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Didier A. Mandelbrot; ... et. al

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Expression of B7 Molecules in Recipient, Not Donor, Mice Determines the Survival of Cardiac Allografts¹

Didier A. Mandelbrot,^{2*†} Yutaka Furukawa,[‡] Alexander J. McAdam,^{*} Stephen I. Alexander,^{*} Peter Libby,[‡] Richard N. Mitchell,^{*} and Arlene H. Sharpe^{*}

Blockade of the CD28/CTLA4/B7 costimulatory pathway using CTLA4-Ig has great therapeutic potential, and has been shown to prolong allograft survival in a variety of animal models. To gain further insight into the mechanism by which costimulatory blockade prevents allograft rejection, we studied cardiac allograft survival in the complete absence of B7 costimulation using mice lacking B7-1 and B7-2 (B7-1/B7-2^{-/-} mice). To determine the role of B7 on donor vs recipient cells, we used B7-1/B7-2^{-/-} mice as either donors or recipients of allografts. Wild-type (WT) recipients acutely reject fully allogeneic hearts from both WT and B7-1/B7-2^{-/-} mice. In contrast, B7-1/B7-2^{-/-} recipients allow long-term survival of grafts from both WT and B7-1/B7-2^{-/-} mice, with minimal histologic evidence of either acute or chronic rejection in grafts harvested after 90 days. The B7-1/B7-2^{-/-} mice acutely reject B7-1/B7-2^{-/-} allografts if CD28 stimulation is restored by the administration of Ab to CD28 and can mount an alloresponse in mixed lymphocyte reactions. Therefore, B7-1/B7-2^{-/-} mice are capable of generating alloresponses both in vivo and in vitro. Our results demonstrate that in the alloresponse to mouse heterotopic cardiac transplantation, B7 molecules on recipient cells rather than donor cells provide the critical costimulatory signals. The indefinite survival of allografts into B7-1/B7-2^{-/-} recipients further shows that the absence of B7 costimulation alone is sufficient to prevent rejection. *The Journal of Immunology*, 1999, 163: 3753–3757.

The CD28/CTLA4/B7 costimulatory pathway plays a crucial role in the regulation of T cell activation. Two B7 molecules, B7-1 (CD80) and B7-2 (CD86), are expressed on APCs and provide a critical costimulatory signal to T cells by engaging CD28. B7 molecules also can provide a negative regulatory signal to T cells by binding to CTLA4 (CD152). Blockade of the B7-CD28 interaction in vitro can produce Ag-specific anergy (1), and use of the CTLA4-Ig fusion protein to block B7 molecules has shown great promise as a treatment for allograft rejection (2). However, current understanding of this pathway is limited by its complexity, because B7 signaling through CD28 and CTLA4 has opposing effects, and the expression of all four molecules is differentially regulated (3). Further improvement in therapeutic regimens that target this pathway requires better understanding of its function.

The initial observation that CTLA4-Ig can prolong cardiac allograft survival (4) has been confirmed in several animal models. In particular, CTLA4-Ig combined with anti-CD40 ligand (CD40L)³ is effective in inducing long-term survival of cardiac allografts in mice (5). However, CTLA4-Ig alone does not produce

indefinite survival, possibly because of incomplete B7 blockade with the treatment regimens that have been used. Also, it is not known whether CTLA4-Ig acts by blocking B7 on donor or recipient cells. This is an important therapeutic question, because CTLA4-Ig pretreatment of an organ before transplantation would only be effective if recipient cells themselves do not provide sufficient costimulatory signals to cause allograft rejection.

We have demonstrated previously that B7-1 and B7-2 are the only stimulatory ligands for CD28 (6). Here we use mice lacking B7-1 and B7-2 (B7-1/B7-2^{-/-} mice) (7) to study the effect of complete removal of B7 costimulation on allograft survival and to definitively examine the relative importance of B7 expression on the allograft vs recipient cells. We find that recipient B7 expression determines allograft survival, with B7-1/B7-2^{-/-} mice allowing long-term survival of grafts in the absence of any additional treatment. In contrast, the expression of B7 on donor cells has no detectable effect on graft outcome.

Materials and Methods

Mice

B7-1/B7-2^{-/-} mice were generated on the H-2^b 129/SvS4Jae (129) background (7) and backcrossed onto the BALB/c (H-2^d) and C57BL/6 (H-2^b) backgrounds. These studies used two different strain combinations with completely mismatched MHC. F6 backcross generation BALB/c donor hearts were transplanted to 129 recipients, and F3 BALB/c donors were used for F6 C57BL/6 recipients. The BALB/c B7-1/B7-2^{-/-} and wild-type (WT) control mice were all progeny of mice confirmed to be H-2^d haplotype. In all experiments, B7-1/B7-2^{-/-} mice were compared with WT controls of the same backcross generation. CTLA4^{-/-}/B7-1/B7-2^{-/-} mice on a 129 background (6) were generated by breeding CTLA4^{-/-} mice with B7-1/B7-2^{-/-} mice. Brigham and Women's Hospital and Harvard Medical School are American Association of Accreditation of Laboratory Animal Care-accredited institutions, and mice were cared for in accordance with institutional guidelines in a pathogen-free animal facility. All mice were used at 8–12 wk of age.

*Immunology Research Division, Department of Pathology, and [†]Renal and [‡]Cardiovascular Divisions, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

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² Address correspondence and reprint requests to Dr. Didier A. Mandelbrot, 221 Longwood Avenue, LMRC Room 512, Boston, MA 02115. E-mail address: damandelbr@bics.bwh.harvard.edu

³ Abbreviations used in this paper: CD40L, CD40 ligand; B7-1/B7-2^{-/-} mice, mice lacking B7-1 and B7-2; 129, 129/SvS4Jae; WT, wild type; MLR, mixed lymphocyte reaction.

Table I. Role of B7 in cardiac allograft survival

BALB/c Donor	129 Recipient	Survival (days)
WT	WT	14, 10, 10, 9, 9
B7-1/B7-2 ^{-/-}	WT	12, 10, 9, 10, 10
WT	B7-1/B7-1 ^{-/-}	>90, >90, >90, >90
B7-1/B7-2 ^{-/-}	B7-1/B7-2 ^{-/-}	>90, >90, >90, >90, >90

Heart transplantation

Allografts from male donors were placed in male recipients in an intra-abdominal location as described previously (8). Graft function was assessed daily by palpation, with rejection defined as the absence of detectable beating. Allografts failing or graft recipients dying within 48 h of surgery were considered technical failures and were excluded from the analysis.

Histology

Allografts were harvested at the time of graft failure, at 12 days, or at 90 days. Tissue for light microscopy was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard techniques.

Cytokine transcript measurement

Cardiac grafts were harvested at the time of graft failure, at 12 days, or at 90 days. Tissue was homogenized in Trizol reagent (Life Technologies, Grand Island, NY) and frozen at -80°C. Cytokine transcripts were measured by RNase protection assay, using the mCK-1 template from the Riboquant kit (PharMingen, San Diego, CA), according to the manufacturer's suggested protocol. Levels of transcript were quantified and normalized for loading controls using densitometry and are expressed in arbitrary density units.

Mixed lymphocyte reactions (MLRs)

Single-cell splenocyte suspensions were prepared by dissociating tissue with frosted glass slides. RBCs were lysed by incubation in Tris-ammo-

nium chloride for 5 min at 37°C. A total of 5 × 10⁵ 129 responder cells were cultured with an equal number of irradiated (2000 rad) BALB/c stimulator cells in round-bottom 96-well plates in media as described previously (9). Proliferation was assessed on days 3–6 by pulsing with 1 μCi/well of [³H]thymidine for the last 24 h of the indicated day.

Ab treatment of mice

Mice received either PV-1 anti-CD28 mAb (kindly provided by Dr. Vijay K. Kuchroo, Brigham and Women's Hospital, Boston, MA) or hamster IgG (ICN Pharmaceuticals, Costa Mesa, CA). A single dose of 100 μg of Ab was administered i.v. within 24 h posttransplantation.

Results

B7 expression on recipient mice, not donor mice, determines cardiac allograft survival

To analyze the relative role of B7 expression on cardiac allografts and on recipient cells, fully MHC-mismatched heterotopic cardiac transplants were performed using B7-1/B7-2^{-/-} mice as either donors and recipients. Table I shows graft survival using BALB/c donors and 129 recipients. WT recipients acutely rejected both WT and B7-1/B7-2^{-/-} donor hearts, with cessation of palpable beating between 9 and 14 days posttransplantation. Histologic analysis of these hearts showed typical signs of acute rejection, including mononuclear cell infiltration, myocyte necrosis, vasculitis, and interstitial edema (Fig. 1, A and B). In contrast, both WT and B7-1/B7-2^{-/-} grafts transplanted to B7-1/B7-2^{-/-} recipients continued to beat for >90 days, and were considered to have long-term survival. Upon sacrifice of the mice after 90 days, these grafts showed minimal cellular infiltration or graft arterial disease (Fig. 1, C and D).

To confirm our results, and because of potential strain differences in the alloresponse, BALB/c allografts were also transplanted into C57BL/6 recipients. For each of the four donor/recipient combinations of WT and B7-1/B7-2^{-/-} mice shown in Table

FIGURE 1. Histology of cardiac allografts. Allografts from WT or B7-1/B7-2^{-/-} donors placed into WT recipients were analyzed when beating stopped at days 9–14 (A and B, respectively). Grafts from WT or B7-1/B7-2^{-/-} donors placed into B7-1/B7-2^{-/-} recipients were analyzed after 90 days (C and D, respectively) or at day 12 (E and F, respectively). Original magnification was ×40.

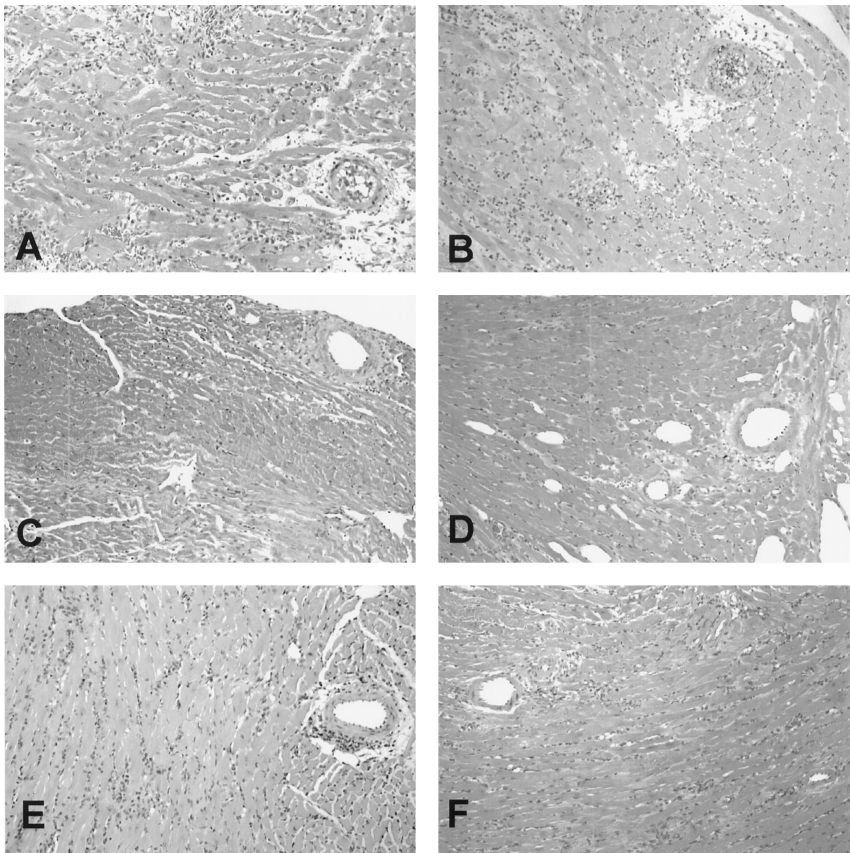


Table II. Role of CD28 and CTLA4 in cardiac allograft survival

BALB/c Donor	129 Recipient	Survival (days)
B7-1/B7-2 ^{-/-}	B7-1/B7-2 ^{-/-} + anti-CD28	10, 12, 11, 13
B7-1/B7-2 ^{-/-}	B7-1/B7-2 ^{-/-} + hamster IgG	>90, >90, >90
WT	CTLA4 ^{-/-} /B7-1/B7-2 ^{-/-}	45, >90, >90, >90
B7-1/B7-2 ^{-/-}	CTLA4 ^{-/-} /B7-1/B7-2 ^{-/-}	>90, >90, >90, >90

I, an additional three allografts were performed using C57BL/6 recipients. The survival and histology of the grafts in these recipients (data not shown) were indistinguishable from the results using 129 recipients. Thus, in two different sets of fully MHC-mismatched mouse strain combinations, the expression of B7 molecules by donor mice does not affect graft survival, but expression of B7 on recipients is critical in determining outcome.

For a temporal comparison with hearts rejected by WT recipients at 9–14 days, WT and B7-1/B7-2^{-/-} donor grafts placed in B7-1/B7-2^{-/-} recipients were analyzed at 12 days rather than after 90 days. Surprisingly, these hearts (Fig. 1, E and F) showed a significant mononuclear cell infiltrate that was comparable with that seen in WT recipients (compare with Fig. 1, A and B). However, the infiltrate found at 12 days in B7-1/B7-2^{-/-} recipients is not associated with the vasculitis and edema found in WT recipients. These histologic findings suggest that part of the initial cellular response to a cardiac allograft is independent of B7, but that this response is insufficient to cause graft failure and dissipates by 90 days.

Role of CD28 and CTLA4 signaling in graft rejection

To demonstrate the importance of B7/CD28 signaling in allograft rejection and to confirm that the B7-1/B7-2^{-/-} mice are capable of rejecting a cardiac graft, B7-1/B7-2^{-/-} recipients of B7-1/B7-2^{-/-} hearts were treated with an activating anti-CD28 Ab on the day of transplantation. This treatment induced acute rejection in the B7-1/B7-2^{-/-} recipients at a rate similar to WT recipients (Table II), and with similar histologic findings (data not shown). Treating B7-1/B7-2^{-/-} recipients with control hamster IgG had no effect on the survival of B7-1/B7-2^{-/-} donor grafts.

To determine whether the presence of CTLA4 in recipients might inhibit responses to low levels of B7 costimulation provided by donor tissue, we transplanted WT hearts into CTLA4^{-/-}/B7-1/B7-2^{-/-} recipients. In vivo, T cells from CTLA4^{-/-}/B7-1/B7-2^{-/-} and B7-1/B7-2^{-/-} mice are naive. With prolonged stimulation in vitro, CTLA4^{-/-}/B7-1/B7-2^{-/-} T cells proliferate more than B7-1/B7-2^{-/-} T cells in response to B7-expressing stimulators, because in CTLA4^{-/-}/B7-1/B7-2^{-/-} cells, CD28 signaling is unopposed by an inhibitory signal through CTLA4 (our manuscript in preparation). Therefore, this strain provides a sensitive tool for detecting B7-CD28 signals. However, graft survival in CTLA4^{-/-}/B7-1/B7-2^{-/-} recipients was not significantly different from that in B7-1/B7-2^{-/-} recipients (Table II), and the histology of donor hearts was similar in the two recipient strains (data not shown), suggesting the absence of B7-CD28 interactions. Thus, in the absence of B7 expression in recipient mice, CTLA4 expression has no detectable effect on graft rejection.

Long-term survival of allografts is not associated with a deviation toward Th2 cytokines

To further characterize the mechanism of T cell responses to allografts, levels of cytokine transcripts in harvested hearts were assessed using an RNase protection assay (Fig. 2A), with quantitation of transcripts performed by densitometry (Fig. 2B). RNA

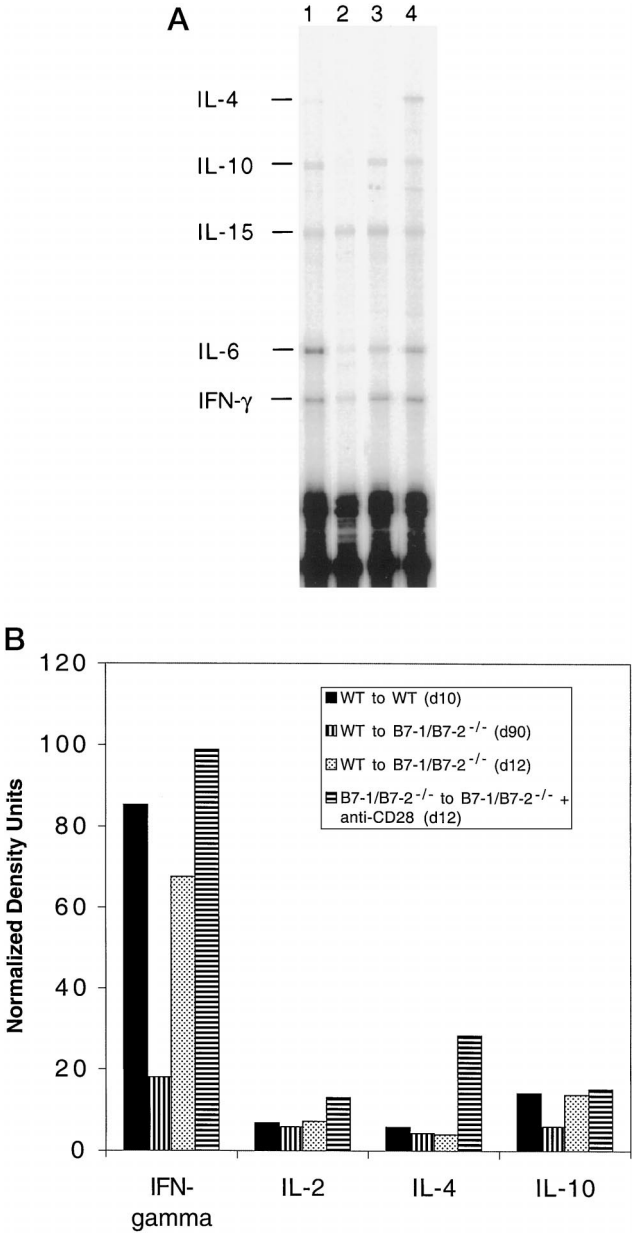


FIGURE 2. Measurement of cytokine transcript levels using RNase protection assays. A, Transcripts typical of WT recipients (lane 1, WT donor, d10), B7-1/B7-2^{-/-} recipients analyzed at day 90 (lane 2, WT donor), B7-1/B7-2^{-/-} recipients analyzed at day 12 (lane 3, WT donor), and B7-1/B7-2^{-/-} recipients treated with PV-1 Ab to CD28 (lane 4, B7-1/B7-2^{-/-} donor, d10). B, Gel bands corresponding to the indicated cytokines were quantitated by densitometry and normalized for loading controls. Each lane is representative of at least four mice.

was analyzed from hearts obtained from four groups of mice: 1) acutely failing hearts in WT recipients, 2) long-term surviving hearts in B7-1/B7-2^{-/-} recipients, 3) hearts in B7-1/B7-2^{-/-} recipients expected to survive long-term but analyzed at day 12, and 4) acutely failing hearts in B7-1/B7-2^{-/-} recipients treated with anti-CD28. In hearts with cellular infiltrates (Fig. 2A, groups 1, 3, and 4), high levels of mRNA for IFN-γ and IL-6 were detected (no statistically significant differences between groups). Much lower levels ($p < 0.01$ by Student's t test) of these cytokine transcripts were present in long-term surviving hearts with minimal cellular infiltrates in B7-1/B7-2^{-/-} recipients (Fig. 2A, group 2). IL-10 levels were also higher in hearts with cellular infiltrates ($p < .01$),

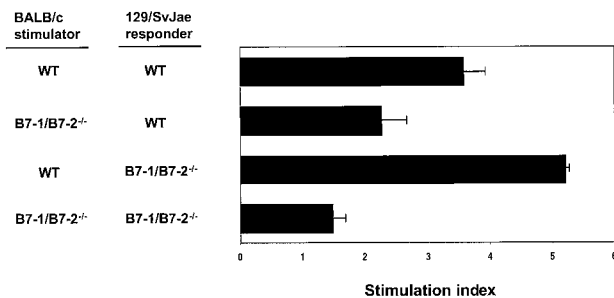


FIGURE 3. MLRs. Day 4 proliferation of 5×10^5 responder splenocytes in the presence of 5×10^5 irradiated stimulators is shown. The proliferation of each responder is divided by proliferation of the responder in the absence of stimulator to determine the stimulation index. Data are representative of four experiments.

whereas IL-4 levels were low in all grafts except from the mouse treated with anti-CD28. IL-2 was only detectable in the mouse treated with anti-CD28. Thus, in hearts surviving for >90 days and in those analyzed at day 12 but expected to survive long-term, there was no increased production of the Th2 cytokines IL-4, IL-5, or IL-10 relative to Th1 cytokines.

Expression of B7 molecules on both stimulator and responder populations affects the outcome of MLRs

To investigate the ability of B7-1/B7-2^{-/-} T cells to mount an alloresponse and to study an in vitro correlate of cardiac transplantation, primary MLRs were performed using splenocytes from naive WT or B7-1/B7-2^{-/-} mice as both stimulators and responders (Fig. 3). In vivo, WT and B7-1/B7-2^{-/-} allografts failed at the same rate. In the MLRs, however, WT stimulators generated stronger responses than B7-1/B7-2^{-/-} stimulators, both in WT and B7-1/B7-2^{-/-} responder cells. This pattern of proliferation was similar on days 3–6. Thus, the expression of B7 on MLR stimulators affects the proliferative response. The strong proliferation of B7-1/B7-2^{-/-} cells to WT stimulators also demonstrates that B7-1/B7-2^{-/-} cells are capable of mounting an alloresponse in vitro, if B7 costimulation is present.

Discussion

The use of B7-1/B7-2^{-/-} mice as both donors and recipients of cardiac allografts clearly demonstrates that B7 expression in recipient mice is critical in determining graft outcome. Without any need for additional treatment, completely allogeneic grafts survive indefinitely in B7-1/B7-2^{-/-} recipients. These results are consistent with previous studies using B7-1-deficient recipients given anti-B7-2 Ab (10). Our results have important therapeutic implications: because of the critical role of B7 expression on recipient cells in the rejection of cardiac allografts, strategies to block B7 on donor hearts before transplantation are unlikely to be successful.

Unlike studies with CTLA4-Ig, which show that donor-specific transfusions (11) or blockade of the CD40-CD40L pathway are also necessary to induce long-term allograft survival, our results suggest that targeting of the B7 costimulatory pathway alone is sufficient to allow indefinite graft survival. Complete blockade of B7 costimulation may be essential and is achieved with B7-1/B7-2^{-/-} mice but not with current protocols using CTLA4-Ig. However, part of the effectiveness of removing B7 may be due to indirect effects on the CD40 pathway. In particular, B7 interactions with CD28 help maintain CD40L expression on T cells, which is required for continued CD40 stimulation (12). T cells from B7-1/B7-2^{-/-} mice, in fact, express reduced levels of CD40L upon

stimulation in vitro (our manuscript in preparation). Therefore, the expression of CD40L may be lower in B7-1/B7-2^{-/-} recipients than in WT mice treated with CTLA4-Ig, and this contributes to the longer survival of allografts in B7-deficient recipients. It is also possible that other indirect effects of B7 deficiency, or subtle developmental effects, may contribute to prolonged allograft survival.

The ability of donor mice to express B7 molecules does not affect the outcome of cardiac allografts, suggesting that few cells in the heart can express B7 even in WT mice. This finding is consistent with previous reports suggesting that in cardiac grafts, unlike skin or islet grafts, passenger leukocytes do not play a significant role in rejection (13). Leukocytes such as dendritic cells and macrophages are scattered throughout the cardiac parenchyma and can express B7. However, our results demonstrate that too few of these cells are present to affect cardiac allograft rejection. These results also suggest that no other cells in a heart, including cardiac myocytes and endothelial cells, express levels of B7 molecules that are functionally significant in allograft rejection. The lack of B7 costimulation from WT hearts is also supported by the lack of rejection observed even in CTLA4^{-/-}/B7-1/B7-2^{-/-} recipient mice, which, because of their CTLA4 deficiency, are more sensitive to B7 costimulation than B7-1/B7-2^{-/-} mice. It is possible that, in contrast to the long-term survival of cardiac grafts into B7-1/B7-2^{-/-} recipients, WT skin allografts would be acutely rejected, because passenger leukocytes in skin may provide sufficient B7 costimulation to mediate rejection. Therefore, the role of B7 costimulation in an alloresponse may depend upon the organ used as the allograft, and optimal approaches for preventing graft rejection may differ between organs.

The acute rejection of B7-1/B7-2^{-/-} donor hearts by WT recipients also raises the question of the relative importance of two potential mechanisms in alloresponse, costimulation in *trans* and indirect allorecognition. In WT recipients of B7-1/B7-2^{-/-} grafts, direct allorecognition of donor MHC would only occur on cells that lack B7 molecules. If allorecognition is direct, costimulation would be provided in *trans* by recipient APCs. If allorecognition is indirect, recipient APCs would present alloepitopes and provide B7 costimulation in *cis*. Although direct allorecognition is generally believed to be more important than indirect allorecognition in acute rejection, and costimulation in *cis* is more potent than costimulation in *trans* (14), no data are available to distinguish the relative strength of indirect allorecognition vs costimulation in *trans*. Studies are in progress to address the relative importance of these two mechanisms.

We also examined the role of Th1 and Th2 cytokines in graft survival in this model. Blockade of B7 costimulation has been reported to be associated with Th2 deviation in allograft models (15), but studies using knockout mice have demonstrated that removal of Th1 cytokines does not always lead to tolerance (16), and absence of Th2 cytokines does not prevent the induction of long-term graft survival by costimulatory blockade (17). Our results show no Th2 skewing in B7-1/B7-2^{-/-} recipients of allografts analyzed at 12 or 90 days. Moreover, early graft failure in mice treated with anti-CD28 is associated with increased IL-4 production. The finding that lesser cellular infiltration is associated with decreased levels of both Th1 and Th2 cytokines is similar to previous results in long-term surviving allografts in mice treated with CTLA4-Ig plus Ab to CD40L (5). However, other explanations besides Th2 deviation may explain why the cellular infiltrate seen at 12 days in these grafts is no longer seen at 90 days. For example, previous reports have suggested that CD28 engagement is not necessary for initiation of Ag-specific immune responses, but is necessary for a sustained response, either to maintain proliferation

(18) or to prevent cell death (19). It is also possible that in the absence of B7 costimulation, the recruitment or effector function of cells such as cytolytic T cells, B cells, and macrophages is impaired.

MLRs performed with the same WT and B7-1/B7-2^{-/-} combinations as allografts show a different pattern of responses, highlighting observations previously made in human and experimental systems that the MLR is not always a good in vitro predictor of the in vivo alloresponse. However, the strong in vitro response of B7-1/B7-2^{-/-} cells in the MLR demonstrates that these cells are capable of mounting an alloresponse in the presence of B7 costimulation. Similarly, in vivo, B7-1/B7-2^{-/-} mice can acutely reject cardiac grafts if anti-CD28 Ab is administered. The MLR results are consistent with the importance of passenger leukocytes in providing costimulation: cardiac allografts contain few passenger leukocytes, so their genetic ability to express B7 does not effect graft outcome. In contrast, the stimulators in MLRs are all leukocytes, so B7-deficient cells elicit much weaker responses than WT cells.

The studies reported here support the critical importance of B7 costimulation in allograft rejection and provide further understanding of the relative role of B7 molecules on donor and recipient cells in cardiac transplantation. Studies are currently in progress to assess the role of passenger leukocytes in the alloresponse, as well as to further characterize responses that lead to rejection vs long-term survival. Manipulating the B7 costimulatory pathway holds great promise for the treatment of allograft rejection, and greater understanding of this pathway is likely to lead to further improvements in therapy.

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