The Role of Cell-mediated Cytotoxicity in Acute GVHD after MHC-Matched Allogeneic Bone Marrow Transplantation in Mice

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Summary

The role of cell-mediated cytotoxicity in the complex pathophysiology of graft-versus-host disease (GVHD) has remained poorly defined for several decades. We transplanted T cells from Fas-ligand (FasL)-defective and perforin-deficient mutant donor mice into lethally irradiated MHC-matched allogeneic recipient mice to characterize the role of cell-mediated cytotoxicity in GVHD. Although recipients of allogeneic FasL-defective donor T cells underwent severe GVHD-associated cachexia, they exhibited only minimal signs of hepatic and cutaneous GVHD pathology. Recipients of perforin-deficient allogeneic donor T cells developed signs of acute GVHD, but the time of onset was significantly delayed. These findings demonstrate that Fas-mediated anti-recipient cytotoxicity may be critical for the development of hepatic and cutaneous GVHD, but is not required for GVHD-associated cachexia. In addition, perforin-mediated anti-recipient cytotoxicity appears to play an important role in the kinetics of GVHD pathophysiology, but is not required for GVHD-associated tissue damage.

Allogeneic bone marrow transplantation (BMT) has greatly expanded as a clinical treatment modality for several disorders of hematopoiesis and certain hematological malignancies (1). Graft-versus-host disease (GVHD) remains a principal complication following allogeneic BMT occurring in up to 75% of recipients of unmanipulated HLA-matched marrow (2). The immunopathophysiology of GVHD is complex, and is generally considered to involve two phases: an afferent (inductive) phase, and an efferent (effector) phase (3). In the afferent phase, mature T cells present in the donor marrow inoculum recognize antigenic disparities expressed on recipient tissues resulting in alloactivation and proliferation of the allogeneic donor T cells. In the efferent phase, inflammatory reactions may develop in specific host target tissues such as skin, liver, and gastrointestinal tract that are characterized by mononuclear cell infiltration and histopathological damage (4). Studies of experimental models of allogeneic BMT using T cell-depletion have demonstrated that mature T cells must be present in the donor marrow inoculum in order to induce GVHD, and several clinical studies have confirmed this finding (5, 6). However, the precise role of T cell-mediated anti-recipient cytotoxicity in the pathophysiology of GVHD remains controversial (7–13).

Recently, it has been demonstrated that perforin-dependent cytolysis and Fas-mediated apoptosis together constitute the major mechanisms of short-term T cell-mediated cytotoxicity (14–16). T cells from mice which are homozygous for the gld (generalized lymphoproliferative disease) mutation are known to express a functionally defective Fas ligand (FasL) molecule which is not capable of transducing an apoptotic signal to Fas-bearing target cells (17). The perforin-deficient (perforin 0/0) mutant mouse strain was developed by homologous recombination in a B6 embryonic stem cell line, and bred to homozygosity on a C57BL/6 background (18). We have transplanted T cells from FasL-defective (gld) and perforin-deficient (perforin 0/0) donor mice into lethally irradiated MHC-matched allogeneic recipient mice to determine the role of these cytotoxic pathways in acute GVHD.

The present studies demonstrate that Fas-mediated cytotoxicity plays an essential role in the pathophysiology of hepatic and cutaneous GVHD, but is not required for GVH-induced cachexia. Furthermore, the absence of perforin-mediated cytotoxicity significantly delays the onset of GVHD, but does not prevent or diminish GVHD. These results suggest that certain local processes of GVHD such as tissue damage are separable from the systemic process of cachexia.

Abbreviations used in this paper: BMT, bone marrow transplantation; DN, double negative; FasL, Fas ligand; GVHD, graft-versus-host disease.

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In addition, the Fas-mediated and perforin-dependent cyto
toxic pathways appear to act during different stages in the
complex pathophysiology of GVHD. The role of inflam-
matory cytokines is discussed.

Materials and Methods

Mice. C57BL/6J (H-2b), B6.SnnC3H-gld (H-2b), LP/J (H-2b),
and C3H.SW (H-2b) mice were obtained from Jackson Labora-
tory (Bar Harbor, ME). Perforin-deficient C57BL/6 mice (B6-
perforin 0/0) were obtained from D. Kägi and H. Hengartner
(University of Zürich, Switzerland) and B. Ledermann and K.
Bürki (Sandoz Pharma Ltd., Basel, Switzerland). B6-perforin 0/0
mice were propagated at the University of Miami School of
Medicine Specialized Animal Facility and were maintained in a
pathogen-free colony until use.

Preparation of Cells. Bone marrow cells were aspirated from
the femurs and tibias of donor mice. T cells were depleted from the
bone marrow by incubation with anti-Thy1.2 mAb (30-H-12
culture supernatant) at 1:5 dilution and 4°C for 30 min followed
by Low-Tox M complement (Accurate Chemical Co., West-
bury, NY) at 1:20 dilution and 37°C for 45 min. Spleen and
lymph node cells were harvested, pooled, and treated with anti-
B220 mAb (14.8 culture supernatant) at 1:2.5 dilution and 4°C
for 30 min, followed by a secondary mouse anti-rat mAb (18.5
ascites) at 1:50 and 4°C for an additional 30 min. The labeled
cells were then treated with rabbit complement at 1:10 dilution
and 37°C for 45 min to remove B cells and enrich for T cells. This
procedure routinely enriches the T cell population to levels of
purity between 75-80% as determined by flow cytometric analysis.

Assay for GVHD. Recipient mice were exposed to 900 cGy
TBI from a 60Co source at a dose rate of 50 cGy/min 24 h before
the BMT. In murine models of GVHD, precise numbers of ma-
ture allogeneic donor T cells are routinely added together with
bone marrow to induce lethal acute GVHD. The severity of
GVHD correlates directly with the number of donor T cells transplanted (5). The number of donor T cells was selected based upon the ability of the wild-type inoculum to induce characteris-
tic signs of severe acute GVHD with an incidence of 100% in ei-
ther donorrecipient strain combination. We transplanted 1 × 107
donor T cells in the B6→LP combination, and 2 × 107 donor T
cells in the B6→C3H.SW combination. To ensure reproducible
results, the number of CD3+ T cells in the donor inoculum was
precisely quantified by flow cytometry for each BMT. Precisely
the same numbers of CD3+ T cells from wild-type, perforin-
deficient, or FasL-defective B6 donors were added to the T cell-
depleted bone marrow cells (5×10^5) from normal wild-type
B6 donors and injected together into irradiated recipient mice in-
travenously via the lateral tail vein in a volume of 0.5 cc. Mice re-
cieving transplants were distributed into groups containing 4–8
mice per group in each experiment. Recipients were maintained on
acidified water (pH 3.0) containing antibiotics (100 mg/L neomy-
cin sulfate, 10 mg/L polymyxin B) from day –3 to day 14 post-
BMT. Recipient mice were monitored for clinical signs of GVHD
including weight loss, skin lesions, alopecia, diarrhea, hunched
posture, and mortality. Representative mice were killed at various
times post-BMT to harvest tissues for histopathological analysis.

Histopathology. Skin and liver sections were harvested from
recipient mice at various intervals after BMT. Tissues were placed in
10% buffered formalin phosphate (Fisher Scientific, Orlando, FL).
The fixed tissues were paraffin embedded, sectioned, and stained
with hematoxylin and eosin by the core service of the Depart-
ment of Comparative Pathology at the University of Miami School
of Medicine. Slides were coded and examined in a blinded fash-
on by NHA, and the tissue histology was graded.

Immunophenotyping. Pooled spleen and lymph node cells were
stained with FITC-conjugated anti-CD3 mAb (145-2C11) or bi-
otinylated anti-B220 mAb (RA3-6B2) obtained from PharMingen
(San Diego, CA). Briefly, 0.5–1 × 10^6 cells were washed in
FACS® buffer (PBS with 1% BSA, 0.02% sodium azide), then
incubated with the FITC-conjugated and biotinylated mAbs for 20
min on ice. Cells were again washed in FACS® buffer and in-
cubated with streptavidin-PE (Fisher Scientific, Orlando, FL). The
stained cells were resuspended in FACS® buffer at 2 × 10^4/ml
and analyzed on a FACScan® flow cyometer (Beckton Dickinson,
San Jose, CA). Data was analyzed within a gate established for lymph-
cocytes using forward (180°) and side (90°) angle light scatter.

Lymphocyte Stimulation Assay. The enriched donor T cell pop-
ulations were cultured 2 d in the presence of either soluble anti-CD3
mAb (2C11-145 culture supernatant) at 10% (vol/vol) or 5 µg/ml
concanavalin A (Sigma Chemical Co., St. Louis, MO) to induce
cellular proliferation. Mixed lymphocyte reactions were performed in
which the enriched donor T cells were cultured 5 d at a 1:1 ratio
with irradiated whole C3H (H-2b) spleen cells to assess alloreac-
tivity. Proliferative responses were determined by measuring [3H]-thy-
midine incorporation following a 6–8-h pulse label period.

Results

Donor T Cells from B6-gld Mice Are Phenotypically and Func-
tionally Normal. Mice homozygous for the gld mutation develop
lymphadenopathy characterized by progressive accumu-
lution of functionally anergic B220+, CD4−, CD8−
double negative (DN) T cells with significant numbers ap-
pearing after 6 wk of age (19, 20). To ensure that we were not
transplanting significant numbers of non-functional
B220+ DN T cells, we used B6-gld mice which were 5–6 wk
of age as donors. In addition, all detectable DN T cells and most B
cells were removed by treating spleen and lymph
node cells with anti-B220 mAb (14.8) and complement that concurrently enriched the phenotypically normal T
cells (Fig. 1 A). Notably, the RA3-6B2 anti-B220 mAb
used for immunophenotyping recognizes an epitope that is
distinct from the epitope recognized by the 14.8 anti-B220
mAb, and binding of 14.8 does not block subsequent bind-
ing of RA3-6B2 (21). Syngeneic donor mice, wild-type al-
logeneic donor mice, and perforin-deficient donor mice
were 6–8 wk of age. All donor T cell populations were
prepared in the same fashion, and exhibited similar cellular
subpopulations as determined by phenotypic analysis (data
not shown).

To confirm that the enriched T cell population purified
from the gld donor mice was functionally competent, these
cells were cultured in vitro in the presence of either soluble
anti-CD3 mAb (2C11-145), concanavalin A, or irradiated
allogeneic (C3H) spleen cells. The proliferative responses of the
gld T cells to these polyclonal and alloantigen-spe-
cific stimuli was within normal limits compared to wild-type
B6 T cells (Fig. 1, B and C).
Figure 1. Phenotypic and functional analysis of B6-gld T cells. (A) Flow cytometric analysis of pooled spleen and lymph node cells from wild-type B6 and B6-gld donor mice before and after treatment with anti-B220 (14.8) mAb and complement. The abnormal CD3+B220+ lymphocytes represent only a small percentage (3.1%) of spleen and lymph node cells from B6-gld donor mice before treatment, and these cells are absent after treatment. The CD3+B220- T cell populations are enriched following this procedure. (B) Polyclonal activation of enriched donor T cell populations. The normal B6 and B6-gld T cells were cultured 2 d in the presence of either soluble anti-CD3 (2Cl1-145) mAb culture supernatant at 10% (vol/vol) or 5 μg/ml concanavalin A. Proliferative responses by both T cell populations were within normal limits. Data are shown from a single representative experiment, and columns represent mean incorporation values among quadruplicate wells. (C) Mixed lymphocyte reaction by the T cell-enriched donor lymphocyte populations. The B6 and B6-gld donor T cells were cultured 5 d at a 1:1 ratio with irradiated C3H (H-2k) spleen cells as a source of alloantigen. Both T cell populations responded similarly to allogeneic stimulation.

plant experiment. All LP recipients of syngeneic T cells and marrow survived and regained normal body weight shortly after the transplant (Fig. 2). In contrast, all LP recipients of wild-type allogeneic B6 T cells developed acute GVHD with severe weight loss and 100% mortality (Fig. 2). Recipients of FasL-defective B6-gld T cells also exhibited severe weight loss and 100% mortality, indicating that they were undergoing a systemic graft-versus-host reaction (Fig. 2).

Mice of the C3H.SW (H-2b) strain were transplanted to confirm the finding that FasL-defective T cells are capable of inducing a systemic GVH reaction in a second MHC-matched allogeneic donor/recipient strain combination. Again, all C3H.SW recipients of syngeneic T cells and marrow regained normal body weight rapidly after transplant. In contrast, all (100%) recipients of wild-type B6 or B6-gld T cells exhibited severe cachexia (data not shown).

Recipient mice were monitored for clinical signs of acute GVHD following the transplant. Neither C3H.SW (Fig 3A) nor LP recipients of syngeneic T cells ever developed any clinical signs of GVHD. The clinical appearance of mice that received wild-type allogeneic B6 T cells was characteristic of acute GVHD including marked weight loss, hunched posture, desquamative rash, and patchy alopecia (Fig. 3B). In striking contrast, none of the mice receiving FasL-defective B6-gld T cells ever exhibited any signs of skin or coat involvement at any time after BMT although all developed severe weight loss (Fig. 3C). Results of clinical observations of groups of C3H.SW and LP mice from five...
independent experiments confirm an absence of clinical cutaneous GVHD (0/32) despite the presence of cachexia (32/32) following BMT with FasL-defective donor T cells (Table 1).

**Marked Delay of GVHD and Mortality after Transplantation of Perforin-Deficient Allogeneic T Cells.** An MHC-matched model of allogeneic BMT was employed to determine whether perforin-deficient T cells could induce severe acute GVHD across minor histocompatibility barriers. Lethally irradiated LP (H-2^b^) mice were transplanted with 1 × 10^7^ T cells and bone marrow from wild-type or perforin-deficient B6 (H-2^b^) donor mice. Recipients of syngeneic (LP) T cells maintained normal body weight and did not develop signs of GVHD (Figs. 4 and 5, A). The mice receiving wild-type B6 T cells developed signs of severe acute GVHD within 28 d after the transplant (Figs. 4 and 5, B). The recipients of T cells from B6-perforin 0/0 donors also developed signs of severe acute GVHD including weight loss (Fig. 4 B), alopecia, hunched posture, diarrhea, and desquamative skin rash with an incidence of 100% (Fig. 5 C). However, the average time of onset of these signs was delayed approximately twofold compared to the recipients of wild-type cells. The delay of onset in the recipients of perforin-deficient T cells was also apparent in the kinetics of mortality. There was a more than twofold increase in the mean survival time (65.3 vs 23.7 d) in the perforin-deficient recipient group compared to the wild-type recipient group (Fig. 4 A). Results from seven independent experiments using either C3H.SW or LP strain mice as recipients confirmed that transplantation of perforin-deficient allogeneic T cells could significantly delay GVHD and increase survival time.
Recipient mice were examined thrice weekly for clinical signs of acute GVHD including cachexia, alopecia, and desquamative rash. Results are expressed as the number of mice exhibiting signs compared to the total number of mice examined. Notably, the recipients of FasL-defective B6-gld T cells all developed cachexia, but none exhibited any signs of alopecia or rash throughout the study.

T cells uniformly produces acute GVHD with a significant delay in time of onset (Table 1).

Cutaneous GVHD Is Markedly Diminished in the Absence of FasL-mediated but Not Perforin-mediated Anti-Recipient Cytotoxicity. Skin sections were harvested from representative mice at 28 d post-transplant to determine if the absence of perforin or Fas-mediated anti-recipient cytotoxicity would effect the incidence or severity of GVHD-associated pathology. Cutaneous sections from recipients of syngeneic T cells were unremarkable (Fig. 6 A). Skin sections from C3H.SW recipients of 2 × 10^7 wild-type B6 T cells exhibited severe inflammation with mononuclear cell infiltrates, dermal fibrosis, loss of hair follicles, and epidermal hypertrophy consistent with cutaneous GVHD (Fig. 6 B). In marked contrast, skin from C3H.SW recipients of B6-gld T cells exhibited evidence of only minimal inflammation (Fig. 6 C). On day 52, cutaneous sections from recipients of perforin-deficient B6 T cells exhibited alterations that were identical in character and severity to those observed in recipients of wild-type B6 T cells on day 28 (Fig. 6 D). A second MHC-matched donor/recipient strain combination (B6→LP) was employed to confirm this differential pattern of tissue damage after transplantation of cytotoxicity-defective T cells. These studies also demonstrated that cutaneous GVHD develops in the absence of perforin-mediated anti-recipient cytotoxicity, but is markedly reduced in the absence of FasL-mediated anti-recipient cytotoxicity (data not shown).

Hepatic GVHD Is Markedly Diminished in the Absence of FasL-mediated but Not Perforin-mediated Anti-Recipient Cytotoxicity. Liver sections were harvested from recipients at various time intervals following transplantation to investigate how the absence of Fas and perforin-mediated cytotoxicity affected the pathophysiology of hepatic GVHD. Sections of liver from C3H.SW recipients of syngeneic T cells 28 d post-transplant were unremarkable (Fig. 7 A). Hepatic sections from recipients of 2 × 10^7 wild-type B6 T cells exhibited a marked infiltrate of predominantly PMNs around bile ducts with associated partial inflammation and fibrosis that was evaluated as severe acute cholangitis consistent with hepatic GVHD (Fig. 7 B). In marked contrast, liver sections from C3H.SW recipients of B6-gld T cells exhibited evidence of only minimal involvement (Fig. 7 C). It is important to note that at no time after transplant of B6-gld T cells did the liver sections of these recipients exhibit significant hepatic pathology. On day 52, hepatic tissues from recipients of perforin-deficient B6 T cells exhibited severe cholangiohepatitis (Fig. 7 D). A second MHC-matched donor/recipient strain combination (B6→LP) was employed to confirm this differential pattern of tissue damage.
after transplantation of the cytotoxically defective T cells. These studies also demonstrated that hepatic GVHD develops in the absence of perforin-mediated anti-recipient cytotoxicity, but is markedly reduced in the absence of FasL-mediated anti-recipient cytotoxicity (data not shown).

There is evidence in the literature that epithelial damage in gastrointestinal GVHD lesions is characterized by apoptosis and DNA fragmentation (22). Therefore, we were interested to examine the role of Fas-mediated cytotoxicity in gastrointestinal GVHD. Clinical evidence of diarrhea was observed in some but not all recipients of wild-type, FasL-defective, and perforin-deficient allogeneic T cells. In addition, histopathological evidence of acute colitis and enteritis was occasionally observed in recipients of wild-type, FasL-defective, and perforin-deficient allogeneic T cells (data not shown). Therefore, defects in individual cytotoxic pathways did not consistently abrogate either clinical or histopathological sequelae associated with gastrointestinal GVHD.

Recipients of FasL-defective Allogeneic T Cells Do Not Exhibit Lymphoid Atrophy. Recipient splenic lymphocytes were harvested and analyzed by flow cytometry 28 d after transplant to examine the status of the lymphoid compartment. Table 2 shows the numbers of spleen cells recovered from these recipients. Mice receiving wild-type or perforin-deficient allogeneic T cells exhibited profoundly decreased spleen cell recoveries that are typically observed in murine models of acute GVHD (Table 2). Notably, recipients of FasL-defective T cells exhibited a somewhat increased spleen cell recovery, but did not exhibit evidence of uncontrolled lymphoproliferative disease (Table 2). These findings have been observed in seven independent transplantation experiments.

Mice that received syngeneic (C3H.SW) T cells exhibited normal percentages of B220+ cells (54.0%) and CD3+ cells (23.1%) in the spleen (Fig. 8 E). Light scatter profiles of spleen cells from recipients of wild-type allogeneic T cells (Fig. 8 F) showed a markedly reduced lymphoid cell population (Region 1), which was comprised of 71.3% CD3+ T cells (Fig. 8 F). This pattern is consistent with acute GVHD (23). A very similar pattern of lymphoid hypoplasia and spleen cell phenotype was observed in spleens from recipients of perforin-deficient allogeneic T cells in which 81.2% of the gated cells were CD3+ (Fig. 8 C and G). In contrast, the degree of lymphoid hypoplasia was less severe in the recipients of FasL-defective T cells as determined by light scatter (Fig. 8 D). In addition, the percentage of B220+ cells was increased to 28.0% in the recipients of FasL-defective T cells (Fig. 8 H) compared to 2.6% in the recipients of wild-type T cells. However, as expected the percentage of CD3+ T cells (54.2%) was elevated in the recipients of FasL-defective T cells consistent with a GVHD-associated expansion of alloreactive donor T cells.

Discussion

The role of cell-mediated anti-recipient cytotoxicity in the pathophysiology of GVHD has remained poorly defined and controversial for several decades. To begin resolving the long standing controversy regarding the role of anti-recipient specific cell-mediated cytotoxicity in GVHD, we have compared the ability of FasL-defective, perforin-deficient, and wild-type allogeneic T cells to induce severe acute GVHD across non-MHC antigenic barriers in mice. The results of the present study demonstrate that Fas-mediated cytotoxicity plays an important role in the pathophysiology of hepatic and cutaneous GVHD after BMT between MHC-matched allogeneic mice. However, Fas-mediated anti-recipient cytotoxicity is not required for the induction of GVHD-associated cachexia. Furthermore, transplantation of perforin-
deficient T cells results in a marked delay in onset of GVHD and mortality compared to GVHD induced by wild-type allogeneic T cells. However, severe acute GVHD ultimately does develop with all of the classical hallmarks of GVHD including hunched posture, weight loss, alopecia, rash, cholangitis, and dermatitis.

The morphological and ultrastructural features of lesions in hepatic, cutaneous, and gastrointestinal GVHD have previously been reported to involve some degree of individual cell necrosis, pyknotic nuclei, and apoptotic bodies consistent with a role for apoptosis in GVHD-associated tissue damage (24–26). In addition, both liver and skin tissues are known to express Fas (27), and mice injected with the anti-Fas monoclonal antibody Jo2 rapidly develop a fulminant lethal hepatitis (28). Furthermore, it has been reported recently that expression of Fas is upregulated in epidermal tissues undergoing certain inflammatory conditions (29). Thus, skin and liver could be potential targets for FasL-bearing cytotoxic donor T cells after allogeneic BMT. Consistent with our findings that GVHD-associated cholangitis and dermatitis is markedly reduced in the absence of Fas-mediated cytotoxicity, we propose that this pathway may be an essential step in the multi-step process that ultimately leads to GVHD-associated inflammation and tissue damage in

Figure 6. Histopathological analysis of skin from C3H.SW recipient mice 28 d after BMT with 2 × 10^7 T cells. Representative recipient mice were sacrificed and skin sections were harvested for histopathological analysis. (A) Skin from recipients of syngeneic (C3H.SW) T cells were unremarkable. (B) Analysis of skin from recipients of wild-type allogeneic (B6) T cells exhibited severe inflammatory infiltrates, dermal fibrosis, loss of hair follicles, and epidermal hypertrophy consistent with cutaneous GVHD. (C) Cutaneous sections from recipients of FasL-defective allogeneic (B6-gld) T cells exhibited evidence of minimal inflammatory changes or no involvement. (D) Skin from recipients of perforin-deficient allogeneic (B6-perforin 0/0) T cells on day 52 post-transplant exhibited severe inflammatory infiltrates, dermal fibrosis, loss of hair follicles, and epidermal hypertrophy consistent with cutaneous GVHD.
recipient liver and skin tissues. However, the findings in the present study do not suggest that Fas-mediated cytotoxicity is the only effector mechanism contributing to hepatic and cutaneous GVHD.

While hepatic and cutaneous pathology was markedly reduced in recipients of FasL-defective donor T cells, these recipients exhibited severe cachexia and lost on average 35% of their initial body weight and virtually all body fat by day 28 post-BMT. Tumor necrosis factor (TNF-α, cachectin) produced by alloactivated FasL-defective donor T cells could account for the profound wasting and mortality observed in the recipients of the B6-gld T cells. TNF-α has been identified as a principal mediator of cachexia in rodents (30). In addition, serum levels of TNF have been shown to be increased in patients undergoing GVHD after allogeneic BMT (31). Notably, Piguet et al. demonstrated that administration of anti-TNF-α anti-sera markedly reduces recipient weight loss and mortality in a mouse model of GVHD (32).

Despite significant work, it has remained difficult to reconcile the classical concept of MHC-restricted T cell-mediated cytotoxicity with the observation that highly purified T cell subset populations (CD4+ or CD8+) are equally capable of inducing identical GVHD pathology irrespective of class I or class II expression by the target tissues (33). Consistent with our results, one interpretation of the finding that either subset alone can induce identical

Figure 7. Histopathological analysis of liver from C3H.SW recipient mice 28 d after BMT with $2 \times 10^7$ T cells. Representative recipient mice were killed and liver sections were harvested for histopathological analysis. (A) Liver sections from recipients of syngeneic (C3H.SW) T cells were unremarkable. (B) Analysis of liver from recipients of wild-type allogeneic (B6) T cells demonstrated severe subacute cholangiohepatitis consistent with hepatic GVHD. (C) Hepatic sections from recipients of FasL-defective allogeneic (B6-gld) T cells exhibited evidence of minimal inflammatory changes or no involvement. (D) Liver sections from recipients of perforin-deficient allogeneic (B6-perforin 0/0) T cells on day 52 post-transplant exhibited severe subacute cholangiohepatitis consistent with hepatic GVHD.
Figure 8. Light scatter and phenotypic analysis of spleen cells from C3H.SW recipient mice 28 d after transplantation of 2 × 10⁷ T cells. Spleen cells were stained with anti-CD3-FITC (FL1) and anti-B220-biotin + avidin-PE (FL2), and analyzed within a gate established for lymphocytes using forward (FSC) and side (SSC) angle light scatter. (A and E) Spleen cells from recipients of syngeneic (C3H.SW) T cells exhibited a normal light scatter and phenotypic pattern of CD3+ and B220+ cells. (B and F) Analysis of spleen from recipients of wild-type allogeneic (B6) T cells demonstrated severe lymphoid hypoplasia and a predominance of CD3+ T cells. (C and G) Spleen cells from recipients of perforin-deficient allogeneic (B6-perforin 0/0) T cells exhibited a pattern of lymphoid hypoplasia and predominance of CD3+ T cells that was similar to the recipients of wild-type allogeneic T cells. (D and H) Analysis of spleen cells from recipients of FasL-defective allogeneic (B6-gld) T cells demonstrated less severe lymphoid hypoplasia (R.I) and increased numbers of B220+ cells compared to recipients of wild-type allogeneic T cells.

GVHD pathology is that cytotoxic T cells (CTL) of either phenotype may effect allogeneic cytotoxicity via FasL. Notably, it has recently been reported that CD4+ CTL may kill primarily through the Fas pathway (34).

It is important to note that we have observed no evidence of lpr-GVH or lymphoproliferative disease in recipients of B6-gld donor T cells. These recipients did not develop lpr-GVH-associated skin or liver inflammation, nor did they develop lpr-GVH-associated lymphoid aplasia (Figs. 6–8). The B6-gld T cells that were transplanted have a defect only in the Fas-ligand molecule resulting in an inability to kill through the Fas pathway. However, the B6-gld T cells express normal levels of functional Fas antigen, and therefore have the potential to be regulated or deleted by cells of host origin such as stromal cells that may be capable of expressing functional FasL (35). Thus, our observations are consistent with the established finding that transplantation of bone marrow from gld/gld mutant donors into wild-type recipients does not induce the lpr-GVH phenomenon (36). Moreover, the transplantation of wild-type B6 marrow in our studies results in the de novo generation of T cells that are able to express normal non-mutant FasL molecules (Baker, M.B. and R.B. Levy, manuscript in preparation). Therefore, wild-type donor marrow-derived T cells that express the non-mutant FasL could also regulate the gld T cells that express a functional Fas molecule.

The finding that recipients of perforin-deficient allogeneic T cells exhibited clinical and histopathological signs of GVHD equivalent in severity to the signs observed in recipients of wild-type allogeneic T cells conclusively demonstrates that perforin-mediated cytotoxicity is not a critical effector function during the effector phase of GVHD.

Table 2. Spleen Cell Recoveries from C3H.SW Mice 28 d after Receiving 2 × 10⁷ Donor T Cells

<table>
<thead>
<tr>
<th>Spleen Cell Recovery</th>
<th>CD3+ B220−</th>
<th>B220+ CD3−</th>
<th>CD3+ B220+</th>
</tr>
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<tbody>
<tr>
<td>C3H.SW→C3H.SW</td>
<td>41.3 × 10⁶</td>
<td>9.5 × 10⁶</td>
<td>22.3 × 10⁶</td>
</tr>
<tr>
<td>B6→C3H.SW</td>
<td>6.9 × 10⁶</td>
<td>4.9 × 10⁶</td>
<td>0.18 × 10⁶</td>
</tr>
<tr>
<td>B6-perforin 0/0→C3H.SW</td>
<td>5.2 × 10⁶</td>
<td>4.2 × 10⁶</td>
<td>0.14 × 10⁶</td>
</tr>
<tr>
<td>B6-gld→C3H.SW</td>
<td>12.6 × 10⁶</td>
<td>6.8 × 10⁶</td>
<td>3.5 × 10⁶</td>
</tr>
</tbody>
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Lymphocyte subpopulation numbers are calculated based on percentages derived from phenotypic analysis (see Fig. 8). Significantly increased numbers of phenotypically normal B cells (B220+CD3−) are recovered from recipients of FasL-defective allogeneic T cells compared to recipients of wild-type and perforin-deficient allogeneic T cells. Notably, mice receiving B6-gld T cells did not exhibit an abnormally expanded population of B220+ DN T cells (B220+CD3+).
We conclude that perforin-mediated donor anti-host cytotoxic function is not an absolute requirement for the development of GVHD across minor histocompatibility barriers.

While perforin-deficient allogeneic T cells are clearly capable of inducing severe acute GVHD, the recipients of these cells have consistently exhibited a significant delay in the time of onset of clinical signs associated with GVHD and a prolonged MST. This finding suggests that perforin-mediated cytotoxicity may be playing an important role in the early post-transplant period during the afferent phase of GVHD that could lead to a shift in kinetics without diminishing the ultimate severity of tissue damage and clinical signs of GVHD. Notably, preliminary results in our laboratory suggest that transplantation of perforin-deficient T cells at a dose twofold higher than the wild-type T cells results in onset of GVHD with no delay compared to the recipients of the lower dose of normal cells. In addition, we have found that when very low numbers of bone marrow-derived perforin-deficient T cells are transplanted the onset of GVHD is delayed indefinitely (37).

One potential explanation for the delayed onset of GVHD in recipients of perforin-deficient T cells is that perforin-mediated cytotoxicity may accelerate and amplify the donor anti-host immune reaction. This may occur as a result of the release of inflammatory cytoplasmic contents following cytolysis of host target cells. When perforin-mediated anti-host cytolytic activity is absent, the resulting alloaggressive reaction might be slowed. Alternatively, perforin-mediated donor anti-host cytotoxic activity may be critical for overcoming residual host resistance in the recipient. Thus, in the absence of perforin-mediated anti-host cytotoxicity, host resistance would remain stronger and persist longer in the recipient diminishing the relative alloaggressive capacity of the perforin-deficient inoculum.

One advantage of employing genetically modified or naturally occurring mutant mouse strains as a source of donor T cells is that only one cytotoxic pathway has been selectively abrogated leaving the other cytotoxic effector functions intact. Cytotoxic T cells and NK cells from perforin-deficient mice exhibit profoundly diminished in vitro cytolytic function, but retain the ability to effect Fas-mediated and TNFα-mediated killing (15). Alternatively, the Fas-ligand molecule expressed by T cells from gld mice is non-functional, but perforin-dependent cytolytic function remains intact. Therefore, these experiments were able to directly examine the role of each cytotoxic pathway by its absence, while simultaneously confirming the function of the other by its presence. Accordingly, consistent with the marked diminishment of hepatic and cutaneous GVHD following transplantation of FasL-defective T cells, GVHD developed in these tissues following transplantation of perforin-deficient T cells because Fas-mediated cytotoxic function remains intact. Another advantage of employing T cells with molecular defects in cytolytic function is that the defect is present in every donor cell of all phenotypes regardless of how these populations may interact and contribute to the development of GVHD.

Previous studies designed to examine effector functions in GVHD have employed transplantation of cell populations that have been negatively selected on the basis of immunophenotype (e.g., CD8+, CD4+, NK1.1+ cells). In studies involving negative selection, all potential cytotoxic and cytokine effector functions of the depleted cell population are removed along with the negatively selected population. In contrast, the present study is designed so that the potential to produce multiple inflammatory cytokines such as IL-1, IFN-γ, and TNFα by the cytotoxically defective T cells remains intact. These cytokines are known to be produced by alloactivated donor T cells during GVHD, and appear to contribute significantly to both inductive and effector phases of GVHD (38).

The results of the present studies represent the first characterization of the role of Fas and perforin-mediated anti-recipient cytotoxic function in the pathogenesis of GVHD after allogeneic bone marrow transplantation across non-MHC genetic disparities. These results demonstrate that Fas-mediated cytotoxicity is required for the development of hepatic and cutaneous GVHD, but is not required for GVHD-associated cachexia. This finding has shown that local and systemic effects of GVHD are separable. Furthermore, the results also demonstrate that absence of perforin-mediated anti-recipient cytotoxicity does not prevent or diminish GVHD, but significantly delays the time of onset. We interpret this finding to indicate that while perforin-mediated cytotoxic function is not required for the effector phase of GVHD, this cytotoxic pathway appears to play a significant role during the inductive phase of GVHD. We conclude that the two major pathways of cell-mediated cytotoxicity play distinct roles in the complex pathophysiology of GVHD, and that these roles appear to be expressed during different stages of GVHD pathogenesis.

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