Ex Vivo Anti-CD3 Antibody-Activated Donor T Cells Have a Reduced Ability to Cause Lethal Murine Graft-Versus-Host Disease but Retain Their Ability to Facilitate Alloengraftment

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Ex Vivo Anti-CD3 Antibody-Activated Donor T Cells Have a Reduced Ability to Cause Lethal Murine Graft-Versus-Host Disease but Retain Their Ability to Facilitate Alloengraftment

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The purpose of this study was to determine whether ex vivo anti-CD3 Ab-activated T cells behaved in a biologically similar manner as naive T cells with respect to causing graft-vs-host disease (GVHD) and facilitating engraftment after allogeneic marrow transplantation. This question was addressed using two well-defined MHC-incompatible murine models of GVHD (C57BL/6 (H-2b)→B10.BR (H-2k)) and engraftment (C57BL/6 (H-2b)→AKR/J (H-2k)). Transplantation with anti-CD3-activated T cells significantly reduced GVHD compared with that in animals transplanted with equivalent numbers of naive T cells. Protection from GVHD was not T cell subset dependent, as highly enriched populations of either activated CD4⁺ or CD8⁺ T cells caused less lethal GVHD than comparable numbers of purified naive CD4⁺ or CD8⁺ T cells. Transplantation with activated T cells also resulted in protection from LPS-mediated GVH lethality in unirradiated F₁ recipients. Analysis of immune recovery indicated that animals transplanted with activated T cells had thymic and splenic B cell reconstitution that compared favorably to that in non-GVHD control mice. When engraftment was analyzed, equivalent degrees of donor cell engraftment were observed when animals were transplanted with limiting numbers (5 × 10⁵) of naive vs activated B6 T cells. Further studies indicated that activated CD8⁺ T cells were exclusively responsible for enhancing engraftment and that facilitation of engraftment was dependent upon the direct recognition of host MHC alloantigens. Collectively, these data demonstrate that transplantation with anti-CD3 Ab-activated T cells results in a reduction in GVHD, but these cells retain their ability to facilitate alloengraftment. The use of this approach in allogeneic marrow transplantation may represent an alternative strategy to mitigate GVHD without compromising engraftment. The Journal of Immunology, 1998, 161: 2610–2619.

Graft-vs-host disease (GVHD) is a major cause of morbidity and mortality in patients undergoing allogeneic bone marrow (BM) transplantation (BMT). GVHD is a complex pathophysiologic process resulting from the cooperative interaction of multiple effector cell populations resident in the donor graft and persistent in the host after the conditioning regimen (1). An essential requirement for donor-derived T cells has been substantiated by studies in which removal of T cells from the donor graft has resulted in the amelioration or complete prevention of the GVHD syndrome (2–4). Secondary immune effector cell populations that are recruited by T cells along with dysregulated cytokine production are also thought to be important elements in the pathophysiology of GVH reactivity (5–9). Although various components of the immune system are known to participate in the pathogenesis of GVHD, the presence and persistence of mature nontolerant donor T cells in the host serve as the critical inciting stimuli for the amplification of immune reactivity that characterizes the GVH reaction and enables it to become a self-sustaining life-threatening process.

Most therapeutic approaches designed to reduce GVHD have focused on the ex vivo removal of alloreactive donor T cell populations from the marrow graft (3, 4). While this has significantly reduced GVHD, there has been a corresponding increase in the rate of graft rejection (10–12) due to the fact that the T cells that are capable of causing GVHD and are removed by these depletion procedures are also necessary to facilitate alloengraftment. Conversely, when T cells are retained in the donor marrow graft, engraftment occurs in the vast majority of patients; however, the incidence and severity of GVHD are significantly increased despite the use of pharmacologic agents designed to suppress GVH reactivity. This is particularly problematic in the case of mismatched related and unrelated BMT, where the greater degree of HLA disparity significantly increases toxicity from GVHD (13–16).

T cell activation after encounter of alloantigen presented in the context of MHC molecules is generally accepted to be the proximate event in GVHD. Once activated, T cells undergo cellular proliferation and are able to secrete a variety of cytokines that contribute to the pathogenesis of GVHD (17). Activation of the T cell, however, also serves to open a cell death pathway that can be triggered by either withdrawal of growth factor or reiligation of the TCR (18–23). This process, termed activation-induced cell death, results in apoptosis and is thought to be an important mechanism by which immune responses are regulated and peripheral tolerance is maintained in vivo (24, 25). Prior studies have also shown that signaling via the TCR/CD3 complex with anti-CD3 Abs or other mitogenic stimuli in vitro can serve as a priming stimulus for T cells to undergo subsequent apoptosis (19–21).
Whether T cells that have been ex vivo activated before transplantation into recipients behave in a biologically similar manner as naive T cells with respect to GVH pathophysiology has not been studied. This question is of emerging clinical relevance given that activated T cells are now being transplanted into allogeneic marrow transplant recipients, most notably in gene therapy protocols designed to modulate GVH and graft-vs-leukemia reactivity (26, 27). We reasoned that the propensity of ex vivo activated T cells to undergo apoptosis after either cytokine withdrawal or reigation of the TCR could be advantageous in allogeneic marrow transplantation, since this might translate into a shortened life span and therefore a reduced ability to cause GVHD. However, since activated T cells possess cytotoxic capability and produce cytokines implicated in the pathophysiology of GVHD (28), it is possible that these cells could conversely exacerbate GVH reactivity. Moreover, if GVHD were reduced, the ability of these cells to promote engraftment might be compromised, since the presence of donor T cells is necessary to eradicate or inactivate host immune cells capable of causing graft rejection (29, 30). The purpose of this study was to examine these questions by comparing the relative effects of naive and activated T cells inmediating GVHD and facilitating alloengraftment in murine recipients transplanted with MHC-mismatched marrow grafts.

Materials and Methods

**Mice**

C57BL/6 (B6) (H-2^b_), B10.BR (H-2^b_), B6.PL (H-2^b_), AKR/J (H-2^b_), and (C57BL/6 × AKR/J)F1 (B6AF1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 × AKR/JF1 mice were bred at the Medical College of Wisconsin (Milwaukee, WI). All animals were housed in the American Association for Lab Animal Care-accredited Animal Resource Center of the Medical College of Wisconsin. Mice received regular mouse chow and acidified tap water ad libitum.

**Ex vivo activation and expansion of T cells**

Spleen cells were obtained from either C57BL/6 or B6.PL mice and passed through nylon wool columns (Robbins Scientific, Sunnyvale, CA) to remove B cells. Cells were then resuspended in complete DMEM (CDMEM) plus 5% FCS and cultured in flasks precoated with an immobilized T cell-specific mAb (either anti-CD3, clone 145-2C11 (provided by J. Bluestone, University of Chicago, Chicago, IL), or anti-TCR-αβ, clone H57-597 (American Type Culture Collection, Rockville, MD)) at a concentration of 5 to 10 × 10^6 cells/ml. Twenty-four hours after the initiation of culture, human IL-2 (Cetus, Norwalk, CT) was added at a concentration of 20 to 40 U/ml (Cetus units). All cultures were split into fresh flasks as needed to maintain a cell concentration of 0.5 to 1.5 × 10^7 cells/ml. Cells were exposed to immobilized mAb for the first 3 days of culture and thereafter grown only in medium plus IL-2 in fresh flasks for an additional 3 to 5 days to allow for re-expression of the TCR. The continuous presence of IL-2 was necessary for optimal cell proliferation. After a total of 6 to 8 days in culture, cells were counted, and the percentage of T cells was analyzed by flow cytometry. Routinely, 90 to 95% of viable cells had reexpressed the CD3/TCR complex at the time that these cells were transplanted into recipients. Approximately 75% of T cells were CD8^+ and 5 to 10% were CD4^+ after ex vivo expansion. The remaining cells were either double positive (CD4^+CD8^+) or expressed neither Ag. The absolute number of activated T cells was quantified by multiplying the percentage of Thy1.2^+ αβ T cells as determined by flow cytometry by the total number of cells. For the purpose of clarity, T cells activated with anti-CD3 Ab are henceforth referred to as anti-CD3-activated T cells.

**CD4^+ and CD8^+ T cell subset enrichment and activation**

To obtain highly enriched populations of activated CD4^+ T cells, B6 or B6.PL spleen cells were passed through nylon wool columns, and then CD4^+ T cells were positively selected using the MACS magnetic cell separation system (Miltenyi Biotech, Auburn, CA). The resulting population was then ex vivo expanded in immobilized anti-CD3 Ab-coated flasks and grown in medium plus 20 to 100 U/ml of IL-2 for 6 to 8 days as described above. Since ex vivo anti-CD3 activation under these conditions results in the preferential outgrowth of CD8^+ T cells, the resulting population was first depleted of CD8^+ T cells and then positively selected for CD4^+ T cells before performing the transplant experiments. After the enrichment procedure, CD4^+ T cells represented ≥90% to 95% of the entire cell population, and CD8^+ T cells represented ≤5% of the total cell population. Activated CD8^+ T cells were obtained by culturing nylon wool-purified T cells for 7 to 8 days, depleting the cell population of CD4^+ T cells, and then positively selecting for CD8^+ T cells. After these procedures were performed, CD8^+ T cells represented <0.5% of the entire cell population. Highly enriched populations of naive CD4^+ or CD8^+ T cells were obtained by negative selection followed by positive selection of nylon wool-purified spleen cells and resulted in >95% purity of the respective T cell subsets.

**Bone marrow transplantation**

BM was flushed from donor femurs and tibias with CDMEM and passed through sterile mesh filters to obtain single cell suspensions. BM T cells were depleted in vitro with anti-Thy1.2 mAb plus low toxicity rabbit complement (C6 Diagnostics, Mequon, WI). The hybridoma for 30-H12 (anti-Thy1.2, rat IgG2b) Ab was obtained from the American Type Culture Collection (Manassas, VA) and grown in CDMEM plus 5% FCS. The culture supernatants was then harvested, precipitated in ammonium sulfate, and dialyzed against PBS before use in in vitro depletion experiments. BM cells were then washed and resuspended in DMEM before injection.

Naïve T cells were obtained by pressing spleens through wire mesh screens. Erythrocytes were removed from spleen cell suspensions by hypotonic lysis with sterile distilled water. T cells for admixture with TCD BM before transplantation were then obtained by passing spleen cells once or twice through nylon wool columns to remove B cells. The number of naive T cells was quantified by multiplying the percentage of Thy1.2^+ αβ T cells, as determined by flow cytometry, by the total number of cells. The average number of naive αβ T cells in the spleen cell suspensions after nylon wool depletion was 70 to 85%. BM T cells were always >90% viable as assessed by trypan blue dye exclusion. Total body irradiation was administered as a single exposure at a dose rate of 75 cGy using a Shepherd Mark I Cesium Irradiator (J. L. Shepherd and Associates, San Fernando, CA). Irradiated recipients then received a single i.v. injection of TCD BM (10–20 × 10^6) with or without added naïve or ex vivo activated T cells.

For studies of GVHD in a fully allogeneic system, B10.BR recipients were lethally irradiated (900 cGy) and transplanted with 10 to 20 × 10^6 TCD B6 BM alone or admixed with 2 to 25 × 10^6 naïve or activated T cells from B6 background donors. In some experiments, congenic B6.PL donors were employed to allow for the determination of the fate of spleen-derived and BM-derived naive or activated T cells. In other experiments, GVHD was also assessed in a parent→F1 model as described by Nestel and colleagues (9). Unirradiated B6AF1 mice were transplanted with equivalent numbers of naïve or ex vivo activated B6 T cells. Donors of naive T cells were freshly in vivo depleted of NK cells with mAb anti-NK 1.1, 36 (anti-NK 1.1; American Type Culture Collection) as previously described (31) before T cells were harvested for the transplant experiments. This was done so that recipients in both the naive and activated experimental groups received only T cells, since NK cells have been implicated in the pathophysiology of GVHD in this model (9) and might therefore have been a confounding variable. Syngeneic control mice were transplanted with an identical number of naive B6AF1 T cells. Seven days after injection, all recipients were administered 10 µg of LPS i.v. and assessed for mortality within 48 h. For studies of engraftment in a fully allogeneic system, AKR recipients were sublethally irradiated (850 cGy) and transplanted with 10 to 10^6 TCD B6 alone or admixed with graded doses of naive or activated B6 T cells. In the engraftment model, donor T cells were ex vivo activated with immobilized anti-CD3/AlloAb to 145-2C11 Ab. As a control, and recipient were mismatched at both class I and II loci to simulate unrelated and mismatched related human transplantation where HLA class I and II disparities are often present, and the risk of GVHD and graft rejection is highest (14, 16).

**Flow cytometric analysis**

mAb conjugated to either FITC or phycoerythrin (PE) were used to assess chimerism in BMT recipients. FITC-anti-CD8 (clone 53-6.7, rat IgG2a) was purchased from Collaborative Biomedical Products (Bedford, MA). FITC-anti-Ly-5 (B220, rat IgG2a) and PE-anti-CD8 (clone CD8a, rat IgG2a) were obtained from Caltag (San Francisco, CA). PE-anti-TCR-αβ (clone H57-597, hamster IgG), FITC-anti-Thy1.2 (clone 30-H12, rat IgG2b), PE-hamster IgG (isotype control), PE-anti-CD3 (clone 145-2C11, hamster IgG), PE-anti-Thy1.1 (clone OX-7, mouse IgG1), FITC-anti-CD4 (clone RM4-4, rat IgG2b), and FITC-anti-H-2K^k^ (clone AF6-88.5, mouse IgG2a) were all purchased from PharMingen (San Diego, CA). Spleen and lymph node T cells were obtained by negative selection using nylon wool columns. Spleen and lymph node T cells were then depleted of CD8^+ T cells and then positively selected for CD4^+ T cells before performing the transplant experiments. After the enrichment procedure, CD4^+ T cells represented ≥90% of the entire cell population, and CD8^+ T cells represented ≤5% of the total cell population. Activated CD8^+ T cells were obtained by culturing nylon wool-purified T cells for 7 to 8 days, depleting the cell population of CD4^+ T cells, and then positively selecting for CD8^+ T cells. After these procedures were performed, CD8^+ T cells represented <0.5% of the entire cell population. Highly enriched populations of naive CD4^+ or CD8^+ T cells were obtained by negative selection followed by positive selection of nylon wool-purified spleen cells and resulted in >95% purity of the respective T cell subsets.
thymus cells were obtained from chimeras at defined intervals posttransplant processed into single cell suspensions and stained for two-color analysis. Red cells were removed when necessary by hypotonic lysis. Cells were analyzed on a FACS analyzer (Becton Dickinson, Mountain View, CA) with Consort 32 computer support and LYSIS II software. Donor T cell chimerism was determined by analyzing cells within the lymphocyte gate. Donor T cells were defined as H-2b/CD3+ . The percentages of donor cells and B cells were determined by analyzing cells within gates that included the entire spleen cell population after exclusion of red cells and nonviable cells by forward and side scatter settings. Donor cells were defined as H-2b+, and B cells were defined as Ly5+. Thyocytes were analyzed within gates that included the entire thymus cell population. At least 10,000 cells were analyzed for each determination whenever possible.

Isolation and quantification of intrahepatic lymphocytes

Livers were perfused with PBS to wash out red cells and pressed through wire mesh screens to obtain single cell suspensions. Liver cell suspensions were then incubated in collagenase VIII (Sigma, St. Louis, MO) at a concentration of 50 μg/ml for 30 min at 37°C. Cells were then washed, passed through a nylon wool column to remove debris, and centrifuged on a 44 to 67% Percoll gradient for 20 min. Lymphocytes were collected at the 44 to 67% interface, washed, counted, and then analyzed by flow cytometry. Forward and side scatter characteristics were used to gate on the lymphocyte population and exclude liver parenchymal cells.

Radioactive labeling of T cells with 111indium oxine and in vivo detection of radioactive T cells

Naive and activated T cells were individually labeled in vitro with 111indium oxine by incubating 125 × 106 cells with 50 μCi of 1.85 MBq of 111indium oxine. Animals injected with 5 μCi of radiolabeled T cells were sacrificed 18 h posttransplant, and tissue samples from the spleen, liver, lung, kidneys, and thymus were removed. Tissue samples and whole organs were individually weighed, and the amount of incorporated radioactivity was determined in a sodium iodide scintillation counter using the 150 to 500 keV window. The biodistribution of radiolabeled T cells was calculated as the normalized uptake of radioactivity in the whole organ and expressed as the percentage of total injected dose per organ.

Histologic studies

Liver tissue was obtained from control and experimental animals, fixed in 10% neutral buffered formalin, and processed into paraffin blocks. Four-micron-thick sections were prepared from each block and were cut at two levels to optimize sampling. Routine tissue hematoxylin and eosin sections were prepared. For the evaluation of GVHD, tissue sections were screened with the examiner blinded to the treatment received by each animal. Tissues were scored positive for GVHD if there was evidence of bile duct necrosis with or without infiltration in the liver.

Statistics

Group comparisons of donor T cell chimerism, overall donor cell chimerism in the spleen and thymus, splenic B cell content, and thymic T cell subsets were performed using unpaired Student’s t test. Survival curves were constructed using the Kaplan-Meier product limit and were compared using the log rank test. A two-tailed p ≤ 0.05 was deemed significant.

Results

Anti-CD3 Ab-activated T cells have a reduced ability to mediate GVHD

Initial experiments were performed to assess the potential of naive and anti-CD3-activated T cells to induce GVHD. Lethally irradiated B10.BR recipients were transplanted with TCD B6 BM and equivalent numbers of either naive or activated T cells. Mice transplanted with 2 × 10^6 naive T cells quickly developed GVHD, and only 10% of the animals survived to day 80 (Fig. 1). In contrast, animals transplanted with an equivalent number of activated T cells had significantly enhanced survival (p = 0.0001), with all mice surviving until the conclusion of the experiments. When weight loss, which is a sensitive indicator of GVHD in this model, was assessed, we observed only a slight reduction in weight in these mice compared with that in TCD BM control animals. To determine whether this observation was T cell dose dependent, the dose of T cells administered to recipients in each cohort was increased. As expected, mice transplanted with 5 × 10^6 naive T cells all died within 35 days of GVHD. Transplantation with 5 × 10^6 activated T cells significantly prolonged survival (p < 0.0001), but weight loss in these mice was more pronounced than that at a dose of 2 × 10^6 cells, indicating that GVHD was not completely abrogated.

To confirm that the effects observed could not be ascribed to the Ab used for ex vivo activation, experiments were conducted using T cells similarly activated through the TCR/CD3 complex but with an alternative Ab (H57-597). Animals transplanted with equivalent numbers of H57-597-activated CD8+ T cells (either 2 × 10^6 or 5 × 10^6) had a similar outcome, as assessed by survival and weight curves, compared with mice transplanted with 145-2C11-activated T cells (data not shown). These data indicated that the observed results were not Ab dependent. Moreover, these data collectively demonstrated that anti-CD3 Ab-activated T cells had a reduced ability to mediate GVH reactivity compared with naive T cells, which translated into significantly prolonged survival.
Activation of T cells with anti-CD3 Ab qualitatively alters the ability of both activated CD4$^+$ and CD8$^+$ cells to mediate GVHD

Ex vivo activation and expansion with anti-CD3 Ab under the conditions used in this study resulted in the preferential expansion of CD8$^+$ T cells (see Materials and Methods). Therefore, the percentage of CD4$^+$ and CD8$^+$ T cells administered to animals was not the same in mice transplanted with activated vs naive T cells (i.e., mice transplanted with activated T cells received more CD8$^+$ T cells and fewer CD4$^+$ T cells than mice reconstituted with naive T cells). To correct for this potential bias in assessing GVHD mortality, we performed a series of experiments to evaluate the ability of activated CD4$^+$ and CD8$^+$ T cells both individually and collectively to cause GVHD compared with that of equivalent numbers of naive CD4$^+$ and/or CD8$^+$ T cells. In initial studies, animals were transplanted with the same absolute number of a mixture of either activated or naive CD4$^+$ and CD8$^+$ T cells. This was achieved by mixing purified activated CD4$^+$ and CD8$^+$ T cells in the same CD4:CD8 ratio as that obtained after nylon wool purification of naive T cells. Mice transplanted with 2 x 10^6 activated T cells had significantly enhanced survival compared with animals transplanted with an equivalent number of naive T cells (67% vs 10% survival on day 60; p = 0.0005; Fig. 2A). Weight curves, however, again demonstrated that surviving mice transplanted with activated T cells were not free from GVHD, as these mice had less weight gain than control animals (Fig. 2B).

CD4$^+$ T cells have been postulated to be primarily responsible for inducing GVHD in this model (32). We therefore evaluated the relative ability of purified activated or naive CD4$^+$ T cells to cause GVHD. In these experiments, animals were transplanted with a higher dose of cells (4 x 10^6 CD4$^+$ T cells) to achieve a sufficiently intense GVH reaction, since only one T cell subset was being administered, and GVHD intensity has been shown to be dependent upon the cooperative interaction of CD4 and CD8 T cells in this strain combination (32). Mice transplanted with activated CD4$^+$ T cells had significantly improved survival (82%) compared with animals transplanted with equivalent numbers of naive CD4$^+$ T cells (43%) when assessed 60 days after transplant (p = 0.006; Fig. 3). Mortality in TCD BM control mice was somewhat higher than that in previous experiments and was accompanied by antemortem weight loss in deceased animals, which was ascribed to graft failure. When similar experiments were performed using highly enriched naive vs activated CD8$^+$ T cells, there was a trend toward improved survival at 60 days in mice transplanted with activated T cells (93%; n = 15) vs naive T cells (67%; n = 15; p = 0.067). These data substantiated that both CD4$^+$ and CD8$^+$ T cells activated with anti-CD3 Ab were qualitatively altered in their ability to mediate GVH reactivity in an MHC-incompatible donor/recipient strain combination.

Activated T cells cause significantly less GVHD than naive T cells when recipients receive no conditioning regimen

Murine studies of GVHD have generally involved the use of irradiation in the conditioning regimen or have alternatively employed...
activated T cells were inherently less capable of causing GVHD than naive T cells. The data indicated that in the absence of a conditioning regimen, activated T cells had significantly enhanced donor T cell engraftment than control animals \((p = 0.03; \text{Table II})\). Specifically, 10 of 12 mice in this group had >95% donor T cell chimerism in the spleen, while the remaining two animals had predominant host T cell reconstitution with <50% donor T cells. The presence of a small percentage (5%) of Thy1.1+ (congenic B6.PL) T cells in the spleen of these mice indicated that some activated T cells were able to persist in these chimeras. Neither thymic size nor the percentage of double-positive thymocytes was different between control mice and mice transplanted with activated T cells. With respect to thymic size in the latter group, we did observe some heterogeneity, as 3 of 12 animals had nonmeasurable thymi, while the remaining mice reconstituted with \(\geq50 \times 10^6\) thymocytes. B cell repopulation, however, was inferior to that observed in TCD BM animals \((p = 0.056)\), consistent with some ongoing GVHD, which was also substantiated by serial weight curves (Fig. 2B).

Activated T cells preferentially localize to the liver early after transplant

T cell activation has been shown to affect the regulation of adhesion molecules as well as lymphocyte homing in vivo compared with those in unactivated T cells (36, 37). We therefore considered that one possible cause of the reduction in GVHD mediated by activated T cells might be the fact that these cells had a different trafficking pattern in vivo compared with that of naïve T cells. Experiments were therefore performed to determine whether anti-CD3-activated T cells had altered trafficking. To address this question, naïve and anti-CD3-activated B6 T cells were specifically labeled with \(^{111}\)indium in vitro and subsequently transplanted with unlabeled TCD B6 BM into cohorts of lethally irradiated B10.BR recipients. Animals were then analyzed 1 day after transplant to assess the trafficking pattern of lymphocytes to representative organs (Table III). Since the binding of this radiolabel to lymphocytes is stable, the amount of detectable radioactivity in each of the respective organs was indicative of specific lymphocyte trafficking. Approximately 50% of the administered radioactivity was detected in both liver and spleen in each of the two groups. However, while naïve T cells preferentially localized in the spleen (31.7%, vs 13.5% in the liver; Table III), transplantation with anti-CD3-activated T cells resulted in the majority of isotopic labeling appearing in the liver (36%, vs 16.4% in the spleen). There was also increased radioactivity in the lungs of these mice, while comparable amounts of radioactivity were detected in kidney and thymus of these animals. These data indicated that ex vivo activated T cells do not cause LPS-induced GVHD lethality

### Table I. Activated T cells do not cause LPS-induced GVHD lethality

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Donor T Cells Added</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>Naive B6AF1</td>
<td>0/5 (0%)</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
<td>Naive B6</td>
<td>9/11 (82%)*</td>
</tr>
<tr>
<td>III</td>
<td>11</td>
<td>Activated B6</td>
<td>0/11 (0%)</td>
</tr>
</tbody>
</table>

* Unirradiated B6AF1 recipients were transplanted with \(25 \times 10^6\) naïve B6AF1, naïve B6, or activated B6 T cells. The donor T cell inoculum in groups II and III was adjusted so that animals received equivalent numbers of CD4+ and CD8+ T cells. Donors of naïve T cells were in vivo depleted of NK cells prior to transplantation of splenic-derived donor T cells into recipient mice (see Materials and Methods). Seven days after transplant, animals were challenged with 10 μg lipopolysaccharide and assessed for mortality over the next 48 h. Data are derived from two separate experiments.

### Table II. Immune cell recovery in mice transplanted with both CD4+ and CD8+ activated T cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Donor T Cells Added</th>
<th>Spleen</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Percent donor T cells(^a)</td>
<td>Percent CD4+ T cells which coexpress Thy 1.1</td>
</tr>
<tr>
<td>I</td>
<td>7</td>
<td>None</td>
<td>57 ± 33</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>12</td>
<td>(2 \times 10^6)</td>
<td>88 ± 24</td>
<td>5 ± 6</td>
</tr>
</tbody>
</table>

* Lethally irradiated (900 cGy) B10.BR mice were transplanted with TCD B6 BM alone (group I) or together with \(2 \times 10^6\) activated (A) B6.PL T cells (group II) as shown in Figure 2. Animals that survived 60 to 75 days were sacrificed and assessed for splenic and thymic reconstitution. Data are shown as the mean ± 1 SD and are derived from two of the three experiments depicted in Figure 2.

\(^a\) Group I vs II, \(p < 0.05\).

\(^\text{Group I vs II,} p = 0.056.\)

\(^\text{Group I vs II,} p = 0.17.\)
T cells had an altered migratory pattern early posttransplant relative to that in naive T cells. Since the measurement of incorporated radioactivity was not a direct quantitation of the number of lymphocytes in the liver, we performed additional experiments to address this question. Lethally irradiated B10.BR recipients were transplanted as described above, except that naive or anti-CD3-activated T cells were obtained from B6.PL donors (Thy1.1). Cohorts of animals (n = 2/day) in each experimental group were sacrificed on 3 consecutive days, beginning 2 days after transplant, and the total number of lymphocytes as well as the percentage of Thy1.1 intrahepatic lymphocytes were determined. There was a significant three- to sixfold increase in the number of intrahepatic lymphocytes in the livers of animals transplanted with activated T cells (Fig. 4). There was also an early increase in the percentage of lymphocytes that were Thy1.1 in animals transplanted with activated T cells, although by day 3 the relative percentages in each of the two groups was equivalent. However, since there was a much greater absolute number of intrahepatic lymphocytes in the former group, the absolute number of Thy1.1 lymphocytes in these mice was higher. By day 4 posttransplant, approximately 50% of the total number of transplanted Thy1.1-activated T cells were detectable in the livers of these chimeras, implicating the liver as a major site of anti-CD3 activated T cell accumulation posttransplant. Histologic analysis of livers from representative animals from each cohort 4 days posttransplant (n = 3/group) revealed no significant tissue damage or incipient evidence of GVHD (data not shown). Thus, despite the three- to sixfold greater number of lymphocytes in animals transplanted with anti-CD3-activated T cells, there was no early evidence of tissue destruction.

Activated T cells are able to facilitate alloengraftment
The fact that activated T cells had a reduced ability to induce GVHD raised the possibility that these cells might also be compromised in their ability to facilitate alloengraftment. We therefore examined the relative abilities of naive and activated T cells to promote engraftment in a model (B6→AKR) where TBI exposures of ≈850 cGy result in rejection of TCD marrow grafts and where donor engraftment is dependent upon the presence of mature T cells in the marrow graft (38). In this model, transplantation with at least 1 to 5 × 10^5 naive αβ T cells is required to significantly enhance donor T cell engraftment. Dose titration studies with activated T cells demonstrated that equivalent numbers of activated and naive αβ T cells (5 × 10^5) were required to significantly enhance donor T cell and overall donor engraftment relative to those in control animals (Table IV). Although the percentage of donor T cells in the spleen was increased in mice transplanted with 5 × 10^5 naive vs activated T cells, overall donor engraftment was equivalent between these two groups (p = 0.18), indicating that activated T cells were comparable to naive T cells on a cell-to-cell basis in their ability to promote donor engraftment. There was also a trend toward increased B cell reconstitution in these chimeras (p = 0.06), which was further evidence that equivalent doses of naive T cells were more likely to cause GVHD and impair immune reconstitution than activated T cells even in this sublethal irradiation model.

Activated CD8^+ but not CD4^+, T cells facilitate alloengraftment
Since activated CD4^+ and CD8^+ T cells were both less likely to cause lethal GVHD compared with comparable numbers of naive CD4^+ and CD8^+ T cells, we examined the relative ability of each of these T cell subsets to prevent graft rejection in the same engraftment model. Highly enriched populations of activated CD4^+ T cells were ineffective in facilitating donor T cell (p = 0.36) or donor cell (p = 0.84) engraftment when compared with TCD BM alone (Table V). The number of splenic B cells in these chimeras, which is another indicator of donor cell engraftment (38), was also

![Figure 4](http://www.jimmunol.org/DownloadedFrom/.../460x245)
Table IV. Activated T cells facilitate allograftment

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<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Donor T Cells Added</th>
<th>Percent Donor T Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent Donor (H-2K&lt;sup&gt;b&lt;/sup&gt;) Cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Number of splenic B Cells (×10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9</td>
<td>None</td>
<td>32 ± 17</td>
<td>42 ± 25</td>
<td>5 ± 5</td>
</tr>
<tr>
<td>II</td>
<td>9</td>
<td>1 × 10&lt;sup&gt;5&lt;/sup&gt; (N)</td>
<td>56 ± 36</td>
<td>68 ± 36</td>
<td>16 ± 19</td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>5 × 10&lt;sup&gt;3&lt;/sup&gt; (N)</td>
<td>95 ± 9</td>
<td>98 ± 3</td>
<td>17 ± 13</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>1 × 10&lt;sup&gt;5&lt;/sup&gt; (A)</td>
<td>39 ± 35</td>
<td>47 ± 34</td>
<td>12 ± 18</td>
</tr>
<tr>
<td>V</td>
<td>14</td>
<td>5 × 10&lt;sup&gt;5&lt;/sup&gt; (A)</td>
<td>75 ± 29</td>
<td>88 ± 24</td>
<td>35 ± 22</td>
</tr>
<tr>
<td>VI</td>
<td>13</td>
<td>1 × 10&lt;sup&gt;6&lt;/sup&gt; (A)</td>
<td>79 ± 33</td>
<td>91 ± 18</td>
<td>31 ± 20</td>
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<sup>a</sup> Irradiated (850 cGy) AKR recipients were transplanted with 10 × 10<sup>6</sup> TCD B6 BM with or without the indicated number of naive (N) or activated (A) T cells. Animals were sacrificed 23 to 33 days posttransplant and chimerism in the spleen was assessed. Data are presented as the mean ± 1 SD and represent the combined data from three individual experiments. Donor T cells were defined as H-2K<sup>b</sup>/CD3<sup>+</sup>, donor cells as H-2K<sup>b</sup><sup>-</sup>, and B cells as Ly5<sup>b</sup> (B2<sup>20</sup>) positive.

<sup>b</sup> Group I vs III, V, and VI, p < 0.01; III vs VI, p = 0.12; II vs V, p = 0.03.

<sup>c</sup> Group I vs III, V, and VI; p < 0.0003; III vs V, p = 0.18; II vs VI, p = 0.06.

<sup>d</sup> Group I vs III, p = 0.04; I vs V and VI, p < 0.0005; II vs VI, p = 0.06.

Discussion

The purpose of this study was to determine whether ex vivo activated T cells had the same capacity as naive T cells to mediate GVHD. The impetus for these studies derived from previous observations that T cells activated through the CD3/TCR complex undergo apoptosis after the TCR is religated (19–21). We reasoned therefore that activation of T cells before transplantation might render these cells more susceptible to accelerated cell death in vivo and thereby alter the capacity of these cells to initiate and sustain a clinically significant GVH reaction. Whether this would ameliorate GVHD was uncertain, however, since other effector functions (e.g., cytokine secretion, etc.) that were retained by these cells might result in an exacerbation of GVHD. Furthermore, since activated T cells also undergo apoptosis upon cytokine withdrawal (18), it was conceivable that ex vivo activated T cells might die in vivo without affecting any discernible functional activity and that this would compromise allograftment even if GVHD was mitigated.

The results of this study demonstrated the T cells activated with anti-CD3/TCR Abs had a markedly reduced ability to mediate GVHD in an MHC-incompatible donor/reipient strain combination. Similar results were observed when an alternative anti-CD3/TCR Ab was employed for ex vivo activation, indicating that the protective effect was not due to a unique property of one specific not significantly different from that in control animals. In contrast, mice transplanted with equivalent numbers of activated CD8<sup>+</sup> T cells had significantly enhanced donor T cell (p < 0.0002) and donor cell (p < 0.0004) engraftment relative to control animals. The degree of donor T cell and donor cell engraftment was comparable to that in mice transplanted with equivalent numbers of naive T cells that were comprised of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These data indicated that activated CD8<sup>+</sup> T cells were primarily responsible for facilitating engraftment and that activated CD4<sup>+</sup> T cells were ineffectual in this model.

Engraftment facilitated by activated T cells is dependent upon recognition of host MHC alloantigens

Experimental data support direct MHC-restricted recognition of residual host T cells by naive donor T cells as one mechanism by which T cells facilitate engraftment (39). Whether activated T cells facilitate engraftment in a similar fashion is unknown. We therefore performed experiments using an F<sub>1</sub>-parent model designed to assess whether activated T cells that were tolerant of the recipient and therefore unable to recognize host alloantigens could enhance donor T cell engraftment. Analysis of the experimental control groups revealed significantly enhanced donor T cell engraftment by naive T cells and a minimal, but statistically significant, improvement in engraftment by naive F<sub>1</sub> T cells relative to that in TCD BM animals. When mice transplanted with activated T cells were analyzed, activated nontolerant T cells were again observed to enhance engraftment relative to that in control mice (group I vs III; p < 0.001; Table VI), but activated T cells from F<sub>1</sub> donors failed to improve donor engraftment (p = 0.11). Notably, there was a trend toward improved donor T cell engraftment after transplantation with 5 × 10<sup>5</sup> naive nontolerant B6 T cells compared with 5 × 10<sup>5</sup> nontolerant activated T cells (p = 0.06), similar to what we had previously observed (Table IV). These results indicated that facilitation of donor engraftment under these conditions required that activated donor T cells be capable of recognizing recipient alloantigens.

Table V. Facilitation of allograftment by ex vivo activated T cell subsets

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Donor T Cells Added</th>
<th>Percent Donor T Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent Donor (H-2K&lt;sup&gt;b&lt;/sup&gt;) Cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Number of Splenic B Cells (×10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4</td>
<td>None</td>
<td>13 ± 6</td>
<td>19 ± 6</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>5 × 10&lt;sup&gt;5&lt;/sup&gt; (N)</td>
<td>68 ± 43</td>
<td>73 ± 41</td>
<td>26 ± 24</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>5 × 10&lt;sup&gt;5&lt;/sup&gt; (CD8)</td>
<td>62 ± 41</td>
<td>73 ± 37</td>
<td>25 ± 18</td>
</tr>
<tr>
<td>IV</td>
<td>11</td>
<td>5 × 10&lt;sup&gt;6&lt;/sup&gt; (CD4)</td>
<td>8 ± 9</td>
<td>17 ± 16</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Irradiated (850 cGy) AKR recipients were transplanted with 10 × 10<sup>6</sup> TCD B6 BM with or without the indicated number of naive (N) or activated (A) T cells. Activated CD8<sup>+</sup> and CD4<sup>+</sup> T cells were obtained as previously described (see Materials and Methods). Mice were sacrificed at 27 days posttransplant, and chimerism in the spleen was assessed. Donor T cells were defined as H-2K<sup>b</sup>/CD3<sup>+</sup> and donor cells as H-2K<sup>b</sup><sup>-</sup>. Data are presented as the mean ± 1 SD and are derived from two separate experiments.

<sup>b</sup> Group I vs III, p < 0.0004; I vs II, p < 0.0002; II vs III, p = 0.74; I vs IV, p = 0.36.

<sup>c</sup> Group I vs II, p = 0.003; I vs III, p < 0.0001; II vs III, p = 0.97; I vs IV, p = 0.84.

<sup>d</sup> Group I vs II, p = 0.01; I vs III, p < 0.0001; II vs III, p = 0.93; I vs IV, p = 0.49.
The reduction in GVHD was manifested not only as significantly improved survival and weight gain relative to those in GVHD control mice, but also by a relative preservation of B cell and thymic reconstitution, which are both sensitive immunologic parameters of GVHD (40, 41). Protection from GVHD, however, was not complete, as evidenced by the fact that some irradiated animals transplanted with activated T cells did succumb to GVHD or experienced weight loss despite prolonged survival. Transplantation with either highly enriched activated CD4<sup>+</sup> or CD8<sup>+</sup> T cells both resulted in a reduction in GVHD, indicating that ex vivo activation and expansion with anti-CD3 Abs plus IL-2 qualitatively altered the ability of both T cell subsets to mediate GVH reactivity. This effect therefore was not T cell subset dependent.

We reasoned that the relative ability of activated vs naive T cells to cause GVHD might be confounded somewhat by the fact that mice in these experiments were preconditioned with irradiation, which is known to contribute to the pathophysiology of GVHD (42). To circumvent this potential bias, we examined these cells using a parent→F<sub>1</sub> model in which transplantation with alloreactive donor T cells results in subsequent susceptibility to LPS-mediated death in unirradiated recipients (9). Animals transplanted with activated T cells had no mortality, while nearly all mice transplanted with naive T cells died of GVHD. Within the context of this model, these results indicated that transplantation with activated T cells resulted in significantly less priming of macrophages, such that the latter were not poised to release TNF after LPS challenge. Transplantation with activated T cells therefore appeared to result in less recruitment of secondary effector cell populations that could contribute to the pathophysiology of GVHD. These studies also underscored the fact that these cells had an inherently reduced ability to cause GVHD, when examined in the absence of a conditioning regimen.

There are several possible explanations for why GVHD was abrogated in mice transplanted with activated T cells. One is that activated T cells had an altered trafficking pattern and were less likely to migrate to lymphoid-rich organs where they would encounter APCs and MHC alloantigens that could trigger GVHD. Prior studies have shown that activated T cells have altered migratory properties in vivo compared with unactivated T cells (43, 44). Our studies demonstrated that activated T cells did indeed preferentially traffic to the lungs and liver in contrast to naive T cells, which migrated primarily to the spleen early after transplant. However, the facts that activated T cells were able to facilitate engraftment and that engraftment in this model was dependent upon recognition of recipient MHC alloantigens (38) were evidence that a significant portion of these cells had to directly interact with and eliminate host immune cells capable of rejecting the graft. Thus, an altered trafficking pattern does not appear to be a sufficient explanation for why activated T cells caused less GVHD, since the finding that activated donor T cells had to interact with host cells to promote engraftment should have been a similarly sufficient stimulus to induce clinically significant GVHD.

An alternative mechanism is that ex vivo activated T cells were more susceptible to apoptosis than naive T cells. Either cytokine withdrawal occurring after activated T cells were removed from culture and transplanted into recipients or religation of the TCR after T cell encounter with host alloantigens in vivo could have served as stimuli for activated T cells to undergo apoptosis. Apoptosis secondary to growth factor withdrawal has been shown to be mediated in part by a deficiency of cytokines that signal through the IL-2 γ-chain, such as IL-2, IL-4, and IL-7 (18). Removal of IL-2 from culture after T cell activation results in a rapid decline in Bcl-X<sub>L</sub> protein levels (45). Since Bcl-X<sub>L</sub> inhibits apoptosis (46), activated T cells are predisposed to undergo cell death. Alternatively, the interaction between donor T cell TCR and host APCs could have triggered activation-induced cell death. Apoptosis in activated T cells occurring as a sequela of TCR religation has been shown to be mediated primarily by way of Fas/Fas ligand interactions (47, 48), although other pathways also appear to play a role (49). This latter process could explain how GVH reactivity could be mitigated without compromising engraftment, since a population of activated donor T cells could have itself also undergone cell death after elimination of host T cells. Concurrent cell death of both responder and target cells has been previously described to occur in vitro in cytotoxic activated γδ T cells (50). The facts that a minority of activated T cells was still detectable in the spleens of recipients 2 mo posttransplant (Table II) and that these cells were still capable of inducing GVHD (Figs. 2 and 3), however, indicated that not all activated T cells would have had shortened survival.

The accumulation of anti-CD3-activated T cells in the liver is not inconsistent with a mechanism of accelerated in vivo T cell death. While it is possible that this observation could have been merely a trafficking event, an alternative explanation is that at least some of these cells specifically migrated to the liver because they were undergoing apoptosis. Huang and colleagues (51) have shown that previously activated murine T cells undergoing apoptosis in vivo are eliminated in the liver, implying that the liver is a site of peripheral T cell deletion. The daily incremental increase in activated T cells that we observed early posttransplant in the liver therefore could have been due to the fact that these cells localized in the liver because they were undergoing apoptosis. The observation that a significant portion of the activated T cells transplanted into recipients resided in the liver 4 days after transplant indicated that this organ was the major site of lymphocyte accumulation. While our attempts to document apoptosis in these cells by flow cytometry were unsuccessful (data not shown), the rapidity with which apoptotic cells are cleared by macrophages may have precluded us from detecting this event (52, 53). Studies are currently underway to define whether accelerated cell death of anti-CD3-activated donor T cells in vivo is indeed the mechanism that mitigates GVH reactivity in this setting.

Despite causing less GVHD, activated T cells were capable of facilitating donor engraftment and were comparable to unseparated naive T cells on a cell-to-cell basis. Thus, these cells retained functional competency such that they could prevent graft rejection. Activated CD8<sup>+</sup> T cells were responsible for promoting engraftment, as CD4<sup>+</sup> T cells had no graft facilitatory capability in this model. These results are consistent with those of prior studies (29, 54), which have demonstrated that nontolerant CD8<sup>+</sup> T cells are far more effective in facilitating engraftment of MHC-disparate donors.
marrow than CD4+ T cells. This is presumably due to the fact that host T cells capable of rejecting the marrow graft do not express class II molecules and therefore cannot be recognized by donor CD4+ T cells. Both CD4+ and CD8+ T cells, which are tolerant of the recipient, however, have been shown to be capable of enhancing engraftment, presumably by a veto mechanism (30, 39), although donor engraftment is generally less complete than when nontolerant T cells are employed (39). To determine whether a similar mechanism might be operable in activated T cells, we examined whether tolerant activated T cells could promote engraftment. Using an F→parent model in which donor T cells were incapable of recognizing recipient alloantigens, these studies clearly indicated that activated T cells had to be able to recognize host alloantigens for engraftment to occur under these experimental conditions. Consequently, if activated T cells did have a shortened life span in vivo, they were still able to persist long enough in recipients to eliminate host immune cells capable of causing rejection.

In summary, this study demonstrates that ex vivo activation of donor T cells before transplantation into recipients resulted in a significant reduction in GVHD. This study was limited to evaluating T cell activation with anti-CD3/TCR Abs, although we have observed similar protection from lethal GVHD with mitogen-stimulated (Con A) T cells as well (unpublished observations). While the mechanism(s) for GVHD protection has not been completely defined, altered T cell trafficking and/or shortened T cell survival are two possibilities that require further examination. In particular, if the latter mechanism proves to be primarily responsible for the reduction in GVHD, then therapeutic strategies that are able to more completely and selectively eliminate T cells capable of mediating GVHD may represent an effective clinical strategy. The fact that activated T cells, in particular CD8+ T cells, were able to facilitate allograft engraftment indicated that these cells retained functional competency. The more complete selective elimination of these cells at a critically defined time point might therefore allow for the preservation of engraftment and the mitigation of GVHD. This might be accomplished by the use of agents that are capable of inducing apoptosis in preactivated CD8+ T cells or by incorporating suicide genes into T cells (55, 56), allowing them to be selectively eliminated at defined points posttransplant when engraftment will have occurred but before GVHD is clinically significant. This approach would be of particular therapeutic advantage in nonmalignant disorders where there is no risk of relapse. In this setting, donor T cells that subsequently reconstituted recipients would be BM derived and tolerant of the host after undergoing clonal selection in the thymus. This strategy might therefore allow for immune reconstitution to occur in a more ordered fashion in the absence of both the immune dysregulation induced by GVHD and the continued need for immunosuppressive agents to treat GVH reactivity.

Acknowledgments
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References