Alloreactive T Cell Responses and Acute Rejection of Single Class II MHC-Disparate Heart Allografts Are under Strict Regulation by CD4^+CD25^+ T Cells

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Skin but not vascularized cardiac allografts from B6.H-2bm12 mice are acutely rejected by C57BL/6 recipients in response to the single class II MHC disparity. The underlying mechanisms preventing acute rejection of B6.H-2bm12 heart allografts by C57BL/6 recipients were investigated. B6.H-2bm12 heart allografts induced low levels of alloreactive T cell priming in C57BL/6 recipients, and this priming was accompanied by low-level cellular infiltration into the allograft that quickly resolved. Recipients with long-term-surviving heart allografts were unable to reject B6.H-2bm12 skin allografts, suggesting potential down-regulatory mechanisms induced by the cardiac allografts. Depletion of CD25+ cells from C57BL/6 recipients resulted in 15-fold increases in alloreactive T cell priming and in acute rejection of B6.H-2bm12 heart grafts. Similarly, reconstitution of B6.Rag−/− recipients with wild-type C57BL/6 splenocytes resulted in acute rejection of B6.H-2bm12 heart grafts only if CD25+ cells were depleted. These results indicate that acute rejection of single class II MHC-disparate B6.H-2bm12 heart allografts by C57BL/6 recipients is inhibited by the emergence of CD25+ regulatory cells that restrict the clonal expansion of alloreactive T cells.}


It therefore becomes essential to understand those factors that determine the size of the effector T cell pool following a given transplant stimulus. The peak size of the effector T cell pool is influenced by the ability of the responding precursor cells to optimally expand during Ag priming. For example, studies performed in infectious disease models have shown that extremely low precursor frequencies can expand to up to 30% of the T cell precursor pool in response to certain stimuli (11–13). Such clonal expansion is influenced by the number of Ag-expressing dendritic cells (DC), the presence of effective costimulatory signals, the dose of Ag used, and the tissue mass of the transplanted organ. Larger numbers of graft-reactive T cells are needed to reject a heart vs a skin graft, providing an explanation for the preferential rejection of skin vs heart grafts in situations in which low numbers of effector T cells are induced posttransplant.

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presence of amplifying signals/cytokines (i.e., IFNα and/or IL-12) produced by the innate immune system, and potentially, by inhibitory signals that could lead to apoptosis or clonal exhaustion. In the present study, we investigated the relationship between priming and regulation of alloreactive T cells to B6.H-2<sup>bm12</sup> cardiac allografts in C57BL/6 recipients. We provide evidence that regulatory T cells play an important inhibitory role in controlling the size of the effector T cell pool following transplantation of B6.H-2<sup>bm12</sup> heart grafts into C57BL/6 recipients. The findings have important implications for therapies aimed at prolonging allograft survival in other model systems.

Materials and Methods

**Animals**

B6.C-H-2<sup>bm12</sup> (B6.H-2<sup>bm12</sup>) and C57BL/6 (H-2<sup>b</sup>) mice were obtained through Dr. C. Reeder at the National Cancer Institute (Frederick, MD). Adult males, 7–12 wk of age, were used throughout this study.

**Skin transplantation**

Full-thickness trunk skin transplantation was performed using a modification of the protocol of Billingham and Medawar (14). Briefly, trunk skin was excised from B6.H-2<sup>bm12</sup> donor mice, the s.c. fat was removed, and 12-mm-diameter circles of full-thickness skin were prepared using a punch. The skin allograft was placed in a slightly larger graft bed prepared over the chest of the recipient and secured with Vaseline gauze and adhesive bandage. After 7 days, the bandage was removed, and each graft was examined daily and was considered rejected when 70% or more of the graft tissue was destroyed as assessed by visual examination.

**Heterotopic cardiac transplant**

Cardiac transplants were performed using the method of Corry et al. (15). Briefly, donor and recipient mice were anesthetized with phenobarbital. Donor hearts were harvested and placed in chilled lactated Ringer’s solution while the recipient mice were prepared. The donor heart was anastomosed to the recipient abdominal aorta and vena cava using microsurgical techniques. Upon completion of the anastomoses and organ reperfusion, the heart grafts resumed spontaneous contraction. The strength and quality of cardiac impulses were graded daily by palpation as previously described (9). Rejection of cardiac grafts was considered complete by the cessation of impulse and was confirmed visually by laporotomy for each graft. Cardiac isografts in C57BL/6 recipients functioned for >100 days. The significance in allograft survival between recipient groups was analyzed by log-rank test, and significance in allograft survival between recipient groups was analyzed by log-rank test.§

**Histology and immunohistochemistry**

Heart grafts were retrieved from recipients at various times posttransplantation, embedded in OCT compound (Sakura Finetek), and frozen at −80°C. Sections were cut at 8 μm and mounted onto slides. For immunohistochemistry, sections were fixed in acetone for 10 min and air-dried. Sections were stained with hematoxylin for 3 min and rinsed with tap water. The slides were dehydrated, viewed under light microscopy, and the images were captured using ImagePro Plus (Media Cybernetics).

**Flow cytometry**

Spleen cells were obtained from anti-CD25 mAb–treated or control B6 recipients on day +17 after transplantation of B6.H-2<sup>bm12</sup> heart allografts. The cells were washed twice with staining buffer (eBioscience’s PBS with 2% FCS/0.2% NaN<sub>3</sub>) and 1 × 10<sup>6</sup> cells aliquots were incubated in ice on 150 μl of rat serum (Rockland). After 30 min, the cells were washed twice and stained with fluorochrome-labeled mAb at 10 μg/ml. After 30 min on ice, the cells were washed five times, resuspended in staining buffer, and analyzed by two-color flow cytometry using a FACScan and CellQuest software (BD Biosciences). Sample data were collected on 20,000 gated cells, and cells staining positive for CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>rat IgG<sup>+</sup> were expressed as the percentage of CD4<sup>+</sup> cells.

**ELISPOT assays**

Priming of donor-specific T cells to IFN-γ-producing cells was quantified by ELISPOT assays as previously described (8, 10). Briefly, ELISA spot plates (Unifilter 350; Whatman) were coated with 2 μg/ml IFN-γ-specific mAb and incubated overnight at 4°C. The plates were blocked with 1% BSA/PBS and then washed four times with PBS. Spleen cell suspensions from graft recipients were prepared on day 7 posttransplant and used as responder cells. Spleen cells from C57BL/6 and B6.H-2<sup>bm12</sup> mice were prepared and treated with mitomycin C for use as stimulator cells in the assay as described above. Responder and stimulator cells (1:2) were cultured in serum-free HL-1 medium (BioWhittaker) supplemented with 1 mM l-glutamine. After 24 h of cell culture at 37°C in 5% CO<sub>2</sub>, cells were removed from the plate by extensive washing with PBS. Biotinylated anti-IFN-γ (2 μg/ml) or anti-IL-4 (4 μg/ml) mAb was added, and the plate was incubated for 6 h at room temperature. The plate was washed three times with PBS/0.05% Tween 20, and streptavidin-conjugated alkaline phosphatase was added to each well. After 2 h at room temperature, the plates were washed with PBS, and NBT-5-bromo-4-chloro-3-indolyl substrate (Kirkegaard & Perry) was added for the detection of IFN-γ-producing cells. The resulting spots were counted with an ImmunoSpot Series I analyzer (Cellular Technology) that was designed to detect ELISA spots with predetermined criteria for spot size, shape, and colorimetric density.

**CD25<sup>+</sup> cell depletion and adoptive transfer**

For adoptive transfers experiments, CD25<sup>+</sup> cells were depleted from spleen cell suspensions by magnetic cell sorting using a CD4<sup>+</sup>CD25<sup>+</sup> T cell isolation kit (Miltenyi Biotec) following the manufacturer’s protocol. Briefly, splenocytes were isolated from naive B6 mice and washed in buffer (PBS, 0.5% BSA, 2 mM EDTA) after lysis of remaining erythrocytes. The cells were incubated with PE-labeled anti-CD25 mAb (Biotech; 10 μl/10<sup>7</sup> cells) for 15 min at 4–8°C. The cells were washed and resuspended, and 10 μl of anti-PE microbeads were added. Following another 15 min of incubation at 4°C, the cells were washed, resuspended in 500 μl of buffer, and loaded onto the MACs columns. Cells passing through the column were collected as the CD25<sup>+</sup> fraction. The CD25<sup>+</sup> depleted and nondepleted cells were adoptively transferred into RAG<sup>+</sup> recipients of B6.H-2<sup>bm12</sup> heart grafts (20 × 10<sup>6</sup> cells/mouse) on day 3 posttransplantation.
Results

Rejection of B6.H-2^{bm12} skin and heart allografts

The acute rejection of single class II MHC-mismatched skin and cardiac B6.H-2^{bm12} allografts by C57BL/6 recipients was compared. All skin allografts were rejected between days 15 and 19 posttransplant (Fig. 1). In contrast, all cardiac allografts were maintained past day 25 posttransplant, and after day 30, 80% of the grafts continued to survive beyond day 100, with median survival time (MST) >100 days (Fig. 1). Histological inspection of long-term-surviving heart allografts indicated little-to-no cellular infiltration when examined at day 80 posttransplant (Fig. 2).

Skin grafts contain many DC that may promote a stronger alloreactive immune response than the response to cardiac allografts (17, 18). Previous studies have shown that donor-specific priming with DC can accelerate acute rejection of complete MHC-mismatched heart allografts (16). The ability of DC priming to provoke acute rejection of B6.H-2^{bm12} heart allografts was tested. C57BL/6 mice were primed with B6.H-2^{bm12} DC 3 days before heart graft transplantation. This priming resulted in a slight increase in heart allograft rejection, with a 50% loss of cardiac allografts by day 42, but no allografts were rejected beyond this time point (Fig. 1). When compared with nonprimed recipients, the decrease in allograft survival in DC-primed recipients did not reach statistical significance. Similar results in heart allograft survival were observed when C57BL/6 mice received B6.H-2^{bm12} skin allografts 14 days before B6.H-2^{bm12} heart allografts (data not shown).

Cardiac allografts retrieved from recipients that were not primed with DC had little cellular infiltration at day 7 posttransplant, and on day 21 this infiltration increased in some but not all allografts (Fig. 2). In contrast, cardiac allografts retrieved from DC-primed cardiac-grafted recipients on day 7 were heavily infiltrated with mononuclear cells, and this infiltrate was associated with marked myocyte necrosis. By day 21 posttransplant, however, the intensity of this cell infiltration into allografts was absent in most of the heart allografts analyzed.

In parallel to histological analyses, T cell development to effector cells in unprimed and DC-primed recipients of B6.H-2^{bm12} cardiac allografts was compared by enumerating the number of alloreactive T cells producing IFN-γ on day 7 posttransplant in ELISPOT assays (Fig. 3). The number of alloreactive cells producing IFN-γ in cardiac allograft recipients that were not primed with DC was increased ~3-fold over the background response observed in naive mice (74 IFN-γ-producing cells per 6 × 10^5 cells vs 22 per 6 × 10^5 cells). Priming with donor DC before receiving the cardiac allograft increased this 1.5-fold further on day 7 posttransplantation (110 IFN-γ-producing cells per 6 × 10^5 cells; p < 0.01). As expected with a response to a single class II MHC disparity, the number of primed alloreactive T cells producing IFN-γ was considerably lower than observed in recipients of complete MHC-mismatched heart allografts at the time of rejection, typically >1000 per 6 × 10^5 cells. On day 21 posttransplantation, the numbers of alloreactive T cells producing IFN-γ were slightly lower in cardiac allograft recipients not primed with DC (61 per 6 × 10^5) and were detected at even lower frequencies (21 per 6 × 10^5; p < 0.05) in DC-primed allograft recipients. Overall,
these results indicated a low-level T cell response to a single allogeneic class II MHC determinant that is poorly sustained over time posttransplant and is reflected by low-level cellular infiltration into the heart allografts that also subsides with time posttransplant.

**Regulation of alloreactive T cell responses and rejection of B6.H-2\textsuperscript{bmi12} cardiac allografts**

We next investigated whether negative signals might be restraining acute rejection of B6.H-2\textsuperscript{bmi12} cardiac allografts. One obvious candidate was CTLA-4, which is implicated both as a negative regulator of naive and effector T cells as well as an effector mechanism expressed by CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T cells (19–22). Thus, we tested the effects of a blocking anti-CTLA-4 mAb on the survival of B6.H-2\textsuperscript{bmi12} cardiac allografts. C57BL/6 mice were treated with control rat IgG or anti-CTLA-4 mAb every other day from days 0 to 10 posttransplant (Fig. 4). Blockade of CTLA-4 induced acute rejection of all B6.H-2\textsuperscript{bmi12} cardiac allografts by day 15 posttransplant, demonstrating a role for CTLA-4 in tempering rejection in this allogeneic response.

The rapid rejection of B6.H-2\textsuperscript{bmi12} cardiac allografts by blocking CTLA-4 suggested a role for CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T cells but could also be explained by the known intrinsic inhibitory functions of CTLA-4 on nonregulatory T cells. The next sets of experiments were designed to discriminate between these two possibilities and to specifically address the hypothesis that T cell responses to B6.H-2\textsuperscript{bmi12} cardiac allografts failed to lead to rejection as a consequence of the induction of CD25\textsuperscript{+} regulatory cell activity.

First, groups of C57BL/6 recipients of B6.H-2\textsuperscript{bmi12} heart allografts were treated with control rat IgG or with rat anti-CD25 mAb from day −1 to day 9 posttransplant. When spleen cells were examined at day 17 posttransplant, treatment with anti-CD25 mAb led to an almost 75% decrease in CD4\textsuperscript{+}CD25\textsuperscript{+} T cells (Fig. 5). There was no increase in CD4\textsuperscript{+} T cells staining positively with anti-rat IgG, indicating that the decrease was due to deletion of CD25\textsuperscript{+} T cells and not to blocking of CD25 by the Ab treatment.

Recipient treatment with anti-CD25 mAb led to a 70% loss of the B6.H-2\textsuperscript{bmi12} cardiac allografts by day 25 ($p < 0.001$ vs control) (Fig. 6A). All grafts of anti-CD25 mAb-treated recipients were severely infiltrated with mononuclear cells on days 14, and this...
increased on day 21 posttransplantation (International Society of Heart and Lung Transplantation grade 3B–4), with CD4+ T cells constituting the majority of the infiltrating cells (Fig. 7). At day 21 posttransplant, three of four cardiac allografts of anti-CD25 mAb-treated animals had high-grade acute cellular rejection, whereas allografts from control IgG-treated recipients showed minimal perivascular and interstitial cellular infiltrates and were similar to syngeneic grafts. Recall assays to compare levels of T cell priming on day 21 posttransplantation revealed a 15-fold increase of alloreactive T cells producing IFN-γ when compared with untreated B6 recipients (750 per 6 × 10⁵ vs 41 per 6 × 10⁵; p < 0.01) (Fig. 8). The increased alloreactive T cell response to B6.H-2bm12 cardiac allografts in recipients treated with anti-CD25 mAb was not accompanied by an increase in self-reactive T cells, because low numbers of spots (<8 per 6 × 10⁵ cells) were observed when recipient cells were cultured with C57BL/6 spleen cells as stimulators in the ELISPOT assay (data not shown).

To eliminate the reconstitution of the CD4+CD25+ T cell compartment by newly developed thymic emigrants, adult mice were thymectomized, and then 2 wk later, the mice were treated with anti-CD25 mAb every other day for a total of four doses of the Ab. This treatment resulted in a similar depletion of CD4+CD25+ T cells as observed above in nonthymectomized mice. Ten days after the last Ab treatment, control thymectomized and anti-CD25 mAb-treated thymectomized mice received B6.H-2bm12 cardiac allografts. In nonthymectomized recipients, 80% of the B6.H-2bm12 cardiac allografts survived longer than 100 days posttransplant, and in adult thymectomized recipients, 65% of the allografts survived longer than 100 days posttransplant (Fig. 6B). Depletion of CD25+ cells from the thymectomized recipients resulted in rejection of >70% of the B6.H-2bm12 cardiac allografts by day 38 posttransplant, and all were rejected by day 80.

Next, adoptive transfer experiments were conducted to further test the role of CD25+ cells in promoting acceptance of single
class II MHC-mismatched grafts. B6.RAG-1−/− mice were transplanted with B6.H-2bm12 hearts, and 3 days later, the recipients were reconstituted with naive B6 splenocytes, which did or did not contain CD25+ cells. Adoptive transfer of CD25+ depleted cells precipitated acute rejection of all grafts by day 20 posttransplantation, whereas only one of five grafts was rejected in recipients reconstituted with undepleted B6 splenocytes (p < 0.01) (Fig. 9). At the time of rejection, cardiac allografts from recipients reconstituted with CD25-depleted spleen cells were heavily infiltrated with CD4+ T cells, whereas allografts from recipients reconstituted with whole spleen cells had little cell infiltration at all times examined (data not shown). Thus, the presence or absence of CD25+ cells influences the intensity of cellular infiltration and the acceptance vs rejection of vascularized allografts expressing a single class II MHC disparity.

Finally, we investigated the consequence of the inability to reject B6.H-2bm12 cardiac allografts on the subsequent acute rejection of B6.H-2bm12 skin allografts. Our results to this point had demonstrated that CD4+CD25+ T regulatory cells were responsible for the failure to reject the B6.H-2bm12 cardiac allografts. Given the known ability of Ag stimulation of these cells to lead to expansion and increased potency of regulation, we postulated that enhanced regulatory capacity in the B6.H-2bm12 cardiac allograft recipients might lead to skin allograft acceptance. Groups of naive C57BL/6 mice and B6.H-2bm12 cardiac allograft recipients were challenged with B6.H-2bm12 skin allografts 100 days after the cardiac allograft transplantation. Whereas naive C57BL/6 mice acutely rejected B6.H-2bm12 skin allografts, recipients of the long-term-surviving cardiac allografts were unable to reject the B6.H-2bm12 skin allografts (Fig. 10).

**Discussion**

The underlying mechanisms that result in failure to reject heart allografts when skin grafts from the same donor are rejected include both graft- and recipient-derived factors. Similar to the rejection of B6.H-2bm12 allografts, recipients reject MHC-matched/minor Ag-mismatched skin grafts but fail to reject heart allografts in most instances. When compared with the effector T cell response induced to complete MHC-mismatched cardiac and skin allografts, the response induced to minor Ag and single MHC-mismatched disparities is considerably smaller. Studies from this and other groups have indicated that a threshold number of effector T cells is required for the rejection of allografts and that the required number of cells is higher for the acute rejection of cardiac allografts than for skin allografts (8–10). Furthermore, increasing the tissue mass of single minor histocompatibility-disparate skin allografts abrogates acute rejection, and decreasing the tissue mass of heart allografts promotes acute rejection. These studies predict that cardiac allografts of large tissue mass will not be acutely rejected unless a threshold number of effector T cells develop in response to the allograft. The current report indicates that removal of regulatory constraints on the expansion of the alloreactive effector T cell pool overcomes the inability to reject heart allografts with a single class II MHC disparity.

The short duration of the T cell response in recipients of B6.H-2bm12 cardiac allografts with or without donor DC priming suggested the presence of a highly regulated alloreactive response to the single class II-disparate grafts. Recent studies in rodent models have indicated the ability of CD4+CD25+ regulatory cells to inhibit allograft rejection (23–26). The function of these regulatory T cells is inhibited by Abs to CD25 or to IL-2 (27–29). However, CD25 is also expressed by activated T cells during Ag priming including alloreactive T cells responding to allografts, and under specific conditions IL-2 binding to its receptor on activated T cells induces cell death (30). Recent studies by Sho et al. (2) have indicated that acute rejection of complete MHC-disparate heart allografts was inhibited by treatment with anti-CD25 mAb. In contrast, MHC-matched/multiple minor histocompatibility-disparate heart allografts normally accepted were rejected when recipients were treated with anti-CD25 mAb. Although it is unclear whether the Ab inhibited the activity of CD4+CD25+ regulatory T cells or acted directly on minor histocompatibility-reactive effector T cells, the Ab treatment was associated with decreased T cell apoptosis and sustained alloreactive T cell responses to the allograft.
In the current studies, treatment of C57BL/6 recipients of B6.H-2<sup>bm12</sup> heart allografts with anti-CD25 mAb also promoted acute rejection of the grafts. This rejection was accompanied by a substantial increase in the number of alloreactive T cells primed to the allograft and intense and sustained T cell infiltration into the grafts. The removal of CD25<sup>+</sup> T cells before transfer to RAG-deficient recipients of B6.H-2<sup>bm12</sup> heart allografts resulted in the ability of the wild-type T cells to reject the grafts. Thus, CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells constrain the clonal expansion of B6.H-2<sup>bm12</sup>-reactive T cells, resulting in low alloreactive T cell responses and low grades of cellular infiltration into the allografts that is not sustained over time. Several recent studies have reported the expansion of CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells in response to self and exogenous Ags and the restriction of the Ag-specific effector T cell response by the emergence of the regulatory T cells (31–35). Similarly, both CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells and pathogenic effector CD4<sup>+</sup> T cells are likely to be activated and expand in response to the B6.H-2<sup>bm12</sup>-alloantigen. The regulatory cells halt or attenuate clonal expansion of the effector CD4<sup>+</sup> T cells during the course of the immune response to the allograft so that the numbers of effector T cells are not sufficient to mediate rejection of the cardiac grafts. The realization that a controlled balance between alloreactive effector and regulatory T cells will promote allograft survival has prompted the recent design of a novel strategy that decreases the effector compartment while maintaining the regulatory compartment and successfully establishes tolerance to MHC-mismatched allografts in murine models (36).

If discrepancies in the numbers of allograft-derived DC accounted for the difference in rejection vs survival of skin vs heart allografts from B6.H-2<sup>bm12</sup> donors, then recipient priming with donor DC should have increased the size of the effector T cell response and overcome the failure to acutely reject the heart allografts. In the current studies, priming of C57BL/6 mice with B6.H-2<sup>bm12</sup> DC did result in a modest increase in primed alloreactive T cells but did not significantly increase acute rejection of the heart allografts. Furthermore, similar numbers of alloreactive T cells are primed in response to both skin and heart allografts expressing single class I MHC or single minor histocompatibility disparities, arguing against differences in graft-derived DC as a factor influencing acute rejection vs acceptance (9, 10). The current results emphasize the strict control imposed on the expansion of alloreactive effector T cells in response to the B6.H-2<sup>bm12</sup> cardiac allografts with or without additional alloantigen priming by DC. The rapid decrease in alloreactive effector T cell numbers observed from days 7 to 21 posttransplant in the spleens of B6.H-2<sup>bm12</sup> cardiac allograft recipients primed with donor DC may be indicative of the potency of this regulation in response to the early increase in the effector T cell pool induced in the DC-primed recipients. Another aspect of these studies that warrants consideration and further investigation is that the interstitial DC in the B6.H-2<sup>bm12</sup> cardiac allograft, but not DC from skin allografts or the bone marrow-derived DC used to prime the allograft recipients, may express tolerogenic properties that induce or promote this regulation. This regulation was expressed in DC-primed cardiac allograft recipients as well as in cardiac allograft recipients subsequently challenged with a donor skin graft, indicating the dominant nature of this regulation in response to B6.H-2<sup>bm12</sup> cardiac allografts. Acute rejection of the cardiac allografts was only quickly and consistently observed when this regulation was removed.

An additional factor that may facilitate the acute rejection of allografts is the induction of an alloreactive Ab response. The close homology of the I-A<sup>b</sup> and I-A<sup>bm12</sup> molecules is reflected by the reactivity of most anti-I-A<sup>b</sup> Abs with I-A<sup>bm12</sup> (37). This cross-reactivity suggests that, if induced, a humoral response to B6.H-2<sup>bm12</sup> allografts by C57BL/6 recipients would result in autoactivity. The absence of an Ab response to B6.H-2<sup>bm12</sup> allografts may also be indicative of the absence of T cells activated through the indirect alloantigen presentation pathway, which is proposed to be an important factor initiating alloantigen-specific Ab responses (38, 39). Consistent with this, we have been unable to detect an indirect T cell response in C57BL/6 recipients of B6.H-2<sup>bm12</sup> heart or skin allografts to peptides incorporating the 3-aa substitutions of I-A<sup>bm12</sup> or by immunizing C57BL/6 mice with the peptides (S. Schenk, unpublished results). The response to B6.H-2<sup>bm12</sup> allografts by C57BL/6 recipients appears to be stimulated entirely through the direct alloantigen presentation pathway, which may also limit the number of effector T cells primed in response to the allograft. The low-level T cell response to B6.H-2<sup>bm12</sup> skin and cardiac allografts might be indicative of a restricted repertoire of alloreactive T cells to the I-A<sup>bm12</sup> alloantigen. However, rigorous investigation of the TCR repertoires expressed by B6.H-2<sup>bm12</sup>-reactive T cells indicates a diverse population of T cells generated in response to the single class II MHC disparity (40).

Collectively, the data of the current report provide new insights into factors that control the size of the T cell repertoire posttransplant. The effector T cell response induced to B6.H-2<sup>bm12</sup> grafts under normal conditions is relatively low in number, well below that required to reject heart allografts but high enough to reject a smaller skin allograft. The B6.H-2<sup>bm12</sup>-reactive T cells are primed to express a pathogenic phenotype but cannot be amplified effectively, and the development of these effector T cells is not sustained. The number of induced effector cells posttransplant is theoretically dependent on the precursor frequency, the proinflammatory signals that activate the innate immune system subsequently amplifying the adaptive response, and the presence or absence of factors that regulate T cell expansion and function. The low precursor frequency cannot solely account for the lack of rejection because the number of alloreactive T cells can be increased significantly. Our data clearly show that naturally developing regulatory T cells are capable of limiting the expansion of transplant-reactive T cells in this strain combination. Interference with the presence or function of CD4<sup>+</sup>CD25<sup>+</sup> T cells leads to a significant expansion of proinflammatory antitumor T cells and precipitates rejection of the heart allografts. These results suggest that, following transplantation, both pathogenic and regulatory T cells are activated. If the alloreactive T cell repertoire is not too large, the regulatory T cells have the capability to control the expansion of the pathogenic effector T cells and limit the extent and duration of T cell infiltration into the allograft.

Disclosures
The authors have no financial conflict of interest.

References
CORRECTIONS


*The Journal* received the following letter from Roderich E. Schwarz requesting correction of this article, which was published in the November 15, 1988 issue:

To the editor:

This is a formal request for a correction to a paper which appeared in *The Journal of Immunology* in November 1988 (R. E. Schwarz and J. C. Hiserodt), in response to a recommendation by the Office of Research Integrity (ORI) of the Public Health Service, Department of Health and Human Services. In 1994, the ORI had performed an investigation against the senior author of the manuscript titled “The expression and functional involvement of laminin-like molecules in non-MHC restricted cytotoxicity by human Leu-19+/CD3− natural killer lymphocytes,” the summary of which has been tagged to the PubMed reference of this article (http://grants1.nih.gov/grants/guide/notice-files/not94-105.html). In this ORI report, a requirement to correct the journal article, namely the indication that Fig. 2 of the article cannot be relied upon, was issued. Such recommendation remains sensible today, albeit 16 years after the original publication, as the investigation apparently failed to identify documented experimental data upon which the figure had been generated, and is hereby formally requested. However, the general ability to inhibit human adherent lymphokine-activated killer (A-LAK) cell cytotoxicity by F(ab′)2 of anti-laminin Ab, as stated in the legend of Fig. 2, should not be questioned. A figure, which is based on actual experimental data and reflects the inhibitory effect, is therefore added to this correction request, to replace the original Fig. 2.

**FIGURE 2.** Inhibition of cytotoxicity by two different human A-LAK cell populations through F(ab′)2 of affinity-purified anti-laminin Ab. Sorted Leu-19+/CD3− and Leu-19+/CD3+ A-LAK cell populations were mixed with Cr-labeled target cells in the continued presence of 150 μg/ml Ab for 4 h, and the resulting lytic activity was calculated as specified in Materials and Methods.
The Journal received the following letter requesting correction of this article, which was published in the August 15, 2002 issue:

The authors would like to alert the scientific community to the fact that we have been unable to reproduce one of the findings presented in this manuscript. In Fig. 5B of this manuscript we showed the results of flow cytometric studies designed to measure the levels of surface expression of FcγRIIB on splenic germinal center (GC) B cells (defined as B220⁺, IgD⁻, GL7⁺) as compared with splenic non-GC B cells (defined as B220⁺, IgD⁻, GL7⁻) using the anti-FcγRIIB mAb K9.361. These cells were isolated from C57BL/6 mice that had been immunized i.p. 8 days earlier with 3 × 10⁸ sheep RBC (SRBC) per mouse. Fig. 5B illustrated ~5-fold lower levels of K9.361 staining on GC B cells as compared with non-GC B cells. In Fig. 6, we presented the results of the semiquantitation of FcγRIIB mRNA levels, via RT-PCR and in gel hybridization, in these two populations of B cells that had been purified by FACS. This figure indicated ~6-fold lower levels of FcγRIIB mRNA in GC, as compared with non-GC B cells.

In multiple recent experiments designed to extend these published studies, neither the reduced levels of K9.361 surface staining of B220⁺, IgD⁻, GL7⁺ splenic B cells detected by flow cytometry or the reduced levels of FcγRIIB mRNA in such cells isolated by FACS 8 days after i.p. immunization of C57BL/6 mice with SRBCs (evaluated via real-time RT-PCR) have been observed.

In several other figures in the above-referenced manuscript, the results of immunohistological analysis of FcγRIIB expression in the GCs of SRBC immunized C57BL/6 mice were illustrated and interpreted to corroborate the results of the studies presented in Figs. 5 and 6. Due to the relative insensitivity of immunohistology as compared with flow cytometry, whether GC B cells stained 5- to 6-fold less intensely with anti-FcγRIIB mAbs as compared with non-GC B cells could not have been unequivocally determined using the former approach. Nonetheless, our previous interpretations of these immunohistological data with regard to levels of FcγRIIB on GC B cells appear to have been incorrect. In addition, arguments we forwarded in Discussion based on the conclusion that GC B cells express lower levels of FcγRIIB than non-GC B cells may no longer hold merit.

We currently can provide no compelling explanation for why our previously published results on the expression levels of FcγRIIB on GC B cells and the results of our more recent studies differ, but are actively investigating several possibilities. We should hasten to point out that our failure to reproduce the results presented in Figs. 5B and 6 does not influence the validity of any of the data or conclusions presented in the above-referenced manuscript regarding the expression and function of FcγRIIB on follicular dendritic cells.


The fifth author’s name, Emma J. O’Neill, was inadvertently omitted. The correct list of authors and affiliations is shown below.


The fifth author’s name, Emma J. O’Neill, was inadvertently omitted. The correct list of authors and affiliations is shown below.

Per O. Anderson,* Anette Sundstedt,† Zihni Yazici,§ Sophie Minaee,§ Emma J. O’Neill,§ Richard Woolf,§ Kirsty Nicolson,§ Nathaniel Whitley,§ Li Li,§† Suling Li,§† David C. Wraith,§ and Ping Wang*†

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In Results, Fig. 2A1 was incorrectly published in black and white. The error has been corrected in the online version, which now differs from the print version as originally published. The correct color figure is shown below.

Also in Results, Fig. 3 fails to mention the headings “NFAT2” and “NF-κB (RelA)” on both panels a and b. The legend to this figure is written with the presumption that the headings are present on each of the two panels. The correct figure is shown below.


The second author’s last name is misspelled. The correct name is Stephen J. Pollock.
In the last summary of In This Issue titled “Helicobacter pylori-neutrophil interactions,” an error was made in citing the page number of the original article titled “Helicobacter pylori disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release” by Lee-Ann H. Allen, Benjamin R. Beecher, Jeffrey T. Lynch, Olga V. Rohner, and Lara M. Wittine. The correct page number associated with the article is 3658. The error has been corrected in the online version, which now differs from the print version as originally published.


The sixth author’s first name is misspelled. The correct name is Chuangqi Chen.