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REVIEW

PD-1/PD-L1, PD-1/PD-L2, and other co-inhibitory signaling pathways in transplantation

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Summary

Transplantation of cells, tissues and vascularized solid organs is a successful therapeutic intervention for many end-stage chronic diseases. The combination of co-stimulatory blockade with the delivery of negative signals to T cells through co-inhibitory receptors would provide a robust approach to modulating T-cell receptor signaling and improving alloantigen-specific control of transplant rejection. This approach based on fundamental knowledge of APC/T-cell interactions may complement conventional therapies in the near future to reinforce long-term allograft survival, and permit minimal immunosuppression. The focus of this review was primarily on two major co-inhibitory signaling pathways, namely PD-1/PD-L1/PD-L2 and BTLA/CD160/HVEM/LIGHT that have been thoroughly characterized in murine models of transplantation using genetically modified mice, specific monoclonal antibodies and fusion proteins.

Introduction

Organ transplantation is currently the only therapeutic choice for the treatment of end-stage organ failure, but it requires the continuous administration of immunosuppressive drugs to abrogate the host immune response against the graft. Furthermore, despite the efficacy of immunosuppression in preventing and reverting acute episodes of rejection, chronic rejection still occurs and more research must be carried out to fully understand the mechanistic basis of this pathological process.

The field of transplantation has benefited from the development of chemical compounds with improved immunosuppressive potency [1–4]. However, a new era of more specific therapies based on the use of monoclonal antibodies (mAb) and recombinant fusion proteins is now emerging in clinical transplantation. These therapeutic approaches are initially scrutinized in genetically modified murine models of transplantation. As a result of these studies, an increasing number of engineered humanized mAb and recombinant fusion proteins have been tested in clinical trials, and many of them are entering clinical practice with great expectations [5–8]. The proper application of these therapeutic strategies should enable clinicians to minimize the dosage of immunosuppressants and promote the establishment of a state of donor-specific transplantation tolerance [9,10].

Another research area that has created enormous expectations in the field of transplantation is the manipulation of signals (receptor/ligand interactions) that are exchanged during the encounter of antigen-presenting cells (APC) with T cells. The balance between positive and negative signals that an APC delivers to a T cell upon encounter determines the outcome of the alloimmune response. Alloreactive T cells expressing a T-cell receptor (TCR), specific for donor-derived antigens and stimulated

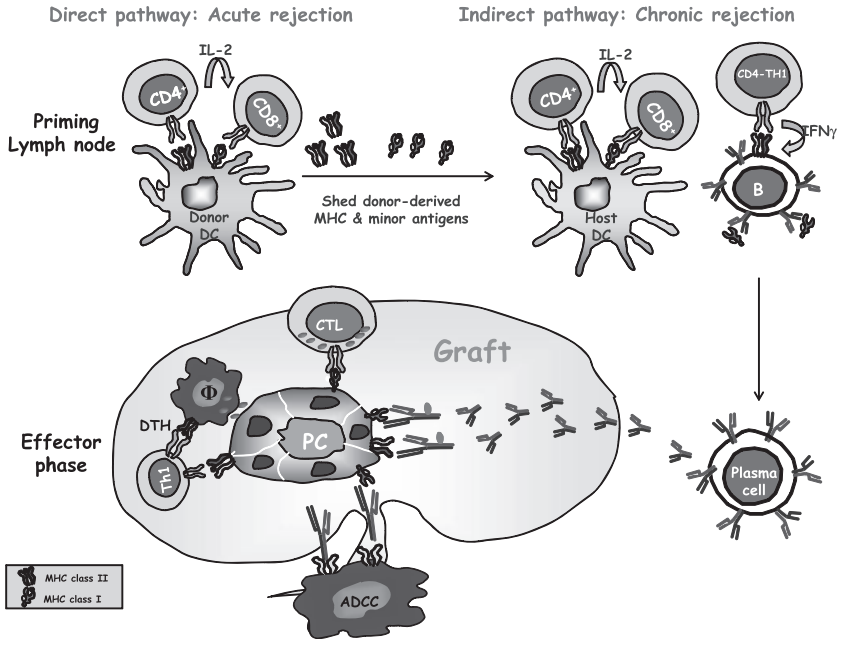

Figure 1 Antigen-presenting cell (APC), T cell, and B-cell collaboration in the alloimmune response Donor and recipient APCs stimulate alloreactive T-cell proliferation, clonal expansion, and differentiation into effector CD4 Th1 and CTL cells that will migrate to the graft during the rejection process. CD4 T cells help B cells recognizing conformational epitopes on donor MHC to differentiate into plasma cells secreting anti-donor MHC antibodies. These antibodies will bind MHC on the target parenchymal cells of the graft where they will mediate effector functions through complement or antibody-dependent cellular cytotoxicity.

by the direct or indirect pathway of antigen presentation differentiate into effector T cells following a multistep biological process that involves a sequence of signals (TCR signaling, co-stimulation and cytokine-mediated proliferation) [11,12] (Fig. 1).

Co-stimulatory and co-inhibitory molecules belong to one of two different superfamilies (SF): Immunoglobulin superfamily (Ig SF) and Tumor Necrosis Factor Receptor superfamily (TNFR SF). Based on the functional outcome, co-stimulatory molecules are distinguished from co-inhibitory molecules because the former enhance TCR-mediated responses, whereas the latter inhibit TCR-mediated responses [13–16] (Fig. 2). The B7/CD28 pathway is the most widely studied ligand/receptor interaction that provides positive signals to T cells [15,17]. B7 family members belong to the Ig SF and are characterized by two Ig-like extracellular domains [18]. In studies carried out during 1990, CD28 was described as the first surface molecule to function as a co-stimulatory receptor for naïve T cells [19,20]. CD28 is constitutively expressed on T cells and synergizes with TCR signaling, leading to increased cytokine production, T helper (Th) differentiation and antibody production. Unlike CD28, CTLA-4 (CD152) is not expressed on resting T cells, but it is transcriptionally up-regulated upon T-cell activation [21]. CD28 and CTLA-4 share binding to the ligands B7-1 (CD80) and B7-2 (CD86), although CTLA-4 binds to B7 ligands with higher affinity than CD28 does [15]. This difference in binding affinity is translated into selective recruitment of CD28 and CTLA-4 at the immunological synapse, with B7-1 being the major ligand capable of mediating CTLA-4 localization and B7-2 in recruiting CD28 [22]. CTLA-4 acts as a negative regulator of T-cell activation, delivering negative signals to T cells by inhibiting interleukin (IL)-2 synthesis and cell cycle progression [23]. CTLA-4.Ig fusion protein inhibits CD28/B7 co-stimulation pathway in several models of transplantation, autoimmune diseases and allergy [24,25] (Fig. 3).

The purpose of this review was to emphasize the relevance of the novel co-inhibitory pathways in the field of transplantation immunology and to explain their contribution to the design of novel strategies aimed at prolonging graft survival and promoting immunological tolerance. We will pay particular attention to the co-inhibitory pathways induced by PD-1/PD-L1 and PD-1/PD-L2 interactions as well as the unique example of a co-inhibitory pathway in which a molecule of the Ig SF (B- and T-lymphocyte attenuator, BTLA) and TNFR SF (Herpesvirus entry mediator, HVEM) form a ligand/receptor pair. Other pathways, such as B7-H3/unidentified ligand and B7-H4/unidentified ligand need to be investigated further to determine their role in the course of the allogeneic immune response. The hybridoma cell lines secreting mAb against the co-inhibitory molecules described in this review are listed in Table 1.

The role of PD-1/PD-L1 and PD-1/PD-L2 pathways in regulating allogeneic T-cell responses

Expression of PD-1 and its ligands under resting and inflammatory conditions

PD-1 (also termed CD279) is a member of the Ig SF, originally isolated by subtractive hybridization using T-cell hybridoma (2B4.11) cells undergoing apoptosis.

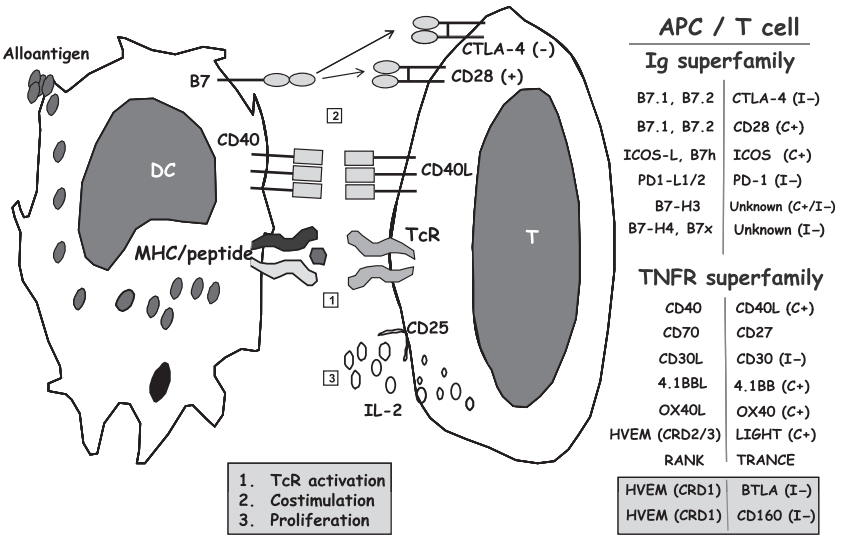

Figure 2 The three-signal paradigm: a simplistic view of T-cell activation. Donor MHC presented on the surface of donor APC (direct pathway) or donor-derived MHC peptides presented by recipient APC (indirect pathway) activate T cells through the T-cell receptor (signal 1). This is followed by a second signal, or co-stimulatory signal, that induces T cells to enter the cell cycle and secrete IL-2 (signal 2). IL-2 acts as an autocrine factor on IL-2 receptor, and clonal expansion and differentiation are initiated leading to the acquisition of effector T-cell function (signal 3). Co-stimulatory and co-inhibitory molecules and their respective ligands could transiently modulate TCR signaling, influencing the outcome of the immune response. These co-signaling pathways involve molecules belonging to the Immunoglobulin (Ig) and Tumor Necrosis Factor Receptor (TNFR) superfamilies. For reasons of clarity, despite the fact that some co-signaling molecules are expressed on both APC and T cells and potentially can signal back to the APC (reverse signaling), the focus of this review is on signals delivered exclusively from the APC to the T cell. C (+) stands for co-stimulation whereas I (–) means co-inhibition. HVEM/BTLA and HVEM/CD160 are highlighted to indicate receptor/ligand interactions, in which one molecule of the immunoglobulin superfamily is recognizing a ligand or receptor that belongs to a different family of molecules (TNFR superfamily).

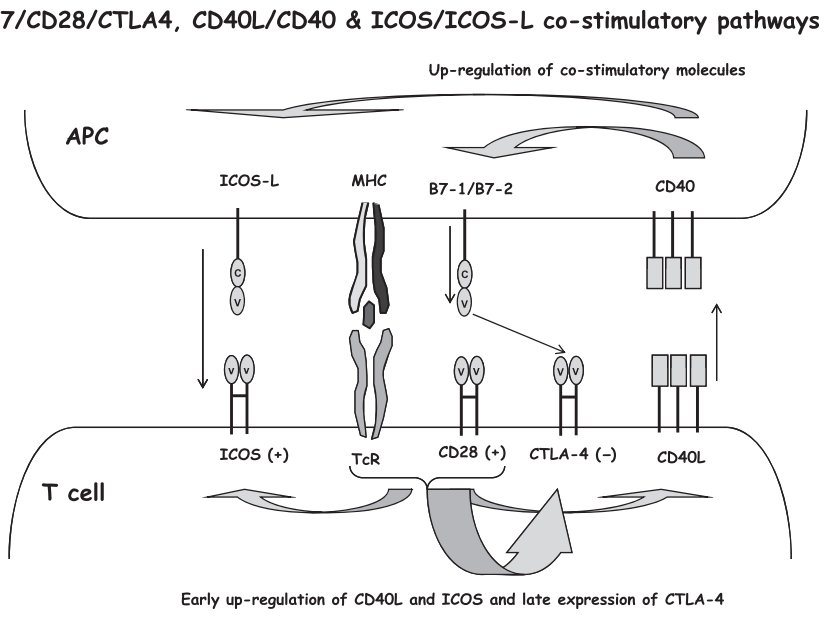

Figure 3 B7/CD28/CTLA-4, CD40L/CD40, and ICOS/ICOSL classical co-stimulatory pathways of T-cell activation. TCR interaction with donor MHC or donor-derived MHC peptides presented in the context of recipient MHC prompts T-cell activation and subsequent up-regulation of co-stimulatory molecules such as CD40L and ICOS. CD40L interacts with CD40 on the APC, inducing up-regulation of B7.1/B7.2 and ICOS-L. These two receptors on the APC deliver co-stimulatory signals to T cells through CD28 and ICOS. In response to these stimuli, T cells enter the cell cycle. Late expression of CTLA-4 on T cells and subsequent binding to B7 with higher affinity than the binding of B7 to CD28 attenuates co-stimulatory signaling, and the immune response returns to baseline.

Table 1. Hybridoma clones secreting monoclonal antibodies against molecules of co-inhibitory pathways and their functional activity.

Murine molecule	Clone and isotype	Reactivity	Functional activity	Reference
PD-1	J43 (Hamster IgG)	Mouse	Blocking	Agata *et al.* [100]
	RMP1-14 (rat IgG2a)	Mouse	Blocking	Yamazaki *et al.* [32,101]
	4F10 (rat IgG2a, k)	Mouse	Blocking	Del Rio *et al.* [102]
	29F.1A12	Mouse	Blocking at high doses	Liang *et al.* [103]
	RMP1-30 (rat IgG2b)	Mouse	Not blocking	Matsumoto *et al.* [104]
PD-L1	MIH6 (rat IgG2a)	Mouse	Blocking	Yamazaki *et al.* [32,101]
	MIH5 (rat IgG2a)	Mouse	Blocking	Yamazaki *et al.* [32,101]
	10F.9G2 (rat IgG2b)	Mouse	Blocking	Liang *et al.* [103]
	10F.5C5 (rat IgG2b)	Mouse	Blocking	Liang *et al.* [103]
	MIH7 (rat IgG2a)	Mouse	ND	Yamazaki *et al.* [32]
	1-111A (rat IgG2a)	Mouse	ND	Ishida *et al.* [105]
PD-L2	TY25	Mouse	Blocking	Yamazaki *et al.* [32]
	122 (rat IgG2a)	Mouse	ND	Ishida *et al.* [105]
BTLA	PK18 (rat IgG1, k)	B6	Inhibitory (agonist)	Han *et al.* [70]
	PK3 (rat IgM, k)	B6	ND	Han *et al.* [70]
	PJ196 (mouse IgG1, k)	B6	ND	Han *et al.* [70]
	6F7, 6G3, 8F4, 3F9.D12 (mouse Igs)	B6 and BALB/c	ND	Hurchla *et al.* [74]
	6H6 (mouse Ig)	Brighter in B6 than in BALB/c	ND	Hurchla *et al.* [74]
	3F9.C6 (mouse Ig)	B6	ND	Hurchla *et al.* [74]
	6A6 (Armenian hamster IgG)	B6	Blocking	Hurchla *et al.* [74]
HVEM	LBH1	Mouse	Blocking	Xu *et al.* [106]
	14C1.1 (rat IgG)	Mouse		Xu *et al.* [106]
B7-H3	B7-H3 (rat IgG)	Mouse	Blocking	Prasad *et al.* [97]
B7-H4	8H4 (Hamster IgG)	Mouse	Blocking ND	Sica *et al.* [99]
	8H4.1 (Hamster IgG)	Mouse	ND	Sica *et al.* [99]

ND, no data available.

The designation 'programmed death-1' comes from the observation that PD-1 mRNA is up-regulated upon *in vivo* administration of anti-CD3 in dying thymocytes [26]. PD-1 is a 50–55 kDa type I transmembrane receptor consisting of a single immunoglobulin variable-like domain and a cytoplasmic domain composed of two tyrosine-based signaling motifs. The cytoplasmic domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Although PD-1 does not signal unless the TCR is ligated, it is the ITSM that appears to be responsible for the inhibitory signaling by recruiting the Src homology 2-domain-containing tyrosine phosphatase (SHP-2), and thus attenuating TCR signaling. Resting T cells express low amounts of PD-1 receptor, and its expression is inducible on $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) T cells, activated monocytes and B cells [27]. PD-1-deficient mice of C57Bl/6 (B6) or BALB/c background develop lupus-like disease and cardiomyopathy respectively [28–30], which further suggests an inhibitory role of PD-1 in the control of the immune response and in the maintenance of peripheral tolerance to self-antigens.

The two ligands for PD-1, namely PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), are B7 family members with IgV and IgC-like domains in the extracellular region. PD-L1 is expressed constitutively on both hematopoietic cells [resting T cells, B cells, dendritic cell (DC), macrophages and regulatory T (Treg) cells] and on nonhematopoietic cells (parenchymal and endothelial cells) and is up-regulated upon exposure to interferon (IFN)-γ [31–34]. Unlike PD-L1, PD-L2 expression is restricted primarily to DC and macrophages and it is inducible by IL-4 and IFN-γ [34–36]. This pattern of ligand expression suggests that PD-1/PD-L pathway may regulate the immune response in both lymphoid and nonlymphoid organs (Fig. 4).

PD-1/PD-L1 interaction delivers co-inhibitory signals to T cells *in vitro*

Initial *in vitro* investigations defined the inhibitory function of the PD-1/PD-L1 and PD-1/PD-L2 pathways. PD-L1- or PD-L2-transfected CHO cells presenting OVA peptide in the context of IA^d-inhibited T-cell proliferation

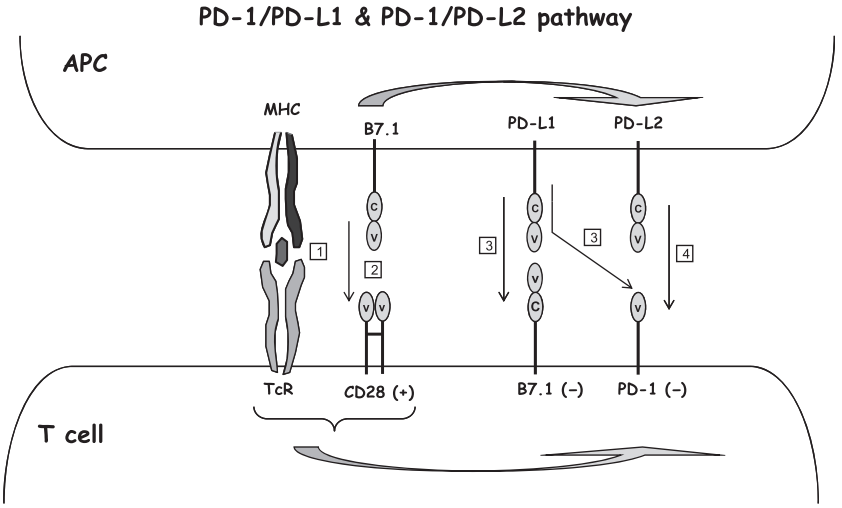

Figure 4 PD-1/PD-L1 and PD-1/PD-L2 pathways. PD-L1 is constitutively expressed on both hematopoietic and nonhematopoietic cells, whereas PD-L2 expression is restricted to antigen-presenting cells (APCs). The expression of PD-1 is positively modulated upon TCR-mediated signaling on the surface of alloreactive T cells. The binding of PD-L1 and PD-L2 to PD-1-expressing T cells delivers a co-inhibitory signal into the T cell that inhibits TCR-mediated proliferation. CD28 co-stimulation can overcome PD-1-mediated inhibition by augmenting IL-2 production. APCs expressing PD-L1 can also deliver negative signals to T cells expressing B7-1, which is a new co-inhibitory receptor/ligand interaction recently reported that adds more complexity to the interactions between APCs and T cells. The numbers on the figure indicate the sequence of signaling events that are exchanged between APC and T cells.

of OVA-specific DO11.10 TCR-transgenic CD4 T cells at relatively low concentrations of antigen [35]. PD-1-deficient $CD8^+$ T cells from 2C TCR transgenic mice recognizing $H\text{-}2L^d$ proliferated more actively than wild-type (WT) 2C TCR transgenic T cells in response to allogeneic ($H\text{-}2^d$) APC [28]. Murine T cells treated with anti-CD3 and PD-L1.Ig-coated beads displayed an attenuated proliferative response and produced less IL-2 compared to control T cells stimulated with only anti-CD3. The proliferative activity could be restored upon addition of soluble anti-CD28 or exogenous IL-2. It has also been shown that PD-1-deficient T cells stimulated with plate-bound anti-CD3 are activated more efficiently than wild-type (WT) T cells [37–40]. Moreover, $CD4^+$ T cells from PD-1-deficient mice stimulated under co-stimulation blockade displayed enhanced proliferation and secreted significantly more Th1-associated cytokines than WT $CD4^+$ T cells [41]. The PD-1 receptor on human T cells also acts as a negative regulator of T-cell activation during primary immune responses, though it inhibits activated and memory T cells more efficiently during secondary immune responses [42].

The role of PD-1/PD-L1 and PD-1/PD-L2 co-inhibitory pathways in regulating alloreactive CD4 and CD8 T-cell responses in murine models of transplantation

The role of the PD-1/PD-L pathways in allogeneic responses has been thoroughly investigated in nonvascularized and vascularized animal models of transplantation. Heart allografts transplanted into fully MHC-mismatched hosts (BALB/c into B6) are quickly rejected (7–10 days), whereas heart allografts transplanted across only MHC class I barriers (bm1 into B6) or only MHC class II barriers (bm12 into B6), survive long term (longer than 3 months) [43]. This outcome observed in vascularized heart allograft transplantation across MHC class I and class II barriers is notable as skin allografts transplanted across the same barriers are rejected in about 15 days and 18–20 days for MHC class I-mismatched and MHC class II-mismatched, respectively [44]. Interestingly, although fully MHC-mismatched cardiac allografts (BALB/c into B6) are rejected more rapidly when PD-1 signaling is blocked, most partially mismatched cardiac allografts (K^{bm1} or IA^{bm12}) survive long-term in PD-1-deficient B6 recipients [45,46]. This experimental observation emphasizes the fact that co-inhibition mediated by PD-1/PD-L interactions plays a more prominent role on T-cell responses wherein T-cell help is required for the generation of $CD8^+$ effector T cells and antibody formation whereas $CD4^+$ and $CD8^+$ T-cell-independent responses are less dependent on this pathway.

Surprisingly, additional studies demonstrated that blockade of PD-L1 accelerates graft rejection (median survival time: 13 days) of class II-mismatched allografts, suggesting that the CD4 T-cell-mediated response is enhanced when PD-L1 is not delivering negative signals to T cells. PD-L1-deficient donor hearts transplanted into

bm12 mice exhibited also accelerated rejection [47]. However, when either PD-1 or PD-L2 was blocked, the course of skin graft rejection was unchanged compared to untreated recipients in this MHC class II mismatch setting. This finding was unexpected because, if PD-L1 were to signal only through PD-1, then PD-1 blockade should have led to a similar outcome to that observed with PD-L1 blockade [47,48]. Overall, these data suggest that PD-1 might not be the unique mediator of negative signals delivered by PD-L1. In support of this observation, the B7-1 molecule expressed on the surface of T cells has recently been identified as a new receptor for PD-L1 that also transmits negative signals to T cells [49,50]. Indeed, the affinity of the PD-L1 and B7-1 interaction is much stronger than that of PD-L1/PD-1 interaction. A substantial overlap of PD-L1/B7-1 regions of interaction with those of B7-1/CTLA-4 and PD-L1/PD-1 has been reported [51]. The overlapping regions between ligand/receptor may account for the distinct outcome in graft survival observed in transplantation depending on whether blocking antibodies or PD-1- or PD-L1-deficient mice were used in the experiments. The more definitive and formal proof of the co-inhibitory role of PD-L1 in modulating T-cell function through B7-1 is that T-cell proliferation is not abrogated in PD-1/B7-1 double deficient T cells upon *in vitro* stimulation with anti-CD3 and PD-L1.Ig fusion protein-coated beads [49].

These findings provided the impetus for rapid testing of PD-L1.Ig and PD-L2.Ig recombinant fusion proteins as potential compounds to deliver negative signals via PD-1 in the setting of fully MHC-mismatched heart allotransplantation. Neither reagent alone prolonged fully MHC-mismatched heart allograft survival in mice; however, the co-administration of PD-L1.Ig plus Cyclosporine A (CsA) or Rapamycin substantially enhanced cardiac allograft survival compared to monotherapy with CsA or Rapamycin alone. In contrast, the combined treatment with PD-L2.Ig and CsA or Rapamycin did not significantly prolong graft survival compared to monotherapy with CsA or Rapamycin alone [39]. Therefore, the PD-1/PD-L1 pathway is more relevant than the PD-1/PD-L2 pathway in delivering *in vivo* inhibitory signals to alloreactive T cells expressing PD-1 and/or B7-1 receptor. The protective effect observed after the administration of PD-L1.Ig fusion protein becomes more relevant in the absence of the CD28/B7 co-stimulatory pathway. Most BALB/c cardiac allografts in PD-L1.Ig-treated CD28-deficient mice survived long term [39].

Unlike CD28-deficient mice, which are still capable of slowly rejecting allografts, B7-1/B7-2 double knockout (KO) recipient mice (i.e., CD28/CTLA-4/B7-independent model) do not reject allogeneic BALB/c to B6 cardiac allografts. Antibody-mediated blockade of PD-1 and PD-L1, but not PD-L2 blockade, accelerated cardiac allograft rejection in this setting [25,52]. Likewise, antibody-mediated blockade of either PD-1 or PD-L1, but not PD-L2, led to more rapid rejection of BALB/c hearts in CD8/CD28 double-deficient recipient mice. However, only anti-PD-L1 blockade, but not anti-PD-1 blockade precipitated the course of rejection of BALB/c hearts in CD4/CD28 double KO recipient mice [45]. Together, this evidence indicates that in the absence of CD28 co-stimulation, the PD-1/PD-L1 interaction is the relevant pathway in controlling both $CD4^+$ and $CD8^+$ T-cell-mediated mechanisms of rejection in solid organ allotransplantation models.

Habicht *et al.* found a remarkable dichotomy of the PD-1/PD-L pathway in modulating alloreactive $CD4^+$ and $CD8^+$ T cells in an *in vivo* murine model of GvHD that consisted of adoptive transfer of CD28-deficient T cells to DBA/2 recipient mice. PD-L2 antibody blockade, but not PD-L1 blockade, resulted in a modest decrease in $CD4^+$ T-cell proliferation and a significant increase in the $CD8^+$ T-cell proliferation rate compared to untreated controls. Similarly, blockade of PD-L2, but not PD-L1, inhibited the proliferation of adoptively transferred CD8/CD28 double deficient T cells ($CD4^+$ T cells) and significantly augmented the proliferation of CD4/CD28 double deficient T cells ($CD8^+$ T cells) compared to nontreated controls [53].

Unlike heart- and skin transplantation models, in which the PD-1/PD-L1 pathway was essential for controlling both $CD4^+$ and $CD8^+$ T cell-mediated responses, in GvHD murine models, the PD-1/PD-L2 pathway is more prominent in efficiently modulating allogeneic $CD8^+$ rather than $CD4^+$ T-cell responses in the absence of CD28 co-stimulation. Although speculative, the reason for these discrepancies may be attributable to the fact that in the GvHD model, alloreactive T cells are responding to alloantigen in a lymphopenic environment, which may affect the level of PD-1 ligand expression (PD-L1 on hematopoietic and nonhematopoietic cells, and PD-L2 on APC) and therefore the frequency and strength of the interactions.

Influence of co-inhibition blockade on co-stimulation blockade-induced tolerance

The induction of allograft tolerance is dependent on the balance of regulatory and effector T cells [54]. Co-stimulation blockade-induced tolerance to fully MHC-mismatched heart- and islet allografts is achieved after the administration of either donor-specific transfusion combined with anti-CD40L and/or CTLA-4.Ig [55–57]. A number of studies point out that co-stimulation blockade-induced tolerance requires an intact PD-1/PD-L axis

as the blockade of PD-1 or PD-L1 accelerates graft rejection. In line with this notion, early as well as delayed mAb-mediated blockade of PD-L1, but not PD-L2, abrogated CTLA-4.Ig-induced tolerance in fully MHC-mismatched BALB/c to B6 cardiac allograft model. Likewise, BALB/c heart allografts in PD-L1 KO B6 recipient treated with CTLA-4.Ig were rejected more rapidly than in CTLA-4.Ig-treated controls. However, heart allografts in CTLA-4.Ig-treated PD-L2-deficient recipients followed a rejection course similar to that of CTLA-4.Ig-treated WT controls [58]. Therefore, only the blockade of PD-1 or PD-L1 affects the survival of allografts in mice treated with CTLA-4.Ig. To examine the role of PD-L1 expression on donor versus recipient, PD-L1-deficient donor hearts were transplanted into WT recipients under the cover of CTLA-4.Ig. These grafts underwent severe chronic rejection and vasculopathy. In contrast, PD-L2-deficient donor hearts in CTLA-4.Ig-treated WT mice were not rejected and survived long-term [45,58].

These data point to the conclusion that PD-L1 expression on recipient APC (indirect pathway) plays a more decisive role in delivering negative signals to T cells than the potential co-inhibitory effect of PD-L1 expression on either donor-derived APC (direct pathway) or on the allograft to promote induction and/or maintenance of CTLA-4.Ig-induced transplantation tolerance.

Anti-CD40L treatment alone prolongs heart allograft survival across MHC barriers but does not induce tolerance as arteriosclerosis, a hallmark of chronic rejection, eventually emerges in these models. This is because of the fact that anti-CD40L therapy only affects CD4, but not CD8, T-cell-mediated rejection [59]. Co-stimulation blockade-induced tolerance subsequent to the administration of donor-specific transfusion and anti-CD40L is abrogated in PD-1- and PD-L1-deficient mice, as well as in WT recipient mice receiving anti-PD-1 or anti-PD-L1 blocking mAb antibody [41]. To account for these observations, the authors postulated that T-cell anergy in CD4 T cells is impaired in PD-1-deficient T cells and, therefore, alloreactive T cells of these mice display enhanced proliferation and cytokine production. PD-L1.Ig fusion protein alone did not prolong fully MHC-mismatched islets or heart allograft survival, but the co-administration of PD-L1.Ig fusion protein and anti-CD40L mAb achieved a synergistic effect that led to prevention of islet and chronic cardiac allograft rejection [39,40].

Moreover, in xenotransplantation, a short course of anti-CD40L mAb therapy induces prolonged survival in a nonvascularized concordant rat-to-mouse islet xenograft model [60,61]. This protective effect observed under the cover of anti-CD40L therapy is abrogated when the PD-1/PD-L1 pathway is blocked with a mAb against PD-1 [62].

The PD-1/PD-L1 pathway in Treg function

PD-1/PD-L receptor-ligand interactions regulate the balance between positive and negative signals required for the maintenance of central and peripheral tolerance. PD-1 expression distinguishes conventional CD4 T cells from $CD4^+CD25^+$ Treg as the former up-regulate CD25 and PD-1 upon activation, while the latter express minimal levels of PD-1 [63]. Although PD-1 mRNA is expressed in resting Treg, this receptor only translocates to the cell surface when these cells are stimulated via the TCR [64].

Several studies have focused their attention on the role of PD-1/PD-L1 pathway in Treg-cell-mediated down-regulation of effector T-cell responses in transplantation. For instance, PD-1/PD-L1 blockade using a blocking anti-PD-L1 mAb had only a partial effect on the ability of Treg to down-regulate the proliferation of $CD4^+CD25^-$ T cells co-cultured with anti-CD3 mAb. However, incubation of Treg with allogeneic APC in the presence of anti-PD-L1 mAb, inhibited the suppressive activity of Treg [65]. More importantly, antibody-mediated PD-L1 blockade abrogated Treg-mediated suppression in GvHD and allograft rejection in Rag2-deficient B6 mice grafted with BALB/c skin and adoptively transferred with syngeneic conventional CD4 T cells and Treg [65]. In line with these studies, Sandner *et al.* [48] reported that PD-L1 blockade induced a more rapid rejection of MHC class II-mismatched skin allografts by enhancing allogeneic T-cell proliferation and Th1 cell differentiation. PD-L1 blockade also influenced the balance between pathogenic T cells and Treg in the graft site as the concomitant treatment of CTLA-4.Ig with blocking anti-PD-L1 led to a significant decrease in the percentage of cells expressing Foxp3 in heart allografts compared to recipient mice treated only with CTLA-4.Ig [58]. Another example of suppressor cell involvement in controlling allogeneic immune responses comes from the observation that delayed blockade of ICOS-B7h pathway augments cardiac allograft survival across a full MHC barrier in WT mice but not in CD8-deficient mice by enhancing the generation of Ag-specific $CD8^+CD28^-PD\text{-}1^+$ regulatory T cells capable of suppressing allogeneic $CD4^+$ T-cell-mediated responses without affecting $CD8^+$ T-cell-mediated responses [66]. Together, all this evidence suggests that the PD-1/PD-L1 pathway may contribute to the maintenance of peripheral transplantation tolerance by limiting the expansion of alloreactive T cells via an active regulatory mechanism.

PD-1/PD-L pathway and T-cell co-stimulation

Growing evidence reinforces the notion that PD-1 ligands, particularly PD-L1, deliver negative signals to T cells; however, it cannot be ignored that some experimental data

suggest that PD-1 ligands might also contribute to co-stimulation of T cells to some extent either through PD-1 or through other unidentified receptors. Dong *et al.* [31] have reported that PD-L1.Ig moderately stimulated human T-cell proliferation and markedly up-regulated IL-10 production. Similarly, PD-L1.Ig preferentially co-stimulated CD4 T cells independently of CD28 and enhanced allogeneic responses in mixed lymphocyte reactions [67]. PD-L2.Ig stimulated the proliferation of murine T cells and exhibited a potent co-stimulatory activity, which correlated with high levels of IFN-γ production [36]. More evidence has come from experiments with PD-L1 mutant molecules that were unable to bind to PD-1 but surprisingly stimulated T-cell proliferation and cytokine production with or without the expression of PD-1 on T cells as well as in the presence of soluble PD-1.Ig soluble fusion protein [51]. Another example that supports the co-stimulatory role of PD-L molecules is that PD-L2-deficient mice exhibit diminished IFN-γ production by naïve CD4 T cells and impaired IFN-γ-dependent humoral and cytotoxic T lymphocyte (CTL) responses [68]. The co-stimulatory activity of PD-1 ligands and the identification of receptors other than PD-1 are active areas of research.

Contribution of the BTLA/CD160/HVEM/LIGHT pathway to regulation of allogeneic T-cell responses

Pattern of expression of molecules implicated in the BTLA/CD160/HVEM/LIGHT pathway

B- and T-lymphocyte attenuator (CD272) is a member of the Ig SF that contains two ITIM domains in its cytoplasmic tail that are involved in the recruitment of the tyrosine phosphatases SHP-1 and SHP-2. The BTLA gene was initially isolated from a cDNA clone expressed mainly in Th1 cells [69]. The expression pattern of the BTLA receptor is more restricted than that of PD-1. BTLA expression appears in the thymus during positive selection and at low levels in the bone marrow during pro-B and pre-B stages. It is also present in more advanced stages of differentiation such as in peripheral naïve B cells, and is induced in activated Th1 T cells [69–74]. The immunosuppressive drug CsA (a calcineurin inhibitor) prevents BTLA expression on T cells activated with polyclonal stimulators and addition of IL-2 does not restore its expression [75].

Murine BTLA is a polymorphic molecule, and three different allelic variants differing in their distribution of expression on leukocyte subsets have been described among 23 murine strains tested thus far. The main distinction is that the BALB/c BTLA allele is not expressed on macrophages or NK cells whereas B6 BTLA allele is expressed on macrophages and NK cells [74] and binds with slightly higher affinity to HVEM than BALB/c BTLA does [76].

BTLA-deficient mice display an increased antigen-specific antibody response and susceptibility to experimental allergic encephalomyelitis (EAE) [69]. BTLA was first postulated to bind to a B7-like Ig family molecule (B7x/B7-H4) based on the observation that B7-H4.Ig fusion protein did not bind to activated BTLA-deficient T cells but did bind to similarly activated WT T cells [77]. However, this initial description was misinterpreted, and recent work shows that the BTLA ligand is actually a member of the TNFR SF called HVEM [71]. HVEM interacts not only with BTLA but also with two other molecules, namely LIGHT and lymphotoxin alpha (LT-α) [78]. BTLA and herpes virus gD protein recognize the same domain of the HVEM protein, namely the co-inhibitory cysteine-rich domain (CRD1), the most membrane-distal domain on this protein, whereas the interaction between HVEM and LIGHT is located at the CRD2 and CRD3 domains (co-stimulatory domains). HVEM is constitutively expressed on T cells and in many immune cells, such as B cells, DC, NK cells, neutrophils and peripheral blood monocytes [79]. To explain the functional importance of this receptor, two different scenarios have been postulated. First, in the absence of HVEM expression on T cells, HVEM expressed on other cell types would deliver negative signals through BTLA to T cells (co-inhibition). Second, if BTLA is absent, but HVEM is present on T cells, then LIGHT would interact with T cells via HVEM as LTβR, the other receptor for LIGHT, is not expressed on T cells [80,81]. This hypothesis, however, does not account for the phenotype of HVEM-deficient mice, which are more susceptible to autoimmune-induced diseases, suggesting that HVEM itself may also deliver negative signals to T cells [82] (Fig. 5).

CD160, a molecule of the immunoglobulin SF has been added to the already complex BTLA/HVEM/LIGHT pathway. CD160 is expressed on NK cells, NKT cells, intraepithelial T cells, γδ T cells, memory/activated effector $CD8^+$ T cells and on a small subset of memory/activated $CD4^+$ T cells, and on 8% of unstimulated $CD4^+$ T cells [83,84]. It is also up-regulated on $CD4^+$ T cells at about 3 days after polyclonal T-cell activation. Engagement of CD160 by antibody cross-linking prevents CD4 T-cell activation even in CD4 T cells that do not apparently express the molecule on their surface. CD160 has been shown to interact with the CRD1 of HVEM at potentially overlapping sites likewise recognized by BTLA. However, despite the higher affinity of HVEM for LIGHT than for BTLA or CD160, the inhibitory function of HVEM is dominant over its co-stimulatory activity [83,84].

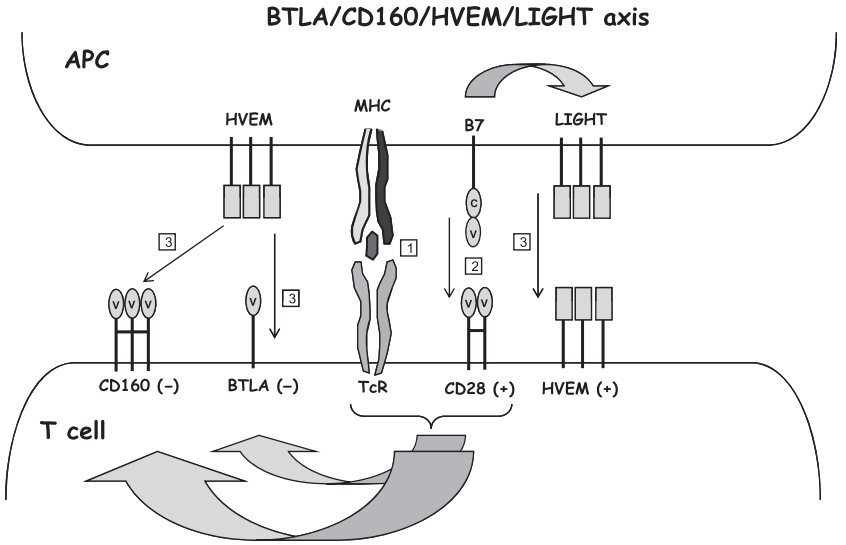

Figure 5 BTLA/CD160/HVEM/LIGHT co-inhibitory/co-stimulatory axis. Although BTLA is constitutively expressed on naïve B cells, CD28 co-stimulation and T-cell activation are required in order for BTLA to be up-regulated on $CD4^+$ Th1 cells. While BTLA is inducible on T cells, the ligand HVEM (that can also act as one of the LIGHT receptors) is constitutively expressed on a large array of immune cells. CD160 is a novel molecule expressed on T cells upon T-cell activation that like BTLA, binds to CRD1 domain of HVEM. It has been postulated that in the absence of HVEM expression on T cells, HVEM expressed in other cell types would deliver negative signals through BTLA to T cells (co-inhibition). If BTLA is absent, but HVEM is present on T cells, then LIGHT would interact with T cells expressing HVEM to co-stimulate them, as the other receptor for LIGHT, LTβR, is absent on T cells. The numbers on the figure illustrate the sequence of signaling events that follow upon encounter APC and T cell.

Therefore, CD160 is a novel molecule joining the BTLA/HVEM/LIGHT axis that is expected to bring novel insights into this co-inhibitory signaling pathway. Certainly, the development of CD160 KO mice in the near future would clarify their *in vivo* functional activity in different murine models of disease.

Transplantation models using BTLA KO mice and blocking monoclonal antibodies

The second most prominent and well-studied co-inhibitory pathway identified so far is the BTLA/CD160/HVEM/LIGHT pathway. While partially MHC-mismatched (K^{bm1} or IA^{bm12}) cardiac allografts survive long-term (longer than 3 months) in B6 mice, they are rejected in 2–3 months (class I disparity) or 2–3 weeks (class II disparity) in BTLA-deficient B6 mice [46]. In agreement with this report, the administration of blocking anti-BTLA mAb to WT B6 mice also accelerated the rejection of cardiac allografts across MHC class II barriers with rejection observed around day 30 after transplantation, whereas untreated control allografts survived longer than 3 months. MHC class II-mismatched cardiac allografts in mice deficient in both BTLA and PD-1 were also rejected more rapidly than in mice deficient in BTLA alone or in WT mice [46]. Furthermore, support for the hypothesis that HVEM delivers negative signals to BTLA-expressing T cells is given by the observation that MHC class II-mismatched heart allografts in HVEM-deficient B6 mice are rejected by 2–3 weeks after transplantation [46]. Overall, this indicates that the BTLA/HVEM axis is required for the long-term survival of heart allografts across class I or class II MHC barriers, affecting both CD4 and CD8 T cell-mediated mechanisms of rejection.

Unexpectedly and unlike the results across class I or class II MHC barriers, fully mismatched cardiac allografts in BTLA-deficient mice or in anti-BTLA mAb-treated mice displayed a slightly prolonged graft survival compared to WT controls. Prolonged survival of fully MHC-mismatched cardiac grafts was also seen in BTLA/PD-1 double KO mice and in BTLA-deficient mice treated with anti-PD-1 mAb [46]. Therefore, PD-1 and BTLA signaling seem to regulate the T-cell responses, in scenarios in which rejection requires CD4 help for the generation of humoral and cytolytic responses but not in circumstances in which CD4 or CD8 T cells act independently as effectors of rejection (i.e., not in partially mismatched settings). An unexpected role of BTLA has also been documented in a murine model of GvHD (nonirradiated parental into F1 model). In this model, BTLA-deficient splenocytes transferred into F1 recipients or WT splenocytes transferred into anti-BTLA-treated F1 recipients disappeared in the periphery of the recipient mice. This suggests that parental

BTLA-deficient T cells were unable to maintain the GvHD response rather than demonstrating an increased alloresponse. This points to the fact that the interaction of BTLA with HVEM is indispensable to sustain the survival of donor lymphocytes in GvHD [85].

With regard to islet allotransplantation, BALB/c islets grafted in B6 mice are rejected in about 20–25 days [86,87]. However, the administration of CTLA-4.Ig every other day for 10 days significantly increased graft survival [88]. In contrast to the outcome observed in heart allografting, the administration of anti-BTLA mAb did not accelerate the rejection of islet allografts in CTLA-4.Ig-treated mice. Unexpectedly, anti-BTLA treatment synergized with CTLA-4.Ig to prolong graft survival compared to CTLA-4.Ig-treated control mice [89,90]. The reason for this discrepancy may have to do with the fact that islet engrafting requires neovascularization whereas heart is a vascularized transplant.

HVEM/LIGHT signaling pathway in transplantation

Unlike other members of the TNFR SF, LIGHT is not required for normal development of lymphoid organs. However, LIGHT-deficient $CD8^+$ T cells have attenuated cytotoxic activity and cytokine production, and LIGHT-deficient $CD4^+$ T cells are defective in IL-2 secretion [91]. This immunity defect is reflected to some extent in LIGHT-deficient mice, in which fully MHC-mismatched cardiac allografts survived slightly longer than those in WT control mice (10 vs. 7 days). The treatment of WT mice with HVEM.Ig fusion protein alone did not promote prolongation of graft survival. However, CsA (10 mg/kg for 10 days) synergized with HVEM.Ig, prolonging graft survival to 21 days [92].

Other co-inhibitory pathways in transplantation

B7-H3 delivers negative signals to T cells *in vitro*

B7-H3 is another member of the B7 family and is expressed on lymphoid and nonlymphoid tissues with a typical IgV–IgC structure in both human and mice. It binds to an unknown receptor on activated T cells distinct from CD28, ICOS, CTLA-4 or PD-1. It is induced by inflammatory cytokines on DC and macrophages [93,94]. Although it has been described as a co-stimulatory molecule in humans, B7-H3 delivers co-inhibitory signals to murine T cells *in vitro* [93,95–97]. B7-H3 inhibits anti-CD3-mediated T-cell proliferation of both CD4 and CD8 T cells in a dose-dependent fashion, but this inhibitory effect is overcome by CD28-mediated co-stimulation. The presence of B7-H3 on APC negatively regulates allogeneic T-cell proliferation, as evidenced by increased proliferation of T cells in MLR assays when stimulated with APC from B7-H3-deficient mice relative to when stimulated with APC from WT mice. Overall, these facts point out that, at least *in vitro*, B7-H3 is involved in the delivery of negative signals to murine T cells [95].

In vivo co-stimulatory role of B7-H3 receptor

B7-H3-deficient B6 mice and WT B6 mice reject fully MHC-mismatched BALB/c cardiac allografts with similar kinetics [98]. Surprisingly, the administration of CsA led to prolonged graft survival in B7-H3-deficient mice compared to WT mice. Similarly, the administration of rapamycin induced long-term protection of cardiac and islets allografts in B7-H3-deficient mice while WT mice treated with rapamycin survived only an additional 2 weeks relative to the untreated controls. Furthermore, anti-CD40L administration to B7-H3-deficient mice induced long-term survival, while WT control mice receiving only anti-CD40L rejected heart allografts by 90 days [98]. These *in vivo* results do not reflect the *in vitro* situation. It would be expected that in the absence of negative signals delivered by B7-H3 to activated T cells, rejection would be accelerated in these mice. This paradoxical observation suggests that, as in humans, B7-H3 receptor may be involved in delivering positive signals to alloreactive T cells *in vivo* in murine systems.

B7x receptor and its orphan ligand

B7x (B7-H4/B7-S1) receptor is a recently described member of the B7 family that is expressed on lymphoid and nonlymphoid tissues. Based on studies using a B7x.Ig fusion protein, B7x appears to recognize an unidentified ligand expressed on activated T cells. In the presence of increasing amounts of immobilized B7x.Ig fusion protein, anti-CD3-induced proliferation and IL-2 secretion is down-regulated in a dose-dependent manner. This effect is mediated by a reduction in the number of $CD4^+$ and $CD8^+$ T cells that enter the cell cycle and by reducing their division rate. It also inhibited cytolytic responses against allogeneic targets *in vivo* [77,96,99].

Conclusions and perspectives

Skin, heterotopic heart, and islet allograft transplantation across different histocompatibility barriers have provided an enormous amount of information on the role of co-stimulatory and co-inhibitory pathways. Data obtained from rodents have contributed to a better understanding of $CD4^+$ and $CD8^+$ T-cell-mediated mechanisms of rejection and of the potential for novel therapeutic interventions that can be translated to the clinic. The enhancement

of inhibitory pathways by anti-PD-1 and anti-BTLA agonist mAbs or agonistic molecules such as PD-L1.Ig and HVEM.Ig, combined with immunosuppressive drugs, may assist clinicians in modulating and suppressing T-cell-mediated immune responses more specifically while reducing the load of immunosuppressants required to achieve appropriate protection of the graft.

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