Published in final edited form as:

Clin Immunol. 2010 August ; 136(2): 174–187. doi:10.1016/j.clim.2010.04.012.

Induction of transplantation tolerance to fully mismatched cardiac allografts by T cell mediated delivery of alloantigen

Chaorui Tian^{1,2}, Xueli Yuan^{1,2}, Peter T. Jindra², Jessamyn Bagley², Mohamed H. Sayegh², and John Iacomini^{2,3}

²Transplantation Research Center, Renal Division, Brigham and Women's Hospital and Children's Hospital Boston, Harvard Medical School, Boston, MA, USA.

Abstract

Induction of transplantation tolerance has the potential to allow for allograft acceptance without the need for life-long immunosuppression. Here we describe a novel approach that uses delivery of alloantigen by mature T cells to induce tolerance to fully allogeneic cardiac grafts. Adoptive transfer of mature alloantigen-expressing T cells into myeloablatively conditioned mice results in long-term acceptance of fully allogeneic heart transplants without evidence of chronic rejection. Since myeloablative conditioning is clinically undesirable we further demonstrated that adoptive transfer of mature alloantigen-expressing T cells alone into mice receiving non-myeloablative conditioning resulted in long-term acceptance of fully allogeneic heart allografts with minimal evidence of chronic rejection. Mechanistically, tolerance induction involved both deletion of donor-reactive host T cells and the development of regulatory T cells. Thus, delivery of alloantigen by mature T cells induces tolerance to fully allogeneic organ allografts in non-myeloablatively conditioned recipients, representing a novel approach for tolerance induction in transplantation.

INTRODUCTION

Transplantation tolerance, defined as long-term allograft survival without ongoing immunosuppression, is a major but elusive goal in the field of transplantation medicine. Tolerance to self is maintained through central mechanisms involving negative selection of self-reactive T cells in the thymus and peripheral mechanisms involving regulatory T cells (Tregs) as well as other mechanisms [1;2]. Indeed, it is clear that tolerance to self requires both a central and peripheral component [3]. Building on these observations it is reasonable to suggest that strategies to induce transplantation tolerance should also involved central and peripheral mechanisms to induce and maintain tolerance. Bone marrow derived hematopoietic cells have been shown to be capable of inducing transplantation tolerance [4;5]. Indeed, tolerance to allogeneic transplants in adults can be established by inducing a state of mixed hematopoietic chimerism through allogeneic bone marrow transplantation [5;6]. Mixed chimerism leads to specific tolerance, and permits transplantation of organs matched to the donor bone marrow without immunosuppression [7]. However, the use of

© 2010 Elsevier Inc. All rights reserved.

³Corresponding Author: John Iacomini, Ph.D., Transplantation Research Center, Brigham and Women's Hospital, 221 Longwood Ave, Boston, MA 02115, Phone: (617) 525-8008, Fax: (617) 264-5196, jiacomini@rics.bwh.harvard.edu.

¹C.T. and X.Y. contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

allogeneic bone marrow transplantation to induce tolerance is associated with serious complications, such as graft-vs-host disease (GvHD), the inability to recover complete immunocompetence, engraftment failure, and infectious complications [8;9;10;11;12;13]. Furthermore, it has been difficult to reliably establish a stable state of mixed host-donor hematopoietic chimerism in primates [14;15;16;17], in most cases resulting in full donor chimerism or a lack of chimerism. Full donor chimerism has been suggested to potentially result in immunoincompetence because developing T cells are selected on host thymic epithelium, but must respond to pathogens in a donor-MHC restricted fashion [18]. Moreover, it has been reported that induction of mixed chimerism through bone marrow transplantation is unable to prevent chronic allograft rejection [19], currently the major factor limiting long-term survival of transplants [20;21;22].

Despite the clinical limitations of mixed bone marrow chimerism, it is clear that donor bone marrow derived cells have potent tolerogenic properties. Therefore, one could hypothesize that if bone marrow derived lineages capable of inducing tolerance could be identified it may be possible to use these cells to induce tolerance without the need for an allogeneic bone marrow transplant. To date, hematopoietic lineages required for tolerance induction in the context of chimerism have been poorly defined. It had been suggested for a number of years that bone marrow derived antigen presenting cells (APCs) are critical for inducing tolerance to MHC antigens [23;24;25;26;27]. Others reported that high levels of donor type T cell chimerism correlated best with long-term maintenance of donor specific tolerance [28]. It has also been shown that thymocytes are able to induce tolerance to MHC class I antigens [29], and MHC class I antigens expressed only in CD2⁺ cells induce CD8 T cell tolerance [30]. T cells have been shown to induce tolerance to minor antigens in neonatal mice [31], and donor bone marrow derived CD8 and CD4 T cells, or CD3e expressing cells are important for inducing tolerance in mixed chimeras [32].

We and others have shown that expression of donor alloantigen on T cells can result in tolerance in the context of hematopoietic chimerism [32;33;34]. Using a gene therapy model we have shown that expression of a MHC class I allo-antigen (*H2-K^b* (*K^b*)) on donor bone marrow derived T cells is required to induce tolerance to MHC class I disparate donor skin grafts [35]. Notably, expression of donor antigen on bone marrow derived APCs alone was not sufficient to induce antigen specific tolerance [35]. Further studies demonstrated that adoptively transferred alloantigen expressing mature CD4⁺ and CD8⁺ T cells, but not B cells, can induce central tolerance to single MHC class I mismatched skin grafts in recipients treated with either myeloablative or non-myeloablative conditioning [36;37]. The demonstration that T cells are able to induce tolerance provides a rational basis for studying the tolerance inducing capability of T cells in order to develop approaches that can exploit this property for eventual clinical application. It is unlikely that all donor antigens expressed in specialized tissues such as the heart could be expressed in host T cells. Thus a major question is whether tolerance to antigens expressed by T cells will generate active regulation to other minor antigens, or linked tolerance. We therefore set out to examine the extent to which delivery of alloantigen by T cells can be used to induce tolerance to fully allogeneic heart transplants when combined with myeloablative or nonmyeloablative host conditioning. Mechanistically, tolerance induced by this approach appears to involve both a deletional and regulatory component, which combine to allow long-term acceptance of fully mismatched heart allografts.

METHODS

Mice

C57BL/6J (*H-2^b*), BALB/cJ (*H-2^d*), CB6F1/J (C57BL/6J × BALB/cJ F1, referred to as CB6F1 hereafter), and CBA/CaJ (*H-2^k*) mice were purchased from The Jackson Laboratory,

Bar Harbor, ME. Mice were housed using micro-isolator conditions in autoclaved cages and maintained on irradiated feed and autoclaved acidified drinking water. All sentinel mice housed in the same colony were viral antibody-free. Four to ten week old mice were used in all experiments. All experiments involved were conducted in accordance with Institutional guidelines.

Antibodies

Monoclonal antibodies specific for *H-2K^d* (SF1-1.1), *H-2K^b* (AF6-88.5), CD3 (2C11), CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD11b (Mac-1, M1/70), CD49b (DX5) and CD11c (HL3), were obtained from BD Biosciences (San Jose, CA). Monoclonal antibody specific for FoxP3 (FJK-16s) was obtained from eBioscience, Inc. (San Diego, CA).

T cell isolation and purification

Spleens from donor mice were harvested and single cell suspensions were prepared. T cells were stained with antibodies specific for CD4 and CD8 followed by goat anti-rat IgG conjugated microbeads. Cells were then positively selected according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). CD4⁺/CD8⁺ B220⁻CD11c⁻CD11b⁻CD49b⁻ cells were then obtained by fluorescence activated cell sorting. After double purification, cells were >99% T cells.

Adoptive transfer of mature T cells into myeloablatively conditioned recipients

Mice received 11 Gy whole body irradiation (WBI) one day prior to bone marrow transplantation. Bone marrow cells were harvested from donor mice treated 7 days prior with 5-fluorouracil (5-FU) (150mg/kg). To prevent contamination of bone marrow with T cells, a depleting dose of anti-CD8 (2mg of 2.43 [38]) and anti-CD4 (2mg of GK1.5 [39]) were given 4 days before bone marrow harvest to eliminate alloreactive T cells *in vivo*. Bone marrow was then harvested and 0.7×10^6 bone marrow cells were transplanted into each recipient as described in [40]. At the time of bone marrow transplantation, mice also received purified donor T cells as described in the text.

Non-myeloablative conditioning regimen

Prior to adoptive cell transfer, recipients treated with the non-myeloablative conditioning regimen received 2.5 Gy WBI. The following day mice received purified donor T cells as described in the text. Recipient mice were also treated with 1.5 mg/kg/day rapamycin (Wyeth, Madison, NJ) diluted in carboxymethylcellulose and polysorbate solution by i.p. injection on days 0–13 relative to T cell transfer and 0.2 mg/day anti-CD154 [41] in PBS on days 0–5, 8, 11 and 14.

Flow cytometry

Cell surface staining and flow cytometry was performed as described previously [40]. Expression of K^b on the cell surface was evaluated as described in [35].

Heart transplantation

Heterotopic vascularized cardiac transplantation was performed by standard microvascular techniques [42]. Graft function was monitored by daily palpation of the transplanted hearts. All heart grafts were harvested on day 120 when experiments were terminated or upon rejection indicated by loss of heart beat measured by palpation.

Histology and immunohistochemistry

Heart grafts were fixed in 10% formaldehyde, embedded with paraffin and cut into 5 μ m sections. The tissue sections were then stained with H&E for evaluation of cellular infiltration. The sections were also stained with anti-CD3 and B220 antibodies (BD Biosciences, San Jose, CA) for cell infiltration as described in [43] as well as Van Giesen staining of elastic tissue fibers for arteriosclerosis scoring. Arteriosclerosis was assessed by light microscopy. The severity of arteriosclerosis was graded according to the percentage of luminal occlusion by intimal thickening using a scoring system previously described [44]. Briefly, a vessel score of 0 indicated a normal artery; 1, <10% luminal occlusion; 2, 20–50% luminal occlusion; 3, >50% luminal occlusion. Only vessels that were cut orthogonally and displayed a clear internal elastic lamina were scored. Images were acquired using a Nikon TE300 microscope and SPOT software (Diagnostic Instruments, Sterling Heights MI). Images were adjusted for brightness contrast and color balance only using Photoshop (Adobe Systems, San Jose, CA) software.

Luminex analysis

Mice were bled upon heart graft rejection or 120 days after heart transplantation. Serum samples were collected and diluted 1:1 in the assay buffer provided, and then were plated on the Millipore (Billerica, MA) Milliplex Mouse Cytokine/Chemokine Panel - 22 Plex 96 well plate. The cytokine profile was analyzed on a Luminex 100™ (Luminex Corporation, Austin, TX) based on manufacturer's instructions. The plate was washed twice and then run on a Luminex 100™. Duplicate wells were used for each sample.

Statistics

Statistics and survival curves were calculated using GraphPad Prism software. P values were calculated using 2-tailed unpaired Students t-test. Significance of the differences observed in Figure 8 were performed using a one sample t-test using a theoretical mean calculated from pooled samples from 4 CB6F1 mice.

RESULTS

Long-term survival of alloantigen expressing T cells after adoptive transfer into mice conditioned using lethal whole body irradiation

To examine the ability of mature T cells to induce tolerance we made use of (C57BL/6J $\times$ BALB/c) F1 (referred to as CB6F1, *H-2^{bxd}*) mice. We reasoned that by adoptively transferring purified CB6F1 T cells into conditioned C57BL/6 mice, it would be possible to specifically deliver BALB/c major (*H-2^d*) and minor histocompatibility antigens to conditioned C57BL/6 recipients and examine their ability to induce tolerance. Using CB6F1 mice makes it possible to deliver alloantigen without inducing a graft vs. host (GvH) response. T cells were purified from the spleens of naïve CB6F1 mice as described previously [36]. All T cell preparations were greater than 99.5% pure based on cell surface staining and flow cytometry (not shown). We first examined the ability of mature CB6F1 T cells to survive long-term after adoptive transfer into mice receiving myeloablative conditioning. C57BL/6 mice were lethally irradiated (11 Gy) and reconstituted the next day with 0.7×10^6 T cell depleted syngeneic C57BL/6 bone marrow cells. At the time of bone marrow transplantation the mice also received 10^7 purified CB6F1 T cells. Mice receiving syngeneic bone marrow cells alone served as controls.

Analysis of H-2K^d expression, which is expressed only on donor cells, in recipient mice revealed that adoptively transferred CD4 and CD8 T cells were detectable in the blood of reconstituted C57BL/6 mice and survived for longer than 24 weeks (Fig. 1A). We did not detect expression of H-2K^d on B220, CD11b, or CD11c expressing cells in recipient mice at

4 weeks (Fig. 1B). The percentage of cells in blood expressing H-2K^d was stable after 8 weeks, with no significant difference detected between 8 and 24 weeks ($P>0.05$). We did not detect expression of H-2K^d on B220, CD11b, or CD11c expressing cells in recipient mice at 24 weeks after transfer (Fig. 1B), indicating that the transferred CB6F1 T cells were not contaminated with hematopoietic progenitors or other cell lineages. T cells expressing H-2K^d were also detected in the thymus (Fig. 1C) and lymph nodes (Fig. 1D) as well as bone marrow and spleen (not shown) 24 weeks after transfer. H-2K^{d+} T cells also expressed H-2K^b indicating that they were derived from the adoptively transferred CB6F1 cells (not shown). These data suggest that CB6F1 T cells survive long-term after transfer into lethally irradiated mice undergoing a syngeneic bone marrow transplant, circulate and re-enter peripheral lymphoid tissue and the host thymus.

Expression of alloantigen on adoptively transferred T cells is sufficient to induce tolerance to fully allogeneic cardiac allografts

We next examined whether the presence of CB6F1 donor T cells in conditioned recipients resulted in tolerance to fully allogeneic BALB/c (H-2^d) allografts. To this end, lethally irradiated C57BL/6 mice undergoing a syngeneic bone marrow transplant received CB6F1 T cells by adoptive cell transfer as described above. Mice receiving T cell-depleted syngeneic bone marrow alone served as controls. Eight to ten weeks after reconstitution, mice received a fully allogeneic BALB/c heart graft. C57BL/6 recipients of adoptively transferred CB6F1 T cells accepted their BALB/c heart grafts long-term (Fig. 2, Median Survival Time (MST) >120 days at which point the experiment was terminated, $n = 6$). In contrast, control mice rapidly rejected BALB/c heart grafts (Fig. 2, MST=8 days, $n = 7$, $P < 0.001$), indicating the mice were immunocompetent and that delivery of alloantigen by T cells was required to achieve long-term survival of fully allogeneic hearts. These results suggest that expression of alloantigen on mature T cells alone is sufficient to induce tolerance to fully allogeneic heart grafts in hosts undergoing myeloablative conditioning.

Long-term persistence of adoptively transferred CB6F1 T cells in hosts receiving non-myeloablative conditioning

We have recently shown that delivery of alloantigen by T cells can be used to induce tolerance to MHC class I mismatched allografts in hosts receiving non-myeloablative conditioning [37]. We therefore next examined the ability of adoptively transferred CB6F1 T cells to persist in hosts receiving non-myeloablative conditioning and induce tolerance. To this end, C57BL/6 mice were given 2.5Gy WBI. The following day the mice received 10^7 purified T cells from CB6F1 mice. On the day of T cell transfer, the mice also received rapamycin (1.5mg/kg) and anti-CD154 (0.2mg). We continued to give rapamycin daily from days 0–13 relative to T cell transfer and anti-CD154 on days 0–5, 8, 11 and 14. As observed in the myeloablative setting, adoptively transferred H-2K^{d+} T cells were detectable in the blood and survived for longer than 16 weeks (Fig. 3A). T cells expressing H-2K^d were also detected in the thymus (Fig. 3B) and lymph nodes (Fig. 3C) as well as bone marrow and spleen (not shown) 42 weeks after transfer. These data suggest that CB6F1 T cells survive long-term after transfer into mice receiving nonmyeloablative conditioning and are able to circulate and re-enter peripheral lymphoid tissue and the host thymus as observed in the myeloablative setting.

Expression of alloantigen on adoptively transferred T cells is sufficient to induce tolerance to fully allogeneic hearts in hosts receiving nonmyeloablative conditioning

We next examined whether the presence of CB6F1 donor T cells in mice receiving nonmyeloablative conditioning resulted in tolerance. To this end, C57BL/6 mice underwent nonmyeloablative conditioning as described above and received 10^7 CB6F1 T cells by adoptive cell transfer. Controls underwent conditioning but did not receive adoptively

transferred T cells. Eight to ten weeks after adoptive T cell transfer, mice in each group received a fully allogeneic BALB/c heart graft. C57BL/6 recipients of adoptively transferred CB6F1 T cells accepted their BALB/c heart grafts long-term (Fig. 4A, Median Survival Time (MST) >120 days at which point the experiment was terminated, $n = 6$). In contrast, control mice rejected BALB/c hearts by 11 days after transplantation (Fig. 4A, MST=9 days, $n = 8$, $P < 0.001$). We then performed the converse experiments in which BALB/c mice received nonmyeloablative conditioning as described, and CB6F1 T cells by adoptive transfer. Eight to ten weeks later the mice received fully allogeneic C57BL/6 heart grafts. Conditioned BALB/c mice that received CB6F1 T cells accepted their C57BL/6 heart grafts indefinitely (Fig. 4B, MST>120 days when the experiments terminated, $n = 5$). In contrast, control mice receiving conditioning alone rejected C57BL/6 hearts within 11 days after transplantation (Fig. 4B, MST=9 days, $n=6$, $P < 0.001$). These results suggest that tolerance induction is not strain specific. Thus, expression of alloantigen on mature T cells alone is sufficient to induce tolerance to heart grafts across a full MHC barrier using a non-myeloablative conditioning regimen.

Tolerance induction through the adoptive cell transfer of mature T cells prevents chronic rejection in fully mismatched heart grafts

One of the major causes of heart allograft loss is chronic rejection. We therefore examined whether the induction of tolerance through the adoptive transfer of mature T cells expressing alloantigen was able to prevent chronic rejection. Lethally irradiated C57BL/6 mice undergoing a syngeneic bone marrow transplant received CB6F1 T cells by adoptive cell transfer, or syngeneic bone marrow only as a control. Eight to ten weeks after T cell transfer, mice received fully-mismatched BALB/c heart grafts. Donor heart grafts were harvested upon rejection or 120 days after transplantation, when experiments were terminated. As expected, cardiac grafts from control recipients that did not receive T cells rejected within ten days. Histological analysis showed substantial CD3⁺ T cell and B220⁺ B cell infiltration of the graft consistent with acute rejection (Fig. 5). In contrast, cardiac grafts from recipients that received allogeneic mature T cells following myeloablative conditioning showed no significant cell infiltration (Fig. 5). When grafts were examined by H&E staining, we were unable to detect significant evidence of chronic rejection.

We next examined grafts from mice receiving T cell transfer and non-myeloablative conditioning as described above. Control mice that received non-myeloablative conditioning, but no T cells, rejected acutely and showed substantial infiltration of CD3⁺ and B220⁺ cells (data not shown) consistent with acute rejection. In contrast, hearts from non-myeloablatively conditioned mice that received mature allogeneic T cells were relatively free of cellular infiltrate, consistent with long-term acceptance of these grafts. In addition, when grafts were examined, minimal evidence of chronic rejection could be detected (Fig. 5).

Tolerance induction through the adoptive cell transfer of mature T cells results in decreased serum levels of pro-inflammatory cytokines

To further characterize the tolerance induced by the adoptive transfer of mature allogeneic T cells, we next examined the cytokine profile in the sera of mice that received cardiac transplants. Serum samples were collected from heart graft recipients 120 days after transplantation, or when the heart graft was rejected. We observed that recipients of allogeneic T cell transfer had significantly less serum IFN- γ when compared with controls (23 ± 3 pg/mL vs. 9 ± 3 pg/mL, $P < 0.001$ Fig. 6A). No significant differences were seen in the concentrations of IL-2 or IL-4. In addition, the serum concentration of IL-15 (23 ± 3 vs. 18 ± 3 $P < 0.03$), IL-9 (83 ± 19 vs. 25 ± 6 $P < 0.001$), IL-1 α (45 ± 2 vs. 35 ± 7 $P < 0.03$), IL-1 β (11 ± 1 vs. 8 ± 2 $P < 0.03$) and IL-6 (375 ± 89 pg/mL vs 21 ± 16 pg/mL, $P < 0.001$)

was significantly higher in control animals that received cardiac grafts but did not receive T cells when compared with mice that received allogeneic T cells in addition to cardiac allografts. Similar results, in which serum levels of IFN- γ , IL-15, IL-9, IL-1 α , IL-1 β and IL-6 were significantly decreased were observed in mice that received non-myeloablative conditioning and adoptive T cell transfer prior to receiving a cardiac allograft when compared with controls that did not receive T cells. However, we additionally observed that the serum concentration of IL-17 was decreased in mice that received non-myeloablative conditioning and adoptive T cell transfer prior to receiving a cardiac allograft when compared with controls that did not receive T cells (Fig 6B, $P < 0.04$).

Adoptive cell transfer of mature T cells causes central deletion

We have previously shown that adoptively transferred mature allogeneic T cells re-enter the thymus and mediate negative selection of newly developing alloreactive T cells [36]. To determine if adoptive transfer of mature fully allogeneic T cells induces central deletion of alloreactive T cells, we made use of differences in mouse endogenous superantigen expression in BALB/c and C57BL/6 mice. BALB/c and CB6F1 mice express the endogenous superantigen Mtv6 [45], resulting in the central deletion of T cells expressing T cell receptors containing V β 3 in these mice. In contrast, V β 3⁺ T cells make up 3–5 percent of the T cell repertoire in naïve C57BL/6, which do not express Mtv6 (Fig. 7A). We examined the frequency of V β 3⁺ T cells in C57BL/6 mice that were lethally irradiated and received syngeneic bone marrow in addition to 10⁷ CB6F1 T cells. Controls underwent conditioning and received syngeneic bone marrow transplants but did not receive CB6F1 T cells. Four weeks after T cell transfer, the frequency of V β 3⁺ CD4⁺ and CD8⁺ T cells was significantly reduced in mice receiving T cells when compared to that in control mice that did not receive T cells (Fig. 7B, $P < 0.001$). As expected, the frequency of V β 3⁺ cells in control mice that received bone marrow alone was similar to that observed in naïve C57BL/6 mice (Fig. 7A, $P > 0.05$). Similar results were observed when the frequency of V β 3⁺ T cells was examined 20 weeks after T cell transfer (data not shown). Furthermore, when mice were treated with the non-myeloablative regimen described above, mice that received T cells had a significantly lower frequency of V β 3⁺ T cells when compared with controls that received conditioning but no T cells (Fig. 7C, $n = 4$, $P < 0.05$). These data suggest that mature allogeneic T cells can induce deletion of T cells in the thymus after adoptive transfer into conditioned hosts.

Adoptive cell transfer of mature T cells increases the frequency of regulatory T cells

We next examined whether peripheral regulation was also involved in induction of tolerance to cardiac grafts following the adoptive transfer of mature allogeneic T cells into conditioned recipients. C57BL/6 mice were lethally irradiated and reconstituted the next day with 0.7 $\times$ 10⁶ T cell depleted syngeneic C57BL/6 bone marrow cells. In addition, mice received 10⁷ CB6F1 T cells, or syngeneic bone marrow only as a control. Four weeks after T cell transfer, we examined the frequency of either donor derived H-2K^{d+} CD4 T cells, or recipient derived H-2K^{d-} CD4 T cells expressing FoxP3 in the peripheral blood. The frequency of donor-derived K^{d+} CD4⁺ cells expressing FoxP3 increased significantly in mice following adoptive T cell transfer when compared with the frequency of CD4⁺ cells expressing FoxP3 in naïve CB6F1 mice (Fig. 8A, $n=4$ $P < 0.05$). In contrast, the frequency of recipient-derived K^{d-} CD4⁺ cells expressing FoxP3 was not significantly different when conditioned mice that received adoptively transferred T cells were compared with mice that received syngeneic bone marrow transplant alone. Similar results were found when recipients of 2.5 Gy irradiation, rapamycin and anti-CD154 in addition to T cell transfer were compared with mock controls. Taken together, there was a significant increase in the frequency of FoxP3 expressing donor-derived cells (Fig. 8B $P < 0.05$).

To further examine whether regulatory T cells are involved in tolerance induction following adoptive transfer of mature T cells, we examined grafts for the presence of FoxP3⁺ cells. C57BL/6 mice were conditioned using lethal irradiation and syngeneic bone marrow transplantation and CB6F1 T cells. Alternately, groups of C57BL/6 mice were conditioned using 2.5Gy total body irradiation, rapamycin and anti-CD154 as described above in addition to CB6F1 T cells. Eight to ten weeks later, all mice received BALB/c heart allografts. 120 days after heart transplantation, grafts were harvested and sections were stained for the presence of FoxP3⁺ cells. Consistent with the general lack of cellular infiltrates in grafts from mice that received myeloablative conditioning, we were able to detect relatively few FoxP3 positive cells in grafts harvested from these mice (Fig. 9). In contrast, when we examined heart grafts from mice that received non-myeloablative conditioning, we were able to detect substantial numbers of FoxP3 positive cells, indicating a greater presence of regulatory cells (Fig. 9).

DISCUSSION

Induction of mixed allogeneic bone marrow chimerism can lead to robust tolerance to donor antigen. However, engraftment of allogeneic bone marrow requires stringent recipient conditioning [46], and is complicated by the potential for graft-versus-host disease [47] or engraftment failure. These drawbacks severely curtail the clinical utility of this approach to tolerance induction. Therefore, it would be beneficial to identify the cell lineages responsible for the induction of tolerance in mixed chimeras so that these cell types rather than whole bone marrow could be used to induce tolerance, potentially circumventing the drawbacks of total bone marrow transplantation.

Towards this end, we have previously shown that expression of an allogeneic MHC class I antigen, H2-K^b, on lymphocytes, but not antigen presenting cells, induced tolerance to skin grafts expressing H2-K^b expressing antigen [35]. Further studies showed that both mature CD4⁺ and CD8⁺ T cells, but not B cells, delivered allogeneic MHC class I antigen to the thymus and mediated central deletion of K^b-reactive T cells [36;37;48]. Thus, adoptive transfer of mature CD4⁺ and CD8⁺ T cells expressing H2-K^b led to tolerance induction, and indefinite survival of skin grafts expressing H2-K^b. These observations raised the possibility that adoptive transfer of mature allogeneic T cells could be used to induce tolerance to organ allografts.

The use of non-myeloablative conditioning regimens would be desirable for clinical applications. We therefore showed that adoptive transfer of mature T cells into mice non-myeloablatively conditioned with 2.5Gy total body irradiation, rapamycin and anti-CD154 at the time of T cell transfer resulted in a state of durable tolerance. We wish to point out that tolerance induced by adoptive transfer of mature T cells is mechanistically distinct from tolerance induced by donor specific transfusion (DST) in conjunction with co-stimulatory blockade. Tolerance induced by adoptive transfer of mature T cells is dependent on central deletion of developing alloreactive T cells [36;37;48]. In contrast, tolerance through DST/co-stimulatory blockade is primarily dependent on the generation of peripheral regulatory mechanisms [49]. This difference may explain why tolerance induction through the adoptive transfer of mature T cells results in durable tolerance to skin grafts, while co-stimulatory blockade/DST prolongs, but does not induce tolerance to, skin grafts.

Having shown that adoptive transfer of mature T cells is able to induce robust tolerance to skin allografts, we set out to extend these findings. We have examined the ability of mature T cell transfer to induce tolerance to fully allogeneic cardiac allografts transplanted into conditioned recipients that received adoptive transfer of mature allogeneic T cells. In conditioned recipients of T cell transfer and syngeneic bone marrow transplantation, fully

mismatched solid organ allografts survived indefinitely. When heart grafts in recipients of mature T cell transfer were examined by histology, no evidence of chronic rejection was observed. Since chronic rejection is one of the major barriers to successful heart transplantation, this observation is significant for the potential clinical utility of this approach. In this context, it is interesting to note that we observed significantly decreased systemic levels of pro-inflammatory cytokines such as IL-1 α , IL-1 β and IL-6, which have been associated with the development of chronic rejection [50;51;52;53;54]. These data indicate that adoptive transfer of mature T cells can induce tolerance to heart grafts across full allogeneic mismatches. Thus we show here for the first time that adoptive transfer of mature T cells induces tolerance to both MHC class I and MHC class II alloantigens.

To use this strategy for tolerance induction in a clinical setting, it would be desirable to avoid stringent conditioning regimens. We therefore examined whether non-myeloablative conditioning was sufficient to allow engraftment of adoptively transferred allogeneic T cells, and induction of tolerance to organ allografts. Since it has recently been shown that alloantigen persistence is required to maintain transplant tolerance [55], we examined the persistence of allogeneic T cells transferred into recipients conditioned with 2.5 Gy whole body irradiation combined with transient injection of rapamycin and co-stimulatory blockade. Adoptively transferred donor derived T cells were able to survive long-term in all recipient lymphoid organs, demonstrating that this regimen is sufficient to allow T cell engraftment. In addition, we demonstrate that fully-allogeneic heart transplants survive indefinitely in non-myeloablatively conditioned mice that receive adoptively transferred T cells, and display minimal evidence of chronic rejection. This indicates that adoptive transfer of allogeneic T cells is able to induce tolerance to organ allografts in the absence of treatments that ablate the pre-existing T cell repertoire. These data suggest that there may be a peripheral component to tolerance induction in mice that receive non-myeloablative conditioning. Indeed, we were able to detect evidence of both central deletional mechanisms of tolerance, and generation of regulatory T cells which may affect peripheral tolerance.

To examine the role of central deletion in tolerance induction in this model, we utilized endogenous expression of murine mammary tumor virus. In BALB/c mice, V β 3⁺ cells are centrally deleted in the thymus due to expression of the endogenous superantigen Mtv6 [45]. It has previously been shown that purified peripheral T cells are potent tolerance inducers to endogenous mouse mammary tumor virus super-antigens (vSAGs). Mature T cells expressing vSAGs are able to re-circulate to the thymus and deliver the soluble vSAGs to APCs expressing MHC class II which then mediate negative selection of vSAG reactive V β 6⁺ T cells [56]. When mature T cells from CB6F1 donors, which express Mtv6 superantigen [57] were adoptively transferred to either myeloablatively or non-myeloablatively conditioned C57BL/6 hosts, V β 3⁺ cells were partially deleted. Since murine T cells do not express cell surface MHC class II [58], it appears likely that the superantigen expressed by T cells is able to bind both V β 3⁺ T cell receptors, and MHC class II present on endogenous cells in the thymus to mediate negative selection. These data imply therefore, that intracellular as well as cell surface antigens delivered to thymus by mature T cells can mediate negative selection. While we have previously shown that mature T cells are able to mediate negative selection of alloreactive T cells developing in the thymus, here we observed only partial deletion. We suggest that this may be due in part to the absence of I-E expression in recipient C57BL/6 mice, which has been shown to negatively affect the efficiency of superantigen mediated V β deletion [59]. Thus, while central deletion occurred in the presence of adoptively transferred mature allogeneic T cells, it was not complete despite achieving complete tolerance to cardiac allografts. These data suggest that in addition to central deletion, peripheral regulation of T cell responses may also play a role in tolerance induction.

To examine the role of peripheral mechanisms of tolerance, we analyzed the frequency of regulatory CD4⁺ FoxP3 expressing T cells. We observed that donor-derived FoxP3⁺ cells were enriched in the peripheral blood of recipients of mature allogeneic T cells when compared with naïve donor T cells. We have previously shown that homeostatic expansion is required for the re-entry of mature T cells into the thymus, and is a necessary component of mature T cell transfer induced tolerance. Homeostatic expansion also preferentially increases regulatory T cells [60], and therefore may contribute to the generation of peripheral regulatory T cells as well as allowing for thymic re-entry and central deletion. We further show that FoxP3 expressing cells could be detected in grafts harvested from recipients of mature T cell transfer, suggesting that regulatory T cells infiltrate these grafts and may control allogeneic immune responses. These cells were especially prevalent in grafts from animals that received non-myeloablative conditioning, perhaps as a result of failing to ablate the pre-existing T cell repertoire.

We have previously demonstrated that the adoptive transfer of mature T cells alone results in central deletion of alloreactive T cells in both myeloablative and non-myeloablative systems [36;37]. Furthermore, the adoptive transfer of mature T cells following non-myeloablative conditioning results in peripheral deletion of alloreactive T cells [37]. Here our data suggest that, regulatory T cells may play an important role in the establishment of tolerance following adoptive T cell transfer. This observation is similar to what is observed in molecular chimeras, with the generation of a strong regulatory T cell component in addition to central deletion in both myeloablative and non-myeloablative settings [61;62;63]. In contrast, in models of mixed bone marrow chimerism using myeloablative conditioning or non-myeloablative conditioning, the dominant mechanism appears to be central deletion, with little or no role for peripheral mechanisms such as regulatory T cells [64;65]. Even when conditioning that strongly favors the development of regulatory T cells is used, Treg do not appear to be required for or involved in the maintenance of tolerance [66] in mixed bone marrow chimeras (Table I).

Together our data demonstrate that mature allogeneic T cells are able to induce and maintain tolerance to heart grafts across a full MHC barrier. The observation that T cells can induce tolerance to MHC class I, MHC class II, as well as to minor histocompatibility antigens substantially increase the potential clinical utility of this approach. In addition, we show that non-myeloablative conditioning regimens are sufficient to allow for engraftment of allogeneic T cells, raising the possibility that T cells may be used to induce tolerance without the drawbacks associated with bone marrow transplantation. Thus, mature T cells are able to actively participate in the induction and maintenance of tolerance to heart grafts across a full MHC barrier, in which both central deletion and peripheral regulation of alloreactive cells may play an important role.

Acknowledgments

The authors wish to thank Jonathan Godwin for technical assistance. C.T. is supported by an American Society of Transplantation (AST)/Roche Basic Science Faculty Development Grant. J.B. is supported in part by a grant from the American Heart Association. P.J. was supported by a Ruth L. Kirschstein Institutional National Research Service Award (T32AI070085) and an AST/Wyeth Basic Science Fellowship Grant. This work was supported by National Institutes of Health Grants P01 AI041521 and P01 AI056299 to M.H.S.; R01 AI043619, R01 AI053666 and R01 AI070601 to J.I. Support for this work was also provided to M.H.S. by The Juvenile Diabetes Research Foundation Center Grant on Immunological Tolerance in Type I Diabetes.

REFERENCES

1. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008;133:775–787. [PubMed: 18510923]

2. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. *Cell* 1987;49:273–280. [PubMed: 3494522]
3. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* 2007;8:191–197. [PubMed: 17136045]
4. Billingham RE, Brent L, Medawar PB. Activity acquired tolerance of foreign cells. *Nature* 1953;172:603–606. [PubMed: 13099277]
5. Slavin S, Strober S, Fuks Z, Kaplan HS. Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts. *J Exp Med* 1977;146:34–48. [PubMed: 17647]
6. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature* 1984;307:168–170. [PubMed: 6361574]
7. Wekerle T, Sykes M. Mixed chimerism and transplantation tolerance. *Annu Rev Med* 2001;52:353–370. [PubMed: 11160784]
8. Socie G, Blazar BR. Acute graft-versus-host disease: from the bench to the bedside. *Blood* 2009;114:4327–4336. [PubMed: 19713461]
9. Daly A, McAfee S, Dey B, Colby C, Schulte L, Yeap B, Sackstein R, Tarbell NJ, Sachs D, Sykes M, Spitzer TR. Nonmyeloablative bone marrow transplantation: Infectious complications in 65 recipients of HLA-identical and mismatched transplants. *Biol Blood Marrow Transplant* 2003;9:373–382. [PubMed: 12813445]
10. Parkman R, Weinberg KI. Immunological reconstitution following bone marrow transplantation. *Immunol Rev* 1997;157:73–78. [PubMed: 9255623]
11. Williams MA, Adams AB, Walsh MB, Shirasugi N, Onami TM, Pearson TC, Ahmed R, Larsen CP. Primary and secondary immunocompetence in mixed allogeneic chimeras. *J Immunol* 2003;170:2382–2389. [PubMed: 12594261]
12. Clift RA, Storb R. Histoincompatible bone marrow transplants in humans. *Annu Rev Immunol* 1987;5:43–64. [PubMed: 3297107]
13. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol* 2007;25:139–170. [PubMed: 17129175]
14. Knechtle SJ. Knowledge about transplantation tolerance gained in primates. *Curr Opin Immunol* 2000;12:552–556. [PubMed: 11007358]
15. Fehr T, Sykes M. Tolerance induction in clinical transplantation. *Transpl Immunol* 2004;13:117–130. [PubMed: 15380542]
16. Toh HC, Spitzer TR, Preffer F, Alexander SI, McAfee S, Dombkowski D, Clark JS, Colby C, Saidman S, Sackstein R, Sykes M. Fluctuating lymphocyte chimerism, tolerance and anti-tumor response in a patient with refractory lymphoma receiving nonmyeloablative conditioning and a haploidentical related allogeneic bone marrow transplant. *Cytokines Cell Mol Ther* 2002;7:43–47. [PubMed: 12607794]
17. Dey BR, McAfee S, Colby C, Cieply K, Caron M, Saidman S, Preffer F, Shaffer J, Tarbell N, Sackstein R, Sachs D, Sykes M, Spitzer TR. Anti-tumour response despite loss of donor chimaerism in patients treated with non-myeloablative conditioning and allogeneic stem cell transplantation. *Br J Haematol* 2005;128:351–359. [PubMed: 15667537]
18. Zinkernagel RM, Althage A, Callahan G, Welsh RM Jr. On the immunocompetence of H-2 incompatible irradiation bone marrow chimeras. *J Immunol* 1980;124:2356–2365. [PubMed: 6444970]
19. Russell PS, Chase CM, Sykes M, Ito H, Shaffer J, Colvin RB. Tolerance, mixed chimerism, and chronic transplant arteriopathy. *J Immunol* 2001;167:5731–5740. [PubMed: 11698446]
20. Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B. Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. *Am J Transplant* 2004;4:378–383. [PubMed: 14961990]
21. Womer KL, Lee RS, Madsen JC, Sayegh MH. Tolerance and chronic rejection. *Philos Trans R Soc Lond B Biol Sci* 2001;356:727–738. [PubMed: 11375075]
22. Wood K, Sachs DH. Chimerism and transplantation tolerance: cause and effect. *Immunol Today* 1996;17:584–587. discussion 588. [PubMed: 8991291]

23. Ferguson TA, Kazama H. Signals from dying cells: tolerance induction by the dendritic cell. *Immunol Res* 2005;32:99–108. [PubMed: 16106062]
24. Enk AH. Dendritic cells in tolerance induction. *Immunol Lett* 2005;99:8–11. [PubMed: 15894104]
25. Lin HH, Faunce DE, Stacey M, Terajewicz A, Nakamura T, Zhang-Hoover J, Kerley M, Mucenski ML, Gordon S, Stein-Streilein J. The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J Exp Med* 2005;201:1615–1625. [PubMed: 15883173]
26. Jenkinson EJ, Jhittay P, Kingston R, Owen JJ. Studies of the role of the thymic environment in the induction of tolerance to MHC antigens. *Transplantation* 1985;39:331–333. [PubMed: 3156439]
27. Gao EK, Lo D, Sprent J. Strong T cell tolerance in parent----F1 bone marrow chimeras prepared with supralethal irradiation. Evidence for clonal deletion and anergy. *J Exp Med* 1990;171:1101–1121. [PubMed: 2139098]
28. Tomita Y, Khan A, Sykes M. Mechanism by which additional monoclonal antibody (mAb) injections overcome the requirement for thymic irradiation to achieve mixed chimerism in mice receiving bone marrow transplantation after conditioning with anti-T cell mABs and 3-Gy whole body irradiation. *Transplantation* 1996;61:477–485. [PubMed: 8610364]
29. Shimonkevitz RP, Bevan MJ. Split tolerance induced by the intrathymic adoptive transfer of thymocyte stem cells. *J Exp Med* 1988;168:143–156. [PubMed: 2899619]
30. Schulz R, Mellor AL. Self major histocompatibility complex class I antigens expressed solely in lymphoid cells do not induce tolerance in the CD4+ T cell compartment. *J Exp Med* 1996;184:1573–1578. [PubMed: 8879232]
31. Weissman IL, Jerabek L, Greenspan S. Tolerance and the H–Y antigen: Requirement for male T cells, but not B cells, to induce tolerance in neonatal female mice. *Transplantation* 1984;37:3–6. [PubMed: 6364485]
32. Umemura A, Morita H, Li XC, Tahan S, Monaco AP, Maki T. Dissociation of hemopoietic chimerism and allograft tolerance after allogeneic bone marrow transplantation. *J Immunol* 2001;167:3043–3048. [PubMed: 11544287]
33. Anderson CC, Matzinger P. Immunity or tolerance: opposite outcomes of microchimerism from skin grafts. *Nat Med* 2001;7:80–87. [PubMed: 11135620]
34. Bagley J, Tian C, Iacomini J. New approaches to the prevention of organ allograft rejection and tolerance induction. *Transplantation* 2007;84:S38–S41. [PubMed: 17632412]
35. Tian C, Bagley J, Iacomini J. Expression of antigen on mature lymphocytes is required to induce T cell tolerance by gene therapy. *J Immunol* 2002;169:3771–3776. [PubMed: 12244171]
36. Tian C, Bagley J, Forman D, Iacomini J. Induction of central tolerance by mature T cells. *J Immunol* 2004;173:7217–7222. [PubMed: 15585843]
37. Tian C, Yuan X, Bagley J, Blazar BR, Sayegh MH, Iacomini J. Induction of transplantation tolerance by combining non-myeloablative conditioning with delivery of alloantigen by T cells. *Clin Immunol* 2008;127:130–137. [PubMed: 18280792]
38. Sarmiento M, Glasebrook AL, Fitch FW. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytotoxicity in the absence of complement. *J Immunol* 1980;125:2665–2672. [PubMed: 6159417]
39. Dialynas DP, Quan ZS, Wall KA, Pierres A, Quintans J, Loken MR, Pierres M, Fitch FW. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J Immunol* 1983;131:2445–2451. [PubMed: 6415170]
40. Bagley J, Tian C, Sachs DH, Iacomini J. Induction of T-cell tolerance to an MHC class I alloantigen by gene therapy. *Blood* 2002;99:4394–4399. [PubMed: 12036867]
41. Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA, Aruffo A. A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proc Natl Acad Sci U S A* 1992;89:6550–6554. [PubMed: 1378631]
42. Corry RJ, Winn HJ, Russell PS. Heart transplantation in congenic strains of mice. *Transplant Proc* 1973;5:733–735. [PubMed: 4572133]
43. Tian C, Bagley J, Cretin N, Seth N, Wucherpfennig KW, Iacomini J. Prevention of type 1 diabetes by gene therapy. *J Clin Invest* 2004;114:969–978. [PubMed: 15467836]

44. Lee RS, Yamada K, Houser SL, Womer KL, Maloney ME, Rose HS, Sayegh MH, Madsen JC. Indirect recognition of alloptides promotes the development of cardiac allograft vasculopathy. *Proc Natl Acad Sci U S A* 2001;98:3276–3281. [PubMed: 11248069]
45. Acha-Orbea H, MacDonald HR. Superantigens of mouse mammary tumor virus. *Annu Rev Immunol* 1995;13:459–486. [PubMed: 7612231]
46. van Bekkum DW. Conditioning regimens for marrow grafting. *Semin Hematol* 1984;21:81–90. [PubMed: 6377501]
47. Ferrara JL, Cooke KR, Teshima T. The pathophysiology of acute graft-versus-host disease. *Int J Hematol* 2003;78:181–187. [PubMed: 14604275]
48. Tian C, Bagley J, Iacomini J. Homeostatic expansion permits T cells to re-enter the thymus and deliver antigen in a tolerogenic fashion. *Am J Transplant* 2007;7:1934–1941. [PubMed: 17617856]
49. Sho M, Kishimoto K, Harada H, Livak M, Sanchez-Fueyo A, Yamada A, Zheng XX, Strom TB, Basadonna GP, Sayegh MH, Rothstein DM. Requirements for induction and maintenance of peripheral tolerance in stringent allograft models. *Proc Natl Acad Sci U S A* 2005;102:13230–13235. [PubMed: 16150717]
50. Rao DA, Pober JS. Endothelial injury, alarmins, and allograft rejection. *Crit Rev Immunol* 2008;28:229–248. [PubMed: 19024347]
51. Vamvakopoulos JE, Taylor CJ, Green C, McNeil K, Wallwork J, Goodman R, Metcalf SM. Interleukin 1 and chronic rejection: possible genetic links in human heart allografts. *Am J Transplant* 2002;2:76–83. [PubMed: 12095061]
52. Lattmann T, Hein M, Horber S, Ortmann J, Teixeira MM, Souza DG, Haas E, Tornillo L, Munter K, Vetter W, Barton M. Activation of pro-inflammatory and anti-inflammatory cytokines in host organs during chronic allograft rejection: role of endothelin receptor signaling. *Am J Transplant* 2005;5:1042–1049. [PubMed: 15816884]
53. Booth AJ, Csencsits-Smith K, Wood SC, Lu G, Lipson KE, Bishop DK. Connective Tissue Growth Factor Promotes Fibrosis Downstream of TGFbeta and IL-6 in Chronic Cardiac Allograft Rejection. *Am J Transplant*. 2009
54. Diaz JA, Booth AJ, Lu G, Wood SC, Pinsky DJ, Bishop DK. Critical role for IL-6 in hypertrophy and fibrosis in chronic cardiac allograft rejection. *Am J Transplant* 2009;9:1773–1783. [PubMed: 19538487]
55. Tian C, Bagley J, Iacomini J. Persistence of Antigen Is Required to Maintain Transplantation Tolerance Induced by Genetic Modification of Bone Marrow Stem Cells. *Am J Transplant*. 2006
56. Webb SR, Sprent J. Induction of neonatal tolerance to Mlsa antigens by CD8+ T cells. *Science* 1990;248:1643–1646. [PubMed: 1973003]
57. Waanders GA, Lees RK, Held W, MacDonald HR. Quantitation of endogenous mouse mammary tumor virus superantigen expression by lymphocyte subsets. *Eur J Immunol* 1995;25:2632–2637. [PubMed: 7589137]
58. Schooten E, Klous P, van den Elsen PJ, Holling TM. Lack of MHC-II expression in activated mouse T cells correlates with DNA methylation at the CIITA-PIII region. *Immunogenetics* 2005;57:795–799. [PubMed: 16235089]
59. Beutner U, McLellan B, Kraus E, Huber BT. Lack of MMTV superantigen presentation in MHC class II-deficient mice. *Cell Immunol* 1996;168:141–147. [PubMed: 8640859]
60. Zhang H, Chua KS, Guimond M, Kapoor V, Brown MV, Fleisher TA, Long LM, Bernstein D, Hill BJ, Douek DC, Berzofsky JA, Carter CS, Read EJ, Helman LJ, Mackall CL. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. *Nat Med* 2005;11:1238–1243. [PubMed: 16227988]
61. Kang ES, Iacomini J. Induction of central deletional T cell tolerance by gene therapy. *J Immunol* 2002;169:1930–1935. [PubMed: 12165518]
62. Forman D, Kang ES, Tian C, Paez-Cortez J, Iacomini J. Induction of alloreactive CD4 T cell tolerance in molecular chimeras: a possible role for regulatory T cells. *J Immunol* 2006;176:3410–3416. [PubMed: 16517709]
63. Forman D, Tian C, Iacomini J. Induction of donor-specific tolerance in sublethally irradiated recipients by gene therapy. *Mol Ther* 2005;12:353–359. [PubMed: 15939670]

64. Khan A, Tomita Y, Sykes M. Thymic dependence of loss of tolerance in mixed allogeneic bone marrow chimeras after depletion of donor antigen. Peripheral mechanisms do not contribute to maintenance of tolerance. *Transplantation* 1996;62:380–387. [PubMed: 8779687]
65. Kurtz J, Shaffer J, Lie A, Anosova N, Benichou G, Sykes M. Mechanisms of early peripheral CD4 T-cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion but not regulatory cells. *Blood* 2004;103:4336–4343. [PubMed: 14962909]
66. Domenig C, Sanchez-Fueyo A, Kurtz J, Alexopoulos SP, Mariat C, Sykes M, Strom TB, Zheng XX. Roles of deletion and regulation in creating mixed chimerism and allograft tolerance using a nonlymphoablative irradiation-free protocol. *J Immunol* 2005;175:51–60. [PubMed: 15972631]

Figure 1. Long-term survival of adoptively transferred mature T cells in fully MHC mismatched recipients

(A) Lethally irradiated C57BL/6 mice were reconstituted with 0.7×10^6 T cell depleted syngeneic bone marrow cells and received either 10^7 purified CB6F1 T cells (squares) or no T cells as a mock control (diamonds). Peripheral blood mononuclear cells were harvested from 4 to 24 weeks after reconstitution and stained with antibodies specific for H-2K^d (K^d). Shown are representative data from one experiment of twelve. Three to four mice were used in each group for each experiment. (B) Lethally irradiated C57BL/6 mice were reconstituted with 0.7×10^6 T cell depleted syngeneic bone marrow cells and received either 10^7 purified CB6F1 T cells (T cell) or received no T cells as a mock control (Mock). Blood was harvested at 4 weeks and 24 weeks after reconstitution and stained with antibodies specific for K^d, CD4, CD8, B220, CD11b and CD11c prior to analysis by flow cytometry. Shown is mock at 4 weeks (left panels) and T cell transfer groups at 4 weeks (middle panels) and 24 weeks (right panels). (C) Thymus and (D) lymph nodes were harvested 4 weeks after reconstitution, and stained with antibodies specific for K^d, CD4 and CD8 prior to analysis by flow cytometry. In each panel, the frequencies of cells in each quadrant are shown. Quadrants in which the percentage of K^d⁺ cells could not be detected (<0.1%) are marked "U". Shown are representative data from one experiment of twelve.

Figure 2. Mature T cells induce tolerance to fully mismatched donor heart grafts

Lethally irradiated C57BL/6 mice were reconstituted with 0.7×10^6 T cell depleted syngeneic bone marrow cells and received either 10^7 purified CB6F1 T cells (open squares, $n = 6$) or syngeneic bone marrow alone (closed squares, $n = 7$). Eight to ten weeks after T cell transfer, mice received a BALB/c heart graft. Shown are combined results of heart graft survival from two independent experiments.

Figure 3. Long-term survival of adoptively transferred mature T cells in non-myeloablatively conditioned fully MHC mismatched recipients
 (A) C57BL/6 mice were given 2.5Gy WBI, rapamycin (1.5mg/kg) and anti-CD154 (0.2mg). The following day the mice received 10^7 T cells from CB6F1 mice (squares) or no T cells as a mock control (diamonds). Peripheral blood mononuclear cells were harvested from 6 to 16 weeks after reconstitution and stained with antibodies specific for K^d. Shown are representative data from one experiment of six. Three to four mice were used in each group of each experiment. (B) C57BL/6 mice were given 2.5Gy WBI, rapamycin (1.5mg/kg) and anti-CD154 (0.2mg). The following day the mice received 10^7 T cells from CB6F1 (T cell) or no T cells as a mock control (Mock). (B) Thymus and (C) lymph nodes were harvested 42

weeks after T cell transfer and stained with antibodies specific for CD4, CD8 and K^d prior to analysis by flow cytometry. Because of the low frequency of donor derived T cells present in the thymus, data are presented gated on single positive CD4 or CD8 T cells using a logarithmic scale. In each panel, the frequencies of cells in each quadrant are shown. Quadrants in which the percentage of cells could not be detected (<0.1%) are marked “U”. Shown are representative data from one experiment of six.

Figure 4. Non-myeloablative conditioning is sufficient to allow mature T cells to induce tolerance to donor heart grafts across a full MHC barrier

(A) C57BL/6 mice were given 2.5Gy WBI, rapamycin (1.5mg/kg) and anti-CD154 (0.2mg). The following day the mice received 10^7 T cells from CB6F1 mice (open squares $n = 6$) or no T cells as a mock control (closed squares $n = 8$). Eight to ten weeks after T cell transfer, mice received a BALB/c heart graft. Shown are combined results of heart graft survival from two independent experiments. (B) BALB/c mice were given 2.5Gy WBI, rapamycin (1.5mg/kg) and anti-CD154 (0.2mg). Mice received either 10^7 purified CB6F1 T cells (open squares, $n = 5$) or received no T cells as mock controls (closed squares, $n = 6$). Eight to ten weeks after T cell transfer, mice received a C57BL/6 heart graft. Shown are combined results of heart graft survival from two independent experiments.

Figure 5. Tolerance induction through the adoptive cell transfer of mature T cells prevents chronic rejection in fully mismatched heart allografts

Eight to ten weeks after adoptive T cell transfer, mice treated with either myeloablative regimen (middle column) or non-myeloablative regimen (right column) received a fully mismatched heart graft as described in the text. Heart grafts were harvested 120 days after heart transplantation. Allogeneic heart grafts in mock control mice treated with myeloablative regimen (left column) or non-myeloablative regimen (not shown) were all rejected by 11 days after transplantation and were also harvested upon rejection. Heart grafts were then fixed, paraffin-embedded, sectioned, and stained with H&E (first row), or anti-CD3 (second row), anti-B220 (third row) antibodies. Shown are representative sections from 6 independent experiments.

Figure 6. Adoptive transfer of mature T cells results in decreased serum levels of pro-inflammatory cytokines

Eight to ten weeks after adoptive T cell transfer, C57BL/6 mice received a fully mismatched heart graft as described in the text. Serum samples were collected 120 days after heart transplantation, or when heart grafts were rejected. Cytokine profiles were analyzed using a 22 analyte Multiplex kit. A. Shown are the concentrations of cytokines in the serum of mice receiving T cells (white bars) or no T cells (black bars). B. Shown are the concentrations of cytokines in the serum of mice receiving non-myeloablative conditioning and T cells (white bars) or no T cells (black bars). Experiments were performed in triplicate. Standard error is shown. Cytokine concentrations which differ significantly ($P < 0.05$) are indicated by an asterisk. Shown are representative data from one experiment of two.

Figure 7. Adoptive transfer of mature T cells results in central deletion

Lethally irradiated C57BL/6 mice were reconstituted with 0.7×10^6 T cell depleted syngeneic bone marrow cells and received either 10^7 purified CB6F1 T cells (T cells) or syngeneic bone marrow alone (Mock). Four weeks after T cell transfer, peripheral blood mononuclear cells were stained with antibodies to Vβ3, CD4 and CD8. (A) Shown are representative flow cytometric data from one experiment of two. Six to seven mice were used in each experiment. Naïve C57BL/6 and CB6F1 were used as staining controls. Plots are gated on live CD4 or CD8 positive cells. The frequency of Vβ3 positive CD4 or CD8 T cells is shown in the upper right quadrant. (B) Shown are frequencies of Vβ3⁺ CD4 or CD8 T cells in recipients of T cells (white bars) or controls that did not receive T cells (black bars) four weeks after T cell transfer. Shown are combined data from two experiments. Six to seven mice were used in each experiment. (C) C57BL/6 mice were given 2.5Gy WBI, rapamycin (1.5mg/kg) and anti-CD154 (0.2mg). The next day mice received CB6F1 T cells (T cells) or no cells (Mock) as a control. 4 weeks after T cell transfer peripheral blood mononuclear cells were harvested and stained with antibodies specific for Vβ3, CD4 and CD8 prior to analysis by flow cytometry. Shown are representative flow cytometry data from one of two independent experiments. The frequency of Vβ3⁺ cells is noted in the upper right quadrant of each graph.

Figure 8. FoxP3 expressing CD4 T cells are increased following mature T cell transfer

Peripheral blood mononuclear cells were harvested from C57BL/6 mice 8 weeks after T cell transfer and stained with antibodies specific for H-2K^d, CD4 and FoxP3. (A) Shown are donor derived K^d⁺ T cells (top row) or recipient derived K^d⁻ T cells (bottom row). Naïve CB6F1 and mock mice were used as controls (left column). The frequency of FoxP3⁺ cells in CD4⁺ T cells is shown. Shown are representative data from one experiment of two. (B) Frequency of FoxP3 expression in donor derived K^d⁺ CD4 T cells. Shown are combined results of peripheral blood mononuclear cells harvested from mice that were conditioned myeloablatively, and non-myeloablatively (n=8 black bar) 8 weeks after T cell transfer.

Pooled blood from naïve CB6F1 mice was used as a control (n=4 white bar). $P = 0.045$ indicated by asterisk.

Figure 9. FoxP3⁺ T cells can be detected in heart grafts from recipients of mature T cell transfer C57BL/6 mice were conditioned with 11Gy irradiation (left panel), or 2.5Gy irradiation, rapamycin and anti-CD154 as described in text (right panel) and received mature T cells purified from the spleens of CB6F1 mice. Eight to ten weeks after T cells transfer, mice received BALB/c heart allografts. 120 days later, heart grafts were harvested and analyzed for the presence of FoxP3 expressing cells. Shown is representative data from two independent experiments.

Table 1

Mechanisms of Tolerance Induction

Method		Tolerance induction		
		Central	Peripheral	
Treatment	Conditioning	Deletion	Deletion	Regulatory T cells
Bone marrow chimerism	Myeloablative [64]	+++	–	–
Bone marrow chimerism	Non-myeloablative [65,66]	++	++	+/-
Molecular chimerism	Myeloablative [61,62]	++	–	++
Molecular chimerism	Non-myeloablative [63]	++	++	++
T cell transfer	Myeloablative [36]	++	–	+
T cell transfer	Non-myeloablative [37]	++		++
Donor specific transfusion	Co-stimulatory blockade [49]	–	–	+++