

Comprehensive Review

Role of the PD-1 Pathway in the Immune Response

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Understanding immunoregulatory mechanisms is essential for the development of novel interventions to improve long-term allograft survival. Programmed death 1 (PD-1) and its ligands, PD-L1 and PD-L2, have emerged as critical inhibitory signaling pathways that regulate T cell response and maintain peripheral tolerance. PD-1 signaling inhibits alloreactive T cell activation, and can promote induced regulatory T cell development. Furthermore, the upregulation of PD-L1 on nonhematopoietic cells of the allograft may actively participate in the inhibition of immune responses and provide tissue-specific protection. In murine transplant models, this pathway has been shown to be critical for the induction and maintenance of graft tolerance. In this review, we discuss the current knowledge of the immunoregulatory functions of PD-1 and its ligands and their therapeutic potential in transplantation.

Key words: Alloimmunity, costimulation, PD-1, PD-L1, PD-L2, regulatory T cells, tolerance, transplantation

Abbreviations: BCR, B cell receptor; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; MHC, major histocompatibility complex; mOVA, membrane-bound chicken OVA; NOD, nonobese diabetic; PD-1, Programmed cell death-1; PD-L1, Programmed cell death-1 ligand; TCR, T cell receptor; TLR, Toll-like receptor.

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Introduction

T cells play a major role in coordinating the immune response against alloantigens during organ transplantation. While T cell activation depends on the initial antigen-specific signal provided to T cell receptors via the antigen-

loaded MHC complex, additional signals provided by costimulatory molecules fine-tune this response, determining its strength, nature and duration. Some costimulatory interactions potentiate the activation and proliferation of naïve T cells, while others inhibit T cell activation and promote regulation (1,2). The founding members of the B7:CD28 costimulatory family are the CD28 and CTLA-4 coreceptors that both bind to the B7-1 (CD80) and B7-2 (CD86) molecules. CD28 acts as a strong positive costimulatory receptor and CTLA-4 as a potent coinhibitory receptor. The programmed death 1 (PD-1) receptor: PD-Ligand (PD-L) pathway is another major receptor–ligand network that functions primarily to provide a coinhibitory signal. PD-1:PD-L interactions maintain peripheral tolerance and are exploited by tumors and viruses that cause chronic infection to evade immune eradication. As such, this pathway has emerged as a potential therapeutic target for either enhancing or dampening the immune response. This review will summarize our current understanding of the immunoregulatory functions of the PD-1:PD-L pathway and its therapeutic potential, focusing on its relevance to the field of transplantation.

Structure and Expression of PD-1 and its Ligands

The inhibitory receptor PD-1 (CD279) is a cell surface molecule with a single immunoglobulin (Ig) superfamily domain and a cytoplasmic domain containing two tyrosine-based signaling motifs: a tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (Figure 1) (3). PD-1 has two ligands, PD-L1 (B7-H1; CD274) (4) and PD-L2 (B7-DC; CD273) (5,6), both of which have Ig-V-like and Ig-C-like extracellular domains and a short intracellular domain. PD-1 is inducibly expressed by T cells and B cells after activation (7) as well as natural killer T (NKT) cells, NK cells, activated monocytes and some subsets of dendritic cells (DCs) [reviewed in (8)]. PD-1 is upregulated after TCR or BCR engagement on naïve lymphocytes and persistent antigen stimulation maintains high PD-1 expression (9). The common γ -chain cytokines (IL-2, IL-7, IL-15 and IL-21), TLRs and interferons also can potentiate PD-1 expression on T cells (10). Expression of PD-1 is in part mediated by the recruitment of the nuclear factor of activated T cells c1 (NFATc1) to the nucleus (11). NFATc1 together with AP-1 and NF- κ B constitute the most critical transcription factors activated upon antigen recognition by T cells. Interestingly, the calcineurin inhibitor Cyclosporine A markedly reduces PD-1 expression through its effect

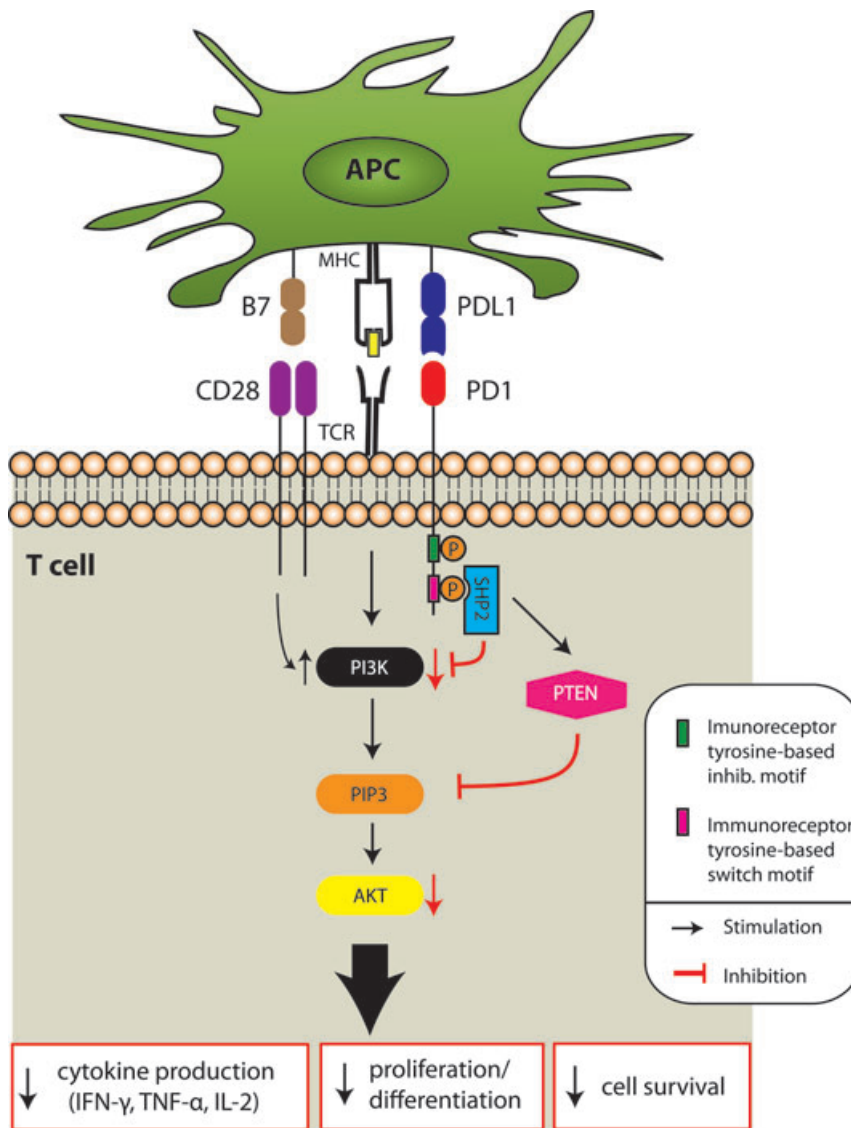


Figure 1: Effect of PD-1 signaling in T cells. PD-1 signaling dephosphorylates proximal signaling molecules and augments PTEN expression, inhibiting PI3K and AKT activation. The consequences include decreased T-cell proliferation, cytokine production and cell survival. PD-1 inhibition can be overcome by strong TCR signaling, CD28 signaling or IL-2 signaling (not shown). SHP2, protein tyrosine phosphatase; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; AKT, serine/threonine protein kinase; PIP3, phosphatidylinositol (3,4,5)-triphosphate.

on NFATc1 (11). While PD-1 upregulation on naïve T cells peaks at 48 h after anti-CD3 or anti-CD3/anti-CD28 stimulation *in vitro* (12), PD-1 on allogeneic CD4⁺ T cells progressively increases over time following skin transplantation *in vivo*, reaching the highest levels on day 10 posttransplant (13). Determination of the level of PD-1 expression in different immune cell subsets at various time points in transplantation remains to be explored. Last, certain subsets of T cells express high levels of PD-1, including CD4⁺Foxp3⁺ regulatory T cells (Tregs) (14), T follicular helper cells (T_{FH}) (15), memory T cells (16) and “exhausted” CD8 cells (17).

Of the PD-1 ligands, PD-L1 has very broad expression, whereas PD-L2 is inducibly expressed in a more restricted fashion. PD-L1 is expressed constitutively on murine T and B cells, DCs, macrophages, mesenchymal stem cells and bone marrow-derived mast cells (12,18), and induced to

higher levels by inflammation. In addition, it can be upregulated on nonhematopoietic cells, including vascular endothelial cells, epithelial cells, muscle cells, hepatocytes, placental cells and pancreatic islet cells (19,20). In humans, PD-L1 is mainly an inducible molecule. PD-L2 is upregulated on DCs, macrophages, bone marrow-derived mast cells and on a subset of peritoneal B1 B cells as well as on germinal center B cells (21). PD-L2 is also expressed on human, but not mouse, vascular endothelial cells and T cells (6,22). Interferon-γ (IFN-γ) potently upregulates PD-L1, and to a lesser extent PD-L2. IL-4 and GM-CSF are the strongest known stimuli for inducing PD-L2 expression (5,12,19,23,24). In addition to binding PD-1, PD-L1 can also bind the B7-1 (CD80) molecule, thus connecting the PD-1:PD-L1 pathway with the B7-1:CD28/CTLA-4 pathway (25). PD-L2, however, does not bind to B7-1. There are data to suggest that PD-L2 may have another receptor, the identity of which is still unknown (26).

Function of PD-1:PD-L Pathway

A major role of the PD-1:PD-L pathway is the inhibition of T cell function by engagement of the PD-1 receptor on T cells by PD-L1 or PD-L2 on antigen presenting cells (APCs). APCs transfected with PD-L1 or PD-L2 inhibit T cell responses, while blockade or genetic ablation of PD-L1 or PD-L2 on DCs or other APCs enhances their capacity to stimulate T cell responses *in vitro* as compared to wild-type (WT) APCs (27,28). Conversely, T cells that lack PD-1 are hyperresponsive relative to WT T cells (5,27,29–31). These inhibitory interactions not only suppress T cells during the priming phase of an immune response in secondary lymphoid tissues, but also modulate effector T cell responses, either during migration to the site of inflammation or in the target tissue itself (8,32).

PD-1 transduces an inhibitory signal when it is bound by its ligands in the presence of TCR or BCR activation (5,33,34). Phosphorylation of a tyrosine residue in the ITSM of PD-1 appears to have a key functional role in mediating PD-1 immunoinhibition. Phosphorylation of the ITSM motif leads to the recruitment of SH2-domain containing tyrosine phosphatase 2 (SHP-2), and possibly SHP-1, to the cytoplasmic domain of PD-1, which then down-regulates CD28-mediated PI3K activity and consequently, leads to less activation of Akt (Figure 1) (35). The exact mechanism of PD-1-mediated antagonism of the PI3K pathway is not yet clear (35). PD-1 ligation also inhibits the phosphorylation of other signaling molecules including CD3, ZAP70 and PCK (35). Thus, a major function of PD-1 signaling is to directly inhibit antigen receptor signaling.

Signaling through PD-1 exerts major effects on cytokine production by T cells, inhibiting production of IFN- γ , tumor necrosis factor- α and interleukin-2 (IL-2). PD-1 can also inhibit T cell proliferation (5,36), and inhibit the upregulation of Bcl-xL, an antiapoptotic protein (33). Last, PD1 signaling decreases the expression of the transcription factors GATA-3, Tbet and Eomes, which are associated with T cell effector function (37). However, a strong positive signaling through CD28 and/or IL-2 receptor can overcome PD-1 inhibitory effects on T cell proliferation, differentiation and survival (5,18,37,38). PD-1 signaling has also been implicated in reversal of the “stop signal” that is mediated by TCR signaling (39). This means that in the presence of PD-1, T cells have a shortened dwell time in their interactions with APCs, which can lead to decreased T cell activation and may also favor the induction of Tregs.

PD-1 can also inhibit signaling through B cell receptor. The role of PD-1 in controlling antibody production may be directly related to PD-1 on the B cells or secondary to effects of PD-1 on T cells. T cell interactions with B cells involve recognition of antigen by helper T cells, which then stimulate B cell expansion, isotype switching and affinity maturation. Among T cells, T_{FH} have emerged as key sup-

porters of the B cell response (40). T_{FH} express high levels of PD-1 (15,41), and PD-L1 and PD-L2 are upregulated on germinal center B cells (42). PD-1 has been shown to be important for the regulation of the germinal center B cell response; PD-1^{-/-} BALB/c mice have a reduced number of long-lived plasma cells after immunization with (4-hydroxy-3-nitrophenyl) acetyl-chicken- γ -globulin (42). In contrast, in two immunization models with either keyhole limpet hemocyanin or extract of *Schistosoma mansoni* eggs in B6 background mice, PD-L1 deficiency led to a significant expansion of T_{FH} cells and enhanced Ag-specific antibody responses (43). PD-1 deficiency can lead to generation of increased numbers of T_{FH} cells with aberrant phenotypes that lead to dysregulated selection of B cells and antibody diversity in germinal centers (44). Further studies are needed to delineate the functions of this pathway in regulating T_{FH} cell function and B cell responses in the germinal center.

Recently described roles for PD-1 expression on DCs and monocytes highlight the possibility that PD-1 signaling may also occur independently of T cell or B cell antigen receptor signaling, possibly by impinging on other receptor signaling pathways (45,46). For example, PD-1 ligation in monocytes has been shown to stimulate the production of IL-10 during HIV infection, which in turn contributes to reducing T cell function (45). These findings demonstrate that PD-1 expression on a nonlymphocyte population also may influence T cell immune function in HIV infection and this finding may extend to other settings.

In addition to PD-1-mediated signaling, there are data to suggest that signals may be transduced by PD-1 ligands. However, the cytoplasmic tail of PD-L1 has no known function. The cytoplasmic domains of human and mouse PD-L2 differ, with the mouse version being only 4 amino acids long and the human bearing 30 amino acids. While this longer form has no known signaling motifs, it is conserved in a number of mammals (but not rodents), which suggests functional significance. Data supporting PD-L1 and/or PD-L2 signaling are primarily based on experiments using soluble PD-1 reagents that engage PD-L1/2 and lead to up-regulation of IL-10 production and reduction in DC function (4,47). The physiological roles of signaling through PD-L1 or PD-L2 remain to be explored.

PD-1: PD-L Pathway in Peripheral Tolerance

The immune system has the daunting task of responding to foreign antigens, while avoiding self-reactivity. Central tolerance mechanisms prevent the emergence of most self-reactive T lymphocytes from the thymus, but some self-antigen-specific T cells escape into the periphery. To prevent the development of autoimmunity, multiple mechanisms of peripheral tolerance have evolved, including T cell deletion, anergy and the suppressive function of

Tregs. Failure of any of these mechanisms might result in autoimmunity. The PD-1:PD-L1 pathway plays a critical role in regulating the delicate balance between protective immunity and tolerance.

Initial evidence suggesting an important role for PD-1 in tolerance came from studies using blocking antibodies and knockout mice (27,48,49). In contrast to the CTLA-4 knockout mouse, which dies of an aggressive autoimmune lymphoproliferative disease within 3 to 4 weeks of birth (50,51), the PD-1 knockout mouse is viable on a number of different backgrounds. However, susceptibility to autoimmune disease is exacerbated in PD-1^{-/-} mice on an autoimmune-prone background (52,53). PD-1^{-/-} and PD-L1^{-/-} NOD mice develop accelerated spontaneous diabetes, which manifests in 100% of male and female mice by 10 weeks (36,54). Similarly, PD-L1 deficiency on the MRL-Fas^{lpr} background results in development of myocarditis and pneumonitis (55). These studies indicate that the PD-1 pathway is critical for self-tolerance.

PD-1 regulates both thymic selection and peripheral tolerance. PD-1 plays a role in inhibiting positive selection of thymocytes during the transition from the "double negative" to CD4⁺CD8⁺ "double positive" stage (56,57). The role of PD-1 in negative selection is less clear. Two studies using the alloreactive 2C TCR transgenic model showed increased negative selection in the absence of PD-1 (53,58), while analysis of HY-specific CD4 and CD8 TCR transgenic mice revealed no role for PD-1 in negative selection (59). In addition, PD-1 is critical for peripheral tolerance, restraining the development of self-reactive CD4 and CD8 T cells. PD-1 has an important role in controlling the outcome of initial encounters between naive self-reactive T cells and DCs. PD-1:PD-L1 interactions are required for both the induction and maintenance of CD4 T cell tolerance. For example, the PD-1 pathway is a critical mediator of tolerance induced by administration of antigen-coupled fixed splenocytes, which can reverse diabetes in NOD mice (60). PD-1 deficiency on self antigen-specific T cells increases CD8 T cell responses to antigen-bearing resting DCs, and abrogates CD8 T cell tolerance to peripheral self-antigen expression *in vivo* (30,31,61). PD-L2 is required for oral tolerance, as shown by failure of oral tolerance in PD-L2^{-/-} mice (62).

The use of T cell depletion therapy is expanding in solid organ transplantation. Upon lymphodepletion, regulatory mechanisms, including PD-1 signaling, prevent the emergence of autoreactive T cells during homeostatic proliferation. In a lymphopenic environment, a subpopulation of PD-1^{high} T cells develop that have skewed TCR repertoires and appear to be preapoptotic (63). It has been speculated that PD-1 may inhibit expansion of potentially pathogenic self-reactive CD8 T cells during homeostatic reconstitution of lymphopenic environments (63). Using an adoptive cell transfer model, Thangavelu et al. demonstrated that the reconstitution of Rag^{-/-} recipients with PD1^{-/-} hematopoi-

etic stem cell (HSC) precursors, but not WT HSC, causes severe autoimmunity, which does not develop when mature WT or PD-1^{-/-} T cells are used as donor cells (59). These findings support a critical role for PD-1 in regulating homeostatic proliferation, in particular of recent thymic emigrants. Therefore, preservation of an intact PD-1 pathway might be important for the prevention of autoimmunity in patients undergoing reconstitution of their immune system after lymphoablation in solid organ and hematopoietic stem cell transplantation.

Regulatory T cells

Regulatory T cell populations are critical for the maintenance of peripheral tolerance, are potent inhibitors of many immune responses and play an important role in the prevention of graft rejection (64,65). CD4⁺Foxp3⁺ Tregs, the most widely studied suppressive T cell population, are critical for peripheral tolerance as illustrated by the fatal autoimmune condition of scurfy mice and the human immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), both caused by mutations in the *foxp3* gene (66,67). Foxp3⁺ Tregs can be divided into "natural" Treg (nTreg) that arise as committed regulatory cells from the thymus, and "induced" Treg (iTreg) that are generated in the periphery by polarization of naive CD4⁺ T cells in a TGFβ- and IL-2-dependent fashion (68–76).

Foxp3⁺ Tregs highly express PD-1 and PD-L1 (1) and a role for the PD1:PD-L pathway in the generation of Tregs has been supported by a number of studies (77–81). PD-L1^{-/-} DCs were shown to be defective at supporting the TGFβ-induced conversion of naive T cells to Tregs *in vitro*, and conversion and maintenance of iTreg function *in vivo* was also dependent on PD-L1 (79). PD-L1 engagement of its receptors (PD-1, and possibly B7–1) on naive T cells leads to iTreg development, at least in part, by inhibiting mTOR/Akt signaling, as shown by experiments using microbeads coated with anti-CD3, anti-CD28 and PD-L1 fusion protein as artificial APCs (77). There are multiple studies that have identified AKT as a strong repressor of Tregs (82–84). It is proposed that AKT diminishes TGFβ-induced Foxp3 expression in a kinase-dependent manner and via a rapamycin-sensitive pathway (82). There is also evidence that weak TCR signals during T cell differentiation, and limited costimulation, can favor Foxp3 induction (85–88). Using human Th1-polarized T cells in a human-into-mouse graft-versus-host disease model (xGVHD), Amarnath et al. showed that PD-L1:PD-1 signaling was critical for the conversion of human Th1 cells into Tregs *in vivo*, preventing the development of xGVHD (81). The role of PD-1 in Treg conversion was partially mediated by the activation of intracellular SHP1/2 and consequent reduction of STAT1 phosphorylation, thereby abrogating IFN-γ-mediated maintenance of T-bet and favoring Foxp3 expression (81). Thus, the PD-1 pathway may promote tolerance induction *in vivo* by inducing and sustaining iTreg.

The role of PD-L1 in natural or existing Tregs is less well-understood. Antibody blockade studies suggest that this pathway inhibits expansion of established Tregs, as well as effector T cells (89,90). In the setting of hepatitis C virus infection, it has been suggested that PD-L1 negatively regulates Tregs by blocking STAT5 phosphorylation (89). However, PD-L1 maintains Foxp3 expression and enhances the suppressive capacity of induced Tregs *in vitro* (77). It may be that the PD-1 pathway exerts distinct effects on induced Tregs and natural Tregs. Further work is needed to clarify this issue as well as the question of whether PD-1 plays a role in regulating the dichotomy between iTreg and Th17 differentiation. In addition, a subpopulation of regulatory allo-specific CD8⁺ T cells expressing PD-1, which is induced by ICOS: B7h blockade, was found to be important for immune regulation (91). Thus, it appears that the PD-1 pathway may regulate the generation and/or functions of multiple types of Tregs.

PD-1:PD-L Pathway in Transplantation

Understanding immunoregulatory mechanisms is essential for the development of novel interventions that might help improve long-term graft survival. Coinhibitory signals play a key role in the regulation of the alloimmune response against the transplanted organ. In particular, a number of studies have demonstrated that an intact PD-1:PD-L interaction is important for the induction and maintenance of graft tolerance (13,32,92–96).

PD-1, PD-L1 and PD-L2 mRNA expression are significantly increased in cardiac allografts during rejection, while expression is minimal in syngeneic grafts or naïve hearts, indicating that upregulation is related to the host alloresponse, rather than a consequence of ischemia/reperfusion injury (97). A similar observation was made in mouse liver transplantation, where allogeneic livers had a significantly higher expression of PD-L1, both in the sinusoidal and parenchymal areas (92). One of the unique characteristics of PD-L1, compared to other costimulatory molecules, is its pattern of broad expression, not only on hematopoietic cells, but also on nonhematopoietic cells such as endothelium, placental trophoblasts and islet cells (1). After transplantation, there is significant upregulation of PD-L1 on endothelial cells in heart allografts (32). The potential function of PD-L1 in the endothelium is particularly intriguing, since the vasculature is the first interface between the immune cells and the target graft, residing in an optimal location for controlling the alloimmune response.

Manipulation of the PD-1:PD-L1 Pathway in Alloimmunity

Animal models of solid organ transplantation

Functions of the PD-1:PD-L1 pathway have been investigated in multiple different murine solid organ transplant models (Table 1), as well as in bone marrow transplan-

tation. Blockade of PD-1 and PD-L1, but not PD-L2, using an antibody approach, significantly accelerated cardiac graft rejection in a fully MHC mismatched allogeneic model (BALB/c into B6), particularly in the absence of CD28 costimulation (98). In less allogeneic models, such as the MHC class II mismatched model (bm12 into B6), blockade of PD-L1 also precipitated rejection, whereas PD-1 and PD-L2 blockade had no effect on graft survival (96,99). The absence of a significant effect of PD-L2 blockade in transplantation suggests that PD-L1 and PD-L2 may have different roles in tolerance induction, possibly related, in part, to diverse expression of these ligands. For example, PD-L1 expression is high in tolerogenic subsets of DCs, Tregs and mesenchymal stem cells (32,100,101), whereas the expression of PD-L2 is mostly restricted to professional APCs.

PD-L1 plays a critical role in the induction and maintenance of peripheral transplant tolerance. In a fully MHC mismatched cardiac transplant model in which tolerance was induced by multiple doses of CTLA-4-Ig, early administration of an anti-PD-L1 mAb prevented tolerance induction, while delayed administration abrogated graft survival (94). Accelerated rejection was associated with a significant increase in the frequency of CD8⁺ effector memory T cells and IFN- γ -producing alloreactive T cells in the periphery, while Foxp3⁺-graft infiltrating Tregs were decreased (94). This finding has been confirmed in PD-L1-deficient recipients. Furthermore, blockade or elimination of PD-L1 also abrogates other tolerogenic strategies, such as anti-CD154 mAb combined with donor-specific transfusion (95). In summary, the PD-1:PD-L1 pathway is key for tolerance development, however, strong TCR signaling might overcome PD-1-mediated inhibition, which may occur in non-immunosuppressed recipients of fully MHC-mismatched transplanted organs.

In order to more finely assess the effects of PD-1 and PD-L1 in allo-specific T cells, our group has used the anti-bm12 (ABM) transgenic (tg) model, in which CD4⁺ TCR tg cells are specifically reactive to I-A^{bm12} (13). Following bm12 skin transplantation, both PD-1 and PD-L1 blockade enhanced alloreactive T cell proliferation and Th1-cell differentiation, although PD-L1 blockade led to a greater effect compared to PD-1 and was unique in its capacity to inhibit alloantigen-specific T cell apoptosis. The effects of PD-L1 blockade were dependent on the presence of CD4⁺CD25⁺ Tregs *in vivo* (13). Another group used a single-antigen-mismatched transplant model where allo-specific CD8⁺ T cells can recognize mOVA skin grafts and be tracked over time (102). Similarly, blockade of PD-1 resulted in rapid expansion of donor-specific T cells and accelerated skin graft loss, even in the presence of combined CD28/CD40L blockade (102), reinforcing the pivotal role of PD-1:PD-L1 interactions in the regulation of alloreactive T cells.

Mouse models of liver transplantation (103) are very interesting to investigate, since the majority of liver grafts are

Table 1: PD-1: PD-L1 function in various transplant models

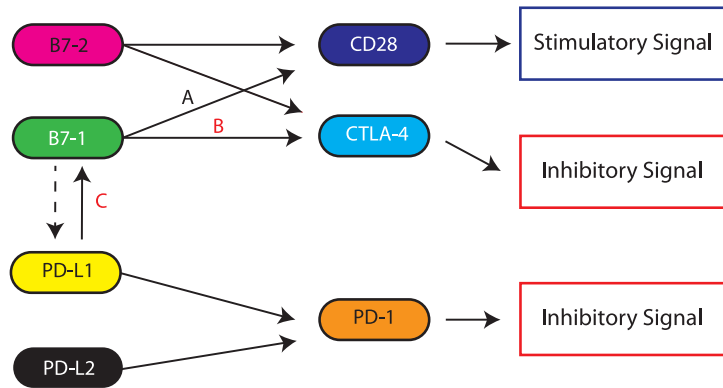
Transplanted organ	Model	Intervention	Effect	Potential mechanisms	Ref.
Heart	BALB/c → B6	PD-L1.Ig or PD-L2.Ig ± cyclosporine or rapamycin	PD-L1.Ig prolongs graft survival in combination with limited immunosuppression	↓ IFN-γ ↓ RANTES, MIP-1, IL10, CXCR3, CCR5	(97)
	BALB/c → B6	1) CTLA-4-Ig+ anti-PD-L1 or 2 mAb 2) CTLA-4-Ig in PD-L1KO or PD-L2KO recipients	PD-L1 blockade or PD-L1KO recipient prevents tolerance development (early and late)	↑CD8 eff mem ↑ IFN-γ, IL4 ↓ Tregs in grafts	(94)
	BALB/c → B6	1) Anti-PD-1 mAb + CD154mAb/DST 2) PD-1KO recipients + CD154mAb/DST	PD-1 blockade or PD-1KO recipient prevents tolerance development	↑IFN-γ, IL2, IP10, RANTES, CXCR3, CCR5	(95)
	BALB/c → B6 CD28KO or B7DKO	Anti-PD-1 or anti-PD-L1 mAb	PD-L1 blockade accelerates rejection at a faster tempo than PD-1 blockade	↑CD4 and CD8eff mem ↑IFN-γ	(98)
	BALB/c PD-L1 chimera → B6	PD-L1 chimeric donor	Graft PD-L1 expression on both hematopoietic and nonhematopoietic cells are essential for tolerance	↑CD8 eff mem ↑IFN-γ, GrB	(32)
	Bm12→ B6 B6→ bm12 B6 → bm1	1) Anti-PD-L1 or Anti-PD-L2 mAb 2) PD-L1KO or PD-L2KO recipients 3) PD-L1KO or PD-L2KO donor hearts	On bm12 model: PD-L1 blockade accelerates rejection; PD-L1KO donor accelerates rejection	↑CD4 eff mem ↑IFN-γ, IL4	(96)
	Bm12→ B6 Bm12→ B6 B7-1KO or B7-2KO or PD-1KO	Selective blockade of PD-L1: B7-1 pathway via antibody approach (2H11) or dual blockade with anti-PD-L1 mAb (MIH-6)	PD-L1:B7-1 blockade exacerbates chronic injury; dual blockade of PD-L1 has a greater deleterious effect.	↓Tregs ↑IFN-γ, IL4, IL6	(99)
	F344→ Lewis (rat model)	Overexpression of PD-L1 on donor heart via adenovirus ± cyclosporine	Slight delay in rejection in combination with cyclosporine	↓CD4 graft infiltration	(119)
Skin	Bm12→B6 and bm12→ABM transgenic mice	1) Anti-PD-1, anti-PD-L1 or anti-PD-L2 mAb	Only PD-L1 blockade accelerated rejection; CD25-dependent effect	↑ allogeneic T cell proliferation ↑IFN-γ ↓ apoptosis of alloreactive CD4 cells	(13)
	mOva skin →B6	Anti-PD-1 and anti-PD-L1 mAb ± anti-CD28 and anti-CD154 mAb	PD-1 and PD-L1 blockade accelerated skin rejection	↑ allogeneic T cell proliferation ↑IFN-γ	(102)
Islet cells	Syngeneic islets from WT or PD-L1KO → NOD mice	PD-L1 and/or PD-L2 deficiency on islets	PD-L1 deficiency accelerates diabetes development PD-L1 expressed on islets protects against infiltration of autoreactive T cells	PD-L1KO islets: ↑ CD4+ T cells infiltrating islets ↑IFN-γ, TNF-α	(36)
	Allogeneic islets from DBA/2→ B6 mice	PD-L1.Ig and anti-CD154mAb	PD-L1.Ig prolonged islet allograft survival	↓ T cell activation	(118)
Liver	B6 → C3H (85% tolerance)	1) Anti-PD-L1 mAb 2) Anti-PD-1 mAb 3) PD-L1 deficiency in liver	PD1 and PDL1 blockade precipitates rejection; PDL1KO liver prevents tolerance development	↑cytotoxic T cell infiltration ↑GrB, FasL and perforin in grafts	(92)

DST, donor-specific transfusion; ABM, anti-bm12 (ABM) transgenic (tg) model; NOD, nonobese diabetic mice.

accepted across MHC barriers without the requirement for immunosuppressive therapy. When B6 WT livers are transplanted into allogeneic C3H recipients, more than 85% of transplanted livers survive long-term (>100 days). PD-L1

deficiency in the liver graft (B6) or administration of anti-PD-1 or anti-PD-L1 blocking antibody prevented tolerance development in all C3H recipient mice (92), and was associated with significantly higher infiltration of cytotoxic T

Figure 2. Costimulatory signaling network. Integration of both positive and negative costimulatory signals during and after initial T cell activation will determine the fate and intensity of the alloimmune response. The complexity of the costimulatory network is exemplified by the potential effects of B7-1 targeting (such as accomplished by CTLA-4-Ig)—while blockade of B7-1 decreases positive costimulatory signaling through CD28 (A), it may affect two inhibitory signaling pathways, preventing B7-1 engagement of CTLA-4 (B) and PD-L1 (C). Dashed line represent the still controversial issue of reverse signaling through PD-L1 or PD-L2.



lymphocytes and increased levels of inflammatory cytokines (92). These findings suggest that PD-1:PD-L1 interactions may be sufficient for the development of spontaneous liver allograft tolerance in mice.

In light of the increased awareness of donor-specific alloantibodies as contributors to chronic rejection, interest in T_{FH} in alloimmune responses has expanded. T_{FH} cells provide B cell help necessary for the generation of an effective antibody-mediated response (40) and express high amounts of PD-1 ($CD44^+CXCR5^{high}PD-1^{high}$) (104). However, as discussed earlier in this review, the precise role of the PD-1 pathway in regulating T cell-dependent antibody responses is still unclear (42,43). Similarly, the importance of this pathway in T_{FH} generation in alloimmunity remains to be determined.

Murine models of bone marrow transplantation

GVHD is one of the most feared complications of bone marrow transplantation, and PD-1⁺ infiltrating cells are found at increased frequency in multiple GVHD target organs (spleen, colon and liver). Blazar et al. have shown that deficiency of the PD-1:PD-L1 pathway increases the lethality of GVHD in a model where B6 bone marrow cells and splenocytes are transferred into lethally irradiated B10.BR recipients (105). The accelerated GVHD was dependent on IFN- γ , and additional blockade of CTLA-4 further hastened the disease, supporting a nonredundant role of CTLA-4 and PD-1 in GVHD (105). Furthermore, in a model of acute myeloid leukemia, adoptively transferred AML-reactive cytotoxic T cells were more effective at eradicating the tumor after PD-L1 blockade (106). In sum, although blockade of the PD-1 inhibitory pathway may enhance antitumor responses, blocking this pathway might be deleterious in bone marrow transplantation due to worsening GVHD.

Novel Role of PD-L1:B7-1 in Alloimmunity

Our studies revealed a significantly greater effect of a blocking anti-PD-L1 mAb as compared to a blocking anti-PD-1 mAb in alloimmunity (98,99). This finding was initially hypothesized to be related to the differences in expression

of PD-1 and PD-L1, or half-lives/affinity of the antibodies. Subsequently, the discovery that PD-L1 could bind to B7-1 in addition to PD-1 suggested a different reason for this difference: PD-L1 interactions with B7-1 also might be important in regulating alloimmune responses (Figure 2) (25). Our group dissected the importance of PD-L1:B7-1 interactions in transplantation with the use of knockout animals ($B7-1^{-/-}$, $B7-2^{-/-}$, $PD-L1^{-/-}$, $PD-1^{-/-}$) and a newly characterized anti-PD-L1 antibody that selectively blocks the PD-L1:B7-1 interaction (10F.2H11) (99). Selective blockade of the PD-L1:B7-1 pathway led to significantly worse vasculopathy of bm12 allografts in B6 recipients and was associated with an increase in alloreactivity and a decrease in Tregs (99). However, the sole blockade of this pathway (PD-L1:B7-1) was not as powerful as dual blockade (PD-L1:PD-1/PD-L1:B7-1) with the anti-PD-L1 MIH-6 mAb in enhancing the alloimmune response, suggesting nonredundant roles of PD-L1:B7-1 and PD-L1:PD-1 interactions.

Since the affinity of the interaction between PD-L1 and B7-1 is higher than the affinity of B7-1 for CD28, it is possible that this interaction achieves sequestration of B7-1 from CD28 (25). However, *in vitro* studies suggest that the PD-L1:B7-1 interaction may function bidirectionally to inhibit T cells responses, by signaling through PD-L1 and/or B7-1 (25). Since both PD-L1 and B7-1 can be expressed on T cells, we further analyzed PD-L1:B7-1 interactions in an allo-setting using a combination of sensitized T cells and allo-DCs from WT and $PD-L1^{-/-}$ mice in an *in vitro* culture assay in the presence of either IgG control or 2H11 Ab (selective B7-1:PD-L1 blocking Ab) (99). In this setting, a potential dominant direction of effect was seen by the ligation of B7-1 on T cells by PD-L1 on DCs compared to PD-L1 on T cells and B7-1 on DCs, suggesting active, inhibitory signaling of B7-1 in T cells (99). A similar predominant direction of signaling was observed in a model of tolerance, in which OVA-reactive OT-I T cells undergo activation in response to injection of high-dose OVA₂₅₇₋₂₆₄ peptide (107). In a minor mismatched GVHD model, Yi et al. showed that IFN- γ leads to upregulation of PD-L1 on host APCs, and PD-L1 on these cells interacts with B7-1, and not PD-1, to expand donor-derived Tregs (90). A function for B7-1 on T cells has been suggested previously (108). However,

there are some studies indicating that B7-1 may signal into DCs and lead to the upregulation of IDO (109,110). In sum, the PD-L1:B7-1 interaction plays a nonredundant inhibitory role compared to PD-L1:PD-1 interactions. How CTLA-4-Ig, which blocks B7-1 and B7-2 ligands, affects the balance among B7-1 interactions with its possible binding partners (CD28, CTLA-4 and PD-L1) remains to be determined (Figure 2), but the affinity of CTLA-4 for B7-1 is the highest of these interactions (25), so presumably CTLA-4-Ig has a significant ability to prevent B7-1:CD28 and B7-1:PD-L1 interactions.

Fetal maternal tolerance

Pregnancy is the most physiological model of tolerance to alloantigens, in which the mother accepts the fetus expressing allogeneic paternal antigens. The placenta is the central organ involved in immune regulation during pregnancy, since it contains both maternal and fetal cells in close proximity (111). However, some maternal cells have been shown to cross the placenta and reside in fetal lymph nodes, inducing the development of Tregs that suppress antimaternal immunity in the fetus (112). PD-L1 is highly expressed on Tregs and in trophoblasts lining the decidua layer of the placenta (111). Interestingly, PD-L1 blockade or deficiency in B6 females mated with male allogeneic CBA mice results in decreased allogeneic fetal survival rates (113). The decrease in litter size and number upon PD-L1 blockade was shown to be dependent on CD25⁺ Tregs. Using an adoptive transfer model with alloantigen-specific TCR transgenic T cells in a bm12 × B6 mating, PD-L1 blockade was capable of breaking fetomaternal tolerance through a decrease in Treg induction, an increase in Treg apoptosis and a shift toward Th17 cells (114). Therefore, PD-L1 seems to play a role, not only in the regulation of effector T cells, but also in the promotion of Ag-specific Tregs in the context of fetomaternal tolerance. Further dissection of the importance of PD-L1 in different subpopulation of cells, including trophoblasts, is required to fully understand the role of PD-L1 in the fetomaternal immune interaction.

Importance of Donor Tissue PD-L1 Expression

One of the most intriguing aspects of the PD-1:PD-L1 pathway is related to the expression of PD-L1 by a variety of parenchymal cells, including heart, lung, kidney, pancreas, endothelium and placenta (1). This expression pattern raises the possibility that nonhematopoietic cells actively participate in the regulation of immune responses and can protect tissues against excessive inflammation and/or alloimmune responses. Indeed, nonhematopoietic expression of PD-L1 contributes to prevention of diabetes in the NOD mouse model (36) and regulates self-reactive CD8⁺ T cell responses in other models of self-tolerance (30,115).

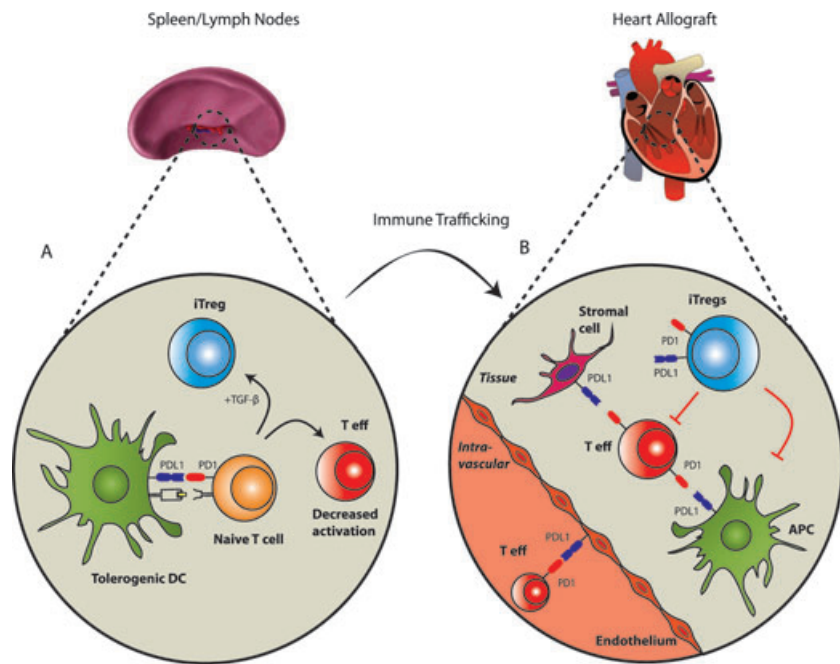
To address the question of whether donor PD-L1 on APCs and/or nonhematopoietic cells are important for tolerance development in transplantation, we generated PD-L1 chimeric mice on the B6 background and transplanted their hearts into BALB/c recipients treated with CTLA-4-Ig for tolerance induction (32). Both donor expression of PD-L1 on hematopoietic and nonhematopoietic cells (mainly endothelium) was essential for tolerance induction in this fully MHC mismatched model. However, PD-L1 deficiency on nonhematopoietic cells led to an earlier and more aggressive rejection with higher frequency of IFN- γ -producing alloreactive cells and CD8⁺ effector T cells (32).

Similarly, in a single MHC class II mismatched model of chronic rejection (B6 into bm12), PD-L1 deficiency in the donor heart also accelerated rejection (MST = 16 vs. >56 days on WT control hearts) and enhanced alloreactivity in recipients (96). In the setting of IFN- γ and/or allostimulation, the endothelium is known to upregulate PD-L1 (19,32,116). Endothelial PD-L1 is capable of downregulating CD8⁺ T cell responses and promoting the generation of allogeneic Tregs (19,78,116). Furthermore, in human kidney transplantation, tubular epithelial cells were found to upregulate PD-L1 during acute allograft rejection and PD-L1 on human tubular epithelial cell cultures was capable of inhibiting proliferation and cytokine production of CD4⁺ and CD8⁺ T cells (117). These findings illustrate how donor PD-L1 expression on nonhematopoietic cells can play a functional role in alloimmune regulation. PD-L1 on nonhematopoietic cells may be important for the induction of allograft tolerance and prevention of rejection by inhibiting T cells previously activated in secondary lymphoid organs within the allograft (Figure 3).

Modulation of PD-1:PD-L1 to Improve Allograft Survival

All of the above experiments dissected the role of PD-1:PD-L1 pathway in transplantation with the use of loss-of-function approaches, with either blocking antibodies or knockout mice. Another way to investigate PD-1:PD-L1 function in transplantation is by potentiating its interaction. Several studies have used a PD-L1-Ig fusion protein that apparently signals through PD-1. This PD-L1-Ig was able to prolong cardiac allograft survival in a fully MHC-mismatched transplant model (BALB/c into B6) treated with concomitant low-dose immunosuppression (cyclosporine A or rapamycin), however, it did not affect graft survival when PD-L1-Ig was used alone (97). PD-L1-Ig in combination with low-dose rapamycin for 2 weeks was able to induce long-term graft survival in all B6 recipients of BALB/c hearts (MST > 100 days vs. 14 days on rapamycin alone group, $p < 0.0001$) (97). Similarly, PD-L1-Ig prevented islet allograft rejection and facilitated tolerance induction when combined with anti-CD154 or suboptimal doses of rapamycin (118). Moreover, PD-L1-Ig was also capable of

Figure 3. PD-1: PD-L1 signaling in allograft tolerance. This figure illustrates some of the potential interactions of this pathway in lymphoid organs and in a cardiac allograft. (A), Tolerogenic DCs interact with naïve T cells and may induce either alloantigen-specific Tregs (iTregs) or decreased activation of effector T cell (T eff), depending on the presence (or absence) of TGF- β . (B), After activation by DCs in secondary lymphoid organs, alloantigen-specific effector T cells migrate to the graft where initial interaction with PD-L1 in the endothelium might inhibit T effector responses. Additional T eff cells that infiltrate the graft may be suppressed by iTregs or local cells expressing PD-L1 (APCs or nonhematopoietic cells). Whether PD1/PDL1 on iTregs play a significant role in the direct suppression of T eff remains to be determined. Other potential costimulatory interactions are not depicted in this figure for simplicity.



minimizing transplant arteriosclerosis in combination with anti-CD154 in another cardiac transplant model (97). These observations confirm that solely targeting the PD-1 pathway is probably not sufficient to inhibit the alloimmune response, and that additional control of T cell activation is required. Nonetheless, enhancing PD-1 signaling might be an important adjuvant approach for the protection of the graft against chronic injury.

Recent studies suggest another therapeutic strategy to improve graft survival could be enhancing the expression of PD-1 or PD-L1. Dudler et al. constructed an adenoviral vector expressing a PD-L1-Ig fusion protein and transfected F344 rat donor hearts, which were then transplanted into Lewis recipients (119). Cardiomyocytes were the predominant cell type transduced by the adenovirus, while only rare endothelial cells were transduced. Even though PD-L1 expression was only transient, graft rejection was slightly delayed, especially in combination with subtherapeutic cyclosporine (MST = 25 vs. 15, $p < 0.05$) (119). Moreover, DCs transfected with PD-L1 recombinant adenovirus and injected into kidney recipients also minimized proteinuria and delayed renal allograft loss in a rat transplant model, in which Fisher 344 kidneys were transplanted orthotopically into a Lewis host after native kidneys were removed (120). While all control rats died prior to 12 days after transplant, PD-L1-DC-treated rats had greater than 60% survival at 3 weeks posttransplant (120). These observations suggest that PD-1:PD-L1 interactions are important in tissue-specific tolerance and methods to enhance PD-1/PD-L1 expression could be a promising approach in the induction of long-term tolerance.

Clinical Translation

While the therapeutic potential of promoting the PD-1 pathway has not yet been realized, the PD-1:PDL1 pathway has become an attractive therapeutic target in the setting of chronic infection and cancer. The critical role for the PD-1 pathway in mediating T cell exhaustion was first revealed by blocking studies in models of chronic viral infection (121). PD-1 is highly expressed on "exhausted" T cells that develop during chronic viral infections in animals and humans (122). These T cells develop in the setting of chronic antigenic stimulation (17) and progressively lose effector functions, such as cytokine production and proliferation, leading to a significant deficit in viral clearance (123). Blockade of PD-L1 or PD-1 can reinvigorate the function of "exhausted" T cells and reduce viral load (123). Though PD-1 is highly expressed on "exhausted" T cells, other T cell subsets, including effector memory T cells present in peripheral blood of healthy humans, might also express high PD-1 levels (16). Therefore, additional markers such as CD223 (LAG-3), CD244 (2B4) and TIM-3 might be required to correctly identify this subset of T cells (121). Whether "exhausted" T cells develop in the setting of alloimmunity is not known, and is a subject for future investigation (124).

In the oncology field, there are a number of clinical trials in progress examining antibody-mediated blockade of PD-1 (anti-PD-1 mAb; Bristol-Myers Squibb [New York, NY, USA], CureTech/Teva [Yavne, Israel] and Merck [Boston, MA, USA] (125) and PD-L1 (anti-PD-L1 mAb; Bristol-Myers Squibb, Genentech [San Francisco, CA, USA]) in the treatment of refractory solid tumors,

including melanoma, renal cell carcinoma, colorectal cancer and nonsmall cell lung cancer as well as in hematologic malignancies [reviewed in (126)]. Based on the preclinical data, blockade of PD-1 has the potential to lead to immunopathology and activation of self-reactive T cells. Initial safety reports from a phase I clinical trial with anti-PD-1 mAb in refractory malignancies (BMS-936558) revealed that therapy was well-tolerated and the most common side effects reported were fatigue and diarrhea (127).

At present, there are no PD-1 agonist reagents in clinical trials. Several approaches are being investigated, including development of agonist antibodies, PD-L1 or PD-L2 fusion proteins, or enhanced local induction of tissue PD-1 ligand expression. How PD-1 engagement would fit in the currently available immunosuppressive armamentarium and what additional benefits it might promote are still undetermined. However, preclinical data suggest that PD-1 agonists alone may not be sufficient to prevent graft rejection, and that combination therapy with CTLA-4Ig, reagents targeting other coinhibitory receptors or administration of antigen-specific therapies may be needed. Preliminary work in mice from our group suggests that enhanced signaling of PD-1 is able to protect the allograft against chronic rejection and induce long-term graft survival in combination with single-dose CTLA-4Ig in a fully allogeneic HLA mismatched murine cardiac transplant model (Riella et al., unpublished data). Therefore, the simultaneous blockade of B7:CD28 costimulation with enhancement of the coinhibitory PD-1:PD-L1 might translate into an effective immunomodulatory strategy.

Concluding Remarks

PD-1 and its ligands play critical and diverse regulatory roles in the immune system. There are multiple potential mechanisms by which PD-1:PD-L1 interactions might participate in the induction of allograft tolerance. PD-L1 can limit effector T cell function and expansion, as well as induce Tregs, providing several means by which this pathway can tip the balance away from immunity, toward tolerance. The upregulation of PD-1 on T cells and PD-L1 on hematopoietic and nonhematopoietic cells might serve as an important negative feedback mechanism for controlling the alloimmune response and limiting allo-specific T cell activation and proliferation against the allograft. An agonist agent would have the potential to simultaneously inhibit function of effector T cells and promote *de novo* Treg generation. Such a PD-1 agonist not only could be beneficial in the prevention of allograft rejection, but also has the potential to ameliorate autoimmunity. The fundamental and therapeutic importance of the PD-1:PD-L1 pathway in immune regulation gives impetus to further investigation in transplantation.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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