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CD28-independent Costimulation of T Cells in Alloimmune Responses

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T cell costimulation by B7 molecules plays an important role in the regulation of alloimmune responses. Although both B7-1 and B7-2 bind CD28 and CTLA-4 on T cells, the role of B7-1 and B7-2 signaling through CTLA-4 in regulating alloimmune responses is incompletely understood. To address this question, we transplanted CD28-deficient mice with fully allogeneic vascularized cardiac allografts and studied the effect of selective blockade of B7-1 or B7-2. These mice reject their grafts by a mechanism that involves both CD4+ and CD8+ T cells. Blockade of CTLA-4 or B7-1 significantly accelerated graft rejection. In contrast, B7-2 blockade significantly prolonged allograft survival and, unexpectedly, reversed the acceleration of graft rejection caused by CTLA-4 blockade. Furthermore, B7-2 blockade prolonged graft survival in recipients that were both CD28 and CTLA-4 deficient. Our data indicate that B7-1 is the dominant ligand for CTLA-4-mediated down-regulation of alloimmune responses in vivo and suggest that B7-2 has an additional receptor other than CD28 and CTLA-4 to provide a positive costimulatory signal for T cells.


Cytolytic T lymphocyte-associated Ag-4-mediated negative signaling of T cells (1, 2) plays a role in physiological termination of immune responses (3, 4) and may play a key role in regulating immune responses after induction of tolerance to nominal Ags (5, 6), autoantigens (7), and alloantigens (8). Although B7-1 and B7-2 bind to CTLA-4 with higher affinity than to CD28 (9, 10), the relative importance of B7-1-CTLA-4 vs B7-2-CTLA-4 interactions is unknown. Indeed, the effects of B7-1 vs B7-2 blockade in modifying autoimmunity (11–13) and alloimmune responses (8, 14–16) have not been consistent. This may be due to the complexity of the pathways involved, with CD28 and CTLA-4 transmitting opposing signals to T cells and with the two B7 molecules having different binding affinities and kinetics of expression (1, 17, 18).

In this study, we used mice lacking CD28 to examine the role of interactions between CTLA-4 and B7-1 vs B7-2 in regulating alloimmune responses in a model of vascularized cardiac transplantation. Our results indicate that B7-1 plays a dominant role in the down-regulatory interaction with CTLA-4 and provide evidence suggesting that B7-2 may mediate a positive costimulatory signal through a previously unrecognized third receptor that is distinct from CD28 and CTLA-4.

Materials and Methods

Animals

C57BL/6J (H-2b) (B6), BALB/cJ (H-2d) and BALB/c background CD28-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 background B7-1-deficient, B7-2-deficient, B7-1/B7-2 double-deficient mice (19), and BALB/c background CD28/CTLA-4 double-deficient mice (20) were generated in the laboratory of Dr. A. H. Sharpe. Animals were used at 6–14 wk of age.

Heterotopic heart transplantation

Vascularized heart grafts were transplanted using microsurgical techniques essentially as described by Corry et al. (21). Briefly, the harvested donor heart was placed in 4°C saline until transplantation. The recipient mouse was anesthetized by i.p. injection of 4% chloral hydrate. The donor aorta was sutured to the recipient aorta and the donor pulmonary artery to the recipient inferior vena cava end-to-side using 10-0 suture. Transplant function was evaluated by daily abdominal palpation. Rejection was defined as complete cessation of cardiac contractility as determined by direct visualization. Loss of graft function within 48 h of transplantation was considered a technical failure (<5% on the average), and these animals were omitted from further analysis.

Reagents, Abs and in vivo T cell depletion

The anti-B7-1 (CD80) mAb 1G10 and anti-B7-2 (CD86) mAb 2D10 hybridomas were a gift from Dr. G. D. Powers (Roche Research Laboratories, Nutley, NJ). Anti-B7-2 Fab was also prepared from hybridomas 2D10 (Bio Ex, West Lebanon, NH). The fusion protein murine CTLA4ig (a gift from Dr. R. Peach, Bristol-Myers Squibb, Princeton, NJ) has been described previously (18). CTLA4igY100F, a mutated form of CTLA4ig that binds B7-1 but not B7-2 (22), was also a gift from Dr. R. Peach. The blocking anti-CTLA-4 mAb hybridoma 4F10 (23) was a gift from Dr. J. Bluestone (University of California, San Francisco, CA). Anti-CD4- and anti-CD8-depleting mAbs were prepared from hybridomas GK1.5 (rat anti-mouse CD4) and 2.43 (rat anti-mouse CD8), respectively, obtained from American Type Culture Collection (Manassas, VA). All treated mice received 0.1 ml i.p. unpurified ascites of the appropriate Ab (roughly equivalent to 100 μg purified Ab) on −3, −5, and −7 days before transplantation (24). This regimen insures >95% depletion of the respective cell type in the peripheral blood on the day of transplantation. Cell counts start recovering by −2 wk after the last injection with complete recovery occurring within 10 wk.

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**Ab-mediated complement-dependent cytotoxicity assay**

Assays were performed in two stages with minor modifications from techniques previously described (25). Sera were collected from recipients around day 14 after transplantation and were heat inactivated at 56°C for 30 min and stored at −20°C until tested. A 125I-labeled mouse splenocytes (10⁶) of donor type were incubated with serially diluted sera in a final volume of 50 µl in U-bottom wells for 15 min at 37°C. Cells were washed with medium, pelleted, resuspended, and incubated with rabbit complement (C-6 Diagnostics, Mequon, WI) at a final dilution of 1/12 for 30 min at 37°C. Cells were pelleted at 75 × 10⁶ g for 10 min and counted on a gamma counter (Gamma 4000; Beckman Instruments, Fullerton, CA). Percentage of lysis was calculated by comparison to the 125I release obtained using a rabbit anti-mouse lymphocyte serum made in our laboratory.

**In vitro MLR**

Untreated responder spleen cells (4 × 10⁶) and stimulator spleen cells (4 × 10⁶) (2000 Gy) were added in a final volume of 200 µl tissue culture medium to U-bottom wells in triplicate. The cultures were incubated at 37°C in a humidified air containing 5% CO₂ for 3 days. [3H]Thymidine uptake was measured by β scintillation counting on a RackBeta model 1209 counter (Wallac, Gaithersburg, MD).

**ELISpot assay**

ELISPOT plates (Polyfiltronics, Rockland, ME) were coated with the capture Abs in sterile PBS overnight: R46A2 at 2 µg/ml for IFN-γ; 11B11 at 2 µg/ml for IL-4; and TRFK5 at 4 µg/ml for IL-5 (PharMingen, San Diego, CA). The plates were then blocked for 1.5 h with sterile PBS-1% BSA and washed three times with sterile PBS. Spleen cells (1.2 × 10⁶ cells/well) in 100 µl AIM-V medium were placed in each well with or without stimulator cells (1.2 × 10⁶ cells/well; 1:1 ratio) and cultured for 24–48 h at 37°C in 5% CO₂. After washing, the detector Abs (IFN-γ: XM1G1.2; IL-4: BV4D1; IL-5: TRFK4; PharMingen, San Diego, CA) diluted in PBS-0.025% Tween containing 1% BSA were added overnight. After washing, HRP avidin D diluted 1/200 was added for 1.5 h at room temperature. The plates were developed using 800 µl 3-amin-9-ethylcarbazole (Sigma, St. Louis, MO; 10 mg dissolved in 1 ml dimethylformamide) mixed in 24 ml 0.1 M sodium acetate, pH 5.0, plus 12 µl H₂O₂. The resulting spots were counted on a computer-assisted ELISpot image analyzer (T Spot Image Analyzer; Cellular Technology, Cleveland, OH) (26, 27).

**Statistics**

Kaplan-Meier survival graphs were constructed, and the log rank comparisons of the groups were used to calculate p values. Significant differences between experimental groups in ELISPOT assay were analyzed using Student’s t test. Differences were considered to be significant at p < 0.05.

**Results**

**Allograft rejection in CD28-deficient mice**

Cardiac grafts from C57BL/6 (B6) donors (H-2b) were transplanted into fully allogeneic wild-type or CD28-deficient BALB/c (H-2b) recipients. While wild-type recipients acutely rejected their grafts between 7 and 11 days (median survival time (MST), 9.5 ± 0.5 days, n = 7; Fig. 1), CD28-deficient recipients had significant prolongation of graft survival as compared with wild-type controls (MST 38.5 ± 12.3 days, n = 6, p < 0.002; Fig. 1).

To study the mechanisms of graft rejection in CD28-deficient mice, we treated the animals with depletion anti-CD4 or anti-CD8 mAbs based on a previously established protocol in wild-type recipients (24). In wild-type animals, and as previously published (28–30), transient depletion of CD8⁺ T cells resulted in marginal prolongation of graft survival (MST 14.3 ± 0.8 days, n = 6, p < 0.0005 compared with untreated controls), whereas transient CD4 depletion resulted in significant and more pronounced prolongation of graft survival (MST 46.8 ± 11.3 days, n = 6, p < 0.0005 compared with untreated controls) with 33% of the animals having long term (> 60 days) graft survival. However, transient CD4 (MST > 100 days, n = 5) or CD8 (MST > 100 days, n = 6) depletion resulted in indefinite allograft survival in all CD28-deficient recipients. These data, unlike that of wild-type recipients, indicate that transient CD4⁺ or CD8⁺ T cell depletion is sufficient to induce a state of prolonged hyporesponsiveness in CD28-deficient recipients. In addition, our data support an essential role of both cell types in mediating allograft rejection in CD28-deficient recipients.

**CTLA-4 or B7 blockade accelerates cardiac allograft rejection in CD28-deficient recipients**

To examine the roles of CTLA-4 and the B7 molecules in the absence of CD28 costimulation, we next studied the effect of CTLA-4 or B7 blockade in CD28-deficient recipients of vascularized cardiac allografts. Administration of CTLA4 Ig (250-µg single dose on day 2 posttransplant), which binds both B7-1 and B7-2, or a blocking anti-CTLA-4 mAb (250 µg on days −1, 0, and 1), significantly accelerated allograft rejection in CD28-deficient recipients (Fig. 2, A and B). In both instances, the effect was more pronounced when the frequency of administration of either CTLA4 Ig (days 0, 2, 4, and 6) or anti-CTLA-4 mAb (days 0, 2, 4, 6, 8, and 10) was increased (Fig. 2, A and B). This is in contradiction to their effects in wild-type recipients, in which anti-CTLA-4 treatment resulted in only a small degree of acceleration of allograft rejection whereas CTLA4 Ig induced indefinite allograft survival (Fig. 2C). The contrasting effects of CTLA4 Ig in wild-type vs CD28-deficient recipients highlight the importance of...
CTLA-4-mediated down-regulation of alloimmune responses in vivo. In the absence of CD28-mediated costimulation, CTLA-4 on T cells interacts with B7 on APC, resulting in down-regulation of alloimmune responses and prolongation of allograft survival observed in CD28-deficient animals (see above and Fig. 1). Administration of CTLA4Ig to CD28-deficient animals blocks B7 interaction with CTLA-4, resulting in acceleration of graft rejection. Similarly, administration of the anti-CTLA-4 mAb blocks an inhibitory signal, also resulting in acceleration of graft rejection that is more pronounced in CD28-deficient mice. These observations confirm the CD28-independent down-regulatory function of CTLA-4 in alloimmune responses in vivo (31).

**Differential effect of B7-1 vs B7-2 blockade in CD28-deficient recipients in vivo**

To study whether B7-1 and B7-2 have distinct or overlapping roles in CTLA-4-mediated down-regulation of alloimmune responses, we next examined the effect of selective blockade of either B7-1 or B7-2 in CD28-deficient recipients. For B7-1 blockade, we treated the recipients with a blocking anti-B7-1 mAb or a mutant form of CTLA4Ig, CTLA4IgY100F, which selectively binds and blocks B7-1 (13, 22, 32). Administration of either the anti-B7-1 mAb or CTLA4IgY100F resulted in significant acceleration of graft rejection (Fig. 3A). In contrast, and to our surprise, administration of a blocking anti-B7-2 mAb significantly prolonged allograft survival (Fig. 3B). Indeed, administration of a single dose of anti-B7-2 mAb resulted in 67% indefinite allograft survival, and administration of multiple injections (days 0, 2, 4, and 6) resulted in 83% indefinite allograft survival (Fig. 3B). However, blockade of B7-1 had no effect, and B7-2 was only marginally effective in prolonging allograft survival in wild-type recipients (Fig. 3C). These results indicate that B7-1 is the dominant ligand for CTLA-4 in mediating an inhibitory signal to T cells during alloimmune responses in vivo. Moreover, they show that even in the absence of CD28 costimulation, B7-2 can deliver a positive signal that promotes graft rejection.

The results of Ab-mediated complement-dependent cytotoxicity assay with sera collected from recipients around day 14 were consistent with graft survival (Fig. 4); control CD28-deficient recipients produced very low level of anti-donor alloantibodies. Anti-B7-1 mAb, CTLA4Ig, and anti-CTLA-4 mAb treatment of CD28-deficient recipients resulted in restoration of alloantibody production up to a level comparable to that of wild-type recipients with no treatment. Anti-B7-2 had no effect on the already low alloantibody levels detected in sera of CD28-deficient recipients.

Finally, we used donor grafts from B7-1, B7-2, or B7-1/B7-2 double-knockout animals to investigate the role of donor B7 interactions with CTLA-4 in CD28-deficient recipients. Graft survival of B7-1 (MST 16.3 ± 3.8 days, n = 4), B7-2 (MST 18.6 ± 3.8 days, n = 6), B7-1/B7-2 double-knockout (MST 19.3 ± 3.8 days, n = 6) grafts did not result in accelerated graft rejection, and indeed, no significant acceleration in allograft survival was observed with any of the donor graft types (Fig. 5A).

**FIGURE 3.** Cardiac allograft survival in CD28-deficient (CD28KO) recipients with either B7-1 mAb or anti-CTLA-4 mAb treatment. A, B7-1 blockade with either anti-B7-1 mAb 4 dose on days 0, 2, 4, and 6 (MST 12.8 days, n = 6, p < 0.001 vs CD28 untreated controls) or CTLA4IgY100F 4 dose on days 0, 2, 4, 6, and 10 (MST 16.2 days, n = 6, p < 0.05 vs CD28 untreated controls) resulted in 83% indefinite allograft survival. Similarly, administration of either the anti-B7-1 mAb or CTLA4IgY100F resulted in significant acceleration of graft rejection (Fig. 3A). In contrast, and to our surprise, administration of a blocking anti-B7-2 mAb significantly prolonged allograft survival (Fig. 3B). Indeed, administration of a single dose of anti-B7-2 mAb resulted in 67% indefinite allograft survival, and administration of multiple injections (days 0, 2, 4, and 6) resulted in 83% indefinite allograft survival (Fig. 3B). However, blockade of B7-1 had no effect, and B7-2 was only marginally effective in prolonging allograft survival in wild-type recipients (Fig. 3C). These results indicate that B7-1 is the dominant ligand for CTLA-4 in mediating an inhibitory signal to T cells during alloimmune responses in vivo. Moreover, they show that even in the absence of CD28 costimulation, B7-2 can deliver a positive signal that promotes graft rejection.

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Differential effect of B7-1 vs B7-2 blockade in CD28-deficient recipients in vitro

To determine the role of B7-1 and B7-2 molecules in the costimulation of T cells from CD28-deficient mice in vitro, we performed primary MLR with wild-type BALB/c or CD28-deficient responders. It has previously been reported that the allograft- and mitogen-induced proliferation of CD28-deficient T cells is reduced compared with that of wild-type responders (4). As shown in Fig. 5B, selective blockade of B7-1 with either anti-B7-1 mAb or CTLA4IgY100F augmented the proliferative response of CD28-deficient responders, whereas anti-B7-2 mAb had no effect. These results are consistent with our in vivo findings that B7-1 is the dominant ligand for CTLA-4-mediated inhibition of T cells.

We also measured the frequency of cytokine producing cells after secondary allostimulation by ELISPOT. For this purpose, we used responder spleen cells from either wild-type or CD28-deficient recipients of cardiac allografts. As seen in Fig. 5C, selective blockade of B7-1 or CTLA-4 augmented the frequency of IFN-γ-producing cells, whereas selective B7-2 blockade resulted in a slight decrease. We also measured the frequency of IL-4- and IL-5-producing cells, but there was no significant difference between selective B7-1 blockade and B7-2 blockade (data not shown) as compared with untreated controls. These results suggest that B7-1 blockade augments IFN-γ production in CD28-deficient mice after allostimulation, whereas B7-2 blockade inhibits its production. These data support our in vivo data indicating a differential role for B7-1 vs B7-2 in down-regulating alloimmune responses in the absence of CD28 costimulation.

Evidence for a B7-2-mediated positive costimulatory signal provided through a third receptor on T cells

The finding that B7-2 blockade prolongs allograft survival in CD28-deficient recipients could be interpreted in one of three ways. First, it is possible that B7-2 may be delivering a positive signal through a yet unidentified third receptor on T cells. We found that B7-2 blockade prolongs allograft survival in CD28-deficient recipients after B6 heart transplantation. Recipients were sacrificed on days 12–14 after transplantation, and spleen cells from these recipients were used as responders and irradiated spleen cells from naive B6 mice were used as stimulators in an ELISPOT. The finding that B7-2 blockade prolongs allograft survival in CD28-deficient recipients after B6 heart transplantation suggests that B7-2 blockade augments IFN-γ production in CD28-deficient mice after allostimulation, whereas B7-2 blockade inhibits its production. These data support our in vivo data indicating a differential role for B7-1 vs B7-2 in down-regulating alloimmune responses in the absence of CD28 costimulation. The
Fab resulted in prolongation of graft survival (MST 78.6 ± 13.2 days, n = 5) that was not significantly different from the whole Ab (MST 88.0 ± 9.5 days, n = 6, NS).

Therefore, to distinguish the first two possibilities in our model (Fig. 6), we treated CD28-deficient recipients with anti-B7-2 mAb plus anti-CTLA-4 mAb. As shown in Fig. 7, B7-2 blockade overcomes the effects of CTLA-4 blockade in accelerating graft rejection. In contrast, grafts treated with a combination of CTLA4IgY100F to block B7-1 plus anti-CTLA-4 mAb rejected their grafts within the same tempo as animals treated with each reagent alone (Fig. 7). These data suggested that B7-2 is not delivering a positive signal to T cells through CTLA-4. They raise the possibility that B7-2 has an alternative third receptor that is delivering a positive signal to T cells. To further confirm this hypothesis, we used BALB/c background CD28/CTLA-4 double-deficient mice that have recently generated in the laboratory of Dr. A. H. Sharpe (20). CD28/CTLA-4 double-deficient recipients rejected cardiac allografts with the same tempo as wild-type recipients (MST 10.3 ± 0.8 days, n = 6, vs MST 9.0 ± 0.5 days, n = 7, NS). Anti-B7-2 mAb treatment resulted in significant (Fig. 8, MST 45.3 ± 18.6 days, n = 4, p < 0.005 compared with untreated controls) prolongation of graft survival in CD28/CTLA-4 double-deficient recipients. These results provide further evidence that B7-2 has a costimulatory receptor other than CD28 and CTLA-4 on T cells.

**Discussion**

Our primary conclusion from the in vivo and in vitro data is that B7-1 is the dominant ligand for CTLA-4 negative signaling in alloimmune responses. This is consistent with recent data published by our group in the autoimmune encephalomyelitis model (34), where B7-1 or CTLA-4 blockade resulted in expression of clinical and pathological disease in otherwise protected CD28-deficient animals. Interactions between B7-1 and CTLA-4 are likely to play an important role in, and may even be required for, induction of tolerance in allogeneic transplantation. The fact that B7-1 has a higher tendency to dimerize than B7-2, and consequently that the avidity of B7-1 to CTLA-4 is higher than that of B7-2 (35), supports our finding. In addition, previous studies using B7-1 transgenic mice suggest that B7-1 may contribute to the down-regulation of T cell immune response (36). There are also in vitro studies with B7-1 transfectants showing that B7-1 inhibits proliferation and cytokine production in primed CD28-deficient T cells (37). Our results document the first direct demonstration that B7-1 has a functionally dominant role compared to B7-2.
with B7-2 in CTLA-4-mediated down-regulation of T cell alloimmune responses in vivo (8). Neither B7-1 nor CTLA-4 is constitutively expressed, but both are up-regulated several days after activation. In contrast, CD28 and B7-2 are constitutively expressed, and B7-2 is rapidly up-regulated on APCs after activation. These patterns of expression suggest not only that B7-1 is the dominant functional ligand for CTLA-4 but also that B7-2 may be the dominant ligand for CD28 (14, 38, 39). Also consistent with this, anti-B7-2 mAb, but not anti-B7-1 mAb, prolonged allograft survival in wild-type recipients in some models, and this treatment is most effective if administered to recipients at the time of transplantation (8).

The second conclusion from our data is that B7-2 may have an additional receptor other than CD28 that provides a positive costimulatory signal for T cells. Our observation that B7-2 blockade in CD28-deficient recipients resulted in significantly prolonged allograft survival raised two main possibilities. First, B7-2 may provide a stimulatory signal through CTLA-4 on T cells. Indeed, it has been reported that CTLA-4 may provide a positive costimulatory signal under some circumstances (40). Second, B7-2 may have an additional receptor other than CD28 which mediates T cell costimulation. To address this question, we blocked both CTLA-4 and B7-2 in CD28-deficient recipients at the same time after transplantation. If B7-2 provided a positive signal through CTLA-4, the acceleration of rejection produced by anti-CTLA-4 mAb would not be affected by addition of anti-B7-2 mAb. If B7-2 blockade reversed the accelerated graft rejection produced by CTLA-4 blockade in CD28-deficient recipients, this would support the notion of an additional receptor for B7-2. Indeed, the latter case proved true, providing evidence for the occurrence of a third receptor for B7-2 costimulation. This is also supported by the finding that concomitant blockade of both B7-1 and CTLA-4 resulted in rejection of the graft (Fig. 7), presumably by B7-2-third receptor-mediated costimulation. Interestingly, B7-2 blockade not only overcomes the effect of CTLA-4 blockade but also increases graft survival compared with untreated recipients (Fig. 7). Therefore, CTLA-4-negative signaling is not absolutely required for the induction of long term graft survival, in the absence of CD28. Furthermore, our results with B7-2 blockade in CD28/CTL-2 double knockout animals showing increased graft survival provide further support to the existence of a third receptor other than CD28 or CTLA-4. This third receptor is unlikely to be inducible costimulator (ICOS) (41), because ICOS ligand is B7RP-1 (41) and ICOS does not appear to bind B7 molecules (42).

Finally, our in vitro cytokine studies show that in the absence of CD28, B7-1-CTLA-4 but not B7-2-CTLA-4 interactions inhibited IFN-γ production. These data parallel our in vivo results with graft survival and alloantibody production in that B7-1-CTLA-4 interactions down-regulate alloimmune responses in CD28-deficient animals.

We conclude that in the absence of CD28 during alloimmune responses, B7-1 provides a negative signal through CTLA-4, whereas B7-2 provides a positive signal through a third receptor. Determining the exact identity of the putative third receptor and its role in normal animals should help to further our understanding of the mechanisms of CD28-independent costimulation of T cells in transplant rejection. Preclinical studies in primates indicate that CD28-B7 blockade alone is not sufficient to induce long term allograft survival and tolerance (43, 44). Whether this is due to inefficient blockade of B7-1-B7-2 interactions with CD28 and/or the third receptor requires further investigation, particularly if the putative third receptor is proved to exist and to play an important role in normal animals and humans. Therefore, understanding the mechanisms of CD28-independent costimulation of T cells in alloimmune responses is clinically relevant and should help in developing new therapies aimed at inducing long term allograft survival and tolerance.

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References


