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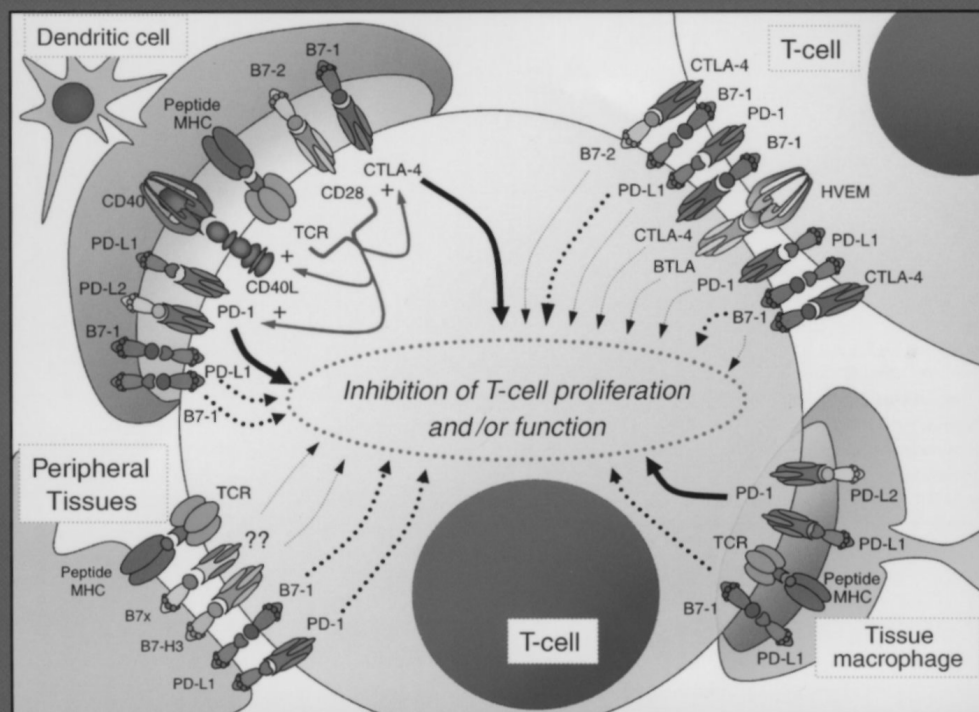
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Inhibition of Immune Cell Function

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Brian T. Fife
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Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways

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Summary: Classically, the CD28/cytotoxic T-lymphocyte antigen-4 (CTLA-4) and B7 families of cell surface molecules regulate complex signaling pathways that profoundly affect T-cell responses. The recent identification and characterization of additional CD28 and B7 family members including programmed death-1 (PD-1), programmed death ligand-1 (PD-L1) (B7-H1), and PD-L2 (B7-DC) has added to the complexity and greater appreciation of how surface molecules control T-cell activation and peripheral tolerance. CD28/B7 interactions mediate co-stimulation and significantly enhance peripheral T-cell responses. CTLA-4, in contrast, interacting with the same B7 molecules, results in decreased T-lymphocyte activity and regulates the immune response. Similarly, PD-1 interactions with PD-L1 and PD-L2 downmodulate T-cell immune responses. Despite these similarities, the regulatory roles of the CTLA-4 and PD-1 pathways are distinct. This may be due, at least in part, to the differential expression patterns of the CTLA-4 and PD-1 ligands both temporally and spatially. This article examines the role of CTLA-4 and PD-1 in limiting autoreactivity and establishing peripheral self-tolerance with the hypothesis that CTLA-4 signals are required early in the lymph node during initiation of an immune response and PD-1 pathways act late at the tissue sites to limit T-cell activity.

Keywords: autoimmunity, tolerance, regulation, CTLA-4, PD-1, PD-L1

Introduction

The immune system has evolved to help protect the body from foreign invading pathogens. To accomplish this critical role, T lymphocytes must discriminate between self and non-self. This property translates into the immune recognition and elimination of infectious invaders while leaving host tissues intact. This highly selective response occurs through several complicated mechanisms to regulate T-lymphocyte activity. The first step is the elimination of self-reactive cells during T-cell development in the thymus. The goal of central tolerance is to achieve a T-cell repertoire that is 'self-tolerant.' However, this process is incomplete, and it is well accepted that autoreactive T cells that escape central tolerance are controlled by peripheral mechanisms (1, 2). Autoimmunity results when these peripheral mechanisms

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fail. The past few years have witnessed significant progress toward understanding the processes involved in autoimmune disease pathogenesis and the breakdown of T-cell tolerance. These advances include the identification and characterization of the key cell types and surface receptors involved in the induction and prevention of autoimmunity. A great deal of information about CD4⁺ effector T cells has been learned from these studies including the role of T-helper 1 (Th1), Th2, and Th17 subpopulations during normal immune responses and autoimmunity. These cells play a pivotal role in directing other inflammatory cells and drive differentiation by creating microenvironments filled with inflammatory cytokines and chemokines (3). Our understanding of antigen-presenting cells (APCs), especially dendritic cells (DCs), has also increased and demonstrates that early interactions with T lymphocytes can have profound effects on the immune response. The activation state of DCs is also important and can be influenced by environmental signals. During an inflammatory response, the APCs act as master regulators and drive adaptive immunity, affecting all T-effector cell subsets. A number of pro-inflammatory cytokines, such as interleukin-12 (IL-12), and cell surface molecules, such as CD28, promote T-cell activation and immunity (3). By contrast, alternative classes of DCs, some with immature phenotypes and others that produce suppressive factors such as IL-10 and indoleamine 2,3 dioxygenase (IDO), favor immune tolerance, possibly through anergy induction and suppressor T-cell activation. Among the most important suppressor T cells, the CD4⁺CD25⁺ Forkhead box protein 3 (FoxP3)⁺ regulatory T-cell (Treg) subset plays a vital role in maintaining immune homeostasis and limiting immune responses (3). In addition to the role of different cell types, we have also learned a great deal about the surface receptors and signaling pathways that regulate T-lymphocyte activity. To date, two key inhibitory receptors that critically affect peripheral T-cell tolerance and T-cell function have been identified: cytotoxic T-lymphocyte antigen-4 (CTLA-4) (CD152) and programmed death-1 (PD-1) (CD279). This article focuses on our current understanding of the negative regulatory molecules CTLA-4 and PD-1, their function in controlling autoimmunity, and how these molecules regulate autoreactive peripheral T cells that have escaped central tolerance. CTLA-4 and PD-1-mediated T-cell intrinsic and extrinsic control of effector cells is also discussed as influenced by the expression of their critical ligands. In addition, we address the consequences of PD-1 and CTLA-4 signaling as it relates to T-cell activation, T-cell mobility (stop signaling), cytokine production, T-cell receptor (TCR) signaling, and function. Lastly, opportunities for

clinical intervention in areas of autoimmunity, tolerance, transplantation, and chronic viral infections are discussed.

CD28, CTLA-4, PD-1: T-cell activation, tolerance, and autoimmunity

The most widely recognized costimulatory molecule is CD28, which is expressed on T cells and interacts with the ligands B7-1 (CD80) and B7-2 (CD86) on the surface of APCs (3). CD28 signaling positively influences T-cell proliferation, IL-2 production, promotes cell survival via Bcl-x_L transcription, and prevents anergy (4). Antibodies against B7-1 and B7-2 can inhibit T-cell activation *in vitro*, demonstrating an important role for CD28 (3). Furthermore, CD4⁺ T-cell proliferation is severely impaired in CD28-deficient mice (5). The extent or requirement for CD28 costimulation of CD8⁺ T cells is more controversial; however, CD8⁺ T cells are less CD28 dependent than CD4⁺ T cells (6–9). These findings suggest that the requirement for CD28 costimulation may be different for CD4⁺ and CD8⁺ T cells. To complicate matters, negative regulators have been added to the mix of costimulation and include the family members CTLA-4, PD-1, and B and T lymphocyte attenuator-4 (BTLA-4), among others (reviewed in 3, 10). CTLA-4 has been well established as a negative regulator of T-cell function (11, 12). CTLA-4 is rapidly expressed on the surface of T cells following activation and is highly upregulated by CD28 engagement (11). In fact, CD28 engagement not only enhances activation and T-cell function but also positions the system for self-regulation by rendering the T cells susceptible to homeostatic signals later in the course of an immune response. The costimulatory signal through CD28 primes the system for regulation by increasing T-cell expression of CTLA-4 and PD-1 (11, 13). The presence of positive and negative costimulatory molecules provides the body a mechanism to control the immune system, which is a difficult balancing act.

CTLA-4 ligation antagonizes early T-cell activation, leading to decreased IL-2 production, inhibition of cell cycle progression, decreased cyclin expression, and modulation of TCR signaling (12, 14, 15). CTLA-4 shares the ligands B7-1 and B7-2 with the positive costimulatory molecule CD28, adding to the complex biology of CTLA-4 (16). Mice deficient for CTLA-4 develop lymphoproliferative disease and die within 3–4 weeks of birth (17, 18), adding further evidence for the critical role of CTLA-4 inhibition of T-cell responses and immune homeostasis. More recently, CTLA-4 has also been implicated in the function of Tregs, which suppress effector T-cell activation and function (19, 20). CTLA-4 has also been

identified as a gene implicated in the susceptibility of several autoimmune diseases including diabetes, multiple sclerosis, rheumatoid arthritis (RA), myasthenia gravis, pancreatitis, thyroiditis, systemic lupus erythematosus (SLE), colitis, as well as several others (reviewed in 3, 10). Thus, many efforts have focused on developing therapeutic approaches to selectively engage this pathway to treat autoimmunity and prevent organ transplant rejection.

Similarly, PD-1 has been described as a negative regulator of immunity that limits T- and B-cell function. PD-1 is a cell surface molecule expressed on activated T and B cells (reviewed in 10). PD-1 interacts with at least two ligands: programmed death ligand-1 (PD-L1) and another closely related molecule, PD-L2 (21–23). The expression patterns of PD-L1 and PD-L2 are somewhat distinct. PD-L1 is expressed on leukocytes, non-hematopoietic cells, and in non-lymphoid tissues including islets (21, 24, 25), while PD-L2 is expressed exclusively on DCs and monocytes (21, 22, 26, 27). The differential expression patterns of PD-L1/PD-L2 and B7 molecules are key differences between these two regulatory receptors and are central to the hypothesis that CTLA-4 acts early for tolerance induction and PD-1 acts late for long-term tolerance maintenance. Signaling through PD-1 limits T-cell function including interferon- γ (IFN- γ) production, proliferation, and increased T-cell apoptosis (28, 29). Notably, negative signals through PD-1 on T cells control autoimmunity. Disruption of the gene encoding PD-1 in BALB/c mice resulted in autoimmune cardiomyopathy (30), while C57BL/6 PD-1 knockout (KO) mice develop progressive arthritis and a lupus-like glomerulonephritis disease (31), and NOD PD-1 KO mice develop accelerated autoimmune diabetes (32). The role of PD-1 and its ligands has been further investigated in autoimmune models such as diabetes and multiple sclerosis using blocking reagents. Administration of anti-PD-L1 monoclonal antibodies (mAb) to young NOD mice, 1–10 weeks old, resulted in rapid onset of diabetes (26). Moreover, PD-1 plays a critical role in murine experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. In EAE, PD-1 blockade resulted in accelerated and more severe clinical disease (33). It was reported recently that a polymorphism in the PD-1 gene is associated with susceptibility to the autoimmune diseases SLE, RA, diabetes, Graves' disease, and multiple sclerosis, linking this important inhibitory receptor to autoimmunity in humans (34–41).

CTLA-4 and PD-1 deficient mice both develop autoimmune phenotypes, however, the severity and timing is significantly different. It is possible that PD-1 and CTLA-4 act differently on a fundamental level to shape the T cell repertoire. Early during

life, T cells are generated and rapidly expand to fill the lymphatic environment (42). In mouse experiments, tolerized or anergic cells proliferate and expand in an 'empty' host (42). It is unclear what signals control this expansion. CTLA-4 might play a critical role in this homeostatic expansion, given the observation that CTLA-4-deficient mice develop multi-organ disease and die within 3 weeks, independent of genetic background (3). PD-1-deficient mice, however, take several months to develop autoimmunity, and this disease is targeted to specific organs (43). Perhaps genetic factors and strain-specific nuances predispose hosts to autoimmunity by having a higher precursor frequency of autoreactive T cells. The absence of PD-1 may allow these cells to overcome tissue-specific barriers and cause disease. It is tempting to speculate that these molecules play distinct roles in limiting T-cell function; if the roles were overlapping, the absence of either CTLA-4 or PD-1 would not result in autoimmunity. This sets up the possibility and hypothesis that differential expression patterns of the CTLA-4 and PD-1 ligands control T-cell functions within different microenvironments. PD-1 ligand expression limits T-cell function within tissue-specific sites based on constitutive expression, while CTLA-4 limits T cells in lymphoid structures based on APC expression of B7 molecules.

CD28 and CTLA-4 broaden the T-cell response

CD28 costimulation provides signals to enhance T-cell activation and lower the threshold for full activation. By lowering the threshold, CD28 allows lower affinity T-cell clones to become activated, thus broadening the polyclonal response to any given antigen. Similarly, CTLA-4 signaling affects the polyclonal T-cell response. It has been well established that CTLA-4 expression is upregulated following T-cell activation (reviewed in 3). It has also been described that T cells with the highest affinity to a given antigen have the highest level of CTLA-4 expression (44). CTLA-4 signaling results in diminished T-cell responses and helps bring an inflammatory response back down to homeostatic levels. Therefore, the highest affinity cells expressing the highest surface levels of CTLA-4 will be preferentially inhibited, allowing the lower affinity clones to respond and participate in the immune response (44). This CD28/CTLA-4 sliding threshold hypothesis might act to ensure that the body mounts a diverse T cell response and increase the potential to clear invading pathogens. This theory makes sense from an evolutionary point of view for host survival. By activating T cells that would likely target invading pathogens, but at the same time broadening the repertoire, the body has effectively

'covered the bases' to maximize an effective immune response to eliminate the threat (3).

Role of CTLA-4/CD28 and PD-1 pathways in T cell intrinsic and extrinsic regulation

Once autoreactive T cells escape central tolerance and leave the thymus, peripheral mechanisms must control their activation or autoimmunity will develop (3, 10). The first mechanism to maintain peripheral tolerance is to remove autoreactive T cells or delete them. This process occurs primarily through the Fas cell death pathway, as deficiencies or deletions in the genes *lpr* or *gld* result in an abundance or accumulation of activated cells in the periphery with increased rates of autoimmune disease (45–47). Cell death, especially activation-induced cell death (AICD) mediated by the Fas/Fas ligand and tumor necrosis factor (TNF)/TNF-receptor pathways, is a critical mechanism for the immune response to return to homeostasis during the refractory phase of a response. Under normal circumstances, T cells respond and clonally expand in response to antigen recognition and positive costimulatory signals. This expansion is followed by a contraction phase. CD28 costimulation provides pro-survival signals through the upregulation of anti-apoptotic genes, namely Bcl-x_L and the growth factor IL-2 (48, 49). In addition, CD28 activates phosphatidylinositol 3-kinase (PI3K), which in turn signals through AKT and promotes survival (50–52). The nature of positive costimulation is to enhance survival and T-cell function, including the induction and survival of Tregs. CTLA-4, while not directly pro-apoptotic, might influence cell fate by suppressing CD28-positive signals, resulting in cell death.

CTLA-4-deficient animals have an accumulation of activated cells in multiple tissue sites; however, CTLA-4 engagement has not been linked to Fas-mediated death or other forms of apoptosis (12, 53). Similarly, PD-1 has not been linked to AICD despite its name. PD-1 was originally identified from T-cell hybridomas undergoing programmed cell death (54). Further studies have shown that PD-1 is more likely to function on cellular activation and not directly on death (55, 56). PD-1 ligation inhibits the induction of Bcl-x_L, an important cell survival factor. PD-1 is expressed on thymocytes and plays an important role in central tolerance both during positive and negative selection (13, 57). PD-1/PD-L1/PD-L2 interactions affect cell fate decisions at several phases of thymocyte maturation. Double-negative (CD4[−]CD8[−]) thymocytes express PD-1, which was shown to be important for selection of thymocytes that have rearranged TCR-β genes productively, a process required for positive selection (13, 58).

PD-1 expression increases upon TCR ligation of double-positive (CD4⁺CD8⁺) thymocytes, and PD-1 may help broaden the TCR-αβ repertoire by regulating the threshold for activation and subsequent selection (59). The ligands for PD-1 are also important for this process based on their expression patterns within the thymus. PD-L1 is broadly expressed in the thymus cortex, while PD-L2 is limited to medullary stromal cells (24, 57). This selective expression, like that in the periphery, may have dramatic consequences for tissue-specific regulation of T-cell function, including positive selection (58). In addition, PD-1-deficient mice have been shown to have dramatic defects in negative selection, indicating again that PD-L expression may be critical to regulate T-cell development and shape the T-cell autoreactive repertoire (60). Therefore, PD-1 may contribute to autoimmunity by an increase of autoreactive T cells escaping selection and central tolerance in the thymus, contributing to the phenotype of the KO mice. Although the precise role for CTLA-4 and PD-1 in central tolerance are debated, it is clear that these receptors are critical for regulating peripheral tolerance.

Role of CTLA-4/CD28 and PD-1 pathways in T-cell anergy

A second form of T-cell tolerance is described as a state of non-responsiveness or anergy, marked by a dramatic reduction in IL-2 production and other effector functions (61–63). Intrinsic mechanisms include the delivery of inhibitory signals through receptors expressed by activated T cells, such as PD-1 (64) and CTLA-4 (65). In this setting, self-reactive T cells are not deleted but persist in an altered and decreased state of responsiveness to subsequent antigen challenges (63). The seminal observation by Jenkins et al. (62) demonstrated that T cells receiving signal 1 (antigen recognition), in the absence of signal 2 (costimulation), resulted in T-cell anergy or non-responsiveness led to the quest to understand mechanisms of peripheral tolerance. In vivo, several methods of antigen delivery have been found to induce a state of T-cell unresponsiveness or anergy. These data suggest that anergy is one potential mechanism the body uses for peripheral tolerance. The knowledge and understanding that autoreactive T cells are present in healthy populations yet remain inert or held in check was, and still is, the center of much research. Both CTLA-4 and PD-1 have been implicated as critical regulators of peripheral tolerance (reviewed in 3, 10).

The direct engagement between PD-1 and PD-L1/PD-L2 to specifically limit T-cell function is intrinsic to the T cell. The mechanism for action of PD-1 signaling has been shown to require an antigen (TCR engagement) and occurs in *cis*

(receiving PD-1 and TCR signals from the same APC) (66). PD-1 signaling does not limit T-cell responses when PD-L1/PD-L2 receptor engagement occurs with neighboring cells, *in trans*. PD-1 has both an immunoreceptor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM) (67). PD-1 is phosphorylated on two intracellular tyrosines upon PD-L1/PD-L2 engagement, which can bind Src homology 2 (SH2) domain-containing tyrosine phosphatase 1 (SHP-1) and SHP-2 (56, reviewed in 67). The binding of SHP-1 and SHP-2 can directly affect T-cell activation by dephosphorylating key TCR signaling intermediates, thus terminating early TCR signaling. PD-1 signaling can cause a redistribution of uniform cell surface expression to the immunological synapse and may potentially bring SHP1 and SHP2 with it (68, 69). However, the concentration of PD-L1 must be extremely high for PD-1 migration into the synapse (70). In addition, PD-L1 has been shown to function even at low levels, and so it is unclear whether PD-1 inhibition depends on re-distribution to the synapse (71). PD-1/PD-L1 interactions may alternatively inhibit TCR signaling via competition for accessory signaling machinery and simple competition for key substrates to limit and terminate TCR signaling. This may in fact reflect the ability of PD-1 to destabilize the synapse by competing for signaling components or by dephosphorylating the TCR signaling components as they move into the immunological synapse. These effects are cell-intrinsic mechanisms for PD-1 mediated T-cell inhibition.

CTLA-4 similarly functions to limit T-cell signaling, cytokine production, cell cycle progression, and biochemical signals including activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B) (reviewed in 65). There are currently at least four different models describing how CTLA-4 inhibits T cells: ligand competition, proximal blockade, distal blockade, and a ligand-independent model (reviewed in 65, 72). The ligand competition model has already been described above, where CTLA-4 directly competes for B7 proteins with CD28. This model is attractive, because CTLA-4 has significantly higher avidity for the B7 ligands combined with a zipper-like multivalency of CTLA-4 with B7 (reviewed in 65). Ligand competition is a result of direct interference for B7s. If B7s are engaged by CTLA-4, CD28 cannot physically interact to mediate the positive signals required for efficient activation. Therefore, CTLA-4 prevents the positive effects of CD28 signaling. Because CTLA-4 binds to B7 with a higher affinity than CD28, CTLA-4 signaling would prevail under conditions with low B7 expression.

CTLA-4 ligand-dependent intrinsic regulation of T cells is thought to act at the immunological synapse by directly

binding to the TCR- ζ complex (65, 73, 74). This intrinsic regulation is the most attractive mechanism and includes both proximal and distal TCR signaling blockade. In this model, CTLA-4 regulation is mediated indirectly through unique negative signals. These negative signals antagonize early T-cell activation, leading to decreased essential growth factors, namely IL-2, and limit cell cycle progression (65). Like PD-1, CTLA-4 has been shown to interact with the phosphatases SHP-1 and SHP-2 (65). Distinct from PD-1, CTLA-4 has also been shown to interact with PP2a, suggesting an additional inhibitory pathway (75). Another significant difference between these two inhibitory receptors is that PD-1 has an ITSM (56). Chemnitz *et al.* (56) reported that mutation of the ITIM had little effect on PD-1 signaling or functional activity. In contrast, mutation of the ITSM abrogated the ability of PD-1 to limit T-cell expansion (56). This observation suggests that PD-1 and CTLA-4 use different structural motifs to bind and recruit phosphatases for signal blockade. Along this line, several studies support the proximal blockade model in which CTLA-4 acts in close proximity to the primary TCR signal (reviewed in 65). We have reported that CTLA-4 functions in the immunological synapse to terminate T-cell signaling (72). Understanding the precise mechanism for CTLA-4-mediated T-cell homeostasis will aid in our understanding and development of therapeutics.

In addition to direct engagement of CTLA-4 with B7 proteins, there is evidence of a ligand-independent model for CTLA-4 inhibition. It has been shown recently that the mouse CTLA-4 gene has an alternatively spliced form, ligand-independent CTLA-4 (liCTLA-4), which encodes a molecule completely lacking a ligand-binding domain (76, 77). The liCTLA-4 splice variant contains a single nucleotide polymorphism (SNP) within exon2 of CTLA-4 and is found in NOD mice, a strain prone to developing spontaneous autoimmune diabetes (77). The experimental evidence for the ligand-independent function of CTLA-4 comes from the following observations using CTLA-4-deficient mice. CTLA-4 KO mice die early (3–4 weeks) in life from polyclonal lymphocyte activation and proliferation, resulting in multi-organ lymphocytic infiltrates and uncontrolled autoimmunity (17, 18). CTLA-4^{-/-} B7-1^{-/-} B7-2^{-/-} triple KO mice survive, unless anti-CD28 is administered. This result indicates that the autoimmunity occurring in CTLA-4 KO mice is dependent on CD28 activation of T cells, which requires B7 engagement. In contrast, CTLA-4-sufficient, B7-1^{-/-} B7-2^{-/-} double KO mice survive, even if anti-CD28 is administered, suggesting that even in the absence of B7, CTLA-4 can function to limit lymphoproliferative disease of CD28-activated T cells

(reviewed in 65, 72). The concept of ligand-independent signaling is not novel for immune receptors as several molecules including CD45, pre-TCR, and pre-B-cell receptor (pre-BCR) have been suggested to signal in a ligand-independent fashion (65, 78). However, this feature of CTLA-4 is unique for negative regulators, as PD-1 inhibition requires engagement with the PD-L1/PD-L2 ligand to mediate T-cell suppression (67). An alternative explanation for this observation is that a third, unidentified ligand could exist and bind to CTLA-4 and not CD28 to mediate this inhibition (65).

Role of CTLA-4/CD28 and PD-1 pathways in T-cell regulation

Tregs inhibit T-helper responses by extrinsic mechanisms. The recent surge of reports on Tregs has led to a large body of knowledge and a better understanding of immune homeostasis and control of aberrant, self-reactive leukocytes. $CD4^+CD25^+FoxP3^+$ Tregs may function as extrinsic regulators through the production of soluble factors including IL-10 and transforming growth factor- β (TGF- β) (79, 80). Extrinsic regulation of homeostasis has centered on the development and mechanistic characterization of these $FoxP3^+$ Tregs. $FoxP3$ is a transcription factor that marks Tregs and also has a critical role for inhibitory function (81, 82). The deletion of Tregs leads to multi-organ autoimmunity (83). Mice lacking effective Tregs as seen in the Scurfy mutation, $CD28^{-/-}$, $IL-2^{-/-}$, and $CD25$ -deficient mice all demonstrate this phenotype (reviewed in 84). Further evidence for the critical role of Tregs has been demonstrated with the selective deletion at various time points in life (82). There are several key steps in which Tregs may have an effect on these destructive T cells, including activation, differentiation, migration, and suppression.

The precise mechanism and role for PD-1 and CTLA-4 Treg function is a much-debated topic. We, and others, have shown mixed requirements for CTLA-4 in Treg function (reviewed in 84). In one study (19), we showed that blocking CTLA-4 on wild-type Tregs abrogated their suppressive activity *in vitro*. Although CTLA-4 blockade prevented Treg suppression, the result is complex, given that CTLA-4 is expressed on both Tregs and T-effector cells. In this same study, Tregs from CTLA-4-deficient mice developed and functioned normally, demonstrating CTLA-4-independent regulatory capabilities. These CTLA-4-deficient Tregs expressed increased levels of the suppressive cytokines IL-10 and TGF- β , and *in vitro* suppression mediated by CTLA-4 $^{-/-}$ Tregs was reduced by neutralizing TGF- β (19, reviewed in 84). These findings, along with the fact that CTLA-4 KO T-effector cells cannot be regulated, argue

that CTLA-4 inhibition is mediated by intrinsic mechanisms as opposed to extrinsic Treg control. In other studies, we have shown that PD-1 Treg interactions are important for controlling maternofetal tolerance. PD-L1 expression on Tregs was critical to maintain fetal tolerance (85). These results suggest that PD-1/PD-L1 inhibition is complex and regulation may occur through engagement of PD-L1 on APCs or through Tregs. It is unknown whether Tregs from PD-1 KO mice can suppress T-effector cell functions.

Anti-CD3 therapy has been shown to induce tolerance by at least two distinct mechanisms: autoreactive T-cell death through the induction of apoptosis and Treg suppression (86). In a recent study, we tested the effect of PD-1–PD-L1 blockade during anti-CD3-induced tolerance. Our results demonstrated that anti-CD3-induced tolerance was controlled by PD-1, as blocking this pathway resulted in rapid precipitation of autoimmune diabetes (87). We asked whether this effect was acting directly on effector cell function alone (intrinsic) or via extrinsic regulation by Treg control. In our diabetes system, BDC2.5 T cells were sorted into Tregs ($CD4^+CD25^+CD62L^+$) and effector T (Teff) cells ($CD4^+CD25^-CD62L^+$) and infused into NOD.RAG KO recipient mice. Two groups were analyzed: one group received Teff cells alone and the second group received Teff together with Tregs. The addition of Tregs with Teff suppressed the development of diabetes. PD-L1 blockade did not alter Tregs' ability to control this disease, indicating that Tregs did not depend on PD-1–PD-L1 interactions for tolerance induction and prevention of autoimmune diabetes (87). Thus, in this model, the PD-1–PD-L1 pathway reversed anergy in islet antigen-specific T cells by a direct T-cell intrinsic regulation. PD-1 blockade restored antigen-specific T-cell proliferation, inflammatory cytokine production, and rapid disease (87). We demonstrated that this effect was cell intrinsic; however, the role for PD-1 and Tregs is still unclear. Using a different autoimmune model, we demonstrated that tolerance was effective in the absence of Tregs, and this protection was controlled by PD-1 signals. In this system, we transferred $CD4^+CD25^-$ (Treg cell-depleted) pathogenic T cells into Treg cell-deficient hosts (87). Tolerance was induced using ethylene carbodiimide (ECDI)-fixed antigen-coupled splenocytes (antigen-SP) depleted of Tregs. Using this treatment, we did not detect an increase of $FoxP3^+$ TCR transgenic (Tg) $^+$ T cells, indicating that antigen-SP tolerance did not induce adaptive Treg cells, and yet PD-1 blockade precipitated rapid diabetes (87). These results suggest that the PD-1–PD-L1 pathway functions in a Teff cell-intrinsic manner to modulate IFN- γ production and direct T-cell function. Taken together, data from different models

for T-cell tolerance suggest that PD-1 functions through both T-cell-intrinsic and Treg-extrinsic mechanisms.

CTLA-4 acts early while PD-1 acts late to control tolerance

Immune homeostasis is a state of defensive readiness in the absence of active inflammation or immunity. The immune system has the difficult task of being ready and able to respond to any environmental insult. It must act quickly to respond and destroy the threat, and yet control local damage to preserve the host tissue. As a result, the process to fully activate a naive T cell is a selective and complex process with potentially dangerous side effects. The immune system has several mechanisms built in to help control T-cell responses once effective immunity has occurred. Among these mechanisms are Tregs, inhibitory cytokines, and negative signaling molecules. CTLA-4 and PD-1 limit T cells both early during the initiation of the immune response and late for the long-term maintenance of tolerance. Our hypothesis is that CTLA-4 plays a dominant role during tolerance induction, while PD-1 plays the dominant role during the maintenance of tolerance.

Animal models using gene deletions have demonstrated the critical role that negative regulatory molecules play in immune homeostasis. For a given genetic background, a hierarchy for negative regulation has emerged. CTLA-4 seems to be the dominant-negative regulator as deletion of this molecule despite different genetic backgrounds has led to a similar phenotype of rapid multi-organ tissue infiltration and death, within 3–4 weeks (17, 18). PD-1 has also been reported to cause severe tissue-specific autoimmunity which depends on the genetic background (3), but has slower kinetics than CTLA-4 KO disease (31, 43). PD-1-deficient mice on the C57BL/6 mouse background develop lupus-like glomerulonephritis and arthritis disease but not until late in life (50% at 14 months) (31). Similarly, PD-1 KO BALB/c mice develop cardiomyopathy (50% incidence by 5 months) (30). NOD.PD-L1 KO mice develop accelerated spontaneous diabetes (100% incidence by 10 weeks) (32). These reports suggest that PD-1 deficiency potentiates the autoimmune predisposition of the given genetic background, leading to the induction of different forms of autoimmune diseases. CTLA-4, in contrast, dominates and spreads multi-organ infiltrate and autoimmunity despite the genetic background.

CTLA-4 and PD-1 ligand expression and distribution may be the deciding factor for the timing and localization of T-cell control and development of autoimmunity. The autoimmune phenotype in the absence of PD-1 may be a consequence of

predisposed condition or underlying genetic propensities. Alternatively, the lack of tissue-specific PD-L1/PD-L2 expression could help tip the balance, allowing autoreactive T-cell activity that would otherwise be silent. PD-1 ligands, PD-L1 and PD-L2, unlike CTLA-4 ligands B7-1 and B7-2, are constitutively expressed in peripheral tissue sites during homeostasis and can become elevated in response to tissue insult (3, 67). Inflammatory cytokines and chemokines have been shown to upregulate tissue-specific expression of the inhibitory ligands (25, 88). Additional support for a tissue-specific role is demonstrated by PD-L1 expression in immune privileged sites including the eye and the placental vascular endothelial cells (24, 89). Blocking PD-1 in each of these tissues has demonstrated a significant effect on T-cell function for both fetal survival and corneal allograft rejection (85, 90). PD-L1 expression is also found in a large number of non-hematopoietic cells including vascular endothelium, non-parenchymal liver cells, pancreatic islets, placental syncytiotrophoblasts, keratinocytes, and lung cells (reviewed in 67). Functional data demonstrated that PD-L1 expression on non-bone marrow-derived cells including islets cells controlled tolerance in a spontaneous diabetes model (87, 91). Thus, PD-L1 expression may be the critical tissue-specific mechanism for autoreactive T-cell control.

Autoimmune diabetes: PD-1 and the maintenance of T-cell anergy

We have reported recently that both PD-1 and CTLA-4 have important roles in the induction of peripheral tolerance using an adoptive transfer model of autoimmune diabetes (87). We have also demonstrated that PD-1, but not CTLA-4, is critical for the long-term maintenance of tolerance in this model (87). Our system relies on using an antigen-specific approach to induce selective T-cell tolerance. Antigen-fixed ECDI-coupled cells were injected into recipient mice receiving antigen-specific activated T cells. The idea behind this strategy is that the TCR/antigen-specific signal is presented in the absence of costimulation (signal 1 only). CTLA-4 blockade has been reported to prevent tolerance induction using this tolerance model (87, 92). Interestingly, anergic T cells could not be 'released' using anti-CTLA-4 (87). In this setting, CTLA-4 did not inhibit cellular trafficking of autoreactive T cells in a delayed-type hypersensitivity response but did prevent their ability to proliferate and produce effector cytokines (92). It is possible that cells can migrate with CTLA-4 blockade, but once within tissue sites, PD-1/PD-L1 interactions dominate, given the tissue-specific basal level of PD-L1 expressed. Only

blocking the tissue-specific PD-L1 releases the cells and allows them to gain full effector function. This was true in the diabetes tolerance model; only PD-1/PD-L1 blockade released these cells and precipitated rapid progression of autoimmune diabetes (87). These results illustrate a complex regulatory control for peripheral tolerance induction and maintenance. It was interesting to discover that PD-1 blockade resulted in both the prevention of tolerance induction and also the rapid release of anergic T cells, several months after anergy was established. We demonstrated that even 6 months following tolerance using insulin-coupled cells to treat new onset spontaneously diabetic mice, PD-1/PD-L1 blockade resulted in rapid autoimmune diabetes and 100% incidence (87).

We predicted that PD-L1/PD-L2 temporal and spatial expression is critical for its inhibitory role. Using the ECDI-fixed tolerance model, we determined that PD-L1 must be present within the tissue for tolerance and not on the initial fixed antigen-coupled cell. Antigen-coupled ECDI-fixed cells from PD-L1 KO mice were used to induce tolerance in the adoptive transfer diabetes model. We found that PD-L1 KO APCs induced 100% tolerance, supporting the idea that only PD-L1 expression within the islets is critical for maintaining tolerance (87). These data, along with the studies from Keir *et al.* (91), demonstrate that PD-L1 is required within the islet tissue and not the peripheral lymph nodes to prevent diabetes. In PD-1-sufficient NOD mice, infiltrating T cells form a cluster surrounding the pancreatic islets (peri-insulitis) and rarely invade the islets, forming a ring as if there were a protective barrier (93). Strong PD-L1 expression on the β cells adjacent to these infiltrating T cells may serve as a barrier to suppress the effector function of diabetogenic T cells (32). In NOD-PD-1^{-/-} mice, this barrier is effectively lost, and T cells invade deep into the pancreas islets, causing rapid tissue destruction and clinical disease (32, 91). These results support the hypothesis that PD-L1 is critical for maintaining tolerance within local tissue sites.

It is interesting that the PD-1–PD-L1 interaction was not critical in the direct engagement of the activated Teff cells and p31-SP ECDI-treated APCs, given the integral role of this pathway in controlling T-cell activation in several systems (reviewed in 10). Rather, the PD-1 pathway acted downstream of the initial antigen encounter. Thus, the PD-1 pathway is not critical for induction but rather the maintenance of tolerance in the target tissue. This was true for both the antigen-coupled fixed APCs and anti-CD3 models, where antigen-specific T cells continue to reside in the target organ (although they are not pathogenic) (94). Therefore, we speculate that the PD-1–PD-L1 interactions maintain tolerance within the local infiltrated tissues. In this regard, we have observed that anti-PD-L1

treatment did not precipitate diabetes in models such as the NOD B7-2KO, where there is no infiltrate (16) and, thus, the endogenous host CD4⁺ T cells were not able to induce disease when PD-1 was blocked. In contrast, blockade of PD-1 on the tolerized BDC2.5 T cells resulted in rapid reversal of anergy, including a dramatic increase in inflammatory cytokines and proliferation, indicating that induction of tolerance through TCR-mediated pathways induces a state of T-cell anergy that is maintained by PD-1–PD-L1 engagement (87).

Both CTLA-4 and PD-1 inhibit T-cell proliferation, cytokine production, and proximal TCR signaling (reviewed in 10). Thus, it is critical to understand the apparent paradox that each molecule is both necessary and yet not sufficient for tolerance. The model suggests that this apparent contradiction can be best reconciled if the two negative regulatory pathways function at different stages and sites during autoimmunity. For instance, unlike PD-L1, which is highly expressed in islets and other stromal cells (57), expression of the CTLA-4 ligands B7-1 and B7-2 is largely restricted to lymphoid tissues (reviewed in 10). Therefore, CTLA-4 may act early during initial T-cell activation in the draining lymph nodes via B7-1 and B7-2 engagement, whereas the PD-1–PD-L1 pathway may function within tissue sites distal to initial activation. PD-1 may provide an additional opportunity to limit T-cell function and prevent tissue damage downstream of CTLA-4.

CTLA-4 and PD-1 effects on T-cell stop signals and reversal of tolerance

Although the PD-1 pathway is a critical regulator of immunity and plays a significant role in tolerance in normal and therapeutic settings, the precise mechanistic basis for tolerance *in vivo* remains unclear. In spite of the biochemical studies, there has been little understanding of the effects of PD-1 engagement in T-cell activation as there is no good *in vitro* model of the inhibitory activity of PD-1/PD-L1 interactions and *in vivo* studies have been limited to global effects on immunity. PD-1 has been shown to limit extracellular signal-regulated kinase (ERK) phosphorylation, which is a signature of anergized T cells (66). We and others (95, 96, B. T. Fife and J. A. Bluestone, unpublished observation) have found that ERK phosphorylation is decreased in anergic T cells.

Recent imaging advances have allowed the use of multi-photon laser-scanning microscopy (MPLSM) to visualize T cells in lymph nodes and non-lymphoid tissues *in vivo* on the single cell level. Several groups have described the speed and movement characteristics of CD4⁺, CD8⁺ T cells, and DCs during T-cell priming and tolerance induction (97–101).

Using this approach, the contact time between CD4⁺ or CD8⁺ T cells with DCs was determined to be a critical factor for full activation, proliferation, and cytokine production (102). However, dynamic T-cell movement and velocity during the long-term maintenance of tolerance has not been studied. Therefore, we examined the effects of CTLA-4 and PD-1 blockade both biochemically and by MPLSM. In a recent study, we took advantage of a robust anergy-inducing regimen to examine the biological basis of fixed APC-induced tolerance and the effects of CTLA-4 and PD-1 engagement in real time. We demonstrated that PD-1/PD-L1 interactions have a direct role in regulating the T-cell stop signaling leading to inhibition of T-cell migration (B.T. Fife and J.A. Bluestone, unpublished observation). BDC2.5 T cells were activated, tolerized, and transferred to a recipient mouse for imaging. PD-1 blockade decreased T-cell movement, velocity, and prolonged the interactions with DCs in the antigen-containing pancreatic lymph nodes but not in the non-antigen-bearing inguinal lymph nodes (B.T. Fife and J.A. Bluestone, unpublished observation). This observation suggested that antigen was required. The prolonged interactions with DCs resulted in a release from anergy and the restoration of calcium signaling, phosphorylation of key TCR signaling molecules including ERK, and IFN- γ production (B.T. Fife and J.A. Bluestone, unpublished observation). Moreover, PD-1 blockade resulted in decreased antigen-specific T-cell movement within the islets and accelerated diabetes. Previous reports by Rudd and colleagues (103) have suggested that CTLA-4 engagement actually led to decreased movement and velocities of CD4⁺ T cells, concluding that CTLA-4 normally enhances the stop signal and firm T-cell interactions with DCs. Our data, using the diabetes adoptive transfer model and long-term tolerance, yielded the opposite finding that PD-1 blockade enhanced T-cell stop signals. Our model for the maintenance of peripheral tolerance is distinct from that reported for CTLA-4 and suggests a fundamentally distinct model for the inhibitory receptors CTLA-4 and PD-1.

PD-1, CTLA-4, and chronic viral responses

Chronic viruses have developed mechanisms for the evasion and/or the modification of host immune responses, resulting in persistent infection. Recent work investigating chronic human immunodeficiency virus (HIV) infections has demonstrated a critical role for PD-1 and the suppression of CD8 anti-viral responses, termed 'exhaustion' (104, 105). T-cell exhaustion is defined as a gradual loss of critical anti-viral effector functions, including IFN- γ , TNF- α , proliferation, and cytotoxicity, and ultimately an inability to clear viral load

(reviewed in 106, 107). PD-1 blockade has been shown to release HIV-specific CD8⁺ T cells to produce inflammatory cytokines and re-invigorate the anti-viral response (104, 105). Similar reports for PD-1 have been demonstrated in chronic hepatitis B virus (HBV), hepatitis C virus (HCV), simian immunodeficiency virus (SIV), and lymphocytic choriomeningitis virus (LCMV) (104, 107–109). In LCMV chronic viral infection, exhausted CD8⁺ T cells do not proliferate, have decreased production of TNF- α , IL-2, IFN- γ , perforin, granzyme granules, decreased cytotoxic potential, are resistant to apoptosis, and persist long term (104, 105). It is interesting to note that the phase or extent of exhaustion has different features, several of which are shared with anergic CD4⁺ T cells. Elevated T-cell PD-1 expression is the first correlation, but interestingly 'partially exhausted' CD8⁺ T cells make IFN- γ (reviewed in 106, 107). There have been several reports of tolerogenic/inhibitory effects of IFN- γ from anergic CD4⁺ T cells as well (110, 111). This similarity, along with the ability of these cells to resist apoptosis and persist, but not proliferate, corresponds with diminished anti-viral responses and is reminiscent of anergic CD4⁺ T cells (104). Blocking PD-1 in this setting has been shown to release these antigen-specific cells, allowing full restoration of CD8 function including proliferation, IFN- γ , TNF- α , and most importantly efficiently decreased viral load (reviewed in 106, 107). Interestingly, CTLA-4 blockade did not restore CD8⁺ T-cell function; the cells remained exhausted and unresponsive. This was also true in the CD4⁺ T-cell autoimmune model of tolerance induced using ECDI-fixed APCs for tolerance induction. CTLA-4 blockade did not restore proliferation, cytokine production, and did not result in rapid autoimmune disease (87). It is quite possible that similar mechanisms regulating CD8⁺ T-cell-exhausted states regulate anergy in CD4⁺ T cells, and PD-1 signaling is central to this non-responsiveness.

It has also been suggested that exhausted CD8⁺ T cells move from partial to full exhaustion as CD4⁺ T-cell help decreases and antigen load and PD-1 increase (112). We would like to speculate that not only does the antigen load help determine the outcome of CD4⁺ and CD8⁺ T-cell responses, but the context of the antigen is critical. In the antigen-fixed tolerance model, CTLA-4 blockade does not break tolerance with *in vivo* administration of neutralizing antibody; however, if CTLA-4 blockade is administered along with a high dose of antigen in a priming condition (in the context of complete Freund's adjuvant), tolerance is broken (92). The production of inflammatory cytokines and pattern recognition stimuli may be critical for CTLA-4 abrogation of tolerance. Additional studies are needed to determine the effects for PD-1 and

CTLA-4 regulation on T and B cells in response to these environmental factors and cytokines during induction of immunity and tolerance.

Clinical opportunities

The establishment of immune homeostasis and return to normal health has been the quest of immunologists and clinicians treating autoimmunity, transplant graft rejection, cancer, and chronic viral infections. In each of these conditions, cellular interactions play a critical role either through undesirable T-cell attack of self-tissue in the case of autoimmunity and graft rejection, or insufficient T-cell activation to target tumor cells or chronic virally infected cells. In either case, regulating T-cell activity seems to be at the heart of therapeutic strategies. PD-1 and CTLA-4 are attractive targets for therapeutic intervention, given their critical role in regulating T-cell activation and tolerance. This next section summarizes the clinical experiences with agents specifically designed to target PD-1 and CTLA-4, along with future clinical opportunities targeting these inhibitory pathways.

Therapeutic strategies targeting CTLA-4: antagonism versus agonism

In the past several years, many efforts have focused on developing therapeutic approaches to selectively engage CTLA-4 to treat autoimmunity and organ transplant rejection (11, 12, 113). Unfortunately, early attempts were largely unsuccessful due to the complex biology of CTLA-4 (3). Early approaches used antibodies targeting CTLA-4, B7-1, and B7-2 for immune regulation. These attempts were unsuccessful, because anti-CTLA-4 or anti-B7 prevents CTLA-4/B7 engagement and cross-linking. As a result, the antibody acts as an antagonist and prevents negative signaling and thus promotes enhanced T-cell activation, expansion, and differentiation. This was demonstrated *in vivo* in an effort to treat autoimmunity. Administration of anti-CTLA-4 antibodies acted as an antagonist of the CTLA-4 function, resulting in exacerbation of clinical autoimmune diseases (114, 115). Thus, a new approach to directly engage CTLA-4 to act as an agonist, to induce the negative signal would be desirable to treat autoimmunity.

T-cell inhibition through CTLA-4 signaling requires cross-linking and must coincide with TCR engagement. This dual requirement is a direct consequence of the fact that CTLA-4 functions by directly influencing TCR-mediated signal transduction by altering TCR- ζ chain phosphorylation and immune synapse formation (15, 72, 116). Thus, any effective therapeutic approach would depend on CTLA-4 engagement,

both physically and temporally, together with the TCR signal from the same APC (15). This most likely explains why *in vivo* administration of anti-CTLA-4 acts as an antagonist. Most CTLA-4/antibody complexes would occur in the absence of antigen-bearing APCs. We hypothesized that effective delivery of a CTLA-4 agonist signal would depend on presentation of a specific CTLA-4 ligand (i.e. anti-CTLA-4 antibody) on a relevant APC-bearing cognate antigen (117). Thus, we developed a single-chain, membrane-bound anti-CTLA-4 Ab that could be expressed directly on an APC with the goal to selectively engage antigen-specific TCR and CTLA-4, simultaneously (118, 119). Using this approach, we demonstrated that B-cell expression of an anti-CTLA-4 single chain (sc α -CTLA-4) was effective in diminishing autoimmune diabetes in the NOD mouse strain (119). This approach was effective in the absence of Tregs, suggesting that the mechanism for this regulation had a direct effect of inhibiting T-cell proliferation and differentiation toward IFN- γ -producing cells. We also demonstrated that this approach was effective in regulating CD8⁺ T-cell-mediated tumor clearance in an *in vivo* tumor model (120). CD8⁺ T cells isolated from animals bearing sc α -CTLA-4 tumor cells were hyporesponsive *ex vivo* upon antigen re-challenge, indicating that CTLA-4 engagement resulted in tolerance (120). Further development of this approach for CTLA-4 agonism in clinical applications will depend on efficient gene transfer technology and expression of anti-CTLA-4 on autologous APCs *in vivo*.

CTLA-4Ig: a potent inhibitor of CD28-mediated T-cell costimulation

CTLA-4 fused to an immunoglobulin chain (CTLA-4Ig), a soluble CD28 antagonist therapeutic reagent, was developed to avoid the complex problems associated with anti-CTLA-4 therapy. CTLA-4Ig functions to prevent the interaction of CD28 with B7 during activation of naive and activated helper or cytotoxic T cells (121). During the past 17 years, the mechanistic basis for CTLA-4Ig function *in vivo* has been investigated. The results from these studies have demonstrated that CTLA-4Ig blocks the engagement of CD28 with its ligands B7-1 and B7-2, inhibiting the early phases of T-cell activation, including cell cycle progression, proliferation, T-effector differentiation, and cell survival (reviewed in 3, 122–124). Despite the original observations *in vitro* demonstrating that CTLA-4Ig treatment resulted in the induction of T-cell anergy, these findings did not translate to *in vivo* models (7). Rather, CTLA-4Ig seemed to mediate its profound effects through the prevention of activation, limiting clonal expansion, and induction of passive cell

death through growth factor withdrawal via pro-apoptotic activity (125–128). Additionally, CTLA-4Ig has been shown to trigger APC production of IDO, an intracellular enzyme that breaks down tryptophan to produce immunoregulatory kynurenines which suppresses T-cell activation (129).

The development of CTLA-4Ig as an effective antagonist of CD28 signaling *in vitro* prompted *in vivo* studies to assess efficacy. In multiple transplant settings, we and others demonstrated that CTLA-4Ig therapy led to tissue and organ graft survival, suppression of alloantibody responses, and the induction of tolerance (123, 130, 131). In addition, experiments in autoimmune models suggested that CD28 blockade not only functioned at the initiation of an immune response but could also ameliorate ongoing disease (3). Most importantly, CTLA-4Ig therapy could be withdrawn after a short time in several of these studies, but the beneficial effects remained long term.

CTLA-4Ig is now a Food and Drug Administration-approved drug for use treating patients with RA (132). Early indicators suggest that CTLA-4Ig is effective and binds the B7 ligands to inhibit T-cell function. At least five studies have demonstrated safety with CTLA-4Ig therapy and significant clinical improvements in RA (133–138). Patient response rates in several studies were comparable to blocking TNF- α (e.g. etanercept, infliximab, adalimumab), which arguably represent the most important advance in the treatment of RA over the past decade (121). Moreover, CTLA-4Ig therapy was shown to significantly inhibit radiographic progression of disease, a surrogate measure of joint damage (reviewed in 121). These results are exciting and represent a significant biological success for RA, by specifically targeting T cells. Previous strategies designed to deplete T cells including anti-CD4, anti-CD5, and anti-CD52 yielded disappointing results and suggested that global T-cell depletion was not the best approach for RA treatment (reviewed in 121). These encouraging results warrant further trials and mechanistic studies to better understand the disease process.

As a cautionary note, CTLA-4Ig treatment may negatively affect Tregs, which are critically important for controlling autoimmunity and maintaining transplant tolerance (3). The development and peripheral survival of Tregs is CD28 dependent; thus, it is not surprising that blockade of the CD28 pathway using CTLA-4Ig in animal models of autoimmunity has resulted in a precipitous decrease in Tregs (139). However, it is unclear whether these potential adverse CTLA-4Ig effects occur in humans following CTLA-4Ig therapy. It likely depends on the dose, location, timing, and the cellular composition during disease pathogenesis. In a recent clinical report, Tregs were unaffected with CTLA-4Ig therapy following renal transplantation

(140). This result did not support the observations from animal models, indicating that CTLA-4Ig may function differently in mice and humans (140). With a greater understanding of the role of Tregs during immunity and homeostasis, combinational therapeutic approaches may be more effective.

PD-1 opportunities in chronic viral infection

Persistent viral infections such as HIV, SIV, HBV, HCV, and LCMV are associated with functional exhaustion of virus-specific CD8⁺ T cells (104, 141, 142). Recently, *in vivo* blockade of PD-1 has been shown to restore the function of virus-specific CD8⁺ T cells, resulting in enhanced viral clearance. These findings highlight the attractive nature of this pathway for therapy in chronic infections (reviewed in 107). PD-1 blockade has been shown to release HIV-specific CD8⁺ T cells to produce inflammatory cytokines and re-invigorate the anti-viral response (104, 105, 141, 142). Similar reports for PD-1 have been demonstrated in chronic HBV, HCV, SIV, and LCMV (104, 108, 109). In these studies, PD-1 expression is elevated on CD8⁺ virus-specific T cells and correlates with a high viral load and reduced CD4⁺ T-cell counts (reviewed in 106, 107). Although anti-viral T cells would be released from exhaustion with systemic PD-1 blockade, autoreactive CD4⁺ and CD8⁺ T cells may also be released and result in autoimmunity. Thus, a targeted or antigen-specific release would be highly desirable. In a recent study, Ha et al. (143) have shown a combinational approach to specifically target the immune system to enhance viral-specific responses during PD-1 blockade. In this setting, chronic LCMV-infected mice were given a vaccination consisting of vaccinia virus expressing an LCMV-dominant epitope glycoprotein (gp) 33 (143). Following vaccination, mice received PD-L1 blockade causing the release from PD-1 exhaustion and effective viral clearance (143). This enhanced viral response was specific as only gp33 responses but not gp276 responses were elevated, indicating epitope-specific responses to therapeutic vaccination (143). These results suggest that blockade of the PD-1/PD-L1 pathway enhances anti-viral responses, and yet this response was a focused release allowing viral-specific T cells to escape exhaustion. Although this study shows clinical promise, potentially harmful side effects should be considered and investigated before translating this therapy into human clinical trials (144).

Therapeutic targeting of PD-1/PD-L1: clinical approaches for tumor immunology

The immune system is designed to identify targets and destroy potential threats. Tumor cells have evolved to escape immune

recognition for survival. Tumor cells express antigens that can be recognized by the immune system; however, tumors often create microenvironments through soluble factors and surface ligands to inhibit T-cell function. PD-L1 expression on tumor cells is one of these inhibitory mechanisms. Several reports have demonstrated PD-L1 expression on the majority of human and rodent cancers as a mechanism to limit T-cell anti-tumor responses (reviewed in 67, 145). Immune escape likely occurs because infiltrating T cells have elevated levels of PD-1, and upon ligand binding, T cells are shut down. In fact, PD-1 expression levels have been shown to correlate with tumor prognosis; higher PD-1 levels are associated with poorer prognosis (146, 147). These data suggest that even if tumor-infiltrating lymphocytes can recognize neoplastic target cells, they may be rendered unresponsive in a PD-1-dependent fashion. A recent report by Azuma *et al.* (148) has provided an additional mechanism for PD-L1's role during immune escape. PD-L1 signaling in tumor cells was shown to enhance resistance to apoptosis induced by both immune effectors and pro-apoptotic drugs (148). In this model, the tumor cells receive PD-L1-induced signals for survival during interactions with PD-1 'ligand' engagement on the tumor-infiltrating T cells (148). This reverse signaling may lead to two critical events: the induction of apoptosis resistance for PD-L1 tumor cells and T-cell inhibition through PD-1-induced signals. This finding may impact our targeting strategy to selectively block PD-L1-induced signals in addition to enhancing T-cell tumorigenicity. The ablation of PD-1/PD-L1 interactions has indeed been shown to restore CTL-mediated lysis of tumor cells *in vitro* (reviewed in 67, 145). Therefore, anti-tumor therapeutic approaches including soluble PD-1, PD-1Ig, and PD-L1Ig have been examined *in vivo* (21, 55, 149–151). Results from these studies have demonstrated mixed efficacy in animal tumor models (152, 153). Treatment with anti-PD-L1 *in vivo* delayed tumorigenesis but did not halt myeloma tumor cell growth (154). However, gene therapy with plasmid DNA encoding sPD-1 was successful in suppressing the progression of pulmonary metastatic melanoma, suggesting that this pathway is a promising target for therapeutic intervention (155). The differences of PD-L1 blockade versus PD-L1 reverse signaling may critically change the outcome for tumor survival and susceptibility to apoptosis. In this case, antagonistic therapies would be beneficial, thus preventing PD-L1-induced protection in tumor cells. Clinical trials examining the use of PD-1 blockade are underway; thus, we will learn whether the temporary interruption of this pathway is feasible and safe for humans suffering from chronic illnesses and mechanisms by which tumor cells escape T-cell immunity.

Genetic manipulation of PD-1 signals as a therapeutic approach for autoimmunity

PD-1/PD-L1/PD-L2 interactions play a critical role during the induction and progression of autoimmunity. PD-1 and PD-L1/PD-L2 genetic deficiencies result in severe autoimmunity. PD-L1 tissue expression is enhanced in response to inflammatory cytokines (67). PD-L1 expression acts as a natural 'balance' to limit tissue-specific responses to inflammation in an effort to limit T-cell destruction. Several studies have addressed whether augmenting this natural response could limit autoimmune disease progression. This approach was through enhanced tissue-specific PD-L1 expression. Hirata *et al.* (156, 157) genetically modified mouse embryonic stem (ES) cells using vectors to express surface PD-L1 and myelin oligodendrocyte glycoprotein (MOG) in MHC class II. They next differentiated the cells into DCs and transferred the cells into mice with EAE, a model for human multiple sclerosis. In their study, the PD-L1-expressing DCs were able to limit spinal cord inflammation and decrease clinical disease severity, demonstrating a therapeutic effect. The authors concluded that this therapy induced anergy rather than the induction of a regulatory population because the transfer of these anergic T cells could not prevent subsequent clinical EAE symptoms (156).

More recently, Ding *et al.* (158) observed suppression of nephritis in a lupus-like syndrome in mice treated with adenovirus expressing the full-length PD-L1 gene. The authors detected a strong expression of PD-L1 on renal proximal tubular epithelial cells after viral infection and concluded that the protective effect was due in part to selective tissue expression of PD-L1 (158). Other studies selectively targeting PD-L1 to tissue-specific sites have found very different clinical outcomes. Although PD-1 has been overwhelmingly characterized as a negative regulator of T-cell function, the role during T-cell activation is still controversial. To test the idea that islet cell-specific expression would protect islets from autoimmune diabetes and prevent islet graft rejection, Subudhi *et al.* (159) created genetically modified mice expressing the PD-L1 gene under control of the rat insulin promoter. Surprisingly, these mice developed spontaneous disease with accelerated kinetics. In fact, PD-L1-expressing islets were not protected in transplant experiments; rather, these PD-L1-expressing islets were quickly rejected and enhanced T-cell activation (159). The reasons for these contradictory results are not yet clear. These findings illustrate the complexities of costimulatory blockade, and only with a better understanding of the molecules involved, along with the critical timing of ligand expression, will we be able to

Peripheral Tolerance Tissue Specific Inhibition

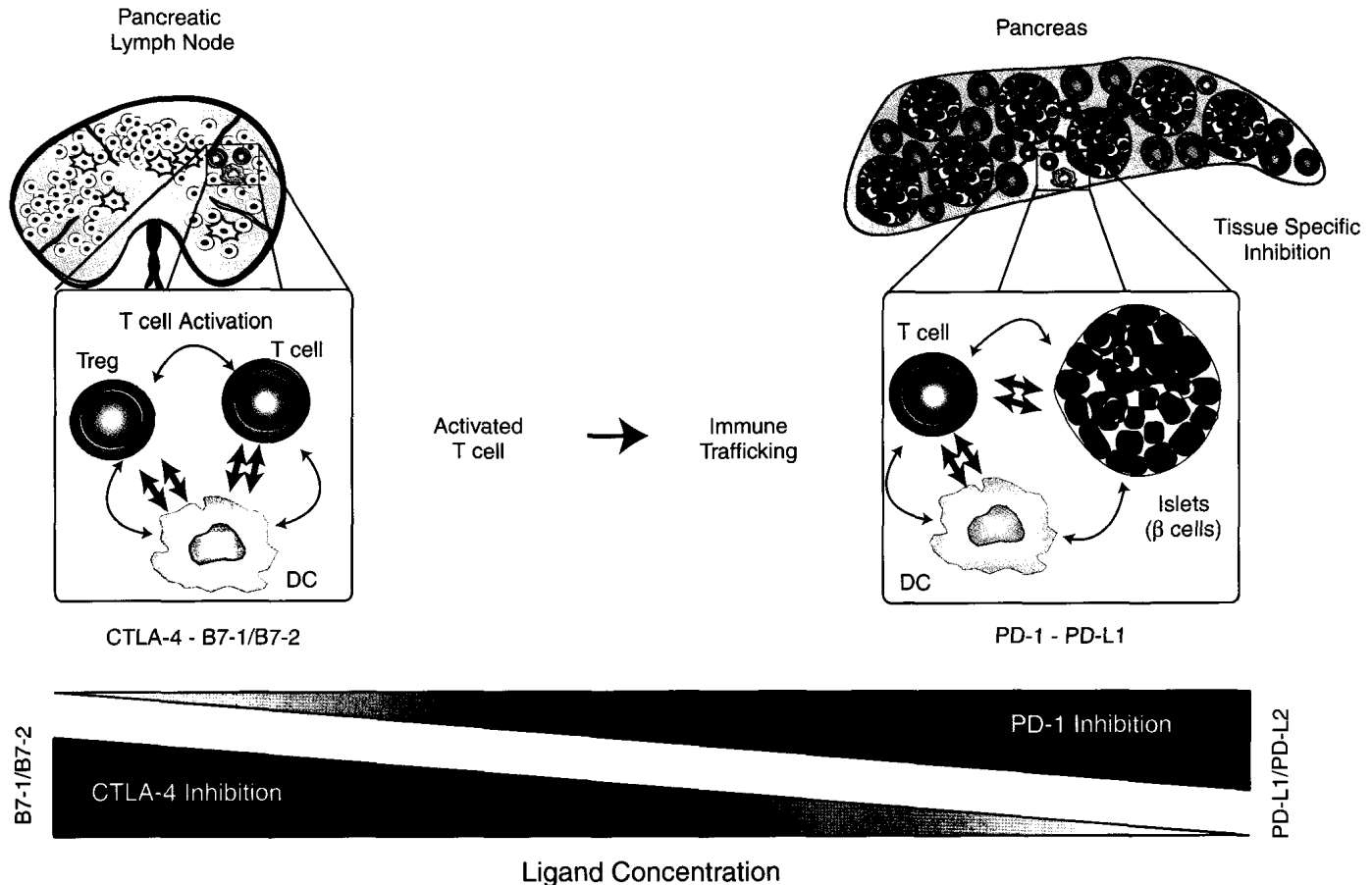


Fig. 1. Peripheral tolerance mediated by negative regulators. This figure represents the differential effects for CTLA-4 and PD-1 for limiting T-cell function during T-cell activation and the maintenance of peripheral tolerance. CTLA-4 and PD-1 are rapidly expressed following T-cell receptor engagement and CD28 co-stimulatory signals. B7-1 and B7-2 are constitutively expressed in the lymph nodes at low levels on antigen-presenting cells and upon activation are highly upregulated. PD-L1 and PD-L2 are constitutively expressed in peripheral tissue sites and upregulated in response to inflammatory signals. The temporal and spatial patterns for ligand expression may help to explain the distinct differences between these two inhibitory molecules. CTLA-4 blockade prevents tolerance induction and therefore may act in the earliest stages of immunity, particularly those within the lymphatic system. PD-1 blockade acts in tissue sites to release anergic T cells and cause autoimmunity, suggesting the inhibitory functions are within the target tissue. Therefore, PD-1 and CTLA-4 can play critical yet non-redundant roles in the maintenance of peripheral tolerance.

administer these types of reagents as therapeutics during autoimmune disease progression.

Concluding remarks

PD-1 and CTLA-4 are critical regulators of T-cell function in diverse processes ranging from autoimmunity, transplantation tolerance, chronic viral infections, and tumor immunity. *In vitro* and *in vivo* animal models as well as clinical trials have demonstrated great therapeutic opportunities by effectively targeting these pathways. T-cell activation is a complicated biological process with several checkpoints and fail-safe processes built in to limit aberrant or unfaithful T-cell activation, including autoimmunity. T-cell signaling through CD28, CTLA-4, and PD-1 controls complex but critical checkpoints for T-cell activation and homeostasis. CD28 signaling induces a

balance of positive and negative signals establishing mechanisms for T-cell expansion and control. CTLA-4 is important for limiting T-cell activity for cells early during the immune responses. PD-1, on the other hand, limits T-cell activity in peripheral tissues and may be the last chance to prevent T-cell destruction of self-tissue resulting in autoimmunity (Fig. 1). PD-1 and PD-L1 are also expressed on Tregs and may help control their suppression of effector T cells. Recent studies indicate that PD-L1 and PD-L2 can signal bi-directionally by engaging PD-1 on T cells. PD-L1/PD-L2 signaling may aid in protection from apoptosis and promote tumor cell growth. The local environment of cytokines and costimulatory molecule expression on professional APCs will be vital for dictating T-helper outcomes. These pathways offer exciting new therapeutic possibilities to selectively silence destructive T cells or

selectively enhance host immune responses to restore immune homeostasis. The key for targeting these pathways may lie within the local tissue microenvironment through cytokine and costimulatory molecular expression patterns. The idea to limit only a subset of T cells resides in antigen recognition, and

therefore antigen-specific approaches to engage or silence CD4⁺ and CD8⁺ T-cell responses would be the ultimate goal. Given the complexity of immune activation and quiescence, it is likely that several pathways and combinatorial approaches will be required to accomplish this goal.

References

- van Noort JM, van Sechel A, Boon J, Boersma WJ, Polman CH, Lucas CJ. Minor myelin proteins can be major targets for peripheral blood T cells from both multiple sclerosis patients and healthy subjects. *J Neuroimmunol* 1993;**46**:67–72.
- Lohmann T, Leslie RD, Londei M. T cell clones to epitopes of glutamic acid decarboxylase 65 raised from normal subjects and patients with insulin-dependent diabetes. *J Autoimmun* 1996;**9**:385–389.
- Salomon B, Bluestone JA. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu Rev Immunol* 2001;**19**:225–252.
- Boise LH, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 1995;**3**:87–98.
- Green JM, et al. Absence of B7-dependent responses in CD28-deficient mice. *Immunity* 1994;**1**:501–508.
- Szot GL, et al. Different mechanisms of cardiac allograft rejection in wildtype and CD28-deficient mice. *Am J Transplant* 2001;**1**:38–46.
- Szot GL, et al. Absence of host B7 expression is sufficient for long-term murine vascularized heart allograft survival. *Transplantation* 2000;**69**:904–909.
- Williams MA, et al. Genetic characterization of strain differences in the ability to mediate CD40/CD28-independent rejection of skin allografts. *J Immunol* 2000;**165**:6849–6857.
- Green JM, et al. T cell costimulation through the CD28 receptor. *Proc Assoc Am Physicians* 1995;**107**:41–46.
- Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* 2005;**23**:515–548.
- Walunas TL, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994;**1**:405–413.
- Walunas TL, Bakker CY, Bluestone JA. CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med* 1996;**183**:2541–2550.
- Nishimura H, et al. Developmentally regulated expression of the PD-1 protein on the surface of double-negative (CD4[−] CD8[−]) thymocytes. *Int Immunol* 1996;**8**:773–780.
- Luhder F, Chambers C, Allison JP, Benoist C, Mathis D. Pinpointing when T cell costimulatory receptor CTLA-4 must be engaged to dampen diabetogenic T cells. *Proc Natl Acad Sci USA* 2000;**97**:12204–12209.
- Chikuma S, Imboden JB, Bluestone JA. Negative regulation of T cell receptor-lipid raft interaction by cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 2003;**197**:129–135.
- Salomon B, et al. Development of spontaneous autoimmune peripheral polyneuropathy in B7-2-deficient NOD mice. *J Exp Med* 2001;**194**:677–684.
- Waterhouse P, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctlα-4. *Science* 1995;**270**:985–988.
- Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 1995;**3**:541–547.
- Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-β in CD4⁺CD25⁺ regulatory T cell function. *Eur J Immunol* 2004;**34**:2996–3005.
- Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* 2005;**6**:152–162.
- Freeman GJ, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;**192**:1027–1034.
- Ishida M, et al. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunol Lett* 2002;**84**:57–62.
- Latchman Y, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;**2**:261–268.
- Brown JA, et al. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol* 2003;**170**:1257–1266.
- Yamazaki T, et al. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* 2002;**169**:5538–5545.
- Ansari MJ, et al. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* 2003;**198**:63–69.
- Loke P, Allison JP. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc Natl Acad Sci USA* 2003;**100**:5336–5341.
- Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4⁺CD25⁺ high regulatory cells in human peripheral blood. *J Immunol* 2001;**167**:1245–1253.
- Sandner SE, et al. Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo. *J Immunol* 2005;**174**:3408–3415.
- Nishimura H, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 2001;**291**:319–322.
- Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999;**11**:141–151.
- Wang J, Yoshida T, Nakaki F, Hiai H, Okazaki T, Honjo T. Establishment of NOD-Pdcd1^{−/−} mice as an efficient animal model of type 1 diabetes. *Proc Natl Acad Sci USA* 2005;**102**:11823–11828.
- Salama AD, et al. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med* 2003;**198**:71–78.
- Nielsen C, Hansen D, Husby S, Jacobsen BB, Lillevang ST. Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. *Tissue Antigens* 2003;**62**:492–497.
- Prokunina L, et al. Association of the PD-1.3A allele of the PDCD1 gene in patients with rheumatoid arthritis negative for rheumatoid factor and the shared epitope. *Arthritis Rheum* 2004;**50**:1770–1773.
- Prokunina L, et al. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nat Genet* 2002;**32**:666–669.
- Ferreiros-Vidal I, et al. Association of PDCD1 with susceptibility to systemic lupus

- erythematosus: evidence of population-specific effects. *Arthritis Rheum* 2004;**50**:2590–2597.
38. Wang SC, et al. Programmed death-1 gene polymorphisms in patients with systemic lupus erythematosus in Taiwan. *J Clin Immunol* 2006;**26**:506–511.
 39. Lin SC, et al. Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis, but not systemic lupus erythematosus. *Arthritis Rheum* 2004;**50**:770–775.
 40. Kroner A, et al. A PD-1 polymorphism is associated with disease progression in multiple sclerosis. *Ann Neurol* 2005;**58**:50–57.
 41. Hayashi M, Kouki T, Takasu N, Sunagawa S, Komiya I. Association of an A/C single nucleotide polymorphism in programmed cell death-ligand 1 (PD-L1) gene with Graves' disease in Japanese patients. *Eur J Endocrinol* 2008, Jun; **124**:277–293.
 42. Marleau AM, Sarvetnick N. T cell homeostasis in tolerance and immunity. *J Leukoc Biol* 2005;**78**:575–584.
 43. Okazaki T, Honjo T. The PD-1–PD-L pathway in immunological tolerance. *Trends Immunol* 2006;**27**:195–201.
 44. Allison JP, et al. A role for CTLA-4-mediated inhibitory signals in peripheral T cell tolerance? *Novartis Found Symp* 1998;**215**:92–98.
 45. Mixter PF, Russell JQ, Budd RC. Delayed kinetics of T lymphocyte anergy and deletion in *lpr* mice. *J Autoimmun* 1994;**7**:697–710.
 46. Adachi M., et al. Enhanced and accelerated lymphoproliferation in Fas-null mice. *Proc Natl Acad Sci USA* 1996;**93**:2131–2136.
 47. Mountz JD, Bluethmann H, Zhou T, Wu J. Defective clonal deletion and anergy induction in TCR transgenic *lpr/lpr* mice. *Semin Immunol* 1994;**6**:27–37.
 48. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 1996;**14**:233–258.
 49. August A, Dupont B. CD28 of T lymphocytes associates with phosphatidylinositol 3-kinase. *Int Immunol* 1994;**6**:769–774.
 50. Pages F, et al. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature* 1994;**369**:327–329.
 51. Prasad KV, et al. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc Natl Acad Sci USA* 1994;**91**:2834–2838.
 52. Truitt KE, Hicks CM, Imboden JB. Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T cells. *J Exp Med* 1994;**179**:1071–1076.
 53. Krummel MF, Allison JP. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* 1996;**183**:2533–2540.
 54. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* 1992;**11**:3887–3895.
 55. Carter L, et al. PD-1: PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. *Eur J Immunol* 2002;**32**:634–643.
 56. Chemnitz JM, Parry RV, Nichols KE, June CJ, Riley J. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 2004;**173**:945–954.
 57. Liang SC, et al. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol* 2003;**33**:2706–2716.
 58. Nishimura H, Honjo T, Minato N. Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. *J Exp Med* 2000;**191**:891–898.
 59. Keir ME, Latchman YE, Freeman GJ, Sharpe AH. Programmed death-1 (PD-1): PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes. *J Immunol* 2005;**175**:7372–7379.
 60. Blank C, Brown I, Marks R, Nishimura H, Honjo T, Gajewski TF. Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells. *J Immunol* 2003;**171**:4574–4581.
 61. Schwartz RH. T cell clonal anergy. *Curr Opin Immunol* 1997;**9**:351–357.
 62. Jenkins MK, Pardoll DM, Mizuguchi J, Quill H, Schwartz RH. T-cell unresponsiveness *in vivo* and *in vitro*: fine specificity of induction and molecular characterization of the unresponsive state. *Immunol Rev* 1987;**95**:113–135.
 63. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J Exp Med* 1987;**165**:302–319.
 64. Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol* 2001;**22**:265–268.
 65. Chikuma S, Bluestone JA. CTLA-4 and tolerance: the biochemical point of view. *Immunol Res* 2003;**28**:241–253.
 66. Bennett F, et al. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. *J Immunol* 2003;**170**:711–718.
 67. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008;**26**:677–704.
 68. Sheppard KA, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKC θ . *FEBS Lett* 2004;**574**:37–41.
 69. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci USA* 2001;**98**:13866–13871.
 70. Pentcheva-Hoang T, Chen L, Pardoll DM, Allison JP. Programmed death-1 concentration at the immunological synapse is determined by ligand affinity and availability. *Proc Natl Acad Sci USA* 2007;**104**:17765–17770.
 71. Saunders PA, Hendrycks VR, Lidinsky WA, Woods ML. PD-L2: PD-1 involvement in T cell proliferation, cytokine production, and integrin-mediated adhesion. *Eur J Immunol* 2005;**35**:3561–3569.
 72. Chikuma S, Bluestone JA. CTLA-4: acting at the synapse. *Mol Interv* 2002; **2**:205–208.
 73. Chuang E, et al. Regulation of cytotoxic T lymphocyte-associated molecule-4 by Src kinases. *J Immunol* 1999;**162**:1270–1277.
 74. Lee KM, et al. Molecular basis of T cell inactivation by CTLA-4. *Science* 1998;**282**:2263–2266.
 75. Parry RV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* 2005;**25**:9543–9553.
 76. Ueda H, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 2003;**423**:506–511.
 77. Vijaykrishnan L, et al. An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity* 2004;**20**:563–575.
 78. Irving BA, Alt FW, Killeen N. Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. *Science* 1998;**280**:905–908.
 79. Levings MK, Bacchetta R, Schulz U, Roncarolo MG. The role of IL-10 and TGF- β in the differentiation and effector function of T regulatory cells. *Int Arch Allergy Immunol* 2002;**129**:263–276.
 80. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the tran-

- scription factor Foxp3. *Science* 2003;**299**:1057–1061.
81. Gavin MA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 2007;**445**:771–775.
 82. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 2007;**8**:277–284.
 83. Brunkow ME, et al. Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 2001;**27**:68–73.
 84. Tang Q, Bluestone JA. The Foxp3⁺ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008;**9**:239–244.
 85. Habicht A, et al. A link between PDL1 and T regulatory cells in fetomaternal tolerance. *J Immunol* 2007;**179**:5211–5219.
 86. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L. TGF- β -dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* 2003;**9**:1202–1208.
 87. Fife BT, et al. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1–PD-L1 pathway. *J Exp Med* 2006;**203**:2737–2747.
 88. Lee I, et al. Blocking the monocyte chemoattractant protein-1/CCR2 chemokine pathway induces permanent survival of islet allografts through a programmed death-1 ligand-1-dependent mechanism. *J Immunol* 2003;**171**:6929–6935.
 89. Sabapatha A, Gercel-Taylor C, Taylor DD. Specific isolation of placenta-derived exosomes from the circulation of pregnant women and their immunoregulatory consequences. *Am J Reprod Immunol* 2006;**56**:345–355.
 90. Guleria I, et al. A critical role for the programmed death ligand 1 in fetomaternal tolerance. *J Exp Med* 2005;**202**:231–237.
 91. Keir ME, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 2006;**203**:883–895.
 92. Eagar TN, Karandikar NJ, Bluestone JA, Miller SD. The role of CTLA-4 in induction and maintenance of peripheral T cell tolerance. *Eur J Immunol* 2002;**32**:972–981.
 93. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 2005;**23**:447–485.
 94. Chatenoud L. CD3-specific antibody-induced active tolerance: from bench to bedside. *Nat Rev Immunol* 2003;**3**:123–132.
 95. Li W, Whaley CD, Mondino A, Mueller DL. Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4⁺ T cells. *Science* 1996;**271**:1272–1276.
 96. Fields PE, Gajewski TF, Fitch FW. Blocked Ras activation in anergic CD4⁺ T cells. *Science* 1996;**271**:1276–1278.
 97. Bousoo P, Robey EA. Dynamic behavior of T cells and thymocytes in lymphoid organs as revealed by two-photon microscopy. *Immunity* 2004;**21**:349–355.
 98. Hugues S, Fétler L, Bonifaz L, Helft J, Amblard F, Amigorena S. Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat Immunol* 2004;**5**:1235–1242.
 99. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 2004;**427**:154–159.
 100. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002;**296**:1869–1873.
 101. Shakhar G, et al. Stable T cell-dendritic cell interactions precede the development of both tolerance and immunity in vivo. *Nat Immunol* 2005;**6**:707–714.
 102. Scholer A, Hugues S, Boissonnas A, Fétler S, Amigorena S. Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8⁺ T cell memory. *Immunity* 2008;**28**:258–270.
 103. Schneider H, et al. Reversal of the TCR stop signal by CTLA-4. *Science* 2006;**313**:1972–1975.
 104. Day CL, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006;**443**:350–354.
 105. Barber DL, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006;**439**:682–687.
 106. Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH. Reinvigorating exhausted HIV-specific T cells via PD-1–PD-L1 ligand blockade. *J Exp Med* 2006;**203**:2223–2227.
 107. Martinic MM, von Herrath MG. Novel strategies to eliminate persistent viral infections. *Trends Immunol* 2008;**29**:116–124.
 108. Petrovas C, et al. PD-1 is a regulator of virus-specific CD8⁺ T cell survival in HIV infection. *J Exp Med* 2006;**203**:2281–2292.
 109. Trautmann L, et al. Upregulation of PD-1 expression on HIV-specific CD8⁺ T cells leads to reversible immune dysfunction. *Nat Med* 2006;**12**:1198–1202.
 110. Knoechel B, et al. Functional and molecular comparison of anergic and regulatory T lymphocytes. *J Immunol* 2006;**176**:6473–6483.
 111. Burstein HJ, Shea CM, Abbas AK. Aqueous antigens induce *in vivo* tolerance selectively in IL-2- and IFN- γ -producing (Th1) cells. *J Immunol* 1992;**148**:3687–3691.
 112. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 2007;**8**:239–245.
 113. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995;**182**:459–465.
 114. Vanderlugt CL, Karandikar NJ, Lenschow DJ, Dal Canto MC, Bluestone JA, Miller SD. Treatment with intact anti-B7-1 mAb during disease remission enhances epitope spreading and exacerbates relapses in R-EAE. *J Neuroimmunol* 1997;**79**:113–118.
 115. Luhder F, Hoglund P, Allison JP, Benoist C, Mathis D. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes. *J Exp Med* 1998;**187**:427–432.
 116. Egen JG, Allison JP. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity* 2002;**16**:23–35.
 117. Falcone M, Lee J, Patstone G, Yeung B, Sarvetnick N. B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. *J Immunol* 1998;**161**:1163–1168.
 118. Griffin MD, et al. Blockade of T cell activation using a surface-linked single-chain antibody to CTLA-4 (CD152). *J Immunol* 2000;**164**:4433–4442.
 119. Fife BT, Griffin MD, Abbas AK, Locksley RM, Bluestone JA. Inhibition of T cell activation and autoimmune diabetes using a B cell surface-linked CTLA-4 agonist. *J Clin Invest* 2006;**116**:2252–2261.
 120. Hwang KW, et al. Cutting edge: targeted ligation of CTLA-4 *in vivo* by membrane-bound anti-CTLA-4 antibody prevents rejection of allogeneic cells. *J Immunol* 2002;**169**:633–637.
 121. Bluestone JA, St Clair EW, Turka LA. CTLA4Ig: bridging the basic immunology with clinical application. *Immunity* 2006;**24**:233–238.
 122. June CH, Ledbetter JA, Linsley PS, Thompson CB. Role of the CD28 receptor in T-cell activation. *Immunol Today* 1990;**11**:211–216.
 123. Lenschow DJ, et al. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science* 1992;**257**:789–792.
 124. Larsen CP, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* 2005;**5**:443–453.
 125. Lakkis FG, et al. Blocking the CD28–B7 T cell costimulation pathway induces long term

- cardiac allograft acceptance in the absence of IL-4. *J Immunol* 1997;**158**:2443–2448.
126. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med* 1999;**5**: 1298–1302.
 127. Wells AD, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med* 1999;**5**:1303–1307.
 128. Finck BK, Linsley PS, Wofsy D. Treatment of murine lupus with CTLA4Ig. *Science* 1994;**265**:1225–1227.
 129. Grohmann U, et al. CTLA-4-Ig regulates tryptophan catabolism *in vivo*. *Nat Immunol* 2002;**3**:1097–1101.
 130. Larsen CP, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 1996;**381**:434–438.
 131. Lin H, et al. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J Exp Med* 1993;**178**:1801–1806.
 132. Dumont FJ. Technology evaluation: abatacept, Bristol-Myers Squibb. *Curr Opin Mol Ther* 2004;**6**:318–330.
 133. Genovese MC, et al. Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N Engl J Med* 2005;**353**:1114–1123.
 134. Moreland LW, et al. Costimulatory blockade in patients with rheumatoid arthritis: a pilot, dose-finding, double-blind, placebo-controlled clinical trial evaluating CTLA-4Ig and LEA29Y eighty-five days after the first infusion. *Arthritis Rheum* 2002;**46**: 1470–1479.
 135. Kremer JM, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med* 2003;**349**:1907–1915.
 136. Weinblatt M, Combe B, Covucci A, Aranda R, Becker JC, Keystone E. Safety of the selective costimulation modulator abatacept in rheumatoid arthritis patients receiving background biologic and nonbiologic disease-modifying antirheumatic drugs: a one-year randomized, placebo-controlled study. *Arthritis Rheum* 2006;**54**:2807–2816.
 137. Kremer JM, et al. Treatment of rheumatoid arthritis with the selective costimulation modulator abatacept: twelve-month results of a phase iib, double-blind, randomized, placebo-controlled trial. *Arthritis Rheum* 2005;**52**:2263–2271.
 138. Rozelle AL, Genovese MC. Efficacy results from pivotal clinical trials with abatacept. *Clin Exp Rheumatol* 2007;**25** (Suppl.): S30–S34.
 139. Tang Q, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 2003;**171**: 3348–3352.
 140. Chavez H, et al. Absence of CD4CD25 regulatory T cell expansion in renal transplanted patients treated *in vivo* with Belatacept mediated CD28-CD80/86 blockade. *Transpl Immunol* 2007;**17**:243–248.
 141. Urbani S, et al. Restoration of HCV-specific T cell functions by PD-1/PD-L1 blockade in HCV infection: effect of viremia levels and antiviral treatment. *J Hepatol* 2008;**48**: 548–558.
 142. Boettler T, et al. Expression of the interleukin-7 receptor alpha chain (CD127) on virus-specific CD8+ T cells identifies functionally and phenotypically defined memory T cells during acute resolving hepatitis B virus infection. *J Virol* 2006;**80**: 3532–3540.
 143. Ha SJ, et al. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. *J Exp Med* 2008;**205**:543–555.
 144. Riley JL, June CH. The road to recovery: translating PD-1 biology into clinical benefit. *Trends Immunol* 2007;**28**:48–50.
 145. Dong H, Chen L. B7-H1 pathway and its role in the evasion of tumor immunity. *J Mol Med* 2003;**81**:281–287.
 146. Thompson RH, et al. Costimulatory B7-H1 in renal cell carcinoma patients: indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci USA* 2004;**101**:17174–17179.
 147. Thompson RH, et al. PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. *Clin Cancer Res* 2007;**13**:1757–1761.
 148. Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. *Blood* 2008;**111**: 3635–3643.
 149. Kuipers H, et al. Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated CD4+ T cell activation. *Eur J Immunol* 2006;**36**:2472–2482.
 150. Onlamoon N, et al. Soluble PD-1 rescues the proliferative response of simian immunodeficiency virus-specific CD4 and CD8 T cells during chronic infection. *Immunology* 2008;**124**:277–293.
 151. Dudley J, Li J, Pagnotta M, Pascual M, von Segesser LK, Vassalli G. Gene transfer of programmed death ligand-1. Ig prolongs cardiac allograft survival. *Transplantation* 2006;**82**:1733–1737.
 152. He YF, et al. Blocking programmed death-1 ligand-PD-1 interactions by local gene therapy results in enhancement of antitumor effect of secondary lymphoid tissue chemokine. *J Immunol* 2004;**173**: 4919–4928.
 153. Hirano F, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 2005;**65**:1089–1096.
 154. Liu J, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN- γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* 2007;**110**:296–304.
 155. Geng H, et al. HSP70 vaccine in combination with gene therapy with plasmid DNA encoding sPD-1 overcomes immune resistance and suppresses the progression of pulmonary metastatic melanoma. *Int J Cancer* 2006;**118**:2657–2664.
 156. Hirata S, Senju S, Matsuyoshi H, Fukuma D, Uemura D, Nishimura Y. Prevention of experimental autoimmune encephalomyelitis by transfer of embryonic stem cell-derived dendritic cells expressing myelin oligodendrocyte glycoprotein peptide along with TRAIL or programmed death-1 ligand. *J Immunol* 2005;**174**: 1888–1897.
 157. Senju S, et al. Genetically manipulated human embryonic stem cell-derived dendritic cells with immune regulatory function. *Stem Cells* 2007;**25**: 2720–2729.
 158. Ding H, et al. Delivering PD-1 inhibitory signal concomitant with blocking ICOS costimulation suppresses lupus-like syndrome in autoimmune BXSB mice. *Clin Immunol* 2006;**118**:258–267.
 159. Subudhi SK, et al. Local expression of B7-H1 promotes organ-specific autoimmunity and transplant rejection. *J Clin Invest* 2004;**113**:694–700.