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Research Article

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Perforin/granzyme-dependent and Independent Mechanisms are Both Important for the Development of Graft-versus-host Disease After Murine Bone Marrow Transplantation

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Abstract

Graft-versus-host disease (GvHD) is the major limiting toxicity of allogeneic bone marrow transplantation. T cells are important mediators of GvHD, but the molecular mechanisms that they use to induce GvHD are controversial. Three effector pathways have been described for cytotoxic T lymphocytes: one requires perforin and granzymes, the second Fas (APO-1; CD95) and its ligand. Thirdly, secreted molecules (e.g., TNF-α, γ-IFN) can also mediate cytotoxicity. Together, these mechanisms appear to account for virtually all cytotoxicity induced by activated CTL in standard in vitro lytic assays. Using transplants across histocompatibility barriers, we were able to analyze the contributions of these effector molecules to cell-mediated cytotoxicity in vivo in a GvHD model. We found that Fas ligand is an important independent mediator of class II–restricted acute murine GvHD, while perforin/granzyme-dependent mechanisms have only a minor role in that compartment. In contrast, perforin/granzyme-dependent mechanisms are required for class I–restricted acute murine GvHD, while Fas ligand is not. The perforin/granzyme pathway may therefore represent a novel target for anti-GvHD drug design. In support of this approach, we provide additional data suggesting that specific perforin/granzyme inhibitors should not adversely affect hematopoietic recovery after transplantation. (*J. Clin. Invest.* 1997. 100:904–911.) Key words: graft-versus-host disease • hematopoiesis • perforin • granzyme B • Fas

Introduction

Bone marrow transplantation across major or minor histocompatibility barriers carries a significant risk of death from graft-versus-host disease (GvHD).[1] The elimination of GvHD by depleting bone marrow grafts of T cells provided strong evidence that T cells mediate GvHD (1). T cell depletion, however, is associated with an increased risk of graft failure (2) and relapse of malignancy after transplantation (3). Efforts to explain the molecular basis of T cell cytotoxicity are motivated by a desire to develop more precise strategies for manipulating donor T cells, while preserving a favorable balance between their beneficial and harmful effects in the host.

Murine bone marrow transplant models developed in the past decade have demonstrated that $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTL) can both mediate lethal GvHD (4, 5). These effector populations induce GvHD syndromes with similar natural histories and dose-response relationships. In vitro experiments, however, suggest that two distinct cytotoxic mechanisms exist and may be used differently in $CD4^+$ and $CD8^+$ CTL. Both pathways culminate in the apoptotic death of target cells.

The first apoptotic mechanism is the granule exocytosis pathway. In this model, the specific recognition and binding of a CTL to a susceptible target cell leads to discharge of the lymphocyte's secretory granules into the extracellular space. One of the granule components is perforin, which undergoes Ca^{++}-dependent polymerization on the target cell membrane, creating a complement-like lesion (6, 7). This membrane damage is not sufficient to cause apoptotic death of the cell (8), but may allow other granule constituents (including the granzymes) to gain access to the target cell cytoplasm. There, the granzymes trigger a series of biochemical events that result in apoptosis (9–13). CTL from mice deficient in perforin have a severe defect in their ability to induce apoptosis (14–17). Granzyme B is required for the early phase of perforin-dependent cytotoxicity (18).

The second pathway involves the Fas/Fas ligand interaction. Activated CTL upregulate expression of Fas ligand (FasL), a membrane-bound protein with homology to TNF (19). Binding of FasL to its receptor (APO-1; CD95), present on the surface of target cells, triggers apoptosis through a death domain on the cytoplasmic tail of the Fas receptor via a series of docking proteins. These proteins transmit the death signal through intermediates that include cysteine proteinases of the caspase family (20–24). Mice with a naturally occurring loss of function mutation of FasL (*gld*/*gld*) accumulate double negative ($CD4^-CD8^-$) T cells in the periphery (25) due to a failure of activation-induced cell death of mature T cells (26, 27). Reduced cytotoxicity of *gld*/*gld* effectors has demonstrated the importance of a Fas/FasL-dependent mechanism of cytotoxicity in vitro (28), particularly in $CD4^+$ CTL (28–30).

Allogeneic transplants from mice doubly deficient in perforin and FasL (31), but not FasL alone, have been shown to cause less mortality from GvHD. Similarly, the onset of lethal GvHD is delayed using perforin-deficient effectors, while hepatic and cutaneous pathology is absent after transfer of FasL-deficient effectors in transplants across an MHC-matched allogeneic barrier (32). These studies, however, did not define the

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1. *Abbreviations used in this paper:* BFU-E, burst-forming unit erythroid; CFU-GM, colony-forming unit granulocyte macrophage; CTL, cytotoxic T lymphocytes; $CFU\text{-}S_8$, day 8 spleen colony-forming unit; FasL, Fas ligand; GvHD, graft-versus-host disease.

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T cell compartments affected by the mutations. We previously reported that granzyme B is an important mediator of cytotoxicity in vivo mediated by $CD8^+$, but not $CD4^+$ CTL (33). This study was conducted to define further the role of perforin/granzyme-dependent and independent mechanisms in the development of GvHD across class I and class II barriers. We found that a perforin/granzyme-independent mechanism (i.e., the Fas system) is an important mediator of GvHD across a class II barrier in this model. The perforin/granzyme pathway does not contribute significantly to class II-restricted GvHD, but it is essential for class I-restricted GvHD. This observation suggests to us that inhibition of the perforin/granzyme pathway may represent a novel target for GvHD therapy.

Until recently, the expression of perforin and granzyme B had not been detected outside the lymphoid compartment (34, 35). Berthou, et al. (36), however, found evidence of perforin and granzyme B expression in KG1a (human acute myelogenous leukemia) cells and in $CD34^+$ peripheral blood cells from patients mobilized with chemotherapy and granulocyte colony-stimulating factor (G-CSF). This report followed the earlier finding (37) of granzyme B expression in undifferentiated FDCP-Mix (murine multipotent hematopoietic) cells. Together, these data raised the possibility that perforin and granzyme B have a role in hematopoietic progenitor cell development and mobilization. Since this would seriously undermine the utility of perforin/granzyme inhibitors for the treatment of GvHD in the transplant setting (when patients have limited hematologic reserve), we undertook an evaluation of hematopoiesis and progenitor mobilization in perforin and granzyme B–deficient mice. Our data suggest that hematopoiesis, peripheral blood progenitor cell mobilization, and engraftment of radioprotective cells proceed normally in the absence of functional perforin or granzyme B. Therefore, perforin/granzyme pathway inhibition may represent a new strategy to mitigate GvHD.

Methods

Mice. Granzyme B–deficient mice were derived in a C57Bl/6 × 129/Sv ($H\text{-}2^b$) background, as previously described (18). Perforin-deficient mice (B6 × 129) were kindly provided by Dr. William Clark (15). B6.C-H-2^{bm1}, B6.C-H-2^{bm12}, B6Smn.C3H-*FasL*^{gld}, and 129/SvJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice doubly deficient for perforin or granzyme B and FasL were produced by crossing perforin or granyzme B −/− mice with *gld*/*gld* mice, and subsequently intercrossing the F1 heterozygotes. All transplants were performed between sex-matched animals at 6–8 wk of age. The granzyme B and perforin genotypes were determined by Southern blot analysis of tail DNA (15, 18). PCR amplification of tail DNA was used to identify the *gld* point mutation, as previously described (38).

Human peripheral blood progenitor cell samples. A standard mobilizing regimen was administered to four normal human donors (G-CSF 10 μg/kg QD × 5–8 d) and eleven patients with hematologic malignancies (G-CSF alone [$n = 2$], or G-CSF with cyclophosphamide 3 g/m^2 [$n = 9$]). The leukopheresis product was affinity-purified on a miniMACS column (Miltenyi Biotec Inc., Auburn, CA). The efficiency of progenitor cell purification was analyzed by flow cytometry using phycoerythrin-conjugated anti-CD34 pool and a cocktail of lineage markers (CD3, CD11b, CD14, and CD19; Coulter Immunology, Hialeah, FL).

S1 nuclease protection assay. Total cellular RNA was prepared from 1–2 × 10^6 affinity-purified human $CD34^+/lin^-$ cells by guanidinium lysis as previously described (39). Activated CTL (obtained from normal human peripheral blood cultured in 5 μg/ml concanavalin A (Sigma Chemical Co., St. Louis, MO) and 50 U/ml rhIL-2 for 48 h) were used as a source of positive control RNA; T cells acquire a CTL phenotype under these culture conditions, as characterized by significant upregulation of granzymes A and B (40) and perforin (41) expression. Negative control samples (no RNA) were processed in parallel. Specific end-labeled DNA probes (100,000 total cpm) were used to detect correctly processed human β-actin and granzyme B mRNAs as previously described (39). The perforin probe was generated by PCR from genomic K562 (human erythroleukemia cell) DNA. The fragment was end-labeled at a BamH1 site; correctly processed perforin mRNA protects a 413-nucleotide exon 2 probe fragment from S1 nuclease digestion.

Acute GvHD model. Lymphocytes were isolated from the mesenteric lymph nodes of sex-matched littermate donors after passage through a stainless steel mesh. T cell subsets were then prepared as previously described (33). In brief, antibody-mediated complement lysis was performed by adsorbing antibodies to B cells (MoAb J11d) and $CD4^+$ cells (MoAb RL.172) or $CD8^+$ cells (MoAb 3.155) at 4°C for 30 min at a concentration of 10^7 lymphocytes/ml in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 5% (vol/vol) heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 15 mM Hepes, 2 mM l-glutamine, nonessential amino acids at 0.1 M each, 25 U/ml penicillin, 25 μg/ml streptomycin, and 75 μM β-mercaptoethanol. The lymphocytes were then incubated at 37°C for 1 h in the presence of complement (rabbit Low-Tox-M; Cedarlane Laboratories Ltd., Hornby, ON, Canada). T cells were recovered by centrifugation through a 1.119 g/cm^3 Ficoll gradient (Sigma Chemical Co.), and were washed twice in complete media before counting (> 95% viable by Trypan blue exclusion), and combined at a dose of 1.0 × 10^6 cells with the marrow graft before infusion. Unpooled T cells derived from single donors were used in all transplants.

Bone marrow grafts were prepared from the hosts by flushing both femora with 1 ml of 1× Hebs (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose) through a 22G needle. After settling debris, cells were washed once in PBS and counted. A dose of 2 × 10^6 viable nucleated marrow cells was combined with the T cell graft in a volume of 450 μl of PBS and infused via a lateral tail vein 24 h after irradiation of the recipient.

Hosts were conditioned with 1,000 cGy from a ^{137}Cs source at a rate of 95.2 cGy/min 24 h before transplantation. Animals were housed in a pathogen-free barrier facility in a manner approved by the Animal Investigation Committee at Washington University. Mice were observed daily and killed if moribund or unable to take food. Survival after transplant was analyzed by the Kaplan-Meier method. The groups were compared using Log-Rank analysis.

Baseline hematopoiesis evaluation. Bone marrow cells were flushed from both femora with 1× Hebs. Total cellularity was determined by duplicate counts of Trypan blue–excluding cells on a hemocytometer grid. 500 cell differentials were performed in duplicate from Wright-stained cytospin preparations. Complete blood counts were performed on samples obtained from the retroorbital sinus of anesthetized mice, and were run on a Baker-1000 automated cell counter.

Before progenitor enumeration, red cells were removed by incubation in hypotonic lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) for 5 min at 4°C. 5 × 10^4 cells were plated in duplicate in alpha-MEM containing 0.9% methycellulose with 30% FBS, 1% BSA, 3 U/ml rhEpo, and 2% pokeweed mitogen-stimulated murine spleen cell conditioned medium (MethoCult M3430; StemCell Technologies, Vancouver, Canada) and cultured at 37°C in 5% CO_2. Colony-forming unit granulocyte macrophage (CFU-GM) and burst-forming unit erythroid (BFU-E) colonies consisting of 40 or more cells were scored by morphology on day 8 with an inverted microscope.

For the day 8 spleen colony-forming unit ($CFU\text{-}S_8$) assay, syngeneic (B6 × 129) sex-matched hosts were conditioned with 1,000 cGy and then infused intravenously with 2 × 10^5 red blood cell-depleted donor marrow cells. Duplicate $CFU\text{-}S_8$ assays were run in parallel from each donor. Colonies were counted at 8 d after transplant with a

dissecting microscope after fixing the spleens for 24 h in Tellesniczky's fixative (87% ethanol, 4% acetic acid, 9% formalin).

A two-tailed *t*-test was used with all measurements to compare mutant and wild-type mice.

Mobilization regimen. Mice received a single intraperitoneal injection of cyclophosphamide (200 mg/kg in sterile H_20; Sigma Chemical Co.) on day −6. rhG-CSF (25 μg/kg in PBS with 0.1% BSA; Amgen Inc., Thousand Oaks, CA) was administered subcutaneously once daily on days −4 to 0. 1 ml of peripheral blood was obtained by cardiac puncture 2 h after the last dose of rhG-CSF.

Radioprotection assay. Syngeneic (B6 × 129) sex-matched hosts were conditioned with 1,000 cGy 24 h before receiving 2×10^6 mobilized peripheral blood cells from wild-type, perforin −/−, or granzyme B −/− donors. Nonmobilized wild-type donors were used as negative controls. At 30 d after transplant, engraftment in the survivors was documented by complete blood count measurement (not shown). Cumulative probability of survival after transplant was calculated by the Kaplan-Meier method.

Flow cytometric analysis. Freshly isolated peripheral lymphocytes were analyzed before selective T cell depletion for the GvHD studies. 1×10^6 cells were stained with directly conjugated monoclonal antibodies specific for murine CD3, CD4, or CD8 (PharMingen, San Diego, CA) and analyzed on a FACScan running CellQuest version 1.22 (Becton Dickinson, Mountain View, CA). T cell subsets were compared using the Student's *t* test.

Red blood cell–depleted bone marrow or peripheral blood cells prepared for the hematopoiesis and mobilization studies were stained with saturating amounts (≤ 1 mg/10^6 cells) of a cocktail of lineage markers, consisting of fluorescein isothiocyanate-conjugated anti-B220, CD3, and CD11b (PharMingen), and biotinylated anti-CD34 (Pharmingen) or its isotype control followed by streptavidin-phycoerythrin (Sigma Chemical Co.). After three washes, 100,000 intermediate FSC/low SSC events were acquired and analyzed.

Results

Production of granzyme B −/− × gld/gld and perforin −/− × gld/gld mice. Mice doubly deficient for FasL and perforin or granzyme B were viable and produced litters of normal size with the expected frequency of wild-type and mutant alleles, as previously described (38).

Because of the characteristic age-dependent accumulation of double negative T cells in mice homozygous for the *gld* mutation, lymph nodes were harvested from young (< 8-wk-old) mice. To ensure that any phenotype observed in the experiments was not due to quantitative differences in the lymphoid compartments, T cell subsets were analyzed from each donor before complement-mediated lysis. There were no significant differences in the single positive CD3, CD4, or CD8 subsets among the five groups of mice used in these experiments (not shown).

Class I-dependent acute GvHD. A single antigen class I mismatched acute GvHD model was used, as previously described (33). The hosts were (bm1 × 129) F1 hybrids, which share all minor histocompatibility loci with the (Bl/6 × 129 [H-2^b]) T cell donors, ensuring that the only allogeneic stimulus derives from the mutant H-2^{bm1} class I allele. Irradiated hosts were rescued with syngeneic marrow combined with CD4-depleted, $CD8^+$ T cells from sex-matched littermate animals singly or doubly deficient for functional FasL and granzyme B or perforin. Wild-type $CD8^+$ T cells used in this model will recognize the mismatched marrow within the host, become activated, and kill the graft, leading to death of the hosts from pancytopenia. If the *gld*, granzyme B, or perforin mutations significantly disarm the T cells, the syngeneic marrow should

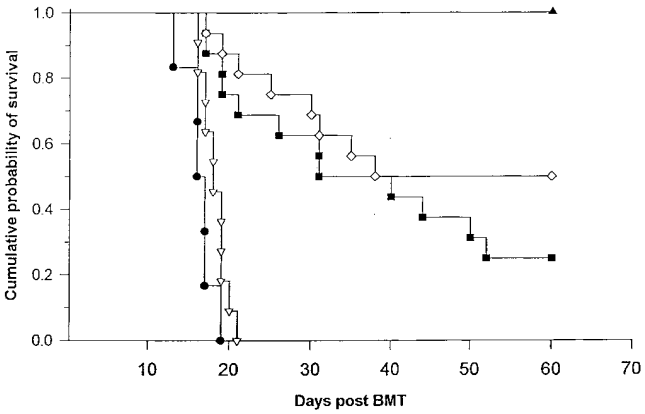

Figure 1. Granzyme B is an important mediator of class I–restricted GvHD, while FasL is not. Bm1 × 129 mice were lethally irradiated and were then reconstituted with 2×10^6 syngeneic bone marrow cells mixed with 1×10^6 class I–mismatched $CD8^+$ T cells. Kaplan-Meier estimates demonstrate significant prolongation of survival when granzyme B–deficient T cell donors (■, $n = 16$) were used compared to wildtype (●, $n = 6$) or FasL-deficient (▽, $n = 11$) donors ($P < 0.01$). Survival after transfer of doubly deficient cells (◇, $n = 17$) was not significantly different from survival after infusion of $CD8^+$ T cells deficient in granzyme B alone ($P = 0.11$). All control animals receiving syngeneic marrow cells without mismatched T cells survived (▲, $n = 4$).

engraft, allowing for the survival of some hosts. Mock-transplanted (PBS only) negative control hosts should die uniformly of marrow aplasia within 2 wk. Complete survival in positive controls (marrow only, without T cells) would confirm that death in the experimental groups is entirely due to the lymphocyte infusion, rather than another effect of the conditioning regimen.

$CD8^+$ T cells from wild-type or *gld/gld* donors caused 100% mortality from GvHD when infused into class I–mismatched bm1 × 129 hosts (Fig. 1). Significantly fewer animals developed fatal GvHD when granzyme B −/− $CD8^+$ T cells

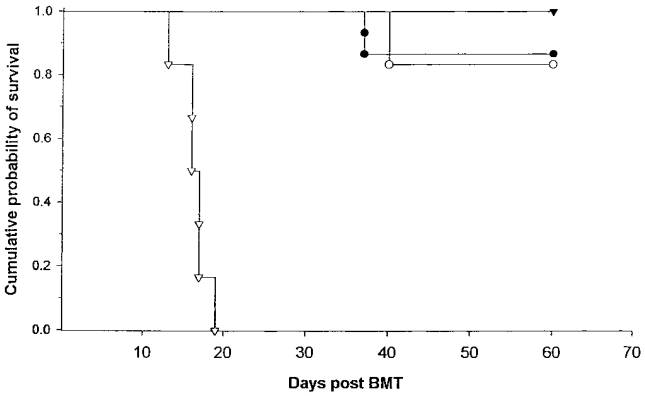

Figure 2. Perforin/granzyme-dependent cytotoxicity is essential for class I–restricted GvHD. Class I–mismatched transplants were performed, as in Fig. 1. The survival of mice receiving $CD8^+$ T cells deficient in perforin (○, $n = 6$) or perforin and FasL (●, $n = 15$) was indistinguishable from controls receiving syngeneic marrow only (▼, $n = 10$). All hosts receiving wild-type class I–mismatched $CD8^+$ T cells succumbed to GvHD (▽, $n = 6$).

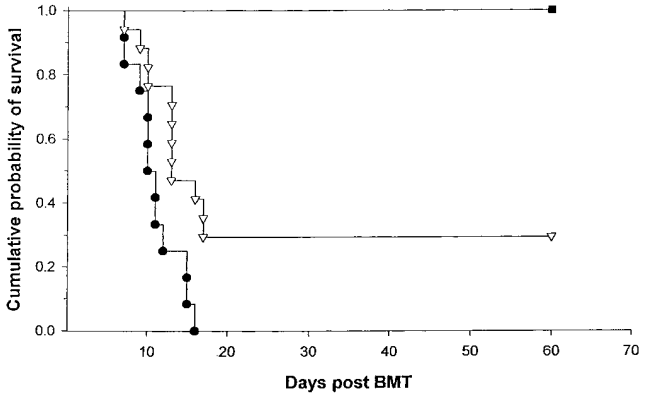

Figure 3. FasL is an important mediator of class II–restricted GvHD. Bm12 × 129 mice were lethally irradiated and then reconstituted with 2×10^6 syngeneic bone marrow cells mixed with 1×10^6 class II-mismatched $CD4^+$ T cells. Survival was significantly prolonged when FasL-deficient T cell donors (▽, $n = 17$) were used compared to wild-type (●, $n = 12$) donors ($P < 0.01$). All controls receiving syngeneic marrow alone survived (■, $n = 4$).

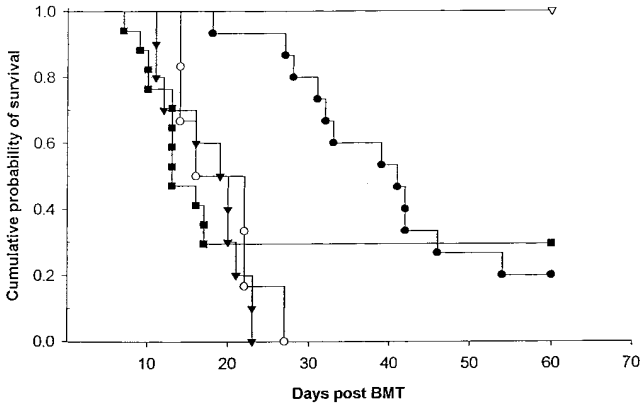

Figure 4. The Perforin/granzyme pathway is not required for $CD4^+$ CTL-mediated cytotoxicity in vivo. Class II–mismatched transplants were performed, as in Fig. 3. All hosts receiving $CD4^+$ T cells from granzyme B–deficient (▼, $n = 10$) or perforin-deficient (○, $n = 6$) donors died rapidly from GvHD. FasL-deficient T cell transfers (■, $n = 17$) were associated with an increase in survival, as seen in Fig. 3 (the FasL-deficient cohort is reproduced in this figure for clarity). Infusion of $CD4^+$ T cells doubly deficient for perforin and FasL (●, $n = 15$) delayed death from GvHD, but did not significantly alter the outcome compared to transplants with FasL-deficient cells. All controls receiving syngeneic marrow alone survived (▽, $n = 4$).

were infused (25% vs. 0%, $P < 0.01$), consistent with our prior observations (33). $CD8^+$ T cells from animals doubly deficient for functional FasL and granzyme B had a phenotype that was not significantly different (50% vs. 25%, $P = 0.11$) from the granzyme B −/− cohort, implying that FasL is not an important contributor to granzyme B–independent cytotoxicity in this model. Perforin −/− $CD8^+$ T cells permitted significantly higher survival rates (Fig. 2) when compared to the wild-type ($P < 0.001$) or granzyme B −/− ($P < 0.001$) groups, whether functional FasL was present (83% probability of survival) or absent (87% probability of survival). These results suggest that the perforin/granzyme pathway is required for cytotoxicity of $CD8^+$ T cells in vivo, while FasL is not.

Class II–dependent acute GvHD. A single antigen class II–mismatched acute GvHD model was created using (bm12 × 129) F1 hosts. CD8-depleted, $CD4^+$ T cells from donors singly or doubly deficient for functional FasL and granzyme B or perforin were infused with syngeneic marrow into lethally irradiated sex-matched bm12 × 129 hosts. Mortality in these cohorts can be attributed to GvHD provoked by the single allogeneic H-2^{bm12} class II allele.

In this case, wild-type, granzyme B −/−, or perforin −/− CD8-depleted, $CD4^+$ T cells caused 100% mortality (see Fig. 4). When *gld/gld* CD8-depleted, $CD4^+$ T cell donors were used, however, a significant proportion of the hosts survived (29% vs. 0%, $P < 0.01$) (Fig. 3). FACS analysis confirmed that the relative numbers of input $CD4^+$ T cells were similar in these two groups (not shown). The perforin −/− mutation superimposed on the *gld/gld* background delayed death from GvHD (Fig. 4), but had no significant effect on overall survival ($P = 0.17$) compared with the *gld/gld* group.

Perforin and granzyme B expression in human mobilized peripheral blood progenitor cells. Normal human activated CTL express abundant amounts of both perforin and granzyme B mRNA (Fig. 5). In contrast, none of the mobilized peripheral blood samples from normal donors (lanes *3*, *5*, *6*, and *8*) or patients with malignancies (lanes *4*, *7*, and *9*) contained detectable amounts of either mRNA. These samples were routinely > 89% $CD34^+$ and > 87% $CD34^+/lin^-$ (not shown), although one product (lane *5*) was 92.8% $CD34^+$ and 70.1% $CD34^+/lin^-$. Analysis of eight additional peripheral blood samples from patients mobilized with cyclophosphamide and G-CSF also failed to demonstrate granzyme B mRNA (not shown) despite adequate amounts of total RNA, and CD34 purity > 90%. Probe specificity is demonstrated by the absence of a perforin or granzyme B signal in samples from resting human PBL (not shown), and the lack of all protected fragments in the samples containing no RNA (lane *1*). β-actin serves as a control for RNA quality and content (no attempt was made to normalize the total RNA load because of limitations in the available clinical material). Spot densitometry yielded an estimate of the total RNA present in each clinical sample (7.1, 5.6, 1.7, 1.7, 9.0, 12.7, and 5.2 μg) relative to the CTL positive control (10 μg). Previous work from our laboratory has established that with 10 μg of total RNA, these probes are sufficiently sensitive to detect transcripts present at ≥ 10 copies per cell (42).

Basal hematopoiesis in perforin and granzyme B–deficient mice. The peripheral white blood cell counts, total bone marrow cellularity, and nucleated cell differentials were similar among wild-type, granzyme B −/−, and perforin −/− mice (Table I). Although light microscopic interpretation of murine bone marrow differentials can be technically difficult, these data are internally controlled by comparison with the wild-type mice. The very small reduction in relative lymphocyte numbers in perforin −/− mice is of uncertain significance. This phenotype was not observed in previous descriptions of perforin −/− mice (14).

The precursor/progenitor pool was examined using morphologic and functional assays. The frequency of $CD34^+$ and $CD34^+/lineage^-$ cells from bone marrow of wild-type mice was 12.71% (range: 7.82–18.81) and 4.22% (range: 2.48–7.19), re-

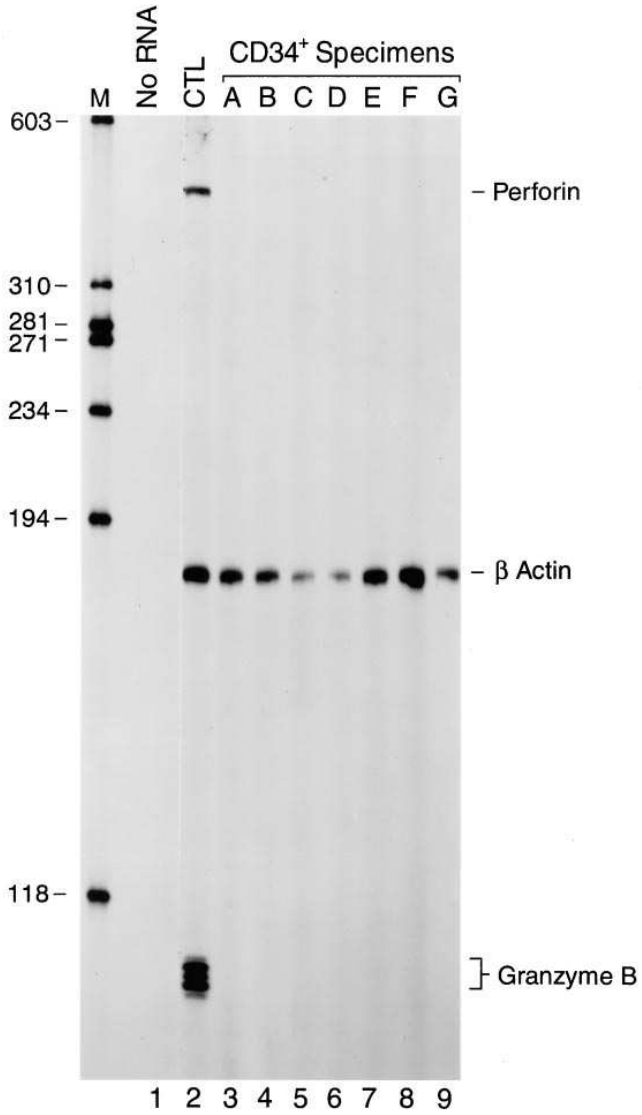

Figure 5. Perforin and granzyme B mRNA expression are not detectable in mobilized human $CD34^+/lin^-$ cells. Probe fragments protected from S1 digestion by correctly spliced transcripts for perforin (413 nucleotides), granzyme B (106 nt) and β-actin (177 nt) are detected in total cellular RNA extracted from activated human CTL (lane *2*). In contrast, there is no detectable perforin or granzyme B mRNA in any of the human mobilized $CD34^+/lin^-$ samples (lanes *3–9*), despite the presence of intact RNA (reflected by the β-actin signal). Unhybridized probes subjected to S1 nuclease digestion (lane *1*) produce no false positive signals.

spectively. The granzyme B −/− and perforin −/− $CD34^+$ and $CD34^+/lineage^-$ counts were indistinguishable from wild-type values (Table II). The sensitivity and specificity of CD34 detection in our assay was confirmed by analyzing NIH3T3 cells (which express CD34) and 32D cells (which do not) (43) (not shown). The number of erythroid and myeloid colonies grown in methylcellulose was not significantly different among the three groups of mice (Table II). Finally, the frequency of day 8 CFU-S cells was similar in the marrows of all three groups (Table II). These data collectively establish that the precursor/progenitor pools are quantitatively normal in granzyme B −/− and perforin −/− mice.

Mobilization of peripheral blood progenitor cells in perforin and granzyme B–deficient mice. After mobilization with cyclophosphamide and G-CSF, all three groups reached similar total numbers of peripheral WBCs (Table III). The frequency of colony-forming cells in mobilized peripheral blood was similar in the three groups examined (Table III). There was no significant difference in the frequency of $CD34^+/lineage^-$ cells present in the peripheral blood of mobilized wild-type, granzyme B−/−, or perforin −/− mice (Table III). Similar results were obtained when higher (250 μg/kg every day) or lower (10 μg/kg every day) doses of G-CSF were used (not shown).

When mobilized cells from the mutant mice or wild-type controls were transplanted into lethally irradiated hosts, 100% short-term multilineage engraftment was noted (Fig. 6). When only 1×10^6 mobilized peripheral blood cells were used, survival fell to 80–90% in all three groups (not shown), suggesting that the dose chosen was close to the threshold for radioprotection. Therefore, this assay should detect (conservatively) a 10-fold reduction in mobilization efficiency due to the perforin or granzyme B mutation. None of the negative control hosts transplanted with nonmobilized wild-type cells survived, indicating that cells with short-term radioprotective ability circulate at a low frequency in the baseline state. Therefore, committed progenitors and short-term radioprotective cells can be mobilized in the absence of perforin or granzyme B.

Discussion

The morbidity and mortality attributable to GvHD remains a major limitation of allogeneic bone marrow transplantation. Progress in defining the T cell subsets responsible for disease has translated into the current practices of selective depletion and graft engineering. Improvements in pharmacologic immunosuppression, however, have been hampered by an incomplete description of the molecular mediators of GvHD. This study provides evidence that the perforin/granzyme pathway is required for class I–restricted GvHD, and that FasL is an important mediator of class II–restricted GvHD.

Cytotoxic T lymphocytes require perforin and granzyme B for the rapid induction of apoptosis in susceptible target cells (14–18). Activated CTL from mice homozygous for a granzyme B null mutation lack > 90% of their ability to induce ^{125}I-DNA release (a measure of target cell DNA fragmentation) during the first 2 h of incubation with susceptible target cells (18). The lytic activity of natural killer and lymphokine-activated killer cells from these animals is even more severely compromised (44). Perforin-deficient CTL induce dramatically less ^{51}Cr release (a measure of target cell membrane damage) than do wild-type controls (14–17), and have a marked reduction in ^{125}I-DNA release (presumably reflecting the inability of granzyme B and perhaps other granzymes to gain access to the target cell cytoplasm). Most of the residual cytotoxicity in perforin −/− CTL is mediated by FasL (45, 46).

Bone marrow transplantation across a class II barrier in the model used here provides strong evidence that functional FasL is required for full in vivo cytotoxicity of $CD4^+$ CTL. Furthermore, these experiments show that the Fas/FasL interaction is important for killing allogeneic target cells in vivo. Activation-dependent upregulation of Fas on T cells marks them for fratricidal or suicidal deletion (47), providing a means of eliminating potentially deleterious activated cells. Similarly, expression of FasL by stromal cells in the testis (48) and eye (49) induces

Table I. Bone Marrow Differentials and Peripheral Blood Counts are Normal in Perforin or Granzyme B–Deficient Mice

Compartment	Wild-type	Granzyme B −/−	Perforin −/−
Bone marrow	$n = 2$	$n = 2$	$n = 2$
Cellularity	Normocellular	Normocellular	Normocellular
Myeloblasts (%)	0.4±0.2	0.5±0.2	0.1±0.1
Promyelocytes (%)	1.6±0.3	2.8±0.6	2.5±0.8
Myelocytes (%)	1.5±0.2	1.9±0.8	1.6±0.7
Metamyelocytes (%)	8.4±1.7	6.3±0.9	5.9±1.6
Bands/neutrophils (%)	45.4±5.6	50.0±1.9	53.2±1.0
Eosinophils (%)	5.3±0.7	3.1±0.7	6.4±0.5
Lymphocytes/plasma cells (%)	1.8±0.4	1.0±0.4	0.6*±0.2
Normoblasts (%)	35.6±3.9	34.5±1.0	30.4±1.4
Megakaryocytes	Adequate	Adequate	Adequate
Myeloid:erythroid ratio	1.9	1.9	2.3
Peripheral blood	$n = 3$	$n = 3$	$n = 3$
WBC ($\times 10^{-3}/\mu l$)	4.3±1.0	5.2±0.7	6.1±2.3
Hb (g/dl)	14.6±1.0	14.2±0.4	15.3±0.7
Plt ($\times 10^{-3}/\mu l$)	975±105	1123±81	1052±122

Total bone marrow cellularity, differentials, and complete blood counts from wild-type, granzyme B–deficient, or perforin-deficient mice (C57Bl/6× 129/Sv). Data shown are mean±SD. 500 cell differentials were performed in duplicate on each bone marrow sample. WBC, white blood cells; Hb, hemoglobin; Plt, platelets. *$P < 0.05$ compared to wild-type.

death of infiltrating activated autologous lymphocytes, perhaps accounting for the immune-privileged status of those organs (50). Destruction of class II–mismatched allogeneic bone marrow grafts apparently lies within the repertoire of FasL-dependent killing as well.

The perforin/granzyme pathway may account partially for FasL-independent killing by $CD4^+$ CTL, since death from GvHD was delayed in the group receiving transplants of $CD4^+$ T cells deficient for both FasL and perforin compared to FasL deficiency alone. Nevertheless, there is a FasL and perforin/granzyme-independent mechanism(s) that accounts for the residual mortality (50% of animals) in the class II–mismatched transplants (Fig. 7). Non-contact-dependent mechanisms of cytotoxicity (i.e., cytokines) that have been implicated in GvHD in several studies (for review see reference 51) may account for perforin/granzyme and FasL-independent allogeneic cell killing by $CD4^+$ T cells. Indeed, CTL doubly deficient for perforin and FasL are able to induce cell lysis after prolonged incubation with TNF-α–sensitive targets (31).

We provide evidence here and elsewhere (33) that an intact perforin/granzyme pathway is required for the in vivo cytotoxicity of $CD8^+$ T cells. Transplants across a class I H-2 barrier resulted in significant prolongation of host survival when the T cells came from a granzyme B −/− donor. Although we note a trend towards further impairment in cytotoxicity when granzyme B −/− × *gld*/*gld* donor cells were used, our data do not conclusively show that Fas is an important mediator of cytotoxicity in the $CD8^+$ compartment in the absence of granzyme B. Perforin-deficient $CD8^+$ CTL, on the other hand, are essentially unable to cause death from GvHD in the setting of a class I mismatch. The *gld*/*gld* mutation had no additional effect in this model when superimposed on the perforin-deficient

Table II. Progenitor Frequencies are Normal in Untreated Bone Marrow from Perforin or Granzyme B–deficient Animals

	Wild-type	Granzyme B −/−	Perforin −/−
	n = 5	*n = 5*	*n = 5*
CFU-GM ($\times 10^{-5}$ cells)	122±30	100±29	120±48
BFU-E ($\times 10^{-5}$ cells)	10±5	10.6±7	10.8±4
CFU-S_8 ($\times 10^{-6}$ cells)	112±30	125±27	105±20
$CD34^+/lin^-$ (%)	4.22±2.0	3.22±0.9	3.36±1.0

Frequency of colony-forming cells and $CD34^+/lineage^-$ (CD3, CD11b, B220) cells in untreated bone marrow from wild-type, granzyme B–deficient, or perforin-deficient mice. Results (±SD) were similar in three independent experiments. Progenitor assays were plated in duplicate from each animal. No significant differences exist between the three groups.

Table III. Peripheral Blood Progenitor Frequencies are Normal in Perforin or Granzyme B–deficient Mice Mobilized with Cyclophosphamide + G-CSF

	Wild-type	Granzyme B −/−	Perforin −/−
	n = 4	*n = 3*	*n = 4*
Day 0 total peripheral WBC ($\times 10^{-3}/\mu l$)	22.5±2.4	19.8±4.5	27.3±4.1
CFU-GM ($\times 10^{-5}$ cells)	53.3±7.6	56.6±8.3	73.4±11.6
BFU-E ($\times 10^{-5}$ cells)	8.3±1.2	10.4±1	9.2±1.6
$CD34^+/lin^-$ (%)	0.93±0.2	0.62±0.1	0.84±0.1

Analysis of peripheral blood from wild-type, granzyme B–deficient, and perforin-deficient mice mobilized with cyclophosphamide (200 mg/kg) followed by 4 d of G-CSF (25 μg/kg every day). Results (±SD) were similar in four independent experiments. Progenitor assays were run in duplicate on each animal. No significant differences exist among the three groups.

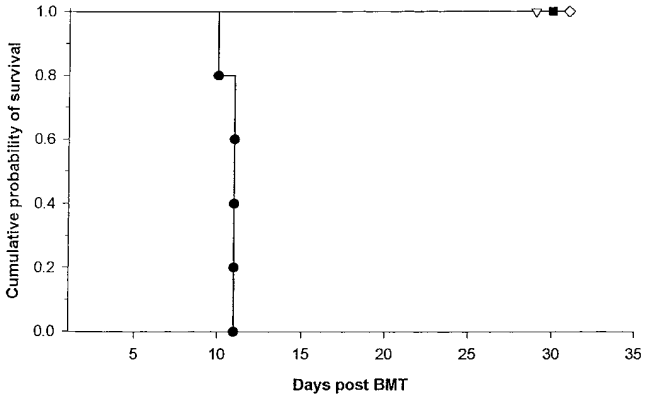

Figure 6. Survival after transplant of mobilized peripheral blood progenitor cells from perforin or granzyme B–deficient donors. Lethally irradiated B6 × 129 mice were transplanted with 2×10^6 peripheral blood cells from syngeneic animals mobilized with cyclophosphamide and G-CSF. Negative control animals transplanted with peripheral blood cells from untreated wild-type donors (●) all died as a consequence of pancytopenia. In contrast, all recipients of mobilized wild-type (◇), granzyme B −/− (■), or perforin −/− (▽) peripheral blood cells survived. $N = 4$ mice in each group. This experiment was performed a second time with similar results.

background. These findings suggest that the perforin/granzyme pathway accounts for virtually all cytotoxicity mediated by $CD8^+$ T cells in vivo (Fig. 7). This observation is consistent with the recent report of substantially reduced GvHD mortality with transplantation into a completely mismatched host using bone marrow doubly deficient for perforin and FasL (31).

The substantial perforin-dependent, granzyme B–independent mortality in the class I–restricted GvHD model may be mediated by perforin itself, or by other primary granule contents. Granzyme A is a candidate for this missing component of cytotoxicity since it is expressed at levels comparable to granzyme B in CTL (40), and since it is an enzyme that can slowly induce apoptosis. Granzyme A–deficient mice (52) have a minimal defect in cytotoxicity; however, redundant granzymes (e.g., granzyme D or K), or the presence of the fast-acting granzyme B cluster may mask the cytotoxic phenotype. We are currently analyzing mice deficient for granzyme A or granzymes A and B in these GvHD models to evaluate this possibility.

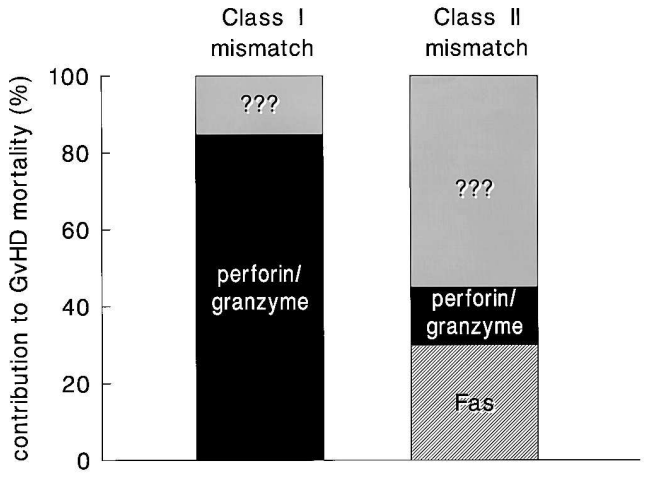

Figure 7. Schematic summary of GvHD results. The perforin/granzyme pathway appears to be essential for the development of GvHD across a class I barrier in this model. FasL is important for the development of GvHD across a class II barrier, but other pathways are extremely important in this compartment as well. The contribution of the perforin/granzyme pathway is shown as a small fraction, since it appears to play only a minor role in the development of class II–restricted GvHD in the absence of FasL. Additional, undefined pathways must account for the residual $CD4^+$ dependent cytotoxicity in vivo.

We chose to study the endpoint of hematopoietic failure resulting from a graft-versus-host reaction in these experiments since it provides a more rapid, reproducible, and quantitative readout than do other manifestations of experimental GvHD (e.g., weight loss, immune dysfunction, histopathology of skin, liver, and intestine). Models designed to study the role of perforin and/or FasL in mediating class I– or class II–dependent cellular cytotoxicity against these other target organs could add to the significance of our findings.

One important implication of this finding is that inhibitors of the perforin/granzyme pathway, currently under investigation (53), could have a role in the treatment or prophylaxis of GvHD. Data from other investigators (36, 37) suggesting that perforin and granzyme B are expressed in hematopoietic progenitors, however, raised an important caveat. If these proteins were critically involved in hematopoiesis, inhibition of their function could have devastating consequences in the peritransplant period. Our analysis, however, suggests that neither basal hematopoiesis nor the mobilization and engraftment of peripheral blood progenitor cells requires perforin or granzyme B. Our data may conflict with the first study (37) because of biological differences between primary cells and the transformed line FDCP-Mix, which may express granzyme B aberrantly as a consequence of genetic alterations. We also cannot confirm the expression of perforin or granzyme B in mobilized human progenitors, probably due to limited specificity of the reagents used in the prior study (36). Our analysis of the murine models, however, suggests that even if perforin and granzyme B are sometimes expressed in mobilized progenitors, they are required neither for hematopoiesis nor mobilization. It remains a formal possibility that perforin and granzyme B are important for hematopoiesis and progenitor cell mobilization, but that a redundant mechanism can fully substitute in their absence. Nonetheless, these data provide proof of principle that specific inhibition of the perforin/granzyme pathway could benefit patients at risk for GvHD without adversely affecting hematopoietic recovery after bone marrow transplantation.

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