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Role of the Programmed Death-1 Pathway in Regulation of Alloimmune Responses In Vivo

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Programmed death-1 (PD-1), an inhibitory receptor up-regulated on activated T cells, has been shown to play a critical immunoregulatory role in peripheral tolerance, but its role in alloimmune responses is poorly understood. Using a novel alloreactive TCR-transgenic model system, we examined the functions of this pathway in the regulation of alloreactive CD4+ T cell responses in vivo. PD-L1, but not PD-1 or PD-L2, blocked accelerated MHC class II-mismatched skin graft (bm12 (I-Abm12) into B6 (I-Ab)) rejection in a similar manner to CTLA-4 blockade. In an adoptive transfer model system using the recently described anti-bm12 (ABM) TCR-transgenic mice directly reactive to I-Abm12, PD-1 and PD-L1 blockade enhanced T cell proliferation early in the immune response. In contrast, at a later time point preceding accelerated allograft rejection, only PD-L1 blockade enhanced T cell proliferation. In addition, PD-L1 blockade enhanced alloreactive Th1 cell differentiation. Apoptosis of alloantigen-specific T cells was inhibited significantly by PD-L1 but not PD-1 blockade, indicating that PD-1 may not be the receptor for the apoptotic effect of the PD-L1-signaling pathway. Interestingly, the effect of PD-L1 blockade was dependent on the presence of CD4+CD25+ regulatory T cells in vivo. These data demonstrate a critical role for the PD-1 pathway, particularly PD-1/PD-L1 interactions, in the regulation of alloimmune responses in vivo. The Journal of Immunology, 2005, 174: 3408–3415.

T he balance of positive and negative costimulatory signals plays a key role in determining the outcome of the alloimmune response (1–3). Positive costimulatory signals are required for clonal expansion of naïve T cells in response to alloantigenic challenge and their subsequent acquisition of effector functions (1, 4). In contrast, negative costimulatory signals delivered to T cells serve to limit alloantigenic responses and to prevent inappropriate immune responses against self-Ags (1, 5). The B7/CD28/CTLA-4 pathway is the best characterized T cell costimulatory pathway and is involved in T cell activation and tolerance induction (6, 7). CTLA-4 engagement delivers a negative signal to T cells, inhibiting TCR- and CD28-mediated signal transduction. Recently, a new costimulatory pathway involving the CD28 homologue programmed death-1 (PD-1) receptor has been characterized. PD-1 is expressed by activated, but not unstimulated, T cells, B cells, and myeloid cells, in contrast to the predominantly T cell-restricted expression of CD28 and CTLA-4 (8–10). The ligands for PD-1 have been identified as PD-L1 (B7-H1), which is expressed on all hemopoietic cells and many nonhemopoietic tissues, and PD-L2 (B7-DC), whose expression is restricted primarily to dendritic cells and macrophages (11–14). PD-1−/− mice display a variety of autoimmune pathologies, including dilated cardiomyopathy (in BALB/c mice) and a lupus-like syndrome (in B6 mice), suggesting a critical role for PD-1 in the maintenance of peripheral tolerance to self-Ags analogous to that of CTLA-4 (15, 16). In addition, recently published data from our group in experimental autoimmune diabetes in NOD mice and experimental autoimmune encephalomyelitis (EAE) have established an important role for the PD-1 pathway in the regulation of autoimmune responses in vivo (17, 18). In contrast to autoimmunity, there is a paucity of data on the role of the PD-1 pathway in allograft rejection and tolerance. Previous work has shown that ligation of PD-1 by PD-L1.Ig significantly prolonged cardiac allograft survival in both CD28−/− recipients of fully mismatched cardiac allografts and wild-type (wt) recipients in conjunction with cyclosporine. This improvement in allograft survival was associated with a reduction in intragraft expression of IFN-γ and proinflammatory chemokines (19). PD-L1.Ig also has been demonstrated to synergize with anti-CD154 mAb to promote long-term islet allograft survival (20). The exact mechanisms of regulation of alloimmune responses in vivo by the PD-1 pathway and the specific role of the ligands PD-L1 and PD-L2 remain unknown.

We have established recently an adoptive transfer model system using anti-bm12 (ABM) TCR-transgenic (tg) mice directly reactive to the MHC class II (MHC-II) molecule I-Abm12 that facilitates the surveillance of clonal expansion and differentiation of alloantigen-specific CD4+ T cells in vivo (21). This approach provides a useful model to investigate the role of the PD-1/PD-L pathway in CD4+ T cell-mediated alloimmune responses in vivo. In this study, we demonstrate that blockade of PD-L1 but not PD-1...
or PD-L2 accelerates rejection of MHC-II-mismatched skin allografts. This was associated with enhanced T cell activation and proliferation, an inhibition of alloantigen-specific T cell apoptosis and polarization of the alloimmune response toward a predominantly Th1-type response.

Materials and Methods

Mice

C57BL/6 (B6) and B6.C-H2<sup>b</sup>K1/H2<sup>k</sup> (bm12) mice were purchased from The Jackson Laboratory. Nude B6 mice were purchased from Taconic Farms. ABM TCR-tg mice have been described previously and were maintained as a breeding colony in our animal facility (21–23). All mice were used at 6–12 wk of age and were housed in accordance with institutional and National Institutes of Health guidelines.

In vivo Abs and treatment protocol

The anti-mouse PD-1 mAb (J501) has been described previously (8). The anti-mouse PD-L1 mAb (MH6) and the anti-mouse PD-L2 mAb (TY25) were also described recently (10, 24). We have previously demonstrated the blocking properties of the mAbs against PD-1, PD-L1, and PD-L2 (18, 24). The anti-CTLA-4 mAb (4F10)-producing hybridoma was provided by Dr. J. Bluestone (University of California, San Francisco, CA). All mAbs were manufactured and purified by Bioexpress Cell Culture. mAbs were given i.p.: 500 μg on day 0 (relative to transplantation), followed by 250 μg on days 2, 4, 6, 8, and 10. The depleting anti-CD25 mAb (PC61) (Bioexpress) was given i.p.: 300 μg on day −5 and on day 0. Splenic depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells was consistently >85% as assessed by flow cytometry.

Skin transplantation

Full-thickness skin grafts harvested from bm12 donors (~1 cm²) were transplanted onto the dorsal thorax of recipient mice, sutured with 6-0 prolene, and secured with Vaseline gauze and a bandage for 7 days (21, 23). Skin graft survival was monitored daily thereafter, and rejection was defined as complete necrosis of the skin graft. In the adoptive transfer experiments, bilateral skin grafts were placed onto the dorsal thorax of recipient nude mice so as to maximize the recovery of alloantigen-specific T cells from draining lymph nodes.

Adoptive transfer of TCR-tg T cells

Adoptive transfer of ABM TCR-tg T cells was performed as described previously (21). Briefly, spleens and lymph nodes were harvested from ABM TCR-tg mice, and pooled single-cell leukocyte suspensions were prepared. CD4<sup>+</sup> T cells were purified by magnetic bead negative selection (Miltenyi Biotech). Typically, CD4<sup>+</sup> T cells were isolated to >85% purity. An aliquot of cells was stained with anti-CD4, anti-TCR V<sub>α</sub>2.1, and anti-TCR V<sub>β</sub>8.1, 8.2, and analyzed by flow cytometry to determine the percentage of ABM TCR-tg CD4<sup>+</sup> T cells. Typically, >90% of CD4<sup>+</sup> T cells expressed the tg TCR. A total of 2 x 10<sup>6</sup> ABM TCR-tg T cells were then injected i.v. into nude B6 mice 1 day before skin transplantation. Bilateral axillary and lateral axillary draining lymph nodes were collected subsequently on days 3, 7, and 10 following skin transplantation, and ABM TCR-tg T cells were identified by flow cytometry.

Labeling of ABM TCR-tg T cells with CFSE and in vivo quantitation of proliferating ABM TCR-tg T cells

In vivo CFSE proliferation assays have been described previously (25, 26). ABM TCR-tg T cells were resuspended in HBSS at a concentration of 1 x 10<sup>6</sup> cells/ml and labeled with the tracking dye CFSE at a concentration of 5 μM in HBSS for 6 min (Molecular Probes). The labeling process was terminated by the addition of FCS (10% of the total volume). Cells were washed twice in RPMI 1640 medium, resuspended in HBSS, and injected i.v. into nude B6 mice 1 day before skin transplantation. The responding frequency of ABM TCR-tg T cells (percentage of dividing precursors among the recovered CFSE-positive ABM TCR-tg T cells harvested 72 h after adoptive transfer) was calculated as described previously (27).

Flow cytometry

Following FcR blockade using anti-FcRγIIIa/anti-FcRγIIa, lymph node cells were stained with fluorochrome-labeled mAbs against CD4, TCR V<sub>α</sub>2.1, TCR V<sub>β</sub>8.1, 8.2, CD25, CTLA-4 (intracellular), PD-1, or PD-L1. All mAbs were purchased from BD Biosciences or eBioscience (anti-PD-L1). Cells were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

Intracellular cytokine staining

Lymph node cells were stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h at 37°C with brefeldin A (10 μg/ml) added for the last 2 h. After staining for surface expression of TCR V<sub>α</sub>2.1 and V<sub>β</sub>8.1, cells were washed in PBS containing 2% FCS, fixed, permeabilized with Cytofix/Cytoperm solution (BD Biosciences), according to the manufacturer’s instructions, and incubated with PE-conjugated IFN-γ, IL-10, or isotype control mAbs for 30 min at 4°C. Intracellular cytokine staining for IFN-γ and IL-10 was analyzed differently given the distinct staining patterns generated using the anti-IFN-γ mAb (XMG1.2) and anti-IL-10 mAb (JES5-16E3). The typical pattern observed with anti-IL-10 mAb is a shift in the mean fluorescence to the right rather than the development of a second population of IL-10-producing cells as observed with anti-IFN-γ mAb. Therefore, IL-10 production by ABM TCR-tg T cells is analyzed by the relative change in mean fluorescence intensity (MFI), whereas IFN-γ production is analyzed as percent cytokine positivity. In all experiments, markers for cytokine positivity were set according to negative staining as defined by staining with isotype controls before evaluation of the effect of blockade of the various costimulatory molecules.

Detection of apoptosis

Leukocytes recovered from draining lymph nodes of adoptively transferred mice were stained for surface expression of V<sub>α</sub>2.1 and V<sub>β</sub>8.1, resuspended in apoptosis buffer (BD Biosciences), according to the manufacturer’s instructions, and incubated with 7-aminoactinomycin D (7-AAD) and PE-conjugated annexin V for 15 min at room temperature. A gate was set on V<sub>α</sub>2<sup>+</sup>V<sub>β</sub>8<sup>+</sup> 7-AAD<sup>+</sup> lymphocytes, and the percentage of annexin V<sup>+</sup> cells was determined by flow cytometry.

CD4<sup>+</sup>CD25<sup>+</sup> T cell purification

CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>−</sup> T cell sorting was performed using a MoFlo High-Performance Cell Sorter (Cytomation) after staining with fluorochrome-conjugated anti-CD4 and anti-CD25 mAbs. The purity of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>−</sup> T cell preparations was consistently >90%. Sorted CD4<sup>+</sup>CD25<sup>−</sup> T cells were injected i.v. into nude B6 mice 1 day before skin transplantation.

Statistics

Kaplan-Meier survival graphs were constructed, and a log-rank comparison of the groups was used to calculate p values. Student’s t test was used for comparison of the means. A value of p < 0.05 was considered significant statistically.

Results

Blockade of PD-L1, but not PD-1 or PD-L2, accelerates CD4<sup>+</sup> T cell-mediated skin allograft rejection

First, we evaluated the role of the PD-1/PD-L pathway in skin allograft rejection using a polyclonal MHC-II-mismatched model in which allograft rejection is mediated primarily by CD4<sup>+</sup> T cells (23, 28). The median survival time (MST) of bm12 (I-A<sup>b</sup>bm12) skin grafts transplanted onto untreated wt B6 (I-A<sup>b</sup>) mice was 16 days (Fig. 1). As expected, blockade of the negative costimulatory molecule CTLA-4 significantly accelerated the time course of skin allograft rejection (MST = 10; p = 0.0001 vs untreated controls). Similarly, blockade of PD-L1 also resulted in accelerated rejection of the skin grafts compared with untreated recipients (MST = 13; p = 0.0005 vs untreated controls), suggesting an enhanced CD4<sup>+</sup> T cell response to donor alloantigen. In contrast, administration of anti-PD-1 or anti-PD-L2 mAbs, both previously shown to be active in vivo (17, 18), did not affect significantly the tempo of skin allograft rejection (MST = 16 for both groups), suggesting disparate roles for the individual PD-1/PD-L pathway family members in the control of the alloimmune response in this model.

CTLA-4 and PD-1 exhibit different in vivo expression kinetics on CD4<sup>+</sup> T cells following skin transplantation

CTLA-4 and PD-1 expression is induced on T cells upon activation (8–10, 29). To analyze the kinetics of CTLA-4 and PD-1 expression on alloreactive CD4<sup>+</sup> T cells during skin allograft rejection, we used the recently described ABM TCR-tg system (21).

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Blockade of the PD-1/PD-L pathway enhances and accelerates receptors during the initiation of an alloimmune response in vivo.

In contrast to these results, other investigators have reported that, under certain circumstances, PD-L1 and PD-L2 provide a positive stimulatory (32). Given the discrepancy observed in vitro studies, we sought to study the effect of PD-1, PD-L1, and PD-L2 blockade on alloantigen-specific ABM TCR-tg CD4\(^+\) T cell proliferation and differentiation in vivo.

First, 2 \times 10^6 ABM TCR-tg T cells were labeled with CFSE and injected into nude B6 recipients of bm12 skin grafts. This technique facilitates the evaluation of the proliferative dynamics of the adoptively transferred ABM TCR-tg T cells in response to the allograft. The proliferation profile of ABM TCR-tg T cells was determined 72 h after transplantation (Fig. 3A). Both anti-CTL4-4 mAb and anti-PD-L1 mAbs significantly increased the proliferative response of ABM TCR-tg T cells to bm12 skin grafts compared with untreated recipients (mean increase in responding frequency, 34 and 50\%, respectively; \(p = 0.03\) and 0.01). In contrast, anti-PD-1 mAb and anti-PD-L2 mAbs had no effect on the proliferative response of ABM TCR-tg T cells. Anti-PD-L1 mAb increased the proliferative response of ABM TCR-tg T cells compared with anti-PD-1 mAb (mean increase in responding frequency, 29\%), although this did not reach statistical significance.

To determine the kinetics of alloantigen-specific ABM TCR-tg T cell expansion, 2 \times 10^6 unlabeled ABM TCR-tg T cells were transferred into nude B6 recipients of bm12 skin grafts. We have previously confirmed in a nonlymphopenic system that the T cell response in reconstituted T cell-deficient nude recipients reflects the physiologic immune response (21). Enumeration of alloantigen-specific ABM TCR-tg T cells in the draining lymph nodes of skin graft recipients on days 3, 7, and 10 following transplantation demonstrated a progressive increase in the number of ABM TCR-tg T cells with progression of skin graft rejection (Fig. 3B). Anti-CTL4-4, anti-PD-1, anti-PD-L1, and anti-PD-L2 mAbs all resulted in an earlier and more pronounced increase in the number of ABM TCR-tg T cells present in the draining lymph nodes compared with untreated recipients, indicating that blockade of the PD-1/PD-L pathway accelerated and enhanced alloantigen-driven T cell expansion. Anti-CTL4-4 \((p = 0.04)\), anti-PD-1 \((p = 0.01)\), and anti-PD-L1 \((p = 0.04)\), but not anti-PD-L2, mAbs significantly enhanced the number of ABM TCR-tg cells in the draining lymph nodes by day 7 compared with untreated recipients. However, by day 10, immediately before the development of end-stage rejection, which occurs on days 10–13 in anti-CTL4-4 mAb- and anti-PD-L1 mAb-treated recipients, the cell number in these animals increased dramatically, as shown in Fig. 3B. In recipients treated with anti-CTL4-4 mAb, 189 \pm 43.8 \times 10^4\) ABM TCR-tg T cells were present in the draining lymph nodes compared with 93 \pm 26.1 \times 10^4\) ABM TCR-tg T cells in untreated recipients \((p = 0.03)\). When recipients were treated with anti-PD-L1 mAb, an even

This is a model of direct alloantigen presentation in which >90\% of CD4\(^+\) T cells carry a tg TCR that directly recognizes the mutated MHC-II molecule I-A\(^{bm12}\). The specificity of ABM TCR-tg T cells for bm12 alloantigen has been demonstrated previously both in vitro and in vivo (21, 23, 30). In this study, we i.v. injected syngeneic T cell-deficient nude B6 mouse recipients of bm12 skin grafts with 2 \times 10^6 ABM TCR-tg T cells. Draining regional lymph node cells were harvested at time points of 3, 7, and 10 days after transplantation, and the ABM TCR-tg T cells were identified by TCR V\(^{2.1}\) and TCR V\(^{B8.1}\)-positive staining. Intracellular CTLA-4 expression and surface PD-1 expression on ABM TCR-tg T cells were evaluated by three-color flow cytometry analysis. Before adoptive transfer, neither CTLA-4 nor PD-1 expression was observed on ABM TCR-tg T cells (Fig. 2). Following transplantation, the peak expression of intracellular CTLA-4 was observed on day 3, and expression levels fell toward baseline by day 7. In contrast, the surface expression of PD-1 on ABM TCR-tg T cells increased progressively over the time course examined with the highest expression levels observed on day 10. However, CTLA-4 expression on ABM TCR-tg T cells remained significantly higher than PD-1 expression \((p = 0.04)\). These studies define the temporal expression patterns of both negative T cell costimulatory receptors during the initiation of an alloimmune response in vivo.

Blockade of the PD-1/PD-L pathway enhances and accelerates CD4\(^+\) T cell activation

PD-L1 or PD-L2 expression by APC inhibits proliferation and cytokine production by Ag-specific CD4\(^+\) T cells. These effects are CD28 independent but are dependent on the Ag dose (12, 13, 31). In contrast to these results, other investigators have reported that, under certain circumstances, PD-L1 and PD-L2 provide a positive rather than a negative costimulatory signal to T cells (11, 14). The basis for this inconsistency has not, as yet, been established, but it raises the possibility that signaling through PD-1 can provide positive as well as negative signals, although the exact circumstances of when and how this occurs remain uncertain. It is also possible that PD-L1 and PD-L2 have an alternate receptor that may be stimulatory (32). The discrepancy observed in vivo studies, we sought to study the effect of PD-1, PD-L1, and PD-L2 blockade on alloantigen-specific ABM TCR-tg CD4\(^+\) T cell proliferation and differentiation in vivo.

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FIGURE 1. Anti-PD-L1, but not anti-PD-1 or anti-PD-L2, mAb accelerates skin graft rejection. B6 recipients of bm12 skin grafts were left untreated \((n = 9)\) or treated with blocking mAbs to CTLA-4 \((n = 8)\), PD-1 \((n = 6)\), PD-L1 \((n = 4)\), or PD-L2 \((n = 4)\) as described in Materials and Methods. Kaplan-Meier plots demonstrate accelerated skin graft rejection in anti-CTL4-4 mAb- and anti-PD-L1 mAb-treated recipients compared with untreated recipients \((p < 0.0001\) and \(p = 0.0005\), respectively).

FIGURE 2. CTLA-4 and PD-1 expression on ABM TCR-tg T cells before adoptive transfer and following skin transplantation. At the indicated time points, lymph node cells were stained with anti-TCR V\(^{2.1}\) mAb, anti-TCR V\(^{B8.1}\) mAb, anti-CTL4-4 or anti-PD-1 mAbs (solid lines), or isotype control mAbs (dashed lines). Expression of CTLA-4 (intracellular) and PD-1 was determined by flow cytometry after gating on V\(^{2.1}\) V\(^{B8.1}\) T cells. The histograms shown are representative of at least three separate experiments per time point.

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greater increase in the number of ABM TCR-tg T cells was observed (300/1000 vs 82.2/1000; p = 0.02 vs untreated controls); however, this was not significant statistically when compared with anti-CTLA-4 mAb. In contrast, anti-PD-1 mAb did not increase significantly the number of ABM TCR-tg T cells present in the draining lymph nodes (125/38/1000) compared with untreated recipients. Similarly, anti-PD-L2 mAb did not increase significantly the number of ABM TCR-tg T cells (131/20.6/1000) compared with untreated recipients. These findings indicate that the increase in the alloreactive T cell clone size was most notable in anti-CTLA-4 mAb- and anti-PD-L1 mAb-treated mice in which the time course of skin graft rejection was accelerated significantly in the polyclonal rejection model.

We then examined the effect of PD-1/PD-L blockade on alloreactive Th1/Th2 cell differentiation in vivo by determining the expression of the Th1 cytokine IFN-γ and the Th2 cytokine IL-10 by alloantigen-specific ABM TCR-tg T cells after intracellular staining (21). The kinetics of IFN-γ production paralleled that of
Anti-PD-L1 mAb inhibits apoptosis of alloreactive CD4+ T cells

Induction of stable peripheral allograft tolerance results, at least in part, from a reduction of the alloreactive T cell clone by apoptosis (33, 34). A recent report (35) has implicated the PD-1/PD-L pathway, specifically PD-L1, in apoptosis of activated tumor-reactive T cells. Interestingly, in that study, apoptosis was PD-1 independent, suggesting that PD-L1 may bind a second receptor on T cells. Given the importance of alloreactive T cell clone size in allograft rejection (36), we sought to determine whether blockade of the PD-1/PD-L pathway had any effect on apoptosis of alloantigen-specific TCR-tg T cells and whether a differential effect of anti-PD-1/PD-L pathway had any effect on apoptosis of ABM TCR-tg T cells. Interestingly, in that study, apoptosis was PD-1 independent, suggesting that PD-L1 may bind a second receptor on T cells. Given the importance of alloreactive T cell clone size in allograft rejection (36), we sought to determine whether blockade of the PD-1/PD-L pathway had any effect on apoptosis of alloantigen-specific TCR-tg T cells and whether a differential effect of anti-PD-1/PD-L pathway had any effect on apoptosis of ABM TCR-tg T cells. Interestingly, in that study, apoptosis was PD-1 independent, suggesting that PD-L1 may bind a second receptor on T cells. Given the importance of alloreactive T cell clone size in allograft rejection (36), we sought to determine whether blockade of the PD-1/PD-L pathway had any effect on apoptosis of alloantigen-specific TCR-tg T cells and whether a differential effect of anti-PD-1/PD-L pathway had any effect on apoptosis of ABM TCR-tg T cells.

Effect of CTLA-4 and PD-L1 blockade is dependent on the presence of CD4+ CD25+ T cells

Recent studies have shown that most naive CD4+CD25+ T cells constitutively express CTLA-4 and that CTLA-4 expression is important in mediating the actions of CD4+CD25+ regulatory T cells (37–39). In our model, as shown above, CTLA-4 or PD-L1 blockade significantly accelerated skin graft rejection (Fig. 1). In contrast, following CD25+ depletion in B6 recipients, anti-CTLA-4 mAb, as well as anti-PD-L1 mAb, failed to accelerate rejection of bm12 skin grafts (Fig. 6). Flow cytometric analysis demonstrated significantly higher expression levels of both CTLA-4 (p = 0.0003) and PD-L1 (p = 0.03) on ABM TCR-tg CD4+CD25+ T cells compared with ABM TCR-tg CD4+CD25− T cells (Fig. 7). Therefore, we investigated whether blocking mAbs to CTLA-4 or PD-L1 mAb inhibits apoptosis of ABM TCR-tg T cells following skin transplantation. Following adoptive transfer of 2 × 10^6 ABM TCR-tg T cells into nude B6 mice, recipients were transplanted with bm12 skin grafts and received blocking mAbs to PD-1 or PD-L1. After 3, 7, or 10 days, draining lymph node cells were harvested, and apoptosis of ABM TCR-tg T cells was determined by flow cytometry as described in Materials and Methods. Values represent the mean percent 7-AAD− annexin V+ ABM TCR-tg T cells obtained from three to five separate experiments performed per time point (±SD).

**FIGURE 5.** Anti-PD-L1 mAb inhibits apoptosis of ABM TCR-tg T cells following skin transplantation. Following adoptive transfer of 2 × 10^6 ABM TCR-tg T cells into nude B6 mice, recipients were transplanted with bm12 skin grafts and received blocking mAbs to PD-1 or PD-L1. After 3, 7, or 10 days, draining lymph node cells were harvested, and apoptosis of ABM TCR-tg T cells was determined by flow cytometry as described in Materials and Methods. Values represent the mean percent 7-AAD− annexin V+ ABM TCR-tg T cells obtained from three to five separate experiments performed per time point (±SD).

**FIGURE 6.** Anti-CTLA-4 and anti-PD-L1 mAb fail to accelerate skin graft rejection in the absence of CD25+ T cells. wt B6 mice were depleted in vivo using anti-CD25 mAb and treated with blocking mAbs to CTLA-4 (n = 4) or PD-L1 (n = 6) as described in Materials and Methods. Kaplan-Meier plots demonstrate no acceleration of skin graft rejection in anti-CTLA-4 mAb- and anti-PD-L1 mAb-treated recipients compared with otherwise untreated CD25−-depleted recipients.

**FIGURE 7.** Expression of CTLA-4 and PD-L1 on ABM TCR-tg T cells. Lymph node cells from naive ABM TCR-tg mice were stained with anti-CD4-PerCP, anti-CD25-APC, anti-CTLA-4, or anti-PD-L1-PE (solid lines) or isotype control mAbs (dashed lines). Expression of CTLA-4 (intracellular) and PD-L1 was determined by flow cytometry after gating on CD4+CD25+ or CD4+CD25− T cells. Values represent the mean (±SD) MFI of CTLA-4 or PD-L1 expressing ABM TCR-tg T cells obtained from six separate experiments.
FIGURE 8. Effect of CTLA-4 and PD-L1 blockade is dependent on the presence of CD4^+CD25^- T cells. Following adoptive transfer of 2 × 10^6 CD4^+CD25^- ABM TCR-tg T cells into nude B6 mice, recipients were transplanted with bm12 skin grafts and received blocking mAbs to CTLA-4, PD-1, or PD-L1. Draining lymph node cells were harvested after 10 days. The absolute number of ABM TCR-tg T cells present in the draining lymph nodes of untreated recipients (A) and apoptosis of ABM TCR-tg T cells (B) were determined as described in Figs. 3B and 5, respectively. Values represent the mean ± SD of the data obtained from four separate experiments.

PD-L1 might mediate some of their effects via ABM TCR-tg CD4^+CD25^- regulatory T cells in our model. ABM TCR-tg T cells were sorted into CD4^+CD25^+ and CD4^+CD25^- populations, and 2 × 10^6 ABM TCR-tg CD4^+CD25^- T cells were injected into nude B6 recipients of bm12 skin grafts. On day 10 following transplantation, 108 ± 35.8 × 10^4 ABM TCR-tg T cells were present in the draining lymph nodes of untreated recipients (Fig. 8A). In contrast to the results observed when unsorted ABM TCR-tg T cells were transferred adoptively, anti-CTLA-4 mAb did not increase significantly the number of ABM TCR-tg T cells present in the draining lymph nodes (138 ± 20.9 × 10^4) when only ABM TCR-tg CD4^+CD25^- cells were transferred. Similarly, blockade of PD-L1 failed to increase the number of ABM TCR-tg T cells (119 ± 31.5 × 10^4). These data indicate that the enhanced alloantigen-triggered proliferation of ABM TCR-tg T cells following CTLA-4 and PD-L1 blockade is dependent on the presence of CD4^+CD25^- T cells.

Furthermore, when only ABM TCR-tg CD4^+CD25^- T cells were transferred, blockade of PD-L1 failed to inhibit apoptosis of alloantigen-specific ABM TCR-tg T cells (Fig. 8B). These data provide additional evidence pointing to a key role for CD4^+CD25^- T cells in mediating the effects of anti-PD-L1 mAb in this model.

Discussion

The outcome of the alloimmune response is, in part, dependent on the balance between activating and inhibitory signals that accompany TCR engagement by alloantigen (1). PD-1 is the newest member of the CD28 family and acts as a negative regulator of activated T cells (3, 8, 12, 40). However, in vitro studies of the functions of the PD-1/PD-L pathway have yielded conflicting results regarding the role played by the members of this pathway in T cell activation. Freeman et al. (12) have reported that ligation of the PD-1 receptor by PD-L1 inhibits proliferation and cytokine production by activated T cells consistent with the hypothesis that this pathway delivers a negative signal to activated T cells. In addition, Chinese hamster ovary cell transfectants presenting OVA peptide in the context of I-Ad and expressing either PD-L1 or PD-L2 on dendritic cells trigger enhanced T cell proliferation and cytokine production (31). In addition, both CD4^+ and CD8^+ T cell responses are enhanced markedly in PD-L1^-/- mice compared with wt mice in vitro and in vivo (42). In contrast, other studies have reported that under certain circumstances PD-L1 and PD-L2 deliver a positive costimulatory signal to T cells. Dong et al. (11) and Tamura et al. (43) have shown that ligation of PD-1 by PD-L1.Ig costimulates T cell proliferation and promotes IL-10 production. PD-L2 also augmented murine T cell responses to anti-CD3 stimulation (14). Given the disparate in vitro data, we sought to investigate the function of the PD-1/PD-L pathway in vivo using an adoptive transfer model system involving ABM TCR-tg mice directly reactive to the mutated MHC-II molecule I-A^b_{12} (21).

In this report, we first demonstrated that blockade of PD-L1, but not PD-1 or PD-L2, accelerates the rejection of a MHC-II-mismatched skin allograft in a manner similar to that of the prototypic inhibitory costimulatory molecule CTLA-4. We have previously demonstrated divergent effects of PD-1, PD-L1, and PD-L2 blockade in EAE and in autoimmune diabetes in NOD mice (17, 18). In the NOD model of autoimmune diabetes, PD-1 and PD-L1 but not PD-L2 blockade precipitated disease and insulin (18). In contrast, data obtained in the EAE model demonstrated that PD-1 and PD-L2 but not PD-L1 blockade augmented disease (17). PD-L1 is expressed not only on bone marrow-derived APC but also on parenchymal cells (44), including microvascular endothelial cells (45). Interestingly, a recent report (46) demonstrated expression of PD-L1 on the intima of cardiac allograft arteries and demonstrated that anti-PD-L1 mAb therapy accelerated chronic vasculopathy following cardiac transplantation, emphasizing a key role for this costimulatory molecule as a negative modulator of alloimmune responses in vivo. By contrast, PD-L2 has more limited expression, predominantly on cytokine-activated macrophages and dendritic cells (13). These patterns of expression may, at least in part, explain the divergent outcomes observed following receptor blockade in this study and others. Importantly, the Ab dose used in these studies was substantial; lower dosing protocols have proven effective in other models (17, 18). Furthermore, the results reported in this study are consistent with unpublished data from our laboratory demonstrating accelerated rejection of fully mismatched cardiac allografts in CD28-deficient mice using anti-PD-L1 mAb. Therefore, the observed results reflect true biological effects, or lack thereof, of anti-PD-1, anti-PD-L1, and anti-PD-L2 mAbs, respectively.

We also demonstrated, using the alloreactive ABM TCR-tg model system, that the PD-1 pathway delivers an inhibitory signal and regulates alloimmune responses by limiting clonal expansion and activation of alloreactive T cells. Analogous to the in vitro studies and the results observed in models of autoimmunity, blockade of the different members of the PD-1/PD-L pathway yielded disparate effects. Only treatment with anti-PD-L1 mAb significantly accelerated the tempo of skin graft rejection. Although blockade of PD-1 and PD-L2 initially increased T cell proliferation

in an allospecific tg model in a similar manner to PD-L1, only blockade of PD-L1 resulted in a significant increase in clone size that correlated to accelerated rejection observed in the polyclonal system. These data indicate that PD-L1 may be of greater relevance in the inhibition of alloimmune responses than PD-L2 and suggest that PD-1 may not be the sole mediator of the inhibitory signal delivered by PD-L1. Furthermore, only PD-L1 blockade was associated with enhanced expression of the Th1 cytokine IFN-γ and a concomitant reduction in the Th2 cytokine IL-10, a significant finding given that the allograft rejection process is predominantly a Th1-mediated immune response.

PD-1 was cloned originally from a T cell hybridoma undergoing apoptosis, and it was thought initially that inhibition of the immune response via PD-1 may result from apoptosis of activated T cells following ligation of PD-1 by PD-L1 (47). However, subsequent studies revealed that PD-1/PD-L interactions lead to cell cycle arrest in G_{0}-G_{1} rather than cell death (13). In addition, previous reports suggest that the PD-1/PD-L pathway inhibits T cell proliferation by reducing the production of IL-2 and by restricting the number of T cells that gain entry into the cell cycle as well as their subsequent division rate (41). It has recently been shown that tumor-cell-associated PD-L1 increased apoptosis of tumor-specific T cells in a PD-1-independent manner (35). In keeping with these data, we have demonstrated that apoptosis of alloantigen-specific T cells could be significantly abrogated anti-PD-L1 mAb but not by anti-PD-1 mAb, supporting the hypothesis that PD-1 is not the receptor for PD-L1-mediated T cell apoptosis.

The mechanisms by which tolerance to foreign alloantigen is established are understood incompletely but include active regulation of the alloimmune response by a subpopulation of CD4^{+} cells expressing the IL-2 receptor α-chain, CD25 (48–50). We have unpublished data demonstrating that a subpopulation of CD4^{+} CD25^{+} T cells in the ABM TCR-tg model bears the phenotypic and functional characteristics of regulatory T cells and express both CTLA-4 and PD-L1 (51). In previous studies, CTLA-4 blockade both in vitro and in vivo abrogates CD4^{+} CD25^{+} T cell-mediated suppression of Ag-specific immune responses (37–39). Similarly, Baecher-Allan et al. (52) have shown that when PD-1 engagement is blocked with anti-PD-L1 responses (37–39). Similarly, Baecher-Allan et al. (52) have shown that when PD-1 engagement is blocked with anti-PD-L1 mAb in the polyclonal model is dependent on the presence of CD4^{+} CD25^{+} T cells, we have demonstrated that enhancement of alloantigen-triggered T cell proliferative responses in vivo by anti-PD-L1 mAb therapy is also dependent on the presence of CD4^{+} CD25^{+} regulatory T cells. It has been suggested that CTLA-4 may play two roles in controlling immune responses. One is to transmit a negative signal to activated T cells and the other to transmit a negative signal to activated T cells and the other to mediate the suppressor activity of CD4^{+} CD25^{+} regulatory T cells (53, 54). The effect of CTLA-4 blockade to enhance allograft rejection has been attributed to the former (55–57). However, the failure of CTLA-4 or PD-L1 blockade to significantly increase T cell proliferation when only CD4^{+} CD25^{+} T cells were transferred suggested that the enhanced proliferation seen with transfer of both CD4^{+} CD25^{+} and CD4^{+} CD25^{−} populations can be attributed to blockade of CTLA-4 or PD-L1 on CD4^{+} CD25^{+} regulatory T cells, and consequent abrogation of CD4^{+} CD25^{−} T cells mediated suppression of alloantigen-induced T cell proliferative responses. Additional investigation will be necessary to examine a possible role for PD-L1 like that of CTLA-4 in CD4^{+} CD25^{+} T cell-mediated down-regulation of T cell responses in vivo. Finally, it is likely that the immunoregulatory functions of PD-L1 may be mediated by more than one mechanism that are working in concert or at different time points following alloantigenic challenge.

In conclusion, we demonstrate a critical role for the PD-1 pathway, particularly PD-L1, in the regulation of alloimmune responses in vivo. These functions appear to be complex and may be mediated by two receptors, PD-1 and an as yet unidentified receptor on T cells. Understanding the functions of the inhibitory T cell costimulatory pathways in alloimmunity and transplantation tolerance may allow us to harness the physiologic mechanisms that regulate alloimmune responses, and, perhaps in combination with blockade of positive costimulatory pathways, may provide novel approaches to achieve durable and reproducible transplantation tolerance.

Disclosures

The authors have no financial conflict of interest.

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