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SHP-1 and SHP-2 Associate with Immunoreceptor Tyrosine-Based Switch Motif of Programmed Death 1 upon Primary Human T Cell Stimulation, but Only Receptor Ligation Prevents T Cell Activation¹

Jens M. Chemnitz,* Richard V. Parry,* Kim E. Nichols,[†] Carl H. June,* and James L. Riley^{2*}

To study the *cis*- and *trans*-acting factors that mediate programmed death 1 (PD-1) signaling in primary human CD4 T cells, we constructed a chimeric molecule consisting of the murine CD28 extracellular domain and human PD-1 cytoplasmic tail. When introduced into CD4 T cells, this construct mimics the activity of endogenous PD-1 in terms of its ability to suppress T cell expansion and cytokine production. The cytoplasmic tail of PD-1 contains two structural motifs, an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM). Mutation of the ITIM had little effect on PD-1 signaling or functional activity. In contrast, mutation of the ITSM abrogated the ability of PD-1 to block cytokine synthesis and to limit T cell expansion. Further biochemical analyses revealed that the ability of PD-1 to block T cell activation correlated with recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHP-2, and not the adaptor Src homology 2 domain-containing molecule 1A, to the ITSM domain. In TCR-stimulated T cells, SHP-2 associated with PD-1, even in the absence of PD-1 engagement. Despite this interaction, the ability of PD-1 to block T cell activation required receptor ligation, suggesting that colocalization of PD-1 with CD3 and/or CD28 may be necessary for inhibition of T cell activation. *The Journal of Immunology*, 2004, 173: 945–954.

Activation and expansion of T cells are a necessary component of an effective acquired immune response. The efficiency with which CD4 T cells direct an immune response demands that proper regulatory measures are in place to prevent immune hyperactivation leading to autoimmune disease. The regulation of T cell expansion and activation is a complex process that involves the coordinated interaction of multiple signaling pathways. The CD28 family of cell surface receptors plays a prominent role in controlling T cell activation. Although there is structural similarity between members of the CD28 family, functional heterogeneity is observed. For example, ligation of CD28 and ICOS promotes T cell activation, whereas engagement of CTLA-4, programmed death 1 (PD-1),³ and B and T lymphocyte alternator inhibits T cell activation (1). The mechanisms by which these receptors control T cell function are not clearly defined; however, a few common themes have emerged. First, none of these

receptors has enzymatic activity, but rather their cytoplasmic tails serve as docking stations for signaling proteins that mediate the distinct effects of each of these molecules (2). Second, the phosphorylation status of the cytoplasmic tails of CD28 family members influences which proteins are recruited. For instance, protein phosphatase 2A binds to the unphosphorylated CD28 cytoplasmic tail at a Src homology 2 binding domain (3), whereas the regulatory subunit of PI3K (p85) and growth factor receptor-bound protein 2 family members bind only the phosphorylated CD28 cytoplasmic tail (4–6).

PD-1, originally described as a transcript preferentially expressed in apoptotic cells (7), is thought to play a prominent role during the maintenance of peripheral tolerance (8, 9). To date, all studies that have specifically targeted PD-1 through Ab ligation demonstrate that PD-1 engagement blocks T cell activation (10, 11). Other studies that have used the natural ligands PD ligand 1 (PD-L1) and PD-L2 to engage PD-1 observe that these ligands enhance T cell proliferation under suboptimal CD3 stimulation (12–14), suggesting that these ligands may interact with additional receptors. PD-1-deficient mice demonstrate dysregulated Ig production and develop glomerulonephritis and arthritis in a C57BL/6 background (15, 16) and autoimmune cardiomyopathy in a BALB/c background (17), further supporting the role of this molecule as a negative regulator of T cell activation.

Little is known about the mechanism(s) by which PD-1 ligation blocks primary T cell activation. PD-1 contains two tyrosine molecules within its cytoplasmic tail. The most membrane-proximal tyrosine is located in an ITIM, and the distal tyrosine is located in an immunoreceptor tyrosine-based switch motif (ITSM) (18), a stretch of amino acids that was recently identified by virtue of its ability to bind to the small adaptor Src homology 2 domain-containing molecule 1A (SH2D1A) (19). Using a Jurkat T cell model, Latchman et al. (20) showed that Src homology region 2 domain-containing phosphatase-2 (SHP-2) was recruited to the PD-1 cytoplasmic tail upon receptor engagement with PD-L1. In

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³Abbreviations used in this paper: PD-1, programmed death 1; aAPC, artificial APC; hPD, human PD; ITSM, immunoreceptor tyrosine-based switch motif; mCD28, murine CD28; PD-L1/L2, PD ligand 1/ligand 2; SH2D1A, Src homology 2 domain-containing molecule 1A; SHP, Src homology region 2 domain-containing phosphatase.

addition, Okazaki et al. (11) showed that in a murine B cell tumor line, IIA1.6, the ITSM of PD-1 was essential to block BCR-mediated Ca^{2+} flux and tyrosine phosphorylation of downstream effector molecules. Similar to the Jurkat model system, recruitment of SHP-2 to the PD-1 cytoplasmic tail was observed upon receptor engagement, and this recruitment correlated with inhibition of BCR-mediated activation events, suggesting that SHP-2 is a key downstream signaling molecule that mediates PD-1 signaling. The ITSM, originally defined in CD150, is known to recruit SHP-2 to the CD150 cytoplasmic tail. However, in the presence of the small adapter SH2D1A, the ITSM recruits SHIP to CD150, and hence, it is able to switch which molecules it recruits based on the presence or absence of SH2D1A.

Because many of the previous studies examining PD-1 signaling used transformed cell lines known to be deficient in several key signaling molecules such as SHIP (21), we hypothesized that the study of PD-1 signal transduction in primary T cells would reveal a distinct subset of signaling molecules required for PD-1 function. Our studies using primary human CD4 T cells revealed that only one tyrosine-phosphorylated complex was recruited to the PD-1 cytoplasmic tail upon receptor ligation. Recruitment of this complex was dependent upon an intact ITSM. Unlike previous studies (11, 20), we find that both SHP-1 and SHP-2 are present within this complex. Interestingly, we show that SHP-2 is recruited upon TCR-induced cell activation, even in the absence of PD-1 receptor engagement. However, despite comparable levels of phosphatase recruitment by receptor engagement and cell activation in the absence of receptor engagement, only receptor engagement is able to block T cell activation, suggesting PD-1 must be in close proximity of either CD3 or CD28 to act as a negative regulator of T cell activation. Finally, we show that the ITSM of PD-1 fails to recruit SH2D1A, suggesting that the mechanism by which PD-1 signals may differ from other ITSM-containing receptors.

Materials and Methods

Cell isolation, artificial APC (aAPC) preparation, and cell stimulation

PBLs were isolated from normal volunteer donors following apheresis and elutriation under a protocol approved by University of Pennsylvania Institutional Review Board. CD4 T cells were purified by negative selection using magnetic beads (Dyna Beads, Lake Success, NY), as previously described (22), and were routinely >95% CD3⁺, >95% CD28⁺, and <5% CD8⁺, as determined by flow cytometry. Primary human CD4 T cells were cultured in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics (Invitrogen Life Technologies, Carlsbad, CA). CD4 T cells were stimulated with aAPC at a ratio of 1:3 (cells to beads) comprised of magnetic beads (Dyna Beads) coated with the following Abs: anti-CD3 (OKT3; Orthoclone, Raritan, NJ), anti-human CD28 (9.3) (23), anti-murine CD28 (mCD28) (37.51; BD Biosciences, San Diego, CA), anti-human PD-1-17 (10) (generously provided by B. Carreno, Wyeth Research, Cambridge, MA), and anti-MHC class I (MHC I) (W6/32; American Type Culture Collection, Manassas, VA). For most experiments, these aAPCs were coated with suboptimal anti-CD3 Ab (5%), suboptimal levels of anti-CD28 Ab (10%), and either anti-MHC class I Ab (CD3/28/MHC I), anti-PD-1 Ab (CD3/28/PD-1), or mCD28 CD3/28/mCD28, constituting the remaining 85% of protein added to the bead, as previously described (24). In Fig. 1, CD3/MHC I, CD3/PD-1, and CD3/28 aAPCs were loaded with equal amount of anti-CD3 Ab and either anti-MHC class I, anti-PD-1, or anti-CD28. CFSE staining and FACS analysis were performed, as previously described (25).

Generation of mCD28/human PD-1 (hPD-1) wild-type and mutant cytoplasmic tails

The mCD28 extracellular and transmembrane domains, bases 9–539 of NM_007642, were amplified from a mCD28-encoding plasmid (generously provided by K. Lee, University of Miami, Miami, FL) by PCR, ligated to a PCR product containing bases 645–935 of PD-1 (NM_005018), and placed into the pCLPS lentiviral expression vector (26). Tyrosine to phenylalanine mutations within mCD28/hPD-1 were produced, as previ-

ously described by Wang and Wilkinson (27). DNA sequencing was used to confirm the fidelity of all constructs.

Production of high titer lentiviral vectors and transduction into CD4 T cells

High titer lentiviral vectors were produced, as described previously (26). Lentiviral vectors were transduced into CD4 T cells by first activating 2 million CD4 T cells with CD3/human CD28-coated beads for 24 h. A total of 300–500 μl ($\sim 5 \times 10^7$ – 5×10^8 IFU) of vector was incubated with the activated CD4 T cells and centrifuged at $1200 \times g$ for 2 h (28). The following day, the medium was exchanged and the cells were expanded until they returned to near resting cell volume, as measured by a Coulter Counter Multisizer II (Corixa, Hialeah, FL).

Cell lysis, SDS-PAGE, and Western blotting

Primary human CD4 T cells (15 million cells/sample for pervanadate activation; 25 million cells/sample for bead activation) were washed twice with cold PBS, resuspended in 800 μl of PBS, and incubated at 37°C for 30 min. Pervanadate was prepared using 1 mM NaVO_3 (mixed 1:1 with 17 μl of hydrogen peroxide added to 5 ml of PBS). A total of 200 μl of the $\text{H}_2\text{O}_2/\text{NaVO}_3$ mixture or aAPCs was added to the CD4 T cells and incubated for 5 min. Cells were lysed in 500 μl of 1% Nonidet P-40 lysis buffer (29) for 20 min at 4°C and pelleted for 10 min at 14,000 rpm at 4°C. To detect PD-1 phosphorylation and recruitment of SHP-2 and SHP-1, the cell lysate was immunoprecipitated overnight with 4 μg of anti-mCD28 (37.51). Protein G-Sepharose was added to the immunoprecipitates, incubated at 4°C for 1.5 h, and washed five times with 1% Nonidet P-40 lysis buffer. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with appropriate primary and secondary Ab combinations, and proteins were visualized using an ECL⁺ kit (Amersham Biosciences, Piscataway, NJ). The following mAbs were used: anti-phosphotyrosine (4G10) Ab (Upstate Biotechnology, Waltham, MA), anti-SHP-1 (sc-287) and anti-SHP-2 (sc-280) Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse IgG-HRP (Amersham Biosciences), and anti-hamster IgG-HRP (Southern Biotechnology Associates, Birmingham AL).

For the SH2D1A immunoprecipitation studies, 293 cells were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, glutamine, and antibiotics. For transfection, 1 million cells were plated in 6-cm tissue culture dishes and allowed to adhere overnight at 37°C in 5% CO_2 . Cells were transfected with a total of 3 μg of DNA/plate using the GeneJammer reagent (Stratagene, La Jolla, CA), according to manufacturer's protocols. After 48 h, cells were stimulated with pervanadate, and lysates were prepared. The physical association between mCD28/hPD-1 and SH2D1A was investigated by immunoprecipitation and Western blotting, as described above. Interaction of the GST-CD150 cytoplasmic tail and SH2D1A was determined by precipitation of immune complexes using glutathione-coupled agarose beads, followed by Western blotting, as previously described (30). CD150 GST fusion construct and SH2D1A expression construct were obtained from J. Lewis (Metabolism Branch, National Cancer Institute) (31), and pcDNA 3.1 SHIP was a generous gift of M. Coggeshall (Oklahoma Medical Research Foundation, Oklahoma City, OK). Western blots were probed with the following Abs: anti-phosphotyrosine (4G10) Ab (Upstate Biotechnology), anti-human SHIP (sc-6244; Santa Cruz Biotechnology), anti-GST (Santa Cruz Biotechnology), anti-mCD28, and anti-human SH2D1A (32).

RNA extraction and RT-PCR

RNA was purified and reverse transcribed, as described previously (22). Primers and probes to detect IL-2, IL-10, IL-13, IFN- γ , Bcl-x_L, and 28S ribosomal RNA were designed using Primer Express software (Applied Biosystems, Foster City, CA) and are available upon request. Real-time PCR amplification and product detection were performed using the ABI Prism 7700 (Applied Biosystems), as recommended by the manufacturer. A duplicate cDNA reaction was set up in which reverse transcriptase was left out to confirm the absence of genomic DNA. Results were normalized to 28S RNA levels, and relative expression was determined using the $\Delta\Delta$ cycle threshold method, as recommended by the manufacturer.

Results

PD-1 is a potent inhibitor of CD28-mediated costimulation

To study how PD-1 ligation alters the activation of primary human T cells, we defined a system by which the effects of PD-1 ligation could be observed. Given the controversy surrounding whether PD-1 functions as a negative regulator of T cell activation or as a costimulatory molecule (10–14), we examined both possibilities.

First, we determined whether PD-1 served as a costimulatory molecule. CD3-mediated stimulation of purified primary CD4 T cells results in minimal T cell expansion and IL-2 production (22). Thus, any costimulatory activity PD-1 ligation contributes to T cell activation should be readily detected by measuring IL-2 production and T cell expansion. aAPCs constructed by coating magnetic beads with anti-CD3 Ab and anti-MHC class I Ab (CD3/MHC I), anti-CD3 Ab and anti-PD-1 Ab (CD3/PD-1), or anti-CD3 Ab and anti-CD28 Ab (CD3/CD28) were incubated with freshly isolated CD4 T cells for 24 h, and quantitative RT-PCR was performed for IL-2 production. Anti-MHC class I Abs were used as a negative control because they bind to the T cell surface, but do not initiate signals

that alter the T cell transcriptional profile (33, 34). Only cells activated by CD3/28-coated beads were able to produce significant levels of IL-2 mRNA (Fig. 1A) and expand (data not shown), suggesting that PD-1 was unable to serve as a costimulatory molecule.

Next, we investigated whether PD-1 engagement could block T cell activation. Whereas the inability of CD3 ligation to stimulate primary human CD4 T cells may facilitate the identification of molecules with costimulatory activity, this property serves as a liability in searching for molecules that block T cell activation, because it offers little opportunity to observe inhibitory effects. Previously, we showed that suboptimal CD28 costimulation allows sufficient T cell activation to enable observation of the

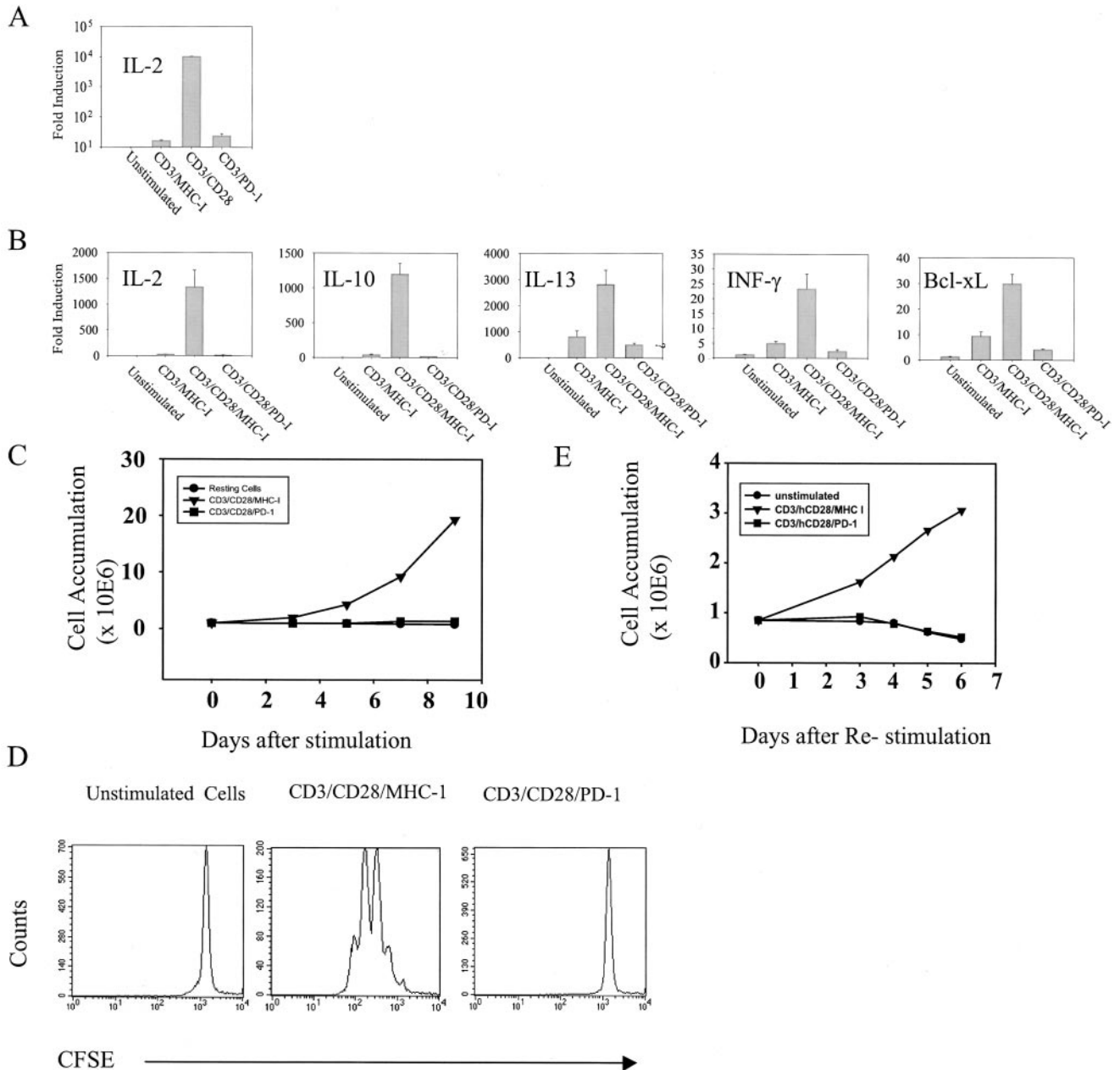


FIGURE 1. PD-1 ligation on primary human CD4 T cells inhibits cytokine production and cell expansion. *A*, Freshly isolated CD4 T cells were left unstimulated or were stimulated with magnetic beads coated with CD3/MHC I, CD3/CD28, or CD3/PD-1 for 24 h. At this time, RNA was harvested, and quantitative RT-PCR for IL-2 was performed. *B*, Purified T cells were similarly stimulated with CD3/MHC I, CD3/CD28/MHC I, and CD3/CD28/PD-1 aAPCs. The relative levels of the indicated cytokines were measured by RT-PCR. *C*, T cell expansion was determined after stimulation with magnetic beads coated with CD3/CD28/MHC I or CD3/CD28/PD-1 by measuring total cell accumulation and *D*, CFSE dilution after 4 days of culture. *E*, T cell expansion was determined after restimulation with magnetic beads coated with CD3/CD28/MHC I or CD3/CD28/PD-1 by measuring total cell accumulation. All data are representative of three independent experiments.

inhibitory effects of CTLA-4 ligation (35). We postulated that a similar system could be engineered to study the potential negative effect of PD-1 ligation in primary CD4 T cells. RT-PCR was performed for a number of effector cytokines and Bcl- χ_L , a CD28-induced cell survival factor (36). As shown in Fig. 1B, we saw a distinct inhibition of CD28-mediated up-regulation of IL-2, IL-10, IL-13, IFN- γ , and Bcl- χ_L when PD-1 was engaged. We also examined the effect that PD-1 engagement had on the ability of T cells to expand upon CD28 costimulation. Cell expansion was measured by two different methods measuring the accumulation of cells within the culture, and dilution of intracellular fluorescent dye CFSE. In contrast to cells stimulated via CD3/28/MHC I aAPCs, we observed no cell accumulation in cultures stimulated by CD3/28/PD-1 aAPCs (Fig. 1C). The lack of dilution of CFSE staining in this population after 4 days of culture (Fig. 1D) suggests that the lack of accumulation of T cells is caused by a failure to divide rather than increased levels of apoptosis. The above experiments were performed on freshly isolated T cells, whereas many of the subsequent experiments are performed on cells that have been activated, rested down, and restimulated. We wanted to compare the ability of PD-1 to inhibit T cell activation and expansion of previously activated cells to ensure that PD-1 had a similar effect on these cells as freshly isolated cells (Fig. 1E). As above, ligation of PD-1 completely blocked the expansion of these cells, indicating that PD-1 can inhibit both primary and secondary T cell responses in a similar manner. These experiments suggest that Ab-mediated ligation of PD-1 blocks, rather than enhances, immune cell activation and establishes a model with which to study the mechanisms used by PD-1 to block T cell function.

PD-1 expression is not rapidly induced on the T cell surface

PD-1 expression is not easily detected on resting T cells, yet we could observe the effects of PD-1 ligation on IL-2 expression as early as 2 h after activation (data not shown). Others have shown that PD-1 expression is up-regulated after B and T cell activation,

and that maximal expression is observed after 48 h (10, 37, 38). To better understand our results, we chose to concomitantly measure the levels of PD-1 mRNA and surface expression to determine whether there was an initial and possibly short-lived spike of PD-1 expression that could explain how this barely detectable receptor could so effectively block CD3/28 costimulation. CD4 T cells were stimulated with CD3/28-coated beads, and PD-1 expression was measured by FACS and quantitative RT-PCR at 2, 4, 8, 24, 48, and 72 h poststimulation (Fig. 2). Consistent with prior reports (10), we observed only minimal PD-1 surface expression until 24 h after T cell stimulation, indicating that PD-1 expression is not biphasic. Moreover, these data suggest that the potent inhibitory effects of PD-1 require only minute levels of PD-1 surface expression. In contrast, CD3/28 costimulation rapidly up-regulated steady state PD-1 mRNA levels, although maximal levels were not achieved until after 48 h of stimulation, suggesting that there may be translational or posttranslational control of PD-1 expression. A similar discordance between PD-1 mRNA levels and surface expression was noted in apoptotic murine B cell lines (37). It should be noted, however, there is a minute, but reproducible increase in PD-1 expression after 2 h of stimulation that slowly increases until 24 h poststimulation. The functional importance of this small change in expression is currently not known.

mCD28/hPD-1 chimeric receptor is able to inhibit T cell activation

To enable structure-function studies of the PD-1 cytoplasmic tail in primary human T lymphocytes, we created a chimeric receptor consisting of the mCD28 extracellular domain and the intracellular PD-1 cytoplasmic tail. The chimeric molecule allows us to distinguish the endogenous PD-1 from the introduced PD-1 cytoplasmic tail by using Ab specific for mCD28. Previously, we used a similar design (mCD28/human ICOS and mCD28/human CD28) to perform structure-function analysis of the CD28 and ICOS cytoplasmic tails in primary human T cells and found that these chimeric

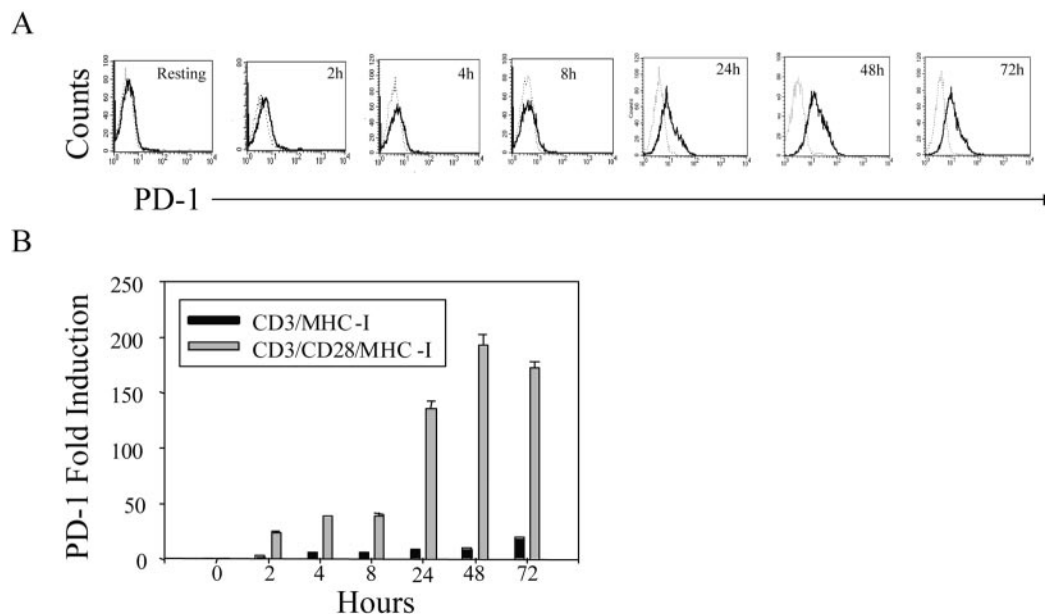


FIGURE 2. PD-1 expression is not rapidly induced on the T cell surface. *A*, CD4 T cells were stimulated with CD3/CD28/MHC I aAPCs. At the indicated times after stimulation, flow cytometry using a FITC-labeled isotype control Ab (spotted line) or FITC-labeled anti-PD-1 Ab (filled line) are displayed. Data are representative of three independent experiments. *B*, CD4 T cells were stimulated with CD3/MHC I or CD3/28/MHC I aAPCs. RNA was prepared at the indicated times, and quantitative RT-PCR was performed using primers and probes specific for PD-1. The same CD3/28/MHC I-stimulated cells used in flow cytometry experiments above were used in these RT-PCR experiments. Errors bars represent the SD from one experiment, and the data are representative of three independent experiments.

receptors were faithful models of endogenous CD28 and ICOS (26). To determine whether the mCD28/hPD-1 construct could inhibit T cell activation, we transduced freshly isolated primary CD4 T cells with a lentiviral vector encoding the mCD28/hPD-1 receptor, and subsequently allowed the cells to expand and rest down. Flow cytometry demonstrated that greater than 90% of the cells expressed mCD28 (Fig. 3A). Next, we stimulated the transduced cells with CD3/28/MHC I- or CD3/28/mCD28-coated beads and measured the total number of cells that accumulated in culture (Fig. 3B). Only cells stimulated with CD3/28/MHC I-coated beads expanded, indicating that the chimeric mCD28/hPD-1 construct was able to suppress T cell proliferation induced by CD3/28 costimulation. We also isolated RNA after 24 h of stimulation and measured induction of IL-2, IL-10, IL-13, IFN- γ , and Bcl-x_L expression (Fig. 3C). Similar to experiments using anti-PD-1 Ab to engage the endogenous PD-1 receptor, ligation of the mCD28/hPD-1 chimeric receptor using specific anti-mCD28 Abs blocked CD28-mediated up-regulation of cytokines and Bcl-x_L. These studies demonstrate that the mCD28/hPD-1 receptor mirrors the endogenous molecule in terms of suppressing T cell expansion and cytokine production.

A single mutation in the ITSM disrupts the ability of PD-1 to block T cell activation

Using a B cell lymphoma cell line, Okazaki et al. (11) demonstrated that disruption of the ITSM blocked the ability of PD-1 to inhibit BCR signaling. To examine PD-1 signaling in primary human CD4 T cells, we created mCD28/hPD-1 chimeric receptors containing single point mutations of each tyrosine (Y223F mutation disrupts the ITIM, and Y248F mutation disrupts the ITSM). A construct containing point mutations of both tyrosine amino acids was also created (223/248F). These vectors were transduced into primary CD4 T cells, and mCD28 expression was examined 3 days after transduction. All cell populations expressed similarly high

levels of mCD28, permitting a direct comparison between each of the mutant cytoplasmic tails (Fig. 4A). These cells were allowed to expand and rest such that their cell volume as measured by a Coulter Counter approached near resting levels (<250 fl) and subsequently restimulated with either CD3/28/MHC I- or CD3/28/mCD28-coated beads, and IL-2 expression and T cell expansion were measured (Fig. 4, B and C). Mutation of the ITIM had no effect on the ability of PD-1 to inhibit CD28 costimulation-mediated IL-2 production and T cell expansion. In contrast, mutation of the ITSM abrogated the ability of PD-1 to block both IL-2 expression and T cell expansion, indicating that factors binding to this site, and not the ITIM, are primarily responsible for the inhibitory function of PD-1.

SHP-1 and SHP-2 are recruited to the PD-1 ITSM

To identify signaling proteins whose binding to the PD-1 cytoplasmic tail correlated with its ability to suppress T cell activation, CD4 T cells were transduced with the mCD28/hPD-1 wild type, mCD28/hPD-1 Y223F, mCD28/hPD-1 Y248F, and mCD28/hPD-1 Y223F Y248F double mutant and allowed to rest. Following stimulation with pervanadate to maximally phosphorylate the tyrosines within the PD-1 cytoplasmic tail, the chimeric receptor was immunoprecipitated and its phosphorylation status and associated molecules were identified by Western blotting. We find that in addition to the phosphorylated chimeric receptor, which migrates at ~50 kDa, only one other band that corresponds to a ~70-kDa tyrosine-phosphorylated protein(s) was immunoprecipitated by the mCD28 Ab in cells transduced with the mCD28/hPD-1 wild-type construct (Fig. 5). This complex was still present in lysates derived from cells expressing a PD-1 cytoplasmic tail that had a mutant ITIM, but was severely reduced in cells expressing the mCD28/hPD-1 Y248F. This complex was absent in cells transduced with mCD28/hPD-1 Y223F Y2248F construct, indicating that the formation of this complex was dependent upon the

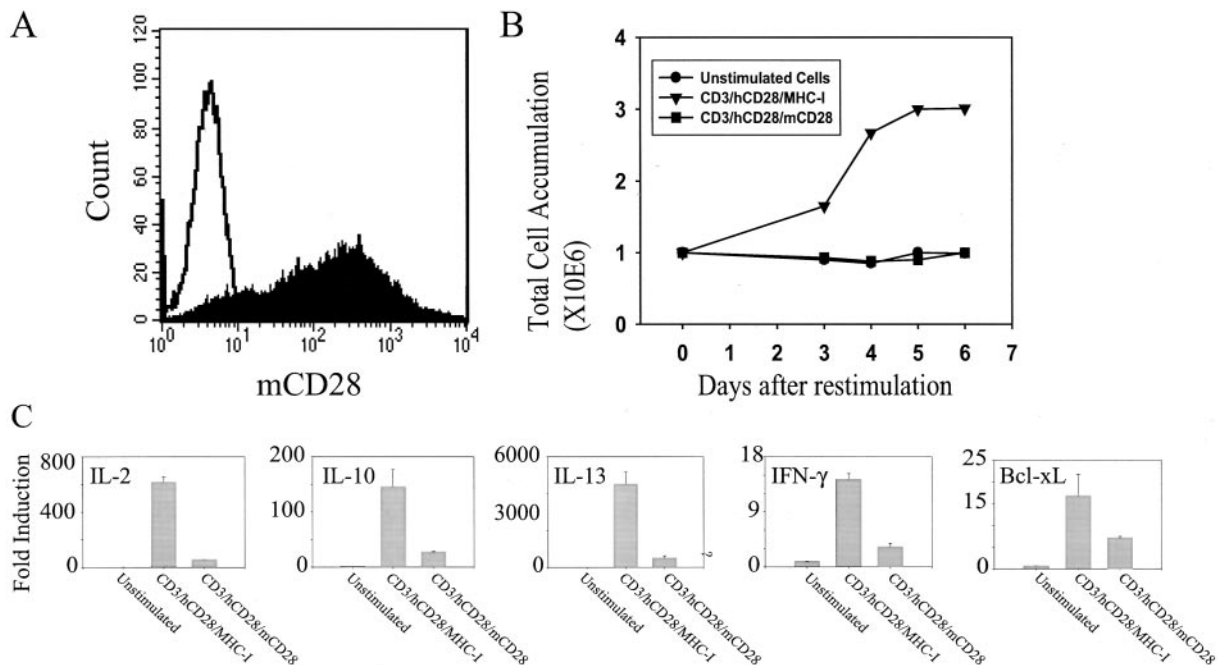
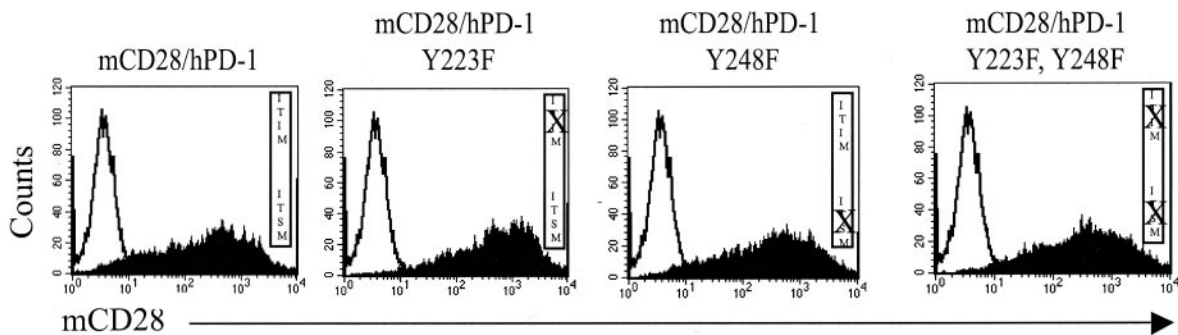
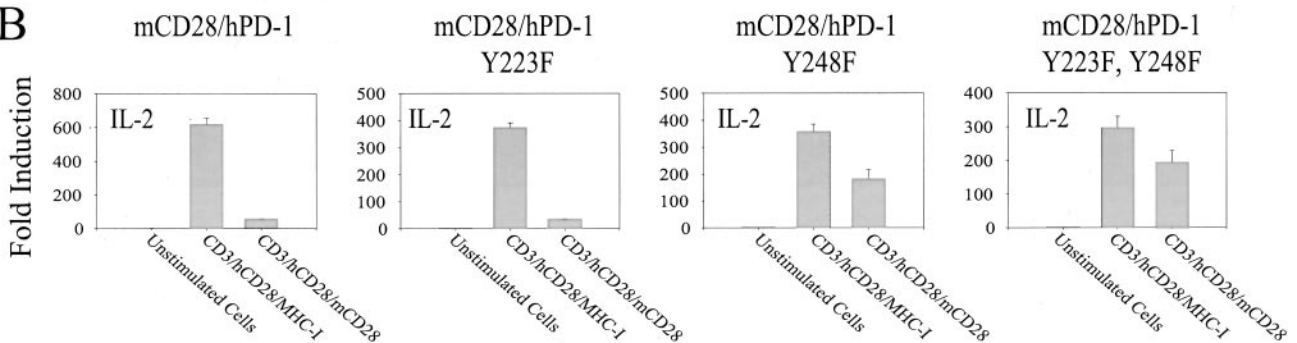


FIGURE 3. Ligation of mCD28/hPD-1 chimeric receptor mirrors the ability of the endogenous PD-1 receptor to inhibit cytokine secretion and cell proliferation. *A*, CD4 T cells were transduced with a lentiviral vector expressing the mCD28/hPD-1 fusion protein. Expression of the chimeric construct was determined by flow cytometry 3 days after transduction using a PE-labeled isotype control Ab (open histogram) and a PE-labeled anti-mCD28 Ab (filled histogram). *B*, mCD28/hPD-1-transduced CD4 T cells were restimulated with the indicated magnetic beads, and T cell expansion was measured by cell counting. *C*, mCD28/hPD-1-transduced CD4⁺ T cells were restimulated with the indicated aAPCs for 24 h, and the indicated transcripts were measured by quantitative RT-PCR. All data are representative of three independent experiments.

A



B



C

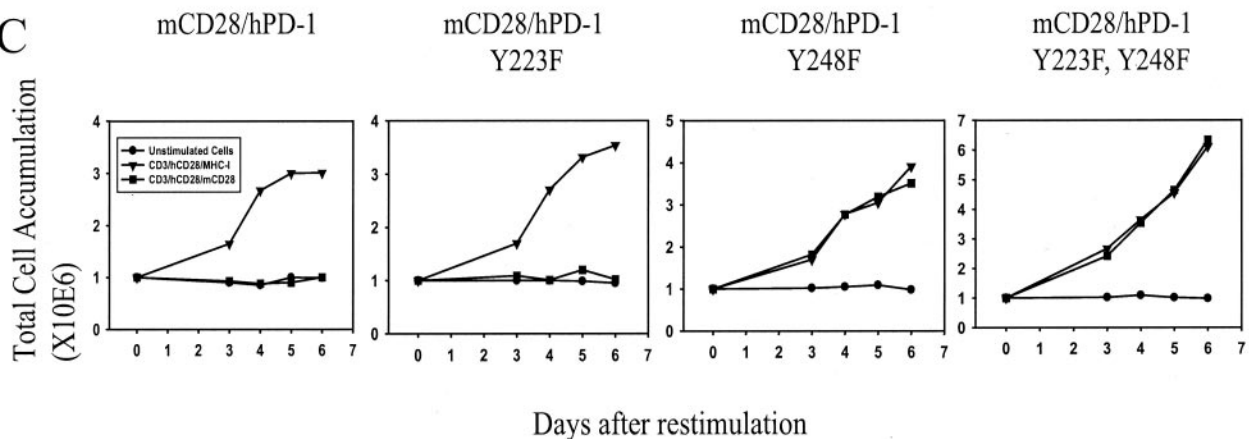


FIGURE 4. Disruption of ITSM of PD-1 interferes with PD-1-mediated suppression of T cell activation. *A*, CD4 T cells were transduced with lentiviral vectors expressing mCD28/hPD-1 wild-type or mutant cytoplasmic tails, and transduction efficiency was determined, as in Fig. 3. *B*, Transduced CD4 T cells were restimulated for 24 h with the indicated magnetic beads, and IL-2 expression was measured by RT-PCR and expressed as fold induction over unstimulated cells. *C*, Transduced CD4 T cells were restimulated with the indicated magnetic beads (same legend is used for all panels), and cell expansion was measured by cell counting. All data are representative for three independent experiments.

PD-1 ITSM. Also, the formation of this ~70-kDa PD-1-associated complex correlated with its ability to regulate T cell expansion and IL-2 production, making it a strong candidate for factor(s) responsible for mediating PD-1 signaling. Others have found that SHP-2 is recruited to the PD-1 cytoplasmic tail in a B cell line (11) and in Jurkat T cells (20). To evaluate whether the same might be true in primary human T cells, we stripped and reprobbed our Western blots using an anti-SHP-2 Ab, and demonstrated that at least one component of this 70-kDa complex is SHP-2 (Fig. 5). SHP-1 also interacts with PD-1 in a modified yeast two-hybrid screen (39), making it another potential candidate for interaction with the PD-1

cytoplasmic tail. To determine whether SHP-1 was recruited to the PD-1 in primary human CD4 T cells, we probed for SHP-1 in these same mCD28 immunoprecipitates and observed that, like SHP-2, SHP-1 was recruited to the PD-1 cytoplasmic tail. Structural mapping demonstrated that SHP-1 also bound to PD-1 at its ITSM, suggesting that the requirements for SHP-1 and SHP-2 binding to the PD-1 cytoplasmic domain overlap. In contrast, using an analogous system in a B cell tumor line, Okazaki et al. (11) did not detect SHP-1 binding to PD-1, indicating that PD-1 signaling differs between B and T cells, and/or primary and transformed cells. Alternatively, the discrepancies on whether SHP-1 binds the PD-1

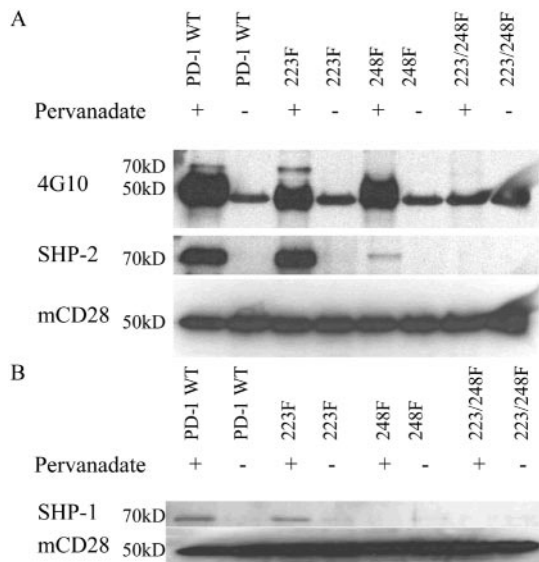


FIGURE 5. Pervanadate activation leads to SHP-1 and SHP-2 recruitment to PD-1 cytoplasmic tail in an ITSM-dependent manner. *A*, CD4 T cells were transduced with lentiviral vectors expressing mCD28/hPD-1 wild-type and mutant cytoplasmic tails, expanded, and rested down. Cells were treated with pervanadate, lysed, and immunoprecipitated using an anti-mCD28 Ab. Tyrosine-phosphorylated proteins were detected using 4G10 by Western blotting of the immunoprecipitated complexes (*upper panel*). The blot was then stripped and reprobed initially with SHP-2 Ab and subsequently with a mCD28 Ab (*lower panel*) to demonstrate equal loading. *B*, The same immunoprecipitates were rerun on a new gel, and SHP-1 recruitment was assessed by Western blotting with a SHP-1-specific Ab. Equal loading is demonstrated by blotting against mCD28 (*lower panel*). The SHP-2 data are representative of three different experiments, and the SHP-1 data are representative of two experiments.

cytoplasmic tail may reflect the ability of different assays to detect the presence of SHP-1 in immunoprecipitation reactions.

SHP-2 is recruited to the PD-1 cytoplasmic tail in absence of receptor ligation

Although pervanadate treatment leads to maximal phosphorylation of tyrosine residues, we were also interested in determining whether SHP-2 was recruited to the PD-1 cytoplasmic tail under more physiological conditions, such as those associated with PD-1-mediated suppression of T cell activation. CD4 T cells were transduced with the mCD28/hPD-1 wild-type and mutant cytoplasmic tail constructs, expanded, and allowed to rest. CD3/28/MHC I- and CD3/28/mCD28-coated aAPCs were used to activate cells for 5 min, cell lysates were made, and mCD28 was immunoprecipitated. Consistent with our prior results using pervanadate, cells stimulated with CD3/28/mCD28 recruited SHP-2 into a single 70-kDa complex in an ITSM-dependent manner (Fig. 6A and data not shown). Interestingly, we also observed that SHP-2 is recruited to the PD-1 cytoplasmic tail in the absence of mCD28 triggering. This result suggests that SHP-2 binds to PD-1 upon T cell activation, and that specific PD-1 receptor engagement is not necessary for this association. Moreover, because PD-1 triggering is necessary to block T cell activation (Fig. 1), these results indicate that SHP-2 recruitment to PD-1 is not sufficient to mediate its suppressive effects on T cell activation, and suggest that PD-1 must be in close proximity to either CD3 or CD28 to alter the response of a T cell to its cognate Ag. An alternative model is that PD-1 engagement is required to recruit an additional factor to SHP-2 that is necessary to suppress T cell activation. It should be noted that

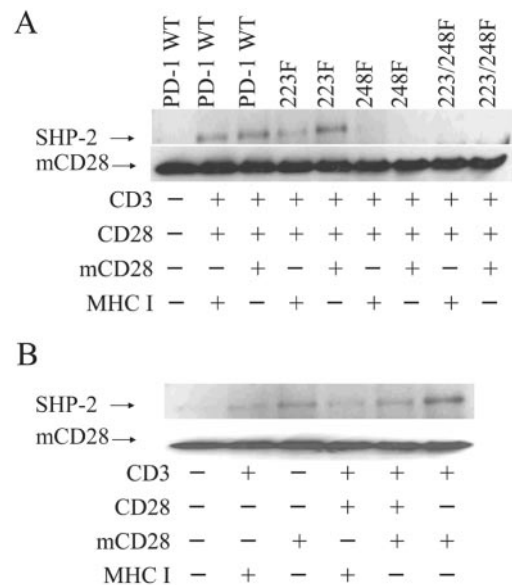


FIGURE 6. T cell activation without specific PD-1 receptor engagement results in SHP-2 recruitment to the PD-1 cytoplasmic tail. *A*, CD4 T cells were transduced with lentiviral vectors expressing mCD28/hPD-1 wild-type or mutant cytoplasmic tails. These cells were restimulated with the indicated magnetic beads and subsequently immunoprecipitated using mCD28 Ab. SHP-2 recruitment was assessed by immunoblotting for SHP-2. Equal loading is demonstrated by blotting against mCD28 (*lower panel*). *B*, CD4 T cells were transduced with a lentiviral vector expressing the mCD28/hPD-1 wild-type cytoplasmic tail. These cells were restimulated with the indicated magnetic beads and subsequently immunoprecipitated with mCD28 Ab. SHP-2 recruitment was assessed by immunoblotting for SHP-2. Equal loading is demonstrated by blotting against mCD28 (*lower panel*). Data are representative of two different experiments.

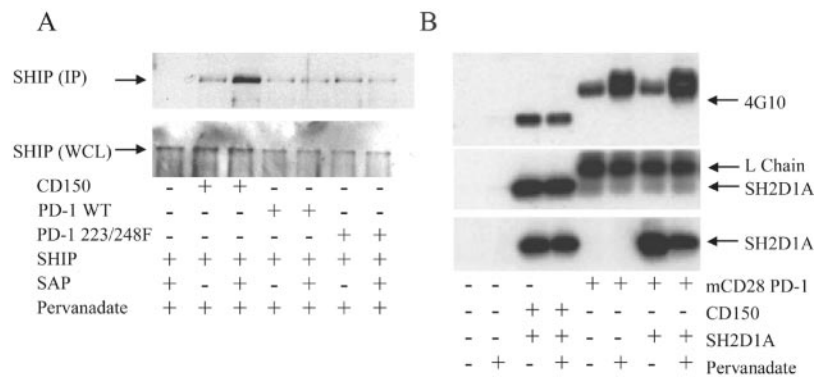
we probed for SHP-1 in these immunoprecipitates and were unable to reproducibly demonstrate the presence of this phosphatase. Using pervanadate to stimulate the mCD28/hPD-1-transduced cells, we detected much less SHP-1 than SHP-2 in the immunoprecipitates. Judging by the relative differences in the amount of SHP-2 bound to mCD28/hPD-1 upon CD3/28/mCD28 and pervanadate stimulation, we suspect that the amount of SHP-1 present is at or below the level of detection of our assay.

To investigate the ability of SHP-2 to bind PD-1 in the absence of receptor ligation, we activated mCD28/hPD-1-transduced cells with CD3/MHC I-, mCD28-, or CD3/mCD28-coated beads and measured the ability of the wild-type PD-1 cytoplasmic tail to interact with SHP-2. We found that SHP-2 was recruited to the PD-1 cytoplasmic tail after CD3/MHC I stimulation (Fig. 6B), indicating that TCR stimulation is sufficient to recruit SHP-2 to the PD-1 cytoplasmic tail. Likewise, cross-linking of mCD28 in the absence of TCR stimulation also recruits SHP-2 to the PD-1, demonstrating that T cell activation is not necessary for SHP-2 recruitment. Simultaneous stimulation of CD3 and mCD28 (CD3/mCD28) resulted in an enhanced recruitment of SHP-2, which suggests that mCD28 and the TCR function in an additive fashion to recruit SHP-2 to the PD-1 cytoplasmic tail. Taken together, our experimental data indicate that both TCR and PD-1 ligation lead to an interaction between SHP-2 and the PD-1 cytoplasmic tail, but only in the context of PD-1 receptor engagement can PD-1 signaling block T cell activation.

ITSM of PD-1 does not recruit SH2D1A

The ITSM was originally defined as a tyrosine-based sequence present within the CD150 cytoplasmic tail that interacts with SHIP

FIGURE 7. PD-1 cytoplasmic tail does not recruit SHIP or SH2D1A. The 293T cells were transiently transfected with constructs encoding human SH2D1A, human SHIP, mCD28/hPD-1, mCD28/hPD-1 223/248F, or GST-CD150 cytoplasmic tail in the combinations indicated. Forty-eight hours following transfection, cells were stimulated with pervanadate. Cells were lysed, and GST-CD150 or mCD28/hPD-1 was precipitated using glutathione-coupled agarose beads or anti-mCD28 Abs, respectively. *A*, Immunoprecipitates and whole cell lysates were probed with SHIP Abs, as indicated. *B*, Immunoprecipitates and whole cell lysates were probed with anti-SH2D1A Abs, as indicated. *Lower panel*, Shows equal loading of protein after stripping the immunoprecipitation membrane and reprobing with GST or mCD28, respectively.



or SHP-2, depending upon the presence or absence of the small adapter SH2D1A (18). Primary human T cells express SH2D1A, yet we did not observe any large complexes (150 kDa) binding to the PD-1 cytoplasmic tail upon pervanadate treatment that would indicate the presence of SHIP. To prove this more definitively, we probed our Western blots with an anti-SHIP Ab and were unable to detect SHIP, suggesting that this molecule does not bind to PD-1 (data not shown). Similar findings were described by others using a B cell line (11). Based on the model proposed by Shlapatska et al. (18), we postulated that the absence of SHIP in PD-1 immune complexes might be due to the inability of the PD-1 cytoplasmic tail to interact with SH2D1A. To test this directly, we transfected 293 T cells with plasmids encoding SHIP and either GST-CD150 cytoplasmic domain, mCD28/hPD-1, or mCD28/hPD-1 223/248F in the presence or absence of SH2D1A. Cells were treated with pervanadate and lysed. Probing of whole cell lysates for SHIP revealed that all cultures expressed equivalent levels of their respective transfected expression vector. Following precipitation, we found a distinct association of CD150 with SHIP in the presence of SH2D1A, but no association with the PD-1 cytoplasmic tail regardless of the presence of SH2D1A (Fig. 7A). This indicates that CD150 can recruit SHIP to its cytoplasmic tail in a SH2D1A-dependent manner, but PD-1 cannot. Next, we wanted to determine whether the PD-1 cytoplasmic tail is able to bind SH2D1A. The 293 T cells were transfected with plasmids encoding SH2D1A and either GST-CD150 cytoplasmic domain or mCD28/hPD-1. Cells were left unstimulated or were treated with pervanadate and lysed. Probing of whole cell lysates revealed that all cultures expressed equivalent levels of their respective transfected expression vectors (Fig. 7B and data not shown). Following precipitation reactions of these lysates using either glutathione-coupled agarose beads or mCD28 Ab, Western blotting using 4G10 and anti-SH2D1A Abs was performed (Fig. 7B). Although CD150 was not phosphorylated well by pervanadate treatment, this had little impact on its ability to interact with SH2D1A. This is consistent with previous data showing that SH2D1A binds to CD150 in a phosphorylation-independent manner (40–42). Importantly, PD-1 was unable to bind to SH2D1A regardless of the phosphorylation status of the mCD28/hPD-1 cytoplasmic tail, suggesting that the CD150 ITSM and the PD-1 ITSM work in fundamentally different ways.

Discussion

The present study was designed to determine the critical residues (*cis* factors) within the PD-1 cytoplasmic tail and the (*trans*) factors that bind these sequences in primary human CD4 T cells. To do this, we constructed a chimeric molecule consisting of the mCD28 extracellular domain fused to the hPD-1 cytoplasmic tail. This molecule was introduced by high efficiency lentiviral medi-

ated gene transfer into primary human CD4 T cells, permitting us to specifically engage our introduced molecule using an anti-mCD28 Ab. We first validated that this model mirrored the effects of cross-linking the endogenous PD-1. In doing so, we demonstrated that PD-1 ligation had no costimulatory activity and was very efficient in preventing suboptimal CD3/28 costimulation from inducing T cell proliferation and cytokine production. These data add to the growing body of evidence that direct ligation of PD-1 inhibits T cell activation (43–46).

Using this model, we were able to show that an intact ITSM was essential to mediate the suppressive effects of PD-1 in primary human T cells. The ITSM sequence TxYxxV/I is found in several immunoreceptors, of which CD150 is the prototype. Interestingly, most of the molecules that contain an ITSM within their cytoplasmic domains also contain one or more membrane-proximal ITIMs. The PD-1 ITIM is conserved in all species in which the PD-1 sequence has been examined (18), suggesting a role for this motif in PD-1 signaling and function. Surprisingly, however, previous data obtained using a murine B cell line (11), and our current data (Fig. 4), show that mutation of this motif has a negligible effect on the ability of PD-1 ligation to block cell activation. These results suggest that either the Y223F mutation does not completely disrupt ITIM function, or that the PD-1 ITIM may serve another signaling role that is not involved in TCR/CD28-mediated T cell activation and effector functions.

Our studies also demonstrate that the PD-1 ITSM and the CD150 ITSM do not function in the same manner. The CD150 ITSM binds to SHIP when the small adapter SH2D1A is present and to SHP-2 when SH2D1A is absent. This was proposed as a mechanism by which CD150 could have both positive and negative effects in regard to B cell activation (18). Because PD-1 engagement has also been shown to play both a positive and negative role during T cell activation (10–14), we postulated that the ability of the ITSM to recruit both positive and negative regulators of cell activation may explain some of these conflicting results. Instead, and in contrast to CD150, we found that PD-1 does not bind to SH2D1A, indicating that there are other structural requirements within the CD150 cytoplasmic tail in addition to the TxYxxV/I that mediate the physical interaction between it and SH2D1A. Moreover, we only observed one phosphorylated complex that was recruited to the PD-1 cytoplasmic tail, questioning whether the ITSM of PD-1 has the ability to switch which factors it binds. To date, two types of ITSMs have been defined: those in which the phosphorylation state of the tyrosine does not affect the binding of SH2D1A (40–42), and those in which the phosphorylation state of the ITSM is critical for SH2D1A binding (47–49). Our studies define a third ITSM that does not bind SH2D1A regardless of the phosphorylation state of the ITSM tyrosine.

We find that the cytoplasmic tail of PD-1 recruits both SHP-1 and SHP-2 in primary human T cells. SHP-1 and SHP-2 are highly related tyrosine phosphatases that serve very distinct roles in signal transduction. SHP-1 expression is largely confined to hemopoietic cells (50), and is thought to act as a negative regulator of immune cell activation (51–53). SHP-2, in contrast, is widely expressed and generally acts in positive manner to transduce signals from receptor protein tyrosine kinases (54, 55). However, SHP-2 also has been shown to inhibit the Jak-STAT signaling pathway initiated by IFN- α and IFN- γ stimulation (56). Several outstanding questions regarding the role SHP-1 and SHP-2 play in mediating PD-1-initiated signal transduction events remain. Do they compete for binding of the ITSM or can they both bind the PD-1 cytoplasmic tail at the same time? Perhaps binding of one modifies the ITIM, allowing binding of the other. Does the ITSM function as a switch motif and toggle the relative ratios of the presumably positive regulator SHP-2 to the presumably negative regulator SHP-1 that binds the PD-1 cytoplasmic tail? Delineation of the role(s) that SHP-1 and SHP-2 play in PD-1-mediated signal transduction will provide further insight into the mechanism by which this receptor modulates T cell activation. It is interesting to note that SHP-1 has not been reported to bind to the PD-1 cytoplasmic domain in a murine B cell line (11) or in Jurkat T cells (20). This inability of the PD-1 cytoplasmic tail to recruit SHP-1 may reflect differences between T and B cells, transformed and primary cells, or sensitivities of different assay systems to detect SHP-1 in PD-1 immunoprecipitation reactions. Moreover, this observation suggests that studies that examine the downstream consequences of PD-1 signal transduction in these models may not accurately reflect what happens in primary human T cells.

Our data also revealed that SHP-2 recruitment to the PD-1 cytoplasmic tail did not always correlate with PD-1-mediated inhibition of T cell activation. T cell activation recruited SHP-2 to the PD-1 cytoplasmic tail, but only when the PD-1 was triggered in tandem with the CD3- and CD28-generated signals was inhibition of T cell activation observed. One explanation of this finding is that PD-1 ligation might recruit other inhibitory molecules in addition to SHP-2 (and SHP-1). Another possibility is that PD-1 must be in close proximity to either the CD3 or CD28 to block a T cell response. Support for the latter possibility is provided by the use of magnetic beads to activate human T cells that have either anti-CD3 and PD-L1 Ig on the same microsphere (*cis* configuration) or anti-CD3 and PD-L1 Ig loaded on distinct microsphere (*trans* configuration) (10). These results demonstrated that only magnetic beads in the *cis* configuration could block T cell proliferation, whereas a 50:50 mixture of anti-CD3- and anti-PD-L1-coated beads had no effect, indicating that PD-1 ligation and signaling must be given in close proximity to anti-CD3 to inhibit T cell activation.

PD-1 shares functional characteristics with the structurally related receptor CTLA-4. Neither is expressed at a significant level on resting T cells, yet engagement of both can inhibit the earliest events in T cell activation. Additionally, both can recruit SHP-2 to their cytoplasmic tails. However, we have noted differences in the downstream consequences of PD-1 and CTLA-4 ligation. In Fig. 1, we observed that PD-1 engagement blocks CD28-mediated up-regulation of the cell survival gene Bcl-x_L, whereas, in a previous report, we demonstrated that CTLA-4 ligation did not inhibit CD28-mediated up-regulation of Bcl-x_L (35), suggesting the ligation of each of these receptors has unique effects on T cell activation. We have recently addressed the issue of functional redundancy between PD-1 and CTLA-4.⁴ We found that both PD-1 and

CTLA-4 engagement limit glucose metabolism and Akt activation. However, each used a unique mechanism to enforce this regulation. CTLA-4 ligation activated protein phosphatase 2A, which directly targeted Akt activation, whereas PD-1 ligation blocked CD28-mediated activation of PI3K. Together, these data indicate that a major target of PD-1-mediated SHP-1 or SHP-2 activation is PI3K. The model established in this study will permit further dissection of the molecular mechanism(s) by which PD-1 engagement modulates T cell function in primary human T cells.

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