RAPID COMMUNICATION

Granulocyte Colony-Stimulating Factor–Mobilized Allogeneic Stem Cell Transplantation Maintains Graft-Versus-Leukemia Effects Through a Perforin-Dependent Pathway While Preventing Graft-Versus-Host Disease

By Luying Pan, Takanori Teshima, Geoffrey R. Hill, David Bungard, Yani S. Brinson, Vijay S. Reddy, Kenneth R. Cooke, and James L.M. Ferrara

Minimization of graft-versus-host disease (GVHD) with preservation of the graft-versus-leukemia (GVL) effect is a crucial step to improve the overall survival of allogeneic bone marrow transplantation (BMT) for patients with hematological malignancies. We and other investigators have shown that granulocyte colony-stimulating factor (G-CSF)-mobilized allogeneic peripheral stem cell transplantation (PBSCT) reduces the severity of acute GVHD in murine models. In this study, we investigated whether G-CSF–mobilized PBSCT maintain their GVL effect in a murine allogeneic transplant model (B6 → B6D2F1). B6 mice (H-2b) were injected subcutaneously with human G-CSF (100 μg/kg/d) for 6 days and their splenocytes were harvested on day 7 as a source of PBSCT. G-CSF mobilization dramatically improved transplant survival compared with nonmobilized controls (95% vs 0%, P < .001). Systemic levels of lipopolysaccharide and tumor necrosis factor-α were markedly reduced in recipients of allogeneic G-CSF–mobilized donors, but cytolytic T lymphocyte (CTL) activity against host tumor target cells p815 was retained in those recipients. When leukemia was induced in recipients by coinjection of p815 tumor cells (H-2k) at the time of transplantation, all surviving recipients of G-CSF–mobilized B6 donors were leukemia-free at day 70 after transplant, whereas all mice who received T-cell-depleted (TCD) spleenocytes from G-CSF–mobilized B6 donors died of leukemia. When splenocytes from G-CSF–mobilized perforin-deficient (pfp−/−) mice were used for transplantation, 90% of recipients died of leukemia, demonstrating that perforin is a crucial pathway mediating GVL effects after G-CSF–mobilized PBSCT. These data illustrate that G-CSF–mobilized allogeneic PBSCT separate GVL from GVHD by preserving perforin-dependent donor CTL activity while reducing systemic inflammation.

© 1999 by The American Society of Hematology.

A LLOGENEIC BONE MARROW transplantation (BMT) is a standard therapy for hematological malignancies. An important benefit of allogeneic BMT is the graft-versus-leukemia (GVL) effect, a process of tumor eradication by donor cells after BMT.1-3 However, GVL effects are closely linked to graft-versus-host disease (GVHD), a major cause of morbidity and mortality after allogeneic BMT.1,4 Results from a series of clinical trials demonstrated that donor T cells play a vital role in both GVL and GVHD, because T-cell depletion (TCD) of the bone marrow reduced the incidence and severity of GVHD, but increased leukemia relapse.5-7 It is also well recognized that leukemia relapse is inversely linked to the severity of GVHD after BMT.1,5 Therefore, separation of GVL and GVHD is a crucial step to improve the overall survival of allogeneic BMT for hematologic malignancy.

Recently, there is increased enthusiasm for the use of granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood stem cell transplantation (PBSCT). Comparison of G-CSF–mobilized PBSCT (containing a 10- to 20-fold increase in donor CD3+ cells) and traditional bone marrow grafts demonstrate a surprisingly similar incidence and severity of acute GVHD.8-11 This relative reduction of acute GVHD may be attributable to immunomodulation of cells in the donor graft. A decrease in interleukin-2 (IL-2) and interferon-γ (IFN-γ) production to allo-antigen stimulation has been reported both in human and animal studies.12-15 Monocytes from G-CSF–mobilized human donors have also been reported to suppress allo-reactivity of T cells in mixed lymphocyte culture,16-18 perhaps through an IL-10–dependent mechanism.19 However, recent studies suggest increased risks of chronic GVHD after G-CSF–mobilized allogeneic PBSCT.20,21 Because donor T cells are major effectors of the GVL effect, it is important to investigate whether G-CSF–mobilized PBSC grafts can maintain GVL effects. In this study of a murine PBSCT-leukemia model, we show that T cells from G-CSF–mobilized PBSC have a markedly diminished capacity to induce acute GVHD, but maintain their GVL function through a perforin-dependent pathway.

MATERIALS AND METHODS

Mice. Female Ly-5 congenic B6.Ly-5 (H-2b, CD45.1+) mice were obtained from the Frederick Cancer Research Facility (Frederick, MD), and female B6D2F1 (H-2b/d, CD45.2+) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Female C57BL/6 (B6, H-2b, CD45.2+) and perforin-deficient mice (pfp−/−) B6x129/SvEv, H-2b, CD45.2+ were purchased from Toconic Laboratory (Germantown, NY). Ly-5 (CD45) alleles are described according to the nomenclature of Morse et al.22 Mice were housed in sterilized microisolator cages and received tap water and normal chow. Mice used for experiments were between the ages of 10 and 14 weeks and received autoclaved hyper-chlorinated drinking water during the first 3 weeks posttransplantation.

G-CSF treatment. Donor mice were injected subcutaneously with recombinant human G-CSF (Amgen Inc, Thousand Oaks, CA) daily at 100 μg/kg body weight or saline (control diluent) for 6 days, and splenocytes were harvested on day 7.

From the Department of Pediatric Oncology, Dana Farber Cancer Institute, Boston, MA; and the Departments of Internal Medicine and Pediatrics, University of Michigan Medical School, Ann Arbor, MI.

Submitted January 18, 1999; accepted March 22, 1999.

Supported in part by National Institutes of Health Grants No. CA 39542 and HL 55709.

Address reprint requests to James L.M. Ferrara, MD, Bone Marrow Transplant Program, University of Michigan Cancer Center, 1500 E Medical Center Dr, Ann Arbor, MI 48109; e-mail: ferrara@umich.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.
RESULTS

G-CSF mobilization reduces the severity of acute GVHD. We first examined the effects of G-CSF mobilization in a murine BMT model (B6 Ly-5 \(^5\) → B6D2F1) that induces acute GVHD to both major and minor histocompatibility antigens. Injections of human G-CSF for 6 days at a dose of 100 \(\mu\)g/kg/d increased the yield of splenocytes by approximately 25\%. As shown in Fig 1, the percentages of T cells (CD4\(^9\), CD8\(^9\)), B cells (B220\(^9\)), and natural killer (NK) cells (NK1.1\(^9\)) were similar in control and G-CSF-treated donors, whereas myeloid cells (Gr-1\(^9\)) were significantly increased in splenocytes from G-CSF-treated donors. The percentage of myeloid cells in the bone marrow doubled in G-CSF-treated donors. B6D2F1 recipient mice were irradiated with 1100 cGy and transplanted with 10 \(\times\) 10\(^6\) splenocytes from either control or G-CSF–treated B6 Ly-5\(^5\) mice. GVHD induced in this model was severe and usually lethal. As shown in Fig 2A, all animals surviving control allogeneic splenocytes died within 2 weeks, with clinical evidence of GVHD (hunched posture, inactivity, and weight loss), whereas 95\% of mice receiving splenocytes from G-CSF–mobilized donors survived at day 70 posttransplant. This survival was markedly superior to that seen in our previous study when splenocytes were mobilized with a lower dose of human G-CSF.\(^{13,14}\) The optimal dose of hG-CSF for PBSC mobilization is approximately 10 times higher in mice than in humans,\(^{15,23,24}\) making the doses used in this study more...
clinically relevant. The mortality of acute GVHD seen in control allogeneic recipients was mediated by donor T cells, because all mice receiving allogeneic TCD-splenocytes survived until the end of experiment. Although the severity of acute GVHD in mice receiving allogeneic G-CSF–mobilized splenocytes was dramatically reduced, these mice did show signs of moderate GVHD, as measured by weight loss compared with recipients of allogeneic TCD-splenocytes or syngeneic splenocytes (Fig 2B).

G-CSF mobilization reduces systemic levels of LPS and TNF-α. Both LPS and TNF-α are known to be important mediators of acute GVHD severity.26-28 Consistent with the severe clinical GVHD in animals receiving control allogeneic splenocytes, the serum LPS levels in these animals were markedly elevated compared with syngeneic controls on day 7 posttransplant, a time of maximal elevation (Fig 3A). By contrast, serum LPS levels in animals transplanted with G-CSF–mobilized allogeneic splenocytes were reduced to levels of syngeneic non-GVHD controls (Fig 3A). Serum TNF-α levels were also significantly reduced in the recipients of G-CSF–mobilized donors, although they remained higher than that seen in syngeneic non-GVHD controls (Fig 3B).

G-CSF mobilization induces a type 2 cytokine profile with preservation of CTL activity. We next examined the effects of G-CSF mobilization on donor T-cell functions. Consistent with previous reports using a lower G-CSF dose,13,14 G-CSF treatment led to an increased production of type 2 cytokines (IL-4 and IL-10) with a decreased production of type 1 cytokines (IL-2 and IFN-γ) in response to host antigen stimulation. This polarization towards a type 2 cytokine profile was maintained in 2° MLR despite the absence of exogenous G-CSF at all times in culture (Table 1). G-CSF also decreased production of IL-12, reflecting its action on the antigen-presenting cells (Table 1). T cells from 1° MLR were then used as effector cells against host type (H-2d) p815 targets or donor type (H-2b) EL4 targets. As shown in Fig 4, G-CSF mobilization did not reduce the CTL activity of splenocytes, despite the shift in cytokine profile.

G-CSF mobilization preserves GVL effects. To examine the effects of G-CSF mobilization on GVL, animals were transplanted as described above and 5,000 p815 tumor cells were injected intravenously together with the donor inoculum. As shown in Fig 5A, syngeneic recipients all died of leukemia by 4 weeks posttransplant with macroscopic evidence of tumor in the liver and spleen. Recipients of allogeneic control donors died within 2 weeks due to severe GVHD, but necropsy showed no
Evidence of tumor. In contrast, 95% of allogeneic recipients of G-CSF–mobilized donor cells were still alive at day 70 posttransplantation. Eradication of leukemia was confirmed by absence of CD45.2 cells in peripheral blood and lack of tumor in liver and spleen by histology. The importance of donor T cells in mediating the GVL effect was confirmed by transplantation of TCD-splenocytes from B6 donors and 5,000 p815 tumor cells. None of recipients showed evidence of GVHD (Fig 2), but they all died by 5 weeks after transplantation with macroscopic evidence of leukemia (Fig 5B).

GVL is mediated through a perforin-dependent pathway. To further delineate the mechanism of GVL after G-CSF–mobilized PBSCT, perforin-deficient (pfp<sup>−/−</sup>) mice were used as donors, because perforin has been shown to be an important effector of Tc2 cytotoxic functions. CTL activity from pfp<sup>−/−</sup> was substantially reduced both in vitro and ex vivo (Fig 6). Splenic T cells after 1° MLR (Fig 6A) or splenocytes from recipients 7 days after transplantation of 10<sup>7</sup> allogeneic splenocytes (Fig 6B) showed significant decrease in lysis of host-type p815 tumor targets, and this was unaffected by G-CSF mobilization (Fig 6A and B). We then examined GVL effects in a murine PSCT-leukemia model. Lethal irradiated B6D2F1 recipient mice received 10<sup>7</sup> splenocytes from G-CSF–mobilized B6 or pfp<sup>−/−</sup> donors with 25,000 p815 tumor cells. As shown in Fig 7, all syngeneic recipients died with macroscopic evidence of tumor within 3 weeks. All recipients of allogeneic G-CSF donors were leukemia-free at day 70 as determined by FACS staining of peripheral blood cells and macroscopic examination of tumor-targeted organs. By contrast, 90% of recipients transplanted with splenocytes from pfp<sup>−/−</sup> donors died with gross evidence of leukemia. Engraftment of pfp<sup>−/−</sup> donor cells was complete by day 60 and expansion of pfp<sup>−/−</sup> donor T cells on day 7 after transplantation was equivalent to wild-type controls (Table 2). Therefore, the

### Table 1. Cytokine Profile After G-CSF Mobilization

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>1°MLR Control</th>
<th>G-CSF</th>
<th>2°MLR Control</th>
<th>G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12 (pg/mL)</td>
<td>16.9 ± 0.20</td>
<td>10.4 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-2 (U/mL)</td>
<td>0.9 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IFN-γ (U/mL)</td>
<td>19.1 ± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5 ± 1.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>485.7 ± 5.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>359.2 ± 17.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-4 (U/mL)</td>
<td>0.4 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.88 ± 1.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3 ± 1.84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>35.7 ± 2.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.2 ± 2.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>343.3 ± 46.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>616.8 ± 11.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

B6 donor mice were injected with human G-CSF (100 µg/kg/d) for 6 days, splenocytes were harvested on day 7, and T cells were enriched by passing through nylon-wool column. Results represent the mean ± SE from 6 to 9 samples/group.

Abbreviations: 1°MLR, splenic T cells from B6 donors were incubated with irradiated peritoneal cells from B6D2F1 mice for 48 hours, and cytokine levels in the culture supernatants were determined by ELISA; 2°MLR, cells from day-6 1°MLR were restimulated with irradiated peritoneal cells from B6D2F1 mice for 48 hours, and cytokine levels in the culture supernatants were determined by ELISA.

<sup>a</sup>P < .01 v control B6 donors.
loss of a GVL effect was not due to diminished donor T-cell expansion or engraftment, but rather lack of perforin activity.

DISCUSSION

In this study, we demonstrate that G-CSF–mobilized allogeneic PBSCT dramatically reduced the severity of acute GVHD while maintaining perforin-dependent GVL effects in a murine PBSCT-leukemia model. CTL activity against host antigens in G-CSF–mobilized donor PBSCT is preserved, although the inflammatory cytokine response is significantly diminished.

The important balance between cytokines derived from type 1 and type 2 T cells in inducing acute GVHD was first demonstrated in the experimental BMT models.31-33 Elevated levels of type 1 cytokines (IL-12, IL-2, and IFN-γ) are associated with severe acute GVHD,34-39 whereas elevated levels of a type 2 cytokine profile (increased IL-4 and IL-10 production) are not.31-33,40,41 A correlation of type 1 and type 2 cytokine profile with the severity of acute GVHD was also reported by Tanaka et al42 in a clinical study of allogeneic BMT. We and other investigators have reported that G-CSF mobilization skews T-cell cytokines toward a type 2 profile upon allo-antigen stimulation and after experimental allogeneic PB-SCt.12-15 G-CSF mobilization also causes a decreased production of type 1 cytokines from PBMC upon allo-antigen stimulation compared with before mobilization,43,44 and increased expression of IL-4 mRNA has also been reported.45

Type 1 cytokines are known to prime mononuclear cells to secrete TNF-α during GVHD.31,33,46,47 Clinical studies have shown that elevated serum TNF-α levels precede clinical symptoms of acute GVHD,28,48 and anti–TNF-α therapy signifi-
Fig 7. Survival after leukemia induction (B6 $\rightarrow$ B6D2F1). Wild-type B6 or pfp$^{-/-}$ donores were injected with G-CSF for 6 days. Total body irradiated B6D2F1 recipients received $1 \times 10^6$ splenocytes plus 25,000 p815 tumor cells from G-CSF-mobilized B6 donors ($n = 10$) or from G-CSF-mobilized pfp$^{-/-}$ donors ($n = 10$) or control B6D2F1 donors ($n = 5$). Survival was monitored daily until day 70 posttransplantation. * $p < .001$ v recipients of splenocytes from G-CSF-mobilized pfp$^{-/-}$ donors and control B6D2F1 donors.

Donor T cells play a vital role in mediating GVL effects, as demonstrated by the effectiveness of donor leukocyte infusion to induce remission after leukemia relapse.54-58 In this study, we have showed that G-CSF–mobilized donor T cells maintain their CTL activity against leukemia targets and preserve GVL effects. An improved GVL effect using G-CSF–mobilized allogeneic PBSC has been reported in another murine leukemia model.59 G-CSF–mobilized PBPC have also been used successfully to treat relapse after allogeneic BMT.60,61 Apoptosis of target cells induced by CTL could be mediated by perforin and/or Fas/FasL pathways, and both pathways may be involved in the development of GVHD.50,62-69 CTL can be divided into Tc1 and Tc2 subpopulations according to their cytokine secretion pattern. Apoptosis mediated by Tc1 cells depends primarily on Fas/Fas ligand pathway.29,70,71 IFN-$\gamma$ secreted by type 1 T cells has been reported to increase expression of Fas and FasL and may thereby enhance apoptosis mediated by the Fas/FasL pathway.72,73 However, apoptosis mediated by Tc2 cells is more dependent on perforin pathway.29,71 Such mechanisms are consistent with the present study, in which G-CSF mobilization amplifies a Tc2 response, reduces acute GVHD, and maintains GVL through a perforin-dependent pathway.

In this study, we demonstrated that G-CSF–mobilized grafts reduce severity of acute GVHD by disruption of cytokine cascade involved in development of acute GVHD. More importantly, G-CSF–mobilized grafts maintain their GVL effects through a perforin-dependent pathway. Therefore, G-CSF mobilization may offer a novel approach to the separation of GVL effects from GVHD. Studies are currently in progress to determine the effects of G-CSF mobilization of donor cells in chronic GVHD and immune reconstitution.

ACKNOWLEDGMENT

The authors thank Dr Anastasia Skandalis for her valuable discussions and Scott Bressler and Vicki Mosher for their technical support.

REFERENCES


| Table 2. Donor T-Cell Expansion and Engraftment After Transplantation |
|---------------------|-------------------|-----------------|-----------------|
| Donors | Day-7 Splenocytes | Day-60 PBC % H2-K$^b$/H-2D$^d$ |
| B6 | 1.48 | 75.0 | ND |
| GCSF-B6 | 1.75 | 60.2 | 99.0 |
| pfp$^{-/-}$ | 1.90 | 69.6 | ND |
| GCSF-pfp$^{-/-}$ | 2.05 | 64.4 | 99.0 |
| B6D2F1 | 2.60 | 13.6 | 0.5 |

Total body irradiated B6D2F1 recipients received $1 \times 10^6$ splenocytes from control B6 donors, G-CSF-mobilized B6 donors, pfp$^{-/-}$ donors, G-CSF-mobilized pfp$^{-/-}$ donors, or control B6D2F1 donors. On day-7 posttransplant, splenocytes were harvested $(n = 5/group)$ and were counted using a hemocytometer. The percentage of T cells was determined by staining with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 Abs. On day 60 postransplant, peripheral blood $(N = 4)$ was collected and stained with FITC-conjugated anti-H-2K$^b$ and PE-conjugated anti-H-2D$^d$.

Abbreviation: ND, not determined.
37. Ferrara JLM, Marion A, McIntyre JF, Murphy GF, Burakoff SJ: Amelioration of acute graft-versus-host disease due to minor histocom-


Granulocyte Colony-Stimulating Factor–Mobilized Allogeneic Stem Cell Transplantation Maintains Graft-Versus-Leukemia Effects Through a Perforin-Dependent Pathway While Preventing Graft-Versus-Host Disease

Luying Pan, Takanori Teshima, Geoffrey R. Hill, David Bungard, Yani S. Brinson, Vijay S. Reddy, Kenneth R. Cooke and James L.M. Ferrara