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Role of the Passive Apoptotic Pathway in Graft-Versus-Host Disease

William R. Drobyski, Richard Komorowski, Brent Logan, and Maria Gendelman

Donor T cells have been shown to undergo apoptosis during graft-vs-host disease (GVHD). Although active apoptosis mediated through Fas/Fas ligand interactions has been implicated in GVHD, little is known about the role of the passive apoptotic pathway. To examine this question, we compared the ability of normal donor T cells and T cells overexpressing the antiapoptotic protein, Bcl-xL, to mediate alloreactive responses in vitro and lethal GVHD in vivo. In standard MLCs, T cells that overexpressed Bcl-xL had significantly higher proliferative responses but no difference in cytokine phenotype. Overexpression of Bcl-xL prolonged survival of both resting and alloactivated CD4+ and CD8+ T cells as assessed by quantitative flow cytometry, accounting for the higher proliferative responses. Analysis of engraftment in murine transplantation experiments demonstrated an increase in donor T cell chimerism in animals transplanted with Bcl-xL T cells, suggesting that overexpression of Bcl-xL prolonged T cell survival in vivo as well. Notably, transplantation of Bcl-xL T cells into nonirradiated F1 recipients also significantly exacerbated GVHD as assessed by mortality and pathological damage in the gastrointestinal tract. However, when mice were irradiated no difference in GVHD mortality was observed between animals transplanted with wild-type and Bcl-xL T cells. These data demonstrate that the passive apoptotic pathway plays a role in the homeostatic survival of transplanted donor T cells. Moreover, the susceptibility of donor T cells to undergo passive apoptosis is a significant factor in determining GVHD severity under noninflammatory but not inflammatory conditions.


Graft-vs-host disease (GVHD) is a major cause of morbidity and mortality after allogeneic bone marrow transplantation (BMT). GVHD is a complex pathophysiological 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. T cell activation after encounter of alloantigen presented in the context of MHC molecules is generally accepted to be the proximate event in GVHD (reviewed in Ref. 1). Once activated, T cells undergo cellular proliferation and are able to secrete a variety of cytokines that contribute to the pathogenesis of GVHD, either directly or indirectly by recruiting secondary effector populations (2, 3). Despite the T cell expansion that occurs during the initial phases of GVHD, severe T cell hypoplasia is a characteristic of long-standing GVHD in both humans and mice (4, 5). One explanation for impaired T cell reconstitution post-BMT is the observation that donor T cells undergo apoptosis during the course of a graft-vs-host (GVH) reaction. In that regard, activation-induced cell death (AICD) of both host-reactive and bystander donor T cells has been demonstrated in murine GVH models (6). Moreover, several studies in man have demonstrated increased apoptosis of donor T cells early after allogeneic BMT, particularly in patients undergoing GVHD (7, 8).

T cell death is regulated primarily through two pathways that have been termed passive and active apoptosis. Active apoptosis, also known as AICD, is mediated primarily (9–11) but not exclusively (12) through Fas/Fas ligand interactions and serves as one mechanism by which the T cell response is down-regulated after exposure to foreign Ags (13). Passive apoptosis occurs through cytokine withdrawal and is regulated by specific cytokines and members of the bcl-2 family (14, 15). One of the critical proteins in the bcl-2 family that affects T cell survival of both resting and activated T cells is Bcl-xL. Bcl-xL is an antiapoptotic protein that is up-regulated after T cell costimulation and makes activated T cells more resistant to proapoptotic stimuli such as growth factor withdrawal (16, 17). Similarly, as Bcl-xL levels decline in activated T cells, these cells are predisposed to undergo programmed cell death through deprivation of cytokines that promote cell growth (18). Conversely, overexpression of Bcl-xL has been shown to protect T cells from death due to cytokine withdrawal when compared with normal nontransgenic T cells (17, 19). While active apoptosis through Fas/Fas ligand interactions has been shown to be one factor that affects T cell survival in GVHD (6), the role of the passive apoptotic pathway has not been examined. The purpose of this study was to examine the role of passive cell death in GVHD by transplantation of donor T cells that overexpressed Bcl-xL and were therefore more resistant to passive apoptosis.

Materials and Methods

Mice
C57BL/6 (B6; H-2b), B10.BR (H-2k), and C57BL/6 × A/J)F1 (B6AF1/J; H-2b/k) mice were bred in the Animal Resource Center at the Medical College of Wisconsin (Milwaukee, WI) or purchased from The Jackson Laboratory (Bar Harbor, ME). B6 mice that overexpress Bcl-xL in T cells were generously provided by Dr. C. Thompson (formerly University of Chicago, Chicago, IL) and bred at the Medical College of Wisconsin. For the purposes of clarity, T cells derived from transgenic Bcl-xL mice on a B6 background were used.
background are henceforth referred to as Bcl-xL T cells and normal non-transgenic T cells from B6 mice as B6 T cells. Screening of offspring from B6 × Bcl-xL mating pairs for the presence of the transgene was performed by PCR amplification of tail DNA extracted from 10- to 14-day-old pups. The primer sequences used for amplification of the Bcl-xL transgene were 5′-GCATCGTTGGCTGCTGAC-3′ (sense) and 5′-CTGAAGTG-GAGCCCCAGCAGAACCC-3′ (antisense) (Operon Technologies, Alameda, CA), and yielded a 382-bp product in positive animals. All mice were housed in the American Association for Laboratory Animal Care-accredited Animal Resource Center of the Medical College of Wisconsin. Animals were fed regular mouse chow and acidified tap water ad libitum.

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

There is an increased percentage of splenic T cells and a lower CD4:CD8 ratio in Bcl-xL vs normal B6 mice. The latter is attributable to the higher percentage of CD8+ T cells in Bcl-xL mice. Therefore, CD4+ and CD8+ T cells were each individually purified from spleens of either B6 or Bcl-xL mice and then admixed so that the same total T cell dose in the same CD4:CD8 ratio was used in all in vitro and in vivo experiments. To obtain highly enriched populations of CD4+ T cells, B6 or Bcl-xL spleen cells were passed through nylon wool columns and then CD4+ T cells were positively selected using the MACS magnetic cell separation system (Miltenyi Biotec, Auburn, CA). A similar procedure was done to isolate highly enriched CD8+ T cells. Typically >90% purity was obtained for the positively selected T cell subset with <2% contamination of the reciprocal subset.

**Mixed lymphocyte culture**

Equivalent numbers of CD4+ and CD8+ responder B6 and Bcl-xL T cells (1 × 10^6 cells/well) were cocultured with 5 × 10^5 B6AF1/J dendraitic cell-enriched stimulator cells in U-bottom microwell plates (BD Biosciences, Lincoln Park, NJ) at 37°C. Responder T cells were obtained by positive selection of CD4+ or CD8+ T cells using the MACS magnetic cell separation system. Stimulators were obtained by collagenase digestion (1 mg/ml, Boehringer Mannheim, Indianapolis, IN) of spleens of females by positive selection of CD11c+ dendraitic cells using the MACS system. An average of 50–60% of cells expressed CD11c after positive selection. Stimulator cells were then irradiated (3000 rad) and seeded into microwell plates. Cells from triplicate wells were harvested each day for 6 consecutive days. A total of 1 μCi of [3H]thymidine was added to wells for the final 12–18 h before harvest. Proliferation was assessed using a liquid scintillation counter. Triplicate wells were analyzed on a flow cytometer (BD Biosciences, Mountain View, CA). Donor T cell chimerism was determined by analyzing cells within a gate that included the entire spleen cell population minus average cpm of triplicate control wells.

**SCDA**

Equivalent number of CD4+ and CD8+ responder B6 or Bcl-xL T cells (1 × 10^6 cells/well) were cocultured with 5 × 10^5 B6AF1/J dendraitic cell-enriched stimulator cells in U-bottom microwell plates at 37°C, as described above. Cells from individual wells were removed on a daily basis, and stained with either PE-anti-CD4 or PE-anti-CD8 Abs. Standard cell dilution analyses (SCDA) were performed as previously described (20, 21) to quantitate the number of responding T cells in these cultures. Briefly, thymocytes from a B10.BR (H-2b, Thyl.2+) mouse were processed into a single-cell suspension and stained with FITC-Thy1.2 mAb, resuspended in 4% paraformaldehyde, and stored at 4°C. These cells served as the standard cells for this assay. PE-stained cells from the MTC reaction were admixed with 50,000 standard cells in PBS/azide containing 1 mg/ml 7-amino actinomycin D (7-AAD). 7-AAD+Thy1.2+ cells were excluded by gating. The remaining cells were then analyzed. The absolute number of viable CD4+ or CD8+ T cells was calculated by multiplying the ratio of PE-CD4+ or PE-CD8+ T cells to FITC-Thy1.2+ standard cells by 50,000 (the absolute number of standard cells). All determinations were done in triplicate.

**Measurement of cytokine levels**

Equivalent numbers of CD4+ and CD8+ responder B6 or Bcl-xL T cells were cocultured with irradiated B6AF1/J dendraitic cell-enriched stimulator cells for 1–6 days in an MLR. Culture supernatant from triplicate wells were obtained and assayed for IL-4, TNF-α and IFN-γ in standard ELISA according to the manufacturer’s instructions (BD PharMingen, San Diego, CA). In other experiments, sera were obtained from transfected recipient animals by tail vein or retroorbital bleeds and assayed for TNF-α and IFN-γ. Experimental values were calculated by interpolation from a regression line constructed from serial dilutions of recombinant murine IL-4, TNF-α, or IFN-γ standards. Experimental values were subtracted from background values in some assays when background values were above the level of assay detection. Assay sensitivities were 4, 15.4, and 10 pg/ml, respectively.

**BM transplantation**

Bone marrow (BM) was flushed from donor femurs and tibias with DMEM and passed through sterile mesh filters to obtain single-cell suspensions. BM was T cell depleted in vitro with anti-Thy.1.2 mAbs plus low-toxicity rabbit complement (C-SIX Diagnostics, Mequon, WI). The hybridoma for 30-H12 (anti-Thy1.2, rat IgG2b) Ab was purchased from the American Type Culture Collection (Manassas, VA). BM cells were washed and resuspended in DMEM before injection. Naïve donor T cells were obtained by passing erythrocyte-depleted spleen cells through nylon wool columns to remove non-T cells. Host mice were conditioned with total body irradiation (TBI) administered as a single exposure at a dose rate of 67 cGy using a Cesium Irradiator (J. L. Shepherd and Associates, San Fernando, CA). Irradiated recipients received a single i.v. injection of T cell depleted (TCD) BM (10^5 cells) with or without added T cells. Nonirradiated recipients received T cells alone without BM.

**Experimental design**

GVHD was first assessed in a parent→F1 model to examine GVH reactivity in the absence of a conditioning regimen (3). In this model, nonirradiated B6AF1/J mice were transplanted with 25 × 10^5 purified B6 or Bcl-xL T cells. In subsequent studies, the effect of a conditioning regimen on GVHD mortality was assessed using two different murine strains, B6AF1/J or B10.BR recipients were lethally irradiated (1000 or 900 cGy, respectively) and transplanted with TCD BM alone or admixed with graded doses of B6 or Bcl-xL T cells.

**Flow cytometric analysis and assessment of chimerism**

mAb conjugated to either FITC or PE were used to assess chimerism in marrow transplant recipients. PE-anti-CD8 (clone CT-CD8a, rat IgG2a) was obtained from Caltag Laboratories (San Francisco, CA). PE-anti-TCRβ (clone H57-597, hamster IgG), FITC-anti-Thy-1.2 (clone 30-H12, mouse IgG2a), PE-anti-CD4 (clone GK1.5, rat IgG2b), FITC-anti-H-2Kb (clone AF3-12.1, mouse IgG1) were all purchased from BD PharMingen. Spleen cells were obtained from chimeras at defined intervals posttransplant, processed into single-cell suspensions, and stained for two-color analysis. RBCs were removed by hypotonic lysis using distilled water. Cells were analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Donor T cell chimerism was determined by analyzing cells within the lymphocyte gate. The absolute number of splenic donor T cells was determined by analyzing cells within a gate that included the entire spleen cell population after excluding debris and RBCs. At least 10,000 cells were analyzed for each determination whenever possible.

**Histological analysis**

Representative samples of skin, lung, and colon were obtained from transplanted recipients and fixed in 10% neutral-buffered formalin. Samples were then embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E. Semiquantitative scoring systems were used to account for histological changes consistent with GVHD and to assess the degree of lymphocytic infiltration in GVHD target organs. Changes in the colon deemed to be compatible with GVHD were crypt cell apoptosis, crypt destruction, goblet cell depletion, and lamina propria lymphocytic infiltration. The scoring system that was used categorized 0 as normal, 1 for mild, 2 for moderate, and 3 for severe for each of these four parameters (maximal score of 12 per mouse). Lymphocytic infiltration in the liver (both portal and sinusoidal) and colon were individually scored as 0 for none, 1 for mild, 2 for moderate, and 3 for severe (maximal score of 9 per mouse). Scores were added to provide a composite score for each animal (maximal score of 18 per mouse because lymphocytic infiltration in the colon was included in both analyses). All slides were coded and read in a blinded manner.

**Statistics**

Group comparisons of parameters of donor T cell engraftment, GVHD scores, and the absolute number of splenic donor T cells were performed using the nonparametric Kruskal-Wallis test. For comparison of IFN-γ measurements and MLC cpm responses between respective groups a mixed effects model was constructed. The absolute number of CD4+ and CD8+ T cells in SCDA was compared using the unpaired Student t test. Survival curves were compared using the log-rank test.
were constructed using the Kaplan-Meier product limit estimator and compared using the log-rank rest. A p value of ≤0.05 was deemed to be significant in all experiments.

Results
Overexpression of Bcl-xL increases the T cell proliferative response but does not affect T cell cytokine phenotype

Initial experiments were performed to determine whether overexpression of Bcl-xL in T cells affected their response to alloantigen in vitro. B6 and Bcl-xL responder T cells were cocultured with irradiated B6AF1/J stimulator cells in MLC assays to assess the effect of Bcl-xL overexpression on T cell proliferation. A representative experiment is depicted in Fig. 1, where a 2-fold increase in thymidine incorporation was observed in Bcl-xL vs B6 T cell cultures beginning on day 2 and persisting throughout the 5 days of culture. Maximal thymidine incorporation occurred on day 4 in both groups, indicating that the tempo of the alloresponse was not altered by overexpression of Bcl-xL. Statistical analysis of the composite results from four experiments, each with triplicate determinations, showed significantly higher thymidine incorporation on days 3–5 for Bcl-xL T cell cultures (p < 0.003) but no difference on day 2 (p = 0.49). We then examined the effect of Bcl-xL overexpression on the production of Th1 and Th2 type cytokines by alloactivated T cells. Both B6 and Bcl-xL T cell cultures produced IFN-γ (Fig. 2) but no detectable IL-4 (data not shown), consistent with a Th1 phenotype. There was no statistically significant difference in the amount or tempo of IFN-γ production in B6 vs Bcl-xL T cells (p = 0.64). TNF-α production also was examined and was below the level of detection in both B6 and Bcl-xL cultures (data not shown). Thus, overexpression of Bcl-xL increased the T cell proliferative response but did not alter the cytokine phenotype of alloactivated T cells or affect production of IFN-γ.

Overexpression of Bcl-xL enhances survival of resting and alloactivated T cells

Prior studies have shown that significant apoptosis can occur in the presence of high thymidine incorporation rates (22). Because Bcl-xL is an antiapoptotic protein that prevents cell death in the context of cytokine withdrawal and after T cell activation, we reasoned that the effect of Bcl-xL overexpression on T cell survival might not be discernible in standard proliferation assays. Consequently, we used SCIDAs to quantitate the absolute number of CD4+ and CD8+ T cells in individual microwells to determine whether overexpression of Bcl-xL in responder T cells conferred a survival advantage on these cells. In initial studies, survival of resting B6 vs Bcl-xL CD4+ and CD8+ T cells was examined in the absence of a stimulator cell population. Within 48 h of culture, the absolute number of both B6 CD4+ and CD8+ T cells decreased to <33% of input numbers indicative of substantial cell death (Fig. 3A). Cell death was greater in CD8+ as compared with CD4+ T cells. By day 5 of culture nearly all CD8+ T cells had died, while CD4+ T cells persisted in culture until day 10, albeit at low levels. In contrast, the absolute number of Bcl-xL CD8+ T cells was higher than B6 CD8+ T cells at all time points and these cells were detectable for a longer period of time (i.e., until day 10). Similarly, there was significantly less cell death of Bcl-xL CD4+ T cells at all time points.

Survival of alloactivated CD4+ and CD8+ T cells was then examined in SCDA assays. The most important finding was that total viable cell numbers of B6 CD4+ and CD8+ T cells declined to significantly lower levels than Bcl-xL T cells within the first 3 days of culture (Fig. 3B). Thereafter, CD4+ T cell numbers increased slightly in both groups for the next 2–3 days and then began to decline with significantly higher numbers of Bcl-xL CD4+ T cells at all time points. Expansion of CD8+ T cells in both B6 and Bcl-xL cultures began on day 4. Maximal expansion was noted on days 5–6 and occurred to a proportionately similar degree after day 3 nadir levels (~2.5-fold by days 5–6), although absolute numbers of Bcl-xL CD8+ T cells remained significantly higher at all time points. Thus, in a standard MLC assay there was early cell

FIGURE 1. Overexpression of Bcl-xL results in an increased T cell proliferative response to alloantigen. Equivalent numbers (1 × 10^5/well) of B6 (hatched bars) or Bcl-xL (filled bars) T cells were cultured with irradiated B6AF1/J CD11c+ dendritic cells (5 × 10^4) in a standard MLC for 5 days. Triplicate wells from each culture were harvested at the indicated times. Cells were pulsed with [³H]TdR for the last 18 h of culture and the percentage of incorporated radioactivity was determined. Data are presented as the mean ± SD of average cpm of triplicate experimental wells minus average cpm of triplicate control wells. Data shown are from one of three experiments that produced similar results.

FIGURE 2. Overexpression of Bcl-xL does not affect the production of IFN-γ by alloreactive T cells. Equivalent numbers (1 × 10^5/well) of B6 (hatched bars) or Bcl-xL (filled bars) T cells were cultured with irradiated B6AF1/J CD11c+ dendritic cells (5 × 10^4) in a standard MLC for 6 days. Triplicate wells from each culture were harvested at the indicated times and the supernatant was assayed for IFN-γ production by ELISA. Data are presented as the mean ± SD. Data are shown from one of four experiments that produced similar results.
FIGURE 3. Overexpression of Bcl-xL enhances survival of resting and alloactivated T cells. Equivalent numbers (1 × 10⁶ well) of B6 or Bcl-xL T cells were cultured alone or with irradiated B6AF1/J CD11c⁻ dendritic cells (5 × 10⁵) in a SCDA for 12 days. At the indicated times, triplicate wells from each culture were stained with either PE-CD4 or CD8 Abs along with 7-AAD. Cells were then analyzed by flow cytometry. Results from T cells cultured in the absence of stimulators are shown in A, while results from T cells cultured in the presence of irradiated stimulators are shown in B. Data are presented as the mean number of CD4⁺ or CD8⁺ T cells in individual wells ± SD. Data shown are from one of three experiments that produced similar results. The absolute number of Bcl-xL CD4⁺ and CD8⁺ T cells was significantly higher than B6 CD4⁺ and CD8⁺ T cells (p < 0.05) at all time points with the following exceptions: B6 v Bcl-xL, CD8, day 3, p = 0.09; B6 v Bcl-xL, CD8, day 9, p = 0.06; and B6 v Bcl-xL, CD4 MLC, day 10, p = 0.06.

death in both CD4⁺ and CD8⁺ T cells, which was significantly attenuated by overexpression of Bcl-xL. This early protective effect was responsible for the greater number of Bcl-xL CD4⁺ and CD8⁺ T cells throughout the entire culture period.

Transplantation with Bcl-xL T cells results in increased donor T cell chimerism and absolute numbers of donor T cells after BMT

In vitro assays indicated that there was increased survival in both resting and alloactivated T cells that overexpressed Bcl-xL. Therefore, we examined whether survival was also prolonged in vivo by performing transplant experiments using a parent→F₁ model in which donor T cell survival could be determined in the absence of a competing host-vs-graft response. This model used donor and recipient mice that corresponded to the same responder/stimulator cell combination used in in vitro assays. Animals transplanted with either B6 or Bcl-xL T cells were sacrificed 11–12 days after transplant and spleen cells were analyzed to determine the extent of donor T cell chimerism. Mice transplanted with Bcl-xL T cells had a significantly greater percentage of donor aβ⁺ T cells when compared with animals reconstituted with B6 T cells (75 vs 63%, p < 0.0001) (Table I). This difference was observed for both CD4⁺ (68 vs 54%, p < 0.0001) and CD8⁺ (78 vs 66%, p < 0.0001) T cell populations. The absolute number of splenic donor T cells was also significantly greater in mice transplanted with Bcl-xL T cells (41 × 10⁶) compared with those transplanted with B6 T cells (26 × 10⁶) (p < 0.01), consistent with the interpretation that T cell survival was augmented in the former cohort of animals.

Transplantation with donor T cells that overexpress Bcl-xL exacerbates GVH lethality in the absence of a conditioning regimen

Studies were then performed to determine whether increased survival of Bcl-xL T cells in transplanted animals affected the severity of GVHD. This question was initially assessed in the absence of a conditioning regimen. Nonirradiated B6AF1/J recipients were transplanted with 25 × 10⁶ B6 or Bcl-xL T cells. Animals in each group were given equivalent doses of CD4⁺ and CD8⁺ T cells so that the increased percentage of CD8⁺ T cells in the spleens of Bcl-xL mice would not be a confounding factor in the assessment of GVHD (see Materials and Methods). Mice transplanted with Bcl-xL T cells all died by 20 days posttransplant due to GVHD and had significantly higher GVHD-induced mortality than mice transplanted with B6 T cells (100 vs 18% mortality at day 60, p < 0.001) (Fig. 4).

As additional parameters of GVH reactivity, we analyzed levels of proinflammatory cytokines and performed histological studies on GVHD target organs in both cohorts of mice. Mean serum IFN-γ levels were 732 ± 84 pg/ml in mice transplanted with B6 T cells (n = 12) and 824 ± 59 pg/ml in animals transplanted with Bcl-xL T cells (n = 13) (p = 0.23) when assayed 11–13 days post-BMT. Measurements of mean serum TNF-α levels were below the limit of assay sensitivity (15.4 pg/ml) in both cohorts of mice (n = 7 mice per group) when assayed over the same time.

Table I. Transplantation with Bcl-xL cells increases donor T cell chimerism and donor T cell reconstitution in nonirradiated recipients

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor aβ⁺ T Cells (%)</th>
<th>Donor CD4⁺ T Cells (%)</th>
<th>Donor CD8⁺ T Cells (%)</th>
<th>Splenic Donor T Cells (× 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>63 ± 1</td>
<td>54 ± 2</td>
<td>66 ± 1</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>75 ± 4</td>
<td>68 ± 1</td>
<td>78 ± 1</td>
<td>41 ± 5</td>
</tr>
</tbody>
</table>

*Nonirradiated B6AF1/J mice were transplanted with 25 × 10⁶ B6 (n = 8) or Bcl-xL (n = 12) T cells. At days 11–12 posttransplant, mice were sacrificed. Spleen cells from individual chimeras were obtained and analyzed to determine cellularity and percentage of donor T cell chimerism. Donor cells were defined as H-2Kd⁻. Data are presented as mean ± 1 SE measurement.

*, p < 0.0001.

**, p < 0.01.
Role of Passive Apoptosis in GVHD

Irradiation is known to exacerbate GVHD through the production of inflammatory cytokines that directly or indirectly contribute to the pathophysiology of GVHD (23). Consequently, we questioned whether differences in survival between mice transplanted with B6 vs Bcl-xL T cells would exist under conditions where GVHD severity was not solely dependent upon donor T cells. To examine this question, lethally irradiated B6AF1/J mice were transplanted with TCD B6 BM alone or together with 2 × 10^6 B6 or Bcl-xL T cells. Mice in each group received 1.2 × 10^6 CD4− and 0.8 × 10^6 CD8+ T cells. Thus, mice in both groups received equivalent ratios of CD4:CD8 T cells. There was no difference in survival in mice transplanted with either B6 or Bcl-xL T cells (Fig. 5A) (p = 0.81).

A similar set of experiments was performed in a second MHC-mismatched murine model (B6→B10.BR). Two T cell doses were tested (i.e., 2 × 10^6/mouse and 0.7 × 10^6/mouse). At a dose of 2 × 10^6 T cells mice received 1.3 × 10^6 CD4+ and 0.7 × 10^6 CD8+ T cells, while at a dose of 0.7 × 10^6 T cell animals received 0.45 × 10^6 CD4+ and 0.25 × 10^6 CD8+ T cells. No difference in GVHD-associated mortality was observed in this strain combination between groups transplanted with either 2 × 10^6 (Fig. 5B) (p = 0.22) or 0.7 × 10^6 (data not shown) (p = 0.53) T cells. Thus, GVHD was not dependent on the absolute number of donor T cells as both groups had 11 days posttransplant and analyzed for donor T cell engraftment (Table I). There was no difference in the percent of total number of donor T cells in mice transplanted with either B6 or Bcl-xL T cells. Lethally irradiated B6AF1/J mice were transplanted with TCD B6 BM together with 2 × 10^6 B6+ or Bcl-xL+ T cells. Mice were sacrificed 11 days posttransplant and analyzed for donor T cell engraftment (Table III). There was no difference in the percentage of donor αβ+, CD4+ and CD8+ T cells, as both groups had complete donor T cell engraftment. However, the total number of splenic donor T cells was significantly higher in mice transplanted with Bcl-xL+ T cells 19 × 10^6 vs 12 × 10^6, p = 0.014, indicating that there was an increased absolute number of donor T cells in the former group of animals. However, despite the higher absolute number of donor T cells, lethally irradiated mice transplanted with Bcl-xL T cells had no statistically significant difference in survival compared with those transplanted with B6 T cells.

Discussion

During the course of a normal immune response, both active and passive mechanisms of apoptosis serve to modulate T cell survival and down-regulate T cell immune reactivity (24–27). These mechanisms also appear to be critical in the maintenance of transplantation tolerance where interference with either pathway abrogates tolerance to solid organ and BM allografts (28, 29). In the context of GVHD, donor T cell expansion is limited despite the fact that Ags to which donor T cells can respond persist in the host. Prior studies have demonstrated that donor T cells undergo apoptosis after allogeneic marrow transplantation and this process appears to have an important role in regulating T cell homeostasis (7, 8).

Active apoptosis is one mechanism that has been shown to limit the survival of both allogeneic and bystander donor T cells in GVHD (6). However, the role of the passive apoptotic pathway has not been examined in the context of GVHD. The purpose of this

Table II. Histological analysis of GVHD target organs

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymphocytic Infiltration</th>
<th>Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>2.6 ± 0.2</td>
<td>5.9 ± 0.7</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>5.4 ± 1.0</td>
<td>10.7 ± 1.3</td>
</tr>
</tbody>
</table>

* Nonirradiated B6AF1/J mice were transplanted with 25 × 10^6 B6 or Bcl-xL T cells (n = 7 per group). On day 13 posttransplant, mice were sacrificed and tissues were analyzed for evidence of GVHD and to determine the extent of lymphocytic infiltration (see Materials and Methods). A total GVHD score was calculated that was derived from the GVHD score in the colon plus the degree of lymphocytic infiltration in the liver and colon of each mouse. Data are presented as mean ± 1 SE measurement.

** p = 0.026.

*** p = 0.004.
study was to determine whether the severity of GVHD was affected by transplantation of donor T cells that were resistant to passive apoptosis.

Initial in vitro studies were performed to determine whether survival of either resting or alloactivated T cells was prolonged by overexpression of Bcl-xL. Due to the limitations of proliferation assays, SCDA were performed to quantify the number of viable T cells in microwell cultures. These studies demonstrated that resting Bcl-xL T cells were present in greater numbers at all time points and had longer survival when compared with wild-type B6 T cells, consistent with the interpretation that overexpression of Bcl-xL reduced passive cell death. The protective effect was evident in both CD4+ and CD8+ T cells but was more pronounced in Bcl-xL CD8+ T cells, possibly due to their more obligate requirement for cytokine support to maintain survival. Overexpression of Bcl-xL also increased the number of T cells that survived in MLCs. This was attributable primarily to the fact that Bcl-xL+ T cells were protected from the early cell death that occurred in B6 T cells within the first 2–3 days of culture. Consequently, there was a significantly greater number of CD4+ and CD8+ Bcl-xL+ T cells that could subsequently expand in response to alloantigen (Fig. 3). Therefore, both resting and alloactivated T cells that were resistant to passive apoptosis had greater survival in vitro than normal T cells.

To determine whether the effects observed in vitro were also operative in vivo, experiments were performed to examine whether donor T cells that were resistant to passive apoptosis had prolonged survival in vivo and whether this affected the severity of GVHD. Analysis of donor T cell chimerism early posttransplant revealed significantly greater numbers of donor CD4+ and CD8+ T cells in nonirradiated mice transplanted with Bcl-xL+ T cells (Table I). These animals also had significantly higher mortality due to GVHD when compared with mice transplanted with normal T cells (Fig. 4). Histological studies revealed significantly more GVHD pathology in the colons of Bcl-xL+ transplanted animals (Table II). This was accompanied by more extensive lymphocytic infiltration in both the liver and colon. Because these mice were predominantly donor T cell engrafted (Table I), we infer that the infiltration in these organs was primarily attributable to donor T cells. Given these collective data, we believe that the most likely explanation for the increased GVHD mortality in these animals is that Bcl-xL overexpression enhanced survival of donor T cells after transplantation into recipients, resulting in a larger number of donor T cells that could mediate a lethal GVH reaction. The precise mechanism by which these T cells caused tissue damage is not resolved by these studies, although available data suggest that this is not associated with an increase in proinflammatory cytokine levels. However, we cannot exclude that levels may have been increased locally in the target tissues. Our data are consistent with studies by Issazadeh et al. (30), who demonstrated that T cells that overexpressed Bcl-xL were able to cause more severe chronic experimental encephalomyelitis. Exacerbation of autoimmunity was associated with increased T cell infiltration and decreased numbers of apoptotic cells in the CNS. The implication of this report and the current study is that passive apoptosis is a mechanism by which the severity of T cell-mediated immune reactions are down-regulated, even under pathological conditions. When this pathway is inhibited, T cell immune reactivity is enhanced and pathological damage is exacerbated.

There are at least two possible mechanisms by which overexpression of Bcl-xL T cells may have promoted survival of GVH-reactive donor T cells. One is based on the premise that, early after BMT, a percentage of donor T cells are destined to die due to a lack of appropriate survival signals and overexpression of Bcl-xL is able to compensate for this early cell death. This would be consistent with SCDA data (Fig. 3B) that showed increased numbers of CD4+ and CD8+ Bcl-xL+ T cells within the first 2–3 days of culture. Thereafter, there was no survival advantage for Bcl-xL as opposed to B6 T cells. Extrapolating these data to the in vivo situation, this would result in a greater number of total donor T cells available to clonally expand after exposure to recipient alloantigens. Alternatively, it is formally possible that Bcl-xL overexpression preferentially increased the survival of alloantigen-specific donor T cells. This could occur if transgene expression somehow altered the T cell repertoire in such a manner as to favor survival of host-reactive as opposed to non-host-reactive T cells. To determine whether expression of the transgene affected the
overall T cell repertoire, we performed TCR spectratype analysis on spleen cells from B6 and Bcl-xL mice. These results showed no difference in the T cell repertoire, suggesting that overexpression of Bcl-xL did not affect any dramatic alteration in TCR complexity (W. R. Drobyński, unpublished observations). Presently, we favor the former hypothesis as a more likely explanation for the exacerbation of GVH reactivity in mice transplanted with Bcl-xL T cells.

The relative role of the passive and active apoptotic pathways is not resolved by these studies. In preliminary experiments, we have observed no effect on GVHD-mediated mortality when Fas-deficient T cells are transplanted into nonirradiated recipients (W. R. Drobyński, unpublished observations). These data suggest that inhibition of apoptosis mediated through the Bcl-xL as opposed to Fas/Fas ligand pathway (31) may have a more dominant effect on GVHD severity. However, it is important to note that AICD can occur through other pathways, most notably through TNF (32, 33). Fas-deficient T cells have been reported to have reduced but measurable apoptosis that is mediated by TNF (12, 34). Thus, it is possible that the absence of increased GVHD lethality after transplantation of lpr T cells is due to the fact that apoptosis can occur through alternative pathways. Alternatively, the passive apoptotic pathway may be the more dominant pathway for regulating T cell survival during a GVH reaction. Lenardo et al. (24) have shown that active Ag-induced T cell death serves as a regulatory brake under conditions of high IL-2 and Ag, while passive apoptosis removes T cells under low IL-2 conditions and Ag stimulation. During the course of a GVH reaction, IL-2 levels are elevated for only a short period posttransplant before declining back to baseline levels (35). Consequently, under low IL-2 conditions that predominate for most of the GVH reaction, the passive apoptotic pathway may be more important for the elimination of alloactivated and bystander donor T cells.

The majority of patients that receive allogeneic marrow transplants are treated with a conditioning regimen as part of the transplant procedure. For that reason, we transplanted either B6 or Bcl-xL T cells into irradiated recipients to determine whether the conditioning regimen affected GVHD-induced mortality. We observed that under inflammatory conditions (i.e., TBI) there was no difference in mortality between mice transplanted with B6 vs Bcl-xL T cells. One potential explanation for this observation is that cytokines induced by the conditioning regimen augmented survival of B6 T cells, thereby eliminating any survival advantage that was conferred by overexpression of Bcl-xL. Cytokines such as IL-7, TNF, and LPS that are induced by the conditioning regimen itself (3, 17, 36) have been shown capable of promoting T cell survival. IL-7 has been demonstrated to be a critical factor for the survival of resting T cells (37), while proinflammatory mediators such as TNF and LPS are able to protect activated T cells from apoptotic cell death (38–40) and are important for T cell expansion and survival in vivo (33, 37). If this were the case, though, we would have expected that mice transplanted with B6 and Bcl-xL T cells would have had an equivalent number of donor T cells when analyzed early post-BMT (Table III). However, when similarly transplanted mice were analyzed 11 days posttransplant, mice transplanted with Bcl-xL T cells had a significantly higher absolute number of splenic donor T cells. Thus, T cell survival appeared to be augmented in these animals as well, similar to what was observed in nonirradiated mice. However, this did not correlate with increased GVHD lethality. An alternative explanation that we favor is that TBI induces direct tissue injury and release of inflammatory mediators, such as TNF and LPS, that are able to amplify the severity of the GVH reaction (23, 41, 42) once it has been initiated by donor T cells. These secondary events appear to have a more dominant role in determining the severity of GVHD under inflammatory conditions than factors that interfere with the passive apoptotic pathway.

The potential clinical implications of our data derive from the growing trend in allogeneic marrow transplantation to use nonmyeloablative conditioning regimens. The rationale for this approach is to diminish conditioning regimen-induced inflammation and thereby ameliorate transplant-related mortality (43, 44). Due to the diminished contribution of the conditioning regimen, GVHD severity is primarily determined by the presence of donor T cells in allogeneic peripheral stem cell products. Our data would predict that any factors that influence donor T cell survival might have a more profound effect on GVHD severity under these conditions. Thus, cytokines such as IL-7 that promote T cell survival and may be beneficial in restoring immunity post-BMT (45) or infections that result in release of TNF and LPS may affect the severity of GVHD to a larger extent than when recipients receive intensive conditioning regimens. Additional studies will be required to determine whether this premise is valid.

In summary, our results indicate that the passive apoptotic pathway plays a significant role in the pathophysiology of GVHD by regulating the survival of donor T cells. However, the role of this pathway in modulating the severity of GVHD is critically dependent upon the presence or absence of a concurrent inflammatory milieu. Under noninflammatory conditions, the ability of donor T cells to undergo passive apoptosis directly affects the severity of the GVH reaction. In contrast, under inflammatory conditions, factors (e.g., proinflammatory cytokines, secondary effector populations, direct tissue injury by TBI) other than the susceptibility of donor T cells to undergo passive apoptotic cell death appear to have a more dominant role in determining the severity of GVHD than variables that modulate T cell survival. Given the emerging role of apoptosis in the pathophysiology of GVHD and allogeneic marrow transplantation (7, 8, 46), further studies to investigate the specific mechanisms that regulate apoptosis in BMT recipients are warranted.
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