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J Immunol 2008; 180:6593-6603; doi: 10.4049/jimmunol.180.10.6593
http://www.jimmunol.org/content/180/10/6593
Long-Term Cardiac Allograft Survival across an MHC Mismatch after “Pruning” of Alloreactive CD4 T Cells

Min Hu,* Debbie Watson,* Geoff Y. Zhang,* Nicole Graf,† Yuan M. Wang,* Mary Sartor,‡ Brian Howden,§ Jeffrey Fletcher,* and Stephen I. Alexander2*

Specific tolerance to allografts has been achieved by a variety of means. We have previously shown that ex vivo removal of dividing CD4⁺ T cells from an MLR or “pruning” delays skin allograft rejection. We tested pruning of alloreactive T cells as a strategy for retaining a broad T cell repertoire while removing alloreactive T cells in a model of cardiac allograft transplant. Using CFSE staining of responder BALB/c cells with stimulator C57BL/6 cells in an MLR, SCID mice were reconstituted with either dividing (D) or nondividing (ND) CD4⁺ T cells derived from an MLR and then challenged with heterotopic cardiac allografts. Mice reconstituted with D CD4⁺ T cells rejected cardiac allografts from the stimulator strain with a median survival time (MST) of 29 days, while mice reconstituted with ND CD4⁺ T cells maintained allografts from the stimulator strain (MST of >100 days) while rejecting third-party allografts (B10.BR) (MST = 11 days). ELISPOT assays demonstrate donor-specific hyporesponsiveness of the ND CD4⁺ T cells. TCR β-chain V region (TRBV) repertoire analysis demonstrates clonal expansion within both rejecting D cardiac allografts and ND cardiac allografts surviving for the long-term. Histology showed greater allograft infiltration by the D CD4⁺ T cells. The surviving ND cardiac allografts demonstrated reduced cellular infiltration and reduced incidence of allograft vasculopathy, but with the development of chronic fibrosis. Thus, pruning of alloreactive T cells allows long-term-specific cardiac allograft survival while retaining the ability to reject third-party allografts.

Cardiac transplantation is now a major therapy for cardiac failure. Its use is limited by organ availability and by several factors after transplant. These include infections related to immunosuppression, as well as acute and chronic immune rejection, including cardiac allograft vasculopathy (CAV) and secondary organ morbidity. CAV is a major challenge that occurs in >50% of heart transplant recipients in the first few years after surgery (1, 2). Attempts to develop long-term tolerance for heart transplants have involved central strategies, such as “mixed chimerism”, and peripheral strategies, such as enhancement of regulatory T cells and costimulatory blockade (3–5).

The induction of tolerance processes so as to minimize the need for immunosuppressive drugs is a major goal for transplantation. Central tolerance in transplantation is induced by deleting alloreactive T cells in the thymus before they can be exported to the periphery (6, 7). The use of nonmyeloablative bone marrow transplantation leading to hematopoietic chimerism induces central tolerance and long-term graft survival (7–9). The induction of peripheral tolerance targets peripheral T cells mainly using costimulatory blockade or by inducing/transferring regulatory T cells (5, 10–12). The mechanisms of peripheral tolerance predominantly include clonal deletion, T cell anergy, and regulation. Costimulatory blockade using anti-CD154 and CTLA4Ig can achieve long-term cardiac allograft survival (4), and in certain models regulatory T cells have been effective in achieving cardiac transplant tolerance (5, 13–15). However, the only strategy that has achieved solid organ tolerance in human trials is mixed chimerism (16).

Animal models suggest that mixed chimerism can achieve cardiac allograft tolerance without the development of cardiac allograft vasculopathy (17, 18). However, the risks of mixed chimerism protocols include the need for bone marrow transplantation and the risk of development of graft-vs-host disease and “engraftment syndrome”, as well as the side effects of T cell depletion such as infection and loss of T cell memory with newly engrafting T cells (19–21).

CD4⁺ T cells are central in initiating cardiac allograft rejection. CD4⁺ T cells are capable of rejecting cardiac allografts through direct recognition (22). CD4⁺ T cell-mediated rejection requires donor MHC class II expression by the allograft and also on host hematopoietic cells to initiate CD4⁺ T cell activation (23). Thus, both host and donor interactions are required to initiate alloreactive CD4⁺ T cell-mediated rejection. Many studies have demonstrated that anti-CD4 Ab depletion therapy and grafting into CD4-deficient hosts results in long-term cardiac allograft survival (24–27).

A key feature of alloreactive T cells is their capacity to divide in response to the donor (28, 29). In vitro assays suggest that these cells make up ~1–10% of the T cell repertoire (30–32). Alloreactive T cell frequencies vary 30-fold from 0.71 ± 0.31% to 21.05 ± 3.62% depending on the specific in vivo conditions and the assay used (29). Removal of these cells while retaining the remaining T cells would allow retention of important immune memory T cells that do not have the capacity to reject specific allografts.

Analysis of diversity of the T cell repertoire can be done at a molecular level by analysis of the TCR, the Ag-specific receptor.
on the surface of T cells. TCR diversity is seen predominantly in the CDR3 region. The diversity of the CDR3 region of the TCR β-chain V region (TRBV) usage is generated by rearrangement of V, D, and J genes, and by junctional N diversity (33, 34). Variability in the distribution of the TCR CDR3 length and TRBV family provides information on clonal expansion and diversity in both rejection and tolerance in heart transplantation (35, 36).

The CFSE allows analysis of cell division and precursor frequency both in vivo and in vitro (28, 29, 37–39). One can track T cell proliferation using CFSE fluorescent dye where fluorescence intensity both in vivo and in vitro (28, 29, 37–39). One can track T cells of the proliferating population while retaining the nondividing (ND) and less activated T cells.

We have tested pruning of alloreactive CD4+ T cells as a strategy to allow long-term skin allograft survival while retaining immune memory (40). In murine models of cardiac transplantation across an MHC mismatch, pruning of alloreactive CD4+ T cells leads to long-term cardiac allograft survival while retaining the capacity to reject third-party cardiac grafts.

Materials and Methods

Mice

Female SCID (BALB/c background), BALB/c (H-2d), C57BL/6 (H-2b), and B10.BR (H-2k) mice were obtained from the Animal Resource Center (Perth, Western Australia). Mice between 8 and 12 wk of age were used in all experiments. All mice were certified pathogen-free and regularly monitored for viral disease. The institutional animal care ethics committee of Westmead Hospital approved and reviewed all experiments.

MLR

MLR was performed using mononuclear cells from BALB/c splenocytes stained with 0.5 μM CFSE (BioScientific) stimulated with irradiated (25 Gy 137Cs) mononuclear cells from C57BL/6 splenocytes. The cells were cultured for 6 days in completed RPMI 1640 medium (Invitrogen) containing 10% FCS, 25 mM HEPES buffer solution, 2 mM L-glutamine, 100 IU/ml penicillin, and 50 μM 2-ME in humidified 37°C, 5% CO2 incubator.

Cell sorting and flow cytometric analysis

On day 6 of the MLR, the harvested cells were stained with mouse anti-CD4-PE Ab (BD Pharmingen). Cells were sorted on a FACSDiva (BD Biosciences) into CFSEhighCD4+ T cells (dividing CD4+ T cells) and CFSElowCD4+ T cells (nondividing CD4+ T cells). Sorted cells and harvested cells also were analyzed on a FACScan analyzer (BD Biosciences) for purity and phenotype. Mononuclear cells from peripheral blood (PB) of SCID mice, collected at day 8, day 14, and >100 days after reconstitution were analyzed on a FACScan analyzer. Abs (BD Pharmingen) used included mouse anti-CD4-PerCP Ab, anti-CD25-PE Ab, anti-CD69-PE Ab, anti-CD44-PE Ab, anti-CD45-FITC Ab, anti-H-2Kb-FITC Ab, and H-2K4-PE Ab. CellQuest software was used for analysis (BD Biosciences).

Heterotopic heart transplantation

Heterotopic cardiac allografts were performed according to standard microsurgical techniques (41). Graft function was assessed by daily abdominal palpation, with rejection defined as loss of palpable beating. SCID mice were transplanted with C57BL/6 allogeneic, BALB/c syngeneic, and B10.BR allogeneic (third party) cardiac grafts.

Reconstitution

Six days posttransplantation, SCID mice with C57BL/6 (donor) allografts (n = 12) were reconstituted with dividing (D) CD4+ and ND CD4+ T cells. Control groups included SCID mice with B10.BR allografts (n = 5) that were reconstituted with ND CD4+ T cells, with BALB/c syngeneic grafts that were reconstituted with D CD4+ (n = 5) and ND CD4+ T cells (n = 1), and with C57BL/6 allografts that were reconstituted with naive BALB/c CD4+ T cells (n = 5). Sorted D CD4+, ND CD4+, or naive BALB/c CD4+ T cells (3.25 × 105) were injected by tail vein into each mouse. Reconstitution was confirmed by flow cytometry.

Table I. Mouse TRBV, TRBCuni, TRBCuni probe, and primers

<table>
<thead>
<tr>
<th>IMGT Name</th>
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<tr>
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</tr>
<tr>
<td>TRBV2</td>
<td>TCCAGCTGCTAAAGCCGACAG</td>
</tr>
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<td>TRBV3</td>
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<td>TRBV4</td>
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<td>TRBV12-2</td>
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<td>TRBCuni</td>
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</table>

* Mouse TRBV families are consistent with the international ImMunoGeneTics database (IMGT).
  
* MGB, Minor groove binding protein.
  
* NFQ, Nonfluorescent quencher.

Real-time RT-PCR for TRBV repertoire

Real-time RT-PCR was performed in triplicate on Rotor-Gene (Corbett Research). Mouse TRBV families are consistent with the international ImMunoGeneTics database (http://imgt.cines.fr/) (42). Primer Express software version 1.5 (Applied Biosystems) was used for designs of primers and probes of TRBV families. The primers and probes of mouse TRBV families used in this paper are showed in Table I. TRBV families were amplified with 5′ TRBV primers and a 3′ TCR β-chain C region universal (TRBCuni) primer. Individual TRBV gene usage expression was expressed as a percentage of total TCR signals for TRBV repertoire (43). TRBV repertoires were assessed in D and ND CD4+ T cells, cardiac grafts, and splenocytes of reconstituted mice.

CDR3 spectratyping of PCR products

CDR3 spectratyping was used to analyze the TRBV repertoire diversity in T cell subsets and to screen clonal expansion of T cells in D and ND CD4+ T cells, cardiac grafts, and splenocytes (34, 44). Briefly, a second round of PCR was performed using 1 μl PCR product of real-time RT-PCR from each TRBV family as cDNA. A Fam-labeled TRBCuni instead of TRBCuni was the reverse primer. Forward TRBV primers were as before in Table I. PCR product (1 μl) from this reaction was mixed with Hi-Di Formamide and GeneScan-500 size standard (Applied Biosystems). The sample was denatured and electrophoresed on the ABI Prism 310 genetic analyzer (Applied Biosystems). Genotyper software (Applied Biosystems) was used to analyze the running results.

Proliferation assay

[3H]Thymidine (MP Biomedicals) was used for proliferation assays. MLR was performed using 2 × 105 splenocytes isolated from SCID mice reconstituted with either D or ND CD4+ T cells, stimulated with irradiated 2 × 105 allogeneic C57BL/6, B10.BR, or syngeneic BALB/c in 96-well round-bottom plates in complete RPMI 1640 medium, and incubated at 37°C with 5% CO2 for 72 h. At 56 h, 1 μCi/well [3H]thymidine was added. Cells were measured for thymidine incorporation using a 1450 Microbeta counter (Wallac).

IFN-γ ELISPOT assay

ELISPOT plates (Millipore) were coated with 6 μg/ml mouse anti-IFN-γ Ab (BD Pharmingen) overnight at 4°C. After blocking plates, 2 × 105 splenocyte cells isolated from SCID mice reconstituted with either D or ND CD4+ T cells >100 days were stimulated with 2 × 105 allogeneic
C57BL/6, B10.BR, or syngeneic BALB/c splenocytes, which were treated with mitomycin C (50 μg/ml) and incubated at 37°C with 5% CO2 in complete RPMI 1640 medium for 24 h. Secondary biotinylated anti-mouse IFN-γ (2 μg/ml; BD Pharmingen) was added overnight at 4°C. Plates were washed before streptavidin-alkaline phosphatase (BD Pharmingen) was added and incubated at room temperature for 2 h, then plates were washed and developed with an alkaline phosphatase conjugate substrate kit from Bio-Rad. Plates with spots were counted using the computer program KS ELISPOT (Zeiss).

**Histological examination**

Tissues were fixed in 10% formalin and embedded in paraffin before staining with H&E for cellular infiltration, Verhoeff’s von Giesen for vascu-lopaphy of arteriosclerosis, and Masson’s trichrome for evaluation of f-brosis. Frozen OCT sections were used for examining infiltrating CD4+ T cells of cardiac grafts by immunohistochemistry. Primary rat anti-mouse CD4 (L3T4) (BioLegend) and the second Ab biotinylated rabbit anti-rat Ig (DakoCytomation) were used. Five-micrometer sections were cut and fixed in 95% ethanol. H2O2 solution (0.3% (v/v)) was employed for blocking endogenous peroxidase activity. Endogenous avidin binding activity was blocked by incubating the sections with a biotin-blocking system (Zymed Laboratories). A three-step staining procedure was used in combination with biotinylated rabbit anti-rat Ig as the secondary Ab and streptavidin-HPR (Zymed Laboratories) with the two-step DAB detection system (Sigma-Aldrich).

**Statistical analysis**

Prism software (GraphPad) was used for statistical analysis to comparisons of matched groups to detect significance. The log-rank test was employed for comparison of survival data between groups. Comparison of means for ELISPOT data and proliferation was performed using two-tailed Student’s t test. Survival is shown by median survival time (MST).

**Results**

**Phenotype of ND and D CD4+ T cells after MLR**

After 6 days of MLR, the responding BALB/c CD4+ T cells were segregated into two populations, ND CD4+ T cells and D CD4+ T cells, based on the degree of the fluorescent cytoplasmic dye CFSE, which decreases upon cell division (Fig. 1, AII and AIII). The D CD4+ T cells were 38% of total CD4+ T cells, while ND CD4+ T cells were 62% of total CD4+ T cells (Fig. 1AI).

Flow cytometric analysis of the ND and D CD4+ T cells showed that D CD4+ T cells express higher levels of CD25+, CD69+, and CD44+ than do ND CD4+ T cells. The percentage of CD25+, CD69+, and CD44+ in D CD4+ T cells was 67.8% (Fig. 1AIIV), 57.4% (Fig. 1AV), and 91.4% (Fig. 1AVI) compared with 35.6% (Fig. 1AIIV), 12.5% (Fig. 1AV), and 14.6% (Fig. 1AVI) in ND D CD4+ T cells (Fig. 1AI).

Flow cytometric analysis of the ND and D CD4+ T cells (Fig. 1BIIV) showed that D CD4+ T cells express higher levels of CD25+, CD69+, and CD44+ than do ND CD4+ T cells. The percentage of CD25+, CD69+, and CD44+ in D CD4+ T cells was 67.8% (Fig. 1AIIV), 57.4% (Fig. 1AV), and 91.4% (Fig. 1AVI) compared with 35.6% (Fig. 1AIIV), 12.5% (Fig. 1AV), and 14.6% (Fig. 1AVI) in ND D CD4+ T cells (Fig. 1AI).

Cultured cells were stained with mouse anti-CD4-PerCP Ab (III), then anti-CD25-PE Ab, anti-CD69-PE Ab, or anti-CD44-PE Ab for flow cytometric analysis. After gating on CD4+ T cells, the percentage of CD25+ was 67.8% of D CD4+ T cells and 35.6% in ND CD4+ T cells (IV), while the percentage of CD69+ was 57.4% in D CD4+ T cells and 12.5% in ND CD4+ T cells (V). Of D CD4+ T cells, 91.4% were CD44+, compared with 14.6% for ND CD4+ T cells (VI). Stimulator cells were necrotic by day 6 at an irradiation dose of 25 Gy137Cs in an MLR (B). Mouse anti-CD4-PerCP, anti-H2-Kb-FITC, and H2-Kd-PE Abs were used in the experiments. C57BL/6 splenocytes cultured with Con A for 6 days showed that the percentage of CD4+ H2-Kb+ was 30.4% of total H2-Kd+ cells (I) and no expression of responder H2-Kb+ cells (II). BALB/c splenocytes as responder cells were stimulated with irradiated C57BL/6 splenocytes in an MLR for 6 days. The percentages of D CD4+ H2-Kb+ (III) and D CD4+ H2-Kd+ cells (IV) relative total mononuclear cells were ~37%. There are no irra- diated stimulator H2-Kb+ cells in the MLR system after 6 days culture (III and IV). BALB/c splenocytes stained with CFSE as responder cells were cultured with irradiated C57BL/6 splenocytes (V and VI). At day 6 of MLR, the percentage of CFSE+H2-Kb+ in total CD4+ cells was 35%, with 65% for CFSE+H2-Kd+ in total CD4+ cells. (The experiments for A and B were performed separately.)
CD4⁺ T cells, respectively. While as expected the dividing T cells had high levels of activation markers such as CD69 and CD25 and also expressed higher levels of CD44, there was also significant expression of these markers in the ND CD4⁺ T cells (40, 45).

Stimulator H-2Kb⁺ cells were rendered necrotic at an irradiation dose of 25 Gy³/²Cs in a 6-day MLR (Fig. 1B). CD4⁺ and CD4⁻ fractions of C57BL/6 unirradiated splenocytes after culturing for 6 days with Con A express only class I H-2Kb⁺ cells (Fig. 1B) and show no expression of H-2Kd⁺ cells (Fig. 1BII). BALB/c splenocytes (H-2Kd⁺ cells) stimulated with irradiated C57BL/6 splenocytes (H-2Kb⁺ cells) in an MLR have only responder BALB/c cells in both CD4⁺ and CD4⁻ cell fractions (Fig. 1BIV). No stimulatory H-2K⁺⁺ cells are found in the MLR system in the 6-day culture (Fig. 1BII). Further evaluation of D and ND populations show that they are entirely responder derived; D CFSElow and ND CFSEhigh were all H-2K⁺⁻ within the CD4⁺ population (Fig. 1, BV and BVII). This demonstrated that ND and D CD4⁺ T cells were not contaminated by stimulatory cells in the MLR.

Prolongation of cardiac allograft survival in SCID mice reconstituted with ND CD4⁺ T cells

We tested whether ND CD4⁺ T cells could reject cardiac allogeneic grafts and whether there was specific delay in rejection of the stimulator strain. Heterotopic cardiac grafts were performed on SCID mice receiving C57BL/6, BALB/c, and third-party B10.BR cardiac grafts at day −7 using the protocol outlined in Fig. 2. SCID mice reconstituted with D CD4⁺ T cells rejected C57BL/6 cardiac allografts (n = 6), which was the same as stimulator cells in MLR, with an MST of 29 days (Fig. 3). However, SCID mice reconstituted with ND CD4⁺ T cells did not reject C57BL/6 cardiac allografts (n = 6), with an MST of >100 days (p = 0.0025) (Fig. 3). Furthermore, SCID mice reconstituted with ND CD4⁺ T cells rejected third-party B10.BR cardiac allograft (n = 5) with an MST of 11 days (p = 0.0007) (Fig. 3), and SCID mice reconstituted with naïve CD4⁺ T cells rejected C57BL/6 cardiac allograft (n = 5) with an MST of 12 days (p = 0.0008) (Fig. 3). Mice reconstituted with naïve CD4⁺ T cells rejected C57BL/6 hearts at a similar rate as those reconstituted with D CD4⁺ T cells (p = NS). BALB/c syngeneic grafts in SCID mice reconstituted with D (n = 3) (Fig. 3) and ND (n = 1) (data not shown) CD4⁺ T cells had long-term graft survival (>100 days). Thus, ND CD4⁺ T cells significantly and specifically did not reject cardiac allografts across an MHC mismatch while retaining the ability to reject third-party grafts.

Equivalent numbers of ND and D CD4⁺ T cells at different time points in vivo

We examined PB CD4⁺ T cells of SCID mice reconstituted with ND or D CD4⁺ T cells by flow cytometry using CD45⁺ cells as the denominator (Fig. 2). CD4⁺ T cells gradually increased presenting in SCID mice reconstituted with D and ND CD4⁺ from an average 3.5 and 4.3% at day 8, 6.4 and 7.6% at day 14, and 17.4 and 19.2% at day >100 (n = 6) (Fig. 4A). The T cell reconstitution after day 100 is shown in Fig. 4B. There were no CD4⁺ T cells in SCID before reconstitution (Fig. 4BII). The percentage of CD4⁺CD45⁺ cells was 26.7% in SCID mice reconstituted with D CD4⁺ cells at day >100 of reconstitution in one representative mouse (Fig. 4BII), and was 19.6% in SCID mice reconstituted with ND CD4⁺ cells at day >100 of reconstitution in another representative mouse (Fig. 4BIII). Our data demonstrate similar numbers of ND and D CD4⁺ T cells that increase over time and are
Reduced cellular infiltration was observed in C57BL/6 cardiac allografts in SCID mice reconstituted with D CD4+ T cells (Fig. 5AIV) and day 35 (Fig. 5AVII) in SCID mice reconstituted with D CD4+ T cells compared with controls, which included a naive C57BL/6 heart (Fig. 5A), and BALB/c syngeneic cardiac grafts in SCID mice reconstituted with D and ND CD4+ T cells (Fig. 5, AII and AIII). Reduced cellular infiltration was observed in C57BL/6 cardiac allografts in SCID mice reconstituted with ND CD4+ T cells, which included one at day 35 (Fig. 5AVIII) and day >100 allografts (Fig. 5AIX), compared with rejecting C57BL/6 cardiac allografts in SCID mice reconstituted with ND CD4+ T cells, which had massive cellular infiltration and more severe myocardial necrosis (Fig. 5AV). Most long-term surviving C57BL/6 cardiac allografts in SCID mice reconstituted with ND CD4+ T cells had minimal vasculopathy (Fig. 5, BVIII and BIX) similar to naive (Fig. 5BI) and BALB/c syngeneic controls (Fig. 5, BII and BIII). However, one long-term surviving C57BL/6 cardiac allograft in a SCID mouse reconstituted with ND CD4+ T cells had thickened vessel intima (data not shown). Day 14 C57BL/6 allografts show progressive vasculopathy (Fig. 5BVI), and a day 35 C57BL/6 cardiac allograft in SCID mice reconstituted with D CD4+ T cells had thickened vessel intima (Fig. 5BVII). Vasculopathy was present in C57BL/6 allografts in SCID mice reconstituted with naive CD4+ T cells (Fig. 5BIV), although it less severe than with D CD4+ T cells. Furthermore, rejecting third-party cardiac allografts in SCID mouse reconstituted with ND CD4+ T cells revealed vascular damage in all examined allografts (Fig. 5BV). Most C57BL/6 cardiac allografts in SCID mice reconstituted with ND CD4+ T cells had varying degrees of fibrosis (Fig. 5CVIX), but not in one at day 35 (Fig. 5CVIII), compared with naive and syngeneic controls (Fig. 5, CI–CIII). Interestingly, fibrosis was seen in all third-party cardiac allografts (Fig. 5CV) in SCID mice reconstituted with ND CD4+ T cells, and a rejected C57BL/6 cardiac allograft (day 35) (Fig. 5CVII) was seen in a SCID mouse reconstituted with D CD4+ T cells. C57BL/6 cardiac allografts in SCID mice reconstituted with naive CD4+ T cells showed edema with limited fibrosis (Fig. 5CVI). The ND C57BL/6 cardiac allograft group had minimal cellular infiltration and less allograft vasculopathy, but it did develop chronic fibrosis.

Infiltration of ND CD4+ T cells in long-term-surviving cardiac allografts

We examined the infiltrating CD4+ T cells of cardiac allografts by immunohistochemistry. As expected, there was massive CD4+ T cell infiltration in third-party B10.BR cardiac allografts (Fig. 6D) in SCID mice reconstituted with ND CD4+ T cells compared with native hearts (Fig. 6A). The day 14 (Fig. 6B) and day 35 (Fig. 6C) rejecting C57BL/6 cardiac allografts that were harvested from SCID mice reconstituted with D CD4+ T cells revealed infiltration of CD4+ T cells. There are scattered CD4+ T cells in surviving C57BL/6 cardiac allograft at day 35 (Fig. 6F) and day >100 (Fig. 6F) in SCID mice reconstituted with ND CD4+ T cells.

Diverse TRBV repertoires in ND and D CD4+ T cells and skewing of TRBV repertoires in cardiac allografts in both groups

Similar and diverse TRBV repertoire patterns were observed between the ND and D CD4+ T cells after MLR. Twenty-one of 22 families, except TRBV23, with only 0.04% expression in ND CD4+ T cells and 0.19% expression in spleen from SCID mice reconstituted with D CD4+ T cells, were detected in D CD4+ T cells, while all 22 TRBV families were detected in ND CD4+ T cells (data not shown). A close correlation of TRBV repertoire except TRBV13-1, which has significantly increased expression ($p < 0.05$) in ND CD4+ cells, was found between the D and ND CD4+ T cells (Fig. 7AI). TRBV repertoires of spleen (21 TRBV families) from SCID mice reconstituted with D CD4+ T cells showed similar TRBV repertoire patterns as did D CD4+ T cells from MLR (Fig. 7AII). There is a close correlation in TRBV repertoire between spleen (22 TRBV families) from SCID mice reconstituted with ND CD4+ T cells and ND CD4+ T cells from MLR, except TRBV20 and TRBV23, which are significantly increased ($p < 0.05$) with >5% expression in spleen from SCID mice reconstituted with ND CD4+ T cells (Fig. 7AIII). Skewing of TRBV repertoires were observed in cardiac allografts from SCID mice reconstituted with D and ND CD4+ T cells compared with D or ND CD4+ T cells. All 22 TRBV families were detected in two C57BL/6 rejecting cardiac allografts in SCID mice reconstituted with D CD4+ T cells, while 21 TRBV families were found in a third allograft. There is significantly increased expression of TRBV4 (>5% of the TRBV repertoire) ($p <$
0.0001) in rejecting cardiac allograft 1, TRBV13-3 (p/H11021 0.05), TRBV15 (p/H11021 0.05), and TRBV17 (p/H11022 5% in TRBV repertoires) in rejecting cardiac allograft 2, and TRBV13-2 (p/H11021 0.05) and TRBV17 (p/H11021 0.0001) (each /H11022 5% of the TRBV repertoire) in rejecting cardiac allograft 3, compared with D CD4 T cells (Fig. 7AIII). All 22 TRBV families were detected in C57BL/6 cardiac allograft surviving 100 days in SCID mouse reconstituted with ND CD4 T cells. Significantly increased expression of TRBV13-2 (p < 0.0001) and TRBV19 (p < 0.05) (each /H11022 5% of the TRBV repertoire) was observed in the allograft (Fig. 7AIV).

Rejecting cardiac allografts (from SCID mice with reconstituted with D CD4 T cells) of CDR3 spectratyping revealed that there were more restricted TRBV4 spectratypes in rejecting cardiac allograft 1 (Fig. 7BI), more restricted TRBV13-3, TRBV15, and TRBV17 spectratypes in rejecting cardiac allograft 2 (Fig. 7BII), and TRBV17 spectratypes in rejecting cardiac allograft 2 (Fig. 7BII), from SCID mice reconstituted with ND CD4 T cells. Representative histological cardiac allografts and naive heart (H&E; ×100) (A), vasculopathy of cardiac allografts vessel and naive heart (Verhoeff’s von Giesen; ×400) (B), and fibrosis of cardiac allografts and naive heart (Masson’s trichrome; ×200) (C) are shown, including a naive C57BL/6 heart (I); BALB/c syngeneic cardiac grafts from SCID mice reconstituted with D (II) and ND (III) CD4 T cells (>100 days); rejecting C57BL/6 cardiac allograft from SCID mice reconstituted with naive BALB/c CD4 T cells (IV); rejecting third-party B10.BR from SCID mice reconstituted with ND CD4 T cells (10 days) (V); rejecting C57BL/6 cardiac allograft from SCID mice reconstituted with D CD4 T cells at days 14 (VI) and 35 (VII); and surviving C57BL/6 cardiac allografts from SCID mice reconstituted with ND CD4 T cells at day 35 (VIII) and >100 days (IX).
FIGURE 7. TRBV repertoires and particular TRBV families of CDR3 spectratyping. TRBV repertoires were performed by real-time RT-PCR (A). The TRBV repertoires were similar between D and ND CD4+ T cells after 6 days MLR, except TRBV13-1 was significantly greater in ND CD4+ T cells than in D CD4+ T cells (p < 0.05) (I). Similar TRBV repertoires were observed between D CD4+ T cells and spleen from SCID mice reconstituted with D CD4+ T cells (day >100). However, TRBV20 and TRBV23 families were increased by >5% (p > 0.05) in spleen from SCID mice reconstituted with ND CD4+ T cells compared with ND CD4+ T cells (II). TRBV repertoires were slightly altered in rejecting cardiac allografts (n = 3) from SCID reconstituted with D CD4+ T cells (III).
and more restricted TRBV13-2 and TRBV17 spectratypes in rejecting cardiac allograft 3 (Fig. 7BIII), compared with D CD4⁺ T cells from MLR. Restricted TRBV13-2 and TRBV19 spectratypes also were observed in surviving cardiac allograft (represented one) from SCID mouse reconstituted with ND CD4⁺ T cells after MLR (Fig. 7BIV).

TRBV4 (p < 0.0001) in allograft 1, TRBV13-3 (p < 0.05), TRBV15 (p < 0.05), and TRBV17 (p < 0.05) in allograft 2, and TRBV13-2 (p < 0.05) and TRBV17 (p < 0.05) in allograft 3 were all significantly increased (>5%) in rejecting cardiac grafts compared with D CD4⁺ T cells after MLR. Significantly increased expression (>5%) of TRBV13-2 (p < 0.0001) and TRBV19 (p < 0.05) were found in long-term surviving cardiac allograft (represented one) from SCID mouse reconstituted with ND CD4⁺ T cells after MLR (IV). The spectratypes of those TRBV families with increased expression are shown in BIII-BIV, and they demonstrated restricted spectratyping in the cardiac allografts as compared with the reconstituting D or ND CD4⁺ T cell populations.

FIGURE 8. CDR3 spectratyping of TRBV families (highly represented families, 4% or more of the repertoire) in ND or D CD4⁺ T cells from MLR. TRBV1, 4, 5, 13-1, 13-2, 13-3, 19, 20, and 31 showed the normal Gaussian distribution of 6-11 peaks in both D (A) and ND (D) CD4⁺ T cells. CDR3 spectratyping of these TRBV families showed more diversity in spleen from SCID reconstituted with D CD4⁺ T cells (B) than with ND (E) CD4⁺ T cells. More restricted or skewed CDR3 spectratyping in TRBV4, 13-3, 19, 20, and 31 were observed in spleen from SCID reconstituted with ND CD4⁺ T cells (E). Also shown is the restricted TRBV4 (shown in one representative) rejecting C57BL/6 cardiac allograft from a SCID mouse reconstituted with D CD4⁺ T cells (C). Restricted or skewed TRBV4, 13-1, 13-2, 13-3, 19, 20, and 31 were seen in long-term surviving C57BL/6 cardiac allograft (>100 days) with ND CD4⁺ T cells (F).
Comparison of splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells to those reconstituted with ND CD4\(^+\) T cells showed that the IFN-\(\gamma\) response to allogeneic stimulation was greater in the D CD4\(^+\) T cells (446 \pm 52-fold) compared with the ND (37 \pm 16-fold) (\(p < 0.0001\)). The splenocytes from SCID mice reconstituted with ND CD4\(^+\) T cells revealed significantly reduced proliferation in response to the stimulator C57BL/6 (4.29 \pm 0.3 fold) compared with the third-party B10.BR (4.86 \pm 0.14-fold) (\(p < 0.05\)), and compared with splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells in response to the stimulator C57BL/6 (\(p < 0.0001\)) (A). The splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells had greater IFN-\(\gamma\) production (446 \pm 58.8 cells) in response to C57BL/6 than did third-party B10.BR (213 \pm 59.6 cells) (\(p < 0.05\), \(p = 0.0085\)). The splenocytes from SCID mice reconstituted with ND CD4\(^+\) T cells had fewer IFN-\(\gamma\)-producing cells in response to C57BL/6 stimulation (126 \pm 15.5) than third-party B10.BR stimulation (181 \pm 8.1) (\(p < 0.05\), \(p = 0.0055\)), and compared with splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells in response to the stimulator C57BL/6 (\(p < 0.05\), \(p = 0.0008\)) (B) (representative of three assays).

**Discussion**

We have previously shown that alloreactive CD4\(^+\) T cells could be removed and the remainder of the T cell repertoire preserved by pruning the T cell population in an MLR using CFSE. Removal of alloreactive CD4\(^+\) T cells specifically prolongs cardiac allograft survival from the in vitro stimulator strain across a major mismatch while maintaining the ability to reject third-party grafts. The MLR classically has been used to measure alloreactivity between donor and recipient pairs in transplant recipients (46). The combination of flow cytometry and CFSE allows characterization of the precursor frequency of alloreactive T cells and identification of the ND population in an MLR. In our model we used CFSE in an MLR system to remove alloreactive D CD4\(^+\) T cells and to achieve long-term cardiac allograft survival, while maintaining an immune response to a third-party and a broad T cell repertoire as shown by TCR studies.

To assess the immune response to donor, self, and a third party we used in vitro MLR proliferation and IFN-\(\gamma\) “ELISPOTs”. These demonstrated a reduced level of proliferation in the splenocytes of mice reconstituted with ND CD4\(^+\) T cells in response to the donor strain compared with proliferative responses to the third-party stimulus. Interestingly, the response to the third-party stimulus was retained in the splenocytes of mice reconstituted with ND CD4\(^+\) T cells, which had a greater response than did the splenocytes of mice reconstituted with D CD4\(^+\) T cells to B10.BR stimulators. There was a more significant reduction in the number of IFN-\(\gamma\)-positive cells by ELISPOT of ND splenocytes stimulated with donor as compared with third-party B10.BR, whereas the splenocytes from mice reconstituted with dividing cells showed both a stronger proliferative response and greater number of IFN-\(\gamma\)-producing cells in response to the stimulator strain than a third-party stimulus. This is in keeping with other groups where IFN-\(\gamma\) is a requirement for CD4\(^+\) T cell-mediated rejection (47). It also in keeping with tolerance studies where lower donor-induced IFN-\(\gamma\) production rather than proliferation is associated with tolerance, suggesting that rejection is a function of the phenotype of the T cells as well as their proliferative ability (48).

In our model, ND cardiac allografts had reduced cellular infiltration as compared with rejecting D cardiac allografts and ND third-party allografts. No vasculopathy was seen in the majority of

**FIGURE 9.** Reduced proliferation (A) and IFN-\(\gamma\) production (B) in response to allogeneic stimulation in splenocytes from SCID mice reconstituted with ND CD4\(^+\) T cells after day 100. The splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells had a greater proliferation in response to the stimulator C57BL/6 (8.4 \pm 0.52-fold) compared with the third-party B10.BR (3.7 \pm 0.16-fold) (\(p < 0.0001\)). The splenocytes from SCID mice reconstituted with ND CD4\(^+\) T cells revealed significantly reduced proliferation in response to the stimulator C57BL/6 (4.29 \pm 0.3 fold) compared with the third-party B10.BR (4.86 \pm 0.14-fold) (\(p < 0.05\)), and compared with splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells in response to the stimulator C57BL/6 (\(p < 0.0001\)) (A). The splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells had greater IFN-\(\gamma\) production (446 \pm 58.8 cells) in response to C57BL/6 than did third-party B10.BR (213 \pm 59.6 cells) (\(p < 0.05\), \(p = 0.0085\)). The splenocytes from SCID mice reconstituted with ND CD4\(^+\) T cells had fewer IFN-\(\gamma\)-producing cells in response to C57BL/6 stimulation (126 \pm 15.5) than third-party B10.BR stimulation (181 \pm 8.1) (\(p < 0.05\), \(p = 0.0055\)), and compared with splenocytes from SCID mice reconstituted with D CD4\(^+\) T cells in response to the stimulator C57BL/6 (\(p < 0.05\), \(p = 0.0008\)) (B) (representative of three assays).

**Diversity of TRBV spectratypes in ND and D CD4\(^+\) T cells and restriction of some TRBV families in allograft infiltrates**

Spectratyping of an individual TRBV family gives an overview of the diversity of this family by visually representing the random variation of CDR3 lengths. With clonal expansion, the normal distribution of lengths is skewed or restricted. To examine each individual family with diversity or clonal expansion, CDR3 spectratyping was employed to analyze the 22 TRBV families. Our data demonstrate that diverse TRBV families were found in both ND and D CD4\(^+\) T cells from MLR. CDR3 spectratyping of TRBV families (nine families) in which the percentage of individual TRBV family was >4% in repertoires in ND or D CD4\(^+\) T cells is shown in Fig. 8. These families are TRBV1, 4, 5, 13-1, 13-2, 13-3, 19, 20, and 31 in both ND and D CD4\(^+\) T cells. CDR3 spectratyping of these families showed the normal Gaussian distribution of 6–11 peaks with each being separated by three nucleotides in both D (Fig. 8A) or ND (Fig. 8D) CD4\(^+\) T cells (from MLR). Diverse TRBV families were observed in these families of spleen from SCID mice reconstituted with D CD4\(^+\) T cells (Fig. 8B). Diverse TRBV families also were found in most TRBV families except for a restricted TRBV4 in one rejecting allograft (Fig. 8C). The spleen (Fig. 8E) and long-term-surviving allograft (Fig. 8F) (represented) from one SCID mouse reconstituted with ND CD4\(^+\) T cells showed that most of these TRBV families had some restriction compared with ND CD4\(^+\) T cells from MLR.
long-term ND cardiac allografts; however, interstitial fibrosis was observed in most cardiac allografts after 100 days in ND mice. Chronic heart rejection is defined by a progressive, obliterative myointimal hyperplasia, known as CAV (49–51). Clinically, chronic cardiac allograft rejection involves predominantly vascular damage with or without parenchymal fibrosis (51, 52). CAV appears to have components of humoral, cellular, and NK cell-mediated mechanisms (1, 53). After pruning of alloreactive CD4⁺ T cells while there was chronic fibrosis with some cellular infiltration, there was minimal CAV. However, rejecting D cardiac allografts and ND third-party allografts all showed marked CAV.

ND CD4⁺ T cells at day 6 MLR demonstrated diverse TRBV repertoires, as did D CD4⁺ T cells. However, as the precursor frequency of the lymphocyte population involved in allosponses is between 1 and 10%, D CD4⁺ T cells will become less diverse than ND CD4⁺ T cells (31, 32). This is exacerbated by selection for expansion of higher affinity T cells leading to ongoing restriction of the T cell repertoire in the allografts and spleens as higher affinity T cells out-competed low-affinity T cells (54). Altered TRBV repertoires have been observed in rejecting and in tolerant cardiac allografts (36). Restricted TRBV has been shown in tolerant cardiac allografts with T cell infiltration including regulatory T cells (35). Our data showed a degree of skewing of TRBV repertoires in rejecting cardiac allografts from SCID mice reconstituted with D CD4⁺ T cells and long-term-surviving cardiac allografts from SCID mice reconstituted with ND CD4⁺ T cells. CDR3 spectratyping demonstrated diversity of TRBV spectratypes both in D and ND CD4⁺ T cells, with restriction in some families in rejecting cardiac allografts and more restriction in long-term-surviving cardiac allografts, suggesting either a smaller reactive clone population or expansion of reactive clones over time.

Equivalent numbers of D and ND CD4⁺ T cells were found at different time points in SCID mice after reconstitution in our mouse model. However, the cardiac allografts from stimulator C57BL/6 mice still have prolonged survival in mice reconstituted with ND CD4⁺ T cells. While differences may exist between the two groups in proliferation, survival, and trafficking, the similar numbers of transferred cells and rate of increase in the mice suggest that homeostatic expansion leading to differences between the groups is not apparent in our model. However, the increase in numbers suggests that there is some homeostatic proliferation that is likely to increase the memory and functional phenotype of the adoptively transferred cells and may explain the fibrosis found in long-term allografts in the SCID mice reconstituted with ND CD4⁺ T cells (55).

Ex vivo pruning of proliferating alloreactive T cells may have clinical value. Two major barriers to transplant success are the infectious risk and the persistence of alloreactive memory cells in many models (55–57). Pruning removes the major component of the alloreactive population while retaining immune memory to other pathogens. Therefore, pruning in combination with other strategies may reduce the alloreactive burden while maintaining adequate general immune function. These studies are performed in immunodeficient mice, and further studies in depleted wild-type mice are necessary to assess the role of ongoing thymic emigrants in the maintenance of graft survival crucial to preclinical evaluation.

In conclusion, pruning of alloreactive CD4⁺ T cells allows long-term specific cardiac allograft survival while retaining the ability to reject third-party grafts.

Acknowledgments
We thank Tony Henwood (Department of Pathology, the Children’s Hospital at Westmead, University of Sydney, Australia) for advice on histological examination, Louie Lu and Megan Cameron for animal care (Westmead Hospital Animal House, University of Sydney, Australia), and Sandra Lum (Westmead Institute for Cancer Research, University of Sydney, Australia) for cell sorting.

Disclosures
The authors have no financial conflicts of interest.

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