Multilineage Engraftment with Minimal Graft-Versus-Host Disease Following In Utero Transplantation of S-59 Psoralen/Ultraviolet A Light-Treated, Sensitized T Cells and Adult T Cell-Depleted Bone Marrow in Fetal Mice

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Multilineage Engraftment with Minimal Graft-Versus-Host Disease Following In Utero Transplantation of S-59 Psoralen/Ultraviolet A Light-Treated, Sensitized T Cells and Adult T Cell-Depleted Bone Marrow in Fetal Mice

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Although engraftment following in utero stem cell transplantation can readily be achieved, a major limitation is the low level of donor chimerism. We hypothesized that a lack of space for donor cells in the recipient marrow was one of the primary reasons for failure to achieve significant engraftment, and that donor T cells could make space in an allogeneic mismatched setting. We found that 3 x 10^5 C57BL/6 (B6) naive CD3^+ cells coinjected with B6 T cell-depleted bone marrow (TCDBM) into 14- to 15-day-old BALB/c fetuses resulted in multilineage engraftment (median, 68.3%) associated with severe graft-vs-host disease (GvHD; 62 vs 0% with TCDBM alone). When 1.5 x 10^5 CD4^+ or CD8^+ cells were used, low levels of engraftment were seen vs recipients of 1.5 x 10^6 CD3^+ cells (2.4 ± 1.1 and 6.6 ± 3.9 vs 20.4 ± 10.4%, respectively). To test the hypothesis that proliferation of T cells in response to alloantigen resulted in GvHD and increased engraftment, we pretreated naive T cells with photochemical therapy (PCT) using S-59 psoralen and UVA light to prevent proliferation. GvHD was reduced (60–0%), but was also associated with a significant reduction in engrafted donor cells (53.4 ± 4.2 to 1.7 ± 0.5%). However, when B6 T cells were sensitized to BALB/c splenocytes, treated with PCT, and coinjected with TCDBM, there was a partial restoration of engraftment (13.3 ± 2.4% H2Kb^+ cells) with only one of nine animals developing mild to moderate GvHD. In this study we have shown that PCT-treated T cells that are cytotoxic but nonproliferative can provide an engraftment advantage to donor cells, presumably by destroying host hematopoietic cells without causing GvHD. The Journal of Immunology, 2002, 169: 6133–6140.
without GvHD, presumably by creating “space” in the host marrow and thus providing an engraftment advantage to the donor hematopoietic stem cells.

Materials and Methods

Mice

Adult BALB/c (H2d), C57BL/6 (H2b), and C3H (H2k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained and bred in a pathogen-free environment in the University of California-San Francisco Animal Care Facility following the standard National Institutes of Health guidelines for animal welfare under a protocol approved by the University of California-San Francisco committee on animal research. For timed pregnancies we used estrous suppression in females by crowding them, with subsequent acceleration by caging them with males. The day on which the vaginal plug was identified was designated day 0. Sensitization of mice was performed as previously reported (9) by injecting B6 adult males with three weekly i.p. doses of 10 × 106 BALB/c splenic T cells.

Chemicals and reagents

Cells were cultured in complete culture medium, consisting of RPMI 1640 supplemented with t-glutamine (2 mM/ml), nonessential amino acids (0.1 mM/ml), sodium pyruvate (1 mM/ml), HEPES (10 mM), 2-ME (50 μM), gentamicin (50 μg/ml), and 10% heat-inactivated FBS. Bone marrow, thymocytes, and splenocytes were collected using 10 mM HEPES, 2 mM EDTA, 50 μg/ml gentamicin, and 2% heat-inactivated FBS in RPMI 1640.

Preparation of T cell depleted bone-marrow (TCDBM)

Bone marrow cells were freshly harvested from the tibias and femurs of 2- to 6-month-old male B6 mice. Erythrocytes were lysed with hypotonic lysis buffer (150 mM ammonium chloride, 10 mM sodium bicarbonate, and 1 mM EDTA). T cells were depleted using magnetic bead-coated anti-CD3 Ab (Milteny Biotech, Auburn, CA) following the manufacturer’s instructions. The purity of the preparations was checked by FACS analysis and was found to be <0.05% CD3+.

Purification of T cells

T cells were purified from the spleens of naive and sensitized B6 mice. The purification of T cells was performed by depleting the splenocytes of macrophages, NK cells, granulocytes, stem cells, and B cells by negative selection using a mixture of biotinylated Abs (F4/80, NK1.1, Gran-1, c-kit, Sca-1, B220, and TER 19) and streptavidin-coated magnetic beads. To isolate CD4+ and CD8+ T cell subsets from separate aliquots of splenocytes, biotinylated anti-CD8 and anti-CD4 were added, respectively, to the absorbed cell aliquots. An aliquot of the cell suspension was fixed with 0.5% paraformaldehyde and phenotyped using a FACScan. Control samples were incubated with isotype-matched mAb. The CD4 and CD8 cell preparations were 89–93% pure, with <3% of the other T cell subset present in each preparation.

S-59 psoralen/UVA PCT

S-59, a psoralen (provided by Cerus, Concord, CA) was diluted to 1–8 mM in 5% FBS in PBS. Isolated T cells (5–20 × 106) were suspended in 5 ml of S-59 psoralen solution and placed in a 15 × 60-mm disposable petri dish, which was exposed to UVA (320–400 nm) from an illumination device (Cole Parmer, Vernon Hill, IL) for 6 min.

Characterization of T cells

Anti-CD3 proliferation and FACScan analysis. Anti-CD3+ mAb (clone 145/2C11; BD PharMingen, San Diego, CA) was loaded onto wells of a 24-well, flat-bottom plate and kept overnight at 4°C. Before use the wells were washed three times with complete culture medium. Splenic T cells (2–4 × 105) with or without PCT were added to each well in triplicate and cultured at 37°C in 5% CO2 for 48 h. The cells were harvested, and the level of T cell activation was determined by CD25 and CD69 expression. In addition, 1 × 106 T cell3 cells with and without anti-CD3 stimulation were cultured in triplicate in round-bottom wells (96-well plate) for 24 h. The culture was pulsed on the third day with 0.5 μCi of [3H]thymidine and incubated for 18 h. Cultures were harvested on glass-fiber filters using an automated cell harvester. The [3H]thymidine incorporation was measured by liquid scintillation counting. The incorporation of [3H]thymidine (stimulated minus resting) was expressed as cpm.

Cytotoxicity studies. For CTL activity, lymphoblast targets (B6, BALB/c, C3H) were prepared by incubating 4–6 × 106 splenocytes with 6 μg/ml of Con A for 2 days at 37°C in 5% CO2. The cells were layered on 100 μl of medium. Varying numbers of effectors (naïve and sensitized T cells with or without PCT) and an identical volume of targets were added to 96-well, V-bottom plates. The incubation was performed in triplicate at 37°C in 5% CO2 for 4 h, after which 100 μl of supernatant was removed, and 10 μl of supernatant was added to 250 targets/100 μl of medium. The incorporation of [3H]thymidine (stimulated minus resting) was expressed as cpm.

Graft-vs-host disease

The pups were monitored for survival, weight gain, fur thickness, and rectal prolapse. Clinical signs of GvHD at 15 days of age were graded as mild, moderate, or severe based upon the following criteria provided in Table I. The pups were killed by cervical dislocation. Blood and various tissues (see below) were collected for enzyme and histologic evaluation.

Identification of engrafted donor cells

The percentage and subtype of donor cells were evaluated by two- or three-color fluorescent cell surface staining with mAb and analyzed on a FACScan (BD Biosciences, San Jose, CA). The FITC-, PE-, and Tricolor-conjugated rat or mouse anti-mouse mAb to mouse macrophage (F4/80), granulocytes (gran-1), T cells (CD3e and CD4), B cells (B220), NK cells (pan DX-5), macrophages (F4/80), and donor cells (H2Kb) were purchased from BD PharMingen (San Diego, CA). The FITC-, PE-, and Tricolor-conjugated hamster, rat, or mouse IgG1, IgG1A, IgG1X, IgG2a, and IgG2b isotype standard Abs were used as control Abs. RBC were lysed with whole blood lysis reagent (Coulter, Miami, FL). Cells were collected twice with cold Dulbecco’s PBS containing sodium azide (0.1%) and 0.5% BSA and were incubated with Fc receptor blocker at 37°C for 20 min. After one wash the cells were incubated with the desired combination of marrow and T cells through a 50-μm glass micropipette. The uterus was replaced, and the incision was closed. Bruneck (150 μg; Reckitt & Colman Pharmaceuticals, Richmond, VA) was administered s.c. for pain. The animals were monitored for 4 h after surgery until they were awake and able to drink, then daily thereafter. Recipients were delivered at days 20–21 of gestation and were weaned at 21 days of age.

Intraperitoneal bone marrow transplant and follow-up

IUT was performed as previously described (8). Briefly, 14- to 15-day-pregnant BALB/c mice were placed under general mask anesthesia using 2% isofluorane in an anesthetic machine (Vet Equip, Pleasanton, CA). The abdomen was cleaned with 70% alcohol, and a 2-cm vertical laparotomy incision was made in the abdomen under sterile conditions. The uterus was exposed, and the fetuses were injected i.p. through the uterine wall with 5 μl of the desired combination of marrow and T cells through a 50-μm glass micropipette. The uterus was replaced, and the incision was closed. Bruneck (150 μg; Reckitt & Colman Pharmaceuticals, Richmond, VA) was administered s.c. for pain. The animals were monitored for 4 h after surgery until they were awake and able to drink, then daily thereafter. Recipients were delivered at days 20–21 of gestation and were weaned at 21 days of age.

Collection of blood, marrow, or tissues

Peripheral blood was drawn from the saphenous vein at various time points up to 9 mo of age. Blood was collected into heparinized microcapillary tubes. Spleen, thymus, bone marrow, liver, small and large intestines, and skin were obtained postmortem as previously described (8).

Identification of engrafted donor cells

The percentage and subtype of donor cells were evaluated by two- or three-color fluorescent cell surface staining with mAb and analyzed on a FACScan (BD Biosciences, San Jose, CA). The FITC-, PE-, and Tricolor-conjugated rat or mouse anti-mouse mAb to mouse macrophage (F4/80), granulocytes (gran-1), T cells (CD3e and CD4), B cells (B220), NK cells (pan DX-5), macrophages (F4/80), and donor cells (H2Kb) were purchased from BD PharMingen (San Diego, CA). The FITC-, PE-, and Tricolor-conjugated hamster, rat, or mouse IgG1, IgG1A, IgG1X, IgG2a, and IgG2b isotype standard Abs were used as control Abs. RBC were lysed with whole blood lysis reagent (Coulter, Miami, FL). Cells were washed twice with cold Dulbecco’s PBS containing sodium azide (0.1%) and 0.5% BSA and were incubated with Fc receptor blocker at 37°C for 20 min. After one wash the cells were incubated with the desired mAb mixture in the dark for 30 min. Parallel cultures were incubated with isotype-matched, nonspecific Abs. Lymphocytes were gated based on their forward and side scatter profile for each experiment.

Two-dimensional display of fluorescence channel (FL1) vs FL2 or FL3 vs FL2 plots of the gated population indicated the donor cell subtype, as identified by the CD Ags and H2Kb+ populations, respectively. BALB/c and B6 blood were used as negative and positive controls, respectively.
Donor cell engraftment was defined as >1% H2Kb+ cells based upon results using nontransplanted BALB/c controls.

**Skin grafting**

We used a previously reported procedure for skin grafting (8). Briefly, syngeneic, allogeneic (B6) and third-party (C3H) donor skin was used. The entire graft bed and a small margin of surrounding tissue was covered with a double-thickness square of a nonadherent pad supplemented with antibiotics, and the wound was covered with a sheer band-aid. An elastic bandage was wrapped around the body to restrict the skin graft and band-aid. Each skin-grafted mouse received 0.05 μg/g of Buprenex by s.c. injection to reduce surgical pain. After 9 days the band-aid was removed, and grafts were scored daily thereafter. Animals that rejected donor skin grafts within 14 days of placement were classified as nontolerant.

**Statistical analysis**

Wilcoxon signed-rank test and χ2 analysis were used to analyze nonparametric data, and Student’s two-tailed t test was used for the parametric data. Values (two-tailed) of p ≤ 0.05 were considered significant.

**Results**

*Role of T cells in engraftment and GvHD in utero*

In a previous study (8) we found that in utero recipients (BALB/c) of B6 progenitor dendritic cells plus low numbers (∼1–2 × 10^5) of B6 T cells developed GvHD at an increased rate. This was associated with significantly increased multilineage engraftment. To pursue the role of T cells in engraftment and GvHD in this IUT model we injected varying doses (0.75 × 10^5, 1.5 × 10^5, and 3 × 10^5) of naive CD3+ splenocytes from B6 adult male donors with and without 1.5 × 10^6 TCDBM into 14- to 15-day fetal BALB/c recipients. The end points were survival, weight gain/loss, and engraftment.

Five groups of survivors were evaluated for the presence of durably engrafted donor cells in the blood by FACS at 6-wk intervals. Table II shows the results for the group that received TCDBM alone or 1.5–3 × 10^5 CD3+ cells alone vs those that received three doses of CD3+ cells plus TCDBM. We found significant multilineage engraftment only in those groups that received at least 1.5 × 10^5 T cells and TCDBM (p < 0.01). There also appeared to be a dose response in terms of the number of T cells injected, with 0.75 × 10^5 CD3+ cells having little effect, while 3 × 10^5 CD3+ cells plus TCDBM resulted in a median engraftment of 57% donor cells in the peripheral blood (Table II).

While circulating donor cells were found in the blood of three BALB/c in utero recipients of B6 T cells alone, it was not multilineage (Table II). Fig. 1 shows a representative example of the FACS analysis for various cell lineages in the blood of an IUT BALB/c recipient of TCDBM and/or 3 × 10^5 CD3+ cells. As we have previously seen (1, 3, 8) there was only a low level (1.3% B220+ to 4.6% Mac+ donor cells) of multilineage engraftment in the recipients of TCDBM (Fig. 1B), and the recipient of T cells alone (Fig. 1C) had a small number (1.08%) of CD3+ cells (compared with BALB/c negative control; Fig. 1A). However, the majority (>80%) of the cells were of donor origin in the recipient of TCDBM plus CD3+ cells (Fig. 1D), comparable to the C57BL/6-positive control (Fig. 1E).

When the bone marrow was evaluated for engraftment at the time of death (Table III), there was no significant difference in the percentage of animals with donor cells in the marrow in any group receiving TCDBM. However, the percentage of multilineage donor cells in the marrow was significantly higher in those groups receiving 1.5–3 × 10^5 CD3+ cells plus TCDBM compared with controls with low to no T cells in the inoculum (p < 0.001). Finally, the majority of the animals injected with 1.5–3 × 10^5 CD3+ cells (with or without TCDBM) developed moderate to severe GvHD (Table III).

These results suggest that when “space” can be made in the marrow via allogeneic donor T cells, significant multilineage engraftment can be established in the majority of recipients. However, the use of allogeneic donor T cells also results in fatal GvHD in many recipients.

**CD4+ vs CD8+ T cell subsets and engraftment/GvHD in utero**

We evaluated the effect of coinjecting either CD4+ or CD8+ adult male B6 splenocytes with TCDBM into fetal BALB/c recipients. Table IV shows the results for recipients followed up to 24 wk of age. The overall survival of the animals that were injected with TCDBM plus 1.5 × 10^5 CD3+, CD4+, or CD8+ cells was 30, 45,

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**Table I. Grading of murine GvHD**

<table>
<thead>
<tr>
<th>Criteria at 15 Days</th>
<th>Normal</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>&gt;12 g</td>
<td>9–11</td>
<td>6–9 g</td>
<td>&lt;6 g</td>
</tr>
<tr>
<td>Hair loss</td>
<td>Normal growth</td>
<td>Growth delay &gt;10 days</td>
<td>&lt;80% normal</td>
<td>&lt;50% normal</td>
</tr>
<tr>
<td>Perianal mucosa</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

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**Table II. Kinetics of engraftment of B6 BM with or without naive CD3+ cells in blood of BALB/c in utero recipients**

<table>
<thead>
<tr>
<th>Age Post-IUT</th>
<th>6 wk</th>
<th>12 wk</th>
<th>18 wk</th>
<th>24 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>No. Born</td>
<td>No. Eng</td>
<td>% Don Cells</td>
<td>No. Eng</td>
</tr>
<tr>
<td>TCDM</td>
<td>7</td>
<td>6/7</td>
<td>0.6–3.4 (0.6)</td>
<td>3/7</td>
</tr>
<tr>
<td>T cells (1.5–3.0 × 10^5)</td>
<td>12</td>
<td>5/7</td>
<td>0.3–3.2 (1.2)*</td>
<td>3/5</td>
</tr>
<tr>
<td>TCDM + 7.5 × 10^5 CD3+</td>
<td>21</td>
<td>8/16</td>
<td>0.2–3.2 (1.5)</td>
<td>1/16</td>
</tr>
<tr>
<td>TCDM + 1.5 × 10^5 CD3+</td>
<td>42</td>
<td>ND</td>
<td>ND</td>
<td>42</td>
</tr>
<tr>
<td>TCDM + 3.0 × 10^5 CD3+</td>
<td>26</td>
<td>9/9</td>
<td>0.3–99 (25.4)</td>
<td>3/3</td>
</tr>
</tbody>
</table>

*Number of animals engrafted/number surviving.

*Number of donor cells (median).

*Only donor CD3+ cells identified.

*Multilineage engraftment documented.

*p ≤ 0.05 vs TCDBM.

*p ≤ 0.001 vs TCDBM.
and 29%, respectively (data not shown). While there was a trend toward improved survival in CD4 recipients compared with the other groups, it was not significant ($p/110050.07$). The engraftment rate also appeared greater in the CD3 and CD8 recipients (43 and 44%, respectively) compared with CD4 recipients (19%), although it was not statistically significant ($p/110050.08$). However, the percent donor cells in the engrafted CD3 recipients was significantly greater than that in either CD4 or CD8 subset recipients ($p/110210.03$). While the incidence of GvHD was not statistically significant between any of the three groups, the 11 CD4 recipients all had mild GvHD compared with the CD3 and CD8 recipients.

**Tolerance induction after CD3$^+$, CD4$^+$, or CD8$^+$ T cell injection in utero**

We performed syngeneic, donor, and third-party skin grafts in long term survivors that had received injections of TCDBM plus $1.5 \times 10^5$ CD3$^+$ cells, $1.5 \times 10^5$ CD4$^+$ cells, or $1.5 \times 10^5$ CD8$^+$ cells. Skin grafts were durably accepted in the in utero recipients in one of four, zero of 11 and zero of three, respectively, and were prolonged in two of four, zero of 11, and zero of three, respectively. All animals in all three groups rejected third-party skin grafts.

**Psoralen/UVA light treatment of naive and sensitized T cells: in vitro results**

Initially, we identified the optimal doses of S-59 psoralen (plus UVA light) that inhibited anti-CD3-stimulated T cell proliferation while maintaining CD69 and CD25 activation in naive and sensitized CD3$^+$ adult B6 splenocytes. The doses of S-59 psoralen that resulted in $>95\%$ inhibition of proliferation were 1–2 nM (Table V). Naive T cells had low background compared with that of resting cells from sensitized mice. This is consistent with the state of

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**Table III.** GvHD and multilineage engraftment in marrow of BALB/c in utero recipients of B6 BM with or without naive CD3$^+$ cells at the time of death and/or sacrifice

<table>
<thead>
<tr>
<th>Cells Injected</th>
<th>No. of Fetuses</th>
<th>No. Born</th>
<th>No. Eng$^b$</th>
<th>% Donor Cells$^c$</th>
<th>No. with GvHD/No. Born (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDBM$^d$</td>
<td>14</td>
<td>7</td>
<td>2</td>
<td>0.03–0.2 (0.11)</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>T cells (1.5–3.0 $\times 10^3$)</td>
<td>56</td>
<td>12</td>
<td>0</td>
<td>0 (0)</td>
<td>7/12 (58)</td>
</tr>
<tr>
<td>TCDBM + 0.75 $\times 10^3$ CD3$^+$</td>
<td>40</td>
<td>21</td>
<td>5</td>
<td>0.2–3 (0.8)</td>
<td>2/21 (10)</td>
</tr>
<tr>
<td>TCDBM + $1.5 \times 10^3$ CD3$^+$</td>
<td>66</td>
<td>42</td>
<td>14</td>
<td>9.7–29 (14.2)$^e$</td>
<td>36/42 (86)$^f$</td>
</tr>
<tr>
<td>TCDBM + $3.0 \times 10^3$ CD3$^+$</td>
<td>68</td>
<td>26</td>
<td>9</td>
<td>1.6–95 (68.3)$^f$</td>
<td>16/26 (62)$^f$</td>
</tr>
</tbody>
</table>

$^a$ Number of fetuses injected.
$^b$ Number engrafted in marrow at the time of death/sacrifice.
$^c$ Range in marrow (median).
$^d$ TCDBM.
$^e$ $p \leq 0.001$ vs TCDBM.
activation of the sensitized T cells. In these experiments proliferation was blocked effectively with 2 nM S-59 psoralen and UVA light, although there was only minimal inhibition of activation marker expression (Table VI). Resting cultures of cells from sensitized animals showed higher levels of CD3⁺ cells. These cells also demonstrated higher levels of CD25 and CD69 expression, and the ratio of CD4⁻CD8⁻ cells was skewed toward the CD8⁺ subset following sensitization (Table VI). However, S-59 psoralen/UVA light treatment had little effect on the expression of these markers. The PCT-treated cells expressed similar levels of CD25 and CD69 in resting and stimulated cultures. However, PCT-treated naive cells showed a lower level of CD69 and CD25 expression following anti-CD3 stimulation compared with the sensitized group. This was not due to increased apoptosis, since there was no significant increase in the number of annexin V-positive cells in any of the cultures (Table VI).

To investigate the effect of S-59 psoralen/UVA light treatment on in vitro T cell cytotoxicity we performed 4-h cytotoxicity assays between B6 effectors and B6, BALB/c, or C3H (third-party) lymphoblast targets. Table VII shows the results with a 50:1 E:T cell ratio. There were very low (4–5%) levels of cytotoxicity against the syngeneic (B6) cells. Naive B6 T cells (with or without PCT) also had a low level of cytotoxicity. However, T cells from B6 animals sensitized to BALB/c splenocytes exhibited increased levels of cytotoxicity (46.8%), which remained elevated (31.8%) even after S-59 psoralen/UVA light treatment. Maximal cytotoxicity was seen with the sensitized T cell population at all E:T cell ratios that we evaluated (Fig. 2).

**S-59 psoralen/UVA light treatment of naive and sensitized T cells and colony formation**

To determine whether sensitized B6 T cells that maintained cytotoxic function could affect the hemopoiesis of the targeted recipient we injected these cells i.p. into 2-day-old BALB/c mice. At 7 days post-transplant the animals were sacrificed, and marrow was harvested for colony growth assays. The results of three experiments were expressed as the mean ± SD of duplicate wells from three mice per group (Table IX). BALB/c recipients of B6 naive T cells or naive T cells treated with PCT had colony formation comparable to that of the untreated BALB/c controls. When T cells from sensitized B6 animals were used, there was a significant (p < 0.001) reduction in colony formation that remained essentially unchanged following PCT treatment. The reduction was seen in both erythroid and myeloid CFU, indicating that the cytotoxic cells were targeting an early progenitor population.

**S-59 psoralen/UVA light treatment of naive and sensitized T cells: IUT**

We injected CD3⁺ splenocytes (with or without PCT) from naive or sensitized B6 male donors along with 1.5 × 10⁵ TCDBM into 14- to 15-day fetal BALB/c recipients. The end points were survival at 8 wk, GvHD, and engraftment at 6–8 wk, the results of which are shown in Table IX. The average survival rate of IUT recipients with 3 × 10⁵ naive T cells was 12%, which increased to 53% (p = 0.01) when PCT-treated naive T cells were injected. None of the animals injected with sensitized T cells and TCDBM survived more than a few days following birth, while 43% of the recipients of PCT-treated, sensitized T cells and TCDBM survived (p = 0.002), which was comparable to the recipients of PCT-treated naive B6 T cells (p = 0.53). GvHD was significantly (p < 0.001) reduced in the recipients of B6 T cells that were treated with PCT in both the naive and sensitized groups. However, multilineage engraftment in the blood (at both 6 and 8 wk of age) was only seen in the recipients of TCDBM plus either naive T cells or PCT-treated, sensitized T cells (p ≤ 0.003) compared with TCDBM plus PCT-treated naive T cells.

**Table IV. Comparison of naive CD3⁺, CD4⁺, and CD8⁺ T cells in making space in utero**

<table>
<thead>
<tr>
<th>Cells Injected</th>
<th>Naive born/No. Injected (%)</th>
<th>Naive with GvHD/No. Born (%)</th>
<th>Engraftment in Blood at 12-24 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDBM⁺ + 1.5 × 10⁵ CD³⁺</td>
<td>42/66 (64)</td>
<td>36/42 (86)</td>
<td>23</td>
</tr>
<tr>
<td>TCDBM⁺ + 1.5 × 10⁵ CD⁴⁺</td>
<td>33/73 (45)</td>
<td>11/33 (33)</td>
<td>21</td>
</tr>
<tr>
<td>TCDBM⁺ + 1.5 × 10⁵ CD⁸⁺</td>
<td>10/31 (32)</td>
<td>6/10 (60)</td>
<td>9</td>
</tr>
</tbody>
</table>

* More than 1% H2Kb + cells in blood.
* 1.5 × 10⁵ TCDBM.
* p < 0.01 vs 1.5 × 10⁵ CD4.
* Mean ± SD.
* Multilineage.
* p < 0.03 vs 1.5 × 10⁵ CD8.
* Mild GvHD (thin hair and slow weight gain), but resolving with normal weight at sacrifice.

**Table V. Effect of S-59 psoralen/UVA light PCT on proliferation of naive and sensitized B6 splenic T cells in response to anti-CD3⁺**

<table>
<thead>
<tr>
<th>Sources of CD3⁺ Splenocytes</th>
<th>Naive*</th>
<th>Naive PCT</th>
<th>Sensitized*</th>
<th>Sensitized PCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting*</td>
<td>153 ± 10⁷</td>
<td>131 ± 94</td>
<td>5,127 ± 53</td>
<td>128 ± 32</td>
</tr>
<tr>
<td>Stimulated*</td>
<td>24,439 ± 950</td>
<td>147 ± 39</td>
<td>36,303 ± 8,737</td>
<td>124 ± 19</td>
</tr>
</tbody>
</table>

* CD3⁺ cells from normal B6 male spleens untreated or treated with PCT.
* CD3⁺ cells from B6 males that had been injected three times at weekly intervals with BALB/c splenocytes (sensitized) untreated or treated with PCT.
* T cells (1 × 10⁵) were cultured with 2 nM S-59 psoralen/UVA light without stimulation.
* Average cpm of six experiments, expressed as the mean ± SD. Each sample was cultured in quadruplicate.
* T cells (1 × 10⁵) were cultured with 2 nM S-59 psoralen/UVA light and stimulated with immobilized anti-CD3.
PCT-treated T cells was 15 vs 2% in the recipients of TCDBM-sensitized, PCT-treated naive T cells, and the engraftments of donor T cells, B cells, and macrophages were 14 vs 0.1%, 4 vs 0.3%, and 3 vs 0.3%, respectively (B cell data not shown in Fig. 3). Engraftment could not be determined in the recipients of untreated sensitized T cells because there were no survivors that could be evaluated.

Finally, when we evaluated the tissues at 8 wk of age for donor cell engraftment, the percent donor CD3+ and CD4+ thymocytes in the recipients of TCDBM plus naive T cells or PCT-treated, sensitized T cells was significantly (p < 0.001) greater than that in the recipients of TCDBM plus PCT-treated naive T cells (data not shown). Similarly, the percentages of donor CD3+ splenocytes in recipients of TCDBM plus naive T cells, TCDBM plus PCT-treated naive T cells, and TCDBM plus PCT-treated naive T cells were 12.7 ± 2, 0.3 ± 0.1, and 3.6 ± 1%, respectively (for TCDBM plus naive T cells vs TCDBM plus PCT-treated naive T cells, TCDBM plus naive T cells vs TCDBM plus PCT-treated naive T cells, and TCDBM plus PCT-treated naive T cells vs TCDBM plus PCT-treated naive T cells, p < 0.001).

Discussion

We evaluated the role of donor T cells in promoting engraftment in utero. There are at least two mechanisms by which T cells may function in this capacity. One is as facilitating cells in a myeloablative recipient, presumably through the release of critical cytokines. The other is as a cytotoxic cell targeting host hemopoietic stem cell. The latter is a likely mechanism to explain engraftment in nonmyeloablative transplants in both adult mouse models and humans (13–15). In our study we found that naive allogeneic donor T cells, while associated with GvHD, resulted in multilineage engraftment when co-injected with TCDBM into fetal recipients. When the GvHD was prevented by inhibiting proliferation of the T cells by pretreatment with S-59 psoralen and UVA light, the degree of engraftment also was reduced to control levels. However, when we used sensitized donor T cells that were pretreated with PCT to prevent proliferation, multilineage engraftment occurred with minimal GvHD, although the total percentage of engrafted cells was reduced in the PCT-treated group compared with the recipients of untreated naive T cells and bone marrow. These results are consistent with the hypothesis that space is a critical barrier to successful engraftment in utero.

To further address this concept, we injected newborn BALB/c mice with naive or sensitized B6 T cells (with or without PCT pretreatment) and found a significant reduction in colony formation only in the BALB/c recipients of sensitized B6 T cells. PCT treatment of the sensitized cells did not inhibit their effect on host hemopoiesis. These results are consistent with those from our in vitro studies in which we found that anti-CD3-induced proliferation of sensitized T cells could be virtually eliminated by PCT, while the expression of the activation markers CD25 and CD69 was only slightly reduced, comparable to what has previously been reported (10). In addition, we found that cell-mediated cytotoxicity by these sensitized CD3+ cells remained intact even with PCT treatment. The in vitro experiments showed that the use of very high E:T cell ratios (≥100:1) results in nonspecific cytotoxicity. At the 50:1 E:T cell ratio we can clearly distinguish between the cytotoxic potential of naive and sensitized T cells. We see a similar cytotoxic pattern even at a 25:1 E:T cell ratio, indicating the consistency of the assay. To determine whether PCT treatment results

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**Table VI. Activation marker expression in CD3-stimulated naive and sensitized B6 T cells**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3+</td>
</tr>
<tr>
<td>Naive B6</td>
<td>47 ± 10d</td>
</tr>
<tr>
<td>Naive B6 PCT</td>
<td>42 ± 6.7</td>
</tr>
<tr>
<td>Sensitized B6</td>
<td>82 ± 3.5</td>
</tr>
<tr>
<td>Sensitized B6 PCT</td>
<td>73 ± 5.6</td>
</tr>
</tbody>
</table>

* Anti-CD3 stimulated splenocytes from adult male C57BL/6 (B6) mice that were naive or sensitized by three weekly injections of BALB/c splenocytes. PCT, Splenocytes treated with S59-psoralen and UVA light as described in Materials and Methods. Cells from about three animals were pooled for each group per experiment (n = 3 experiments).

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**Table VII. Effects of S-59 psoralen/UVA light on T cell-mediated cytotoxicity in naive and sensitized T cells**

<table>
<thead>
<tr>
<th>Targets</th>
<th>C57BL/6 effectors</th>
<th>C57BL/6</th>
<th>BALB/c</th>
<th>C3H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>1.1 ± 0.6d</td>
<td>18.1 ± 15</td>
<td>14.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Naive PCT</td>
<td>1.2 ± 0.8</td>
<td>17.8 ± 14.2</td>
<td>15.7 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Sensitized</td>
<td>4.1 ± 2.3</td>
<td>46.8 ± 29.2e</td>
<td>15.0 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Sensitized PCT</td>
<td>2.1 ± 1.5</td>
<td>31.8 ± 20.2f</td>
<td>14.1 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

* Data shown are mean of three experiments. Each assay was set up in triplicate.

**FIGURE 2.** Cytotoxicity of freshly isolated splenic CD3+ effectors with or without PCT from naive and sensitized B6 animals against BALB/c targets at the indicated E:T cell ratios. ●, Naive T cells; ◆, naive PCT T cells; ▲, sensitized T cells; ■, sensitized PCT T cells.
in apoptosis, we evaluated annexin V binding and found no difference between naive and sensitized T cells with or without PCT. There are reports of induction of apoptosis in human lymphocytes following PCT treatment (16–18). However, the rate of apoptosis following PCT treatment is dose and exposure time dependent. Truitt et al. (10) found significant apoptosis at 10 nM S-59 psoralen with an 8-min exposure to UVA light, but the viability was >90% with 1 nM under identical conditions. We had similar results up to 3 nM S-59 psoralen, at which toxicity is induced through apoptosis induction (data not shown). The absence of apoptotic T cells further supports the possibility of cytotoxic T cells being responsible for the increased multilineage engraftment that we have seen in these experiments. Previous studies of naive T cells have shown that the generation of CTL in MLR cultures is also dose dependent; however, the lytic potential of viable cells remains unchanged (10). Our results, showing a moderate, but relatively higher (p < 0.05), cytotoxic potential of PCT-treated sensitized compared with naive donor T cells, are probably due to the presence of a higher frequency of alloantigen-specific CTL in the sensitized splenocytes. Overall, our data indicate that following PCT, T cells remain viable, cytotoxic, and capable of reacting to host alloantigens, although they are unable to proliferate in response to anti-CD3.

We also found that the dose of naive donor T cells needed to generate a GvHD reaction after IUT is higher than expected based on studies in irradiated mice (19). When up to 7.5 × 10^6 CD3^+ B6 splenocytes were added to the marrow inoculum, we found little evidence of GvHD either clinically or by histologic examination (data not shown). This is comparable to ~7.5 × 10^7 T cells/kg (in a 1-g fetus). In fact, significant GvHD did not begin to appear until the dose of T cells reached 1.5–3 × 10^8 or ~1–2 × 10^9/kg; a dose that is at least 10 times the dose needed to induce GvHD in irradiated adult mice (19). The reason(s) for this relative resistance to GvHD in the fetal recipient may be due to altered expression of MHC Ags (20), a reduced ability to present alloantigen, and/or the fact that no conditioning is used for IUT, and therefore there is no tissue damage and subsequent release of cytokines, which are thought to be critical in the pathogenesis of GvHD (21).

We wanted to determine whether CD4^+ or CD8^+ T cells could mediate GvHD and multilineage engraftment in fetal recipients. Both CD4^+ and CD8^+ cells resulted in GvHD, the incidence of which was not statistically different from that in CD3^+ recipients, although the CD4 recipients tended to have more mild disease that resolved by 12–24 wk of age. Engraftment was more likely in the

Table VIII. Colony formation postinjection of CD3^+ allogeneic cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>CFU</th>
<th>E^a</th>
<th>GM^a</th>
<th>GEMM^a</th>
<th>Total^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive PCT</td>
<td>24 ± 8^c</td>
<td>133 ± 45</td>
<td>15 ± 2</td>
<td>172 ± 55</td>
<td></td>
</tr>
<tr>
<td>Sensitized</td>
<td>14 ± 6</td>
<td>47 ± 16</td>
<td>3 ± 1</td>
<td>64 ± 19^f</td>
<td></td>
</tr>
<tr>
<td>Sensitized PCT</td>
<td>15 ± 3</td>
<td>59 ± 8</td>
<td>8 ± 2</td>
<td>81 ± 23^f</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24 ± 2</td>
<td>148 ± 22</td>
<td>18 ± 3</td>
<td>190 ± 35</td>
<td></td>
</tr>
</tbody>
</table>

^a Erythroid CFU counts following CD3 injections as described in Materials and Methods.
^b Granulocyte-macrophage CFU counts.
^c Granulocyte-erythroid-macrophage-megakaryocyte CFU.
^d Sum total of CFU-E + CFU-GM + CFU GEMM.
^e Mean CFU ± SD (three experiments).
^f p < 0.001 vs control.

FIGURE 3. FACS analysis of two cell lineages in the blood of IUT BALB/c recipients of B6 TCDBM and 3 × 10^6 naive vs sensitized PCT-treated CD3^+ cells. The upper panel represents a recipient of TCDBM and naive PCT-treated T cells. The lower panel represents a recipient of TCDBM and S-59 psoralen/UVA light-treated T cells sensitized against BALB/c splenocytes. The cell lineages evaluated on the y-axis are T cells (CD3^+) and macrophages (Mac). The x-axis represents H2Kb^+ (donor) cells.

Table IX. Multilineage engraftment in blood at 6 wk following IUT with sensitized PCT-treated CD3^+ B6 splenocytes and TCDBM

<table>
<thead>
<tr>
<th>Recipient Groups^a</th>
<th>No. Injected/No. Born</th>
<th>Survivors^b</th>
<th>GvHD^c</th>
<th>H2Kb^+ Cells</th>
<th>T Cells</th>
<th>CD4^+ Cells</th>
<th>B Cells</th>
<th>Granulocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive B6^c</td>
<td>17/5</td>
<td>2 (12)</td>
<td>3 (60)</td>
<td>53.4 ± 4.2^d</td>
<td>19.95 ± 1.9</td>
<td>7.6 ± 1.9</td>
<td>21.1 ± 1.8</td>
<td>8.2 ± 3.7</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>Naive B6 PCT^c</td>
<td>15/8</td>
<td>8 (53)</td>
<td>0 (0)^f</td>
<td>1.7 ± 0.5</td>
<td>0.51 ± 0.3</td>
<td>0.51 ± 0.3</td>
<td>0.38 ± 0.1</td>
<td>0.64 ± 0.3</td>
<td>0.37 ± 0.1</td>
</tr>
<tr>
<td>Sensitized B6^d</td>
<td>18/9</td>
<td>0 (0)</td>
<td>9 (100)</td>
<td>13.3 ± 2.4^e</td>
<td>6.3 ± 2.1^f</td>
<td>3.0 ± 1.4^f</td>
<td>2.4 ± 1.4^f</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sensitized B6 PCT^f</td>
<td>21/9</td>
<td>9 (43)^h</td>
<td>1 (11)^i</td>
<td>13.3 ± 2.4^e</td>
<td>6.3 ± 2.1^f</td>
<td>3.0 ± 1.4^f</td>
<td>2.4 ± 1.4^f</td>
<td>5.9 ± 2.2^g</td>
<td>0.97 ± 0.5^w</td>
</tr>
</tbody>
</table>

^a All BALB/c recipient groups received lineage^- BM from B6 donors in addition to naive or sensitized B6 CD3^+ splenocytes that were treated or not treated with psoralen/UVA light.
^b Animals (n (percentage injected)) surviving at 8 wks of age and evaluated for engraftment at 6 wk.
^c 300,000 CD3^+ splenocytes with or without treatment with psoralen/UVA light.
^d Mean ± SD.
^e p < 0.001 vs naive B6.
^f 300,000 CD3^+ splenocytes from B6 donors sensitized to BALB/c (i.e., injected with three weekly doses of BALB/c splenocytes) with or without treatment with psoralen/UVA light.
^g Animals died within 1–3 days of birth and were not evaluable.
^h p = 0.04 vs naive B6, p = 0.53 vs naive PCT; p = 0.002 vs sensitized B6.
^i p < 0.001 vs sensitized B6.
^j 0.6 vs naive B6 PCT.
^k p ≤ 0.001 vs naive B6 PCT.
^l p = 0.003 vs naive B6 PCT.
^m p = 0.007 vs naive B6 PCT.
CD3 and CD8 recipient groups, and the degree of engraftment was significantly greater in the CD3 recipients compared with either CD4 or CD8 recipients, with a trend toward degree of engraftment in the CD8 recipients being higher than that in the CD4 recipients ($p = 0.08$). However, tolerance to donor skin grafts was not induced in either subset recipient group. These results suggest that in the B6→BALB/c IUT model either CD4$^+$ or CD8$^+$ cells are capable of mediating GvHD, at least in fetal recipients, although it appears to be milder with CD8$^+$ cells. This is similar to other reports in irradiated adult recipients in which either CD4$^+$ or CD8$^+$ T cells are capable of generating GvHD (22). Interestingly, when we increased the number of CD4$^+$ or CD8$^+$ cells to $3 \times 10^5$ in the injections, the recipients all died (data not shown). The CD8$^+$ recipients died in utero, and the CD4$^+$ recipients died shortly after birth from severe GvHD. Future studies will evaluate the role of sensitized CD8$^+$ and CD4$^+$ T cell subsets, although we would predict that the sensitized CD8$^+$ T cells should be sufficient to generate space in utero, comparable to what we saw with sensitized CD3$^+$ splenocytes.

In summary, we found that large numbers of allogeneic donor T cells are necessary to induce GvHD in the fetal recipient. We also found that CD4$^+$ or CD8$^+$ naive T cells alone could induce GvHD and multilineage engraftment, although to a lesser degree compared with similar numbers of CD3$^+$ cells. However, IUT with sensitized T cells pretreated with S-59 psoralen and UVA light resulted in multilineage engraftment with minimal GvHD and improved survival compared with untreated naive or sensitized T cells and comparable survival relative to PCT-treated naive T cells, supporting the concept that space is a critical barrier that must be overcome for successful IUT.

References