RAPID COMMUNICATION

The Role of Granzyme B in Murine Models of Acute Graft-Versus-Host Disease and Graft Rejection

By Timothy A. Graubert, John H. Russell, and Timothy J. Ley

A complete molecular description of the syndromes of graft-versus-host disease (GVHD) and graft rejection could have a significant impact on clinical bone marrow transplantation. Recent in vitro experiments (Heusel et al, Cell 76:977, 1994 and Shresta et al, Proc Natl Acad Sci USA 92:5679, 1995) have shown that the putative mediators of these two syndromes, cytotoxic lymphocytes (CTL) and natural killer (NK) cells, respectively, initiate a program of cell death (apoptosis) in susceptible target tissues in a manner critically dependent on the serine protease Granzyme B (gzm B). In the present study, we have analyzed the phenotype of gzm B-deficient mice using experimental transplant models designed to isolate their CD8+ CTL, CD4+ CTL, and NK compartments. We found a significant impairment in class I-dependent GVHD mediated by gzm B −/− CD8+ CTL, whereas class II-dependent GVHD was not altered using gzm B −/− CD4+ effectors. In a hybrid resistance model, gzm B −/− hosts rejected haplo-identical marrow grafts as efficiently as did their wild-type littermates. This result is surprising in light of a severe defect in the ability of gzm B −/− NK cells to induce apoptosis in susceptible targets in vitro. These in vivo data define a significant role for gzm B in cytotoxicity mediated by CD8+ CTL, but not by CD4+ CTL. Furthermore, these results do not support a model of hybrid resistance in which NK cells play a pivotal role.

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In vitro analysis of immune effector cells from mice bearing a null mutation in the gzm B locus has shown profound functional defects. In standard lytic assays, gzm B-deficient CD8+ CTL and lymphokine-activated killer (LAK) cells exhibit a severe defect in their ability to rapidly induce apoptosis in allogeneic target cells, but late cytotoxicity recovery partially.7 In contrast, gzm B-deficient NK cells cannot induce apoptosis in target cells despite high effector:target ratios or prolonged incubation times.7

The Chédiak-Higashi Syndrome, an inherited disorder of humans resulting in increased susceptibility to infections, provides independent confirmation of the critical importance of the granule exocytosis pathway for CTL- and NK-mediated cytotoxicity. CTL, NK cells, and neutrophils from these patients share a common defect in granulopoiesis. The primary and secondary granules fuse aberrantly; consequently, the lytic contents cannot be delivered to a target cell.6 Although Chédiak-Higashi CTL still exhibit some cytotoxicity, Chédiak-Higashi NK cells are virtually devoid of cytotoxicity.6 Mice homozygous for the beige mutation have a similar phenotype.10 These observations support the conclusion drawn from the analysis of perforin and gzm B-deficient mice that the granule exocytosis pathway is important for CTL cytotoxicity and essential for NK cytotoxicity.

Murine transplant models developed in the 1980s dissected the roles of CD4+ and CD8+ CTL in the pathogenesis of GVHD.11-13 Congenic strains of C57BL/6 mice expressing either a mutant class I (B6.C-H-2b(1)) or class II (B6.C-H-2b(2b)) allele served as hosts for these experiments. These mice were reconstituted after lethal irradiation with syngeneic marrow combined with purified T-cell subsets from the partially mismatched parental strain (BL/6, H-2b). After alloattack by T cells on the marrow graft, the hosts succumbed as a result of marrow aplasia at day 10 to 14 (in the case of a class I mismatch) or day 20 to 24 (class II mismatch) post-BMT. These results indicated that single antigen H-2 mismatches that provoke either class I- or class II-restricted responses can lead to 100% mortality from acute GVHD when as few as 107 donor-derived T cells are infused.

The cellular basis of the host-versus-graft (ie, rejection) response has not been as well characterized. The prototypical
host-versus-graft reaction is rejection of parental marrow by the F1 progeny of two inbred strains (a × b), a phenomenon known as hybrid resistance. Recent transgenic experiments support the notion that the effector cell mediating hybrid resistance lyses target cells that do not display the full complement of self histocompatibility determinants (the missing self hypothesis). Circumstantial evidence favors the NK cell as the likely mediator of hybrid resistance, although definitive proof of this is not yet available.

We performed the present study to analyze the contribution of gzm B to cytotoxicity mediated by CTL and NK cells in vivo using these established murine models of acute GVHD and hybrid resistance. Our results suggest that gzm B plays a significant role in acute GVHD mediated by CD8+, but not CD4+ CTL. In contrast, gzm B does not appear to be essential for hybrid resistance. In view of the critical role of gzm B for in vitro NK cytotoxicity, this finding suggests that NK cells are not required for hybrid resistance.

MATERIALS AND METHODS

Mice. gzm B-deficient mice were derived in a C57BL/6 × 129/SvJ background, as previously described. Heterozygote crosses yielded littersmates genotypically −/− or +/+ at the gzm B locus, determined by Southern blot analysis of tail DNA. B6.C-H-2bm12, B6.C-H-2bm12, Balb/c, CBA/J, and 129/SvJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Acute GVHD model. Lymphocytes were isolated from the mesenteric lymph nodes of 8- to 12-week-old gzm B +/+ or −/− sex-matched littermate donors by passage through a stainless steel mesh. T-cell subsets were then prepared, as previously described. In brief, antibody-mediated complement lysis was performed by adsorbing antibodies to B cells (monoclonal antibody [MoAb] J1 Id) and CD4+ cells (MoAb 254D1) at 4°C for 30 minutes at a concentration of 107 lymphocytes/mL in RPMI 1640 (Sigma, St Louis, MO) supplemented with 5% (vol/vol) heat-inactivated fetal calf serum, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 1 mmol/L Na2EDTA, 0.7 mmol/L Na2HPO4; 25 µg/mL streptomycin, and 75 µmol/L L-β-mercaptoethanol. The lymphocytes were then incubated at 37°C for 1 hour in the presence of complement (rabbit Low-Tox-M; Cedarslane Laboratories, Hornby, Ontario, Canada). T cells were recovered by centrifugation through a 1.1 19 g/mL Histopaque gradient (Sigma), washed twice in complete media before counting (>95% viable nucleated marrow cells was combined with 200 cGy 24 hours before intravenous infusion of syngeneic marrow from a sex-matched littermate. With an intact T-cell effector pathway, the target marrow cells are killed, leading to death of the host from pancytopenia. If the gzm B null mutation disarms the T cells, the syngeneic marrow engrafts and the hosts receive a single neutralizing dose (250 µg IP) of monoclonal antitumor necrosis factor α (TNFα) or antimouse γ interferon (γIFN; both provided by Dr R Schreiber, Department of Pathology, Washington University, St Louis, MO) 24 hours before transplantation. The remainder of the protocol was performed as discussed above.

H-2 immunophenotyping. One hundred microliters of whole blood was obtained from the retro-orbital sinus of an anesthetized animal. After 10 minutes of incubation at 4°C in red blood cell lysis buffer (150 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L Na2EDTA, pH 7.1-7.4), the nucleated cells were washed twice in PBS and resuspended in serum-free media. Cells (107) were stained with fluorescein isothiocyanate (FITC) antitumor H-2Kb and phycoerythrin (PE) antitumor H-2Kd (PharMingen, San Diego, CA) and analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Statistics. Survival after BMT in the GVHD model was analyzed by the Kaplan-Meier method. The groups were compared using Log-Rank analysis. The Mann-Whitney test was used to compare engraftment among the experimental groups in the hybrid resistance model.

RESULTS

Acute GVHD model: CD8+ CTL. A single antigen class I mismatch acute GVHD model was created using (bm1 × 129) F1 mice as hosts. These F1 hybrids share all minor histocompatibility loci with the gzm B (BL/6 × 129) T-cell donor, ensuring that the only allogeneic stimulus derives from the mutant H-2bm12 (class I) allele. Balb/c (H-2bm12) mice, mismatched at all major and minor loci relative to the gzm B mice, served as hosts in the fully allogeneic acute GVHD model. The experimental design involved conditioning the hosts with lethal irradiation and then rescuing them with an infusion of syngeneic marrow from a sex-matched littermate, gzm B +/+ or −/−, CD4-depleted, CD8+ T cells from littermate donors were infused with the marrow graft into sex-matched littermate hosts. With an intact T-cell effector pathway, the target marrow cells are killed, leading to death of the host from pancytopenia. If the gzm B null mutation disarms the T cells, the syngeneic marrow engrafts and the animals survive. Mock-transplanted (PBS only) negative control hosts uniformly died of marrow aplasia within 2 weeks. One hundred percent survival of positive control hosts (receiving marrow grafts without T cells) confirmed that the conditioning regimen did not put the animals at risk for death from nonhematologic causes.

CD8+ CTL directed against the single antigen class I mismatched bm1 × 129 hosts caused significantly less death.
from acute GVHD (Fig 1) when the lymphocytes were derived from gzm B -/- donors compared with gzm B +/+ donors (36% v 12% at 40 days; P = .00657). gzm B +/+ and +/- T cell donors were indistinguishable in this analysis and were used interchangeably in these and subsequent experiments. Complete blood counts at day 10 post-BMT documented severe pancytopenia only in those animals that subsequently died of acute GVHD (data not shown).

Similar results were obtained in the fully allogeneic model (Fig 2). Significantly fewer Balb/c hosts survived when gzm B +/-, CD4-depleted, CD8+ T cells from gzm B -/- donors were used compared with gzm B +/- donors (40% v 6% at 40 days; P < .00622). These transplants into hosts mismatched at all H-2 and non-H-2 loci confirm that the data obtained in the bm12 system are accounted for by the single class I mismatch and are not an artifact of minor histocompatibility loci introduced by the BL6 × 129 strain differences.

Of note, mortality in the gzm B +/- cohort did not reach 100%, as seen by Sprent et al.13 This may be due to strain differences in our model or to a reduced susceptibility to death from infection in contemporary barrier facilities.

**Acute GVHD model: CD4+ CTL.** A single antigen class II mismatch acute GVHD model was created using (bm12 × 129) F1 mice as hosts. In this case, gzm B +/- or -/-, CD8-depleted, CD4+ T cells from littermate donors were infused into sex-matched littermate bm12 × 129 hosts, which were histocompatible at all major and minor loci except for the single mutant class II (H-2b129) allele. In contrast to the class I model, there was no difference in survival of the bm12 × 129 hosts when gzm B +/- or -/- CD4+ T cells from littermate donors were infused into sex-matched littermate bm12 × 129 hosts, which were histocompatible at all major and minor loci except for the single mutant class II (H-2b129) allele. In contrast to the class I model, there was no difference in survival of the bm12 × 129 hosts when gzm B +/- or -/- CD4+ T cells from littermate donors were infused into sex-matched littermate bm12 × 129 hosts, which were histocompatible at all major and minor loci except for the single mutant class II (H-2b129) allele. In contrast to the class I model, there was no difference in survival of the bm12 × 129 hosts when gzm B +/- or -/- CD4+ CTL were infused (Fig 3). A lower T-cell dose would be unlikely to bring out a phenotypic difference between the two experimental groups, because mortality in the wild-type group is reduced when fewer than 10⁶ cells are used.13 These findings support the hypothesis that the perforin-granzyme pathway is not essential for CD4+ CTL-mediated cytotoxicity in vivo.

**Hybrid resistance model.** Hosts for the hybrid resistance model were created by crossing CBA/J (H-2k) and gzm B -/- (H-2b) mice. The F1 progeny were then backcrossed to the gzm B -/- parent. F2 hybrids bearing both haplotypes (H-2b/k) and either gzm B genotype (+/- or -/-) were the experimental hosts. Engraftment was measured after the infusion of wild-type BL6 × 129 (H-2b/k) marrow into a lethally irradiated host. The missing self hypothesis predicts that a radioresistant cell within the F2 hybrid hosts will recognize and kill the parental marrow stem cells because those cells do not express the H-2b/k haplotype. Homozygotes (H-2b) within the F2 litter provided concurrent positive controls because they provide no barrier to engraftment of the parental BL6 × 129 marrow. Mock-transplanted animals (PBS only) were used as negative controls.

All syngeneic control animals engrafted, with splenic

13³HdR incorporation (mean, 2.2%) in good agreement with previously published reports.23 As expected, gzm B +/- H-2b/k hosts rejected bone marrow grafts from the H-2b parental

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Figure 1. Probability of survival after transplant of syngeneic marrow combined with class I-mismatched CD8+ T cells from gzm B +/- or -/- donors. Survival is prolonged in recipients of gzm B +/- T cells (P = .00622). Data are from six separate experiments.

Figure 2. Probability of survival after transplant of syngeneic marrow combined with fully mismatched CD8+ T cells from gzm B +/- or -/- donors. Survival is prolonged in recipients of gzm B +/- T cells (P = .00622). Data are from six separate experiments.

Figure 3. Probability of survival after transplant of syngeneic marrow combined with class II-mismatched CD4+ T cells from gzm B +/- or -/- donors. Survival is not significantly different between the groups (P = .31254). Data are from four separate experiments.
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Fig 4. Engraftment (as measured by splenic [3H]UdR incorporation) after transplantation of 8 × 10^6 parental bone marrow cells (H-2^b^) into lethally irradiated (800 cGy) F2 hybrid hosts (H-2^kb^) that are homozygous or heterozygous for the gzm B null mutation. Hybrid resistance was not significantly altered in the gzm B +/- hosts compared with the gzm B +/- hosts (P = .5672). Positive control hosts are syngeneic with the marrow donor, whereas negative control hosts are mock-transplanted with PBS only. Results are the mean ± SD of three separate experiments.

strain, as reflected by very low splenic [3H]UdR uptake (mean, 0.19%; Fig 4). Surprisingly, gzm B +/- H-2^kb^ hosts also rejected the mismatched marrow completely (mean [3H]UdR uptake, 0.08%). Pretreatment of the gzm B +/- or +/- hosts with anti-mouse TNFα (n = 9), γIFN (n = 2), or the combination of both antibodies (n = 2) did not significantly change the outcome, because persistent complete rejection of haplo-identical marrow was observed (data not shown).

DISCUSSION

The models used in the present study provide tools for the in vivo dissection of the role of gzm B in each of the lymphocyte compartments. To summarize the findings, we note a moderate, but significant reduction in acute GVHD mediated by gzm B +/- CD8^+ CTL as compared with wild-type CD8^+ CTL. In contrast, CD4^+ CTL-mediated cytotoxicity and hybrid resistance were not affected by the loss of gzm B activity in these models.

The molecular basis of cellular cytotoxicity is being elucidated using complementary experimental systems. Evidence accumulated thus far implicates two major pathways used by CTL. The importance of the Ca^2+ -dependent granule exocytosis pathway has been shown by the targeted disruption of the key effector molecules, perforin and gzm B. CTL and LAK cells from perforin- or gzm B-deficient mice have a severe defect in their ability to rapidly induce apoptosis in susceptible target cells. NK cells from these mice are completely unable to induce apoptosis in susceptible target cells.

The second mechanism of cellular cytotoxicity involves the Ca^2+ -independent interaction of Fas, a ubiquitously expressed 45-kD transmembrane protein in the TNF receptor family, with its ligand present on the surface of CTL. Although Fas-induced cell death may serve primarily an immunoregulatory role, Fas-dependent cytotoxicity can be shown against nonlymphoid targets as well. CTL from mice with naturally occurring loss of function mutations of Fas or its ligand have compromised cytotoxicity, particularly in the CD4^+ compartment.

Although CTL may also kill target cells through secretion of soluble mediators such as TNFα, experiments with mice genetically deficient in both perforin and Fas suggest that these two pathways account for nearly all T-cell cytotoxicity in standard in vitro lytic assays.

CD8^+ CTL in the experiments described here were able to kill mismatched bone marrow cells and cause aplastic death in over half of the animals analyzed, despite the lack of gzm B. This implies that, just as the in vitro assays have shown, gzm B-independent apoptotic mechanisms are operative in CD8^+ CTL. It further follows from the in vitro experiments that the Fas-Fas ligand interaction probably accounts for much of this residual cytotoxicity in gzm B-deficient mice. CTL deficient in both gzm B and Fas ligand have a killing defect intermediate between gzm B-deficient and perforin/Fas ligand-deficient CTL (S. Shresta and T.J. Ley, unpublished observations). Because perforin is required for delivery of gzm A and B to the target cell cytoplasm, but is unable to induce apoptosis itself, gzm A may account for the difference between the gzm B/Fas ligand-deficient and the perforin/Fas ligand-deficient CTL in these assays. Therefore, we would predict that the gzm B-independent cytotoxicity in this GVHD model is mediated primarily by the Fas pathway with a smaller contribution from gzm A.

Our finding that gzm B is important, but not essential for CD8^+ CTL-mediated attack on allogeneic hematopoietic grafts is consistent with the recent report that perforin-deficient mice have impaired, but not fully disabled resistance to cardiac allografts. In addition, Selvaggi et al have shown that bone marrow grafts from perforin-deficient mice will survive and engraft in fully incompatible hosts with no mortality from GVHD unless the T-cell inoculum is increased substantially. Although this model differs from ours, it further supports the conclusion that the perforin/granzyme pathway is critical for in vivo cytotoxicity in the CD8^+ compartment, but that important redundancy (eg, Fas) remains.

CD4^+ CTL-mediated cytotoxicity, in contrast, appears to be completely gzm B-independent in the class II-restricted GVHD model. This is consistent with the in vitro data that suggests that Fas is the sole mediator in this compartment. This possibility is being formally tested using Fas ligand-defective CTL in similar transplant experiments.

It is harder to reconcile the failure of gzm B deficiency to impact on hybrid resistance with the strong in vivo phenotype of gzm B +/- NK cells. One possible explanation is that the pool of NK cells is heterogeneous, ie, armed with different effector molecules. Similarly, individual NK cells may have redundant pathways (eg, Fas or other granzymes) that are used in vivo, but escape detection in an in vitro assay using homogenous targets such as YAC-1 cells. However, Fas is unlikely to account for gzm B-independent NK cytotoxicity, because Fas ligand-deficient NK cells have normal activity in lytic assays against NK susceptible target
cells (S. Shresta and T.J. Ley, unpublished observations). Furthermore, allogeneic resistance was recently shown to be unaffected by null mutations of perforin and Fas. This experiment provides strong independent confirmation of our observation that mutations that fully disarm NK cells have no effect on hybrid resistance. Finally, the secretion of the cytotoxic cytokines TNFα or γIFN do not seem to account for the gzm B-independent hybrid resistance we have observed, although the in vivo administration of antibodies does not conclusively rule out the possibility that some biologically active cytokine may remain. In summary, it appears that hybrid resistance remains intact when NK cells are fully disarmed.

Alternatively, the paradigm that NK cells mediate hybrid resistance may be incorrect. Complementary experiments from Aguila and Weissman support this conclusion. These investigators found that a sorted population of NK1.1+ cells from an H-2 hybrid or allogeneic background were unable to lyse hematopoietic stem cells in vitro. Furthermore, transgenic animals overexpressing a "gzm A-driven" diphtheria toxin gene had no alteration in hybrid resistance, although these animals contain little, if any, NK activity. This provides strong evidence that NK cells are not essential for hybrid resistance. In this case, another radioresistant immune effector cell (such as the macrophage) may account for hybrid and/or allogeneic resistance. Although it is clear from our work and the work of Aguila et al that hybrid resistance is not altered when NK cells are disarmed or deleted, it remains a formal possibility that NK cells are the primary mediators of hybrid resistance in the intact animal and that an alternative effector cell with allogeneic restriction is able to fully compensate in the absence of an active NK compartment.

Genetic models such as these may ultimately lead to a complete understanding of the molecular basis of immune effector function. Hopefully, this will provide new therapeutic strategies for the clinical problems of GVHD and graft rejection.

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