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Program Death-1 Engagement Upon TCR Activation Has Distinct Effects on Costimulation and Cytokine-Driven Proliferation: Attenuation of ICOS, IL-4, and IL-21, But Not CD28, IL-7, and IL-15 Responses

Frann Bennett,[†] Deborah Luxenberg,[†] Vincent Ling,[†] I-Ming Wang,[†] Kim Marquette,[†] David Lowe,[*] Nighat Khan,[*] Geertruida Veldman,[†] Kenneth A. Jacobs,[†] Viia E. Valge-Archer,[*] Mary Collins,[†] and Beatriz M. Carreno[†]

The program death 1 (PD-1) receptor and its ligands, PD-1 ligand (PD-L)1 and PD-L2, define a novel regulatory pathway with potential inhibitory effects on T, B, and monocyte responses. In the present study, we show that human CD4$^+$ T cells express PD-1, PD-L1, and PD-L2 upon activation, and Abs to the receptor can be agonists or antagonists of the pathway. Under optimal conditions of stimulation, ICOS but not CD28 costimulation can be prevented by PD-1 engagement. IL-2 levels induced by costimulation are critical in determining the outcome of the PD-1 engagement. Thus, low to marginal IL-2 levels produced upon ICOS costimulation account for the greater sensitivity of this pathway to PD-1-mediated inhibition. Interestingly, exogenous IL-2, IL-7, and IL-15 but not IL-4 and IL-21 can rescue PD-1 inhibition, suggesting that among these cytokines only those that activate STAT5 can rescue PD-1 inhibition. As STAT5 has been implicated in the maintenance of IL-2Rα expression, these results suggest that IL-7 and IL-15 restore proliferation under conditions of PD-1 engagement by enhancing high-affinity IL-2R expression and hence, IL-2 responsiveness. ***The Journal of Immunology*****, 2003, 170: 711–718.**

B7 family ligands and their receptors play a critical role in the activation, expansion, and contraction of T cell populations. During T cell activation, interaction of B7-1 (CD80) and B7-2 (CD86) with CD28 increases proliferation, cytokine production, and prolongs survival of T cells (1). Additionally, interaction of inducible costimulatory molecule ligand (ICOS-L)[3] (GL50/B7h) with the TCR inducible costimulatory molecule (ICOS) enhances secondary and Th2 effector T cell responses (2). In contrast, interaction of B7-1 and B7-2 with CTLA-4 leads to inhibition of proliferation, cytokine production, and cell cycle arrest (3). Thus, a balance between activation and inhibitory signals ensures the development of an effective immune response and, importantly, safeguards against the development of autoimmunity.

Program death 1 (PD-1) is an activation-induced inhibitory receptor expressed on T cells, B cells, and monocytes (4, 5). PD-1 ligand (PD-L)1 (B7H1) and PD-L2 (B7-DC) are the ligands for PD-1 (6, 7); these ligands share 38% amino acid identity (7, 8) and 20–27% amino acid identity with other B7 family members, B7-1, B7-2, and ICOS-L (6, 9–11). PD-L1 and PD-L2 expression is induced on monocytes and dendritic cells upon treatment with IFN-γ or IFN-γ/LPS (6), and transcripts for both ligands are detectable on lymphoid and nonlymphoid tissue. PD-1-deficient mice display disregulated Ig production with late onset chronic progressive glomerulonephritis and arthritis in C57BL/6 mice and self-reactive Ig-mediated dilated cardiomyopathy in BALB/c mice (12, 13). These data suggest PD-1 receptor engagement leads to down-regulation of immune responses and its deficiency results in loss of peripheral tolerance. Thus, the PD-1/PD-L pathway has been proposed to attenuate central and peripheral immune responses.

We have previously demonstrated that anti-CD3/PD-L1.Fc activation of wild-type but not PD-1-deficient murine T cells, as well as human T cells, results in decreased proliferation relative to cells activated with anti-CD3 alone (6). Additionally, CD28 costimulation can overcome PD-1-mediated inhibition by augmenting IL-2 production (14). In this study, we examine the effect of ICOS costimulation and γ common receptor cytokine family members on PD-1 inhibition. We determine that IL-2 levels induced by ICOS costimulation are insufficient to fully restore proliferation upon PD-1 engagement. Thus, IL-2 levels induced by CD28 or ICOS costimulation are critical in determining the outcome of the PD-1 engagement. The effect of IL-2, -4, -7, -15, and -21 on TCR/PD-1 activation was examined. We find that among these cytokines, only those that activate STAT5 can rescue PD-1 inhibition.

Materials and Methods

Abs and fusion proteins

Abs to CD3 (clone UCHT-1), CD28 (clone CD28.2), and IL-2 (clone MAB202) were purchased from BD PharMingen (San Diego, CA) or R&D Systems (Minneapolis, MN). Construction of PD-L1.Fc (human (hu)PD-L1-murine (mu)IgG2a), ICOS-L.Fc (GL50-muIgG2a), and B7.2.Fc (huB7.2-huIgG1) has been described previously (6, 10). Murine Abs (J110) to human PD-1 were obtained from e-Biosciences (San Diego, CA). Neutralizing Abs specific for human PD-1 or human PD-L1 were isolated by selection from large (total size greater than 10^{11}), human scFv phage display libraries (15). Human PD-1 or PD-L1 fusion proteins were diluted to

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[2] Abbreviations used in this paper: ICOS-L, inducible costimulatory molecule ligand; ICOS, inducible costimulatory molecule; PD-1, program death-1; PD-L, PD-1 ligand; BCR, B cell receptor; hu, human; mu, murine.

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10 μg/ml (PD-L1) or 20 μg/ml (PD-1) in PBS and coated onto wells of Nunc Maxisorp 96-well microtiter plates (Nunc, Naperville, IL) overnight at 4°C. Two rounds of panning selection were performed using deselection with unrelated fusion protein partners. Ag-specific phage clones were identified by phage binding to Ag in an ELISA, but not to other fusion proteins or unrelated proteins. Unique scFv were identified by DNA sequence analysis of the scFv insert using appropriate vector primers. Purified scFv from PD-1 or PD-L1 binding clones was prepared by nickel chelate chromatography from 500 ml induced *Escherichia coli* cultures using Pharmacia Ni-NTA agarose resin (Amersham Pharmacia Biotech, Piscataway, NJ). ScFv was assayed for the ability to inhibit the binding of 100 ng/ml biotinylated hPD-L1 fusion protein to 1.5 μg/ml huPD-1 fusion protein immobilized on plastic. Bound PD-L1 was detected with streptavidin-AmDEX-alkaline phosphatase (Amdex, Jyllinge, Denmark). Neutralizing scFv were converted to human IgG1 by subcloning of H chain $V_HD_HJ_H$ and L chain V_LJ_L domains into vectors containing the human IgG1 H chain constant domain or appropriate human L chain constant domain. Subcloning was verified by sequence analysis of the constructs, which were cotransfected into eukaryotic cells for IgG expression. IgG was purified from culture supernatants by protein A-Sepharose (Amersham Pharmacia Biotech) affinity chromatography according to manufacturer's directions. Human anti-human Abs PD-1–17 and PD-1–35 were characterized for their ability to inhibit PD-1/PD-L interaction using ELISA and FACS assays. ELISAs were performed as described for scFv. FACS assays were performed using a human PD-1 expressing Jurkat cell line; cells were incubated with increasing concentrations of Ab (0–100 μg/test) followed by incubation with human PD-L.Fc (2 μg/test). Cells were washed and incubated with PE-conjugated goat anti-mouse IgG2a to detect PD-L.Fc binding. A stable Chinese hamster ovary cell line has been established for PD-1–17 Ab production and Ab produced and purified. Requests for this Ab should be addressed to the attention of Dr. B. Carreno (Wyeth Research, Cambridge, MA).

T cell assays

Human $CD4^+$ T cells were purified by negative selection from peripheral blood lymphocytes as described previously (16). Tosyl-activated magnetic microspheres (Dynal Biotech, Great Neck, NY) were coated with anti-CD3 Ab (0.25–3 $\mu g/10^7$ microspheres), and PD-L1.Fc, ICOS-L.Fc, or B7.2.Fc (0.5–4 $\mu g/10^7$ microspheres) as described (17). Murine IgG or an irrelevant fusion protein was used to saturate the binding capacity of the microspheres (total protein = 5 $\mu g/10^7$ microspheres). Protein-coated microspheres were added to purified $CD4^+$ T cells (10^5 cells/well) in flat-bottom 96-well microtiter plates at a ratio of 1:1; 72 h after culture initiation, plates were pulsed with 1 μCi tritiated thymidine/well and incubated for a 6- to 16-h period. For assessing effect of soluble anti-PD-1 Ab on proliferation, $CD4^+$ T cells were preactivated for 48 h with anti-CD3/anti-CD28-coated microspheres, harvested, and restimulated with the indicated concentration of PHA plus 10 ng/ml IL-2 in the presence of the indicated Abs. Proliferation was measured at 72 h. For neutralization of IL-2, 20 μg/ml of either anti-huIL-2 Ab or muIgG1 (R&D Systems) was added at initiation of culture. To assess effect of exogenous cytokines on PD-1-mediated inhibition, cells were activated with anti-CD3 or anti-CD3/PD-L1.Fc-coated microspheres; human cytokines (R&D Systems) were added at the indicated concentrations at the initiation of culture and proliferation was measured at 72 h as described above. Unpaired Student's *t* test statistical analysis was performed on data on Fig. 4. Values of $p \leq 0.05$ were considered significant.

PD-1, PD-L1, and PD-L2 expression

Purified human $CD4^+$ T cells (10^6 cells/ml) were activated with anti-CD3/anti-CD28-coated microspheres as described above, samples were collected at the indicated times and incubated with FITC-conjugated anti-CD25 Ab (clone M-A251; BD PharMingen), or anti-CD69 Ab (clone FN50; BD PharMingen) for 30 min at 4°C. For PD-1 and PD-L1 expression, cells were stained with anti-huPD-1 Ab-biotin conjugate (clone J110) or anti-huPD-L1 Ab-biotin conjugate (clone PD-L1–12) followed by streptavidin-PE (Southern Biotechnology Associates, Birmingham, AL). Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest software (BD Biosciences). For PCR analysis, the following primers were used: hPD-L1 5′ primer, CTGTTGAAGGACCAGCTCTCC; hPD-L1 3′ primer, TTTGGAGGATGTGCCAGAGG; hPD-L1 detection primer, TTTGGAG GATGTGCCAGAGG; hPD-L2 5′ primer, GAAAGAGCCACTTTGCTG GAGGAGC; hPD-L2 3′ primer, TGTTGTGGTGACAGGTCTTT; hPD-L2 detection primer, AACGCTGACGTTTGGCCAGG; and reverse transcription extension primer, CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAG CTTTTTTTTTTTT.

$CD4^+$ T cells were activated with anti-CD3- or anti-CD3/PD-L1.Fc-coated microspheres, harvested at 24 h, and RNA extracted using Qiagen RNeasy kit (Qiagen, Valencia, CA). RT-PCR was performed using the extension primer and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in 20 μl reactions at 42°C, according to manufacturer's protocols. Subsequent PCR was performed using Advantage Polymerase (Clontech Laboratories, Palo Alto, CA) in 30 μl reactions in a Robocycler Gradient 96 (Strategene, La Jolla, CA) set for 51–65°C annealing gradient. Reaction conditions were: denaturation 95°C, 2 min; cycling 95°C, 1 min; annealing gradient 72°C, 1 min (35 cycles); and extension 72°C, 10 min. Five microliters of optimally amplified products were fractionated by electrophoresis on 1% TBE agarose gels followed by alkaline capillary transfer onto zeta-probe GT membranes (Bio-Rad, Hercules, CA). ^{32}P-Radiolabeled probes were generated by 5′ phosphorylation of detection oligonucleotides using T4 polynucleotide kinase (NEB, Beverly, MA) and subsequently purified on Sephadex G-25 spin columns (Amersham Pharmacia Biotech). Blots were hybridized with detection oligonucleotides in 0.4× White Rain Shampoo for 1 h at 42°C followed by washing in 1× SSC, 1% SDS until adequate background noise levels were achieved (18). Blots were imaged on a BAS2000 phosphoimager (Fuji Medical Systems, Stamford, CT).

Quantitative PCR assessment of IL-2 transcripts

$CD4^+$ T cells were activated with microspheres coated with anti-CD3/control Ig (1 μg/4 $\mu g/10^7$ microspheres), anti-CD3/PD-L1.Fc/control Ig, anti-CD3/ICOS-L.Fc/control Ig or anti-CD3/B7-2.Fc/control Ig, anti-CD3/PD-L1.Fc/ICOS-L.Fc or anti-CD3/PD-L1.Fc/B7-2.Fc (1 μg/2 μg/2 $\mu g/10^7$ microspheres), and harvested 24 h after activation. RNA was prepared as described above. Quantitative PCR was conducted using 50 ng RNA in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Data were normalized by GAPDH expression. Oligonucleotide PCR primer pairs and fluorogenic probes for each gene were designed from the published sequences using Primer Express software (Applied Biosystems): IL-2 5′ primer, ACCAGGATGCTCACATTTAAGTTTT; IL-2 3′ primer, GAGGTTTGAGTTCTTCTTCTAGACACTGA; probe, 6FAM-CATGC CCAAGAAGGCCACAGAACTG. The primers were labeled with FAM at the 5′ end and TAMRA at the 3′ end (Applied Biosystems). PCR was conducted in duplicate using a PCR reagent kit (TaqMan PCR Core Reagents kit with AmpliTaq Gold; Applied Biosystems) according to the manufacturer's protocol. In brief, a master mixture including all reagents required for PCR was prepared to give final concentrations of 1× TaqMan buffer A, 5.5 μM deoxynucleotide triphosphates, 5.5 mM manganese acetate, 0.01 U/μl AmpErase UNG, and 0.025 U/μl of *Taq* Gold DNA polymerase. Hybridization probe and primers were added to give a final concentration of 100-nM probe and 200-nM primers, and the total reaction volume was increased to 50 μl. PCR was performed for 40 cycles.

Immunoblot assays

$CD4^+$ T cells were activated with anti-CD3-coated microspheres for 5 or 24 h, followed by addition of 100 ng/ml purified IL-2 or IL-21 for 15 min (R&D Systems). Cells were harvested and lysates prepared as described (19). A total of 10^6 cell equivalent/lane were loaded on 4–20% SDS-PAGE gel, run to resolution, and protein immunoblotted as described (19). Total and phosphorylated STAT-1, STAT-3, and STAT-5 were probed using rabbit anti-human Abs (Cell Signaling Technologies, Beverly, MA and Santa Cruz Biotechnology, Santa Cruz, CA). All blots were developed using goat anti-rabbit HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) and detected with Super Signal West Dura (Pierce, Rockford, IL).

Results

Activation of human T cells results in PD-1 and PD-L expression

Previous studies examined the expression of PD-1 on murine T and B cell populations and demonstrated that expression of this receptor is tightly regulated and induced upon TCR/B cell receptor (BCR) signals (5, 14). We have examined PD-1 and PD-L expression on anti-CD3/anti-CD28-activated human T cells. Cells were harvested at the indicated times, stained using anti-hu PD-1 and anti-hu PD-L1 Abs, and analyzed by flow cytometry. Minimal PD-1 and PD-L1 expression is observed in resting T cells (0 h time point). Over 50% of T cells are positive for PD-1 and PD-L1 expression 48 h after activation (Fig. 1*A*). Similar results are obtained upon anti-CD3 activation, although kinetics are slightly delayed (data not shown). Up-regulation of PD-1 and PD-L1 expression correlates with expression of other activation markers such as

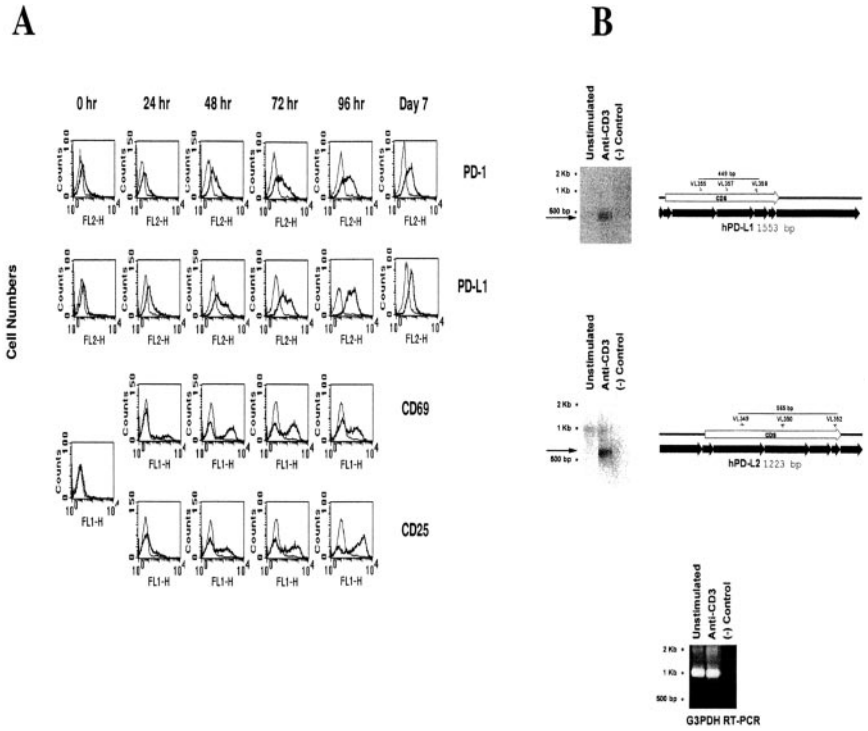

FIGURE 1. PD-1 and PD-L are expressed by human CD4$^+$ T cells upon activation. *A*, CD4$^+$ T cells were activated with protein-coated microspheres as described in *Materials and Methods*. At the indicated times, cells were harvested, washed once, and stained with either anti-huPD-1, anti-huPD-L1, anti-CD25, or anti-CD69 Abs. Control Ab isotype (thin line) was included for each staining. Cell populations were analyzed by flow cytometry using a FACScan. A representative experiment is shown ($n = 3$). *B*, RNA was prepared from unstimulated or 72 h anti-CD3 activated cells. PCR analysis was performed as described in *Materials and Methods*. Control indicates PCR performed in the absence of RNA. All RNA samples were also assessed for expression of GAPDH, a housekeeping gene.

CD25 and CD69 (Fig. 1*A*). PD-L2 was analyzed by PCR using RNA prepared from activated T cells. TCR activation results in expression of transcripts for PD-L1 and PD-L2 (Fig. 1*B*), as well as PD-1 (data not shown). Altogether, these findings indicate that T cell activation results in expression of PD-1 receptor and its ligands and, importantly, suggest that T cells can receive signals through PD-1 as well as deliver signals to other cells through their expression of PD-L.

Abs to PD-1 can act as agonists and antagonists of the pathway

PD-1 engagement by its ligand PD-L1 (PD-L1.Fc) results in inhibition of T cell proliferation and cytokine production (6). In this study, we evaluate anti-PD-1 Abs as agonists and antagonists of the PD-1/PD-L pathway. Abs PD-1–17 and PD-1–35 were characterized for their ability to recognize human PD-1 and selected, using both FACS- and ELISA-based assays, for their ability to neutralize the binding of PD-1 to its ligands PD-L1 and PD-L2 (data not shown). PD-1–17 and PD-1–35 were found to neutralize PD-1/PD-L interaction with K_D's of 5.8×10^{-9} and 6.4×10^{-10} M, respectively (data not shown).

To determine whether anti-PD-1 Abs can signal through PD-1, Ab-coated microspheres were prepared using titrated concentrations of anti-PD-1 Abs and a fixed suboptimal concentration of anti-CD3 Ab. Human CD4$^+$ T cells were stimulated with Ab-coated microspheres and proliferation was determined at 72 h. As shown in Fig. 2*A*, PD-1 engagement by PD-L1.Fc or PD1–17 Ab causes decreased T cell proliferation in a dose-dependent manner. As previously reported using immobilized PD-L1.Fc (6), CD28 costimulation can also modulate the ability of immobilized anti-PD-1 Abs to inhibit T cell proliferation; anti-PD-1 Abs inhibitory effect is observed at suboptimal but not optimal conditions of CD28 costimulation (Fig. 2*B*). These results indicate that neutralizing anti-PD-1 Abs may act as PD-1 agonists when cross-linked and trigger PD-1-mediated inhibition. To further characterize the PD-1 signaling pathway, experiments were performed to determine whether PD-1 down-regulation of T cell responses requires coordinate TCR/PD-1 engagement on a single (CIS) or a separate (TRANS) microsphere surface. CIS microspheres contained anti-CD3 Ab and PD-L1.Fc; TRANS microspheres contained either anti-CD3 or PD-L1.Fc. As shown in Fig. 2*C*, inhibition of proliferation was only observed when cells were activated with CIS microspheres. To rule out steric hindrance in the TRANS experiments, similar assays were set up using anti-CD3 Ab and B7.2.Fc; B7 costimulation of T cell responses was observed with both CIS and TRANS microspheres (data not shown). Altogether, these findings suggest PD-1 proximity to TCR is required for PD-1 modulatory function on T cell activation. Moreover, these data suggest that for the PD-1 pathway to modulate a T cell response, both activating and inhibitory signals must emanate from the same cell.

If engagement of PD-1 delivers a negative signal, we postulated that PD-1 blockade might result in enhancement of T cell responses. To test this hypothesis, PD-1 and PD-L expression were induced on CD4$^+$ T cells by activation with anti-CD3/anti-CD28-coated microspheres (Fig. 1*A*). At 72 h, microspheres were removed and cells restimulated with PHA in the presence of soluble anti-hu PD-1 Abs (Fig. 3). Up to 4-fold increases in proliferative responses are observed in the presence of soluble anti-hu PD1–17 and PD1–35 Abs (Fig. 3). Similarly, increased IFN-γ levels were also measured in these cultures (data not shown). Increased proliferative responses are dependent on the ability of anti-PD-1 Ab to block receptor/ligand interaction, as addition of a nonneutralizing anti-PD-1 Ab has no effect on proliferation or IFN-γ production (data not shown). Thus, PD-1/PD-L interactions may play a role in regulation of T-T cell interaction and anti-PD-1 Abs can act as antagonists to modulate this interaction.

PD-1-mediated inhibition overrides optimal ICOS but not CD28 costimulation

We have previously reported that optimal CD28 costimulation can override PD-1-mediated inhibition (6). In this study, we examine the effect of optimal ICOS costimulation on TCR/PD-1 signaling. Microspheres were prepared using suboptimal anti-CD3 Ab and optimal PD-L1.Fc, ICOS-L.Fc, and B7-2.Fc concentrations and used to stimulate purified CD4$^+$ T cells. Summarized results ob-

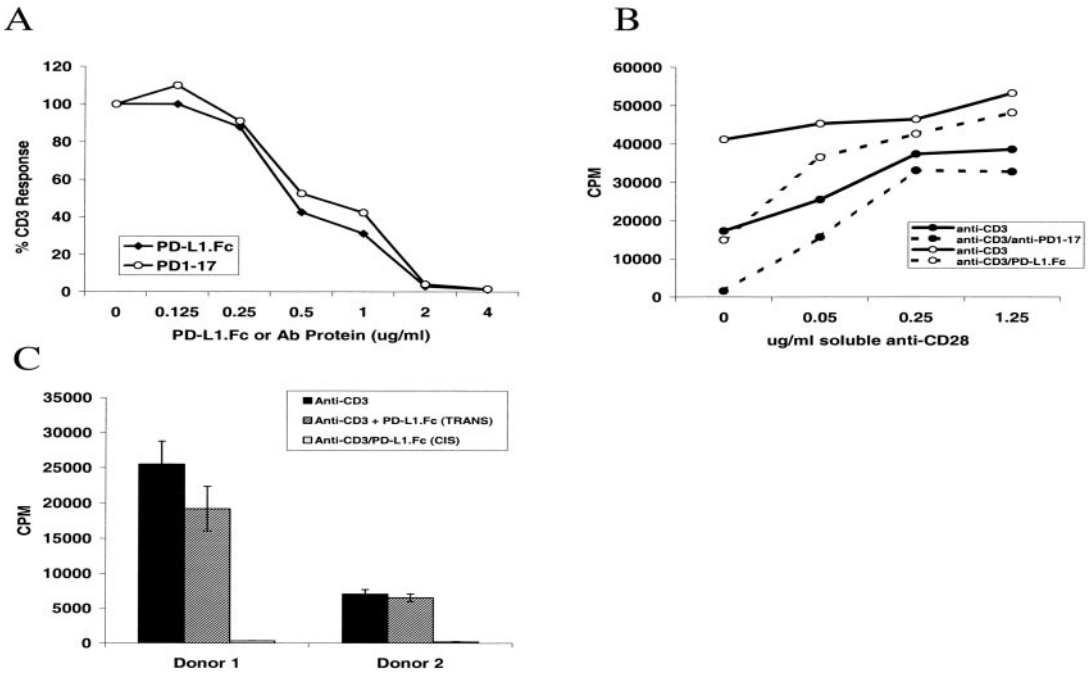

FIGURE 2. Anti-PD-1 Ab can deliver a negative signal to human $CD4^+$ T cells. *A*, Purified $CD4^+$ T cells (10^5 cells/well) were activated with microspheres coated with a fixed concentration of anti-CD3 Ab (1 μg/10^7 microspheres) and increasing concentrations of PD-L1.Fc or PD1–17 Ab. Activation in the absence of PD-1 engagement was determined using microspheres containing anti-CD3 Ab (1 μg) and murine IgG (4 μg/10^7 microspheres). For comparison purposes, values are represented as the percentage of anti-CD3 Ab response, where 100% represent cpm obtained when cells were activated with anti-CD3/murine IgG-coated microspheres. A representative experiment is shown ($n = 3$). *B*, Cells (10^5 cells/well) were activated with anti-CD3, anti-CD3/PD-L1.Fc, and anti-CD3/anti-PD1–17 microspheres in the presence of increasing concentrations of soluble anti-CD28 Ab. Two sets of anti-CD3 microspheres are shown, one for each of the corresponding isotypes (muIgG2a for PD-L1.Fc and huIgG1 for anti-PD1–17). Proliferation was measured at 72 h. Similar results were obtained with multiple donors ($n = 3$). *C*, Cells (10^5 cells/well) were activated with anti-CD3, anti-CD3/PD-L1.Fc (CIS), and anti-CD3 + PD-L1.Fc (TRANS) microspheres. CIS microspheres contained both anti-CD3 and PD-L1.Fc on the same bead, TRANS microspheres consist of two beads, one containing anti-CD3, and one containing PD-L1.Fc. To maintain equal bead to cell ratios in CIS and TRANS conditions, microspheres coated with muIgG control were added to CIS cultures. In all cases, control murine Ig was added to achieve a total protein concentration of 5 μg/10^7 microspheres. Proliferation was measured at 72 h. Similar results were obtained with multiple donors ($n = 6$).

tained with nine donors are shown in Fig. 4*A*. Costimulation through ICOS (anti-CD3/ICOS-L.Fc microspheres) or CD28 (anti-CD3/B7.2.Fc microspheres) engagement resulted in enhanced T cell proliferation compared with stimulation by anti-CD3 alone (Fig. 4*A*). Interestingly, engagement of PD-1 in the presence of optimal CD28 costimulation (anti-CD3/B7.2.Fc/PD-L1.Fc) has minimal effect on proliferation (Fig. 4*A*). In contrast, the ICOS costimulatory effect is negligible in the presence of PD-1 engagement; proliferative responses upon PD-1/ICOS-L activation are similar to those obtained with anti-CD3 alone. Thus, costimulatory signals mediated by ICOS are more susceptible to PD-1-mediated negative regulation than those provided by CD28 costimulation.

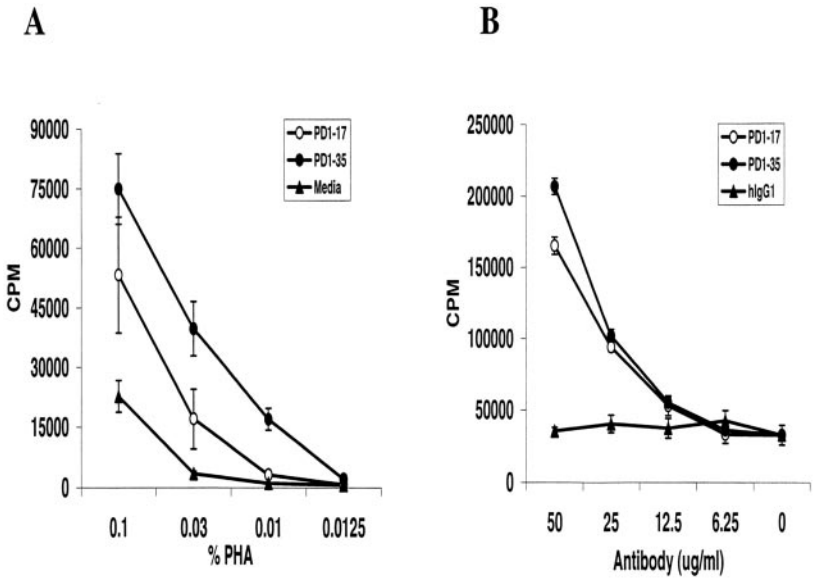

FIGURE 3. Soluble anti-PD-1 Abs enhance T-T cell responses. $CD4^+$ T cell were preactivated for 72 h with anti-CD3/anti-CD28-coated microspheres, harvested, and restimulated with increasing concentrations of PHA in the presence of IL-2 (10 ng/ml) and anti-PD-1 Ab (20 μg/ml) (*A*) or increasing concentrations of anti-PD-1 Ab in the presence of 0.1% PHA + 10 ng/ml IL-2 (*B*). Proliferation was measured at 72 h ($n = 3$).

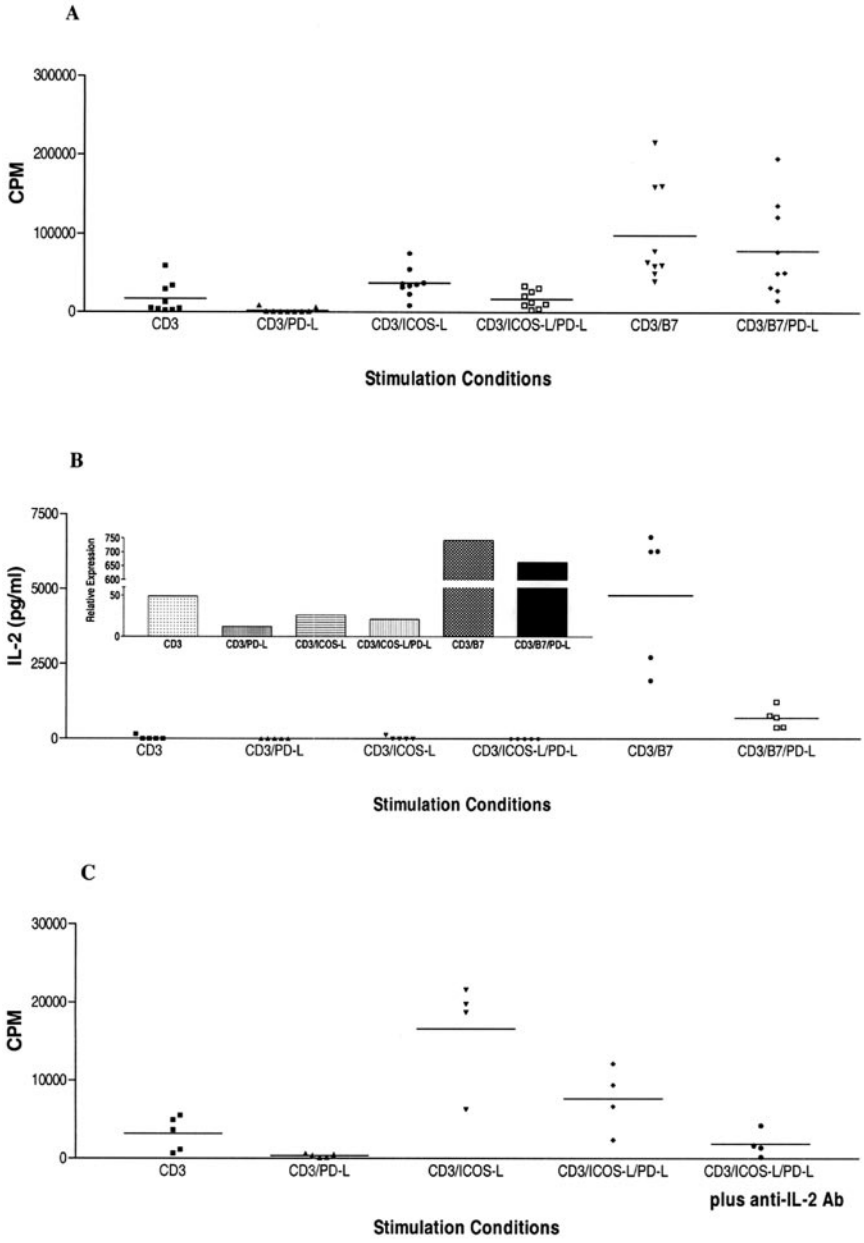

FIGURE 4. ICOS costimulation can be prevented by PD-1 engagement. *A*, $CD4^+$ T cells were activated with microspheres coated with anti-CD3/control Ig (1 μg/4 μg/10^7 microspheres), anti-CD3/PD-L1.Fc/control Ig, anti-CD3/ICOS-L.Fc/control Ig, anti-CD3/B7-2.Fc/control Ig, anti-CD3/PD-L1.Fc/ICOS-L.Fc, or anti-CD3/PD-L1.Fc/B7-2.Fc (1 μg/2 μg/2 μg/10^7 microspheres). Microsphere composition is indicated in the chart below *x*-axis. Proliferation was measured at 72 h. Statistical analysis indicated that CD3/PD-L ($p = 0.0386$), CD3/ICOS-L ($p = 0.0393$), CD3/B7 ($p = 0.0019$), and CD3/B7/PD-L ($p = 0.0099$) responses differed significantly from CD3 responses; in contrast, no statistical significance was observed between CD3/ICOS-L/PD-L ($p = 0.9836$) and CD3 responses. *B*, Supernatants were harvested at 24 h and IL-2 production analyzed by ELISA (sensitivity 20 pg/ml). *Inset*, IL-2 transcript relative expression under stimulation conditions. Values are normalized for GAPDH expression ($n = 2$). *C*, $CD4^+$ T cells were activated as in *A* in the presence of a neutralizing murine anti-human IL-2 Ab (20 μg/ml) or control murine IgG. Microsphere composition and presence of neutralizing IL-2 Ab is indicated in the figure below the *x*-axis. Statistical analysis indicated that CD3/PD-L ($p = 0.0209$), CD3/ICOS-L ($p = 0.0045$) responses differed significantly from CD3 responses; no statistical significance was observed between CD3/ICOS-L/PD-L ($p = 0.0759$) and CD3 responses. CD3/ICOS-L/PD-L plus anti-IL-2 Ab ($p = 0.0419$) responses differed significantly from CD3/ICOS-L/PD-L responses. Proliferation was measured at 72 h. Summaries of results obtained with four to eight donors are shown. Horizontal lines represent mean value for each condition.

ICOS costimulation has been reported to induce low to marginal levels of IL-2 production (20), and exogenous IL-2 rescues PD-1 inhibition (14). Therefore, we reasoned that IL-2 levels induced upon ICOS engagement may be insufficient to override PD-1 inhibition. A summary of IL-2 production levels obtained with five donors under the various stimulatory conditions is shown in Fig. 4*B*. At 48 and 72 h after activation, IL-2 protein could only be measured in cultures activated in the presence of CD28 costimulation (Fig. 4*B*, and data not shown). Under conditions of PD-1 engagement, T cell proliferative responses are rescued by CD28 costimulation despite a dramatic reduction in IL-2 levels (Fig. 4, *A* and *B*); IL-2 levels equal or above 700 pg/ml are sufficient to account for full restoration of proliferation (Fig. 4, *A* and *B*). No IL-2 protein is detected in ICOS-stimulated cultures (Fig. 4*B*). However, IL-2 transcripts are detected in ICOS-stimulated cultures suggesting that IL-2 produced under these conditions is below assay detection limits (Fig. 4*B*, *inset*). Altogether, these findings are consistent with the notion that ICOS costimulation results in IL-2 levels that are sufficient to drive proliferation upon TCR activation but are insufficient to overcome PD-1-mediated inhibition.

Does IL-2 produced during ICOS activation account for the partial rescue of PD-1 inhibition? To address this question, the effect of neutralizing anti-IL-2 Ab on ICOS/PD-1 activation was examined. Anti-hu IL-2 Ab was added at the initiation of cultures at concentrations sufficient to neutralize 20 ng/ml of exogenous IL-2 (data not shown). Anti-IL-2 abrogates the ability of ICOS costimulation to modulate PD-1 inhibition (Fig. 4*C*). Thus, PD-1 engagement can prevent ICOS but not CD28 costimulation. The inability of ICOS costimulation to override PD-1 inhibition is directly related to the low IL-2 levels it induces upon its engagement.

Modulation of the PD-1/PD-L pathway by IL-2 and other members of the γ common receptor cytokine family

The effect of CD28 and ICOS costimulation on PD-1 inhibition suggests that IL-2 plays a crucial role in modulation of the PD-1/PD-L pathway. Indeed, as shown in Fig. 5*A*, exogenous IL-2

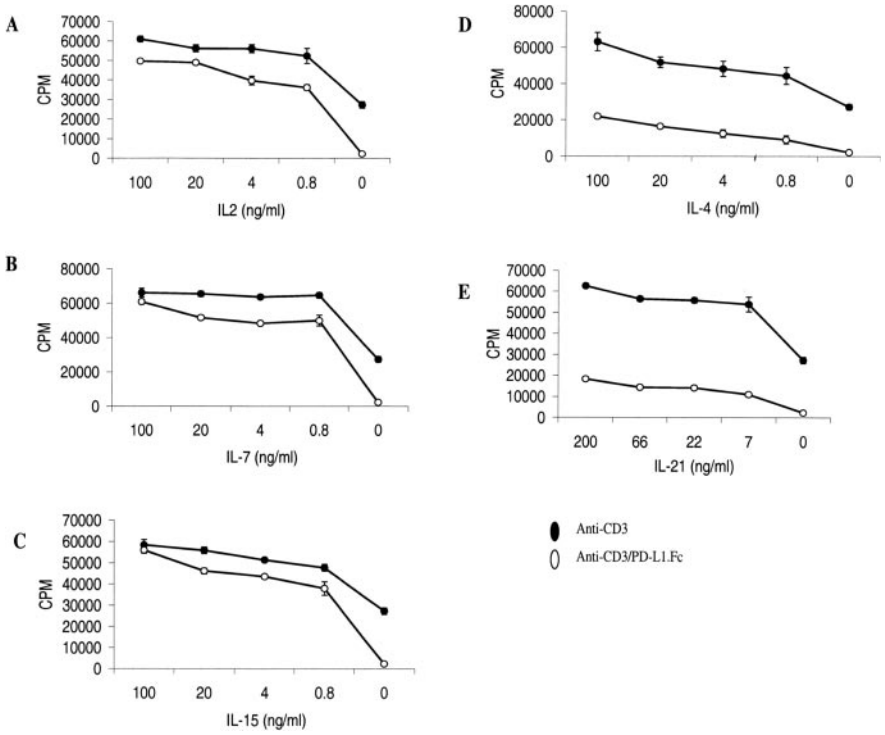

FIGURE 5. IL-2, IL-7, and IL-15 but not IL-4 or IL-21 rescue PD-1-mediated inhibition. $CD4^+$ T cells were activated with microspheres coated with anti-CD3 (●) or anti-CD3/PD-L1.Fc (○) in the presence of increasing concentrations of IL-2 (*A*), IL-7 (*B*), IL-15 (*C*), IL-4 (*D*), and IL-21 (*E*). Proliferation was measured at 72 h. A representative experiment is shown ($n = 6$).

rescues human T cells from PD-1-mediated inhibition, a finding consistent with our report on murine T cells (14). As IL-2 shares structural homology with IL-4, IL-7, IL-15, and IL-21 and these cytokines mediate their effect through receptors that associate with the γ common cytokine receptor chain (21), we examined whether any of these cytokines could rescue PD-1-mediated inhibition. T cell cultures were activated with anti-CD3- or anti-CD3/PD-L1.Fc-coated microspheres in the presence of various concentrations of IL-4, IL-7, IL-15, and IL-21 and proliferation was measured at 72 h. As shown in Fig. 5, *B–E*, all cytokines enhanced T cell proliferation upon anti-CD3 Ab activation. However, upon anti-CD3/PD-L1.Fc activation, only IL-2, IL-7, and IL-15 restored T cell proliferation to levels equal to those obtained with anti-CD3 alone (Fig. 5, *B* and *C*). Neither IL-4 nor IL-21 can fully restore proliferative responses upon PD-1 engagement (Fig. 5, *D* and *E*). These results suggest that differences in signaling by these cytokines may account for their ability or inability to rescue PD-1 inhibition.

IL-4 activates STAT6 phosphorylation, whereas IL-2, IL-7, and IL-15 activate STAT5 phosphorylation upon binding their receptors (21). STATs involved in IL-21 signaling on primary T cells have not been investigated. To gain insight into the mechanism involved in cytokine-mediated PD-1 rescue, we analyzed STAT phosphorylation of cytokines that do (IL-2) and do not (IL-21) rescue PD-1-mediated inhibition. Purified $CD4^+$ T cells were preactivated with anti-CD3-coated microspheres for 5 or 24 h, IL-2 or IL-21 (100 ng/ml) added for 15 min, and lysates prepared. Cell lysates were immunoblotted for total and phosphorylated STAT1, 3, and 5. As shown in Fig. 6*A*, both IL-21 and IL-2 induced phosphorylation of STAT1, although with different kinetics. IL-21-induced STAT1 phosphorylation can be observed as early as 5 h after anti-CD3 activation; IL-2-induced phosphorylation is observed at 24 h. STAT3 phosphorylation is only detected upon IL-21 stimulation, whereas STAT5 activation is only detected upon IL-2 stimulation (Fig. 6, *B* and *C*). Neither IL-4 nor IL-21 activate STAT5, nor can they can rescue PD-1-mediated inhibition. These findings support the notion that within the γ common receptor cytokine family only those that activate STAT5 phosphorylation can modulate the PD-1/PD-L pathway.

STAT5 has been implicated in maintaining IL-2Rα expression (22). Next, we investigated the effect of the various cytokines on IL-2Rα (CD25) expression. As shown in Table I, anti-CD3 and anti-CD3/PD-L1.Fc activation of $CD4^+$ T cells by IL-2, IL-7, and IL-15 results in CD25 up-regulation. In contrast, neither IL-4 nor IL-21 have any effect on CD25 expression. Taken together these results support a link between STAT5 activation, up-regulation of CD25 expression, and the ability of a cytokine to rescue PD-1 inhibition.

Discussion

We (6, 7) and others (8, 23) have previously examined the functional consequences of PD-1 engagement on murine and human T

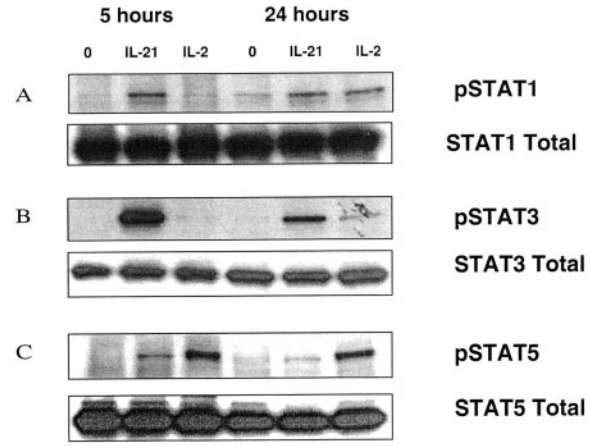

FIGURE 6. IL-21 activation of primary human $CD4^+$ T cells does not result in STAT5 phosphorylation. $CD4^+$ T cells were activated with anti-CD3 Ab-coated microspheres for 5 and 24 h followed by addition of 100 ng/ml IL-2 or IL-21 for 15 min. Cell lysates were immunoblotted and analyzed for total and phosphorylated STAT1 (*A*), STAT3 (*B*), and STAT5 (*C*) as described in *Materials and Methods* ($n = 3$).

Table I. *Treatment of T cells with IL-2, IL-7, and IL-15 but not IL-4 and IL-21, leads to up-regulation of IL-2Rα chain*[a]

	Media		IL-2		IL-4		IL-7		IL-15		IL-21	
	Isotype	Anti-CD25	Isotype	Anti-CD25	Isotype	Anti-CD25	Isotype	Anti-CD25	Isotype	Anti-CD25	Isotype	Anti-CD25
Anti-CD3	3.44	96.75	5.35	**736.86**	3.5	94.12	5.31	**622.07**	4.69	**488.4**	4.16	100.12
Anti-CD3/PD-L1.Fc	3.12	46.38	4.49	**307.03**	4.05	51.41	4.18	**246.99**	4.46	**272**	4.34	51.42

[a] $CD4^+$ T cells were activated with anti-CD3 Ab-coated microspheres in the presence of optimal concentrations of the indicated cytokines. At 72 h, cells were harvested and stained for IL-2Rα (CD25) expression as described in *Materials and Methods* ($n = 2$). Mean fluorescence values are shown, bold numbers indicate increases 4-fold above media control.

cells. In the present study, we have characterized anti-human PD-1 Abs for their agonist and antagonist functions and assessed the effect of ICOS and IL-2 family cytokine members on PD-1-mediated inhibition of T cell proliferation. We find that neutralizing anti-PD-1 Abs act as pathway agonists when cross-linked; attenuation of T cell responses by PD-1 requires coengagement of TCR and PD-1 by the same cell. Additionally, soluble anti-PD-1 Abs can act as antagonists of the pathway and enhance T-T cell responses. ICOS costimulation is more susceptible to PD-1 inhibition than CD28 costimulation and among IL-2 cytokine family members only those that signal through STAT5 can rescue PD-1-mediated inhibition.

PD-1 is expressed on T and B cells (5). The phenotype of PD-$1^{-/-}$ mice suggests that the PD-1/PD-L pathway plays a critical role in T and B cell homeostasis and its deficiency leads to deregulation of T and B cell functions (12, 13, 24). The mechanism by which the PD-1/PD-L pathway regulates homeostasis remains unclear; however, a crucial role has been assigned to the interaction of PD-1 on T or B cells with PD-L on APCs and nonlymphoid tissues. We show in this study that human T cells express both PD-1 receptor and PD-L1 and PD-L2 ligands and demonstrate that T-T responses can be modulated upon PD-1/PD-L engagement. Blockade of PD-1/PD-L interaction with antagonistic anti-PD-1 Abs leads to enhance T cell proliferative responses, a finding consistent with a down-regulatory role for the PD-1 pathway in T-T interactions. This finding is significant in light of human T cells expressing MHC class II upon activation and their reported capacity to present Ag to other T cells. Additionally, one could postulate that T-B cell responses could be regulated bidirectionally by the PD-1 pathway and may serve to limit BCR as well as TCR signaling. Studies using purified PD-$1^{-/-}$ murine T and B cells populations should help clarify which interactions are relevant in vivo for limiting immune responses and maintaining peripheral tolerance.

Engagement of PD-1 by either its ligand or anti-PD-1 Abs leads to inhibition of T cell responses. Only those anti-hu PD-1 Abs able to bind PD-1 at its ligand-binding site could function as agonists. Furthermore, agonistic Ab/ligand function requires both TCR and PD-1 signals to be delivered on the same surface. These results are similar to those reported for CTLA-4 function, as CTLA-4-mediated down-regulation also requires coengagement of TCR and CTLA-4 on the same surface (25). On B cells, Okazaki et al. (26) has shown that BCR down-regulation by PD-1 requires coligation of receptors and results in Src homology 2 protein tyrosine phosphatase-2 recruitment and deactivation of downstream signaling molecules such as phosphatidylinositol 3-kinase and extracellular signal-regulated kinase. Altogether, these results support a "modified proximal signal" model (25) in which PD-1 engagement results in activation of the phosphatase Src homology 2 protein tyrosine phosphatase-2 (7) and modification of early TCR phosphorylation events. Our results suggest that in the case of T cells, physical proximity between positive (TCR) and negative (PD-1) signals is required for PD-1-mediated inhibition, suggesting that PD-1 modulation of Ag responses in vivo requires a strict copresentation of Ag and PD-L by the APC. Altogether, our studies with immobilized and soluble anti-PD-1 Abs strongly suggest PD-1 functions as negative regulator of T cell responses. However, these findings do not rule out the possible existence of a second receptor for PD-L ligands and its potential role in costimulation of T cell responses as shown by Dong et al. (23, 27)and Tseng et al. (8).

Induction of IL-2 upon CD28 engagement has been well documented (28) and $CD28^{-/-}$ T cells have diminished IL-2 production (29). Furthermore, IL-2 production in responses to CD28 engagement is central to CD28 ability to rescue PD-1 inhibition (14). In contrast, ICOS costimulation results in IL-2 levels sufficient for initial proliferation but insufficient for sustained growth (20), and we report in this study that costimulation provided by ICOS signaling is susceptible to PD-1 inhibitory effect. By several criteria, our data suggest that low levels of IL-2 produced upon ICOS costimulation account for the susceptibility of this pathway to PD-1 inhibition: ICOS signals induce negligible levels of IL-2 protein, and the modest modulatory effect of ICOS engagement on PD-1-mediated inhibition is abrogated in the presence of neutralizing anti-IL-2 Ab. Additionally, exogenous IL-2 (as low as 10 pg/ml) can rescue PD-1 inhibition. Similarly, a study by Riley and coworkers (20) demonstrates that ICOS engagement cannot prevent inhibition by CTLA-4, and they have proposed that one of ICOS main functions is to initiate T cell responses from nonprofessional APCs in the periphery. As PD-1 ligands PD-L1 and PD-L2 are expressed at sites of inflammation by nonprofessional APCs (6, 7), our findings suggest that PD-1 may be the main attenuator of T cell activation at these sites. Furthermore, our in vitro results suggest that PD-1 signals will override ICOS costimulation and consequently down-regulate T cell responses in vivo at peripheral sites.

We have shown that exogenous IL-2 rescues PD-1-mediated inhibition in murine $CD4^+$ and $CD8^+$ T cells (14); in this study, we extend these observations to human T cells. Furthermore, by examining other cytokines that use the γ common chain as part of their receptor, we find that IL-2, IL-7, and IL-15 can restore proliferative responses upon PD-1 engagement, whereas IL-4 or IL-21 cannot. Binding of IL-2 and IL-15 to their receptors results in Janus kinase-mediated phosphorylation of IL-2Rβ docking sites, recruitment of STAT5, and its subsequent phosphorylation (21). IL-4, in contrast, activates STAT6 phosphorylation (21). We find that IL-21, a novel cytokine whose receptor shares the common γ chain, activates STAT1 and STAT3 but not STAT5 on primary T cells. Thus, our results in T cells indicate that among IL-2 cytokine family members only those that signal through STAT5 (IL-2, IL-7, and IL-15) can restore proliferative responses upon PD-1 engagement. Furthermore, treatment of cells with IL-2, IL-7, and IL-15 results in up-regulation of IL-2Rα chain expression. As IL-2Rα up-regulation has been reported to be dependent on STAT5 transcriptional regulation (22, 30), our findings suggest that selected

members of the γ common receptor cytokines may be able to modulate the PD-1 pathway by maintaining high-affinity IL-2R expression, thus enhancing IL-2-dependent cell growth.

IL-21, a novel cytokine whose receptor shares the common γ chain, has been reported to activate STAT5 on a pro-B cell line, Ba/F3, expressing a chimeric EPO/IL-21R and primary murine splenic B cells (31–33). In contrast, Asao et al. (34) reports that in an immortalized T cell line, IL-21 treatment activates STAT1 and 3 but not STAT5. In agreement with this latter report, we find that in primary human $CD4^+$ T cells, IL-21 activates STAT1 and STAT3 but not STAT5. Thus, our results and those previously reported (31–34) strongly support the notion that IL-21 activates different STATs in T and B cells, with STAT5 activation only occurring in B cells. Therefore, the effects of IL-21 on the PD-1 pathway may differ between B and T cells; IL-21 may rescue PD-1 inhibition on B, but not T, cells via activation of STAT5.

In summary, our data suggest that the modulation of immune responses by PD-1 will depend upon the type of costimulatory signal delivered, the cytokine milieu, and the signaling pathways activated in the responding cell. PD-1 cannot attenuate immune responses when costimulatory signals induce high IL-2 levels. By contrast, under costimulatory conditions where IL-2 production is limited, i.e., ICOS-ICOS-L, attenuation of immune responses by PD-1 predominates. Interestingly, the cytokine environment may also play a critical role, as cytokines such as IL-15 and IL-7 also rescue PD-1-mediated inhibition. These findings suggest that at peripheral sites of inflammation, PD-1 inhibition can be overcome by expression of non-T cell-derived cytokines, such as IL-15. Finally, the observation that only cytokines that induce STAT5 activation can reverse PD-1-mediated inhibition proposes a connection between inhibitory receptors and cytokine signaling.

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