Programmed Death-1 Targeting Can Promote Allograft Survival

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The two-signal hypothesis of T cell activation (1), involving an MHC-bound peptide on an APC delivering signal 1 via the TCR and the generation of signal 2 via a distinct T cell surface molecule, has remained useful in the light of ongoing research into the molecular basis of T cell activation. CD28 is the best established signal 2-generating receptor on primary T cells (2), and ligands of CD28, B7.1 (CD80), and B7.2 (CD86), are expressed by APC de novo or up-regulated upon activation (3). CD28 expression is constitutive, such that extra steps, including ligation of APC-expressed CD40 by CD154, which is only transiently expressed by activated T cells (4, 5), and ligation of TNFR family members by their respective ligands (6), provide further degrees of control. In contrast, CTLA4 in its Ig variable (IgV)-like domain (15), is expressed by 55-kDa glycosylated surface receptor protein with 23% identity to PD-1, a 50- to 65-kDa glycosylated surface receptor that is normally expressed by circulating T cells by activation through the TCR (16). PD-1, a 50- to 65-kDa glycosylated surface receptor protein with 23% identity to CD28 homolog, programmed death-1 (PD-1), and its two known ligands.

Therapy with cyclosporin A or rapamycin, but was prevented in the early posttransplant period by costimulation blockade using CD154 or anti-inducible costimulator mAb. We prepared PD-L1.Ig and PD-L2.Ig fusion proteins and showed that each bound to activated PD-1+ T cells and inhibited T cell functions in vitro, thereby allowing us to test the effects of PD-1 targeting on allograft survival in vivo. Neither agent alone mediated allograft rejection in wild-type recipients. However, use of PD-L1.Ig administration in CD28+/− recipients, or in conjunction with immunosuppression in fully MHC-disparate combinations, markedly prolonged cardiac allograft survival, in some cases causing permanent engraftment, and was accompanied by reduced intragraft expression of IFN-γ and IFN-γ-induced chemokines. PD-L1.Ig use also prevented development of transplant arteriosclerosis post-CD154 mAb therapy. These data show that when combined with limited immunosuppression, or in the context of submaximal TCR or costimulatory signals, targeting of PD-1 can block allograft rejection and modulate T and B cell-dependent pathologic immune responses in vivo. The Journal of Immunology, 2002, 169: 6546–6553.

Additional inducible CD28 and B7 homologs are also recognized (8). One pathway, involving up-regulation of a third CD28 family member, inducible costimulator (ICOS) (9–11), on activated T cells, and its ligation by B7-related protein-1 (12, 13) on APCs, is important to the generation and maintenance of memory and effector T cell functions (13). Targeting of this pathway can prevent development of acute or chronic cardiac allograft rejection (14). The current work focuses on a recently identified fourth CD28 homolog, programmed death-1 (PD-1), and its two known ligands.

PD-1 cDNA was first cloned from T cells undergoing apoptosis after exposure to phorbol ester and ionomycin (15). PD-1, a 50- to 55-kDa glycosylated surface receptor protein with 23% identity to CTLA4 in its Ig variable (IgV)-like domain (15), is expressed by activated T, B, and myeloid cells. With regard to T cells, PD-1 is normally expressed by <5% of thymocytes in adult mice but is induced on circulating T cells by activation through the TCR (16). PD-1 has a single extracellular Ig-like variable domain (IgV) (17) and an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain (18). ITIMs occur in the cytoplasmic domains of multiple regulatory immune receptors, including CD22 and FcγRIIB on B cells and killer Ig-related receptors on NK cells (19), and bind inhibitory phosphatases (20). Gene knockout studies show that depending upon the background strain, PD-1−/− mice develop increased rates of glomerulonephritis (19), lupus-like arthritis (19), and autoimmune cardiomyopathy (21), indicating a role for PD-1 like that of CTLA-4 in maintenance of self-tolerance.

Abbreviations used in this paper: ICOS, inducible costimulator; PD-1, programmed death-1; IgV, Ig variable; ITIM, immunoreceptor tyrosine-based inhibitory motif; CsA, cyclosporin A; RPM, rapamycin; DST, donor-specific transfusion; RPA, RNase protection assay; MIP, macrophage-inflammatory protein; IP-10, IFN-γ-inducible protein-10; SHP, Src homology protein.
addition, compared with wild-type mice, T cells from PD-1−/− 2C-TCR (anti-H-2Lδ) transgenic mice exhibit increased in vitro proliferative responses to allogeneic cells, suggesting a role for PD-1 in the regulation of alloresponses (19).

Recently, PD-L1 (B7-H1) (22, 23) and PD-L2 (B7-DC) (24, 25) were identified as ligands for PD-1. Both are members of the B7 superfamily, with 38% amino acid identity between murine PD-L1 and PD-L2, and, like other B7 family members, have characteristic extracellular IgV- and Ig constant-like domains (8). With relevance to our study, the in vitro proliferation and cytokine production of CD3 mAb-stimulated PD-1− T cells can be inhibited by the addition of PD-L1.Ig (23) or PD-L2.Ig (24). Like PD-1, neither ligand is well expressed by resting leukocytes, but both PD-L1 (15) and PD-L2 (24, 25) are induced upon culture of monocytes and dendritic cells in the presence of IFN-γ and/or LPS (18).

In this study, we determined the murine tissue distribution of PD-1 and its ligands and analyzed the kinetics of intragraft PD-1, PD-L1, and PD-L2 mRNA expression following transplantation in unmodified recipients, as well as in recipients receiving immunosuppressive therapy with cyclosporin A (CsA) or rapamycin (RPM) or costimulation blockade. Based upon this expression data, we constructed PD-L1.Ig and PD-L2.Ig fusion proteins and tested the effect of targeting PD-1 in vivo on allograft rejection in various models of cardiac and skin transplantation. The results show that ligation of PD-1 can decrease TCR- and CD28-dependent cytokine and chemokine production in vivo and promote allograft survival, especially in models in which full-scale T cell activation is prevented by concomitant reduction in signal 1 or signal 2, or when the effects of cytokine receptor signaling are diminished by concomitant limited immunosuppression with RPM.

Materials and Methods

Reagents and fusion proteins

We purchased CsA and RPM (Sigma-Aldrich, St. Louis, MO), hamster anti-mouse CD154 mAb (BioExpress, West Lebanon, NH), and control human (Sigma-Aldrich), hamster, and rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Rat anti-mouse ICOS mAb was described previously (14). Murine PD-L1.Ig and PD-L2.Ig fusion CDNA clones were prepared by fusing the sequences corresponding to the first 232 aa of PD-L1 and the first 220 aa of PD-L2 to the C terminus of human IgG in a pcdNA3.1 vector (26). Constructs included an upstream Kozak sequence and a stop codon after the Cc coding sequence and were inserted into a mammalian expression vector containing antibiotic selection markers, the SV40 origin of replication, and an EF1α promoter sequence to drive fusion protein expression. Constructs were transiently transfected into 293T cells using Lipofectamine 2000 (Life Technologies, Rockville, MD), and supernatants conditioned for 3 days in serum-free medium were prepared and a log-rank comparison was used to calculate p values.

Cloning of murine PD-1, PD-L1, and PD-L2 cDNA

We used the ProStar RT-PCR System (Stratagene, La Jolla, CA) for cDNA generation and amplification of cDNAs. Full-length PD-1 cDNA was amplified from thymi cDNA by primers 5′-GCAATTCCTCACCATGTCGGGTCGCGCCAGGATCCACTCAGATTCTGCACATGG-3′ and 5′-TCTCTGTTGAATAAAAAAGGCCCAAGAACAATGACCATCC-3′ to introduce an upstream Kozak sequence, stop codon, and upstream EcoRI and downstream XbaI sites. The 895-bp PD-1 cDNA fragment was gel-purified and cloned into a Sf I-cutter pCR-Script (BlueScript) vector (Stratagene). Full-length PD-L1 cDNA was amplified from rejecting cardiac allograft RNA by primers 5′-CCGAATTTCCACTCCAGGATATTTGCTGGCC-3′ and 5′-TGGCTCTAGATCGTCCTCGAATTTGATGCATC-3′ to introduce an upstream Kozak sequence, stop codon, and upstream EcoRI and downstream XbaI sites. The 898-bp PD-L1 cDNA fragment was gel-purified and cloned into a pCR-Script vector. Full-length PD-L2 cDNA was amplified from rejecting cardiac allograft RNA by primers 5′-GGAACCTGATACAGGAGGA-3′ and 5′-TGGCTCTAGATCGTCCTCGAATTTGATGCATC-3′ to introduce an immediately upstream Kozak sequence, stop codon, and upstream EcoRI and downstream XbaI sites. This eliminated extra 5′ and 3′ noncoding sequences in the 1368-bp PD-L2 cDNA clone and reduced the PD-L2 cDNA to 768 bp; the cDNA fragment was gel-purified and cloned into a pCR-Script vector.

Northern analysis of PD-1, PD-L1, and PD-L2 mRNA

We purchased Northern blots containing 2 μg/lane of poly(A)+ RNA from 12 major mouse tissues (OriGene Technologies, Rockville, MD). We also prepared total RNA from each cardiac graft using acid-guanidinium thiocyanate-phenol-chloroform (31); 25 μg RNA was loaded per lane of 1.2% agarose-formaldehyde gels, and a 0.24- to 9.5-kb RNA ladder (Life Technologies) was used as a size control. After electrophoresis, RNA was blotted overnight onto Nytran Supercharge membranes (Schleicher & Schuell, Keene, NH) with 20× SSC and cross-linked to membranes by irradiation with UV light using Stratalinker (Stratagene). 32P-labeled probes were prepared from full-length PD-1 and PD-L1 cDNAs and from a partial-length PD-L2 cDNA fragment. northern blots were hybridized with 32P-labeled probes at 68°C in hybridization buffer (6× SSC, 0.1% SDS) at 68°C. Membranes were washed twice in 2× SSC, 0.1% SDS at 70°C, and exposed to film to assure complete removal of previous hybridization signals.

RNase protection assay (RPA)

Intragraft mRNA levels of cytokines (mTk-1), chemokines (mTk-5), and CCR- (mCr-5) and CXCR-type (mCr-6) chemokine receptors were quantitated by RPA according to the manufacturer’s instructions (BD PharMingen). Briefly, RNA samples (20 μg) were hybridized with complement [32P]UTP labeled riboprobes, digested with RNase A, RNase T1, and proteinase K, separated on denaturing polyacrylamide gels, and detected by autoradiography, followed by normalization to GAPDH gene expression (14).

Transplant protocols

Male 6 wk-old BALB/c (H-2b), C57BL/6 (H-2b), and CD28−/− (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. Heterotopic abdominal cardiac grafts were performed as described (29), and the role of PD-1 in acute allograft rejection was explored by daily i.p. injection of 100 μg of PD-L1.Ig or PD-L2.Ig or control IgG1 alone, or in conjunction with CsA (10 mg/kg/day) or RPM (1 mg/kg/day), for 14 days posttransplant. For studies of PD-1 expression post-costimulation blockade, allografts recipients were treated with 1) CD154 mAb or hamster IgG (250 μg, i.p.) with or without donor-specific transfusion (DST; 5×106 splenocytes, i.v.) at engraftment (30) or 2) anti-ICOS mAb or control rat IgG (200 μg, i.p.) every 48 h from days 1 to 14 posttransplant (14). Involvement of PD-1 in chronic rejection, which develops reproducibly post-CD154 mAb therapy (14), was studied by injection of CD154 mAb i.p. at transplant plus 14 days of therapy with PD-L1.Ig or IgG1. Kaplan-Meier survival graphs were prepared and a log-rank comparison was used to calculate p values.

In vitro studies

Mice expressing the transgene encoding the DO11.10 TCR, which recognizes chicken OVA amino acids 323–339 in association with I-Ak (27), were provided by D. Loh (Washington University, St. Louis, MO). OVA-specific TCR-transgenic CD4+ T cells were isolated from the spleen using negative-selection columns (R&D Systems, Minneapolis, MN). APCs were prepared from mitomycin C-treated (50 μg/ml, 30 min) BALB/c splenocytes. OVA-specific CD4+ T cells (2×106/ml) were stimulated for 5 days with APCs (2×106/ml) and the peptide OVA232-243 (0.3 μg/ml) in 96-well flat-bottom plates precoated overnight with PD-L1.Ig, PD-L2.Ig, or control human IgG1 (5 μg/ml), then washed and aliquoted into new 96-well plates. Activated CD4+ T cells (2×105/well) were restimulated with fresh APCs (2×105/well) and OVA (1 μg/ml) for another 2 days in the presence of immobilized fusion proteins or control Ig. Culture supernatants were collected and tested for IFN-γ, TNF-α, and IL-10 cytokine production using commercial ELISA kits (BD Pharmingen, San Diego, CA) (28).
Paraffin sections were stained with H&E to assess overall cellularity and myocardial preservation, and trichrome and elastin stains to assess interstitial fibrosis and development of transplant arteriosclerosis. Transplant arteriosclerosis within elastin-stained arteries (five sections per graft and five grafts per group) was scored as follows: 0, <5% occlusion; 1, 5–20%; 2, >20–40%; 3, >40–60%; 4, >60–80%; or 5, >80–100% (29).

Expression of PD-1 and its ligands was evaluated by immunoperoxidase staining of cryostat sections with an Envision-peroxidase kit (DAKO, Carpinteria, CA), using hamster anti-mPD-1 mAb (32) (J43; BD PharMingen), rat anti-mPD-L1, and rat anti-mPD-L2 mAbs, plus isotype-matched controls. The mAbs to PD-L1 (1F7, 3H1) and PD-L2 (BH7, 8C11) were generated by s.c. gene-gun immunization with cDNA, followed by fusion, screening of supernatants by ELISA, repeated cloning, and confirmation of specificity using transient cell transfectants (14). These anti-PD-1 ligand mAbs are useful for immunopathology but, as with J43, the only currently available PD-1 mAb, do not appear to block PD-1/ligand binding or generate inhibitory signals within PD-1+ T cells.

**Results**

**Tissue-specific expression of PD-1, PD-L1, and PD-L2**

Northern analysis using poly(A)+ RNA showed differences in the expression of PD-1, PD-L1, and PD-L2 mRNA in murine tissues (Fig. 1). PD-1 mRNA was detected primarily in the thymus, with only weak additional expression in the spleen. PD-L1 mRNA was expressed at the highest level in thymus but was also seen in most of the other 12 tissues examined, including moderate expression in liver and heart. Cardiac expression of PD-L1 in these blots may appear to contrast with the lack of PD-L1 signal in Northern blots of subsequent figures. However, the exposure times for the subsequent blots were very short. If longer exposures were undertaken such that baseline cardiac PD-L1 RNA expression was consistently detected, the signals from allografts developed at the same time were grossly overdeveloped. In contrast to PD-L1, the distribution of PD-L2 mRNA in these multitissue blots was very restricted, with strong expression in thymus, in addition to moderate expression in spleen and weak expression in liver.

**PD-1, PD-L1, and PD-L2 mRNA expression in cardiac isografts and allografts**

To analyze PD-1, PD-L1, and PD-L2 mRNA expression during cardiac allograft rejection across a full MHC disparity, total RNA was prepared from heterotopic cardiac grafts harvested at daily intervals until rejection and analyzed by Northern blot hybridization (Fig. 2a). Negligible amounts of PD-1, PD-L1, and PD-L2 mRNA were present in control hearts, but all three genes were clearly expressed during allograft rejection. A single PD-1-specific message (2.0 kb) was detectable in rejecting hearts at day 5, and this mRNA increased dramatically from day 6, just before the end-stage rejection which occurs on day 7 in this MHC combination. In contrast to PD-1, PD-L1 mRNA rose to significant levels as early as the first day after transplantation. The pattern of expression of PD-L2 was similar to that of its receptor, PD-1; two PD-L2-specific messages, at 1.7 and 8.0 kb, were detected in rejecting allografts. In contrast to the ready detection of PD-1, PD-L1, and PD-L2 mRNA expression within cardiac allografts, no expression was seen within Northern blots of isografts exposed for the same period (Fig. 2a) or by immunohistology (Fig. 2b), indicating that up-regulation was related to the host alloresponse rather than occurring as a result of ischemia/reperfusion injury, anesthesia, or additional surgically related factors. Allografts harvested at day 7 showed considerable PD-1, PD-L1, and PD-L2 protein expression (Fig. 2b). PD-1 and PD-L1 labeling was readily detected on infiltrating leukocytes, whereas PD-L2 staining was somewhat more restricted with regard to the numbers of mononuclear cells stained.

**FIGURE 1.** Limited expression of PD-1 and PD-L2 but broad tissue expression of PD-L1. Northern analysis of PD-1, PD-L1, and PD-L2 mRNA expression using poly(A)+ RNA from 12 mouse tissues (β-actin loading control).

**FIGURE 2.** PD-1, PD-L1, and PD-L2 expression following cardiac transplantation. a, Northern analysis of PD-1, PD-L1, and PD-L2 shows mRNA expression in cardiac allografts harvested on days 1–7 posttransplant, whereas normal control heart (C) and cardiac isografts (Iso) lacked mRNA expression; day-7 allograft mRNA (Allo) was included on the same blot as isograft mRNA to provide a positive control. RNA was obtained from healthy animals. b, Immunohistology shows lack of PD-1, PD-L1, or PD-L2 protein expression in isografts at day 7 (insets). Allografts at day 7 were unstained by a rat IgG isotype control mAb, whereas PD-1 and both ligands were expressed by mononuclear cells. Cryostat sections, hematoxylin counterstain; original magnifications ×600 for allografts and ×300 for isografts.
Immunosuppression or costimulation blockade decrease allograft PD-1, PD-L1, and PD-L2 expression

There are no data reported concerning expression of the components of the PD-1 pathway in subjects treated with immunosuppression or immune tolerance-directed therapies. Northern analysis of allografts harvested at day 7 posttransplantation from untreated recipients or recipients treated with CsA or RPM showed that intragraft expression of both PD-1 and PD-L1 were decreased, but still readily detectable, despite use of either immunosuppressive agent (Fig. 3a). These findings, while clearly far from exhaustive, provided important background data on the effects of CsA and RPM at concentrations that we were later to use in vivo. We also examined the effects of CD154 mAb therapy, with or without concomitant DST, on intragraft expression of PD-1, PD-L1, and PD-L2 mRNA at day 7 posttransplant. PD-1, PD-L1, and PD-L2 mRNA, almost undetectable in normal hearts, were expressed at high levels in cardiac allografts undergoing rejection in the control hamster IgG-treated group (Fig. 3b). Treatment with CD154 mAb, with or without DST, suppressed the intragraft mRNA expression of PD-1 and PD-L2 completely, and markedly decreased that of PD-L1 (Fig. 3b). Similarly, in contrast to levels detected in grafts from rat IgG-treated recipients, anti-ICOS mAb administration led to suppression of PD-1 mRNA and nearly complete suppression of PD-L1 mRNA expression (Fig. 3c).

In vitro effects of PD-L1.Ig and PD-L2.Ig

We prepared recombinant murine PD-L1.Ig and PD-L2.Ig fusion proteins and undertook in vitro studies of their effects on purified CD4+ T cells. Both fusion proteins bound to murine PD-1 cell transfectants (Fig. 4a) and decreased Ag-induced T cell production of the cytokines IFN-γ, TNF-α, and IL-10 (Fig. 4b), indicating their capacity to bind to PD-1 and modulate T cell functions.

Experimental design of in vivo therapeutic studies

Because PD-1 and its ligands were expressed during development of cardiac rejection and targeting of PD-1 using PD-L1.Ig or PD-L2.Ig fusion proteins could modulate T cell function in vitro, we decided to test the effects of fusion protein therapy in vivo. We first analyzed the effects of each protein alone or in conjunction with CsA. The ensuing data led us to focus on the use of PD-L1.Ig in vivo, in combination with RPM, as well as in systems that involved costimulation blockade-resistant cardiac allograft rejection.

Synergistic prolongation of allograft survival by PD-L1.Ig plus CsA

We injected each protein daily (100 μg/dose, i.p.) into allograft recipients until graft rejection occurred, and found that neither agent prolonged survival as compared with untreated controls or mice receiving control fusion protein (Fig. 5a). However, as clinical protocols commonly involve use of CsA, we tested whether targeting of PD-1 might potentiate the effects of a subtherapeutic regimen of CsA (10 mg/kg/day for 14 days). Consistent with previous studies in the model (14, 33, 34), this dose of CsA had only a marginal effect, prolonging allograft survival for only an extra 2 days. However, the combination of 14 days of therapy with PD-L1.Ig plus CsA significantly enhanced allograft survival over that

![Figure 3](http://www.jimmunol.org/)  
**FIGURE 3.** Effects of therapy on allograft-associated increases in intragraft PD-1, PD-L1, and PD-L2 at day 7 posttransplant. *a.* Northern analysis of cardiac allografts harvested from untreated recipients or recipients treated with RPM or CsA shows persistence of PD-1, PD-L1, and PD-L2 expression. *b.* Northern analysis shows hamster IgG had no effect on intracardiac transplant (T) induction of PD-1, PD-L1, and PD-L2 mRNA, whereas CD154 mAb therapy (with or without DST) largely suppressed intragraft expression of PD-1, PD-L1, and PD-L2 at this time point. *c,* Anti-ICOS mAb therapy also suppressed intragraft PD-1 and PD-L1 mRNA expression. In each panel, control (C) cardiac RNA was obtained from healthy animals, along with RNA from native (N) hearts of allograft recipients, and a marine GAPDH cDNA fragment was used as loading control (data representative of two samples per group).

![Figure 4](http://www.jimmunol.org/)  
**FIGURE 4.** In vitro characterization of PD-L1.Ig and PD-L2.Ig fusion proteins. *a.* Flow cytometry shows that both fusion proteins bound to mouse PD-1 cell transfectants (light trace) whereas cells exposed to control IgG1 fusion protein (dark trace) were unstained. *b.* Both fusion proteins suppressed Ag-induced CD4+ T cell cytokine production. Shown are ELISA data (mean ± SD) from two separate experiments, in which OVA-specific mouse TCR-transgenic splenic CD4+ T cells were stimulated with OVA peptide plus APCs in the presence of immobilized fusion proteins or control Ig.
of CsA or PD-L1.1g alone \((p < 0.01)\), with 50% of grafts functioning for \(>25\) days (Fig. 5a). By contrast, 14 days of therapy with PD-L2.1g plus CsA had no beneficial effect on allograft survival over that of CsA alone (Fig. 5a). Histologic comparison of allografts harvested at day 7 from each of the groups showed significant differences between allografts in animals treated with PD-L1.1g plus CsA vs other groups. Allografts in untreated controls, or in recipients treated with CsA only, showed characteristic end-stage rejection with extensive myocyte mononuclear cell infiltration, interstitial hemorrhages, and myocyte necrosis (Fig. 5b); the same morphologic features of severe rejection were seen in allografts harvested from mice treated with PD-L1.1g or PD-L2.1g (data not shown). However, allografts from recipients treated with PD-L1.1g plus CsA showed well-preserved graft morphology with a moderate mononuclear cell infiltrate and interstitial edema but no interstitial hemorrhages or myocyte necrosis (Fig. 5b). By contrast, allografts from animals treated with PD-L2.1g plus CsA showed severe rejection, as seen in untreated recipients or mice receivingCsA alone (Fig. 5b).

**PD-L1.1g plus CsA modulates intragraft immune activation**

We analyzed the expression of a large series of cytokines, chemokines, and chemokine receptors by RPA to explore potential mechanisms responsible for the protective effect of PD-L1.1g on allograft rejection in CsA-treated recipients. As we reported previously (34), use of the low-dose CsA regimen had no demonstrable effect on intragraft cytokine or chemokine or chemokine receptor mRNA expression at 7 days posttransplant as compared with allografts from untreated recipients, and in the current study PD-L2.1g alone, or in combination with CsA, also gave similar RPA results to those of untreated recipients (data not shown). Therefore, we focused our attention on why addition of PD-L1.1g to the CsA regimen enhanced allograft survival and decreased allograft leukocyte infiltration and myocardial injury. Compared with use of CsA alone, the addition of PD-1.1g therapy significantly decreased the intragraft expression of IFN-\(\gamma\) mRNA, as well as that of several chemokines induced by IFN-\(\gamma\), including RANTES (CCL5), macrophage-inflammatory protein (MIP)-1\(\alpha\) (CCL3), MIP-1\(\beta\) (CCL4), and IFN-\(\gamma\)-inducible protein-10 (IP-10; CXCL10), whereas the IFN-\(\gamma\)-independent chemokine, monocyte chemotractant protein-1 (CCL2), was unaffected (Fig. 6). Addition of PD-L1.1g also dampened intragraft expression of the two chemokine receptors most important to allograft rejection in this model (35) (Fig. 6), namely CCR5 (receptor for MIP-1\(\alpha\), MIP-1\(\beta\), and RANTES) and CXCR3 (receptor for IP-10) (33, 36, 37).

**PD-L1.1g plus RPM leads to long-term cardiac allograft survival**

In animals treated with RPM alone (1 mg/kg/day), cardiac allografts across a full MHC disparity were rejected by day 14. Untreated recipients, or those receiving control IgG or PD-L1.1g, rejected their allografts by days 7–8. By contrast, 100% of recipients treated for 14 days with combined RPM and PD-L1.1g, at the same doses as were used individually, had long-term survival \((p < 0.001)\) (Fig. 7a). Combination of PD-L1.1g and RPM was also synergistic when the RPM was given, as before, daily for 14 days, but the PD-L1.1g was injected twice per week; 70% of recipients retained their cardiac allografts long-term \((p < 0.01)\) (Fig. 7b).

**PD-L1.1g causes prolonged cardiac allograft survival in CD28\(^{-/-}\) recipients**

CD28\(^{-/-}\) C57BL/6 recipients rejected fully MHC-disparate BALB/c cardiac allografts with a delayed tempo as compared with wild-type mice; 50% were rejected by day 20 (Fig. 7c). A 14-day course of therapy with PD-L1.1g alone led to prolonged (\(>100\)-day) engraftment in 75% of CD28\(^{-/-}\) recipients \((p < 0.001)\).
Inhibitory effect of PD-L1.Ig on costimulation blockade-resistant chronic rejection

Cardiac allografts harvested at day 30 of transplantation from recipients treated with CD154 mAb plus human IgG1 showed florid transplant arteriosclerosis (vessel score of 2.6 ± 0.6, mean ± SD), interstitial fibrosis, and focal myocyte necrosis in conjunction with a mononuclear cell infiltrate (Fig. 7d). Use of CD154 mAb plus PD-L1.Ig improved the preservation of graft architecture post-transplant, with intact myocardium, minimal interstitial fibrosis, focal mononuclear cell aggregates, and essentially normal elastin-stained vessels (score of 0.3 ± 0.2, p < 0.001) (Fig. 7e), indicating that PD-L1.Ig use markedly reduced the development of transplant arteriosclerosis.

Discussion

Exploration of the in vivo functions of the PD-1 pathway are of interest given that aging PD-1−/− mice progressively develop autoimmune diseases (19). These data indicate that PD-1 is important...
to immune regulation, although with perhaps a lesser role than its homolog CTLA-4, in that CTLA-4−/− mice develop a severe and rapidly fatal T cell proliferative disease (38, 39). Like CTLA-4, PD-1 expression is tightly regulated, being normally primarily restricted to the thymus (16). However, expression of PD-1 is induced in both T and B cells upon activation via their Ag receptors (32). Our study reveals functions of this pathway in normal mice and a role for its therapeutic manipulation post-organ transplantation.

We found by Northern analysis, in the resting state, murine PD-1 and PD-L2 mRNA were largely restricted to the thymus and spleen, whereas moderate levels of PD-L1 expression were detected in most tissues examined. Up-regulation within cardiac allografts but not isografts of all three components of the PD-1 pathway suggests, as with data from PD-1−/− mice, that it may indeed be important to the dampening of ongoing T cell responses and resolution of host inflammatory responses. To determine the extent to which this up-regulation might be exploited therapeutically, it was necessary to develop tools for PD-1 targeting and therapeutic protocols for their testing. In baseline studies, we examined the results of immunosuppression with CsA or RPM on PD-1 expression within cardiac allografts. Although a rigorous and large-scale dose-response analysis was not performed, decreased but persistent expression of all three genes was observed at doses of immunosuppression that by themselves only marginally prolonged allograft survival, suggesting the potential usefulness of combining immunosuppression with targeting of PD-1 using PD-1 ligand fusion proteins.

We prepared PD-L1.Ig and PD-L2.Ig and showed that each bound specifically to PD-1 transfectants and suppressed Ag-induced cytokine production, consistent with the data of Freeman and colleagues (23, 24). Freeman and colleagues (23, 24) showed PD-L1.Ig had no effect on proliferation of PD-1−/− T cells, indicating that PD-L1.Ig attenuates TCR-mediated T cell proliferation via its interaction with PD-1, and also reported that PD-L1.Ig-induced inhibition of T cell proliferation was linked with decreased production of IFN-γ and IL-10. However, Chen and colleagues (22) have reported that ligation of PD-1 by PD-L1.Ig can costimulate T cell proliferation and promote IL-10 production. In addition, Tseng et al. (25) found that PD-L2.Ig could costimulate T cell proliferation and enhance IFN-γ production. Hence, depending upon as-yet-little-understood effects of PD-1 ligation, including the experimental conditions and indeed the nature of the PD-1 ligand used, differing effects may be observed. The effects of the fusion proteins on T cell functions in vitro can also vary considerably depending upon whether the ligands are immobilized or present in soluble form (22–25), such that a future formal side-by-side comparison of their effects under varying conditions may well be informative. Thus, the very limited current knowledge of these pathways is such that an inhibitory effect may be a function of 1) delivery of a negative signal via PD-1 ligation or 2) interruption of a costimulatory signal for T cell activation and function, and may vary in extent depending upon whether PD-1 ligation involves soluble or immobilized ligand.

We began our in vivo studies of the effects of targeting PD-1 by testing each fusion protein alone or in conjunction with CsA, the mainstay of current clinical transplant therapeutic protocols. Neither protein alone affected the tempo of allograft rejection in the fully MHC-mismatched allograft combination. This is not unexpected given the potent, multifactorial nature of alloreponses in this model and data that the inhibitory effects of PD-1 ligation on T cell proliferation and cytokine production are inversely proportional to the strength of TCR- and CD28-mediated signals (23, 24, 40). However, therapy with PD-L1.Ig plus CsA, in contrast to PD-L2.Ig plus CsA, or CsA alone, significantly enhanced cardiac allograft survival. Combined PD-L1.Ig/CsA suppressed intragraft expression of IFN-γ and IFN-γ-induced chemokines and chemokine receptors, but was not associated with evidence of immune deviation such as up-regulation of intragraft IL-4 or IL-10 expression (30). We recently demonstrated the key role of the chemokine receptors CXCR3 and CCR5, expressed by Th1 cells in mediation of allograft rejection in this model (33, 36), and also reported that IFN-γ-induced production of the CXCR3 ligand, IP-10, by cardiac endothelial cells is central to host alloresponses (37). We can now add that, when undertaken in conjunction with a subtherapeutic regimen of CsA, PD-1 targeting in vivo leads to reduced intragraft IFN-γ production, diminished production of multiple chemokines, and correspondingly decreased infiltration by chemokine receptor-bearing host leukocytes.

The mechanisms by which PD-1 ligation diminishes TCR and CD28 signaling are unclear but may include negative signals from the cytoplasmic ITIM domain of PD-1 by recruitment of the tyrosine phosphatase Src homology protein (SHP)-2 to PD-1, increased SHP-2 phosphorylation, and reduction in calcium fluxes and tyrosine phosphorylation of several signaling molecules (23, 24). The C-terminal PD-1 tyrosine is also reportedly essential for binding and activation of SHP-2 (41). CsA addition likely potentiates these effects by suppressing TCR-linked calcineurin activation and production of IL-2, which is essential for prevention of T cell anergy (42). However, the basis for the differential effects of PD-L1.Ig vs PD-L2.Ig in vivo is unknown. Given the beneficial but finite effects of PD-L1.Ig in combination with CsA on the survival of fully MHC-disparate cardiac allografts, we also tested whether its combination with RPM might prove more effective. Indeed, PD-L1.Ig therapy proved synergistic with RPM and induced permanent engraftment (>100 days) when used for the initial 2 wk posttransplant (Fig. 7a) or when given intermittently (Fig. 7b), in agreement with persistence of PD-1 expression despite RPM therapy (Fig. 3a), and again indicative of a beneficial effect of PD-1 targeting on T cell responses.

A need to understand the pathogenetic mechanisms of chronic allograft rejection and to develop therapeutic approaches for its prevention or regulation is paramount in clinical transplantation. Therefore, we investigated whether targeting of PD-1 might contribute to regulation of costimulation blockade-resistant allograft rejection, such as commonly gradually develops following inhibition of CD28/B7 or CD154/CD40 costimulation. We modeled the first scenario by use of CD28−/− allograft recipients of fully MHC-mismatched cardiac allografts and found that therapy with PD-L1.Ig alone led to permanent engraftment, in marked contrast to that of controls (Fig. 7c). These in vivo findings are consistent with in vitro data showing that efficient costimulation can overcome PD-L1-mediated inhibition of T cell activation (40), whereas in the absence of CD28 costimulation, residual pathways leading to T cell activation appear to be effectively controlled by PD-1 targeting.

In a second scenario, we used CD154 mAb plus PD-L1.Ig in a fully mismatched allograft model. We focused on the effects of CD154 mAb, because a humanized CD154 mAb was recently tested clinically. We found that supplementing CD154 mAb therapy with PD-L1.Ig administration suppressed development of transplant arteriosclerosis and other hallmarks of chronic rejection in long-surviving cardiac allografts (Fig. 7). Rejection post-CD154 mAb therapy appears to result from a relative lack of effect of CD154 mAb on CD8+ T cell functions (43, 44). Interestingly, in vitro PD-1 ligation by PD-L1 is more effective in regulating the activation of CD8+ vs CD4+ T cells, perhaps, as suggested (40), because of an inability of the CD8 cells to produce significant quantities of IL-2. It remains to be determined whether this is the
mechanism of action of PD-L1:lg in our in vivo system or whether additional effects of PD-1 ligation on host B cell responses (45) also contribute.

In summary, a detailed understanding of the role of each PD-1 ligand in immune responses will require the development and testing of appropriate blocking mAbs and knockout mice, and is the subject of ongoing studies. However, the current in vivo findings provide a rationale for modulation of host alloresponses underlying acute and chronic allograft rejection by taking advantage of accruing knowledge of physiologic pathways central to immune regulation and homeostasis. The encouraging distillation of our studies in several models is that PD-1 targeting, in some cases alone, and certainly when used in conjunction with agents that are currently used clinically or are in clinical trials, can markedly enhance the outcomes of organ transplantation.

References