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“Pruning” of Alloreactive CD4⁺ T Cells Using 5- (and 6-)Carboxyfluorescein Diacetate Succinimimidyl Ester Prolongs Skin Allograft Survival¹

Debbie Watson,* Geoff Yu Zhang,* Mary Sartor,† and Stephen I. Alexander²*

Removal of alloreactive cells by either thymic deletion or deletion/anergy in the periphery is regarded as crucial to the development of tolerance. Dyes, such as CFSE, that allow monitoring of cell division suggest that in vitro proliferation could be a used as a way of “pruning” alloreactive cells while retaining a normal immune repertoire with retention of memory to previously encountered pathogens. This would overcome the problems occurring as a result of therapies that use massive depletion of T cells to allow acceptance of organ transplants or bone marrow grafts. We therefore used a skin graft model of CD4-mediated T cell rejection across a major H-2 mismatch (C57BL/6 (H-2b) to BALB/c (H-2d) mice) to evaluate whether nondividing CD4⁺ T cells derived from a mixed lymphocyte culture would exhibit tolerance to a skin graft from the initial stimulator strain. We demonstrate that selective removal of dividing alloreactive CD4⁺ T cells resulted in marked specific prolongation of allogeneic skin graft survival, and that the nondividing CD4⁺ T cells retained a broad TCR repertoire and the ability to maintain memory. This novel way of depleting alloreactive T cells may serve as a useful strategy in combination with other mechanisms to achieve transplant tolerance. The Journal of Immunology, 2004, 173: 6574–6582.

CD4⁺ T cells are the major initiating cells of the cognate immune response in rejection (1–3). CD4⁺ T cells alone are capable of rejecting allografts (4). They also appear to be the major cell in the development of tolerance (5, 6). Deletion or induction of anergy of CD4⁺ T cells has been successful for peripheral tolerance induction in rodent transplant models (7), but less so in larger primates, possibly because of their greater memory population (8). Other studies have focused on central tolerance strategies that involve T cell ablation and nonmyeloablative bone marrow transplantation (BMT)³ to achieve mixed chimerism that leads to thymic tolerization (9). Regeneration of a functional T cell population in the recipient involves thymic maturation from T cell precursors, at which stage potential host- and donor-reactive T cells are deleted in the chimeric thymus (10). This has been successful in animal models and in limited clinical studies. T cell ablation in the periphery has been used as part of tolerizing strategies in clinical trials, with OKT-3 and, more recently, Campath and MEDI-507 (11, 12). The Immune Tolerance Network has a number of protocols for solid organ transplantation. Removal of potentially alloreactive T cells is used in these clinical trials of tolerance for solid organ transplants. Either central deletion of alloreactive T cells in mixed chimerism or wholesale ablation using anti-CD52 or specific ablution of peripheral alloreactive T cells using anti-CD25 Abs is the basis for these models (13).

These methods of achieving tolerance by peripheral or central deletion of alloreactive T cells have a number of potential pitfalls. These include the need for BMT, the side effects of T cell depletion, including infections and loss of T cell memory with newly engrafting T cells, and the dangers of engraftment syndrome. Failure of engraftment and host-vs-graft disease may also occur (14–16). Other significant dangers include the development of graft-vs-host disease (GVHD), where donor T cells have not been completely purged and when host T cells have not been completely removed (15). Some of these pitfalls could be overcome if recipient T cells could be obtained before treatment to deplete them, “pruned” of host-vs-graft alloreactive T cells, and injected after T cell depletion had ceased.

Other studies have focused on the direct or indirect pathway of allograft rejection used by the alloreactive T cells. Alloreactive T cells are predominantly activated through the direct pathway by foreign MHC (17). However, indirectly activated alloreactive T cells also have a crucial role in rejection and tolerance despite their lower frequency (17). In the absence of the indirect pathway, it is not possible to develop tolerance in models using costimulatory blockade (18). Prior skin graft models of CD4-mediated rejection on the same strain background using CD8 depletion show that the rate of rejection in this skin graft model is increased by the degree of mismatch (4, 19). Blocking the direct pathway combined with CD8 depletion still allows rejection, but at a slower rate than with the direct pathway intact. The more rapid rejection seen with directly activated cells may be due to their greater frequency or their higher affinity (4).

Recently, use of the fluorescent dye CFSE has led to the ability to define the alloreactive proportion of T cells by assessment of their precursor frequency (20). Cell proliferation has historically been used as the hallmark of T cell activation, and CFSE and other fluorescent dyes have provided the ability to follow cell proliferation by analysis of reduced fluorescence demonstrating cell division (21, 22). Thus, cell proliferation can be used as a marker of...
the alloreactive T cell and to remove these T cells while leaving the majority of the T cell population intact. Ex vivo manipulation of hemopoietic cells has been used in a number of strategies that have reached clinical therapeutic use, including dendritic cells (23), stem cells for gene therapy (24), and ex vivo viral transduction using CD40L in BMT (25). Thus, CFSE and cell sorting can allow ex vivo manipulation of whole T cell populations with the removal of selective proliferating populations.

To test whether nondividing (ND) CD4⁺ T cells could allow restoration of a normal immune repertoire while providing specific tolerance across a major mismatch, we studied a mouse model of skin rejection in immunodeficient mice reconstituted with CD4⁺ T cells pruned of alloreactive CD4⁺ T cells. We used CFSE to separate dividing (D) and ND CD4⁺ T cells after mixed lymphocyte culture (MLC) and demonstrated a specific delay in allograft rejection. We also demonstrated that ND T cells have a diverse T cell repertoire and maintain memory to a specific Ag in vivo. Finally, we investigated the mechanism of allo-activation in rejection.

Materials and Methods

Animals

BALB/c (H-2b), C57BL/6 (H-2b), and B10.BR (H-2b) mice were purchased from the Animal Resource Center (Perth, Western Australia). SCID (BALB/c background) mice were purchased from the Walter and Elisa Hall Institute (Melbourne, Victoria). Female mice, 6–10 wk old, were used in all experiments.

**FIGURE 1.** Allo-stimulated cells can be separated into ND and D CD4⁺ T cell populations using CFSE. Responder BALB/c (H-2b) splenocytes (2 × 10⁶/ml) were stained with 0.5 μM CFSE and were stimulated with irradiated C57BL/6 (H-2b) splenocytes (2 × 10⁶/ml). a. Flow cytometric analysis of splenocytes on day 6 of an MLC stained with PE-conjugated anti-CD4 show a CFSE⁺/CD4⁺ population representing D CD4⁺ T cells (36.3%) and a CFSE⁻/CD4⁻ population representing ND CD4⁻ T cells (63.7%). D and ND CD4⁺ T cell populations were subsequently sorted and injected into immunodeficient mice. Flow cytometric analysis was performed on D (CFSE⁻/CD4⁻) and ND (CFSE⁺/CD4⁻) cells in MLC on day 5 stained with PerCP-conjugated anti-CD4 (b), and activation markers PE-conjugated anti-CD69 (c) and PE-conjugated anti-CD25 (d). The majority of D CD4⁺ T cells express activation markers CD69 (70.8% of D CD4⁺ T cells) and CD25 (88.5% of D CD4⁺ T cells). A proportion of the ND CD4⁺ T cells also demonstrate the expression of the activation markers CD69 (8.5% of ND CD4⁺ T cells) and CD25 (9.1% of ND CD4⁺ T cells).

**Mixed lymphocyte culture**

Responder mononuclear cells were derived from BALB/c (H-2b) mice, and stimulator mononuclear cells were derived from C57BL/6 (H-2b) mice. BALB/c responder cells were stained with 0.5 μM CFSE (BioScientific, Gynoea, Australia) for 8 min at room temperature, and any unbound CFSE was quenched by washing with FCS. Stimulator C57BL/6 cells were irradiated (25 Gy). Cells were washed with PBS and resuspended at 2 × 10⁶/ml in RPMI 1640 medium (Invitrogen Life Technologies, Grand Island, NY) containing 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg of streptomycin, and 5 μM 2-ME. Splenocytes were plated at 2 × 10⁶ cells/well in 24-well plates (Falcon; BD Biosciences, San Diego, CA).

**Flow cytometry and cell sorting**

Cells were harvested on day 6 of the MLC. Cells were stained with mouse anti-CD4-PE-conjugated Ab (BD Pharmingen, San Diego, CA) for 30 min at 4°C, washed with 2% FCS, and resuspended in PBS. Cells were analyzed by flow cytometry and sorted on a FACSCalibur (BD Biosciences) into CFSE⁺/CD4⁺ (ND CD4⁺ cells) and CFSE⁻/CD4⁻ (D CD4⁺ cells) populations. Sorted cells were washed with PBS and resuspended at a concentration of 2 × 10⁶/ml. Flow cytometry for activation markers was performed on day 5 of the MLC. Cells were stained with mouse anti-CD4-PerCP Ab (BD Pharmingen), mouse anti-CD25-PE-conjugated Ab (BD Pharmingen), and anti-CD69-PE-conjugated Ab (BD Pharmingen) for 30 min at 4°C, washed with 2% FCS, and resuspended in PBS. Cells were analyzed by flow cytometry.

**Skin transplant model**

SCID mice (with a BALB/c background) were injected i.v. with 2 × 10⁶ sorted, ND (CFSE⁺/CD4⁻) or D (CFSE⁻/CD4⁺) T cells. SCID mice were grafted with skin from syngeneic BALB/c (H-2d) (●), allogeneic stimulator C57BL/6 (H-2b) (▲), and third-party B10.BR (H-2b) (●) strains. a. Mice reconstituted with 2 × 10⁶ ND (CFSE⁺/CD4⁻) CD4⁺ T cells (n = 6) demonstrate delayed rejection of their stimulator C57BL/6 skin grafts. On day 6 of the MLC, cells were stained with PE-conjugated anti-CD4 and sorted into ND (CFSE⁺/CD4⁻) and D (CFSE⁻/CD4⁺) T cells. Survival curves are shown for SCID mice that were reconstituted with 2 × 10⁶ ND or D CD4⁺ T cells and grafted with skin from syngeneic BALB/c (H-2b) (●), allogeneic stimulator C57BL/6 (H-2b) (▲), and third-party B10.BR (H-2b) (●) strains. a. Mice reconstituted with 2 × 10⁶ ND (CFSE⁺/CD4⁻) CD4⁺ T cells (n = 6) demonstrated delayed rejection of their stimulator C57BL/6 skin graft (MST, 69 days), whereas their third-party B10.BR skin graft is rejected (MST, 16 days) from day 12 posttransplant (p = 0.0007). b. Mice reconstituted with 2 × 10⁶ D (CFSE⁺/CD4⁺) CD4⁺ T cells (n = 8) rejected their stimulator C57BL/6 (MST, 18 days) and third-party B10.BR (MST, 16 days) skin grafts at a similar rate (p = NS). Syngeneic BALB/c skin grafts remain intact for SCID mice reconstituted with ND or D CD4⁺ T cells.
were reconstituted with BALB/c CD4/H11001
/H9262 (pH 8.3), 50 mM KCl, and 1.5 mM MgCl), 5
ers 1–20 and a FAM-labeled C
SEhigh) or D (CFSElow) CD4
dNTPs (Roche, Mannheim, Germany), and 0.5 U of
Taq
stimulator C57BL/6 (H-2b), and third-
in a total volume of 25
CD4
geneic BALB/c (H-2d), allogeneic
and grafted with tail skin from syn-
party B10.BR (H-2 k) strains. Skin
grafts were
5m m2. Grafts were assessed daily for signs of rejection,
and a graft was considered rejected when 90% of the graft had been lost or
affected.

Histology
BALB/c, C57BL/6, and B10.BR skin graft biopsies were obtained on day
12 from SCID mice that had been reconstituted with 2 \times 10^5
ND CD4 T cells and SCID mice that
were reconstituted with BALB/c CD4 T cells were grafted as controls. Skin
grafts were ~5 mm2. Grafts were assessed daily for signs of rejection,
and a graft was considered rejected when 90% of the graft had been lost or
affected.

TCR repertoire analysis
RNA was isolated from spleens and skin grafts of reconstituted SCID mice
using TRIzol (Invitrogen Life Technologies). RNA was reverse-transcribed
into cDNA using SuperScript reverse transcriptase (Invitrogen Life
Technologies) and random primers (Promega, Madison, WI) following the
manufacturer’s instructions. cDNA was measured using GAPDH and C-C \beta
primers (26). \( V \beta \) repertoire analysis was performed with mouse \( V \beta \) primers
1–20 and a FAM-labeled \( C \beta \) primer (27). cDNA was amplified by PCR
in a total volume of 25 \( \mu l \) consisting of reaction buffer (10 mM Tris-HCl
(pH 8.3), 50 mM KCl, and 1.5 mM MgCl), 5 \( \mu M \) of each primer, 200 \( \mu M 
\) dNTPs (Roche, Mannheim, Germany), and 0.5 U of Taq polymerase
(Roche). PCRs were performed in a thermal cycler 9600 (PerkinElmer,
Wellesley, MA). PCRs consisted of an initiation denaturation step of 94°C
for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, an-
ealing at 60°C for 1 min, and extension at 72°C for 1 min. A final ex-
tension was performed at 72°C for 10 min. Ten microliters of PCR product
was analyzed on a 2% agarose gel. For CDR3 spectratyping, PCR products
were analyzed on the PerkinElmer ABI PRISM 373 sequencer.

Delayed-type hypersensitivity (DTH) response
Responder mononuclear cells were derived from BALB/c mice immunized
with 0.5 mg of OVA/CFA in vivo and were stimulated with irradiated
C57BL/6 splenocytes in vitro. SCID mice were reconstituted with 4 \times 10^5
ND CD4 T cells sorted on day 6 of the MLC. Control BALB/c mice and
SCID mice were immunized with 0.5 mg of OVA/CFA in vivo. To mea-
sure the DTH response, the left footpad of mice was injected with OVA,
and the right footpad was injected with PBS. Swelling responses were
measured after 24 h with a standard outside micrometer (Mitutoyo, Tokyo,
Japan).

Proliferation assay
For proliferation assays, 3 \times 10^5 splenocytes isolated from SCID mice
reconstituted with either D or ND CD4 T cells that had rejected their skin
grafts were stimulated with 3 \times 10^5 allogeneic C57BL/6, B10.BR, or sy-
neic BALB/c splenocytes (direct stimulation) or UV-irradiated freeze/
thaw lysates isolated from 3 \times 10^5 allogeneic C57BL/6, B10.BR, or sy-
neic BALB/c splenocytes (indirect stimulation). Cells were grown in
96-well, round-bottom plates (in triplicate) in RPMI 1640 medium and
10% FCS and incubated at 37°C with 5% CO_2 for 4 days. At 96 h, 1
\( \mu Ci/well \) \[^{3}H\]thymidine was added. Cells were harvested, and the amount of \[^{3}H\]thymidine was measured using a scintillation counter.
**IFN-γ ELISPOT**

Immobilon-P plates (Millipore, Billerica, MA) were coated with 6 μg/ml mouse anti-IFN-γ Ab (BD Pharmingen) in carbonate buffer overnight at 4°C. Plates were washed, and responder and stimulator splenocytes were added at 3 x 10^5 cells/well. Responder splenocytes were isolated from mice that had been reconstituted with 2 x 10^5 ND or D CD4^+ T cells that had rejected their skin grafts. Stimulator splenocytes isolated from syngeneic BALB/c or allogeneic C57BL/6 or B10.BR naive mice for direct responses were treated with mitomycin C (50 μg/ml), incubated at 37°C for 30 min, and washed four times in RPMI 1640 medium. Stimulator Ags were prepared from UV-irradiated freeze-thaw lysates of splenocytes from naive C57BL/6, B10.BR, and BALB/c mice (17). Cells were cultured at 3 x 10^6/well for 24 h in 200 μl/well RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FCS, 25 mM HEPES, 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg of streptomycin, and 5 μM 2-ME. Plates were washed, secondary biotinylated anti-mouse IFN-γ was added at 2 μg/ml overnight at 4°C, plates were washed again, and streptavidin-alkaline phosphatase was added for 2 h, then plates were washed and developed with alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA). Spots were counted using the computer program KS ELISPOT (Zeiss, Göttingen, Germany).

**Statistical analysis**

Comparison of survival data between groups was analyzed using the log-rank test. Comparison of means for ELISPOT data, proliferation, and DTH responses was performed using two-tailed Student’s t test. Statistical analysis was performed using PRISM software (GraphPad, San Diego, CA). A difference was considered statistically significant at p < 0.05.

**Results**

**Allo-stimulated cells can be sorted into ND and D CD4^+ T cell populations**

To remove the alloreactive CD4^+ T cell population, we separated C57BL/6 (H-2^d^)-stimulated BALB/c (H-2^b^) cells into ND and D populations on day 6 of an MLC. Using the fluorescent cytoplasmic dye CFSE, which distributes equally to daughter cells upon cell division, D CD4^+ T cells were separated from ND CD4^+ T cells. On day 6 of the MLC, two discrete populations of cells could be identified, including a CFSE^high/CD4^+ T cell population (63.7% of CD4^+ T cells) and a CFSE^low/CD4^+ T cell population (36.3% of CD4^+ T cells). Cell sorting was used to separate these cells into a CFSE^high or ND population and a CFSE^low or D population of CD4^+ T cells (Fig. 1a). Phenotypic analysis of the ND and D populations of CD4^+ T cells using CD69 and CD25 demonstrated that both populations express markers of T cell activation (Fig. 1, b–d). The majority of the D CD4^+ T cells expressed the activation markers CD69 (70.8%) and CD25 (88.5%) as expected, but, interestingly, the expression of CD69 (8.5%) and CD25 (9.1%) was also found in the ND CD4^+ T cell population (Fig. 1, c and d). Therefore, although the ND or CFSE^high population of CD4^+ T cells did not divide, a proportion of T cells in this population was activated, as demonstrated previously (28). Allo-stimulated CD4^+ T cells were sorted into the ND and D populations and adaptively transferred into immunodeficient mice.

**Prolonged allograft survival in mice reconstituted with ND CD4^+ T cells**

To assess whether CD4^+ T cells that do not divide after allo-stimulation had a reduced capacity to reject specific allogeneic grafts, stimulator C57BL/6, third-party B10.BR, and syngeneic BALB/c skin grafts on SCID mice reconstituted with ND or D CD4^+ T cells mice were assessed daily for signs of rejection. Mice reconstituted with ND allogeneic CD4^+ T cells showed prolonged survival of their stimulator C57BL/6 (H-2^b^) grafts up to 70 days posttransplant (median survival time (MST), 69 days) and rejected their third-party B10.BR (H-2^d^) grafts from day 12 posttransplant (n = 6; MST, 16 days; p = 0.0007; Fig. 2a). Three mice had rejected their C57BL/6 grafts by day 70 (days 63, 70, and 70). Two mice had not rejected their C57BL/6 grafts at this time point, but had features of chronic rejection, with shrinkage and loss of hair. One mouse killed at an earlier time point (day 34) showed an intact C57BL/6 skin graft. Mice reconstituted with D allogeneic CD4^+ T cells rejected skin grafts from both stimulator C57BL/6 (MST, 18 days) and third-party B10.BR (MST, 16 days) strains. The time to rejection of allogeneic grafts from mice reconstituted with D CD4^+ T cells was similar to that of third-party B10.BR grafts on mice reconstituted with ND CD4^+ T cells (p = NS; n = 8; Fig. 2b). Both the ND and D groups of mice had not rejected their syngeneic BALB/c (H-2^b^) grafts on day 70 posttransplant (Fig. 2).

SCID mice that were not reconstituted with cells, but were grafted with C57BL/6, B10.BR, and BALB/c skin grafts, showed no signs of rejection of any of their grafts (data not shown; n = 6). SCID mice that were reconstituted with BALB/c CD4^+ splenocytes (n = 4) rejected both their stimulator C57BL/6 (MST, 18 days) and third-party B10.BR (MST, 20 days) skin grafts at a similar rate to SCID mice reconstituted with D allogeneic CD4^+ T cells (data not shown).

To compare the histology of skin grafts before rejection in SCID mice reconstituted with ND and D allogeneic CD4^+ T cells, we obtained skin graft biopsies on day 12 posttransplant. BALB/c syngeneic skin grafts from the control (Fig. 3a), ND (Fig. 3d), and D (Fig. 3g) groups of mice were intact. SCID mice that were not reconstituted with cells showed intact allogeneic stimulator C57BL/6 (Fig. 3b) and third-party B10.BR grafts (Fig. 3c). In mice reconstituted with ND CD4^+ T cells stimulated in the MLC with...
C57BL/6, the histology of skin grafts confirmed minimal lymphocyte infiltration in their stimulator C57BL/6 grafts (Fig. 3e); however, there was significant lymphocyte infiltration in third-party B10.BR skin grafts (Fig. 3j). Lymphocyte infiltration was observed in the MLC stimulator C57BL/6 strain (Fig. 3h) and third-party B10.BR grafts (Fig. 3i) in mice reconstituted with D allogeneic CD4+ T cells. SCID mice that were reconstituted with unseparated BALB/c CD4+ splenocytes demonstrated lymphocyte infiltration in both C57BL/6 and B10.BR skin grafts (data not shown).

The histology of skin grafts in mice reconstituted with ND CD4+ T cells showed a progressive increase in cellular infiltration in their stimulator C57BL/6 grafts; minimal cellular infiltrate was observed on day 12 (Fig. 3e), and it increased on day 35 (Fig. 3j) and again on day 65 posttransplant (Fig. 3k).

### ND CD4+ T cells demonstrate a diverse TCR repertoire

To demonstrate that allo-stimulated ND CD4+ T cells maintain competent immune function, we examined the TCR diversity of cells in the ND and D T cell populations. RNA was isolated from sorted D and ND CD4+ T cells that had been stimulated by C57BL/6 in the MLC. In addition, RNA was prepared from the spleens of SCID mice that had been reconstituted with ND and D allogeneic CD4+ T cells, and splenocytes from BALB/c mice served as a control. RT-PCR analysis of the TCR repertoire of spleen cells from the mice reconstituted with ND and D allogeneic CD4+ T cells was performed for mouse TCR Vβ genes 1–20.

Both the D and ND cells expressed a wide range of TCR Vβ genes (Fig. 4a). A representative Vβ gene spectratype is shown (Vβ14) for splenocytes from the ND and D groups and for sorted cells before injection (Fig. 4, b and c). CDR3 region spectratyping analysis of Vβ genes in the spleens of mice reconstituted with ND and D CD4+ T cells showed a wide range of CDR3 lengths, similar to that observed in control BALB/c mice (Fig. 4b). This range of CDR3 lengths was observed in sorted ND and D CD4+ T cells (Fig. 4c) before injection into mice.

### ND CD4+ T cells maintain specific memory after transfer

To investigate whether ND CD4+ T cells maintain a memory response, DTH responses of mice reconstituted with ND CD4+ T cells from mice prestimulated with OVA were tested. Responder mononuclear cells were derived from BALB/c mice immunized with OVA/CFA in vivo and were stimulated with irradiated C57BL/6 splenocytes in vitro. SCID mice reconstituted with ND CD4+ T cells sorted from the MLC and BALB/c mice were immunized with OVA in vivo. SCID mice reconstituted with ND CD4+ T cells sorted from the MLC established with immunized BALB/c mice (ND(i)) and immunized BALB/c mice (BALB/c(i)) were injected with OVA or PBS (control) in the footpads. BALB/c mice, SCID mice, and SCID mice reconstituted with ND CD4+ T cells (ND) that had not been immunized previously were also injected with OVA or PBS (control) in the footpads. Swelling responses were measured with a micrometer 24 h after injection.

A swelling response to OVA was observed in mice reconstituted with ND CD4+ T cells sorted from the MLC established from mice immunized with OVA (ND(i); 82.4% ± 19.9%; p = 0.0026). A swelling response to OVA was observed in both immunized BALB/c mice (BALB/c(i); 59.8% ± 12.7%) and control BALB/c mice (50.6% ± 25.8%; Fig. 5). Minimal swelling in footpads in response to OVA was observed in control SCID mice (0.5% ± 0.40%). Minimal swelling responses were observed for PBS injections, which were used as a control for all groups (Fig. 5).

### FIGURE 5. ND CD4+ T cells maintain specific memory after transfer.

SCID mice were reconstituted with ND CD4+ T cells sorted on day 6 of the MLC established with responder splenocytes isolated from BALB/c mice immunized with OVA/CFA in vivo that were stimulated with irradiated C57BL/6 in vitro. Control BALB/c mice were immunized with OVA/CFA in vivo. To measure the DTH response, the left footpad of mice was injected with OVA (■) and the right footpad with PBS (□). Swelling responses were measured after 24 h with a standard outside micrometer (Mitutoyo, Tokyo, Japan). A swelling response to OVA (■) was observed in mice reconstituted with ND CD4+ T cells sorted from the MLC established with mice immunized with OVA (ND(i); 82.4% ± 19.9%; n = 3); however, minimal swelling was observed in mice that received ND CD4+ T cells sorted from MLC established from mice that had not been immunized with OVA (ND; 3.9% ± 3.63%; n = 3, p = 0.0026). A swelling response to OVA was observed in immunized BALB/c mice (BALB/c(i); 59.8% ± 12.7%; n = 3) and control BALB/c mice (BALB/c; 50.6% ± 25.8%; n = 3). Minimal swelling in footpads in response to OVA was observed in control SCID mice (SCID; 0.5% ± 0.40%; n = 3). Minimal swelling in footpads was observed with PBS injections in all groups used as a control.* , p < 0.05.

Allo-stimulated ND CD4+ T cells demonstrate an indirect response to the stimulator strain

To determine whether ND CD4+ T cells use the direct or indirect allo-activation pathway, proliferation and IFN-γ production by splenocytes from mice reconstituted with ND and D CD4+ T cells were analyzed after stimulation in vitro with allogeneic splenocytes (direct) or with allogeneic splenocyte lysates (indirect).

Responder splenocytes isolated from SCID mice reconstituted with D and ND allogeneic CD4+ T cells that had rejected their allografts (>80 days posttransplant) were stimulated in vitro for 4 days. ND CD4+ T cells directly activated demonstrated significantly reduced proliferation in response to allogeneic stimulator C57BL/6 splenocytes (1496 ± 219.0), but maintained their proliferative response to allogeneic third-party B10.BR splenocytes (5948 ± 347.5; p = 0.0014; Fig. 6a). This is in contrast to D CD4+ T cells that demonstrated a greater proliferation in response to the stimulator C57BL/6 strain (1715 ± 121.6) compared with the third-party B10.BR strain (784.0 ± 104.0; p = 0.0043) when directly activated (Fig. 6b).

Interestingly, when the ND CD4+ T cells are indirectly stimulated, they demonstrate a similar proliferative response to both allogeneic stimulator C57BL/6 (11,130 ± 1,590) and third-party B10.BR strains (10,250 ± 1,295; Fig. 6c; p = NS). However, D CD4+ T cells again demonstrate a greater proliferative response to the stimulator strain C57BL/6 (8,753 ± 147.5) compared with the third-party B10.BR (2,608 ± 442.6) when indirectly stimulated (p = 0.0002; Fig. 6d). Minimal proliferation was observed in response to syngeneic BALB/c stimulators for both ND and D CD4+ T cells, either directly or indirectly activated (Fig. 6).
IFN-γ production was also assessed when ND and D CD4+ T cells were stimulated with allogeneic splenocytes (direct) or allogeneic lysates (indirect) for 24 h in vitro. IFN-γ production by ND CD4+ T cells was significantly reduced when they were stimulated directly by the allogeneic stimulator C57BL/6 strain (16.0 ± 1.35 spots/3 × 105 cells) compared with the third-party B10.BR (55.5 ± 4.27 spots/3 × 105 cells; p = 0.0001; Fig. 7a). However, D CD4+ T cells demonstrated a similar amount of IFN-γ production when stimulated directly by either allogeneic stimulator C57BL/6 strain (74.3 ± 10.11 spots/3 × 105 cells) or third-party B10.BR (70.5 ± 4.21 spots/3 × 105 cells) splenocytes (p = NS; Fig. 7b). IFN-γ production by ND CD4+ T cells that had been indirectly activated with the stimulator C57BL/6 strain (9.3 ± 2.50 spots/3 × 105 cells) was less than the IFN-γ response to third-party B10.BR stimulators (31.8 ± 1.49 spots/3 × 105 cells; p = 0.0002; Fig. 7c). However, D CD4+ T cells demonstrated a greater amount of IFN-γ production when indirectly activated with the stimulator C57BL/6 strain (61.8 ± 3.28 spots/3 × 105 cells) compared with the third-party B10.BR stimulators (30.8 ± 5.94 spots/3 × 105 cells; p = 0.0038). Minimal IFN-γ production was observed for ND or D CD4+ T cells either directly or indirectly activated with syngeneic BALB/c stimulators (Fig. 7).

Discussion
Skin grafts across major MHC mismatches are rapidly rejected and are more difficult to tolerize than vascular grafts. In our model, the removal of alloreactive CD4+ T cells by pruning the T cell population using CFSE to remove proliferating cells prolongs the survival of skin grafts across a major mismatch in a strain-specific fashion. However, rejection does occur after a prolonged period and appears to be due to a chronic process mediated primarily through the indirect pathway. Previous studies show that in mice depleted of CD8 T cells and grafted with class II knockout skin, loss of the direct pathway caused prolongation of skin graft survival across a few minor mismatches, but had less effect with multiple mismatches and no effect on major MHC-mismatched skin (4). In our model, the marked prolongation of survival across a major mismatch suggests that removal of D cells is much more potent than removal of the direct pathway alone.

This suggests either that a regulatory mechanism is involved or that in addition to directly activated cells, there is the removal of some indirectly activated cells in the D population, leaving a population of T cells that is more highly depleted of alloreactive T cells. ND CD4+ T cells still retain their capacity to reject third-party grafts. Interestingly, the D CD4+ T cells do not reject the C57BL/6 grafts faster than the third-party cells. There are several possibilities that can account for the inability of D cells to more rapidly reject their priming Ag compared with third-party cells. These include the sharing of MHC epitopes between the two strains, the possibility that the B10.BR strain may be more immunogenic than C57BL/6 for BALB/c mice, or that minor epitopes shared between B10.BR and C57BL/6 are responsible. The ELISPOT data suggest that direct responses are equivalent between donor and third-party
cells from the mice reconstituted with D cells, whereas C57BL/6 induces a stronger indirect response than B10.BR. Because B10.BR mice are derived from C57BL/6, they would be expected to share many minor Ags. The stronger indirect response would therefore be due to indirectly presented H-2 molecules. Thus, the indirect results suggest that it is these minor Ags that are being recognized. In contrast, the mice reconstituted with D CD4+ T cells respond indirectly to lysates from C57BL/6, but not B10.BR, suggesting that the indirect response in these cells is predominantly to reprocessed MHC Ags. Although this does not alter the clinical response, it supports the previous reports that the D population makes up 3–5% of the initial T cell population (22). The ND population retained of antigenic memory and T cell repertoire avoids the risks associated with T cell depletion and the requirement for stem cell reconstitution of the T cell compartment.

Depletion of alloreactive T cells has been attempted by a number of methods, in particular for BMT to reduce GVHD. Most have used modified MLCs and have included deletional Abs or immunotoxins against activation markers (32–34), enhancement of activation-induced cell death in the alloreactive population (35), magnetic bead sorting to remove cells expressing activation markers such as CD69 or CD25 (36–38), cell sorting by flow cytometry based on cell size and activation markers (39), viral infection of stimulator cells to enhance activation in the MLC (40), and keratinocytes as stimulator cells (41). Most recently, the use of CFSE

**FIGURE 7.** ND CD4+ T cells demonstrate reduced IFN-γ production when activated with the stimulator strain. Splenocytes (3 × 10^5) were isolated from SCID mice that were reconstituted with ND (CFSEhigh) or D (CFSElow) CD4+ T cells that had rejected their skin grafts and were stimulated in vitro for 24 h with medium, 3 × 10^5 syngeneic BALB/c, allogeneic stimulator C57BL/6, or third-party B10.BR splenocytes (direct) or lysates (indirect). IFN-γ production was measured by ELISPOT analysis (spots per 3 × 10^5 cells). a, ND CD4+ T cells directly stimulated show reduced IFN-γ production in response to the stimulator C57BL/6 strain (16.0 ± 1.35 spots), but maintain a third-party B10.BR response (55.5 ± 4.27 spots; p = 0.0001). b, D CD4+ T cells directly stimulated show reduced IFN-γ production in response to both the stimulator C57BL/6 (74.3 ± 10.11 spots) and third-party B10.BR (70.5 ± 4.21 spots) strains (p = NS). c, ND CD4+ T cells that were indirectly activated demonstrate a reduced amount of IFN-γ in response to the stimulator C57BL/6 strain (9.3 ± 2.50 spots), but maintain a response to the third-party B10.BR strain (31.8 ± 1.49 spots; p = 0.0002). d, D CD4+ T cells that were indirectly activated show increased IFN-γ production in response to the stimulator C57BL/6 strain (61.8 ± 3.28 spots) compared with the response to third-party B10.BR (30.8 ± 5.94 spots; p = 0.0038). *p < 0.05; **p < 0.001.
to remove alloreactive T cells has been shown in BMT to reduce GVHD (42). The removal of a specific alloreactive population has been tried experimentally in the graft-vs-host model and in an allograft model using a suicide gene (43). In this study the removal of activated T cells using a suicide gene prevents the development of GVHD and prolongs allograft survival. We believe that proliferation and pruning of the alloreactive cells may serve as a more effective marker of alloreactivity than the models described above, because this allows positive selection of a nonproliferating population rather than requiring all alloreactive cells to express an individual marker or sensitivity to a specific Ab.

Induction of peripheral tolerance, allowing graft survival in higher primates compared with murine models, appears to be limited by the larger memory population and greater alloreactive pool (44). Other strategies currently in place in man or primates use T cell ablation as part of their regimen to achieve central tolerance (45). Specifically, strategies such as mixed chimerism require significant recipient and donor T cell ablation to avoid GVHD and allow engraftment. Other strategies, such as T cell or other immune ablation using ATG or Campath (anti-CD54), lead to long periods of T cell depletion with their associated risks (45, 46). The method outlined in our studies uses an in vitro method to remove alloreactive T cells that is analogous to central deletion. The additional advantage of reconstitution with allo-depleted peripheral T cells is that it leaves intact a large reservoir of memory T cells to potential pathogens, thereby reducing the risk of infection. Furthermore, regulatory T cells are likely to be included in the ND population, because they do not proliferate in vitro (47).

Regulatory T cells in transplantation appear to be indirectly activated (48). Our strategy purges predominantly directly activated T cells while leaving some residual ND T cells. Thus, there may be T cells with an indirect affinity for the allo-stimulus in the ND CD4+ T cell population. This may reflect the suppression of lower affinity T cell TCRs by higher affinity T cell TCRs (49). Therefore, pruning of alloreactive T cells may allow combination with other strategies to induce regulatory T cells. Flow cytometry demonstrates a CD25 population in the ND T cell population that may have regulatory properties.

In our model we found homoeostatic proliferation, with loss of CFSE staining, to be indicative of proliferation after transfer to SCID mice, which is not seen with transfer to syngeneic BALB/c mice. Recent data suggest that homoeostatic proliferation, by inducing a memory phenotype, makes the development of effective tolerance more difficult, although the graft prolongation found in the presence of homoeostatic proliferation in our model is an indicator of its effectiveness (50).

In conclusion, the reconstitution of mice with CD4+ T cells from an MLC after the removal of a D population suggests that this is a powerful way to reduce the alloreactive population while maintaining normal immune capacity. The predominance of the indirect response in the eventual rejection of the skin suggests that this strategy needs to be developed to eventually remove T cells with both direct and indirect allo-recognition. This might raise the possibility of obtaining long-term acceptance of a transplanted organ in the absence of both direct and indirect allo-recognition or the use of tolerizing strategies that use the indirect pathway and may suppress memory or CD8 alloreactive T cells.

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6581


