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Graft-facilitating doses of *ex vivo* activated γδ T cells do not cause lethal murine graft-vs.-host disease

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ABSTRACT

The purpose of this study was to examine the ability of γδ T cells to cause graft-vs.-host disease (GVHD) after allogeneic bone marrow transplantation (BMT) and to determine whether these cells offered any therapeutic advantages relative to αβ T cells. Due to the paucity of naive γδ T cells in mice and humans, γδ T cells (obtained from αβ T cell–deficient murine donors) were *ex vivo* activated and expanded in interleukin (IL)-2 so as to achieve sufficient cell numbers and to serve as a more clinically feasible strategy. After transplantation into lethally irradiated hosts, donor γδ T cells were detected in target organs of GVHD such as the spleen and intestines 2 weeks after BMT and constituted the primary T cell subpopulation. Large doses (150×10^6) of activated γδ T cells, which we have previously shown capable of facilitating engraftment in MHC-disparate recipients, failed to cause fatal GVHD in lethally irradiated recipients of MHC-incompatible donor marrow grafts (C57BL/6 [H-2^b]→B10.BR [H-2^k] and C57BL/6 [H-2^b]→B6D2F1 [H-$2^{b/d}$]). The absence of GVHD was confirmed by histologic analysis of target organs, splenic B cell reconstitution, and appropriate negative selection in the thymus, that were all comparable to those observed in mice transplanted with T cell–depleted BM only. While early splenic reconstitution was attributable to donor γδ T cells, analysis of durably engrafted chimeras 2 months posttransplant revealed that the vast majority of donor splenic T cells expressed the αβ T cell receptor. The results of secondary adoptive transfer assays showed that these cells were tolerant of recipient alloantigens in vivo, demonstrating that γδ T cells did not prevent the subsequent development of donor anti-host tolerance in BM-derived αβ T cells. When comparatively evaluated, the minimal number of naive αβ T cells necessary for donor engraftment caused significantly more fatal GVHD than the corresponding minimal dose of activated γδ T cells and thus had a superior therapeutic index. These studies indicate that doses of activated γδ T cells that are able to promote alloengraftment do not cause lethal GVHD in mice transplanted with MHC-incompatible marrow grafts.

KEY WORDS:

Allogeneic bone marrow transplantation • Graft-vs.-host disease • γδ T cells

INTRODUCTION

Clinical studies and experimental animal models have established that donor T cell recognition of alloantigen is the primary event in the initiation of GVHD. Thereafter, there occurs an amplification of immune reactivity which results in dysregulated cytokine production and activation of secondary immune effector cell populations [1–4]. Several lines of evidence suggest that donor-derived αβ T cells are the T cell subset that plays the major role in the pathophysiology of GVH reactivity. First of all, T cells initiate GVHD and αβ T cells constitute the vast majority of T cells, indirectly implicating this subpopulation as most responsible for GVHD. Second, immunohistologic studies in humans have shown that infiltrating T cells in GVH lesions almost exclusively express the αβ, as opposed to the γδ, T cell receptor (TCR) when analyzed in skin, liver, or intestines of affected recipients [5–7]. A third line of evidence is that the residual αβ T cell content in the donor marrow graft (as opposed to the γδ T cell content) most closely correlates with the subsequent development and severity of acute GVHD in recipients of T cell–depleted HLA-identical sibling marrow grafts [8].

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The specific role of γδ T cells, which represent a minority of T cells in both humans and rodents, in the pathophysiology of GVHD has been controversial. Whereas some studies have presented indirect evidence that γδ T cells contribute to GVHD pathophysiology [9], others have not found these cells capable of causing GVHD [10]. Under certain experimental conditions, naive γδ T cells derived from transgenic mice have been shown to cause GVHD [11]. The clinical relevance of γδ T cells expressing only a single TCR, however, is unknown. Moreover, the role in GVHD of a polyclonal population of γδ T cells, which is more representative of the clinical situation, has not been examined in a direct fashion. In our clinical transplant program, we have observed high rates of engraftment in recipients of unrelated marrow grafts in which γδ T cells are preferentially spared from the depletion procedure, suggesting that γδ T cells may play a role in overcoming graft resistance [12]. We have further found a direct correlation between the γδ T cell dose administered to patients and the probability of engraftment [8]. This prompted us to examine the role of γδ T cells in facilitating engraftment in a major histocompatibility complex (MHC)-incompatible murine model using transgenic αβ T cell–deficient animals as donors so as to provide sufficient numbers of γδ T cells. We observed that large doses of naive γδ T cells (20×10^6) failed to facilitate engraftment except in animals that also developed clinically significant GVHD [13]. Since further dose escalation of naïve γδ T cells was constrained by cell availability, we examined this question by *ex vivo* activating and expanding γδ T cells to achieve higher cell numbers. This approach was also taken because of the low frequency of these cells in the peripheral blood and bone marrow. Therefore, the only way sufficient numbers of γδ T cells are likely to be obtainable for clinical application is through large-scale expansion. These studies demonstrated that transplantation with these escalated doses of activated γδ T cells was able to facilitate durable donor engraftment and promote hematopoietic recovery [13]. Given that γδ T cells are capable of promoting engraftment, the clinical application of this strategy to allogeneic marrow transplantation is contingent on the cells not being capable of causing significant GVHD. We therefore sought to determine more critically whether activated γδ T cells caused significant GVHD, assess what effect the cells had on splenic and thymic reconstitution, and determine if they possessed any therapeutic advantage relative to αβ T cells.

MATERIALS AND METHODS

Mice

T cell receptor β^-/β^- (αβ T cell–deficient, pure C57BL/6 background, H-2^b), normal C57BL/6 (H-2^b), AKR/J (H-2^k), and B10.BR (H-2^k) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). (C57BL/6 × DBA/2) F1 mice (B6D2, [H-$2^{b/d}$]) were purchased from the National Institutes of Health (Bethesda, MD). All animals were housed in the American Association for Lab Animal Care (AALAC)-accredited Animal Resource Center of the Medical College of Wisconsin. Mice received regular mouse chow and acidified tap water *ad libitum*.

Flow cytometric analysis

Monoclonal antibodies (mAbs) conjugated to either fluoroscein isothiocyanate (FITC) or phycoerythrin (PE) were used to assess chimerism in marrow transplant recipients. FITC anti-Thy1.2 (clone 30-H12, rat IgG2b) was purchased from Collaborative Biomedical Products (Bedford, MA). FITC anti-Ly5 (B220, rat IgG2a) and PE anti-CD8 (clone CT-CD8a, rat IgG2a) were obtained from Caltag (San Francisco, CA). PE anti-TCR αβ (clone H57-597, hamster IgG), PE anti-TCR γδ (clone GL3, hamster IgG), PE anti-CD3 (clone 145-2C11, hamster IgG), PE anti-Thy 1.1 (clone OX-7, mouse IgG1), PE anti-CD4 (clone GK 1.5, rat IgG2b), FITC anti-H-2K^b (clone AF6-885, mouse IgG2a), V_β5-FITC (clone MR9-4, mouse IgG1), and V_β6-FITC (clone RR4-7, rat IgG2b) were all purchased from Pharmingen (San Diego, CA). Spleen, thymus, and intestinal intraepithelial cells were obtained from chimeras at defined intervals posttransplant and stained for two-color analysis. Red cells were removed by lysis in distilled water. Cells were analyzed on a FACS analyzer (Becton Dickinson, Mountain View, CA) with Consort 32 computer support and Lysis II software. Red cells and nonviable cells were excluded using forward and side scatter settings before analysis of spleen and intestinal intraepithelial cell populations. Splenic T cell chimerism was assessed within a lymphocyte gated population, and thymic chimerism was evaluated using open gates. The absolute number of donor T cells in the spleen and intestinal epithelium was calculated by determining the relative percentage of each population within the lymphocyte gate and multiplying by the total number of cells isolated. For quantification of splenic T cells, this calculation was further adjusted through gating to account for the fact that lymphocytes represented only a fractional number of total spleen cells. Ten thousand cells were analyzed for each determination whenever possible.

Ex vivo expansion of murine γδ T cells

Spleen cells were obtained from TCR β^-/β^- donor animals and passed through nylon wool columns to remove B cells. The resulting population typically comprised ~50% cells expressing the γδ TCR. Cells were then resuspended in complete Dulbecco's modified essential medium (CDMEM) plus 5–10% fetal bovine serum (FBS) and cultured in flasks precoated with an immobilized γδ T cell–specific monoclonal antibody (GL4, hamster IgG, Pharmingen) at a concentration of 5–10 μg/mL. Twenty-four hours after the initiation of culture, human IL-2 (Cetus Corp., Norwalk, CT) was added at a concentration of 20–100 U/mL (Cetus units). All cultures were split into fresh flasks as needed to maintain a cell concentration of $0.5–1.5\times10^6$ cells/mL. Cells were exposed to immobilized monoclonal antibody for the first 3–4 days of culture and thereafter grown only in medium plus IL-2 to allow for reexpression of the γδ TCR. After a total of 7–8 days in culture, cells were counted, and the percentage of γδ T cells was analyzed by flow cytometry. Routinely, a total of $5–10\times10^8$ cells was obtained after expansion, with 95–99% of cells expressing the γδ TCR. Typically, 15–25% of activated γδ T cells coexpressed CD8. CD4 expression was not detected on these cells.

Bone marrow transplantation

Bone marrow (BM) was flushed from donor femurs and tibias with CDMEM and passed through sterile mesh filters to obtain single-cell suspensions. BM was T cell–depleted *in vitro* with anti-Thy1.2 monoclonal antibody plus low toxicity rabbit complement (C6 Diagnostics, Mequon, WI). The hybridoma for 30-H12 (anti-Thy1.2, rat IgG2b) antibody was obtained from ATCC (Rockville, MD) and grown in CDMEM plus 5% FBS. The culture supernatant was then harvested, precipitated in ammonium sulfate, and dialyzed against phosphate-buffered saline (PBS) before use in *in vitro* depletion experiments. BM cells were then washed and resuspended in DMEM before injection. To recover naive T cells, spleen cell suspensions were obtained by pressing spleens through wire mesh screens. Erythrocytes were removed from cell suspensions by hypotonic lysis with sterile distilled water. Naive T cells for admixture with T cell–depleted (TCD) BM before transplantation were then obtained by passing spleen cells once or twice through nylon wool columns (Robbins Scientific, Sunnyvale, CA) to remove B cells. The percentages of $\alpha\beta^+$ and $\gamma\delta^+$ T cells from B6 donors were quantified by flow cytometry and defined as Thy1.2$^+$ $\alpha\beta$ TCR$^+$ and Thy1.2$^+$ $\gamma\delta$ TCR$^+$, respectively. In some experiments, naive T cells were defined as Thy1.2$^+$ CD4$^+$ and Thy 1.2$^+$ CD8$^+$.

B10.BR, AKR/J, or B6D2F1 recipient mice were given various doses of lethal total-body irradiation (900 cGy, 1100 cGy, or 1100 cGy, respectively) as a single exposure at a dose rate of 75 cGy using a Mark I Cesium Irradiator (Shepherd, San Fernando, CA). Irradiated recipients then received a single intravenous injection of TCD BM (10×10^6) with or without added naive or *ex vivo* activated T cells. Recipients transplanted with activated $\gamma\delta$ T cells received a dose of 150×10^6 cells. In some experiments, half of the cells were given at the time of transplant and the remaining cells were given 1 hour later to reduce the cell density in the initial inoculum. In other experiments, the total dose was given over 24 hours in four divided doses to reduce immediate toxicity from the infusion of large numbers of activated cells. The total dose employed was based on previous data which had shown that this number of $\gamma\delta$ T cells was in the range required for consistent donor engraftment in sublethally irradiated MHC-incompatible recipients transplanted with TCD marrow grafts [13].

Isolation of intestinal intraepithelial lymphocytes

Intestinal intraepithelial lymphocytes (IELs) were obtained according to the method of Whetsell et al. [14] with minor modifications. Briefly, the small intestine was removed from individual animals and the mesentery and Peyer's patches dissected from the tissue. Each intestine was then cut into 0.5- to 1-cm segments, opened longitudinally to expose the epithelium, and washed extensively in CDMEM and 5% FBS until the supernatant was clear. Intestinal pieces were placed into CDMEM and 5% FBS plus 1 mM EDTA and incubated for 20 minutes ($\times$ 2) at 37°C. The supernatant was collected, centrifuged, and resuspended in medium. Cells were passed through a prewet nylon wool column to remove residual debris, washed, pelleted, and resuspended in a 44% isotonic Percoll solution. Cells were centrifuged for 20 minutes at 1600 rpm. The cell pellet was then resuspended in medium and cells were used for FACS analysis to assess the extent of donor T cell chimerism.

Histological studies

Tissues were obtained from control and experimental animals, fixed in 10% neutral buffered formalin, and processed into paraffin blocks. Sections 4 μm thick were prepared from each block and cut at two levels to optimize sampling. Routine tissue hematoxylin and eosin sections were prepared. For the evaluation of GVHD, tissue sections were scored with the examiner blinded to the treatment received by each animal. Tissues were scored positive for GVHD if there was evidence of single-cell necrosis in the skin, bile duct necrosis with or without infiltration in the liver, crypt cell necrosis with or without infiltration in the colon, or lymphocytic infiltration in the lung with associated cell destruction.

Statistical analysis

Group comparisons of donor T cell chimerism in the spleen and thymus, splenic B cell content, and thymic size were performed using the unpaired Student's *t* test. A two-tailed *p* value ≤ 0.05 was deemed to be significant. Survival curves were constructed using the Kaplan-Meier product limit and compared using the log rank rest. Mice that were killed for secondary transfer experiments were censored from the survival analysis at the time of death.

RESULTS

Activated $\gamma\delta$ T cells traffic to the spleen and intestines early posttransplant

$\gamma\delta$ T cells constitute a minor T cell population in the spleen of mice but represent the majority of T cells in the intestinal epithelium. Since the spleen and the intestines are both target organs in GVHD, we performed initial studies to determine whether transplanted donor $\gamma\delta$ T cells were detectable early posttransplant in lethally irradiated AKR animals in either of these two sites, so as to exclude altered trafficking as a mechanism by which GVHD might be abrogated. Animals were studied at two early time points (3 and 14 days post-BMT). Three days posttransplant, mice transplanted with activated $\gamma\delta$ T cells had a greater absolute number of donor splenic T cells when compared with mice receiving TCD BM only (2.5×10^6 vs. 0.1×10^6) (Table 1). The difference in donor T cell chimerism between these two groups was accounted for solely by the presence of donor-derived $\gamma\delta$ T cells, since the number of donor $\alpha\beta$ T cells was similar in both groups. The percentages of splenic T cells that were donor as opposed to host in origin was 76 and 12%, respectively, in these two cohorts (data not shown). Of note, virtually all of the $\gamma\delta$ T cells in the spleen were of donor origin (94%) in mice transplanted with activated $\gamma\delta$ T cells. Donor-derived $\gamma\delta$ T cells were also detectable in the intestinal epithelium and accounted for the majority of donor T cells. The total number of donor T cells was not substantially different between the two groups at this early time point, however. When mice transplanted with $\gamma\delta$ T cells were analyzed 2 weeks later (days 14–17), the predominant donor T cell population again expressed

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Table 1. *Activated γδ T cells traffic to the spleen and the intestinal epithelium early posttransplant*

	Number of donor cells (H-2K^b) expressing ($\times 10^{-6}$)											
	Day 3						Day 14-17					
	Spleen			Intestine			Spleen			Intestine		
Group	CD3	αβ	γδ	CD3	αβ	γδ	CD3	αβ	γδ	CD3	αβ	γδ
I	0.1	0.1	NE	0.2	0.1	0.1	0.5	0.2	0.2	0.05	0.01	0.01
II	2.5	0.2	2.4	0.3	0.1	0.3	12.5	1.7	12.6	1.9	0.2	1.8

Lethally irradiated (1100 cGy) AKR mice were transplanted with TCD B6 BM alone (group I) or together with 150×10^6 activated γδ T cells from β$^-$/β$^-$ (B6) donors (group II). At defined time points, animals from each cohort were killed (n=*3/group, day 3;* n=*2/group, day 14–17). Pooled spleen and intestinal intraepithelial lymphocyte cell suspensions were obtained and analyzed by flow cytometry to determine the absolute number of donor cells (H-2K^{b+}) that also coexpressed CD3, αβ TCR, or γδ TCR. Data are presented as the mean number of cells from pooled cell suspensions. NE, not evaluable for technical reasons.*

the γδ TCR in both the spleen and intestine. The absolute number of donor γδ$^+$ T cells and CD3$^+$ T cells were substantially higher in these animals when compared to control mice, and γδ$^+$ T cells again accounted for the preponderance of donor T cells. Moreover, 74 and 98% of all γδ$^+$ T cells found in the spleen and intestinal epithelium of these mice, respectively, were donor-derived (data not shown). The percentages of donor as opposed to host T cells in the intestinal epithelium of group I vs. group II animals was 6 and 64%, respectively, while the percentages of donor T cells in the spleen was 53 and 91%, respectively. These data demonstrated that γδ T cells were detectable and persisted in the spleen and intestinal epithelium of recipients for at least several weeks, and that these cells did not uniformly undergo rapid cell death in vivo after transplantation.

Activated γδ T cells do not cause lethal GVHD

To address whether activated γδ T cells could cause GVHD, we performed studies using two well-characterized MHC-incompatible donor/recipient strain combinations. In initial experiments, lethally irradiated B10.BR recipients were transplanted with TCD B6 BM alone or together with 150×10^6 activated γδ T cells. In this model, approximately 2×10^6 naive T cells has been shown to be sufficient for the induction of lethal GVHD in the majority of recipients [15]. Animals transplanted with 150×10^6 activated γδ T cells had survival equivalent to that observed in control mice (Fig. 1). To further examine these mice for subclinical GVHD, we performed detailed histologic analyses on tissues from the mice that had survived for ~90 days. Examination of the skin, lung, liver, and colon of these chimeras revealed no significant differences between mice transplanted with or without γδ T cells. Specifically, three of 28 tissues were positive for GVHD in the former group while zero of 19 tissues were positive in the latter cohort (p = 0.26 by Fisher's exact test). When splenic B cell reconstitution, which is also known to be a sensitive indicator of GVHD in this model, was comparatively analyzed, no significant differences were observed, indicating that γδ T cells did not cause a GVH-associated reduction in B cell repopulation. Mice transplanted with TCD BM alone (n=5) had a mean of 79×10^6 splenic B cells vs. 80×10^6 for those transplanted with activated γδ T cells (n=7). Notably, animals in both groups had equivalent levels of donor T cell chimerism (87 vs. 93%), so the observed results could not be ascribed to differential donor T cell engraftment.

To confirm that the lack of GVHD did not depend on mouse strain, we examined this question using an alternative model of GVHD in which donor and recipient are also MHC-incompatible (B6→B6D2). Lethally irradiated B6D2 recipients transplanted with 5×10^6 naive B6 T cells (GVHD control animals) all developed severe GVHD and died within 25 days of BMT (Fig. 2). In contrast, there was no observed mortality in B6D2 mice reconstituted with activated γδ T cells, and these animals had survival comparable to TCD BM control animals. Weight curves in these mice were also similar to those observed in control mice (data not shown). Thus, large doses of activated γδ T cells failed to cause lethal GVHD in these two different MHC-incompatible donor/recipient strain combinations.

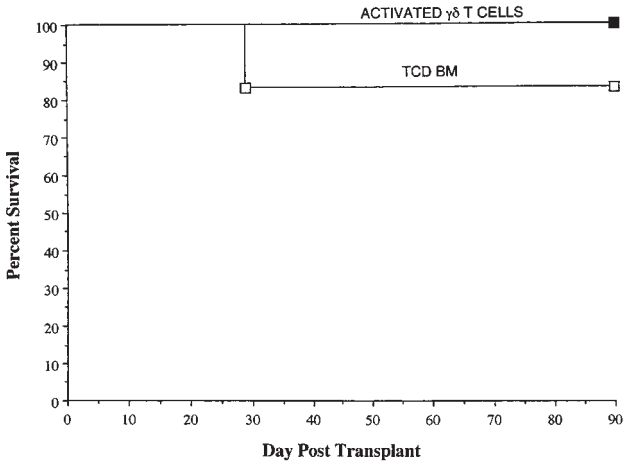

Figure 1. Activated γδ T cells do not cause lethal GVHD in MHC-incompatible recipients

Lethally irradiated (900 cGy) B10.BR recipients were transplanted with TCD B6 BM alone (□, n=*5) or TCD B6 BM plus 150×10^6 activated γδ T cells (■,* n=*7). Survival is depicted. Data are the cumulative results of two similarly performed experiments.*

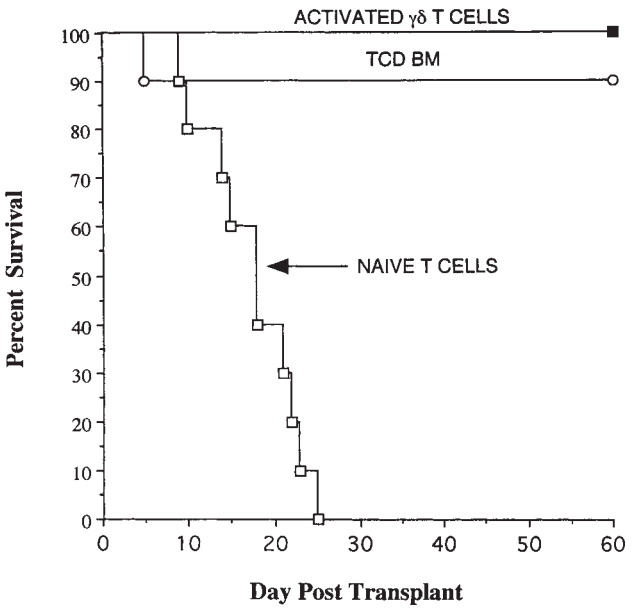

Figure 2. Activated γδ T cells do not cause clinical GVHD in haplotype mismatched recipients

Lethally irradiated (1100 cGy) B6D2F1 recipients were transplanted with TCD B6 BM alone (○, n=10) or TCD B6 BM plus either 5×10^6 naive B6 T cells (□, n=10) or 150×10^6 activated γδ T cells from β^-/β^- donors (■, n=10). Survival is depicted. Data are the cumulative results of two similarly performed experiments.

Long-term splenic donor T cell engraftment is accounted for by αβ and not γδ T cells

We had observed that early posttransplant (within 2 weeks), γδ T cells were the predominant donor T cell population in the spleens of chimeras. Since γδ T cells comprise only a minority of the total splenic T cells in normal mice, we questioned whether donor γδ T cells persisted in the spleens of these animals when examined at later time points after marrow transplantation or whether animals ultimately resembled normal mice with respect to the relative percentages of each T cell subpopulation. When analyzed 60 days after BMT, mice transplanted with TCD BM plus activated γδ T cells had donor T cell engraftment comparable to mice reconstituted with TCD BM alone (data not shown). In TCD BM control mice, nearly all donor T cells were αβ TCR^+ (Table 2), similar to what was observed at early posttransplant time points (Table 1). Conversely, while most splenic donor T cells were primarily γδ TCR^+ in mice transplanted with activated T cells early after BMT, the majority of donor T cells 60 days post-BMT expressed the αβ TCR (Table 2). γδ T cells were present in higher numbers in these animals than in TCD BM control mice (mean 0.1×10^6 vs. 2.8×10^6) but represented only ~10% of all donor T cells. Thus, the relative percentage of γδ T cells detectable in the spleens of chimeras decreased with time and αβ T cells became the predominant donor T cell population. The latter cells were necessarily bone marrow–derived since the transplanted activated γδ T cell population was devoid of any αβ T cells.

Table 2. *αβ T cells are the major splenic donor T cell population in durably engrafted chimeras*

Group	Number of mice	Donor γδ T cells added	Donor cells ($\times10^{-6}$) expressing CD3	αβ TCR	γδ TCR
I	3	None	10.2	8.9	0.1
II	4	150×10^6	23.1	21.4	2.8

Lethally irradiated (900 cGy) B10.BR recipients were transplanted with 10×10^6 TCD B6 BM alone (group I) or together with 150×10^6 activated γδ T cells (group II). Animals were killed 60 days posttransplant, and spleen cells were analyzed from individual chimeras by flow cytometry to determine the absolute number of donor cells (H-$2K^{b+}$) that coexpressed CD3, αβ TCR, or γδ TCR. Data are presented as the mean of individual data points.

Splenic donor T cells in chimeric animals are tolerant of recipient alloantigens

By performing secondary adoptive transfer assays, we evaluated whether reconstituting splenic donor T cells, which were primarily BM-derived αβ TCR^+ in animals transplanted with activated γδ T cells, were specifically tolerant of host alloantigens in vivo. Bone marrow and spleen cells were obtained from B6→B6D2F1 chimeras 60 days posttransplant, and equivalent numbers of each were transplanted into either lethally irradiated B6D2F1 or third-party B10.BR recipients. The percentage of donor-derived splenic T cells in these chimeras averaged 87%, demonstrating that chimeras were primarily engrafted with donor T cells. B6D2 mice transplanted with chimeric (B6) T cells failed to develop clinically significant GVHD, whereas the majority of B10.BR recipients died of this complication within 60 days of transplant (Fig. 3). Overall survival was significantly greater in B6D2 recipients when compared with B10.BR animals ($p = 0.004$). These data indicated that donor-derived αβ T cells that reconstituted in animals transplanted with large doses of activated γδ T cells were tolerant of B6D2 host alloantigens but were capable of mediating an alloresponse against third-party cells in vivo. Transplantation with γδ T cells therefore did not interfere with the subsequent development of donor anti-host tolerance in BM-derived αβ T cells.

Activated γδ T cells do not cause GVHD in the thymus

The thymus is also known to be a target tissue for GVHD, and animals undergoing GVHD have been shown to have an increased number of single-positive CD4 and CD8 cells with a corresponding reduction in double-positive ($CD4^+CD8^+$) thymocytes [16,17]. Since tolerance appeared to have been established as judged by secondary assays, we performed additional experiments to directly examine for the presence or absence of GVHD in the thymus of transplanted recipients. Lethally irradiated B10.BR mice were transplanted as above and analyzed 2 weeks posttransplant. As previously observed, mice reconstituted with activated γδ T cells had a greater percentage of donor T cells in the spleen (mean 82 vs. 23%, $p < 0.00005$), with 94% of donor T cells expressing the γδ TCR. Analysis of thymic reconstitution revealed that these animals had a significantly greater number of thymocytes (mean 71×10^6 vs. 34×10^6, $p < 0.05$), but

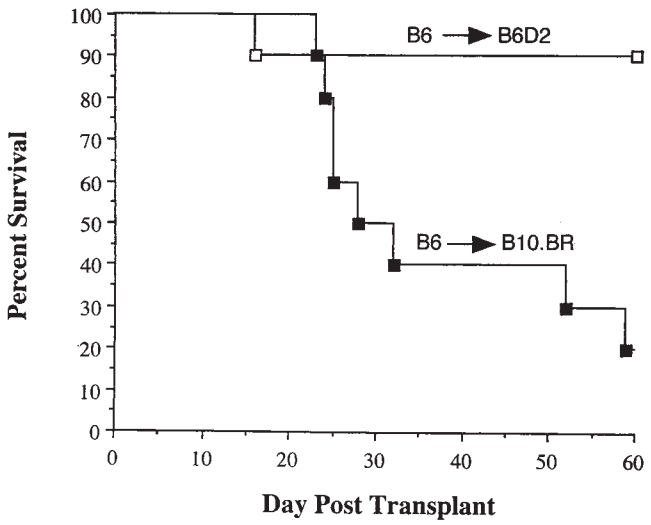

Figure 3. Donor-derived αβ T cells which reconstitute in mice transplanted with activated γδ T cells are tolerant of recipient alloantigens *in vivo*

Lethally irradiated (1100 cGy) B6D2F1 mice were transplanted with TCD B6 BM plus 150×10^6 activated γδ T cells. Sixty days after transplant, representative chimeras (B6) were killed, and pooled suspensions of BM (10×10^6/mouse) and spleen cells (15×10^6/mouse) were transplanted into lethally irradiated B6D2 (□, n=10) and B10.BR (■, n=10) animals. Survival is depicted. Data are cumulative results from two similarly performed experiments.

equivalent percentages of double-positive $CD4^+CD8^+$ cells (mean 75 vs. 77%, $p = 0.66$), when compared with control mice. The percentage of single-positive $CD4^+$ and $CD8^+$ T cells was also equivalent in both cohorts (data not shown). These results demonstrated that transplantation with activated γδ T cells did not effect any of the phenotypic changes associated with GVHD in the thymus.

Negative selection in the thymus in animals transplanted with activated γδ T cells

A characteristic of GVHD in B6→B10.BR chimeras is the failure to delete in the thymus T cells that are $V_\beta5^+$ or $V_\beta11^+$. Consequently, these cells appear in the periphery of recipients due to an absence of negative selection. We evaluated whether negative selection occurred in the thymus of animals transplanted with activated γδ T cells by comparing the splenic T cell phenotype of these mice with non-GVHD and GVHD control animals. Mice transplanted with naive T cells had a significantly higher absolute number of $CD4^+$ and $CD8^+$ $V_\beta5^+$ T cells than mice transplanted with TCD BM only ($p < 0.02$), consistent with the interpretation that these mice had ongoing GVHD (Table 3). The absolute number of $V_\beta6^+$ T cells was similar in both groups of mice, reflecting the fact that these cells are not normally deleted in this strain combination. Animals transplanted with activated γδ T cells had an absolute number of $V_\beta5^+$ CD4 and CD8 T cells not significantly different from that observed in control animals ($p \geq 0.38$). These data indicated that negative selection of $V_\beta5^+$ T cells occurred in these mice. The lack of $V_\beta5^+$ T cell expansion was further evidence of an absence of GVH reactivity in these chimeras. Since the majority of transplanted activated γδ T cells lacked expression of CD4 and CD8, these data indicated that potentially host-reactive bone marrow–derived donor $CD4^+$ and $CD8^+$ T cells underwent appropriate clonal deletion in the thymus.

Graft-facilitating doses of activated γδ T cells cause significantly less GVHD than limiting numbers of naive αβ T cells

We previously had shown that 5×10^5 naive αβ T cells is the minimal number required to facilitate donor engraftment in sublethally irradiated B6→AKR chimeras, while $80–150\times10^6$ activated γδ T cells are necessary for durable engraftment in the same model [13]. We therefore sought to compare the relative ability of these two T cell doses to induce GVHD so as to determine whether transplantation with activated γδ T cells offered any therapeutic advantage over limiting numbers of αβ T cells in this donor/recipient strain combination. These studies were based on the premise that limiting numbers of naive αβ T cells would presumably cause less GVHD than larger doses, and therefore this would be an alternative strategy by which to reduce GVH-related toxicity. When lethally irradiated AKR mice were transplanted with activated γδ T cells, these animals had similar survival and weight gain compared with animals transplanted with TCD BM only (Fig. 4). B cell and thymic recovery in these mice were comparable to that observed in control animals (data not shown). Conversely, animals transplanted with limiting numbers of naive αβ T cells had substantially more GVHD, as evidenced by reduced survival and poorer weight gain posttransplant. These animals were not evaluable for long-term immune reconstitution studies because of early death. Collectively, these data indicated that doses of activated γδ T cells sufficient to facilitate alloengraftment did not cause clinically significant GVHD, while minimal doses of naive αβ T cells necessary for donor engraftment resulted in substantial mortality from GVHD.

DISCUSSION

The clinical role of γδ T cells in allogeneic BMT is undefined. Moreover, the paucity of these cells in the peripheral blood of humans has made potential clinical administration problematic. For that reason, we have evaluated the biological effects of *ex vivo* activated and expanded γδ T cells on engraftment and GVHD as a more clinically feasible strategy. We have previously shown that large numbers of donor activated γδ T cells ($80–160\times10^6$) facilitates engraftment of MHC-disparate TCD marrow grafts in sublethally irradiated recipients [13]. Chimerism studies revealed that 50–80% of splenic T cells and 70–95% of all splenic cells were donor-derived. In contrast, animals transplanted with TCD marrow grafts averaged <10% donor T cell chimerism. Analysis of the donor T cell phenotype in the spleens of these chimeras demonstrated that the vast majority expressed the αβ T cell receptor, indicating that transplantation of activated γδ T cells enhanced the engraftment of BM-derived donor T cells. Hematopoietic reconsti-

Table 3. *Animals transplanted with activated γδ T cells undergo negative selection in the thymus*

Group	Number of mice	Absolute number of splenic CD4 (CD8) cells coexpressing: $V_\beta5$ ($\times10^{-6}$)	$V_\beta6$ ($\times10^{-6}$)
TCD BM	3	0.04 (0.10)*	0.29 (0.16)†
Activated γδ T cells	4	0.09 (0.07)*	0.16 (0.06)†
Naive T cells	4	0.35 (0.85)	0.17 (0.26)
Normal B10.BR	2	0 (0.06)	2.95 (1.55)
Normal C57BL/6	2	0.98 (2.3)	2.55 (1.23)

Lethally irradiated (900 cGy) B10.BR mice were transplanted with TCD B6 BM alone or together with either 150×10^6 activated γδ T cells from β^-/β^- donors or 2×10^6 naive T cells from normal B6 donors. Normal B10.BR and C57BL/6 mice were included as controls. The absolute percentage of splenic CD4 and CD8 T cells coexpressing either $V_\beta5$ or $V_\beta6$ was determined by multiplying the total number of $CD4^+$ or $CD8^+$ T cells in the spleen cell suspension as determined by flow cytometry by the percentage of each of these cell types that coexpressed $V_\beta5$ or $V_\beta6$.

$p \le 0.02$ for both $CD4^+$ and $CD8^+$ cells compared with naive T cells.

†NS compared with naive T cells.

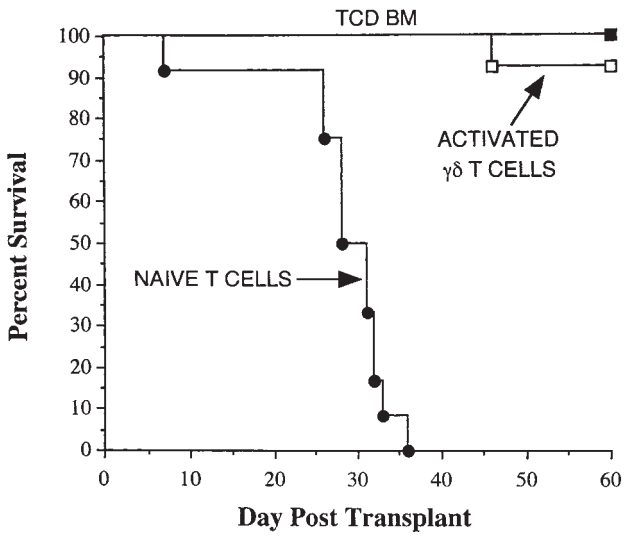

Figure 4. Activated γδ T cells cause significantly less GVHD than limiting numbers of αβ T cells

Lethally irradiated (1100 cGy) AKR recipients were transplanted with TCD B6 BM alone (■, n= 8) or TCD B6 BM plus either 0.5×10^6 naive αβ T cells (●, n=12) or 150×10^6 activated γδ T cells (□, n= 13). Survival is depicted. Data are cumulative results from two similarly performed experiments.

tution was also augmented in these mice, as evidenced by significantly higher white blood cell and platelet counts, further proof for a graft-facilitating effect.

The aim of the current study was to determine whether these cells were capable of causing GVHD across the MHC barrier. The results herein demonstrated that transplantation with activated γδ T cells did not result in clinically significant GVHD when examined in several different MHC-incompatible murine models. Both overall survival and serial weight curves were comparable to those observed in control (non-GVHD) mice. B cell reconstitution, which is a sensitive indicator of GVHD, revealed that long-term donor-engrafted chimeras had equivalent numbers of splenic B cells when compared with control animals not undergoing GVHD. The thymic phenotype in mice transplanted with activated γδ T cells was also normal, indicating a lack of clinically significant GVHD. Furthermore, host reactive $V_\beta5^+$ T cells, which are increased in B6→B10.BR chimeras undergoing GVHD, were present in the same absolute numbers as in control mice, indicating that BM-derived αβ T cells underwent appropriate clonal selection in the thymus. This conclusion was corroborated by secondary adoptive transfer assays which demonstrated that these cells were tolerant of recipient alloantigens but were capable of immune reactivity against third-party cells.

A central question was whether γδ T cells were therapeutically beneficial relative to limiting numbers of naive αβ T cells. This issue spoke to whether similar results could be obtained with respect to GVHD prevention by employing a small number of naive αβ T cells, a number minimally sufficient for alloengraftment and therefore less likely to cause GVHD. To that end, we performed a direct comparative analysis between threshold numbers of naive αβ and activated γδ T cells to determine the ability of these respective cell populations to induce GVHD. These studies clearly indicated that limiting numbers of naive αβ T cells (0.5×10^6) induced significantly more GVHD than large doses of activated γδ T cells (150×10^6). The data therefore provided support for the premise that γδ T cells may have a relative therapeutic advantage in allogeneic marrow transplantation by being capable of promoting engraftment without escalating toxicity from GVHD.

The mechanism by which animals transplanted with activated γδ T cells are protected from lethal GVHD in these donor/recipient strain combinations is not resolved by these studies. Several possible explanations deserve consideration. One is that activation may have altered the migratory capabilities of these cells in such a way as to diminish their capability to cause GVHD. Prior studies have shown that activated T cells have altered migratory properties in vivo when compared with unactivated T cells [18,19]. The fact that these cells were detectable in the spleen and intestines of chimeric mice >2 weeks posttransplant, however, was evidence that these cells were able to traffic to lymphoid tissue as well as target organs of GVHD. In the spleen, specifically, one would expect that γδ T cells would have come into contact with antigen-presenting cells and therefore would have had the ability to recognize host alloantigens.

Another possibility is that γδ T cells are inherently less capable of causing GVHD than αβ T cells. Support for this premise comes from prior studies showing that allorecognition by γδ T cells differs from that observed for αβ T cells [20]. Specifically, recent data indicate that γδ T cells may be more immunoglobulin-like in their recognition properties, and thus have a more flexible immune recognition reper-

toire than αβ T cells [20], which allows them to recognize nonpolymorphic class I–like molecules or mycobacterial nonapeptide antigens [21–26]. The frequency of alloreactive γδ T cell clones has also been shown to be much lower than comparable αβ T cell clones [27]. Therefore, the failure to induce a GVH reaction may have been in part due to the fact that the majority of these cells may not have been capable of mediating an effective alloreactive response necessary to trigger a GVH reaction. Activated γδ T cells have been shown to be capable of facilitating alloengraftment [13], however, suggesting that donor γδ T cells are capable of either eliminating or inactivating host immune cells which can reject the marrow graft. One would presume that such an interaction would also be an effective stimulus to induce GVHD.

A final possibility is that activation of γδ T cells alters the propensity of these cells to induce GVHD without compromising their ability to promote alloengraftment. We have recently documented that activated $CD4^+$ and $CD8^+$ αβ T cells have a reduced ability to mediate GVH reactivity, although animals still exhibit clinical signs of GVHD [28]. This suggests that the activation process (anti-TCR monoclonal antibody plus IL-2) reduces the ability of the T cell to mediate GVHD. Notably, with respect to activated $CD8^+$ T cells, these cells retained their ability to facilitate engraftment, although GVHD was not completely prevented. Prior studies have shown that activation of both αβ and γδ T cells makes these cells susceptible to apoptosis after religation of a T cell receptor or after cytokine withdrawal [29–33]. These data raise the possibility that these cells are likely to have a more limited life span *in vivo*, reducing their ability to sustain a GVH reaction but not necessarily compromising their ability to facilitate engraftment. Supportive data for this premise come from studies by Ferrarini et al. [34], who demonstrated that cytotoxic activated γδ T cells underwent programmed cell death after killing of Daudi lymphoma target cells. The concurrent cell death of both responder and target cells could explain the simultaneous ability to facilitate engraftment without exacerbating GVHD. Studies are currently underway to define if this is a relevant mechanism by which GVHD is abrogated in these animals.

Whether transplantation of activated γδ T cells is of clinical utility is unknown. The administration of γδ T cells to patients will likely require the capability for large scale *ex vivo* expansion because of the need for sufficient cell numbers. To that end, we have recently performed studies which demonstrate that the selective expansion of activated γδ T cells from human peripheral blood is feasible (W.R.D., C. Keever-Taylor, manuscript in preparation). After only 3 weeks in culture conditions designed to stimulate the preferential growth of these cells, a highly purified population of γδ T cells (~90%) free from detectable contaminating αβ T cells is obtainable. The total cell yield from a standard buffy coat sample is $>1.5\times10^8$ γδ T cells. Given that leukapheresis cell yields are at least 10-fold greater, this approach would yield the requisite 10^9–10^{10} activated γδ T cells predicted from the animal studies to be necessary to facilitate alloengraftment in humans.

The ability of γδ T cells to promote engraftment without causing GVHD is an observation that could be therapeutically advantageous if it can be corroborated in humans. A specific advantage of this approach is that the therapeutic index for activated γδ T cells appears to be high in contrast to naive αβ T cells or even activated αβ T cells, for which GVHD is typically not abrogated even at low doses and modest dose escalation can significantly increase the severity of GVHD (Fig. 4) [28]. While the therapeutic index of activated αβ T cells may be better than their resting counterparts [28], activated γδ T cells appear to have an even superior index. This strategy would be particularly attractive in nonmalignant disorders where disease recurrence is not a concern and the amelioration of GVHD would not compromise a graft-vs.-leukemia effect. Even in malignant diseases, however, such an approach might be effective given murine and human studies which have suggested that γδ T cells may play a role in tumor surveillance [35–37]. Clinical studies will ultimately be required to determine if these cells possess any advantages relative to αβ T cells in reducing complications from allogeneic BMT.

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