p < 0.0001. (B) TIRF images showing the time course of the development of a PD-1/CD28/TCR immunological synapse, starting from initial contact with the supported lipid bilayer (0 s), to full spreading (30 s), to a bull eye pattern (145 s). Scale bars: 5 µm. Experiment is representative of four independent experiments. (Right) Histograms from the respective line scan quantifications.
Fig. 4. Intact cell assays confirm CD28 as the preferential target of PD-1 mediated inhibition. (A) (Left) Cartoon illustrating an intact cell assay in which CD28+ PD-1 transduced Jurkat T cells were stimulated with B7.1+. PD-L1 transduced (PD-L1^{High}) Raji B cells preloaded with antigen. (Right) FACS histograms showing the expression of B7.1 and PD-L1 in parental or PD-L1^{High} Raji B cells, and expression of CD28 and PD-1 in parental or PD-1 transduced Jurkat T cells. (B) Bar graph summarizing IL-2 release from a 24 hours Jurkat-Raji co-culture with or without PD-L1/PD-1 signaling, or from each type of cell alone (see Methods). Data are presented as mean ± S.D. from four independent measurements, with each run in triplicates. **p < 0.0001, two-way ANOVA. (C) A representative experiment of Western blots showing the phosphorylation of CD28 and TCR signaling components in Jurkat T cells in response to PD-L1 titration on antigen-presenting Raji B cells; the time after the initial contact of the two cell populations is indicated (see Methods). Different ratios of PD-L1^{High} cells and PD-L1^{Negative} Raji B (both containing pMHC and B7.1) were used to vary the PD-L1 stimulation to the Jurkat cells. Each condition contained identical number of Raji B cells (Raji to Jurkat ratio = 0.75). The phosphorylation states of CD3ζ, ZAP70 and LAT were immunoblotted with phospho-specific antibodies. Due to the lack of CD28-specific phosphotyrosine antibodies, CD28 was co-precipitated with p85α (see Methods), which is dependent upon CD28 phosphorylation. (D) Quantification of phosphorylation data incorporating results from three independent experiments (mean and S.D.).
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