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Analysis of the Role of Negative T Cell Costimulatory Pathways in CD4 and CD8 T Cell-Mediated Alloimmune Responses In Vivo

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Negative costimulatory signals mediated via cell surface molecules such as CTLA-4 and programmed death 1 (PD-1) play a critical role in down-modulating immune responses and maintaining peripheral tolerance. However, their role in alloimmune responses remains unclear. This study examined the role of these inhibitory pathways in regulating CD28-dependent and CD28-independent CD4 and CD8 alloreactive T cells in vivo. CTLA-4 blockade accelerated graft rejection in C57BL/6 wild-type recipients and in a proportion of CD4<sup>+</sup>/CD28<sup>+</sup> but not CD8<sup>+</sup>/CD28<sup>+</sup> recipients of BALB/c hearts. The same treatment led to prompt rejection in CD28<sup>+</sup>/PD-L1<sup>+</sup> and a smaller proportion of CD4<sup>+</sup>/CD28<sup>+</sup> mice with no effect in CD8<sup>+</sup>/CD28<sup>+</sup> recipients. These results indicate that the CTLA-4:B7 pathway provides a negative signal to alloreactive CD8<sup>+</sup> T cells, particularly in the presence of CD28 costimulation. In contrast, PD-1 blockade led to accelerated rejection of heart allografts only in CD28<sup>+</sup>/PD-L1<sup>+</sup> and CD8<sup>+</sup>/PD-L1<sup>+</sup> recipients. Interestingly, PD-1 ligand (PD-L1) blockade led to accelerated rejection in wild-type mice and in all recipients lacking CD28 costimulation. This effect was accompanied by expansion of IFN-γ-producing alloreactive T cells and enhanced generation of effector T cells in rejecting allograft recipients. Thus, the PD-1:PD-L1 pathway down-regulates alloreactive CD4 T cells and enhances generation of effector T cells in rejecting allograft recipients. The PD-1:PD-L1 pathway down-regulates alloreactive CD4 T cells, particularly in the absence of CD28 costimulation. The differential effects of PD-1 vs PD-L1 blockade support the possible existence of a new receptor other than PD-1 for negative signaling through PD-L1. Furthermore, PD-1:PD-L1 pathway can regulate alloimmune responses independent of an intact CD28/CTLA-4:B7 pathway. Harnessing physiological mechanisms that regulate alloimmunity should lead to development of novel strategies to induce durable and reproducible transplantation tolerance.


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4 Abbreviations used in this paper: PD-1, programmed death-1; PD-L1, PD-1 ligand; WT, wild type; DKO, double knockout; CD62L, CD62 ligand; MST, median survival time.

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including dendritic cells, monocytes, and B cells upon activation. In addition, PD-L1 expression has been detected in lymphoid as well as nonlymphoid organs (19, 20, 22). Previous work in transplantation models has shown that ligation of PD-1 using PD-L1 Ig prolonged cardiac allograft survival. This prolongation of survival was associated with reduced intragraft expression of IFN-γ and IFN-γ-induced chemokines both in CD28+/− recipients and in wild-type (WT) recipients in conjunction with immunosuppression (cyclosporine) in fully MHC-mismatched combinations (23). In another study, PD-L1 Ig and anti-CD154 mAb synergized to prolong long-term islet allograft survival (24).

Conventional T cell costimulatory blockade directed at the CD28:B7 and/or CD154:CD40 pathways is not effective in reproducibly inducing tolerance in some stringent murine transplant models and in primates (25–27). Similarly, they do not prevent development of chronic rejection in fully allogeneic transplant models (28, 29). The difficulty in achieving long-term survival in some models has been attributed to resistance of effector CD8+ T cells (26, 30) and/or memory T cells (31, 32) to costimulatory blockade.

There is a paucity of data on the role and interaction between costimulatory and inhibitory receptors and their ligands in allograft survival. In the present study, we first analyzed the role of negative costimulatory pathways in mediating allograft survival. To this end we made use of novel double gene knockout (DKO) mice (CD4+/−CD28−/− and CD8+/−CD28−/−) generated in our laboratory as recipients of fully allogeneic heart transplants. These studies are critical for development of new strategies to harness physiologic mechanisms regulating alloimmune responses in transplantation.

**Materials and Methods**

**Mice**

C57BL/6 (B6, H-2b) and BALB/c (H-2d), B6 background CD28−/−, CD8−/−, CD4−/−, B cell-deficient (MuMT), and B7-1/B7-2 DKO mice were purchased from The Jackson Laboratory. CD8−/−CD28+/− mice previously described (33) and CD4−/−CD28−/− mice were both generated and maintained as a breeding colony in our animal facility. BALB/c B7-1/B7-2 DKO mice are kindly provided by Dr. A. H. Sharpe (Harvard Medical School, Boston, MA) (34, 35). All mice were used at 6–12 wk of age and were housed in accordance with institutional and National Institutes of Health guidelines.

**Abs and in vivo treatment protocol**

The anti-mouse PD-L1 mAb (J43) has been described (11). The anti-mouse PD-1 mAb (J43) has been described (11). The anti-mouse CTLA-4 mAb (4F10)–producing hybridoma was provided by J. Bluestone (University of California, San Francisco, CA). All mAbs were manufactured and purified by Bioexpress Cell Culture. mAbs were given i.p. according to the following protocol: 0.5 mg of mAb on the day of transplantation and 0.25 mg on days 2, 4, 6, 8, and 10 after transplantation.

**Heterotopic heart transplantation**

Vascularized heart grafts were transplanted using microsurgical techniques as described by Corry et al. (36). Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization. Graft survival is shown as the median survival time (MST) in days.

**CD4+ T cell purification for adoptive transfer experiments**

To obtain 100% purified CD4+ CD28− and CD4+ CD28− T cells for adoptive transfer studies, we first prepared a single cell suspension from spleens of naive WT and CD28−/− mice. CD4+ T cells were enriched (>95% purity) using CD4+ T cell-enrichment column (R&D Systems).

**ELISPOT assay**

The technique for ELISPOT analysis has been described recently by our group and others (37–39). Immunospot plates (Cellular Technology) were coated with 4 μg/ml anti-mouse IFN-γ mAb (R4-6A2) in sterile PBS overnight. The plates were then blocked for 1 h with sterile PBS containing 1% BSA-fraction V and washed three times with sterile PBS. Splenocytes (0.5 × 10^6 in 200 μl of HL-1 medium containing 1% γ-glutamine) were then placed in each well in the presence of 0.5 × 10^5 irradiated (30 Gy) syngeneic or allogeneic splenocytes and cultured for 24 h at 37°C in 5% CO2. After washing with PBS followed by washing with PBS containing 0.05% Tween (PBST), 2 μg/ml biotinylated rat anti-mouse IFN-γ detection mAb (XMG1.2) was added overnight. All Abs mentioned were purchased from BD Pharmingen. The plates were then washed four times in PBST, followed by 2 h of incubation with HRP-conjugated streptavidin (DAKO) diluted at 1:2000 in PBS/1% BSA. After washing three times with PBST followed by PBS, the plates were developed using 3-amoino-9-ethyl-carbazole (Sigma-Aldrich). The resulting spots were counted on a computer-assisted enzyme-linked immunospot image analyzer (Cellular Technology), and frequencies were expressed as the number of cytokine-producing spots per 0.5 × 10^5 splenocytes.

**CD8 and CD4 effector T cell enumeration**

Recipient splenocytes were isolated 14 days after transplantation, and red cells were lysed with AKLysis buffer (BioWhittaker). Cells were stained with anti-CD4-FITC or anti-CD8-FITC, anti-CD62 ligand (CD62L)-allophycocyanin, and anti-CD44-PE (all from BD Pharmingen). Flow cytometry was performed using a FACSCaliber flow cytometry system (BD Biosciences) and analyzed using CellQuest software (BD Biosciences). Percentages of effector CD4+ and CD8+ cells expressing the CD44highCD62L−low phenotype were measured, as previously described (39–41). Results are representative of three experiments.

**Flow cytometry**

To study the expression of PD-1 and CTLA-4 on T cell subsets, splenocytes derived from WT or CD28−/− mice are stained with CD8 FITC, CD4 FITC, and PE-conjugated mAb against CD-1 and CD152 (CTLA-4). Intracellular CD4- and CD8-GST staining was performed using the Cytofix/Cytoperm intracellular staining kit. All anti-mouse Ab and the Cytofix/Cytoperm kit were obtained from BD Biosciences.

**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 means between experimental groups examined by ELISPOT assay. Differences were considered to be significant at values p < 0.05.

**Results**

**The role of CD4 and CD8 T cells in cardiac allograft rejection in the absence of CD28**

First, we explored the role of CD4 and CD8 T cells in mediating allograft rejection in the presence and absence of CD28 costimulation. C57BL/6 WT, CD4, CD8, CD4, CD5, CD8/CD28, and CD4/ CD8 deficient mice were used as recipients of BALB/c vascularized heart grafts. As previously published (37–39), immunospot plates demonstrate that in the absence of CD4+ alone cannot reject cardiac allografts in the presence or absence of CD28. However, CD28 signaling seems to be essential for CD4-mediated allograft rejection in our model. To further study the interactions between CD4+ and CD8+ T cells, 10 × 10^6 purified CD4+ T cells isolated from WT mice were injected i.v. into CD4−/− CD28−/− recipients of BALB/c cardiac allografts. Interestingly, none
of the recipient mice rejected their allografts to date (survival days, >78, >78 >78, >55, >58, >58, respectively). Next, the same number of purified CD4+CD28− T cells derived from CD28−/− mice were injected into CD4+/− recipients. All these mice rejected cardiac allografts within 3–6 wk (days 25, 28, 28, and 36). All in all, these findings indicate that CD4+CD28− T cells can provide help to activate alloreactive CD8+ T cells that can then mediate rejection but only in the presence of CD28 signaling.

**CTLA-4 blockade accelerates cardiac allograft rejection in WT and CD4+/− but not CD8+/− mice**

We next studied the role of CTLA-4 in fully allogeneic C57BL/6 recipients (H-2b) of BALB/c hearts (H-2d). The MST of cardiac allografts in untreated group was 8.0 days (Fig. 2a). In mice treated with a blocking anti-CTLA-4 mAb, statistically significant acceleration of allograft rejection was observed (MST = 6 days; n = 6; p = 0.001) (Fig. 2a). To study the effect of CTLA-4 blockade on individual T cell subsets, we next used CD8−/− and CD4−/− mice as recipients. CTLA-4 blockade had no effect on allograft rejection in CD8−/− mice (MST = 11, n = 7 in control group vs MST = 11, n = 8 in treated group) (Fig. 2d). CD4-deficient recipients had indefinite allograft survival as previously published (MST > 130 days; n = 9). Interestingly, CTLA-4 blockade resulted in rejection in a proportion of CD4-deficient recipients within 40 days after transplantation (rejection in 38% of treated mice within 40 days; n = 6; MST = 107; p = 0.03) (Fig. 2c), indicating an important role for CTLA-4 in regulating CD8 T cell-mediated rejection.

**CTLA-4 blockade accelerates cardiac allograft rejection in CD28−/− and some CD4−/−CD28−/− but not CD8−/−CD28−/− mice**

We then explored the role of CTLA-4 blockade in the absence of CD28 costimulation. CD28−/− mice rejected the fully allogeneic
BALB/c cardiac allografts 16–24 days after transplantation (MST = 16.5 days, n = 6), whereas the treated animals demonstrated significant acceleration of the cardiac allografts (MST = 9.5, n = 8, p = 0.023 as compared with untreated controls) (Fig. 3a). To dissect the effects of CTLA-4 blockade on CD4\(^+\) and CD8\(^+\) T cells, we next used blocking anti-CTLA-4 mAb in CD4\(^{+}\)/CD28\(^{-}\)/CD8\(^{+}\)/CD28\(^{-}\) (Fig. 3b) and CD8\(^{+}\)/CD28\(^{-}\) mice (Fig. 3c). Although CTLA-4 blockade had no effect on allograft survival in any CD8\(^{+}\)/CD28\(^{-}\) mice (MST > 100; n = 7), it was able to promote allograft rejection in 30% of CD4\(^{+}\)/CD28\(^{-}\) mice (n = 6; MST > 90; p = not significant). Taken together, these results indicate that the CTLA-4:B7 pathway provides a negative signal to regulate alloreactive CD8\(^{+}\) T cells.

**Blockade of PD-L1, but not PD-1 or PD-L2, accelerates cardiac allograft rejection in WT mice**

Next we aimed to explore the role of PD-1:PD-L pathway in allograft rejection in WT recipients. Administration of PD-1 mAb had no significant effect on graft rejection as compared with the control group (MST = 9 days; p = 6; Fig. 2a). Similarly, administration of PD-L2 mAb did not affect the allograft survival (MST = 10; n = 6; data not shown). In contrast, anti-PD-L1 mAb led to a small but consistent and significant acceleration of allograft rejection (MST = 6.5; n = 6; p = 0.0017; Fig. 2a). We then explored whether B lymphocytes were necessary for the effects of anti-PD-L1 in WT mice, because PD-1 is expressed on B cells (11). Interestingly, B cell-less mice treated with anti-PD-L1 demonstrated significant acceleration of allograft rejection similar to WT animals (MST = 10.5 days in untreated B cell-less mice, n = 4 vs MST = 8 days in treated animals; n = 5; p = 0.04; Fig. 2b). In CD4- and CD8-deficient recipients, neither PD-1 nor PD-L1 blockade results in any significant change in allograft survival (Fig. 2, c and d).

**Role of PD-1:PD-L pathway in allograft rejection in CD28\(^{-}\)/CD4\(^{-}\) recipients**

To determine the role of PD-1:PD-L pathway in CD28\(^{-}\)/CD4\(^{-}\) recipients, we treated these mice with anti-PD-1, anti-PD-L1, and anti-PD-L2 Abs. In contrast to our findings in WT recipients, anti-PD-1 mAb resulted in accelerated allograft rejection in CD28\(^{-}\)/CD4\(^{-}\) recipients (MST = 11 days, n = 9, p = 0.017 compared with controls) (Fig. 3a). Moreover, administration of anti-PD-1 was extremely powerful in inducing acute allograft rejection in CD8\(^{+}\)/CD28\(^{-}\) recipients (MST > 100; n = 4; Fig. 3b). These results and the findings in WT mice together suggest a second negative signal through the PD-1 pathway that mainly downregulates CD4\(^{+}\) T cells. However, CD28 costimulation appears to reverse or overcome PD-1-mediated inhibition of alloreactive CD4 T cells.

Administration of anti-PD-L1 mAb resulted in accelerated graft rejection in CD28\(^{-}\)/CD4\(^{-}\) mice similar to blockade of PD-1, albeit with significantly faster tempo (MST = 7 days, n = 6, p = 0.0008 compared with control group and p = 0.0023 compared with PD-L1-treated group). However, in contrast to PD-1 blockade, PD-L1 blockade resulted in acute allograft rejection in both CD8\(^{+}\)/CD28\(^{-}\} (MST = 9.5; n = 6; p = 0.0002) and CD4\(^{+}\)/CD28\(^{-}\} recipients (MST = 21; n = 6; p = 0.01). Blockade of PD-L2 did not accelerate allograft rejection in any group of treated animals (data not shown). Overall, these data and data in WT mice indicate that PD-L1 signaling can mediate negative regulation of...
both CD4+ and CD8+ alloreceptive T cells, especially in the absence of CD28 costimulation.

**PD-1-PD-L pathways can mediate negative costimulatory signals independent of B7-CTLA-4 pathways**

Our data show that PD-1 blockade with an anti-PD1/anti-PD-L1 mAb accelerates cardiac allograft rejection in CD28−/− recipients to a similar degree to what we have reported for CTLA-4 blockade (37). However, it is not clear whether the two pathways are merely redundant or provide distinct regulatory signals that independently regulate alloimmune responses. Unlike CD28-deficient recipients, B7−/−/B7-2-deficient recipients do not reject allogeneic cardiac allografts (34, 44). Because B7-1 and B7-2 are the only published ligands for CD28 and CTLA4, this model is “truly” independent of CD28/CTLA4-B7 signals. To test whether the PD-1-PD-L1 pathway might play a dominant role in regulating host alloreceptive T cell responses in animals that lack B7 costimulation including a critical CTLA-4 negative signal, we treated B7 DKO recipients of fully allogeneic BALB/c WT heart transplants with blocking mAbs against PD-1, PD-L1, or PD-L2. As shown in Fig. 4, treatment with both anti-PD-1 mAb (MST = 22 days; n = 5; p = 0.004) and anti-PD-L1 mAb (MST = 21 days; n = 5; p = 0.004) but not anti-PD-L2 mAb (data not shown) resulted in acute allograft rejection (MST > 100, n = 4 in control group). Because there is the possibility that B7 molecules expressed on donor tissue may provide a costimulatory signal in trans to alloreceptive T cells (45), we also transplanted B7−/−/B7-2−/− BALB/c hearts into C57BL6 B7−/−/B7-2−/− mice, which accept the allografts indefinitely (34). We then treated these mice with the blocking mAbs against PD-L1. All mice rejected their allografts promptly (days 28, 23, and 22) similar to C57BL6 B7−/−/B7-2−/− mice transplanted with BALB/c WT grafts and treated with anti-PD-L1 mAb. These data provide evidence for nonredundant functions of these two negative pathways (CTLA-4, PD-1) in regulating alloimmunity in vivo.

**In vivo mechanisms of accelerated allograft rejection by the blockade of CTLA-4/B7 and PD-1/PD-L pathways**

We have previously demonstrated that blocking CTLA-4 accelerated the rejection in WT recipients of both the major and the minor mismatched cardiac allografts (38). This was accompanied by an increased frequency of alloreceptive T cells as measured by ELISPOT analysis (38). To address the mechanism by which blockade of PD-1/PD-L pathway accelerates rejection, we first measured the frequency of IFN-γ-producing donor-specific T cells in untreated recipients and recipients treated with blocking Abs, using a previously published ELISPOT assay (37–39). Recipient splenocytes were collected 14 days after transplantation, and the frequency of IFN-γ-producing allospecific cells was measured. We used CD28−/− and B7−/B7-2-deficient recipients because PD-1-PD-L1 blockade in these mice had the most pronounced effects on allograft survival. As shown in Fig. 5a, in the CD8−/− CD28−/− mice treated with the anti-PD-1 mAb (402 ± 183 spots; p = 0.02) or anti-PD-L1 mAb (500 ± 51 spots; p = 0.002), the frequency of alloreceptive IFN-γ-producing CD4+ T cells was significantly increased as compared with the untreated control recipients (41.5 ± 19.2 spots). By contrast, CTLA-4 blockade did not result in significant change in the frequency of alloreceptive CD4+ T cells (98 ± 76 spots; p = 0.13). As expected, the frequency of IFN-γ-producing CD8+CD28− T cells in CD4−/−CD28−/− mice was very low, regardless of whether the mice were left untreated (14.3 ± 3.7 spots per 0.5 × 106 splenocytes), or treated with mAb against PD-1 (49 ± 14.75), PD-L1 (56 ± 8.24), or CTLA-4 (38.6 ± 19.85) (data not shown). Treatment of B7 DKO mice with the anti-PD-1 mAb (272.4 ± 120.8; p = 0.0058) or anti-PD-L1 mAb (229 ± 41.9; p = 0.0073) led to significant increase in the frequency of IFN-γ-producing T cells as compared with untreated mice (76.7 ± 20.6) (Fig. 5b). Interestingly, blockade of PD-1:

![FIGURE 4](https://www.jimmunol.org/content/171/11/7652/F4.large.jpg)  
**FIGURE 4.** Negative costimulatory signals mediated by PD:PD-L pathway are independent of CTLA-4:B7 pathway. B7 DKO recipients of fully allogeneic BALB/c hearts accepted the allografts indefinitely (MST > 100 days; n = 4). Treatment with both anti-PD-1 mAb (MST = 22; n = 5; p = 0.004 as compared with controls) and anti-PD-L1 (MST = 21; n = 5; p = 0.004 vs controls) led to acute allograft rejection in all treated mice.

![FIGURE 5](https://www.jimmunol.org/content/171/11/7652/F5.large.jpg)  
**FIGURE 5.** Frequency of IFN-γ-producing, donor-specific T cells in CD8CD28-deficient C57/BL6 mice and B7 DKO mice treated with isotype control, anti-PD-1, and anti-PD-L1 or anti-CTLA-4 mAb following allogeneic (BALB/c) cardiac transplantation.Recipient mice were sacrificed, and splenocytes were harvested on day 14. Splenocytes (0.5 × 106 cells per well) were incubated with donor irradiated splenocytes. The frequencies of IFN-γ-producing T cells were then determined by ELISPOT assay. Data are expressed as the mean ± SEM of triplicate wells. The results represent three independent experiments. a, Anti-PD-1 (402 ± 183 spots; p = 0.02 vs untreated controls) and anti-PD-L1 mAb-treated CD8CD28-deficient recipients (500 ± 51 spots; p = 0.002) had significantly increased frequencies of IFN-γ-producing alloreceptive T cells, whereas anti-CTLA-4 mAb-treated recipients exhibited a similar frequency of alloreceptive T cells (98 ± 76; p = 0.13) as untreated recipients (41.5 ± 19.2). b, Anti-PD-1 (272.4 ± 120.8 spots; p = 0.0058 vs controls) and anti-PD-L1 mAb-treated B7-deficient recipients (229.8 ± 41.9; p = 0.0073) had significantly increased frequencies of IFN-γ-producing alloreceptive T cells, as compared with control recipients (76.7 ± 20.6).
PD-L pathway did not result in a significant change of the frequency of IL-4-producing T cells in any of above models (data not shown).

Next, we measured the percentage of effector CD4+ T cells (expressing a CD62LlowCD44highb phenotype) generated 14 days after transplantation in CD8−/CD28− recipients (Fig. 6a). Similar to the ELISPOT data, the frequency of effector CD4+ T cells was significantly increased with the blockade of PD-1 (12.6±1.7%) and PD-L1 (17.23±1.4%), as compared with blockade of CTLA-4 (10.28±0.6%) or untreated control animals (6.13±0.22%). These data demonstrate that in the absence of CD28 costimulation, blockade of PD-1-PD-L1 significantly increases the frequency of IFN-γ-producing CD4+ effector T cells, whereas CTLA-4 has no significant effects. Finally, to assess the effect of negative costimulatory blockade on effector CD8+ T cell generation, we measured the percentage of effector CD8+ T cells (expressing a CD62LlowCD44highb phenotype) in treated and untreated CD4−/CD28− animals before transplantation and on days 7 and 14 after transplantation. The frequency of CD8+ effector cells in naive mice (before transplantation) is consistently <3% as expected. On day 7 posttransplantation, there is a slight increase in frequency of CD8+ effector cells in anti-PD-L1-treated mice as compared with control mice (2.8±0.09% vs 1.7±0.69%). These findings are not surprising as PD-L1 blockade in CD4−/CD28− recipients promote acute rejection between days 17 and 23. However, as presented in Fig. 6b, the percentage of effector CD8+ T cells was significantly increased by PD-L1 blockade (10.84±1.6%) as compared with control mice (3±0%) 14 days after transplantation. These data clearly show that PD-L1 can transmit negative signals to alloreactive CD8+ T cells in the absence of CD28 costimulation.

Interestingly, in both CD4−/ and CD4−/CD28− allograft recipients, blocking CTLA-4 resulted in graft rejection in 30% of mice. To understand this intriguing finding we decided to analyze CD8+ T cell activation in these animals, particularly by contrasting rejectors vs acceptors. The main challenge in this experiment is the lack of true control mice with which the rejectors can be compared because after CTLA-4 blockade some of the mice can reject their grafts any time between days 20 and 40. Taking nonrejecting mice as controls for rejecting mice on the day of rejection may not be appropriate because some of these control mice may still go on to rejection a few days later. Thus, we have decided to compare the

![FIGURE 6](image)

**FIGURE 6.** The effect of negative costimulatory pathway blockade on effector CD4+ and CD8+ T cell generation in CD8−/CD28− (a) and CD4−/CD28− (b) heart allograft recipients. Splenocytes obtained from transplant recipients on day 14 were used for staining of effector CD4+ and CD8+ T cells, characterized as CD44highCD62Llowb. This is a representative example of three different experiments. a, The percentage of effector CD4+ T cells. b, The percentage of effector CD8+ T cells.

rejector mice (that completely rejected their grafts) and nonrejector mice with allograft survival >60 days (that we know for sure will not reject their grafts). We transplanted CD4−/− mice with BALB/c heart allografts and treated all recipients with the blocking CTLA-4 mAb. Interestingly, the frequency of CD8+ effector cells in rejecting mice (MST = 34 days, n = 4) sacrificed on the day of rejection was significantly higher compared with mice that did not reject by day 60 (23±1.9% vs 10.5±1.4%; p = 0.02; n = 4). Given the issue of proper control mice discussed, we have also designed a separate experiment in which we treated CD4−/− allograft recipients (n = 2) with higher doses (double the maintenance dose) of anti-CTLA4 mAb (500 μg of i.p. on days 0, 2, 4, 6, 8, and 10) reasoning that this therapy may cause prompt rejection and provide a clear difference vs nonrejector control mice. Interestingly, both mice treated with this protocol rejected their heart grafts promptly by day 14. The frequency of IFN-γ-producing donor alloreactive CD8+ T cells by ELISPOT on day 14 was significantly higher in rejecting mice as compared with nonrejector control animals harvested at the same time point (132±71.1 vs 18.5±9.3 spots per 0.5×106 splenocytes; p = 0.0002). In addition, the frequency of effector CD8+ T cells by flow cytometry was significantly increased with CTLA-4 blockade (41.1±0.3% vs 20.5±0.9% in control mice, p = 0.02). Thus, collectively these data support the notion that the frequency of effector CD8 T cells does correlate with the definite clinical outcome in these mice. These data are in keeping with our in vivo data demonstrating that CTLA-4 can transmit negative signals to alloreactive CD8+ T cells.

We then wanted to explore whether the observed effects of CTLA-4 vs PD-1 pathway blockade can be explained on the expression patterns of these molecules on activated WT vs CD28−/− T cells. Other investigators have demonstrated up-regulation of PD-1 and CTLA-4 on CD4+ and CD8+ T cells after stimulation with anti-CD3 mAb (13, 46). Thus to test and optimize our system, we first stimulated naive WT splenocytes with plate-bound anti-CD3 for 24 h and examined the expression of PD-1 and CTLA-4 (intracellular staining) by flow cytometry. In keeping with previous published results, there was significant PD-1 and CTLA-4 expression on both T cell subsets (12.4% of CD4+ and 53.4% of CD8+ cells for PD-1; 75% of CD4+ and 76% of CD8+ cells for CTLA-4). Next, we examined expression of PD-1 and CTLA-4 on freshly isolated CD4+ and CD8+ cells from WT and CD28−/− mice. None of the molecules were found on any of the T cell subsets, demonstrating that these molecules are not constitutively expressed. Finally, naive WT and CD28−/− splenocytes were stimulated by irradiated BALB/c splenocytes (in vitro allosresponse model) and the expression of PD-1 and CTLA-4 was examined after 12 and 24 h of allostimulation in vitro. Although the expression of PD-1 or CTLA-4 was minimal after 12 h, we found significant up-regulation of PD-1 expression on both T cell subsets in WT (10.8±1.1% of CD4+ and 10.1±1% of CD8+ T cells) and CD28−/− mice (6.3±2.4% of CD4+ and 11.1±1.3 of CD8+ T cells). After 24 h, CTLA-4 expression was slightly up-regulated in CD28−/− splenocytes (1.7±0.72% of CD4+ and 1±1% of CD8+ cells), to a significantly lesser degree than in WT splenocytes (27.6±0.84% of CD4+ and 41.7±1.1% of CD8+ cells) consistent with previously published data. Therefore, our data provide evidence that the differential effects of CTLA-4 vs PD-1 blockade on CD4+ vs CD8+ alloreactive CD4+ cells cannot be explained solely based on expression patterns of these negative costimulatory receptors. There are two caveats for this conclusion; first, there may be differences in the kinetics of expression of the receptors in vivo as compared with in vitro systems; and second, it is possible that expression of the ligands and
not the receptors on APCs or tissues may play a key role in determin- ing fate of immune responses in vivo (2, 47).

**Discussion**

Development of strategies to promote immunologic tolerance is a critical area of research in the field of organ transplantation, given the cost and the toxicities of current immunosuppressive drugs, and the lack of significant improvement in long-term allograft survival (48). After encountering Ag, naive T cells receive signal 1 through TCR-MHC plus antigenic peptide complex and signal 2 through positive costimulatory molecules leading to their full activation (2). The CD28:B7 signaling is the best characterized and perhaps the most important costimulatory pathway for the activation of naive T cells. Interaction of CD28, constitutively expressed on T cells, with B7-1 and B7-2, expressed on APCs, provides the second positive signal that results in full T cell activation including cytokine production, clonal expansion, and prevention of anergy and T cell survival (49–51). However, conventional T cell costimulatory blockade directed at the CD28:B7 pathway, although effective in inducing tolerance in some rodent models (52–54), is not effective in reproducibly inducing tolerance in stringent models such as murine skin and islet transplantation, and in organ transplantation in primates (25, 26). These variable outcomes may be explained by the fact that blockade of CD28:B7 pathway may not be as effective in inhibiting primed effector/memory responses (31, 55) or CD8 T cell responses (26, 30) in some models. In recent studies, it has become apparent that the outcome of the alloimmune response is determined by the interplay between positive stimulatory and negative regulatory signals. CTLA-4, which also binds both B7-1 and B7-2 molecules, is induced after T cell activation and has been shown to play a critical role in down-regulating T cell responses (6, 7). Data from our group have demonstrated that CTLA-4-negative signaling can regulate alloimmune responses in a solid organ transplant model (37, 56). Newer B7 family ligands (PD-L1 and PD-L2) are found more broadly expressed in nonlymphoid tissues and can be up-regulated by inflammatory mediators, whereas their receptor on T cells (PD-1) are induced on activated T cells (19–21). Thus, these novel molecules appear to be critical for regulating effector and memory T cell responses and their broad distribution uniquely positions them to regulate Ag-specific T cell functions at the sites of inflammation. Recent data from our group established for the first time the critical role of the PD-1:PD-L pathway in regulating alloimmune responses in vivo, using the experimental autoimmune encephalitis and the NOD mouse models (16, 17). In addition, PD-1 ligation has been shown to down-regulate graft-versus-host disease through modulation of IFN-γ production (57).

In this study, we systematically dissected the in vivo functions of negative costimulatory molecules in regulating CD4⁺ and CD8⁺ alloreactive T cells. In addition, we elucidated their interactions with each other and their relationship to the B7/CD28-positive costimulatory pathway during alloimmune responses. Our data clearly demonstrated that CTLA-4:B7 pathway provides a critical negative signal to alloreactive CD8⁺ T cells, particularly in the presence of CD28 costimulation. In contrast, PD-1 receptor seems to down-regulate alloreactive CD4⁺ T cells in the absence of CD28 costimulation. These novel data are very important for devising rational strategies to exploit these pathways therapeutically, perhaps in combination with costimulatory blockade. One interesting finding was that blocking PD-L1 accelerated the rejection process not only in CD28-deficient and CD8CD28-deficient recipients (similar to PD-1), but also in WT and CD4CD28-deficient recipients. Unlike PD-L1, PD-L2 blockade did not have any significant effects on allograft survival in any of transplant recipients studied. The divergent results observed following blockade of PD-L1 and PD-L2 may at least in part be due to different patterns of expression. Although PD-L1 is expressed not only on bone marrow-derived APCs but also on parenchymal cells (22), including microvascular endothelial cells (58), the PD-L2 has more limited expression, predominantly on cytokine-activated macrophages and dendritic cells (20). The second interesting observation is the differential effects of PD-1 vs PD-L1 blockade in vivo. This may suggest the possible existence of a new receptor other than PD-1 for PD-L1-mediated suppression. The finding of accelerated allograft rejection by PD-L1 blockade in B cell-less mice (similar to WT recipients) is in keeping with our hypothesis, as the expression of PD-1 can be induced not only on T cells but also B cells (11). Furthermore, it has recently been suggested that tumor cell-assoc- iated PD-L1 could induce apoptosis of tumor-specific T cells in a PD-1-independent manner (59). In fact, using an adoptive transfer system involving anti-bmi12 TCR transgenic T cells directly reactive to the mutated MHC class II molecule I-A/cm12 (60), we have recently demonstrated that apoptosis of alloantigen-specific T cells could be significantly abrogated by 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 (61).

Another important topic with therapeutic implications is the question whether CTLA-4:B7 and PD-1:PD-L1 regulatory pathways provide distinct and independent functions. To answer this question, we made use of B7 DKO mice, which is a true CD28/ CTLA4:B7 independent model. Both PD-1 and PD-L1, but not PD-L2 blockade resulted in cardiac allograft rejection, suggesting that these pathways are not redundant. In fact, simultaneous block- ade of CTLA4 and PD-1 in the graft-versus-host disease model was additive, pointing to the same conclusion (57).

Finally, targeting negative T cell costimulatory pathways may regulate alloreactive CD4⁺ and CD8⁺ T cells by several nonex- clusive and probably complementary mechanisms including induction of anergy, apoptosis, and immunoregulation. The accelerated rejection process caused by PD-1:PD-L pathway blockade was always associated with the expansion of IFN-γ-producing alloreactive T cells and enhanced generation of effector T cells in allograft recipients. Using a novel alloreactive TCR transgenic model system our group recently examined the functions of this pathway in the regulation of alloreactive CD4⁺ T cell responses in vivo (61). In this model we demonstrate that blockade of PD-L1 but not PD-L2 enhanced T cell proliferation and expansion, inhibited alloantigen-specific T cell apoptosis and polarized the alloimmune response toward a predominantly Th1-type response (61). Although further investigation will be necessary to examine the exact mechanisms of down-regulation of T cell responses, our findings are in keeping with recent studies revealing that PD-1:PD-L interactions lead to cell cycle arrest in G₁/G₂ (20, 62), and inhibition of T cell proliferation by reducing the production of IL-2 (46).

In conclusion, we have demonstrated a critical and differential role for the CTLA4:B7 and the PD-1:PD-L1 pathway in the regu- lation of CD4⁺ and CD8⁺ alloimmune responses in vivo. The functions of the latter pathway seem to be independent of intact CTLA4:B7 pathway, more pronounced in the absence of CD28 costimulation, and may be mediated by two receptors, PD-1 and another as yet unidentified receptor on T cells. Understanding the functions of the inhibitory pathways in alloimmunity may allow us to harness the under-appreciated physiologic mechanisms that reg- ulate alloimmune responses, and perhaps in combination with blockade of positive costimulatory pathways, may provide novel approaches to active and durable transplantation tolerance.
Disclosures
The authors have no financial conflict of interest.

References


