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The Contribution of Cytotoxic and Noncytotoxic Function by Donor T-Cells That Support Engraftment after Allogeneic Bone Marrow Transplantation

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ABSTRACT

The present studies were designed for investigation of the requirements for cytotoxic function in donor T-cells transplanted to support engraftment after infusion of allogeneic bone marrow. The experiments examined the capacity of donor CD8$^+$ T-cells lacking Fas ligand and/or perforin function to facilitate donor B6 congenic (B6-Ly5.1) BM engraftment across major histocompatibility complex class I/II barriers after transplantation. T-cell–depleted BM cells from B6-Ly5.1 donors were transplanted into sublethally irradiated (5.5 Gy) BALB/c recipients together with different lymphocyte populations from wild-type B6 (B6-wt) donors or donors lacking functional cytotoxic pathways. Early presence of lineage-committed donor progenitor cells was assessed by the presence of day 5 splenic colony-forming units–granulocyte-macrophage (CFU-GM). Recipients of BMT without donor T-cells did not demonstrate significant CFU-GM activity 5 days post-BMT. Lineage-committed progenitor cells in recipient spleens could be supported by addition to the BM of wild-type (B6-wt) and cytotoxically single- (perforin, B6-pko or FasL, B6-gld) or double-deficient (B6-cdd) CD8$^+$ T-cells. However, B220$^+$-enriched B-cells could not support the presence of day 5 donor CFU-GM. For further assessment of the capacity of cytotoxically impaired T-cells to participate in the engraftment process, the ability of these and normal CD8 cells to support the homing of donor cells to the BM was examined after infusion of carboxyfluorescein diacetete succinimidyl ester–labeled progenitors. In a syngeneic model lacking resistance, cytotoxically impaired donor T-cells supported increased numbers of progenitor cells in the marrow equivalent to the support provided by wild-type donor T-cells. Examination of peripheral chimerism indicated that during the first month after B6→BALB/c BMT, donor chimerism was detected in BMT recipients receiving unfractionated T-cells or CD8$^+$ T-cells from B6-wt donors, and chimerism was maintained at least 80 days after BMT. In contrast, B6-cdd unfractionated or CD8$^+$ T-cells failed to maintain long-term B6 donor chimerism in the host. Experiments with highly enriched populations of positively selected CD8$^+$ T-cells from B6-pko, B6-gld, or B6-cdd donors demonstrated that although each of these T-cell populations could promote the initial presence of donor CFU-GM early post-BMT, B6-pko and B6-cdd CD8$^+$ T-cell populations were not able to support long-term peripheral chimerism. These results demonstrate that donor T-cells lacking major cytotoxic effector pathways have functions that support initial donor progenitor cell presence in the host hematopoietic compartment after BMT. They also demonstrate that support of long-term donor BM engraftment requires CD8$^+$ T-cells with intact cytotoxic, that is, perforin, function. Finally, syngeneic B6→B6 BMT suggests activation of CD8$^+$ T-cells posttransplantation apparently is required to support enhanced progenitor cell activity. This study provides new findings concerning the role of cytotoxic function in the process of facilitating allogeneic donor BM engraftment.

KEY WORDS

CD8$^+$ T-cell cytotoxicity • Engraftment • Facilitation • Bone marrow transplantation

INTRODUCTION

Both experimental and clinical studies have demonstrated that depletion of T-cells from allogeneic donor bone marrow (BM) frequently results in graft failure. The findings support a role for donor T-cell–mediated functions in successful BM engraftment [1]. Two mechanisms that are not mutually exclusive have been proposed by which T-cells may support donor marrow engraftment: (1) donor T-cells can provide necessary lymphokines for donor stem cell differentiation and proliferation [2-4] and (2) donor T-cells can prevent grafted stem cells from being rejected by exerting effector function and eliminating residual host resistance [5]. Engraftment following progenitor cell transplantation appears to take place in 2 phases. Rapid reconstitution early post-BMT followed by a slower but more stable long-term reconstitution has been observed [6]. Some investigators have proposed that the early reconstitution was established by lineage-committed progenitor cells whereas the long-term engraftment was due to the multipotential hematopoietic stem cells (HSC) contained in the transplant. Other investigators, however, have reported that both early and long-term reconstitution can result from the engraftment of donor HSC, not colony-forming committed progenitor cells [7,8].

The requirements for achieving early and long-term reconstitution of donor marrow cells remain to be clearly defined. In this study, a colony-forming assay was used for analysis of the initial seeding into H-2^d recipients of allogeneic H-2^b lineage-committed progenitor cells on days 5 and 12 post-BMT. Long-term reconstitution was evaluated by phenotypic analysis of the donor/host origin of circulating peripheral-blood mononuclear cells (PBMC). Addition of donor T-cells with defined cytotoxic deficiencies to T-cell–depleted (TCD) marrow inoculum enabled evaluation of the role of both Fas ligand and perforin-mediated lytic pathways in promoting the initial seeding of allogeneic donor marrow and long-term engraftment. The results demonstrated that initial seeding and early presence of lineage-committed progenitor cells could be accomplished without the presence of both perforin and Fas ligand function in donor lymphocytes. However, some T-cell function was clearly required for lineage-committed progenitor cell presence posttransplantation because addition of a donor $B220^+$ B-cell–enriched population failed to promote the appearance of donor colony-forming units (CFU) in recipient spleens. To remove the requirement to overcome resistance post-BMT, we performed experiments with a syngeneic model to assess whether cytotoxically impaired T-cells could support the early presence of donor progenitor cells in the host marrow compartment. Such perforin/FasL defective T-cells functioned equivalently to normal T-cells in ability to augment the numbers of donor cells found in the marrow early posttransplantation. In contrast, long-term allogeneic hematopoietic reconstitution required cytotoxic function, specifically perforin function by donor T-cells added to promote marrow engraftment. These results support the notion that facilitating cells employ multiple functions to support the engraftment process. The findings may also suggest that differing functions of "facilitating" T-cells may be important during different phases of the engraftment process.

MATERIALS AND METHODS

Mice

Six- to 8-week-old female BALB/c, B6.SJL-$CD45^a Pep^b$/BoyJ (B6-Ly5.1), and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6Smn.C3H-*Tnfsf6*^{gld}, FasL mutant (B6-gld), and C57BL/6-*Pfp*^{tm1Sdz}, perforin knock-out mice (B6-pko) were originally provided by Dr. Eckhard Podack. B6-GFP [Tg14(act-EGFP)OsbY01] mice were originally obtained from Dr. Masaru Okabe (Osaka University, Osaka, Japan). C57BL/6 cytotoxically double-deficient (perforin and Fas) mice (B6-cdd) were generated from the breeding of B6-*pfp*$^{+/-}$ *Fasl*$^{gld/gld}$ pairings as described previously [9]. To select for B6-*pfp*$^{-/-}$*Fasl*$^{gld/gld}$ (B6-cdd), we used a polymerase chain reaction to screen the offspring for homozygous perforin deficiency [10]. Six- to 8-week-old B6-cdd and B6-*pfp*$^{-/-}$ (B6-pko) mice maintained in pathogen-free conditions in the department of microbiology and immunology at the University of Miami School of Medicine were used as donors for all experiments.

BM Transplantation

A major histocompatibility complex (MHC) class I/II mismatched graft-versus-host disease model was used in which BALB/c mice (H-2^d) were recipients. One day before transplantation, BALB/c (or syngeneic H-2^b) recipients were conditioned with varying doses of total body irradiation (TBI) (cobalt 60 source; dose rate, 44.5 cGy/min). BM cells obtained by flushing of donor B6-Ly5.1 femurs and tibias were resuspended at a concentration of 2.5×10^7/mL in RPMI-1640 medium and incubated with anti-Thy1.2 murine antibodies (mAb) (HO13.2) at 4°C for 30 minutes. The cells were then washed and incubated at 37°C for 45 minutes in the presence of complement (Rabbit Low Tox-M, Cedarlane Laboratories, Hornby, Ontario, Canada). After being washed with RPMI-1640 medium, TCD BM cells were collected, and the viable cells were counted with trypan blue staining. TCD BM cells (5×10^6) from MHC class I/II mismatched B6-Ly5.1 donors were injected intravenously into BALB/c recipients alone (control) or with selected numbers of B6-wt, B6-pko, B6-gld, or B6-cdd T-cells or B-cells. A minimum of 2 BMT recipients per time point were used in each study.

Lymphocyte Preparation

T-cells were prepared from donor spleen and lymph nodes. B-cells were removed by 2 rounds of panning on anti-sIg coated plates. This procedure was followed by incubation with anti-B220 mAb (14.8) plus goat anti-rat mAb (18.5) and complement (Cedarlane). More than 85% purity of T-cells was obtained. Highly enriched $CD8^+$ T-cells were obtained by positive selection with the Miltenyi magnetic cell sorting system (MACS) (Miltenyi, Auburn, CA). Donor spleen and lymph node cells were suspended in phosphate-buffered saline solution (PBS) containing 0.5% bovine serum albumin (BSA) at 1×10^8/mL and labeled with anti-CD8 conjugated magnetic beads by incubation for 15 minutes at 4°C. The cells were then washed and resuspended in PBS/0.5% BSA and loaded onto a MACS separation column in a magnetic field. The unlabeled cells were removed by 3 washes with PBS/0.5% BSA, and $CD8^+$ cells were eluted from the column with the same buffer outside the magnetic field. In some experiments, B-cell populations were prepared by posi-

tive selection with the Miltenyi MACS system. Donor spleen cells were incubated with fluorescein isothiocyanate (FITC) anti-B220 for 15 minutes and then with anti-FITC conjugated magnetic beads for 15 minutes. The $B220^+$ B-cells were then positively selected by binding to the MACS column followed by elution. Enriched cell populations were stained with Cy-Chrome anti-CD8, phycoerythrin (PE) anti-CD4, and FITC anti-B220 (Pharmingen, San Diego, CA) mAbs and examined for purity by flow cytometric analysis with a FACScan system (Becton Dickinson, Franklin Lakes, NJ). The purity of T-cell and B-cell populations obtained after the enrichment protocol was always >90%.

Spleen Colony-Forming Assay

The CFU-GM and CFU–interleukin 3 (IL-3) assays were modified from a previously described method [11]. Recipients were killed on day 5 or day 12 post-BMT, and the nucleated spleen cells were counted. The spleen cells (2.5×10^5) were cultured in 1 mL mixture containing α modification of Eagle's medium with nucleotides (α-MEM), 0.86% methycellulose (Methocult, StemCell Technologies, Vancouver, BC), 30% fetal calf serum, 250μM 2-mercaptoethanol, 2mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 U/mL recombinant murine granulocyte-macrophage colony-stimulating factor, or recombinant murine IL-3 (R&D Systems, Minneapolis, MN). Cultures were established in triplicate and maintained at 37°C in humidified air with 5% CO_2 for 5 days. Cell aggregates containing more than 25 cells were scored as individual colonies (CFU) on day 5 of culture. Results are presented as average CFU-GM/2.5×10^5 spleen cells ± SD or total CFU/spleen ± SD.

Analysis of Colony-Forming Cell Phenotype

Cells from CFU cultures were recovered after the methycellulose medium was dissolved. Cultures were treated with cellulase (1 U/mL) for 30 minutes at 37°C, followed by 2 washes with PBS containing 0.5% BSA and 0.02% NaZ_3. The cells were then stained with FITC-conjugated anti-Ly5.1 and PE-conjugated anti-H-$2K^b$ mAb (Pharmingen). The phenotypes ($Ly5.1^+$H-$2K^{b+}$, donor marrow origin; $Ly5.1^-$H-$2K^{b+}$, donor T-cell/donor spleen origin; H-$2K^{b-}$, host origin) of the colony-forming cells were determined by analysis with a FACScan system.

Peripheral Blood Chimerism Analysis

Blood samples of recipients were collected at various time points after BMT. Red blood cells were removed by 2 rounds of ammonium chloride potassium treatment. Mononuclear cells were stained as described above with anti-H-$2K^b$ and anti-Ly5.1 mAbs. Percentage of donor BM-derived ($Ly5.1^+K^{b+}$) cells was determined with flow cytometry (FACScan). Forward and side-angle light scatter was used to establish analytical gates containing predominantly lymphoid or nonlymphoid (monocyte/granulocytes) populations.

Lineage-Negative Donor Marrow Cell Migration to Host BM

Murine BM mononuclear cells (from B6-Ly5.1 mice) were depleted of cells expressing markers of lineage commitment (CD3, CD4, CD8, Gr-1, Mac-1, Ter-119, and B220). Lineage negative (lin^-) cells were labeled with 0.1 μM carboxyfluorescein diacetete succinimidyl ester (CFDA-SE) (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. These cells then were incubated either alone or with an equal number of Miltenyi MACS purified $CD8^+$ cells (either from wild-type or cdd-deficient T-cells) for 2 to 3 hours. Lin^- cells alone (2×10^5 per mouse) or with 2×10^5 $CD8^+$ T-cells were then injected into the tail vein of 6- to 8-week-old C57BL/6 mice that had been lethally irradiated (10 Gy) 24 hours previously. Mice were then euthanized after 6 hours, and the levels of transplanted cells in the BM were measured through the detection of CFDA-SE^+ cells by flow cytometry.

Statistical Analysis

Data were analyzed with a Student 2-tailed unpaired *t* test and one-way ANOVA. The confidence interval was 95%. A *P* value <.05 was considered significant.

RESULTS

Early Presence of Lineage-Committed Donor Progenitor Cells in Recipient Spleens Is Supported by Wild-Type Donor T-Cells as well as Donor T-Cells That Lack FasL and Perforin Function

After irradiation and BMT, donor progenitor cells rapidly enter the recipient spleen, a temporary site of extramedullary hematopoiesis in the mouse. The initial seeding and expansion of donor marrow cells can therefore be examined by evaluation of the presence and number of colony-forming cells of donor origin in the host spleen early (day 5) post-BMT. To define facilitating activity in the transplantation model, we found it necessary to identify a dose of host TBI such that engraftment of donor progenitor cells required addition of a facilitating cell population. BALB/c recipients initially received transplants of TCD B6-Ly5.1 BM cells 24 hours after receiving varying doses of TBI. The hosts were then assessed by examination of CFU-GM activity in the recipient spleen 5 days after transplantation for detection of the presence of progenitor cells (Figure 1). The results indicated that at a dose of 6.0 Gy, strong host resistance remained intact, evidenced by virtually complete rejection of 5×10^6 donor B6-TCD marrow. However, after 7.5 Gy TBI, significant numbers of CFUs were routinely detected in these recipients (Figure 1). On the basis of these observations, a dose of 7.5 Gy was considered a level that would enable the sensitive detection of donor facilitation activity.

To examine the role of T-cells in this early appearance of donor progenitor cells, we added spleen + lymph node cells containing increasing numbers of T-cells from wild-type or cytotoxically double-deficient (B6-cdd) donors to the wild-type congenic (B6-Ly5.1) BM inoculum. CFU-GM assay revealed that 7.5 Gy–conditioned hosts receiving BM exhibited only a very low number of CFU as predicted from the previous results (Figure 2). However, the addition of donor T-cells, with or without functional Fas ligand and perforin, significantly increased (>1 log) the number of colony-forming cells in the host spleens (Figure 2). Antibody staining conclusively demonstrated that virtually all colonies detected were derived from donor BM (data not shown). At T-cell doses of 1×10^6, both B6-wt and B6-cdd cells supported the presence of donor progenitor cells in the

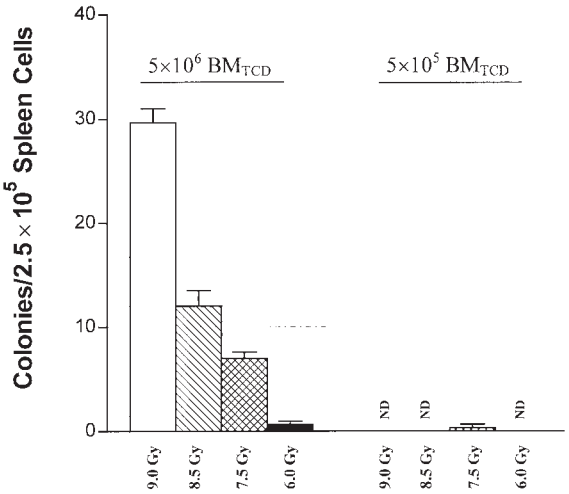

Figure 1. Strong host resistance after 6.0 Gy TBI. BALB/c recipients (n = 3 per group) were irradiated with varying doses of TBI as indicated. Twenty-four hours later, 5×10^5 or 5×10^6 TCD B6-Ly5.1 BM cells were transplanted. Five days after transplantation, the colony-forming activity of recipient spleen cells measured with CFU-GM assay was assessed for determination of the effect of TBI dosage on host resistance. *P* values: 9.0 Gy versus 8.5 Gy, *P* = .0010; 8.5 Gy versus 7.5 Gy, *P* = .036; 7.5 Gy versus 6.0 Gy, *P* = .0007.

BALB/c spleens 5 days post-BMT. The supporting effect remained strong even when the irradiation dose was decreased to 5.5 Gy (Figure 2). The differences between the number of colonies from recipients of wild-type B6 and B6-cdd T-cells were not significant after either 7.5 Gy or 5.5 Gy irradiation (Figure 2). Results of two additional independent experiments with B6-cdd T-cells corroborated these observations (data not shown). These results therefore showed that donor T-cells lacking functional Fas ligand and perforin-dependent pathways could support the initial seeding of allogeneic progenitor cells in recipients post-BMT.

CD8$^+$ Donor T-Cells Support the Persistence of Donor Chimerism

Disparities between the ability of CD4$^+$ and CD8$^+$ T-cell subpopulations to support allogeneic donor BM cell engraftment have been reported [7,12]. Highly enriched B6-wt CD8$^+$ donor T-cells were generated with anti-CD8 mAb conjugated magnetic beads and selection on a MACS column. Spleen colony-forming assays on days 5 and 12 post-BMT showed that the addition of CD4$^+$ T-cells with CD8$^+$ cells did not enhance the support of progenitor cell presence by CD8$^+$ T-cells alone (Figures 3A and 3B). It was important that equivalent numbers of donor B220$^+$ cells failed to support the presence of donor progenitor cells in the host spleen. Compared with the results for recipients receiving BM alone, no significant increase in colony-forming cells was observed post-BMT when the enriched B6 H-2^b B-cell population was added to the syngeneic BM inoculum. These observations demonstrate that T-cell function is required for this process.

Long-term engraftment was assessed by peripheral blood chimerism in the recipients. Previous results indicated that after 7.5 Gy TBI, TCD allogeneic BM established peripheral donor chimerism in approximately 20% of the recipients assessed 12 weeks post-BMT (data not shown). For these studies, recipients were exposed to 5.5 Gy TBI to ensure uniform rejection of all Ly5.1$^+$ H-2^{b+} donor BM grafts post-BMT. Blood samples were collected weekly after BMT, and PBMC were analyzed with anti-H-2K^b and anti-Ly5.1 mAbs for determination of donor marrow–derived chimerism (double-positive staining) for up to 3 months posttransplantation. Our initial observations indicated that both B6-wt and B6-cdd spleen + lymph node inocula containing T-cells were able to support detectable chimerism for the first several weeks post-BMT (Figure 2 and data not shown). However, only recipients of cytotoxically normal B6-wt T-cells maintained long-term, stable donor chimerism. In accordance with the observations early post-BMT (Figures 3A and 3B), findings at examinations of peripheral blood chimerism over a 3-month period demonstrated that only BM grafts containing donor CD8$^+$ T-cells resulted in long-term stable, multilineage donor chimerism (Figures 3C and 3D). Allogeneic marrow grafts without added T-cells or with B220$^+$ (B-cell)-enriched populations did not show persistent chimerism or establishment of engraftment (Figure 3). Donor lymphoid chimerism was extremely transient in recipients of marrow alone or with B220$^+$ or CD4$^+$ or B220$^+$ cells (Figure 3C), whereas granulocytic chimerism persisted for several weeks after transplantation in these groups (Figure 3D). In summary, BM transplanted with CD8$^+$ and CD4$^+$ T-cells or with only CD8$^+$ T-cells efficiently and equally engrafted (Figures 3C and 3D), whereas the addition of only CD4$^+$ T-cells to the BM failed to establish long-term engraftment (Figures 3C and 3D). These findings agreed with previous findings by others [13].

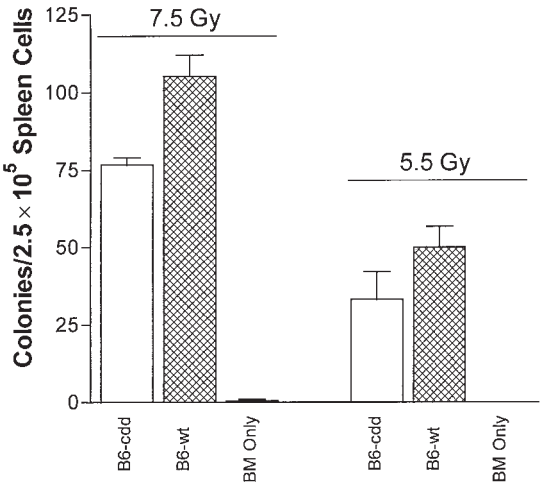

Figure 2. Donor T-cells lacking Fas ligand and perforin can support donor progenitor presence early post-BMT after nonmyeloablative host conditioning. Twenty-four hours after 7.5 Gy (left) or 5.5 Gy (right) TBI, 5×10^6 TCD B6-Ly5.1 BM cells were transplanted into BALB/c recipients (3 per group) together with 1×10^6 T-cells from B6-wt or B6-cdd donors. The spleen cells were analyzed for CFU-GM on day 5. Spleen cells obtained from mice receiving BM cells alone failed to generate significant CFU activity. Total CFU-GM spleen at 7.5 Gy: B6-cdd T-cells, 3535 ± 851; B6-wt T-cells, 3684 ± 928, *P* = .059. Total CFU-GM spleen at 5.5 Gy: B6-cdd T-cells, 2165 ± 148; B6-wt T-cells, 2701 ± 155, *P* = .268.

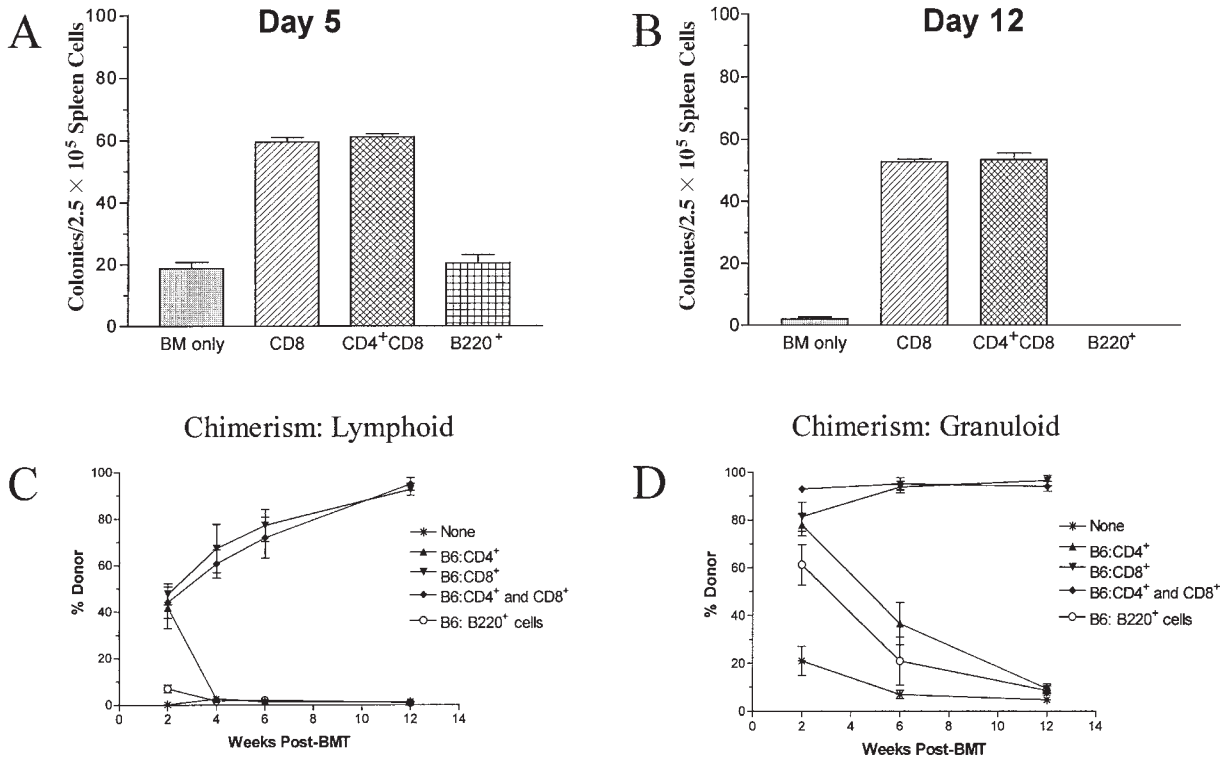

Figure 3. Donor CD8$^+$ T-cells are capable of supporting the initial presence of donor progenitor cells and maintaining long-term engraftment after allogeneic MHC class I/II mismatched BMT. B6-wt CD4$^+$ and CD8$^+$ T-cells and B220$^+$ B-cell–enriched populations were obtained by positive enrichment with Miltenyi MACS magnetic columns. BALB/c recipients were irradiated with 5.5 Gy TBI 4 hours before transplantation with 5×10^6 TCD B6-Ly5.1 BM alone or with addition of 1×10^6 enriched CD8$^+$ T-cells or 1×10^6 CD4$^+$ plus CD8$^+$ (2:1) T-cells or 1×10^6 B220$^+$ cells. Spleen CFU-GM assay was performed 5 days (A) and 12 days (B) later. Peripheral blood samples were collected at different time points and stained for donor chimerism with anti-H-2K^b and anti-Ly5.1 mAbs. Donor chimerism was determined by analysis with FACScan (C). Representative histograms of both lymphoid and granuloid cells (D) demonstrate multilineage peripheral chimerism obtained from recipients of highly purified B6-wt CD8$^+$ T-cells.

CD8$^+$ T-Cell Cytotoxic Function Is Required for Long-Term Engraftment but Not to Support Initial Allogeneic Progenitor Cell Presence in the Recipient's Spleen

We evaluated the importance of the 2 principal cytotoxic T-cell pathways, that is, Fas ligand and perforin-dependent killing, to determine the contribution of these pathways to the promotion of donor BM engraftment. We obtained 1×10^6 highly enriched CD8$^+$ T-cells from wild-type (B6-wt), gld (B6-gld), perforin knock-out (B6-pko) or cytotoxically double-deficient (B6-cdd) donors by positive selection with MACS cell separation columns. The enriched T-cells were transplanted with 5×10^6 TCD B6-Ly5.1 BM cells into 5.5 Gy irradiated BALB/c recipients (Figure 4A). All 4 CD8$^+$ T-cell populations supported donor CFU-GM progenitor cell presence in day 5 recipient spleen regardless of the cytotoxic deficiency (Figure 4A). However, unlike B6-wt and B6-gld donor CD8$^+$ T-cells, both B6-pko and B6-cdd CD8$^+$ T-cells failed to support stable long-term donor lymphoid and nonlymphoid chimerism in the recipients (Figures 4B and 4C). In agreement with these observations, the number of colony-forming cells in the spleen and BM of recipients receiving B6-pko donor CD8$^+$ T-cells decreased in comparison with the number of B6-wt and B6-gld donor CD8$^+$ T-cells observed as early as day 14 posttransplantation (data not shown). By 4 weeks after transplantation, a significant percentage of annexin V$^+$ cells (data not shown) was detected in recipients of B6-cdd T-cells. A very small fraction was detected in recipients of B6-wt T-cells. Identification of the large number of apoptotic donor cells in the former was consistent with the ensuing loss of chimerism after BM transplantation with CD8$^+$ B6-cdd donor T-cells. In total, these results uphold the notion that cytotoxic function by T-cells aiding engraftment is not required to support the initial presence of donor progenitor cells in the recipient; however, perforin-dependent donor T-cell function is required to maintain long-term allogeneic engraftment post-BMT.

CD8$^+$ T-cells from Normal and Cytotoxically Deficient Mice Increase the Homing of Lin$^-$ Progenitor Cells to Recipient Marrow Early Posttransplantation

To determine whether donor T-cells could provide some supportive function in the absence of a host barrier, we prepared CD8 T-cells and transplanted them with enriched lin$^-$ donor BM cells into lethally irradiated syngeneic B6 recipient mice (Table). The number of CFDA-SE–labeled progenitor cells in the BM of recipient mice 6 hours after infusion increased approximately 50% when B6-wt CD8 T-cells were present in the inoculum (Table). CD8$^+$ T-cells from B6-cdd mice increased the numbers of transplanted lin$^-$ cells in recipient BM to virtually identical levels. In separate experiments, CD8 T-cells from peripheral blood yielded essentially

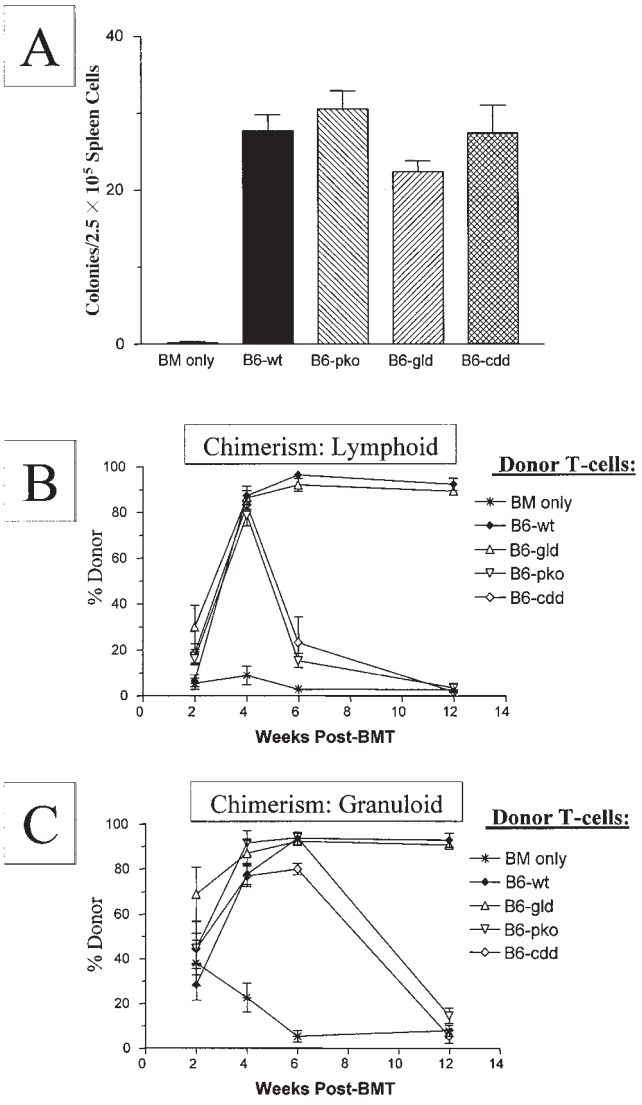

Figure 4. Neither Fas ligand nor perforin function is required to support the early presence of functional donor CFU-GM, but perforin is required for maintaining long-term engraftment of donor marrow. BALB/c recipients were irradiated with 5.5 Gy TBI and given transplants of 5×10^6 TCD B6-Ly5.1 BM cells plus 1×10^6 highly enriched $CD8^+$ T-cells from B6-wt (group B), B6-gld (group C), B6-pko (group D), or B6-cdd (group E) donors. Recipients of BM only (group A) were used as negative controls. On day 5, spleen cells of the recipients were harvested and analyzed for CFU-GM (A). (*P* values: group A versus B, C, D, or E, $P < .0001$; group B versus C, $P = 0.38$; group B versus D, $P = 0.14$; group B versus group E, $P = 0.93$). Peripheral blood samples were collected at different time points and stained with anti $H\text{-}2K^b$ and anti-Ly5.1 mAbs for determination of donor chimerism. The donor chimerism of lymphoid (B) and granuloid (C) lineages was determined by analysis with a FACScan system. Representative histograms of the peripheral chimerism (C) of an individual recipient of B6-pko $CD8^+$ T-cells.

the same results (data not shown). These findings illustrate that donor T-cells can function to support increased homing of progenitor cells to the host marrow early posttransplantation and demonstrate that such function does not require perforin or FasL-mediated cytotoxicity by the CD8 T-cells.

To examine whether CD8 T-cells could enhance the number of colony-forming units–culture in recipients following syngeneic BMT, we transplanted BM-TCD with B6-wt or B6-cdd CD8 T-cells (Figure 5). B6 recipients were conditioned with 5.5 Gy TBI, and 2×10^6 BM cells were transplanted 1 day later alone or together with the purified syngeneic CD8 T-cells. Five days after transplantation, CFU–IL-3 cultures were established from pooled spleen cells (n = 2 or 3 recipients per group). B6-GFP BM was used to verify the donor origin of the colonies detected (data not shown). Cultures established from all groups of recipients contained CFU–IL-3. No differences ($P > .05$) were observed in the CFU–IL-3 numbers between recipients of marrow alone or marrow and CD8 T-cells (Figure 5). An independent experiment examining B6-wt CD8 T-cells was performed to examine CFU–IL-3 support in the marrow and spleen 7 days posttransplantation. The numbers of CFU in the marrow and spleen compartments of recipients of BM only or with syngeneic CD8 T-cells also were not different. In summary, syngeneic CD8 T-cells did not augment the CFU–IL-3 activity of syngeneic BM transplanted into syngeneic, sublethally irradiated recipients.

DISCUSSION

A requisite goal of allogeneic BMT is the accomplishment of long-term multilineage hematopoietic engraftment in the recipient. Marrow/progenitor cell allograft rejection can result from immunological responses, such as host resistance in the recipient after transplantation [14-21]. Despite rigorous host immunosuppression before transplantation of progenitor cell–containing inoculum, considerable resistance remains intact. Experimental studies as well as clinical observations have shown that TCD marrow or progenitor-enriched inoculum has increased susceptibility to host resistance as evidenced by an increased incidence of graft failure with TCD versus non-TCD inoculum [22-26]. These observations support the notion that T-cells can perform necessary functions to support successful engraftment.

Transplantation of Highly Enriched CD8 T-Cells and Lin^- Donor BM Cells into Lethally Irradiated Syngeneic B6 Recipient Mice

Experiment*	Lin^- BM Alone	Lin^- BM + cdd $CD8^+$ T-Cells	Lin^- BM + wt $CD8^+$ T-Cells
1	90	133 (147%)†	140 (155%)†
2	73	133 (182%)	121 (166%)
3	84	119 (142%)	129 (153%)

*Individual mice (10 Gy) in each experiment were injected IV with 2×10^5 CFDA-SE–labeled B6 lineage–negative BM (lin^- BM) cells alone or with purified (2×10^5) $CD8^+$ T-cells. Six hours later the animals were killed, and BM from 2 tibias and 2 femurs of each mouse was collected and analyzed by flow cytometry for injected cells.

†Values represent the number of CFDA-SE–labeled cells per 1×10^5 BM cells. Numbers in parentheses represents the percentage increase in cell numbers compared with the numbers of lin^- BM cells transplanted alone. Combining results from the three independent experiments demonstrates that Lin^-BM + cdd CD8's (123+/–4.7) or Lin^-BM + wt CD8's (130+/–5.5) versus Lin^-BM alone (82.3+/–5.0), $P < .01$, Lin^-BM + cdd CD8's versus Lin^-BM + wt CD8's, $P > .05$.

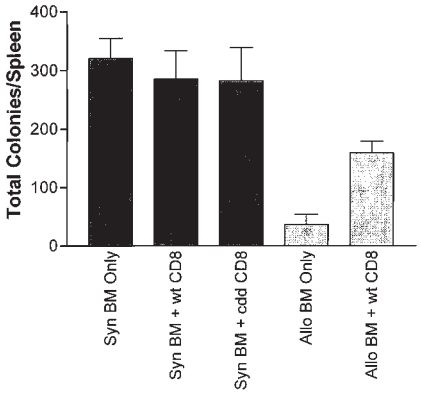

Figure 5. Donor CD8$^+$ T-cells do not support increased progenitor activity after syngeneic BMT following sublethal irradiation of recipients. B6 recipients (n = 2 or 3 per group) were irradiated with 5.5 Gy TBI and given transplants the following day of 2×10^6 TCD B6-GFP BM cells alone or BM plus 2×10^6 highly enriched CD8$^+$ T-cells from B6-wt or B6-cdd donors. On day 5, spleen cells of the recipients were harvested, pooled, and analyzed for CFU–IL-3. Donor origin of colonies was confirmed by GFP fluorescence. Allogeneic BALB/c recipients were also irradiated and given transplants in an identical fashion with either the same B6-GFP BM alone or with B6-wt CD8$^+$ T-cells. Total splenic CFU was obtained by multiplying the CFU frequency by the total number of spleen cells. B6 recipients of BM only (321 ± 34.7) versus BM + B6-wt CD8 (285 ± 49.2) or BM + B6-cdd CD8 (282 ± 57), $P > .05$. BALB/c recipients of BM only (36 ± 17.6) versus BM + B6-wt CD8 (159 ± 20.4), $P < .01$.

A number of studies removing or adding defined cell types to allogeneic stem cell inoculum have shown that nonprogenitor populations can promote or facilitate long-term multilineage engraftment [27-32]. The capacity to facilitate has been primarily assessed by evaluation of the level of engraftment established in the allogeneic recipient 4 or more weeks after progenitor cell transplantation. Although considerable investigation has defined a number of distinct supporting cell populations, the overall process by which facilitation results in long-term engraftment remains unclear. To approach this question, we are investigating the early as well as later period posttransplantation to examine and compare the functional requirements of, and activity effected by, T-cells for promotion of the initial seeding of donor progenitor populations as well as persistent long-term multilineage engraftment. The findings in this study show that T-cells lacking perforin and/or FasL function have the capacity to promote the early presence of MHC class I/II mismatched donor progenitor cells in the recipient's hematopoietic compartment after transplantation into sublethally irradiated hosts. Competent B-cell–enriched lymphoid populations do not have such functions. In contrast to these observations, persisting multilineage donor engraftment was clearly dependent on intact perforin function in the T-cells examined.

The addition of marrow-derived and peripheral TcR$^+$ cells, including both TcRα/β^+ [27] and TcR $\gamma\delta^+$ T-cells [29], as well as NK [28] and TcR$^-$CD8$^+$ cells [32] to progenitor grafts has been demonstrated to promote engraftment in experimental BMT models. CD8$^+$ peripheral T-cells were found by Martin and colleagues to support engraftment of allogeneic marrow as determined by peripheral donor cell chimerism more than 4 weeks post-BMT [13]. Subsequent studies by this group showed that cytotoxically deficient CD8$^+$ T-cells were impaired in their capacity to support this engraftment [12]. Specifically, perforin, but not granzyme B– or FasL-deficient T-cells, failed to support multilineage engraftment 1 month posttransplantation [12]. Findings in the present study corroborated a requirement for perforin function in peripheral T-cell–facilitating populations. In contrast, a recent study showed that the absence of granule-dependent cytotoxicity did not detectably impair the ability of CD8$^+$ BM T-cells to support prolonged engraftment after transplantation of purified MHC-mismatched stem cell populations [32].

It has been reported that after BMT, 2 phases of donor cell engraftment can be observed: an early period of reconstitution (radioprotective) occurring immediately after transplantation followed by a slower but more stable long-term chimerism [7,33]. Experiments underway in this laboratory have shown that in contrast to the splenic compartment in normal adult mice, which possesses <0.1% of the total CFU–IL-3 potential contained in the combined marrow + spleen compartments, the splenic compartment of mice subjected to syngeneic BMT contains approximately 5% to 30% of this potential during the first 1 to 2 weeks after transplantation. These levels diminish to almost homeostatic levels during the next 2 weeks (A. Shatry and R.B.L., unpublished observations). Splenectomy of mice prior to BMT clearly impacted, that is, augmented, the CFU–IL-3 marrow numbers early after BMT (A. Shatry and R.B.L., unpublished observations). These findings may indicate that interactions between these compartments early post-BMT are critical for determining committed donor progenitor cell numbers in recipients and support the notion that the spleen is a functionally important host hematopoietic compartment during the first month after hematopoietic progenitor cell transplantation.

The presence of donor-derived progenitor cells early posttransplantation could be assessed by examination of the presence of CFU-GM in the host spleen. After TCD BMT with wild-type B6 (H-2^b) donor T-cells to facilitate donor BM cell engraftment, the presence or absence of significant numbers of CFU-GM numbers in the host BALB/c (H-2^d) spleen on day 5 post-BMT correlated with successful or failed long-term engraftment of donor marrow. However, when cytotoxically deficient donor T-cells were included with the TCD-BM donor graft, a clear discrepancy was observed between early and late donor cell presence dependent on the individual lytic pathway absent in the facilitating population. The presence of lineage-committed donor progenitor cells in the host spleen 5 days posttransplantation after addition of cytotoxically defective CD8$^+$ T-cells did not necessarily result in long-term donor chimerism. These findings suggested that different functions of donor T-cells could be required to support the early seeding of donor progenitor cells versus those necessary for support of long-term engraftment. Recent studies have shown that addition of peripheral CD8$^+$ T-cells to CD34$^+$ stem cell populations increases the level of stem cell migration to hematopoietic compartments after infusion [34].

Interestingly, in syngeneic lethally irradiated BMT recipients in this study, cytotoxically deficient, as well as normal T-cells, had the capacity to promote the migration of increased numbers of progenitor cells to the marrow compartment soon

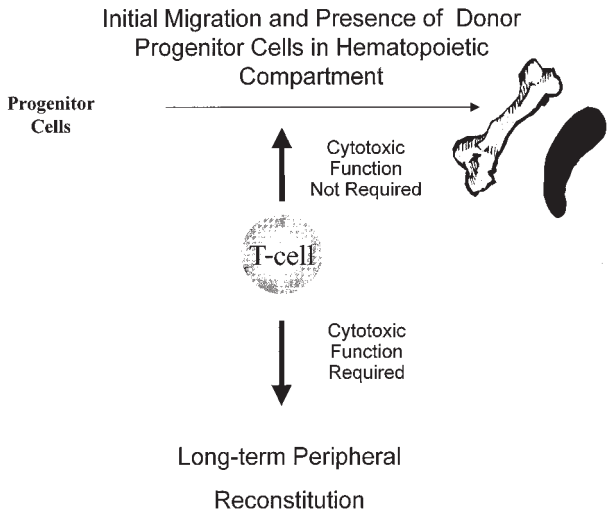

Figure 6. The requirements for cytotoxic function by donor T-cells during the process of progenitor cell engraftment.

after infusion. These observations were made (1) under conditions in which cytotoxic function by the donor T-cells was not required to overcome resistance and (2) with B6-cdd CD8 T-cells. Therefore these data would appear to exclude any role for perforin/FasL cytotoxic function in such T-cell activity. To determine whether syngeneic T-cells had the capacity to support enhanced CFU early after syngeneic BMT in our model, we gave sublethally irradiated B6 mice transplants of B6 BM-TCD alone or BM together with B6-wt or B6-cdd CD8 T-cells. The inability of these cotransplanted populations to increase CFU–IL-3 levels after syngeneic BMT supports the notion that activation of these CD8 T-cells occurring in the B6→BALB/c allogeneic BMT experiments is required for their affect on subsequent (1-2 weeks) progenitor cell activity. It may be interesting to examine CFU–IL-3 after syngeneic BMT under conditions that strongly activate putative syngeneic CD8 supportive populations.

Regarding long-term reconstitution, the present results confirmed that $CD8^+$ peripheral T-cells can be effective in maintaining donor-derived multilineage chimerism in the allogeneic host [12,13]. Consistent with previous findings, T-cell perforin function was crucial for this activity [12]. When recipients in the present study were given transplants of TCD BM with the addition of donor T-cells lacking perforin function, although these T-cells promoted the early presence of donor CFU-GM in the recipient's spleen, donor chimerism began to decrease 4 to 6 weeks posttransplantation. These findings indicated that T-cells must have cytotoxic function if they are to successfully support long-term engraftment. However, such cytotoxic function is apparently unnecessary in support of the initial/early presence and function of progenitor cells in the hematopoietic compartment (Figure 6). Such observations further suggest that the mechanism of facilitation is complex and is likely to involve a multistep/multifunctional process.

The present studies also demonstrated that donor T-cells simultaneously lacking FasL and perforin function were capable of supporting the early presence of donor-derived progenitor cells in the recipient spleen. Thus the 2 major donor cytotoxic T-cell pathways are not required by $CD8^+$ T-cells to promote donor progenitor cell presence early post-BMT. Alternatively, other T-cell cytotoxic functions or cell populations could be important in supporting the presence of donor progenitor cells. Besides FasL and perforin, cytotoxic pathways such as tumor necrosis factor α (TNF-α) [35], TNF-related apoptosis-inducing ligand (TRAIL) [36], and transforming growth factor β (TGF-β) [37] have been suggested to play some role in hematopoiesis. However, the involvement of these molecules in supporting donor BM cell engraftment remains undefined. The failure of enriched B-cells to promote splenic donor CFU activity indicated that a specific T-cell, but not B-cell, function is required for these observations and that the function is not passive, such as "diverting" host resistance by "flooding" donor antigen into recipients. This result is consistent with the finding that although direct antihost responses by facilitating populations cannot be excluded in a number of studies, donor T-cells have been found to support engraftment without direct recognition of host antigen [38,39]. It also has been reported that IL-1α was able to augment donor marrow engraftment and hematopoietic reconstitution [40]. Other investigators have reported that HSC treated with IL-11 and stem cell factor or anti–TGF-β exhibited a dramatic increase in short-term reconstitution after BMT [37,41,42]. The mechanism whereby T-cells promote the initial presence and activity of progenitor cells in the hematopoietic compartments of allogeneic recipients remains unclear and requires further study for elucidation of the pathways involved.

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