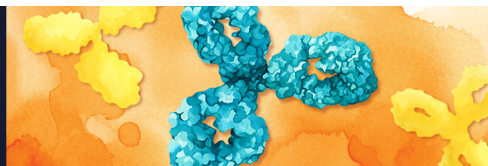


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# Immune Tolerance to Combined Organ and Bone Marrow Transplants After Fractionated Lymphoid Irradiation Involves Regulatory NK T Cells and Clonal Deletion<sup>1</sup>

Masanori Higuchi,\* Defu Zeng,\* Judith Shizuru,<sup>†</sup> Jennifer Gworek,<sup>†</sup> Sussan Dejbakhsh-Jones,\* Masaru Taniguchi,<sup>‡</sup> and Samuel Strober<sup>2\*</sup>

Immune tolerance to organ transplants has been reported in laboratory animals and in humans after nonmyeloablative conditioning of the host and infusion of donor bone marrow cells. We examined the mechanisms of immune tolerance to mouse cardiac allografts in MHC-mismatched hosts that developed mixed chimerism after posttransplant conditioning with a 2-wk course of multiple doses of lymphoid tissue irradiation, depletive anti-T cell Abs, and an infusion of donor bone marrow cells. When CD1<sup>-/-</sup> or J<sub>α</sub>281<sup>-/-</sup> hosts with markedly reduced NK T cells were used instead of wild-type hosts, then the conditioning regimen failed to induce tolerance to the heart allografts despite the development of mixed chimerism. Tolerance could be restored to the CD1<sup>-/-</sup> hosts by infusing enriched T cells from the bone marrow of wild-type mice containing CD1-reactive T cells but not from CD1<sup>-/-</sup> host-type mice. Tolerance could not be induced in either IL-4<sup>-/-</sup> or IL-10<sup>-/-</sup> hosts given the regimen despite the development of chimerism and clonal deletion of host T cells to donor MHC-Ags in the IL-10<sup>-/-</sup> hosts. We conclude that immune tolerance to bone marrow transplants involves clonal deletion, and tolerance to heart allografts in this model also involves regulatory CD1-reactive NK T cells. *The Journal of Immunology*, 2002, 169: 5564–5570.

Immune tolerance to skin allografts was first achieved by establishing chimerism after the injection of donor bone marrow cells into neonatal hosts (1). Subsequently, there have been many attempts to induce tolerance to organ allografts in adult laboratory animals with the ultimate goal of developing protocols that have clinical application. Using the approach of combined organ and bone marrow transplantation in hosts conditioned with nonmyeloablative regimens, tolerance has been achieved in rodents, monkeys, mini-swine, and, more recently, in humans (2–7). However, in some studies acceptance of the marrow transplants as judged by the development of mixed chimerism was associated with the rejection of skin grafts or heart grafts (5, 8). Initial reports of radiation chimeras that rejected donor skin grafts suggested that skin-specific transplantation Ags explained the rejection, since the infusion of epidermal cells into chimeras resulted in skin graft acceptance (9, 10).

The above studies may have important implications for clinical transplantation and suggest that acceptance of bone marrow transplants will not necessarily result in the acceptance of other donor tissue transplants due to either tissue-specific transplantation Ags

or to differences in the immunogenicity or susceptibility of different tissues to immune rejection. However, there is considerable variability of skin and s.c. myocardial graft acceptance in mixed chimeras depending upon the host-conditioning regimen that is used, the level of donor T cell chimerism achieved, and the time interval between transplantation of the donor bone marrow/hemopoietic progenitor cells and the organ graft (2, 3, 11, 12). In some studies, there is uniform acceptance of these organ grafts (2, 3, 11) and in others the majority of the organ grafts are rejected despite high levels of chimerism (5, 12).

The object of the current study in mice was to elucidate the mechanisms by which mixed chimeras accept or reject s.c. heart grafts using a completely posttransplant-conditioning regimen in which donor bone marrow cells are infused after the transplantation of the heart graft. This posttransplant-conditioning regimen consisting of total lymphoid irradiation (TLI)<sup>3</sup> and anti-thymocyte globulin has been previously shown to induce mixed chimerism and tolerance to vascularized heart grafts in completely MHC-mismatched rats (13, 14). A key advantage of the posttransplant regimen is that it can be applied to human cadaver organ transplantation. Because the timing of the availability of cadaver organs cannot be predicted or planned, pretransplant-conditioning regimens cannot be used in the clinical setting. In addition, tolerance induction using the TLI and anti-thymocyte globulin regimen is facilitated by the use of the calcineurin inhibitor, cyclosporine, a frequently used immunosuppressive drug in clinical organ transplantation (14).

We found that the different parts of the host-conditioning regimen, the makeup of the residual host T cell subsets, and host secretion of IL-4 and IL-10 were critical in determining whether tolerance to both the bone marrow and heart grafts was induced. We found a marked increase in the fraction of host T cells that expressed NK cell markers in wild-type recipients, and that the latter T cells, as well as host secretion of IL-4 and IL-10, were required for heart graft acceptance even in mixed chimeras with

Divisions of \*Immunology and Rheumatology and <sup>†</sup>Bone Marrow Transplantation, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305; and <sup>‡</sup>Core Research for Engineering, Science, and Technology and Department of Molecular Immunology, Chiba University Graduate School of Medicine, Chiba, Japan

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<sup>2</sup> Address correspondence and reprint requests to Dr. Samuel Strober, Division of Immunology and Rheumatology, Stanford University School of Medicine, Center for Clinical Science Research Building, MC:5166, 300 Pasteur Drive, Stanford, CA 94305-5166. E-mail address: sstrober@stanford.edu

<sup>3</sup> Abbreviations used in this paper: TLI, total lymphoid irradiation; ATS, anti-thymocyte serum.

clonal deletion of host T cells. Thus, acceptance of marrow transplants and associated clonal deletion does not ensure the acceptance of organ grafts, and additional mechanisms of tolerance such as immune regulation are required for organ graft acceptance in this model.

## Material and Methods

### Animals

Wild-type BALB/c (H-2<sup>d</sup>), C57BL/6 (H-2<sup>b</sup>), and C3H/He(H-2<sup>k</sup>) mice were purchased from the Department of Comparative Medicine, Stanford University (Stanford, CA). Male BALB/c IL-4<sup>-/-</sup> (BALB/c-IL-4<sup>tm2Nnt</sup>), BALB/c IFN- $\gamma$ <sup>-/-</sup> (BALB/c IFN- $\gamma$ <sup>tm1Tts</sup>), C57BL/6 IL-4<sup>-/-</sup> (C57BL/6J-IL-4<sup>tm1Cgn</sup>), and C57BL/6 IL-10<sup>-/-</sup> (C57BL/6J-IL-10<sup>tm1Cgn</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Development of CD1<sup>-/-</sup> founder mice was described previously (15) and those in the current study were maintained on the BALB/c background and kindly provided by Drs. M. A. Exley and S. P. Balk (Harvard University, Boston, MA), and have been backcrossed for more than 10 generations. J $\alpha$ 281<sup>-/-</sup> founder mice were backcrossed for nine generations with C57BL/6 mice (16).

### Cardiac transplantation technique and monitoring

Neonatal hearts were transplanted into a pouch in the ear pinna according to the procedure described by Trager et al. (17), on day 0. Heart grafts were monitored daily for visible contractions and survival was based on the time interval until contractions stopped.

### Irradiation

TLI was delivered to the abdomen, lymph nodes, thymus, and spleen with shielding of the skull, lungs, limbs, pelvis, and tail as described previously (2).

### Rabbit anti-thymocyte serum (ATS)

Rabbit ATS was purchased from Accurate Chemical and Scientific (Westbury, NY). BALB/c recipients were injected i.p. with 0.05 ml of ATS in 0.5 ml of saline on days 0, 2, 4, 8, and 10 in combination with 10 doses of TLI, or on day 0 in combination with 17 doses of TLI.

### Antibodies

Anti-Gr-1-biotin and anti-Mac-1-biotin were purchased from Caltag Laboratories (Burlingame, CA). Anti-H-2K<sup>b</sup>-FITC, anti-H-2K<sup>b</sup>-PE, anti-H-2K<sup>d</sup>-FITC, anti-H-2K<sup>d</sup>-PE, anti-Thy1.2-PE, anti-B220-PE, anti-DX5-biotin, anti-NK1.1-PE, anti-TCR $\alpha\beta$ -APC, anti-CD3-APC, anti-V $\beta$ 2, V $\beta$ 3, V $\beta$ 4, V $\beta$ 5, V $\beta$ 6, V $\beta$ 8, V $\beta$ 9, V $\beta$ 11, & V $\beta$ 12-FITC, and anti-CD16/32 mAb to block FcR- $\gamma$ II/III were purchased from BD PharMingen (San Diego, CA). Streptavidin-PE was obtained from Southern Biotechnology Associates (Birmingham, AL). CD1 tetramer staining reagent loaded with

$\alpha$ -galactosylceramide was kindly provided by Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA).

### Flow cytometric analysis and sorting

Single cell suspensions of blood lysed with ammonium chloride buffer and spleen cells were harvested, stained, and analyzed by flow cytometry as previously described (18). TCR $\alpha\beta$ <sup>+</sup> T cells from the bone marrow were enriched by immunomagnetic-beads, stained, and sorted as before (18).

## Results

### Some mixed chimeras reject heart allografts

To achieve long-term cardiac tissue allograft acceptance and mixed chimerism using BALB/c (H-2<sup>d</sup>) host and C57BL/6 (H-2<sup>b</sup>) donor mice, we conditioned hosts with a nonmyeloblastic post-transplantation regimen of fractionated irradiation of the spleen, lymph nodes, and thymus with marrow shielding (TLI), depletive anti-T cell Abs, and an infusion of donor bone marrow cells that had been reported to tolerize MHC-mismatched rats (13, 14). Host mice received neonatal donor heart allografts transplanted to the ear pinnae on day 0, 5 doses of ATS i.p. starting on day 0, 10 doses of TLI (240 cGy each) between days 1 and 14, and an i.v. injection of donor marrow cells (50 × 10<sup>6</sup>) on day 15. Table I shows that all hosts given the complete regimen became mixed chimeras, and 11 of 13 accepted their heart grafts as judged by continued graft contractions for >100 days. Untreated control hosts rejected all their grafts by 17 days (Table I).

Modest prolongation of heart graft survival without long-term (>100 days) acceptance was observed in hosts given ATS alone (median 28 days), TLI alone (median 20 days), ATS and TLI (median 49 days), ATS and marrow cells (median 21 days), or TLI and marrow cells (median 27 days) (Table I). Surprisingly, mixed chimerism developed in the group given ATS, TLI, and marrow cells, and in the group given TLI and marrow cells without ATS despite the rejection of seven of eight heart allografts in the latter group (Table I). Multilineage chimerism was documented in both groups by immunofluorescent staining of white blood cells for the donor MHC (H-2K<sup>b</sup>) marker vs T cell (Thy-1.2), B cell (B220), and granulocyte and macrophage (Gr-1 and Mac-1) markers 28 days after the bone marrow cell injection. In the representative analyses shown in Fig. 1, hosts given the complete regimen of TLI, ATS, and donor bone marrow cells had 4.2% donor T cells, 54.6% donor B cells, and 14.2% donor granulocytes and monocytes among

Table I. Survival of C57BL/6 heart allografts and chimerism in BALB/c hosts given ATS, TLI, and/or donor bone marrow cells

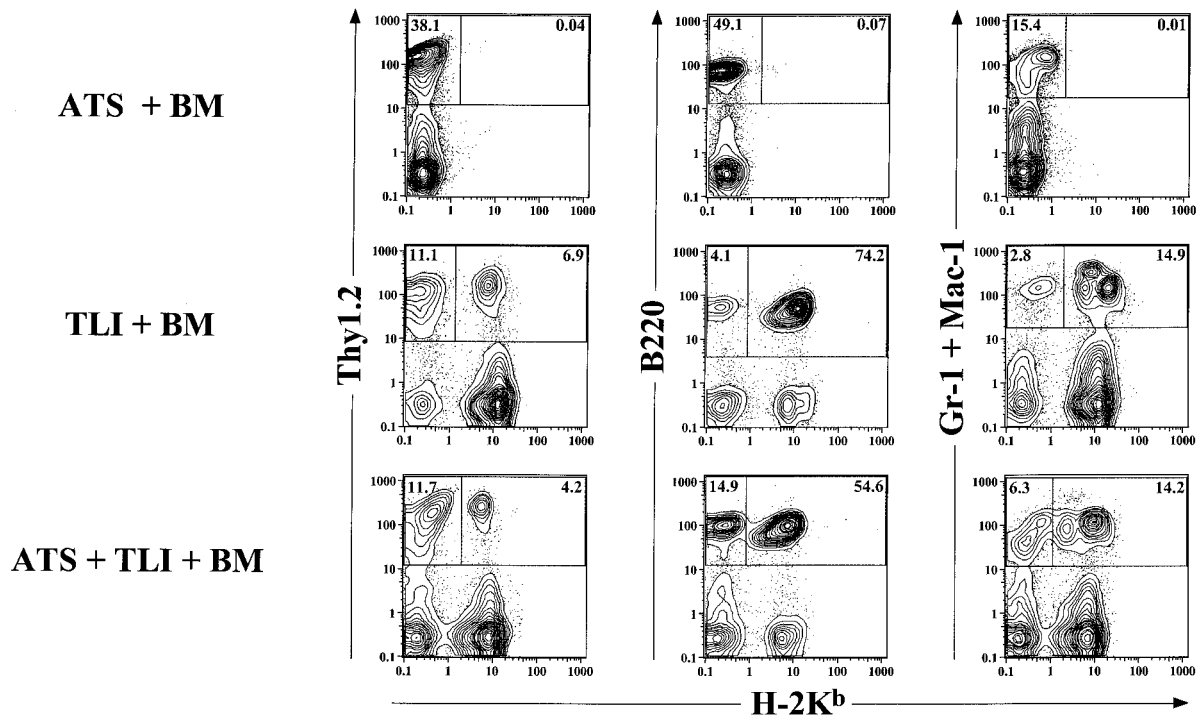
Host Treatment			Heart Allograft Survival (days)	Median Survival (days) (± SD)	Percentage of Donor Type Cells Amongst White Blood Cells of Host <sup>d</sup>
ATS <sup>a</sup>	TLI <sup>b</sup>	Bone marrow cells <sup>c</sup>			
–	–	–	9, 14, 14, 14, 17, 17	14 (±3)	NA
+	–	–	25, 27, 27, 29, 33, 34	28 (±4)	NA
–	+	–	13, 14, 17, 20, 45, 48, 55	20 (±18)	NA
+	+	–	38, 45, 48, 49, 50, 52, 55, 59	49 (±6)	NA
+	–	+	20, 20, 20, 21, 21, 22, 23	21 (±1)	<1, <1, <1, <1, <1, <1, <1
–	+	+	21, 23, 27, 27, 27, 35, 59, >100	27 (±27)	68, 74, 80, 88, 97, >99, >99
+	+	+	35, 77, >100, >100, >100, >100, >100, >100, >100, >100, >100, >100	>100 (±20)	51, 54, 56, 57, 58, 58, 58, 60, 60, 61, 64, 65

<sup>a</sup> Five doses of 50  $\mu$ l, i.p. given on days 0, 2, 4, 6, 8 (heart allograft given day 0).

<sup>b</sup> Ten doses of 240 cGy given on days 1–3, 6–10, 13, and 14.

<sup>c</sup> A total of 50 × 10<sup>6</sup> cells injected i.v. on day 15.

<sup>d</sup> Day 28 after BM cell injection (= day 43 after heart transplantation). Adequate blood samples were not available for analysis in some hosts. NA, not applicable.



**FIGURE 1.** BALB/c hosts given TLI and C57BL/6 bone marrow (BM) cells or TLI, ATS, and marrow cells develop mixed chimerism. White blood cells were stained with fluorochrome-conjugated mAbs and two-color analysis was performed for chimerism amongst T cells (Thy1.2 vs donor H-2K<sup>b</sup> marker), B cells (B220 vs H-2K<sup>b</sup>), or granulocytes and monocytes (Gr-1 + Mac-1 vs H-2K<sup>b</sup>). Donor and host-type cells are enclosed in boxes on the right and left, respectively, of each panel, and percentages in boxes are shown. Hosts were given C57BL/6 heart grafts on day 0 and were conditioned thereafter with ATS, TLI, or ATS and TLI before the injection of  $50 \times 10^6$  marrow cells.

white blood cells. When gated donor T cells (H-2K<sup>b</sup><sup>+</sup>Thy1.2<sup>+</sup>) were analyzed for the presence of NK T cells, <1% were NK1.1<sup>+</sup> (data not shown). Similar percentages of donor T and B cells (6.9 and 74.2%) and granulocytes and monocytes (14.9%) were found in hosts given the TLI and marrow cells without ATS (Fig. 1). Levels of chimerism were stable when reanalyzed at 100 days (data not shown). Hosts in the group given ATS and donor marrow cells had <0.1% donor-type cells in all lineages tested.

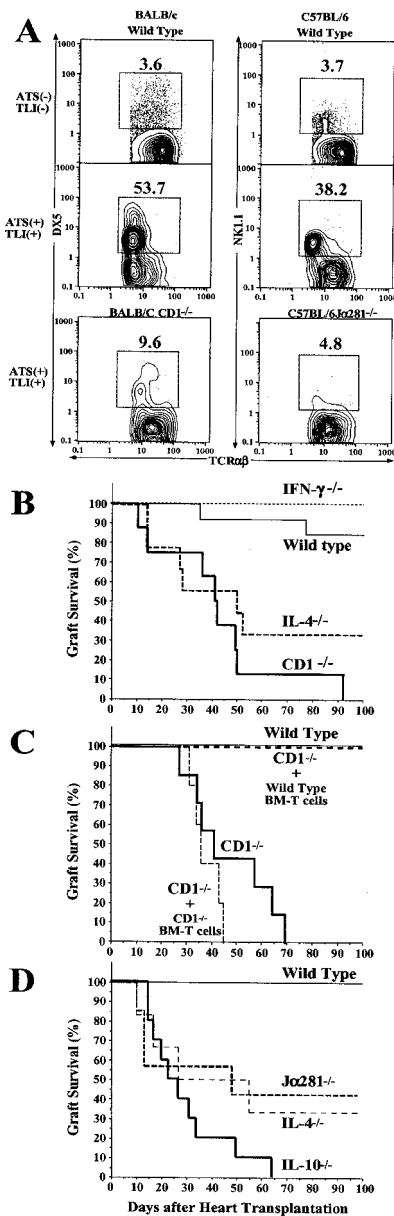
Histopathological studies of heart grafts removed after 100 days from hosts given the complete TLI, ATS, and bone marrow transplantation regimen showed an intact myocardium with little evidence of myocyte necrosis, mononuclear cell infiltration, scarring, or hemorrhage (data not shown). In contrast, heart grafts removed shortly after the cessation of contractions in the other groups showed intense mononuclear cell infiltration, patchy hemorrhage, and diffuse myocyte necrosis consistent with acute rejection.

#### *Role of CD1-reactive T cells and cytokines in heart graft acceptance*

Recent studies showed that the combined regimen of TLI and ATS administered to BALB/c or C57BL/6 mice altered the balance of residual T cell subsets such that the minor (~2%) subset of T cells expressing NK cell markers, DX5<sup>+</sup>TCRαβ<sup>+</sup>, and NK1.1<sup>+</sup>TCRαβ<sup>+</sup> T cells, became the majority of all T cells (19). These unusual T cell subsets were regulatory and prevented graft-vs-host disease (19). The role of CD1-reactive DX5<sup>+</sup>TCRαβ<sup>+</sup> and NK1.1<sup>+</sup>TCRαβ<sup>+</sup> cells in long-term graft acceptance in the current study was examined by comparing graft survival in wild-type, CD1<sup>-/-</sup>, or J<sub>α</sub>281<sup>-/-</sup> hosts given the complete host-conditioning regimen. Previous studies showed peripheral NK1.1<sup>+</sup> T cells are markedly reduced in CD1<sup>-/-</sup> and J<sub>α</sub>281<sup>-/-</sup> mice as compared to wild-type mice due to either the lack of positive selection by CD1

or the inability to generate the invariant CD-1 reactive V<sub>α</sub>14J<sub>α</sub>281 TCRα chain, respectively (20–22). In addition, our studies of bone marrow TCRαβ<sup>+</sup> T cells showed that about 20% of the latter T cells are NK T cells in wild-type mice, and about 4% are NK T cells in CD1<sup>-/-</sup> mice (21). Less than 1% of bone marrow T cells that are CD1-reactive as judged by staining with a CD1-tetramer reagent loaded with α-galactosylceramide are present in CD1<sup>-/-</sup> mice (data not shown).

Immunofluorescent staining and two-color flow cytometric analysis of spleen cells for DX5 vs TCRαβ or NK1.1 vs TCRαβ markers was performed in BALB/c and C57BL/6 wild-type mice before and immediately after treatment with the combined TLI and ATS regimen without organ and marrow transplants (Fig. 2A). Before treatment the DX5<sup>+</sup> and NK1.1<sup>+</sup> T cells accounted for between 3.6 and 3.7% of all T cells in wild-type hosts and no discrete population of cells was observed (enclosed in boxes in Fig. 2A). At that time point, the mean ± SD percentage of TCRαβ<sup>+</sup> T cells was 34 ± 4% in four wild-type BALB/c mice and 26 ± 5% in five wild-type C57BL/6 mice. After TLI and ATS, the mean percentage of T cells in wild-type mice was reduced to 0.4 ± 0.1 and 0.6 ± 0.5%, respectively. The percentage of DX5<sup>+</sup> and NK1.1<sup>+</sup> T cells amongst all residual T cells rose at least 10-fold to about 54 and 38%, respectively, but the absolute numbers of DX5<sup>+</sup> and NK1.1<sup>+</sup> T cells were slightly reduced as compared to pretreatment levels (19). The rise in the percentage of DX5<sup>+</sup> or NK1.1<sup>+</sup> T cells amongst all T cells was attenuated after treatment of CD1<sup>-/-</sup> BALB/c mice (~10%) and of J<sub>α</sub>281<sup>-/-</sup> C57BL/6 mice (~5%) as compared to that of the wild-type mice (Fig. 2A). The CD1 reactivity of the DX5<sup>+</sup> and NK1.1<sup>+</sup> T cells in wild-type BALB/c and C57BL/6 hosts that received TLI and ATS was confirmed by >90% staining positively with CD1 tetramers loaded with α-galactosylceramide (data not shown).



**FIGURE 2.** Long-term heart graft acceptance is more frequent in wild-type as compared to  $CD1^{-/-}$ ,  $J\alpha 281^{-/-}$ ,  $IL-4^{-/-}$ , and  $IL-10^{-/-}$  hosts given ATS, TLI, and a donor bone marrow transplant. *A*, Top panels, two-color analysis of DX5 vs  $TCR\alpha\beta$  markers on gated  $TCR\alpha\beta^{+}$  cells in the spleen of untreated (ATS(-), TLI(-)) wild-type BALB/c mice. NK1.1 vs  $TCR\alpha\beta$  markers in wild-type C57BL/6 mice are also shown. Middle panels, The same two-color analysis on wild-type mice immediately after treatment with 5 doses ATS and 10 doses of TLI (ATS(+), TLI(+)). Bottom panels, The same analyses after treatment of  $CD1^{-/-}$  BALB/c and  $J\alpha 281^{-/-}$  C57BL/6 mice. None of these mice received transplants. Boxes enclose either  $DX5^{+}TCR\alpha\beta^{+}$  or  $NK1.1^{+}TCR\alpha\beta^{+}$  cells. *B*, Survival of C57BL/6 heart allografts in wild-type,  $CD1^{-/-}$ ,  $IFN-\gamma^{-/-}$ , and  $IL-4^{-/-}$  BALB/c hosts given 5 doses of ATS, 10 doses of TLI, and  $50 \times 10^6$  donor bone marrow cells. There were 8–13 mice per group. *C*, Survival of C57BL/6 heart grafts in BALB/c  $CD1^{-/-}$  and wild-type hosts given 1 dose of ATS, 17 doses of TLI, and  $50 \times 10^6$  donor marrow cells. Some  $CD1^{-/-}$  hosts were given an i.v. injection of  $0.5 \times 10^6$  sorted  $TCR\alpha\beta^{+}$  T cells (BM-T cells) from the marrow of wild-type BALB/c mice after 8 doses of TLI. The TLI was stopped for 96 hr thereafter, and then resumed to complete 17 doses. Some  $CD1^{-/-}$  hosts were given the same regimen as the latter hosts except that sorted T cells were obtained from  $CD1^{-/-}$ , instead of wild-type BALB/c, mice. There were 5–7 mice per group. *D*, Survival of BALB/c heart grafts in wild-type,  $J\alpha 281^{-/-}$ ,  $IL-4^{-/-}$ , or  $IL-10^{-/-}$  C57BL/6 hosts given 5 doses of ATS, 10 doses of TLI, and  $50 \times 10^6$  BALB/c bone marrow cells. There were 6–12 mice per group.

Comparison of heart graft survival in  $CD1^{-/-}$  BALB/c vs wild-type BALB/c mice given TLI, ATS, and donor marrow cells showed that all grafts were rejected in the  $CD1^{-/-}$  group by 95 days, but about 85% of grafts survived >100 days in the wild-type group ( $p < 0.001$  as judged by the log-rank test) (Fig. 2*B*). Since previous studies (15, 23) indicated that the regulatory functions of  $NK1.1^{+}$  T cells are mediated at least in part by  $IL-4$  or  $IFN-\gamma$ , the survival of heart allografts was compared in  $IL-4^{-/-}$ ,  $IFN-\gamma^{-/-}$ , and wild-type BALB/c hosts given the complete conditioning regimen. Fig. 2*B* shows that all heart grafts survived >100 days in the  $IFN-\gamma^{-/-}$  group, but that only about one-third of grafts survived during the same period in the  $IL-4^{-/-}$  hosts ( $p < 0.01$   $IL-4^{-/-}$  vs  $IFN-\gamma^{-/-}$ ;  $p < 0.01$   $IL-4^{-/-}$  vs wild-type). Untreated  $IFN-\gamma^{-/-}$  hosts rejected heart grafts within the same time interval as untreated wild-type hosts (data not shown).

Table II shows that the use of  $CD1^{-/-}$  instead of wild-type BALB/c hosts had an impact on the development of chimerism, since three of six  $CD1^{-/-}$  hosts had 1% or less donor-type cells amongst white blood cells (mean 23%). All wild-type hosts had at least 51% donor-type cells (mean 59%) ( $p < 0.001$  by Student's *t* test). However, remaining chimeric hosts in the  $CD1^{-/-}$  group (23–50% donor-type cells) still rejected their heart allografts despite the complete conditioning regimen. Similarly, six of nine hosts in the  $IL-4^{-/-}$  group rejected their heart grafts, and five of six of the latter were chimeras (Table II). Thus, deficiency in  $CD1$  and  $IL-4$  genes had a more robust effect on the rejection of heart as compared to bone marrow allografts.

We theorized that the uniform heart graft rejection observed in  $CD1^{-/-}$  hosts was due to a deficiency in  $CD1$ -reactive  $TCR\alpha\beta^{+}$  T cells. To reconstitute these T cells in the  $CD1^{-/-}$  hosts, we injected them with  $0.5 \times 10^6$  sorted  $TCR\alpha\beta^{+}$  T cells from the bone marrow of wild-type BALB/c mice. Sorted T cells had  $\geq 95\%$  purity as judged by reanalysis. Amongst gated T cells in a wild-type BALB/c bone marrow sample not used for sorting, there were 4.9%  $CD1$ -reactive cells as judged by staining with the  $CD1$ -tetramer reagent, and there were  $< 0.1\%$  in a  $CD1^{-/-}$  marrow sample. Previous studies showed that  $NK1.1^{+}$  T cells depleted from the periphery can be replaced within 48 hr from dividing precursors in the marrow (24). Because the marrow is shielded during TLI, proliferating cells in the marrow are protected from irradiation. To reduce the possibility that ATS injected into hosts would kill the transferred bone marrow T cells, the conditioning regimen was changed to include only 1 dose of ATS on day 0, and 17 instead of 10 doses of TLI. The BALB/c marrow T cells were injected after 8 doses of TLI. Fig. 2*C* shows that wild-type BALB/c mice given the regimen of 1 dose of ATS, and 17 doses of TLI and an injection of donor marrow cells all accepted donor C57BL/6 heart grafts for >100 days. These long-term hosts developed immune tolerance, since four hosts given third-party C3H/He heart grafts rejected them within 8–22 days.

$CD1^{-/-}$  BALB/c hosts all rejected the donor C57BL/6 grafts by day 69 ( $p < 0.001$ ), but  $CD1^{-/-}$  hosts given the sorted wild-type marrow  $TCR\alpha\beta^{+}$  T cells all accepted their grafts for at least 100 days (Fig. 2*C*). A control group of  $CD1^{-/-}$  hosts given sorted marrow  $TCR\alpha\beta^{+}$  T cells from  $CD1^{-/-}$  BALB/c mice rejected all grafts by day 45. Thus, T cells from wild-type, but not  $CD1^{-/-}$  BALB/c, mice prevented the rejection of heart grafts in  $CD1^{-/-}$  hosts.

The  $TCR\alpha$  chain of most peripheral  $CD1$ -reactive  $NK1.1^{+}$  T cells is derived from an invariant rearrangement of  $V\alpha 14$  and  $J\alpha 281$  gene segments (25, 26). To determine whether  $CD1$ -reactive cells that regulate heart allograft rejection express the invariant  $TCR\alpha$  chain, the donor and host mouse strains were reversed so

Table II. Chimerism and survival of allografts in wild-type and gene-deficient hosts given ATS, TLI, and donor bone marrow cells<sup>a</sup>

Host Type	Percentage of Donor-Type Cells Amongst White Blood Cells of Host <sup>b</sup>	Fraction of Hosts with Allograft Surviving >100 Days
BALB/c		
Wild type	51, 54, 56, 57, 58, 58, 58, 60, 60, 61, 64, 65	11/13
CD1 <sup>-/-</sup>	<1, <1, 1, 23, 57, 58	0/8
IL-4 <sup>-/-</sup>	<1, 53, 57, 60, 65, 67, 70	3/9
IFN- $\gamma$ <sup>-/-</sup>	48, 50, 50, 52, 57, 60, 61, 63, 64, 69	10/10
C57BL/6		
Wild type	57, 62, 63, 70, 70, 72, 74, 77, 77, 78, 78, 78	12/12
J $\alpha$ 281 <sup>-/-</sup>	70, 71, 72, 75, 75, 77, 78	3/7
IL-4 <sup>-/-</sup>	66, 66, 70, 71, 74, 76	2/6
IL-10 <sup>-/-</sup>	68, 69, 70, 70, 72, 73, 73, 75, 80, 80	0/10

<sup>a</sup> Day 28 after bone marrow cell injection (= day 43 after heart transplantation). Adequate blood samples were not available for analysis in some hosts.

that wild-type and J $\alpha$ 281<sup>-/-</sup> C57BL/6 host mice could be compared for their ability to reject BALB/c heart grafts. The C57BL/6 hosts were conditioned with five doses of ATS, 10 treatments of TLI, and an infusion of  $50 \times 10^6$  BALB/c marrow cells as before. Fig. 2D shows that the C57BL/6 wild-type hosts accepted all BALB/c heart grafts for at least 100 days, and Table II shows that all hosts were mixed chimeras. In contrast, four of seven J $\alpha$ 281<sup>-/-</sup> hosts rejected their grafts within 50 days ( $p < 0.0001$ ) (Fig. 2D). All of the latter hosts were mixed chimeras with between 70 and 78% donor-type cells amongst white blood cells (Table II). The more complete loss of tolerance in the CD1<sup>-/-</sup> as compared to the J $\alpha$ 281<sup>-/-</sup> hosts may be due to the presence of CD1-reactive T cells in the latter mice that do not express the V $\alpha$ 14-J $\alpha$ 281 invariant TCR $\alpha$  chain (27). We also compared the ability of IL-4<sup>-/-</sup> and IL-10<sup>-/-</sup> C57BL/6 hosts with that of wild-type C57BL/6 hosts given the complete conditioning regimen to accept BALB/c heart grafts. The IL-10<sup>-/-</sup> hosts rejected all heart grafts by day 65, and four of six heart grafts were rejected by day 55 by the IL-4<sup>-/-</sup> hosts (Fig. 2D). The graft survival in the IL-10<sup>-/-</sup> and IL-4<sup>-/-</sup> hosts was significantly reduced ( $p < 0.0001$  and  $p < 0.01$ , respectively) as compared to wild-type mice. All of the gene-deficient hosts were mixed chimeras (Table II).

#### Rejection of second heart grafts in IL-10<sup>-/-</sup> chimeras with clonal deletion

The development of immune tolerance by host T cells to donor MHC Ags in mixed chimeras has been shown to be due to clonal deletion (28, 29). It was possible that the process of tolerance and clonal deletion in mixed chimeras that rejected heart grafts required several weeks, and that rejection occurred before clonal

deletion to donor MHC Ags was complete. This hypothesis was tested by studying IL-10<sup>-/-</sup> chimeric hosts that rejected heart grafts. Six of these hosts received a second donor (BALB/c) heart graft at day 42 (after all first heart grafts had been rejected). All of these hosts had at least 68% donor-type cells amongst white blood cells at day 63 (Table II).

All second heart grafts were rejected within 10 to 28 days (Table III). Chimerism was tested 21 days after the second heart transplantation, and remained in the range of 74–80% donor-type cells (Table III). Chimeras that rejected second heart grafts were tested for clonal deletion of host T cells by staining the spleen cells for the host-type MHC marker (H-2K<sup>b</sup>) vs CD3 and a panel of V $\beta$  receptors. Gated host T cells (H-2K<sup>b+</sup>CD3<sup>+</sup>) were analyzed for the percentage of V $\beta$ 2, 3, 4, 5, 6, 8, 9, 11, and 12 cells. Because the percentage of the nondeleted V $\beta$ 8 T cells amongst all T cells can vary from group to group, analysis of deleted versus nondeleted V $\beta$  subsets was measured as a ratio of V $\beta$ n:V $\beta$ 8 T cells. Table IV shows that in the control untreated BALB/c spleen, V $\beta$ 3, V $\beta$ 5, V $\beta$ 11, and V $\beta$ 12 T cells are deleted (ratio <0.10) as compared to C57BL/6 splenic T cells as judged by the reduced ratios of these V $\beta$  receptors to that of the nondeleted V $\beta$ 8 receptor. The ratios of the V $\beta$ 2, V $\beta$ 4, V $\beta$ 6, and V $\beta$ 9 receptors were similar in the two strains. The gated host-type (C57BL/6) T cells in the IL-10<sup>-/-</sup> chimeras showed significant reductions in the ratios for the V $\beta$ 3, V $\beta$ 5, V $\beta$ 11, and V $\beta$ 12 receptors ( $p < 0.05$ ) as compared to untreated wild-type C57BL/6 mice. Ratios for V $\beta$ 2, V $\beta$ 4, V $\beta$ 6, and V $\beta$ 9 were not significantly different ( $p > 0.1$ ). Similar reductions of the ratios of V $\beta$ 3, V $\beta$ 5, V $\beta$ 11, and V $\beta$ 12 were found in the C57BL/6 wild-type chimeras that had accepted the heart grafts (Table IV). No significant reductions were measured for V $\beta$ 2, V $\beta$ 4,

Table III. Survival of second BALB/c heart allografts and chimerism in IL-10<sup>-/-</sup> C57BL/6 hosts conditioned with ATS, TLI, and given BALB/c heart and bone marrow transplants

Host Type	Second Heart Allograft Survival <sup>a</sup> (days)	Percentage of Donor Type Cells Amongst White Blood Cells of Host After Second Allograft Transplantation <sup>b</sup>
IL-10 <sup>-/-</sup>	10, 10, 13, 22, 22, 28	74, 75, 75, 76, 79, 80

<sup>a</sup> Second heart allograft given on day 42.

<sup>b</sup> Day 21 after second heart transplantation (= day 48 after BM cell injection; = day 63 after first heart transplantation).

Table IV. Clonal deletion in IL-10<sup>-/-</sup> C57BL/6 hosts conditioned with ATS, TLI, and given BALB/c heart and bone marrow transplants

Host Type			V <sub>β</sub> 2 <sup>a</sup>	V <sub>β</sub> 3	V <sub>102</sub> 4	V <sub>β</sub> 5.1/5.2	V <sub>β</sub> 6	V <sub>β</sub> 9	V <sub>β</sub> 11	V <sub>β</sub> 12
BALB/c	Wild type	Control	0.26 ± 0.01	0.02 ± 0.01	0.30 ± 0.04	0.03 ± 0.00	0.40 ± 0.09	0.10 ± 0.00	0.05 ± 0.00	0.03 ± 0.02
C57BL/6	Wild type	Control	0.26 ± 0.03	0.17 ± 0.06	0.29 ± 0.03	0.35 ± 0.04	0.40 ± 0.12	0.13 ± 0.02	0.36 ± 0.04	0.16 ± 0.04
C57BL/6	IL-10 <sup>-/-</sup>	Chimera <sup>b</sup>	0.25 ± 0.04	<b>0.03 ± 0.01</b>	0.47 ± 0.02	<b>0.07 ± 0.03</b>	0.44 ± 0.02	0.11 ± 0.01	<b>0.09 ± 0.04</b>	<b>0.05 ± 0.02</b>
C57BL/6	Wild type	Chimera <sup>b</sup>	0.24 ± 0.02	<b>0.02 ± 0.01</b>	0.40 ± 0.05	<b>0.06 ± 0.04</b>	.28 ± 0.07	0.06 ± 0.01	<b>0.07 ± 0.02</b>	<b>0.03 ± 0.02</b>

<sup>a</sup> The ratio equals the percentage of V<sub>β</sub>n<sup>+</sup> to the percentage of V<sub>β</sub>8<sup>+</sup> T cells. Mean percentage of V<sub>β</sub>8<sup>+</sup> T cells of BALB/c wild-type control was 21.6 ± 1.3%. Mean percentage of V<sub>β</sub>8<sup>+</sup> T cells amongst H-2K<sup>b</sup> T cells of C57BL/6 wild-type control, IL-10<sup>-/-</sup>, and wild-type chimeras was 16.3 ± 1.1, 21.9 ± 1.2, and 20.8 ± 4.2%, respectively. Bold shows V<sub>β</sub> ratios indicative of clonal deletion. Mean and SE of three replicate experiments are shown.

<sup>b</sup> Ratio amongst gated H-2K<sup>b</sup> CD3<sup>+</sup> host-type cells.

V<sub>β</sub>6 T cells ( $p > 0.05$ ). There was a significant reduction ( $p < 0.05$ ) in the ratio of V<sub>β</sub>9 cells in the latter mice as compared to C57BL/6 wild-type mice and the IL-10<sup>-/-</sup> chimeras, but the ratios differed by <2-fold; in contrast, the ratios of deleted V<sub>β</sub>3, 5, and 11 T cells were reduced by at least 5-fold as compared to wild-type mice. Thus, second heart grafts were rejected by the IL-10<sup>-/-</sup> chimeras despite clonal deletion.

## Discussion

The rejection of skin and heart allografts by mixed chimeras has been reported previously in mice, dogs, and mini-swine (5, 8, 12). In the current study, a posttransplant host-conditioning regimen of fractionated lymphoid irradiation, depletive anti-T cell Abs, and an infusion of donor bone marrow cells allowed BALB/c or C57BL/6 wild-type mice to develop mixed chimerism and tolerance to MHC-mismatched heart grafts placed in the ear pinnae. This regimen has been used successfully to induce tolerance to vascularized heart allografts in rats and to kidney allografts in humans (7, 13, 14). Unexpectedly, the removal of the anti-T cell Abs from the regimen in the current study resulted in uniform heart graft rejection with uniform mixed chimerism.

We analyzed the role of residual host NK1.1<sup>+</sup> and/or DX5<sup>+</sup> T cells in the development of tolerance to the heart grafts. NK1.1<sup>+</sup> T and DX5<sup>+</sup> T cells are markedly increased in hosts given TLI, but do not increase significantly after sublethal total body irradiation (19). More than 90% of these C57BL/6 and BALB/c host T cells were reactive to CD1 and expressed the NK T cell invariant TCR $\alpha$  chain as judged by positive staining with a CD1 tetramer loaded with the  $\alpha$ -galactosylceramide ligand (data not shown). NK1.1<sup>+</sup> T cells have been shown to facilitate tolerance to tissue allografts after costimulatory blockade (23), prevent graft-vs-host disease (18, 19), and facilitate tolerance to heterologous proteins in the anterior chamber autoimmune eye disease model (30). The critical role of the host NK1.1<sup>+</sup> and DX5<sup>+</sup> T cells in the current study was shown by the loss of tolerance to heart grafts in the CD1<sup>-/-</sup> or J $\alpha$ 281<sup>-/-</sup>-deficient hosts as compared to wild-type hosts, and the ability to reconstitute long-term heart graft acceptance by the transfer of wild-type host bone marrow T cells containing CD1<sup>-</sup>-reactive T cells, but not by the transfer of CD1<sup>-/-</sup> marrow T cells. Although the C57BL/6 donor marrow cells contained about the same level of CD1-tetramer<sup>+</sup> T cells as the BALB/c marrow cells (data not shown), the CD1-reactive T cells contained in the donor marrow (injected after TLI) did not allow for graft acceptance, and donor-type NK T cells could not be detected in the spleen at day 28 after transplantation. Injection of host marrow T cells was done during rather than after TLI. Long-term heart graft acceptance was markedly reduced in the IL-4<sup>-/-</sup> and IL-10<sup>-/-</sup>, but not in the IFN- $\gamma$ <sup>-/-</sup>, hosts. The results suggest that secretion of both IL-4<sup>-/-</sup> and IL-10<sup>-/-</sup> by CD1-reactive NK T cells and/or conventional T cells in the hosts facilitates tolerance induction to the heart grafts. We did not demonstrate that the IL-4 and IL-10 were se-

creted by the CD1-reactive NK T cells in the current study, but our previous studies showed that the regulatory function of enriched NK T cells was lost when obtained from IL-4<sup>-/-</sup> mice (18). Thus, the latter cells are the likely source of the cytokines required for tolerance. However, the NK T cells may polarize other host or donor T cells toward a Th2-immune response. Thus, tolerance may require IL-4 and/or IL-10 secretion by both NK and non-NK T cells. Determination of the contribution of T cell subsets to cytokine secretion is the subject of a separate study. Despite the failure of heart graft acceptance, IL-10<sup>-/-</sup> hosts developed uniform mixed chimerism associated with clonal deletion of C57BL/6 host T cells. Yet, second BALB/c heart grafts were rejected within 28 days, despite the high levels of chimerism measured at 21 days.

The clonal deletion pattern of the host V<sub>β</sub> T cell subsets indicates that the C57BL/6 (H-2<sup>b</sup>) host cells have lost reactivity to donor (H-2<sup>d</sup>) MHC Ags. Previous studies of mixed chimeras have shown that negative selection of host T cells is due to the presence of donor-derived dendritic cells in the host thymus (28). However, the ability of the chimeric hosts to reject the heart, but not bone marrow, grafts indicated that tissue-specific minor, non-MHC, transplantation Ags are likely expressed by the donor heart cells but are not expressed by the donor bone marrow cells or their progeny. Presumably neither host nor donor-derived dendritic cells in the thymus express these heart-specific minor Ags which are the likely targets of rejection in some chimeric hosts. Reactivity to the latter Ags and associated rejection appears to be prevented by the regulatory cells and the secretion of IL-4 and IL-10.

In conclusion, clonal deletion of host T cells to donor MHC Ags was insufficient to achieve tolerance to the heart grafts. Other tolerance mechanisms were involved that included the contribution of regulatory CD1-reactive T cells with NK cell markers that secrete high levels of IL-4 and IL-10. We previously called these regulatory T cells from TLI-treated mice "natural suppressor" cells (31). The results impact on clinical organ transplantation and suggest that acceptance of bone marrow transplants from an organ donor and associated clonal deletion will not result in uniform organ graft acceptance unless additional mechanisms of immune tolerance are in place.

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