Published in final edited form as:

Transplantation. 2007 June 15; 83(11): 1449–1458. doi:10.1097/01.tp.0000265482.88936.b1.

Spontaneous Allograft Tolerance in B7-Deficient Mice Independent of Preexisting Endogenous CD4+CD25+ Regulatory T-Cells

Todd J. Grazia^{1,4}, Robert J. Plenter^{2,3}, An N. Doan^{2,3}, Brian P. Kelly^{2,3}, Sarah M. Weber², Jonathan S. Kurche², Susan O. Cushing², Ronald G. Gill², and Biagio A. Pietra^{2,3}

¹Department of Medicine, Division of Pulmonary and Critical Care Medicine, University of Colorado Health Sciences Center, Denver, CO

²Department of Medicine and Immunology, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Aurora, CO

³Department of Pediatrics, Division of Cardiology, University of Colorado Health Sciences Center, The Children's Hospital, Denver, CO

Abstract

Background—Acute cardiac allograft rejection requires host, but not donor, expression of B7-1/B7-2 costimulatory molecules. However, acute cardiac rejection requires direct antigen presentation by donor-derived antigen presenting cells to CD4 T-cells and does not require indirect antigen presentation to CD4 T-cells. Given this discrepancy in the literature and that the consequence of allograft exposure in B7-deficient mice is unknown; the goal of the study was to examine the antidonor status of allografted B7-1/B7-2-deficient hosts.

Methods—C57Bl/6 B7-1/B7-2^{-/-} mice were grafted with heterotopic BALB/c hearts. Recipients bearing long-term surviving allografts were used to examine the status of antidonor reactivity in vitro and in vivo. Tolerance was examined in vivo through adoptive transfer of splenocytes from graft-bearing animals to secondary immune-deficient Rag-1^{-/-} hosts bearing donor-type or third-party cardiac allografts and by regulatory T-cell depletion with anti-CD25 antibody.

Results—When transferred to B7-replete Rag-1^{-/-} recipients, cells from naïve B7-1/B7-2^{-/-} mice readily initiated cardiac allograft rejection. However, splenocytes transferred from long-term allograft acceptor B7-1/B7-2^{-/-} hosts failed to reject donor-type hearts but acutely rejected third-party allografts. In addition, such cells did not reject (donor×third-party) F1 allografts. Finally, in vivo depletion of regulatory T-cells did not prevent long-term acceptance.

Conclusions—Results demonstrate that B7-deficient T-cells are capable of acute cardiac allograft rejection in a B7-replete environment. Importantly, results also show that B7-deficient hosts do not simply ignore cardiac allografts, but rather spontaneously develop transferable,

donor-specific tolerance and linked suppression in vivo. Interestingly, this tolerant state does not require endogenous CD4+CD25+ regulatory T-cells.

Keywords

CD80; CD86; B7-1; B7-2; Costimulation; Transplantation; Linked suppression; Tolerance

It is well known that B7 costimulatory molecules play a major role in the adaptive immune response. T-cell activation is a two-signal process involving specific antigen recognition (Signal 1) and a second nonantigen-specific inductive co-stimulatory signal (Signal 2) involving the immunoglobulin (Ig) superfamily molecules B7-1/B7-2 (1–3). The value of such molecules as therapeutic targets is illustrated by the efficacy of blocking reagents such as CTLA4 Ig and/or anti-B7 antibodies to prevent either allograft rejection (4, 5) or autoimmunity (6–9). A prevailing tenet is that T-cell exposure to Signal 1 in the absence of Signal 2 is protolerogenic (10–13). For example, previous studies demonstrate long-term survival of heart allografts in B7-1/2^{-/-} (B7KO) recipients (14, 15). Importantly, these studies show that host but *not* donor B7 expression is necessary for acute rejection. However, it remains unclear what consequence allograft exposure in the absence of B7 plays in alloimmunity. One interpretation of durable heart allograft survival in B7-deficient host is that “indirect” (host antigen-presenting cell [APC] dependent) antigen recognition requiring B7 costimulation is required for rejection (14). However, this concept is perplexing based on our own results from studying CD4 T-cell-mediated cardiac allograft rejection. CD4 T-cells are required (16–22) and sufficient (16) for acute cardiac allograft rejection and, importantly, require *direct* recognition of donor major histocompatibility complex (MHC) class II for this response (16, 23), without a requirement for host MHC class II-restricted antigen (16). Thus, direct T-cell reactivity in B7-deficient hosts would be expected to respond to donor APCs that do express B7 molecules in this model (14).

An alternate explanation for the host B7 requirement for acute rejection may be a role for costimulation in “*trans*” (24). That is, alloreactive T-cells activated by donor-derived APCs may require additional host-derived B7-dependent co-stimulation expressed on donor-type APCs for optimal T-cell activation. Such “bystander” costimulation has been demonstrated previously in vitro (25). In this situation acute rejection may still require the direct pathway of donor antigen presentation, but may also require additional bystander co-stimulation by host APCs to initiate an optimal response, consistent with the results of Mandelbrot et al. (24).

In addition, more recently it has been shown that B7 molecules can also be expressed on T-cells themselves (26), raising the question of T-cell immune-competence in a B7-deficient environment. Ferlazzo et al. demonstrated that T-cells can express B7 molecules after activation and can potentially amplify the acute response to alloantigen via bystander costimulation (26). Furthermore, other studies indicate that B7 expression by T-cells may be important for immunity (26) and/or tolerance induction (27–29). Regarding tolerance, several studies have demonstrated a potential role for B7 signaling in tolerance induction via regulatory cells (27–29). CD28 is required for the generation of Foxp3+CD25+ thymic regulatory cells (30) and B7 costimulatory signals have been shown to regulate autoreactive

T-cells (31). In other studies, T-cell-associated B7 expression has been demonstrated to downregulate alloresponses (31). Therefore, the relative role of generalized B7 deficiency versus B7 deficiency on T-cell alloreactivity is unclear. Taken together, it is unknown what consequence global B7-deficiency has on cardiac allograft recognition.

In the current study, we had two primary goals. Firstly, we determined whether B7 expression by T-cells is required for acute allograft rejection. Secondly, we determined whether allograft exposure in the absence of B7-1/B7-2 expression is “ignored” by the host or rather leads to spontaneous tolerance induction. To address these questions, we utilized B7KO mice as cardiac allograft recipients and as cell donors. Results show that transferred B7-deficient T-cells readily mediate cardiac allograft rejection within a B7-replete B6 *Rag1*^{-/-} host, indicating that T-cell expression of B7 is not required for the response. Furthermore, B7KO allograft recipients are not simply ignorant of the allograft but develop transferable, donor-specific tolerance. That is, spleen cells transferred from B7KO allograft recipients accept donor-type but reject third-party allografts in secondary *Rag1*^{-/-} recipients. Interestingly, (donor×third-party) F1 grafts are accepted by splenocytes from “tolerant” B7KO recipients, illustrating a dimension of linked suppression to the tolerant state, presumably due to active regulation. Further studies demonstrate that this tolerant state does not require preexisting CD4+CD25+ regulatory T-cells (Tregs) in vivo, does not appear to be dependent on transforming growth factor (TGF)- β or interleukin (IL)-10 in vitro, and is not due to the development of T-cell anergy. Taken together, such results support at least a functional or partial deletion of donor reactive T-cells combined with a form of dominant regulatory activity.

MATERIALS AND METHODS

Mice

Inbred female C57BL/6ByJ (B6, H-2^b), C57BL/6-*Rag1*^{tm1/Mom} (B6 *Rag1*^{-/-}, H-2^b), and C57BL/6 B7-1/B7-2 double-deficient B6.129S4-*Cd80tm1Shr Cd86tm1Shr/J* (B6 B7KO, H-2^b) (32) mice were utilized as heart allograft recipients. Inbred female BALB/cByJ (BALB/c, H-2^d), C3H/HeSnJ (C3H, H-2^k) and (BALB/c×C3H)F1 mice were utilized as heart allograft donors. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) except for (BALB/c×C3H)F1 mice that were generated and bred in the Barbara Davis Center Animal Facility under Institutional Animal Care and Use Committee approval. Animals were housed under pathogen-free conditions, according to National Institutes of Health guidelines.

Heterotopic Heart Transplantation

Vascularized grafts were transplanted according to standard microsurgical technique (33). Allograft survival was assessed by daily palpation with rejection defined as loss of palpable beating. Survival differences were determined using the Kaplan Meier log-rank test.

Adoptive Transfer of Naïve or Allograft Conditioned Splenocytes

Spleens from BALB/c heart-engrafted B6 B7KO recipients, with allografts functioning >100 days, were utilized to generate conditioned splenocytes and nonengrafted B6 B7KO mice

were utilized to generate naïve splenocytes. Spleens were homogenized, cells were washed, and red blood cells were lysed with RBC lysis buffer (Sigma, R7757, St. Louis, MO). Then 30×10^6 viable conditioned or naïve splenocytes were adoptively transferred intraperitoneally (I.P.) to B6 *Rag1*^{-/-} mice bearing established BALB/c, C3H, or (BALB/c×C3H)F1 cardiac allografts between days 2 and 7 postcardiac transplantation.

Mixed Lymphocyte Reactions

Mixed lymphocyte reactions (MLR) of BALB/c and C3H splenocyte-stimulator cells with B6 naïve control and conditioned splenocytes were performed. Quadruplicate wells containing 2.0×10^5 responder cells were mixed with 3.0×10^5 irradiated (2,500 Rads) splenic stimulator cells in 96-well flat bottom plates. Cells, cultured in Eagle's Minimal Essential Medium supplemented with 10% FCS, 10⁻⁵ M 2-Me, and antibiotics, were incubated at 37 °C in 10% CO₂. Cultures were then pulsed with 1.0 uCi thymidine for 6 hr on the indicated day of cell culture. Plates were harvested and counted on a Trilux 1450 micro beta scintillation counter (Wallac Inc., Gaithersburg, MD).

Flow Cytometric Analysis

Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson, NJ). A 1:100 dilution of fluorescein isothiocyanate-labeled antimouse CD25 (clone 7D4, Becton Dickinson/Pharmingen), Peridinin-Chlorophyll-Protein (PerCP)-labeled antimouse CD4 (clone L3T4, Becton Dickinson/Pharmingen), or phycoerythrin-labeled antimouse/ rat Foxp3 (clone FJK-16s, eBioscience) antibody was used. Intracellular Foxp3 staining was performed per eBioscience kit standard protocol.

Cytokines and Antibodies

Recombinant transforming growth factor- β 1 (R&D Systems, catalog no. 240-B) at a concentration of 2 ng/mL, antimouse/bovine/human transforming growth factor- β 1 (R&D Systems, clone 1D11) at 50 μ g/mL, recombinant mouse interleukin (IL)-10, (Becton Dickinson, catalog no. 550070) at 20 ng/mL, antimouse IL-10 (Becton Dickinson, clone JES5-2A5) at 10 μ g/mL, and recombinant mouse IL-2 (Pharmingen, catalog no. 19211T) at 50 units/mL were utilized for proliferation assays. Rat antimouse CD25 (PC 61) was prepared as ascites generated in SCID mice and quantitated using an isotype-specific enzyme-linked immunosorbent assay.

Tissue Histological Examination

Transplanted and native hearts were divided in half. One half was fixed in 10% formalin for paraffin embedding and staining with hematoxylin and eosin (H&E). These were examined in a blinded fashion for myocardial damage and cellular infiltration. The other half was fixed in 4% paraformaldehyde. Tissue sections were then stained for CD4 and CD8 as previously published (23).

RESULTS

BALB/c Cardiac Allografts Can Survive Indefinitely in B7KO Recipients

Previous data have demonstrated indefinite survival of fully allomismatched cardiac allografts in B6 B7KO hosts (14, 15). To replicate this data, we transplanted BALB/c cardiac allografts heterotopically into B6 B7KO recipients. Interestingly, using a large cohort of recipients, we found that a significant proportion B7KO recipients could reject cardiac BALB/c allografts, albeit with greatly delayed kinetics, with approximately 50% of allografts surviving >100 days post-transplantation (Fig. 1). Nonetheless, overall survival was strikingly different between B6 control recipients and B6 B7KO recipients ($P<0.0001$). It is noteworthy that results demonstrate that the loss of recipient B7-1/B7-2 costimulation does not always result in long-term acceptance inferring that loss of indirect costimulation does not necessarily result in “ignorance” of the allograft. However, results confirm dramatic cardiac allograft prolongation in B7KO hosts.

B7KO T-Cells Are Capable of Responding to Donor Antigen Both In Vitro and In Vivo

Because B7-1/B7-2 expression by T-cells may play a significant role in T-cell activity by providing “bystander co-stimulation” (26), we first set out to determine whether B7-1/B7-2 expression by T-cells was required for alloreactivity both in vitro and in vivo. We first assessed whether or not B7KO T-cells could respond to BALB/c stimulators in vitro. Mixed lymphocyte reactions clearly demonstrated that both wild type and B7KO T-cells could proliferate equivalently to BALB/c stimulators in vitro (Fig. 2A), consistent with prior findings (14). We then set out to determine whether B7KO T-cells could mediate graft rejection in a B7-replete environment. To accomplish this, B7KO spleen cells were transferred to immune deficient, but B7-expressing B6 *Rag1*^{-/-} recipients. Results show that both wild-type and B7KO spleen cells initiate a similar tempo of cardiac allograft rejection within B6 *Rag1*^{-/-} hosts (Fig. 2B). Taken together, results clearly demonstrate that T-cell derived B7-1/B7-2 expression is *not required* for alloreactivity in vitro or for acute rejection in vivo.

Allograft Conditioned B7KO Splenocytes Demonstrate Nonspecific Hyporesponsiveness In Vitro and Evidence of Donor-Specific Tolerance In Vivo

Because the above data demonstrate that B7KO T-cells are intrinsically capable of rejecting allografts, we then assessed the status of antidonor reactivity in a B7KO host conditioned by a long-term surviving cardiac allograft. To address this issue, we first looked at the ability of splenocytes from B7KO hosts bearing long-term surviving BALB/c hearts (>100 days) to respond to donor and third-party stimulators in vitro. Interestingly, these conditioned B7KO splenocytes had greatly reduced in vitro responses to both donor and third-party stimulator cells relative to naïve B7KO responder cells (Fig. 3A). We then determined whether B7KO hosts conditioned by a cardiac allograft for >100 days resulted in actual tolerance induction in vivo. Donor-type BALB/c (H-2^d) or third-party C3H (H-2^k) heart allografts were established in B6 *Rag1*^{-/-} recipients, with the subsequent adoptive transfer of either naïve or conditioned B6 B7KO splenocytes. B6 *Rag1*^{-/-} hosts reconstituted with control naïve B6 B7KO splenocytes promptly rejected both BALB/c and C3H donor heart allografts (Fig. 3B). However, conditioned B6 B7KO splenocytes failed to reject most of the donor-type

hearts after adoptive transfer (7 of 10 grafts surviving more than 60 days, $P < 0.0001$ vs. naïve controls). Unlike the hyporeactivity found in response to third-party stimulators in vitro, C3H heart allografts were readily rejected after the adoptive transfer of conditioned B6 B7KO splenocytes (Fig. 3C). As such, results demonstrate evidence for spontaneous generation of donor-specific tolerance induction within B7KO cardiac allograft recipients.

Spontaneously Tolerant B7KO Recipients of BALB/c Heart Allografts Demonstrate Evidence of Linked Suppression

We then set out to determine if there was a regulatory component to the donor specific tolerance observed. One means of determining whether regulatory allograft tolerance is present is through assessing the property of linked suppression (34). Linked suppression is the property in which tolerance generated against a specific donor can lead to the protection of new allogeneic determinants to which the recipient is not tolerant, provided that these new antigens are expressed on the same donor cells. This was tested by finding whether (donor×third party)F1 heart allografts were accepted or rejected by tolerant splenocytes. Control naïve B6 B7KO splenocytes were capable of rejecting donor-type BALB/c, third-party C3H, or (BALB/c×C3H)F1 donor hearts in B6 Rag1^{-/-} recipient mice (Fig. 3b). Importantly, although tolerant splenocytes rejected third party C3H allografts (clearly demonstrating the lack of tolerance to this strain), (BALB/c×C3H)F1 hearts were accepted by the tolerant splenocytes with five of six allografts surviving 60 or more days ($P = 0.02$ versus C3H third-party controls; Fig. 3C). These results demonstrate that the tolerant state includes the property of linked suppression to third-party allografts.

B7KO Mice Have Low Levels of Endogenous CD4+CD25+Foxp3+ Tregs and Significantly Increased Levels of Peripheral Tregs After Long-Term Exposure to Cardiac Allografts

The finding of in vivo linked suppression strongly implicated regulatory tolerance. As such, we then sought to determine what role the CD4+CD25+Foxp3+ Treg might play. Flow cytometric analysis shows that B7KO mice have extremely low (nearly undetectable) levels of preexisting endogenous CD4+Foxp3+Tregs relative to wild-type B6 controls (Fig. 4A, mesenteric lymph node cells [MLN]). This was similarly demonstrated in spleen cells and peripheral blood cells (data not shown). Interestingly, after >70 days of exposure to a BALB/c heart allograft, conditioned B7KO mice demonstrated a significant elevation in CD4+Foxp3+MLN cells (Fig. 4A) and in peripheral blood and the spleen (data not shown). Additionally, CD4+CD25+ lymphocytes and Foxp3+CD25+lymphocytes were also similarly increased in all cellular compartments (data not shown). Of note, 99% of CD4+CD25+ lymphocytes were also Foxp3+ (data not shown). These data were repeated three times from three separate long-term acceptor B7KO hosts.

Depletion of Endogenous CD4+CD25+ Tregs Does Not Prevent the Development of Long-Term Acceptance of BALB/c Heart Allografts in B7KO Recipients

Given that a significant expansion of CD4+CD25+ Foxp3+Tregs occurs after exposure to a cardiac allograft in B7KO hosts, we next sought to determine whether endogenous CD4+CD25+Foxp3+Tregswere required for the development of long-term acceptance in vivo. Anti-CD25 (PC61) antibody was used to deplete CD25+ cells in vivo precardiac transplant.

For this, 500 μ g of PC61 antibody was injected via the intraperitoneal route day -3 from the time of cardiac transplantation. Of note, flow cytometric analysis demonstrated a 90% reduction in CD4+CD25+ cells and a 96% reduction in CD25+Foxp3+ cells in the spleen, MLN, and peripheral blood of B6 control mice at day 8 postinjection (data not shown). Interestingly, in vivo depletion of CD4+CD25+ Tregs did not prevent the development of long-term acceptance of BALB/c cardiac allografts transplanted into B7KO recipients (three of five untreated control BALB/c $\rightarrow$ B6 B7KO allografts survived >90 days and three of five anti-CD25 treated BALB/c $\rightarrow$ B6 B7KO allografts survived >90 days; $P=NS$, Fig. 4B).

Conditioned B7KO Splenocytes Are Not Anergic and Do Not Require TGF- β or IL-10 to Exert Their Suppressive Effects

Given the surprising results of linked suppression in vivo and non-specific hyporesponsiveness in allograft conditioned B7KO splenocytes to donor-type and third party stimulators in vitro, we performed further proliferation assays in an attempt to reconcile these contrasting results. Results show that the lack of reactivity of conditioned B7KO splenocytes was not overcome by the addition of IL-2 or by the mixing in of naïve B7KO responder splenocytes at a 1:1 ratio (Fig. 5a). Additionally, the addition of anti-TGF- β or anti-IL-10 did not reverse the non-specific hyporesponsiveness of conditioned B7KO splenocytes (Fig. 5B).

Histological Assessment of Tolerant and Rejected Heart Allografts

Negative control BALB/c heart allografts in B6 Rag1^{-/-} recipients left unreconstituted demonstrated intact architecture without lymphocytic infiltration (Fig. 6A). Positive control BALB/c allografts rejected in wild-type B6 recipients demonstrated severe lymphocyte infiltration and significant cardiomyocyte necrosis (Fig. 6B). Long-term surviving BALB/c heart allografts in B6 B7KO recipients demonstrated mild to moderate lymphocyte infiltration with mild cardiomyocyte damage (Fig. 6C). Conversely, BALB/c allografts that acutely rejected in B6 B7KO recipients demonstrated evidence of severe lymphocytic infiltration and cardiomyocyte necrosis (Fig. 6D). CD4 and CD8 staining in long-term surviving BALB/c allografts in B6 B7KO recipients demonstrated both CD4+ and CD8+ lymphocyte infiltration (not shown). Long-term surviving BALB/c allografts in B6 Rag1^{-/-} recipients after adoptive transfer of conditioned B6 B7KO splenocytes demonstrated mild lymphocyte infiltration and mild tissue damage (Fig. 6E) similar to that seen in long-term surviving BALB/c into B6 B7KO recipients (Fig. 6C). Conversely, when BALB/c engrafted B6 Rag1^{-/-} mice received naïve B6 B7KO splenocytes, the hearts were acutely rejected and demonstrated severe lymphocytic infiltration and cardiomyocyte necrosis (Fig. 6F). Long-term surviving (BALB/c $\times$ C3H)F1 allografts in B6 Rag1^{-/-} recipients demonstrated mild lymphocyte infiltration and mild to moderate cardiomyocyte damage (despite function beyond 60 days) after adoptive transfer of conditioned B6 B7KO splenocytes (Fig. 6G). Finally, third-party C3H heart allografts were acutely rejected in B6 Rag1^{-/-} recipients after adoptive transfer of conditioned B6 B7KO splenocytes with histology revealing significant lymphocyte infiltration and massive tissue necrosis (Fig. 6H).

DISCUSSION

Given our previous results highlighting the significance of direct donor MHC expression in acute CD4 T-cell-mediated rejection (16, 23) we have been intrigued by the finding that the expression of host but not donor B7 costimulatory molecules is required for acute cardiac allograft rejection (14, 15). We therefore set out to determine the status of antidonor reactivity in naïve versus allografted B7KO recipients. Firstly, data demonstrate that B7-1/B7-2 T-cell expression is *not required* for acute cardiac allograft reactivity in vitro or in vivo. This issue was important to clarify since T-cell expression of B7 costimulatory molecules has been implicated as playing a role in T-cell immunity (26). Thus, the failure of B7KO mice to reject cardiac allografts is not primarily due to T-cell-specific B7 deficiency. Secondly, the lack of host B7 expression could result in a loss of requisite costimulation necessary for initial T-cell activation, rendering the host ignorant of the established allograft. However, data demonstrate that host exposure to the allograft in the absence of host B7 expression is not the lack of rejection (e.g., ignorance), but actually results in the spontaneous development of donor-specific tolerance induction. The fact that this tolerant state demonstrates linked suppression, the protection of transplants co-expressing donor and third-party alloantigens, strongly implicates an active regulatory component to the response. Although not yet completely clear, we can say that the tolerant state does not require the presence of endogenous CD4+CD25+Foxp3+ Tregs and does not appear to be dependent on TGF- β or IL-10 (Tr1/Th3 cell types).

That tolerance can occur in B7KO hosts is intriguing due to the recent suggestion that B7-1/B7-2 can play a role in tolerance induction (27–29). It is conceivable that both immunity (14, 15, 32) *and/or* active tolerance (6, 27, 28) might be impaired in the B7KO environment, depending on experimental conditions. Results show that at least some forms of donor-specific tolerance can develop in the absence of host B7-1/B7-2 expression. However, it is important to emphasize that tolerance was tested in vivo using *Rag1*^{-/-} recipients that *do* express B7-1/B7-2 molecules in the innate immune system. Thus, it is possible that B7-1/B7-2 provided by the adoptive transfer recipient could contribute to the expression of the tolerant state in our model system. Therefore, these studies do not preclude a potential role for B7-1/B7-2 in maintaining the tolerant state. Additionally, the demonstration of linked suppression strongly implicates active regulation. In light of this and the fact that many other forms of induced allograft tolerance depend on the presence of endogenous CD4+CD25+ Tregs (35, 36), it was interesting that our results show *no* requirement for preexisting *endogenous* CD4+CD25+Foxp3+ Tregs for the development of cardiac allograft tolerance in B7KO hosts. Despite this, a several-fold increase in peripheral CD4+CD25+Foxp3+ Tregs was noted after long-term exposure in B7KO hosts to cardiac allografts. It is therefore possible that, although not required for the development of tolerance, CD4+CD25+Foxp3+ Tregs may be important for the maintenance of the tolerant state in B7KO recipients of cardiac allografts.

It was curious that the in vitro and in vivo assays of tolerance were not concordant in this study. While tolerant T-cells demonstrated a nonspecific hyporeactivity to allogeneic stimulators in vitro, this was not reflected by the assessment of allograft tolerance in vivo. That is, while tolerant splenocytes showed hyporeactivity to third-party stimulator cells in

vitro, they readily rejected third-party allografts in vivo. It is of interest that in other studies of allograft tolerance we have generally found the reciprocal finding. That is, while operationally tolerant animals can both demonstrate donor and third-party reactivity in vitro, they nevertheless demonstrate donor-specific tolerance in vivo (37, 38). Currently, the disparity between these two assessments of tolerance is not completely clear. It is possible that cross-reacting alloantigens expressed by third-party stimulators could potentially elicit suppressive activity in vitro. In fact, Sakaguchi's group has shown that regulatory T-cells can show strong cross-reactivity to other haplotypes in vitro that may not be reflected in the setting of donor-specific allograft tolerance in vivo (39). The fact that anti-third party (C3H) responses are inhibited in vitro and that (donor×third party)F1 grafts (BALB/c×C3H)F1 are protected in vivo supports this view. Additionally, mixing experiments, whereby naïve B7KO responders were added to conditioned responders (Fig. 5a), did not demonstrate restoration of proliferation to baseline, raising the possibility of both crossreactive Tregs and/or paracrine inhibition by cytokines. Results, however, demonstrate that nonspecific hyporesponsiveness in vitro is *not* dependent on TGF- β or IL-10 individually. This finding makes the possibility of paracrine cytokine inhibition less likely. At present, it is unclear to us why in multiple experiments tolerant B7-1/B7-2 deficient animals demonstrate non-specific hyporeactivity in vitro. We would speculate that T-cells with 'direct' specificity, the primary participants in responses to allogeneic APCs under most in vitro conditions, are gradually deleted/ inactivated without sufficient available host-derived B7 co-stimulation. Under these conditions, functionally inactivated/ deleted effector T-cells may tip the balance in favor of the production of Tregs in the periphery. This is supported by data showing a peripheral expansion of CD4+CD25+Foxp3+ T-cells after exposure of B7KO hosts to cardiac allografts and by the fact that the lack of reactivity in vitro is *not* overcome by the addition of exogenous IL-2, suggesting that alloreactive T-cells are not simply anergic but that there is a functional elimination of a significant proportion of alloreactive T-cells. Finally, it is worth noting that this form of tolerance that develops in B7-deficient mice appears to have some features of both deletional and regulatory tolerance. That is, tolerant animals are largely unresponsive to donor APCs (despite the addition of IL-2) in vitro and yet also inhibit graft rejection. Of note, this combined deletional/regulatory tolerance has also been seen in mixed hematopoietic chimeric animals (40).

An important general issue to be resolved is the potential relationship between host-derived B7-1/B7-2 costimulatory molecule expression and the direct pathway of antigen recognition. While host but not donor B7-1/B7-2 is required for acute cardiac rejection (14, 15), other data clearly indicate that direct donor recognition is sufficient to actually kill the allograft (16, 23). Therefore, an ongoing issue to resolve will be the exact role of host bystander costimulation (24) in the generation and function of donor-specific cells of direct allograft specificity in vivo. Clearly, results indicate (Fig. 2A) that B7KO T-cells can vigorously respond directly to donor-type antigen presenting cells in vitro. Therefore, this implies that the B7KO host does have a repertoire of direct-reactive T-cells that can in fact recognize donor cells. The concept that ongoing costimulation to direct donor-reactive T-cells provided in *trans* from host-derived cells (24) would reconcile this idea. Likewise, how tolerance can be spontaneously generated in the face of such direct, donor-reactive direct T-cells remains to be completely resolved.

In summary, previous results clearly indicate that host B7-1/B7-2 plays an important role for acute cardiac allograft rejection in vivo. However, the current results indicate that transplants are not simply ignored in the B7KO host, but actually can generate donor-specific tolerance and linked suppression in vivo. Furthermore, the lack of rejection in B7KO hosts is not secondary to the lack of B7-1/B7-2 expression on T-cells in that B7KO can readily reject a cardiac allograft in a B7-1/B7-2 replete environment. Importantly, the generation of the tolerant state is independent of B7-1/B7-2 expression, indicating that there are tolerance pathways that do not require costimulation as suggested by other studies of regulatory T-cell activation (4, 5). Finally, spontaneous tolerance in B7KO hosts does not require preexisting endogenous CD4+CD25+Foxp3+ Tregs nor does it appear to require TGF- β or IL-10 (Tr1, Th3 cells).

Acknowledgments

This work was supported by grants from the National Institutes of Health Grants (RO1 HL67976 to B.P. and RO1 DK 33470 to R.G.).

The authors thank Philip Pratt and Leslie Bloomquist for technical assistance.

References

- Freeman GJ, Freedman AS, Segil JM, et al. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J Immunol.* 1989; 143:2714. [PubMed: 2794510]
- Freeman GJ, Gribben JG, Boussiotis VA, et al. Cloning of B7-2: A CTLA-4 counter-receptor that costimulates human T-cell proliferation. *Science.* 1993; 262:909. [PubMed: 7694363]
- Azuma M, Ito D, Yagita H, et al. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature.* 1993; 366:76. [PubMed: 7694153]
- Turka LA, Linsley PS, Lin H, et al. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc Natl Acad Sci U S A.* 1992; 89:11102. [PubMed: 1332070]
- Bluestone JA. Costimulation and its role in organ transplantation. *Clin Transplant.* 1996; 10:104. [PubMed: 8680045]
- Liang B, Gee RJ, Kashgarian MJ, et al. B7 costimulation in the development of lupus: Autoimmunity arises either in the absence of B7.1/B7.2 or in the presence of anti-b7.1/B7. 2 blocking antibodies. *J Immunol.* 1999; 163:2322. [PubMed: 10438978]
- Bagenstose LM, Class R, Salgame P, Monestier M. B7-1 and B7-2 co-stimulatory molecules are required for mercury-induced autoimmunity. *Clin Exp Immunol.* 2002; 127:12. [PubMed: 11882027]
- Linsley PS, Greene JL, Tan P, et al. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J Exp Med.* 1992; 176:1595. [PubMed: 1334116]
- Lenschow DJ, Zeng Y, Thistlethwaite JR, et al. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science.* 1992; 257:789. [PubMed: 1323143]
- Walunas TL, Bluestone JA. CTLA-4 regulates tolerance induction and T-cell differentiation in vivo. *J Immunol.* 1998; 160:3855. [PubMed: 9558090]
- Van Ginderachter JA, Liu Y, Geldhof AB, et al. B7-1, IFN gamma and anti-CTLA-4 co-operate to prevent T-cell tolerization during immunotherapy against a murine T-lymphoma. *Int J Cancer.* 2000; 87:539. [PubMed: 10918195]
- Boussiotis VA, Freeman GJ, Gray G, et al. B7 but not intercellular adhesion molecule-1 costimulation prevents the induction of human alloantigen-specific tolerance. *J Exp Med.* 1993; 178:1753. [PubMed: 7901318]
- Onodera K, Chandraker A, Schaub M, et al. CD28 –B7 T-cell costimulatory blockade by CTLA4Ig in sensitized rat recipients: Induction of transplantation tolerance in association with depressed cell-mediated and humoral immune responses. *J Immunol.* 1997; 159:1711. [PubMed: 9257832]

14. Mandelbrot DA, Furukawa Y, McAdam AJ, et al. Expression of B7 molecules in recipient, not donor, mice determines the survival of cardiac allografts. *J Immunol.* 1999; 163:3753. [PubMed: 10490971]
15. Szot GL, Zhou P, Sharpe AH, et al. Absence of host B7 expression is sufficient for long-term murine vascularized heart allograft survival. *Transplantation.* 2000; 69:904. [PubMed: 10755548]
16. Pietra BA, Wiseman A, Bolwerk A, et al. CD4 T-cell-mediated cardiac allograft rejection requires donor but not host MHC class II. *J Clin Invest.* 2000; 106:1003. [PubMed: 11032860]
17. Shizuru JA, Seydel KB, Flavin TF, et al. Induction of donor-specific unresponsiveness to cardiac allografts in rats by pretransplant anti-CD4 monoclonal antibody therapy. *Transplantation.* 1990; 50:366. [PubMed: 1976282]
18. Onodera K, Lehmann M, Akalin E, et al. Induction of "infectious" tolerance to MHC-incompatible cardiac allografts in CD4 monoclonal antibody-treated sensitized rat recipients. *J Immunol.* 1996; 157:1944. [PubMed: 8757313]
19. Pearson TC, Hamano K, Morris PJ, Wood KJ. Anti-CD4 monoclonal antibody-induced allograft survival is associated with a defect in interleukin-2-dependent T-cell activation. *Transplant Proc.* 1993; 25:786. [PubMed: 8438480]
20. Pearson TC, Darby CR, Wood KJ. Successful secondary heterotopic cardiac transplantation in the mouse. *Transplantation.* 1992; 53:701. [PubMed: 1549874]
21. Orosz CG, Wakely E, Bergese SD, et al. Prevention of murine cardiac allograft rejection with gallium nitrate. Comparison with anti-CD4 monoclonal antibody. *Transplantation.* 1996; 61:783. [PubMed: 8607184]
22. Krieger NR, Yin DP, Fathman CG. CD4+ but not CD8+ cells are essential for allojection. *J Exp Med.* 1996; 184:2013. [PubMed: 8920888]
23. Grazia TJ, Pietra BA, Johnson ZA, et al. A two-step model of acute CD4 T-cell mediated cardiac allograft rejection. *J Immunol.* 2004; 172:7451. [PubMed: 15187123]
24. Mandelbrot DA, Kishimoto K, Auchincloss H Jr, et al. Rejection of mouse cardiac allografts by costimulation in trans. *J Immunol.* 2001; 167:1174. [PubMed: 11466331]
25. Ding L, Shevach EM. Activation of CD4+ T-cells by delivery of the B7 costimulatory signal on bystander antigen-presenting cells (trans-costimulation). *Eur J Immunol.* 1994; 24:859. [PubMed: 7512033]
26. Ferlazzo G, Semino C, Meta M, et al. T lymphocytes express B7 family molecules following interaction with dendritic cells and acquire bystander costimulatory properties. *Eur J Immunol.* 2002; 32:3092. [PubMed: 12385029]
27. Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T-cells that control autoimmune diabetes. *Immunity.* 2000; 12:431. [PubMed: 10795741]
28. Tang Q, Henriksen KJ, Boden EK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+regulatory T-cells. *J Immunol.* 2003; 171:3348. [PubMed: 14500627]
29. Liang S, Alard P, Zhao Y, et al. Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T-cells in vivo requires B7 costimulation, but not the thymus. *J Exp Med.* 2005; 201:127. [PubMed: 15630140]
30. Tai X, Cowan M, Feigenbaum L, Singer A. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T-cell differentiation independently of interleukin 2. *Nat Immunol.* 2005; 6:152. [PubMed: 15640801]
31. Bour-Jordan H, Salomon BL, Thompson HL, et al. Costimulation controls diabetes by altering the balance of pathogenic and regulatory T-cells. *J Clin Invest.* 2004; 114:979. [PubMed: 15467837]
32. Borriello F, Sethna MP, Boyd SD, et al. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity.* 1997; 6:303. [PubMed: 9075931]
33. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation.* 1973; 16:343. [PubMed: 4583148]
34. Davies JD, Leong LY, Mellor A, et al. T-cell suppression in transplantation tolerance through linked recognition. *J Immunol.* 1996; 156:3602. [PubMed: 8621893]

35. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3+CD25+CD4+natural regulatory T-cells in dominant self-tolerance and autoimmune disease. *Immunological Rev.* 2006; 212:8.
36. Jiang S, Lechler RI, He XS, Huang JF. CD4+CD25+ regulatory T-cell therapy for the induction of donor-specific clinical transplantation tolerance. *Exp Opin Biol Therapy.* 2006; 6:1003.
37. Nicolls MR, Coulombe M, Yang H, et al. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J Immunol.* 2000; 164:3627. [PubMed: 10725719]
38. Nicolls MR, Coulombe M, Beilke J, et al. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J Immunol.* 2002; 169:4831. [PubMed: 12391193]
39. Nishimura E, Sakihama T, Setoguchi R, et al. Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ regulatory T-cells. *Int Immunol.* 2004; 16:1189. [PubMed: 15237110]
40. Domenig C, Sanchez-Fueyo A, Kurtz J, et al. Roles of deletion and regulation in creating mixed chimerism and allograft tolerance using a nonlymphoablative irradiation-free protocol. *J Immunol.* 2005; 175:51. [PubMed: 15972631]

FIGURE 1.

BALB/c cardiac allografts are accepted indefinitely in B7KO recipients. BALB/c (H-2^d) heart allografts were transplanted heterotopically into B6 B7KO recipients (H-2^b). Fifty percent of these BALB/c cardiac allografts survive >100 days (18 of 36), whereas all (10 of 10) BALB/c allografts are rejected in B6 wild-type control recipients. This relationship was statistically significant with a *P* value of <0.0001.

FIGURE 2.

B7-1/B7-2 deficient T-cells are capable of responding to donor antigen both in vitro and in vivo. (A) Mixed lymphocyte reactions demonstrate equal proliferation of naïve B6 B7KO lymphocytes (H-2^b) and control B6 lymphocytes (H-2^b) (Groups 2 and 3). Group 1 represents the negative control with wild-type B6 lymphocyte responders in media alone. Error bars represent the standard deviation from quadruplicate wells in the MLR assay. This experiment was repeated in quadruplicate with the current figure representing one typical result. (B) BALB/c (H-2^d) heart allografts were transplanted heterotopically into B6 *Rag1*^{-/-} recipients (H-2^b). Recipients then received either no cells, adoptive transfer of 30×10^6 naïve B6 splenocytes, or 30×10^6 naïve B6 B7KO splenocytes. Unreconstituted B6 *Rag1*^{-/-} recipients of BALB/c hearts survive indefinitely (n=5) and represent the negative control. B6 *Rag1*^{-/-} recipients of BALB/c hearts reconstituted with either naïve B6 (n=4) or naïve B6 B7KO (n=10) cells reject their hearts with equal vigor ($P=NS$).

FIGURE 3.

Conditioned B7KO splenocytes demonstrate non-specific hyporesponsiveness in vitro and are capable of transferring donor-specific tolerance and demonstrate evidence of linked suppression in vivo. (A) Mixed lymphocyte assays demonstrate equal proliferation of naïve B6 B7KO ($H-2^b$) responders to BALB/c ($H-2^d$) and third-party C3H ($H-2^k$) stimulators (Groups 2 and 3). However, conditioned B6 B7KO splenocytes (from B6 B7KO recipients of BALB/c hearts surviving >100 days) fail to respond significantly to either BALB/c or third-party C3H stimulators (Groups 4 and 5). Group 1 represents the negative control with naïve B6 B7KO mixed with media alone. Error bars represent the standard deviation from quadruplicate wells in the MLR assay. This experiment was repeated in quadruplicate with the current figure representing one typical result. (B) 30×10^6 naïve B6 B7KO splenocytes were adoptively transferred into BALB/c cardiac transplanted, C3H cardiac transplanted, or (BALB/c $\times$ C3H)F1 cardiac transplanted B6 *Rag1*^{-/-} recipients. In each situation, all heart grafts were rejected by naïve B6 B7KO splenocytes. This rejection was not statistically different between any of these three groups in any combination. (C) 30×10^6 conditioned B6 B7KO splenocytes were adoptively transferred into BALB/c cardiac transplanted B6 *Rag1*^{-/-} recipients with 8 of 11 allografts surviving >60 days after transfer of conditioned cells, whereas 10 of 10 allografts were rejected after receiving naïve cells (in B. above). This relationship was statistically significant with a *P* value of <0.0001. Additionally, (BALB/c $\times$ C3H)F1 allografts survived indefinitely in B6 *Rag1*^{-/-} hosts after adoptive transfer of

30×10^6 conditioned B6 B7KO splenocytes with five of six allografts surviving 60 days or more. This is in contrast to (BALB/c $\times$ C3H)F1 allografts that received 30×10^6 naïve B6 B7KO splenocytes (eight of eight rejected in B. above). Additionally third party C3H allografts were all acutely rejected in B6 *RagI*^{-/-} hosts after transfer of 30×10^6 conditioned or naïve B6 B7KO splenocytes. Long-term survival in (BALB/c $\times$ C3H)F1 allografts given conditioned B6 B7KO splenocytes was statistically significant ($P=0.02$ vs. C3H given naïve B6 B7KO splenocytes in B., $P=0.02$ vs. C3H given conditioned B6 B7KO splenocytes, and $P=0.01$ vs. [BALB/c $\times$ C3H]F1 given naïve B6 B7KO splenocytes in B.).

FIGURE 4.

Despite an increase in peripheral CD4⁺Foxp3⁺ Tregs, depletion of endogenous CD4⁺CD25⁺ Tregs does not prevent long-term acceptance in B7KO recipients of BALB/c cardiac allografts. (A) Flow cytometric analysis was performed on wild type B6, naïve B6 B7KO, and allograft conditioned B6 B7KO mice (n=3, figure shows representative example). Analysis of mesenteric lymph node cells (MLN) show that B6 B7KO mice have extremely low (nearly undetectable) levels of preexisting endogenous CD4⁺ Foxp3⁺ Tregs (0.02% of lymphocyte gate) relative to wild-type B6 controls (2.1% of lymphocyte gate). After >70 days of exposure to a BALB/c heart allograft, conditioned B7KO mice demonstrated a significant elevation in CD4⁺ Foxp3⁺ MLN cells (1.2% of lymphocyte gate). Additionally, similar significant increases in spleen and peripheral blood derived CD4⁺ Foxp3⁺ lymphocytes were seen (data not shown). CD4⁺CD25⁺ lymphocytes and Foxp3⁺CD25⁺ lymphocytes were also similarly increased in all cellular compartments (data not shown). Of note, 99% of CD4⁺CD25⁺ lymphocytes were also Foxp3⁺ (data not shown). (B) Five hundred micrograms of anti-CD25 antibody (PC61) was injected I.P. day -3 from the time of transplantation. Anti-CD25 treatment did not prevent the induction of long-term allograft acceptance in B6 B7KO recipients of BALB/c cardiac allografts. Three of five allografts survived >90 days in B6 B7KO animals treated with α -CD25 and three of five allografts survived >90 days in B6 B7KO animals left untreated ($P=NS$).

FIGURE 5.

Conditioned B7KO splenocytes are not anergic and do not require TGF- β or IL-10 to exert their suppressive effects. (A) As in Fig. 3A, mixed lymphocyte assays again demonstrate equal proliferation of naïve B6 B7KO (H-2^b) responders to BALB/c (H-2^d) and third-party C3H (H-2^k) stimulators while conditioned B6 B7KO splenocytes are hyporesponsive to both BALB/c and third-party C3H stimulators. The addition of IL-2 (50 units/mL) did not reverse the hyporesponsiveness of conditioned B6 B7KO splenocytes responders to BALB/c or third-party C3H stimulators. The addition of 2×10^5 naïve B6 B7KO responders (1:1 ratio of naïve and conditioned responders) also did not reverse the hyporesponsiveness of conditioned B6 B7KO splenocytes responders to BALB/c or third-party C3H stimulators. Of note, control proliferation experiments were performed with IL-2 that demonstrated the in vitro activity of the cytokine used (data not shown). (B) The addition of α -IL-10 (10 μ g/mL) did not reverse the hyporesponsiveness of conditioned B6 B7KO splenocytes responders to BALB/c or third-party C3H stimulators. Additionally, the addition of α -TGF- β (50 μ g/mL)

also did not reverse the hyporesponsiveness of conditioned B6 B7KO splenocytes responders to BALB/c or third-party C3H stimulators. Of note, control proliferation experiments were performed with IL-10, IL-10+ α -IL-10, TGF- β , and TGF- β + α -TGF- β that demonstrated the in vitro activity of the cytokines and antibodies used (data not shown). Error bars represent the standard deviation from quadruplicate wells in the MLR assay. This experiment was repeated in triplicate with the current figure representing one typical result (A and B).

FIGURE 6.

Histological assessment of cardiac allografts. (A) Negative control BALB/c allograft that survived >100 days in a B6 *Rag1*^{-/-} recipient that did not receive any adoptively transferred cells. (B) BALB/c allograft rejected on day 9 posttransplantation into a wild-type B6 recipient. (C) BALB/c allograft that survived >100 days in a B6 B7KO recipient. (D) BALB/c allograft that rejected on day 22 posttransplantation into a B6 B7KO recipient. (E) BALB/c allograft that survived >60 days after transfer of 30×10^6 conditioned B6 B7KO splenocytes in a B6 *Rag1*^{-/-} host. (F) BALB/c allograft that rejected on day 7 after transfer of 30×10^6 naive B6 B7KO splenocytes in a B6 *Rag1*^{-/-} host. (G) (BALB/c × C3H)F1 allograft that survived >60 days after transfer of 30×10^6 conditioned B6 B7KO splenocytes in a B6 *Rag1*^{-/-} host. (H) Third-party C3H allograft rejected on day 9 after transfer of 30×10^6 conditioned B6 B7KO splenocytes in a B6 *Rag1*^{-/-} host.