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 × 10^5) 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 allopeptide 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 reigation 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 religation 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 in mediating GVHD and facilitating allografting in murine recipients transplanted with MHC-mismatched marrow grafts.

Materials and Methods

**Mice**

C57BL/6 (B6) (H-2b), B10.BR (H-2b), B6.PL (H-2b), AKRJ (H-2b), and (C57BL/6 x AJ)F1 (B6AF1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). (C57BL/6 x AKR/J)F1 mice were bred at the Medical College of Wisconsin (Milwaukee, WI). All animals were housed in the American Association for Laboratory 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 μg/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 density of 5 x 10^5 cells/ml. Erythrocytes were removed from spleen cell suspensions as determined by flow cytometry, by the total number of cells. The viable fraction was measured by trypan blue exclusion. Total body irradiation was administered as a single exposure at a dose rate of 75 cGy using a Cobalt-60 treatment machine. All mice were housed in the American Association for Laboratory Care-accredited Animal Resource Center of the Medical College of Wisconsin. Mice received regular mouse chow and acidified tap water ad libitum.

**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-Ly5 (B220, rat IgG2a) and PE-anti-CD8 (clone CT-CD8) were obtained from Caltag (San Francisco, CA). PE-anti-TCR αβ (clone H57-597, hamster IgG), FITC-anti-Thy1.1 (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-2Kb (clone AF6-88.5, mouse IgG2b) were all purchased from PharMingen (San Diego, CA). Spleen and lymph node cells were used to determine the percentage of T cells, B cells, and NK cells directly ex vivo or after treatment with a given mAb as determined by flow cytometry. The percentage of cells counted was determined by the total number of cells. The viable fraction was measured by trypan blue exclusion. In both cases, the viable cells were counted and expressed as a percentage 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 >90% of the 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 was then 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) 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 vitro deletion experiments. Naive and activated T cells were then washed and resuspended in DMEM before injection.

Naive 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 TCM 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+ 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 and 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 Cobalt-60 treatment machine. All cultures were split into fresh flasks as needed to maintain a cell density of 5 to 10^6 cells/ml. Erythrocytes were removed from spleen cell suspensions as determined by flow cytometry, by the total number of cells. The viable fraction was measured by trypan blue exclusion. Total body irradiation was administered as a single exposure at a dose rate of 75 cGy using a Cobalt-60 treatment machine. All mice were housed in the American Association for Laboratory Care-accredited Animal Resource Center of the Medical College of Wisconsin. Mice received regular mouse chow and acidified tap water ad libitum.

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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\textsuperscript{+}/CD3\textsuperscript{+}. 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\textsuperscript{+}, and B cells were defined as Ly5\textsuperscript{+}. Thymocytes 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
Leukocytes 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 \(^{111}\)indium oxine and in vivo detection of radioative T cells
Naive and activated T cells were individually labeled in vitro with \(^{111}\)indium oxine by incubating 125 × 10\(^6\) cells with 50 μCi of 1.85 MBq of \(^{111}\)indium 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 αβ 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 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 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 × 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). Mortality 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 × 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...
reconstitution is a sensitive indicator of GVHD in this model (un-
published observations). Surviving mice from Figure 2 transplanted with $2 \times 10^6$ activated T cells and those transplanted with TCD BM only were comparatively analyzed 60 to 75 days after BMT (Table II). Analysis of T cell chimerism in the spleen revealed that mice transplanted with activated T cells had significantly enhanced donor T cell engraftment than control animals ($p = 0.03$; 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 Thy.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 re-

parent→F1 models in which no conditioning regimen is used. An advantage of the latter is that the predominant effects of GVHD are immunologic, and these can be examined in the absence of irradiation-induced inflammatory changes that contribute to GVH pathophysiology in lethal irradiation models. Using this approach, Nestel and colleagues (9) have described a model in which GVH reactivity due to alloreactive donor T cells results in macrophage priming that renders recipients susceptible to LPS-mediated lethal-

ity secondary to elevated TNF-α production. As an alternative way to more precisely assess the ability of activated T cells to cause GVHD in the absence of radiation, we performed experiments using this model in which F1 recipients were transplanted with equivalent numbers of naive or activated T cells. The total number of administered CD4$^+$ and CD8$^+$ T cells was again equivalent in each group to control for a subset bias in the induction of GVHD. Recipients of syngeneic naive T cells (F1→F1) had no mortality after LPS challenge (Table I). Conversely, animals transplanted with $25 \times 10^6$ naive T cells had 82% mortality within 48 h after LPS injection. Mice transplanted with activated T cells were similar to TCD BM control mice, as all survived challenge with LPS ($p = 0.0002$, by Fisher’s exact test, vs naive T cell group). These data indicated that in the absence of a conditioning regimen, activated T cells were inherently less capable of causing GVHD than equivalent numbers of naive T cells.

Thymic and B cell reconstitution in mice transplanted with activated T cells

As another index to assess the severity of GVHD in animals transplanted with activated T cells, long term immune reconstitution was examined in chimeras 2 to 3 mo posttransplant. Specifically, thymic reconstitution and splenic B cell repopulation were evaluated, since the thymus has been shown to be a target tissue during the GVH reaction (33–35), and we have also observed that B cell reconstitution is a sensitive indicator of GVHD in this model (un-

<table>
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<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Donor T Cells Added</th>
<th>Percent donor T cells $^a$</th>
<th>Percent CD4$^+$ T cells which coexpress Thy 1.1</th>
<th>B cells ($\times 10^6$) $^b$</th>
<th>Size ($\times 10^{-5}$$^c$)</th>
<th>Percent CD4$^+$CD8$^+$ cells</th>
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<td>I</td>
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<td>57 ± 33</td>
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<td>85 ± 5</td>
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<tr>
<td>II</td>
<td>12</td>
<td>$2 \times 10^6$ (A)</td>
<td>88 ± 24</td>
<td>5 ± 6</td>
<td>27 ± 26</td>
<td>66 ± 52</td>
<td>77 ± 21</td>
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$^a$ 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.

$^b$ Group I vs II, $p < 0.03$.

$^c$ Group I vs II, $p = 0.056$.

$^d$ 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.

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 × 10⁵ 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 × 10⁵) 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 × 10⁵ 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

![Image](https://www.jimmunol.org/article-figures/2615_img4a.jpg)

**FIGURE 4.** Anti-CD3-activated T cells localize to the liver early after transplant. Lethally irradiated (900 cGy) B10.BR recipients were transplanted with TCD B6 BM plus either 25 × 10⁶ naive or anti-CD3 activated B6.PL T cells. Cohorts of mice (n = 2/group) were sacrificed daily on days 2, 3, and 4 posttransplant, and livers were harvested for analysis. The total number of intrahepatic lymphocytes obtained from chimeras transplanted with naive (solid bar) or activated (hatched bar) T cells is shown in A. Data are presented as the total number of recovered cells per two mice. The percentage of Thy1.1⁺ lymphocytes in the livers of chimeras is shown in B. Data are presented as the percentage of Thy1.1⁺ cells in the pooled lymphocyte cell suspension obtained from two mice. One representative experiment is depicted.
from F1 donors failed to improve donor engraftment (mice (group I vs III; again observed to enhance engraftment relative to that in control activated T cells were analyzed, activated nontolerant T cells were to that in TCD BM animals. When mice transplanted with acti-
vated CD8\(^+\) T cells. These data indicated that activated CD8\(^+\) 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\(^+\) and CD8\(^+\) T cells. These data indicated that activated CD8\(^+\) T cells were primarily responsible for facilitating engraftment and that activated CD4\(^+\) 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 F1—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 F1 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 F1 donors failed to improve donor engraftment \((p = 0.11)\). Notably, there was a trend toward improved donor T cell engraftment after transplantation with \(5 \times 10^5\) naive nontolerant B6 T cells compared with \(5 \times 10^5\) 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.

**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 engraftment 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

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**Table IV. Activated T cells facilitate alloengraftment**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Donor T Cells Added</th>
<th>Percent Donor T Cells(^a)</th>
<th>Percent Donor (H-2K(^b)) Cells(^b)</th>
<th>Number of splenic B Cells ((\times 10^{-6})^d)</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 \times 10^5) (N)</td>
<td>56 ± 36</td>
<td>68 ± 36</td>
<td>16 ± 19</td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>(5 \times 10^3) (N)</td>
<td>95 ± 9</td>
<td>98 ± 3</td>
<td>17 ± 13</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>(1 \times 10^5) (A)</td>
<td>39 ± 35</td>
<td>47 ± 34</td>
<td>12 ± 18</td>
</tr>
<tr>
<td>V</td>
<td>14</td>
<td>(5 \times 10^5) (A)</td>
<td>75 ± 29</td>
<td>88 ± 24</td>
<td>35 ± 22</td>
</tr>
<tr>
<td>VI</td>
<td>13</td>
<td>(1 \times 10^5) (A)</td>
<td>79 ± 33</td>
<td>91 ± 18</td>
<td>31 ± 20</td>
</tr>
</tbody>
</table>

\(^a\) Irradiated (850 cGy) AKR recipients were transplanted with \(10 \times 10^6\) 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\(^b\)/CD3\(^+\), donor cells as H-2K\(^b\), and B cells as Ly5 (B220) positive.

\(^b\) Group I vs III, V, and VI, \(p < 0.01\); III vs VI, \(p = 0.12\); III vs V, \(p = 0.03\).

\(^c\) Group I vs III, V, and VI, \(p < 0.0003\); III vs V, \(p = 0.18\); III vs VI, \(p = 0.11\).

\(^d\) Group I vs III, \(p < 0.04\); I vs V and VI, \(p < 0.0005\); III vs VI, \(p = 0.06\).

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**Table V. Facilitation of alloengraftment 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(^a)</th>
<th>Percent Donor (H-2K(^b)) Cells(^b)</th>
<th>Number of Splenic B Cells ((\times 10^{-6})^d)</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 \times 10^5) (N)</td>
<td>68 ± 43</td>
<td>73 ± 41</td>
<td>26 ± 24</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>(5 \times 10^5) (CD8)</td>
<td>62 ± 41</td>
<td>73 ± 37</td>
<td>25 ± 18</td>
</tr>
<tr>
<td>IV</td>
<td>11</td>
<td>(5 \times 10^5) (CD4)</td>
<td>8 ± 9</td>
<td>17 ± 16</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

\(^a\) Irradiated (850 cGy) AKR recipients were transplanted with \(10 \times 10^6\) TCD B6 BM with or without the indicated number of naive (N) or activated (A) T cells. Activated CD8\(^+\) 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\(^b\)/CD3\(^+\) and donor cells as H-2K\(^b\). Data are presented as the mean ± 1 SD and are derived from two separate experiments.

\(^b\) Group I vs II, \(p < 0.004\); I vs III, \(p < 0.002\); II vs III, \(p = 0.74\); I vs IV, \(p = 0.36\).

\(^d\) Group I vs II, \(p = 0.003\); I vs III, \(p < 0.001\); II vs III, \(p = 0.97\); I vs IV, \(p = 0.84\).

\(^d\) Group I vs II, \(p = 0.01\); I vs III, \(p < 0.001\); II vs III, \(p = 0.93\); I vs IV, \(p = 0.49\).
Ab. 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\(^+\) or CD8\(^+\) 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\(_1\) model in which transplantation with allogeneic 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 inter-

### Table VI. Facilitation of engraftment by activated T cells is dependent upon recognition of host MHC alloantigens^a^

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Donor T Cells Added</th>
<th>Percent Donor T Cells</th>
<th>Mean^b</th>
<th>Added Percent Donor T Cells</th>
<th>Mean^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>8</td>
<td>None</td>
<td>25, 25, 24, 11, 31, 2, 2</td>
<td>15 ± 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5 × 10^3 (N)</td>
<td>99, 99, 100, 99, 84, 97, 93</td>
<td>96 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>8 × 10^3 (A)</td>
<td>96, 96, 92, 77, 72, 65, 75, 3</td>
<td>72 ± 30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>5 × 10^3 (N)</td>
<td>30, 36, 24, 32, 22, 16, 17, 25</td>
<td>27 ± 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>5 × 10^3 (A)</td>
<td>39, 22, 35, 28, 3, 41, 43, 7</td>
<td>27 ± 15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Irradiated (850 cGy) AKR recipients were transplanted with 20 × 10^6 TCD B6 BM with or without the indicated number of naive (N) or activated (A) B6 or (B6 × AKR:F1) T cells. Mice were sacrificed 23 to 27 days posttransplant and assessed for donor T cell chimerism in the spleen. Individual data points are presented along with the mean ± SD. Data are derived from two separate experiments.

^b Group I vs II, p < 0.00001; I vs III, p < 0.001; I vs IV, p = 0.04; I vs V, p = 0.11; II vs III, p = 0.06.

One mouse in this group died prior to analysis.
Although donor engraftment is generally less complete than when enhancing engraftment, presumably by a veto mechanism (30, 39), of the recipient, however, have been shown to be capable of en-graftment to occur under these experimental conditions. Consequently, if activated T cells did have a short-ened 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-stimu-lated (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 medi-ating GVHD may represent an effective clinical strategy. The fact that activated T cells, in particular CD8+ T cells, were able to facilitate allograft rejection indicated that these cells retained func-tional 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 incor-porating suicide genes into T cells (55, 56), allowing them to be selectively eliminated at defined points posttransplant when GVHD will have occurred but before GVHD clinically signif-icant. 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.

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References


