RAPID COMMUNICATION

Pretreatment of Donor Mice With Granulocyte Colony-Stimulating Factor Polarizes Donor T Lymphocytes Toward Type-2 Cytokine Production and Reduces Severity of Experimental Graft-Versus-Host Disease

By Luying Pan, John Delmonte Jr, Candice K. Jalonen, and James L.M. Ferrara

The incidence and severity of acute graft-versus-host disease (GVHD) after allogeneic transplantation using peripheral blood progenitor cells mobilized by granulocyte colony-stimulating factor (G-CSF) appear to be no worse than those after bone marrow transplantation, despite the presence of large numbers of T cells in the donor infusion. Experimental studies have shown that type-1 T cells (secreting interleukin-2 [IL-2] and interferon-γ) mediate acute GVHD, whereas type-2 T cells (secreting IL-4 and IL-10) can prevent acute GVHD. We tested the hypothesis that G-CSF modulates T-cell function toward a type-2 response and thus reduces the severity of acute GVHD. B6 mice were injected with G-CSF or diluent for 4 days, and their splenic T cells were stimulated in vitro with alloantigen or mitogen in the absence of G-CSF. T cells from G-CSF–treated mice showed a significant increase in IL-4 production, with a simultaneous decrease in IL-2 and interferon-γ production in response to both stimuli. We also examined the effect of G-CSF pretreatment of donors in a GVHD model (B6 → B6D2F1). Survival was significantly improved in recipients of G-CSF–treated donors. Concanavalin-A–induced cytokine production at day 13 after transplantation also showed an increase in IL-4 along with a decrease in IL-2 and IFN-γ production by splenocytes from recipients of G-CSF–treated donor bone marrow and T cells. These data show that pretreatment of donors with G-CSF polarizes donor T cells toward the production of type-2 cytokines, which is associated with reduced type-1 cytokine production and reduced severity of acute GVHD.

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GRANULOCYTE COLONY-STIMULATING factor (G-CSF) is a natural hematopoietic growth factor that stimulates proliferation and maturation of neutrophil precursors and can mobilize progenitor cells (PCs) from the bone marrow (BM) into the peripheral blood (PB).45 CD34+ cells in PB of normal volunteers are increased about 25- to 30-fold after 4-5 consecutive days of G-CSF injection.6 The paucity of side effects associated with G-CSF administration has made its clinical use attractive and has encouraged recent clinical trials using PBPCs mobilized by G-CSF. In several centers, transplantation of these PBPCs, obtained by leukapheresis from normal donors, resulted in substantial engraftment in high-risk patients.6-12 These leukapheresis products also contained large numbers of T lymphocytes (approximately 10-fold higher than conventional BM harvests), and such donor cell infusions would normally be expected to cause severe graft-versus-host disease (GVHD) after allogeneic transplantation. However, in 30 recipients of allogeneic PBPCs mobilized by G-CSF, 16 patients did not develop acute GVHD, 2 had grade-I acute GVHD, and 10 patients developed grade-II acute GVHD. Only 2 of these 30 patients (7%) developed grade-III acute GVHD, and in both cases cyclosporine prophylaxis had been discontinued.6-12 Thus, the incidence and severity of acute GVHD after PBPC transplantation in these preliminary studies appear to be no worse than after BM transplantation (BMT), despite the fact that PBPC infusions contain 10-fold more T cells than do BM infusions.

Recently, several reports have emphasized the role of cytokines as mediators of acute GVHD.13-19 Cytokines produced by both CD4+ and CD8+ T cells can be characterized into two patterns, type-1 cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2) and type-2 cytokines such as IL-4 and IL-10.20-25 Generally, type-1 responses are considered proinflammatory whereas type-2 responses are considered anti-inflammatory. Recent studies have shown that T lymphocytes with type-1 cytokine responses mediate acute GVHD,13,15 whereas those with type-2 cytokine responses can prevent acute GVHD.17,18 Therefore, we investigated the immunomodulatory effects of G-CSF on T-cell cytokine secretion in response to alloantigens and mitogen. Our studies show that G-CSF administration in vivo has no effect on splenic T-cell number or phenotype, but it does polarize T-cell responses toward production of type-2 cytokines. This polarization is profound and persists in secondary mixed lymphocyte reactions (MLRs) despite the absence of exogenous G-CSF at all times in vitro. Furthermore, splenocytes from allogeneic recipients transplanted with BM and T cells from G-CSF–pretreated donor mice showed identical polarization of concanavalin-A (Con A)–induced cytokine production, which was associated with significantly reduced lethality from acute GVHD.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6, H-2b, Ly-5.1+)* and B6D2F1 (H-2k, Ly-5.2+)* mice were purchased from the Jackson Laboratories (Bar Harbor, ME). B6 Ly-5.1+ (H-2b, Ly-5.1+)* mice were bred in the animal facility at Dana Farber Cancer Institute (Boston, MA). The age of mice used for experiments ranged between 3 and 6 months. Mice were housed in sterilized microisolator cages and received filtered water and normal chow or autoclaved hyperchlorinated drinking water for the first 2 weeks post-BMT.

G-CSF treatment. Recombinant human G-CSF was purchased from Amgen Inc (Thousand Oaks, CA). Mice were injected subcuta-
neously with G-CSF (0.01 μg/g body weight) daily for 4 to 6 days. G-CSF was diluted in 5% dextrose/1% normal mouse serum before injection, as recommended by the manufacturer. Mice from the control group received injection of diluent only. For in vitro mixed lymphocyte culture mice were injected with diluent or G-CSF for 4 days, and splenic T cells were harvested on day 5. For BMT, mice were injected with diluent or G-CSF for 6 days, and BM, splenocytes, and splenic T cells were harvested on day 7. Eye-breed specimens to determine granulocyte population in PB were collected on day 4 of G-CSF injection.

T-cell purification and separation of T-cell subpopulations. Enriched T-cell populations were obtained by passage of splenocytes through nylon wool columns. CD4+ and CD8+ populations were selected by using MiniMACS (Miltenyi Biotec Inc, Sunnyvale, CA) according to the manufacturer’s protocol. Briefly, cells were incubated with rat IgG antimouse CD4 or CD8 for 30 minutes at 4°C, washed twice, and incubated with goat-antirat IgG microbeads for 20 minutes at 4°C. CD4+ or CD8+ cells were then separated from negative cells using a magnetic column and magnetic separator. The purity of positive cells was ≥93% after positive selection.

Mixed lymphocyte culture. All culture media reagents were purchased from Gibco-BRL (Gaithersburg, MD), except normal mouse serum, which was purchased from Pel-Freez ( Rogers, AR). Splenic T cells were cultured with irradiated B6D2F1 splenocytes (20 Gy) at a 1:2 ratio in Dulbecco’s modified Eagle medium supplemented with 1% normal mouse serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acid, 0.02 mmol/L L-β-mercaptoethanol, and 10 mmol/L HEPES (pH 7.75) at 37°C in a humidified incubator supplemented with 7% CO2. For determination of cytokine production, 2 × 10^5 B6 T cells were cultured with 4 × 10^5 irradiated B6D2F1 splenocytes (20 Gy) or 0.5 × 10^5 irradiated B6D2F1 peritoneal cells (32 Gy) or with 0.5 × 10^5 irradiated B6 peritoneal cells (32 Gy) plus 2.5 μg/mL ConA in a 96-well flat-bottom plate. At 48 hours, supernatants were collected for measuring cytokine levels; cells were then pulsed with [3H]-thymidine (1 μCi/well) for an additional 20 hours to determine T-cell proliferation.

BMT. This protocol has been described previously. Donor mice were injected with control diluent or G-CSF for 6 days. Before transplantation, B6D2F1 recipients received 11 Gy total body irradiation, split into 2 doses separated by 3 hours to minimize gastrointestinal toxicity. For experiments to determine cytokine production, 5 × 10^5 BM cells plus 2 × 10^5 splenic T cells from B6 donors were injected intravenously into B6D2F1 recipients. Splenocytes were harvested on day 13 post-BMT. Splenocytes (5 × 10^5) from each recipient were incubated in triplicate with 2.5 μg/mL ConA for 48 hours. Cell proliferation and cytokine production were determined as described above. For survival and GVHD induction experiments, 2 × 10^5 splenocytes from B6 Ly-5+ donors were injected intravenously into B6D2F1 recipients. Recipients transplanted with 5 × 10^5 T-cell–depleted BM (TCO-BM; treated with anti-Thy1.2 and rabbit complement) from control donors were used as non-GVHD controls. Survival was monitored daily, recipients' body weights were measured weekly, and donor cell engraftment was determined by examining the percentage of Ly-5+ cells in PB at day 28 post-transplantation.

Fluorescence-activated cell sorter (FACS) analysis. Fluorescein isothiocyanate (FITC)- or R-phycocerythrin (PE)-conjugated monoclonal antibodies (MoAbs) to mouse Gr-1, CD4, CD8, NK1.1, CD25, and CD69 were purchased from Pharmingen (San Diego, CA). FITC-conjugated antimouse CD3 was purchased from Boehringer Mannheim (Indianapolis, IN). A total of 5 × 10^6 cells/sample were first incubated with MoAb 2.4G2 for 15 minutes at 4°C to block nonspecific binding to Fc receptors and then were incubated with FITC- or PE-conjugated specific MoAbs for 30 minutes at 4°C. Cells were then washed twice with phosphate-buffered saline (PBS)/0.2% bovine serum albumin (BSA) and fixed with PBS/1% paraformaldehyde. Two-color flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) as described previously. For determining the extent of donor cell engraftment, PB cells (PBs) were first incubated with MoAb 2.4G2 for 15 minutes at 4°C and then with anti Ly-5.1 MoAb or anti Ly-5.2 MoAb for 30 minutes at 4°C. After washing with PBS/0.2% BSA twice, cells were then incubated with FITC-conjugated goat antimouse IgG2a (CalTag, San Francisco, CA) at 4°C for 30 minutes. Finally, cells were washed twice with PBS/0.2% BSA and fixed with PBS/1% paraformaldehyde. In control experiments, PBS from donor B6 Ly-5+ mice was 99.5% Ly-5.1+, and PBS from recipient B6D2F1 mice was 99.4% Ly-5.2+ (data not shown).

Enzyme-linked immunosorbent assay. The antibodies used in the assays were purchased from PharMingen (San Diego, CA), and assays were performed according to the manufacturer’s protocol. Briefly, IFN-γ, IL-4, or IL-2 is samples were captured by the specific primary MoAb and detected by the biotin-labeled secondary MoAb. The assays were developed by avidin-peroxidase and its substrate, and plates were read at 405 nm using a microplate reader (Model 3550, Bio-Rad Labs, Hercules, CA). Recombinant murine (m) IL-2 (Genzyme, Cambridge, MA), mIL-4 (Schering-Plough, Kenilworth, NJ), and mIFN-γ (Amgen Biologicals, Thousand Oaks, CA) were used as standards for ELISA assays. The sensitivity of the assays is 0.5 U/mL for all three cytokines.

Statistical analysis. Unpaired two-tailed Student’s t-test was used for the statistical analysis. P = .05 was considered statistically significant.

RESULTS

Effects of G-CSF on murine myeloid and lymphoid cells. We examined the effectiveness of G-CSF in our model by analyzing granulocyte populations in BM and PB in 96 mice after injection. B6 mice were administered a subcutaneous injection of G-CSF (0.01 μg/g body weight) daily for 4 to 6 days, a dose and duration similar to that used in human clinical trials. No changes in physical appearance or behavior

Fig 1. Effect of G-CSF on granulocyte population and T-cell phenotype. B6 mice were injected with control diluent (□) or G-CSF (■) for 4 to 6 days. BM (n = 12 per group) and splenic T cells (n = 20 per group) were harvested the day after the last injection. After lysis of red blood cells, cells were stained with specific antibodies and analyzed by FACS. The results represent mean ± SD from nine experiments. **, P < .0001 versus control mice.
were observed in treated mice. BM was harvested on the day after the last injection. The size of the granulocyte compartment was determined by measuring the number of Gr-1+ cells by FACS.27 Although G-CSF treatment did not change the yield of BM cells, the percentage of granulocytes in BM was significantly increased in the G-CSF group (Fig 1, P < .0001). The percentage of Gr-1+ cells in PB was also significantly increased after G-CSF treatment, ranging between 160% to 210% of the control group (P < .0001; data not shown).

We next examined the phenotype of splenic T cells after G-CSF injection. G-CSF altered neither the ratio of CD4+ and CD8+ T cells after 4 to 6 days of injections (Fig 1) nor the yield of T-enriched splenocytes (data not shown). We then analyzed the effect of G-CSF treatment on T-cell function. Splenic T cells from control or G-CSF-treated B6 mice were cocultured with irradiated B6D2F1 splenocytes (B6D2F1) or unirradiated B6 peritoneal cells plus 2.5 μg/mL ConA (ConA). The supernatants were collected at 48 hours for cytokine measurement. Proliferation was determined by incubation of cells with [3H]-thymidine (1 μCi/well) for an additional 20 hours. The results for one of three similar experiments are shown (mean ± SD).

* P < .05 versus control mice.
† P = .005 versus control mice.

when ConA was used as a stimulus, proliferation of T cells from G-CSF-treated mice was slightly, but significantly, lower than that observed in the control group. In terms of cytokine production, IL-2 secretion in response to both stimuli was reduced in the G-CSF-treated group. A second type-1 cytokine, IFN-γ, was also decreased in response to both stimuli.

To evaluate more precisely the effects of G-CSF on the function of specific T-cell subpopulations, CD4+ and CD8+ T cells were isolated from splenic T cells and stimulated separately; the purity of these populations was ≥ 93% and ≥ 96%, respectively. Proliferation of both CD4+ and CD8+ subsets from G-CSF-treated mice was reduced in response to alloantigen, but no statistical difference in cell proliferation was observed between groups with mitogen stimulation. By contrast, IL-2 and IFN-γ production by both CD4+ and CD8+ cells was reduced in G-CSF-treated mice by at least 25% in response to B6D2F1 stimulators. This reduction in type-1 cytokine secretion was not limited to alloantigen stimulation, but was also observed after stimulation with mitogen. Production of IL-4 by T cells from both groups was under the limit of detection in response to stimulation with both alloantigen and mitogen during primary culture (data not shown).

### Table 1. Effect of G-CSF on T-Cell Function During Primary MLR

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Unseparated T cells</th>
<th>CD4+</th>
<th>CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control G-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6D2F1</td>
<td>33.3 ± 3.3</td>
<td>35.7 ± 0.3</td>
<td>22.5 ± 1.1</td>
</tr>
<tr>
<td>ConA</td>
<td>157.5 ± 7.9</td>
<td>139.1 ± 1.5*</td>
<td>119.7 ± 3.4</td>
</tr>
<tr>
<td>IL-2 (U/mL)</td>
<td>62.9 ± 3.6</td>
<td>51.4 ± 4.6*</td>
<td>55.9 ± 4.9</td>
</tr>
<tr>
<td>ConA</td>
<td>137.5 ± 9.2</td>
<td>110.4 ± 8.0*</td>
<td>261.8 ± 12.3</td>
</tr>
<tr>
<td>IFN-γ (U/mL)</td>
<td>2.7 ± 0.4</td>
<td>1.7 ± 0.2*</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>ConA</td>
<td>22.4 ± 2.2</td>
<td>15.6 ± 2.5*</td>
<td>4.1 ± 0.4</td>
</tr>
</tbody>
</table>

B6 mice were injected with diluent (control) or G-CSF (n = 4 per group) for 4 days; splenic T cells were harvested on day 5. Unseparated T cells and CD4+ and CD8+ cells were stimulated in triplicate with irradiated B6D2F1 splenocytes (B6D2F1) or irradiated B6 peritoneal cells plus 2.5 μg/mL ConA (ConA). The supernatants were collected at 48 hours for cytokine measurement. Proliferation was determined by incubation of cells with [3H]-thymidine (1 μCi/well) for an additional 20 hours. The results for one of three similar experiments are shown (mean ± SD).

* P < .05 versus control mice.
† P = .005 versus control mice.

### Table 2. T-Cell Proliferation and Lymphokine Production After 48-Hour Secondary MLR

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Unseparated T cells</th>
<th>CD4+</th>
<th>CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control G-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation (cpm x 10^-2)</td>
<td>12.6 ± 2.2</td>
<td>13.9 ± 2.7</td>
<td>21.9 ± 3.3</td>
</tr>
<tr>
<td>IL-4 (U/mL)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>IL-2 (U/mL)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>IFN-γ (U/mL)</td>
<td>48.3 ± 4.2</td>
<td>32.0 ± 7.71</td>
<td>75.9 ± 4.0</td>
</tr>
</tbody>
</table>

Splenic T cells from a primary MLR (n = 4 per group) were separated into CD4+ and CD8+ subsets; cells were then restimulated in triplicate with irradiated B6D2F1 peritoneal cells. The supernatants were collected at 48 hours for cytokine measurement. Proliferation was determined by incubation of cells with [3H]-thymidine (1 μCi/well) for an additional 20 hours. The results for one of three similar experiments are shown (mean ± SD).

* P = .01 versus control mice.
† P = .05 versus control mice.
These surprising results led us to examine whether pre-treatment of responders with G-CSF in vivo could cause long-lasting polarization of T-cell function. T cells from control and G-CSF–treated mice were cocultured with irradiated B6D2F1 splenocytes in a primary MLR for 1 week and were then separated into CD4+ or CD8+ populations. Cells were restimulated with irradiated B6D2F1 peritoneal cells or ConA in a secondary culture; cytokine secretion and proliferation assays were performed as in Table 1. As shown in Table 2, in a secondary MLR of unseparated T cells, proliferation was not statistically different between the G-CSF–treated and the control groups, but IFN-γ secretion was significantly reduced by 35%. IL-2 and IL-4 secretion were under the limit of detection in both groups. When T cells were separated into CD4+ and CD8+ populations, the polarization away from type-1 and toward type-2 responses became readily apparent. CD4+ cells from G-CSF–treated mice showed markedly elevated IL-4 production together with complete abrogation of IL-2 production and significant reduction in IFN-γ secretion. Similar alterations of cytokine production were observed in CD8+ responder cells. We also examined the cytokine production after mitogen stimulation because differential responses in cytokine production have been observed after stimulation with alloantigen and mitogen.25 Increases in IL-4 production together with decreases of IL-2 and IFN-γ secretion after mitogen stimulation were observed in unseparated T cells along with CD4+ and CD8+ subsets obtained from G-CSF–treated mice (Fig 2). Thus, 4 days of G-CSF treatment in vivo significantly altered the cytokine response of both CD4+ and CD8+ T-cell subsets; the polarization toward type-2 cytokine production was maintained regardless of stimuli in the secondary culture.

G-CSF pretreatment of donors reduces the ability of donor cells to mediate GVHD. Because IFN-γ is thought to be critical for the induction of acute GVHD,13,15,19 we next examined the effects of G-CSF pretreatment of donors on cytokine production in a BMT model of GVHD to both major and minor histocompatibility antigens. B6D2F1 mice were irradiated with 1,100 cGy, and transplanted with 5 × 10^6 BM and 2 × 10^6 splenic T cells from B6 donor mice treated with either control diluent or G-CSF for 6 days. Splenocytes were harvested on day 13 post-BMT, and T-cell phenotype and cytokine production were analyzed. No difference in the number of CD4+ or CD8+ cells was observed between recipients transplanted with cells from G-CSF–treated and control donors (data not shown). By contrast, a polarization of T-cell cytokine production was again apparent in recipients of G-CSF–treated donors (Fig 3). Splenocytes from mice transplanted with cells from G-CSF–treated donors showed an increased production of IL-4 and a decreased...
production of IL-2 and IFN-γ after stimulation with ConA for 48 hours. Thus, changes in the cytokine profile observed after transplantation of cells from G-CSF-treated donors closely paralleled the changes observed after in vitro stimulation. GVHD induced in this model is severe and often lethal. By day 13 post-BMT, only 54% of recipients of control donors survived (7 of 13), whereas 77% of recipients (10 of 13) of G-CSF-treated donors survived. All animals died with clinical evidence of GVHD (hunched posture, inactivity, and weight loss). Thus, improved survival in recipients of G-CSF-treated donors is associated with increased IL-4 production and decreased IL-2 and IFN-γ production.

We also examined clinical parameters of GVHD and donor cell engraftment for the first month after BMT in this model. Total-body-irradiated B6D2F1 recipients were transplanted with 2 × 10⁷ splenocytes from B6 Ly-5⁺ (Ly-5.1⁺) donors injected with control diluent or G-CSF for 6 days before harvest. Mice transplanted with 5 × 10⁶ TCD-BM from control donors were used as non-GVHD controls. As shown in Fig 4A, all mice receiving splenocytes from control donors died within 8 days from severe GVHD and/or graft failure; BM aplasia can be a feature of severe GVHD across major histocompatibility complex class II barriers, and all mice in this group showed classic signs of GVHD including weight loss, hunched posture, and inactivity. Animals receiving splenocytes from G-CSF-treated donors survived significantly longer, with a median survival time of 21 days (P < .0001). Although animals receiving splenocytes from G-CSF-treated donors showed a definite survival advantage, they showed evidence of clinical GVHD with significant weight loss throughout the month (Fig 4B). The observation of reduced, but significant GVHD in recipients of G-CSF-treated donors is consistent with the substantial amount of type-1 cytokines produced by partially polarized donor splenic T cells from these animals (Fig 4B). Analysis of engraftment at day 28 showed 100% donor origin (Ly-5.1⁺) of PBCs in recipients of G-CSF-treated donors. This level of engraftment was significantly higher than that achieved after transplantation of 5 × 10⁶ TCD-BM from control donors (P < .001). Similar results were obtained in a second experiment in which 2.5 × 10⁷ splenocytes were transplanted (n = 10 per group). Recipients of control donor splenocytes all died at day 8 posttransplantation, whereas 40% of the recipients of G-CSF-treated donors were still alive at day 28 posttransplantation (P < .0001) with complete donor cell engraftment (100% v 84% in recipients of control TCD-BM, P < .001); 20% to 30% weight loss was observed in recipients of G-CSF-treated donor splenocytes throughout the month (P < .005 versus recipients of control TCD-BM). Thus, treatment of donors with G-CSF reduced, although did not completely eliminate, the potential of transplanted splenocytes to cause GVHD, whereas it significantly increased their engraftment potential when compared with transplantation of control TCD-BM.

**DISCUSSION**

In this study, we have shown for the first time that in vivo administration of G-CSF results in a long-lasting polarization of T-cell function toward an anti-inflammatory type-2 cytokine response, and that this polarization is associated with a reduction in the severity of acute GVHD after transplantation of these cells into allogeneic recipients. These results are surprising because G-CSF receptors are rarely detectable on T cells. G-CSF administration has been observed to temporarily increase the number of T lymphocytes in humans without significantly changing the CD4⁺/CD8⁺ ratio. In our study, however, no change in splenic T-cell number or phenotype was observed after administration of G-CSF for 4 to 6 days, despite a significant increase in granulocyte content in BM (Fig 1) and PB. Expression of CD3 antigen on splenic T cells was also different between control and G-CSF-treated mice (data not shown). We then examined effects of G-CSF on other T-cell activation markers, and it did not change the expression of CD25 (IL-2 receptor, α chain) and CD69 (a very early activation antigen) on splenic T cells (data not shown). Therefore, G-CSF treatment does not appear to alter the activation status of these cells in vivo.

It has been reported that the cytokine profile of a T-cell population can shift toward either a type-1 or type-2 response depending on the stimulation and local microenvironment involved. Unseparated T cells along with CD4⁺ and CD8⁺ T-cell subsets obtained from G-CSF-treated mice showed a persistent polarization toward type-2 cytokine production during both primary and secondary stimulation in vitro. Such polarization occurred despite the absence of G-CSF in the culture media (Tables 1 and 2 and Fig 2). Although IL-4 production was below the limit of
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detection during primary stimulation, a marked increase of IL-4 production was observed during secondary stimulation, particularly by CD4+ T cells (Table 2 and Fig 2). A subpopulation of T cells (NK1.1+) has been reported to secrete large amounts of IL-4 upon stimulation.30 However, the expression of NK1.1 on T lymphocytes (CD3+/NK1.1+, or CD4+/NK1.1+) was not statistically different between mice injected with diluent and G-CSF (data not shown).

IFN-γ is believed to play a crucial role in the development of acute GVHD.13-16,19 IFN-γ is responsible for priming macrophages, and increased tumor necrosis factor-α (TNF-α) secretion from these cells results in rapid mortality in response to normally sublethal doses of endotoxin.13 In addition, antibodies to IFN-γ prevent intestinal damage in a murine GVHD model.13 In a recent study, Rus and colleagues19 showed that a very early feature of acute GVHD is the expansion of donor CD8+ cells and the production of IFN-γ by CD8+ and CD4+ donor T cells. Other studies have shown that treatment of donor cells with IL-4 both in vivo and in vitro can polarize T cells toward the type-2 phenotype. Transplantation of these type-2 T cells together with BM into allogeneic recipients markedly reduced the severity of acute GVHD, even though the expansion of donor T cells was unaffected.17,18 In this model of murine GVHD to both major and minor histocompatibility antigens, we observed an increase in IL-4 production along with a decrease in IL-2 and IFN-γ production in response to mitogen by splenocytes from recipients of G-CSF–treated BM and T cells (Fig 3). Pretreatment of donors with G-CSF also improved survival after BMT (Fig 4). G-CSF has a similar effect in an entirely different disease model. Aoki and coworkers31 recently reported that pretreatment of mice with G-CSF reduced serum IL-2 levels and protected them from T-cell–mediated lethal shock triggered by superantigen. It should be noted that type-1 and type-2 cytokine responses in vivo may not be as mutually exclusive as initially observed in T-cell clones.29 Indeed, in an experimental GVHD model in unirradiated recipients, the acute GVH reaction begins as a type-2 response.19 Because G-CSF also has direct anti-inflammatory effects (see below), the mechanisms by which it reduces GVHD may therefore be multiple and not due to an increase or decrease in any single cytokine.

It is important to note that recipients of G-CSF–treated splenocytes showed significantly better engraftment at day 28 posttransplantation than that of mice receiving control TCD-BM. Further experiments are underway to analyze long-term engraftment and the stability of donor hematopoiesis. However, these findings suggest that G-CSF treatment does not inhibit donor T cells from facilitating engraftment across major histocompatibility complex barriers. Donor T cells that are completely polarized toward type-2 function have also been shown to retain significant BM graft-enhancing potential.15

The anti-inflammatory effects of G-CSF have been well documented.13 G-CSF has been shown to enhance anti-in-

Fig 4. Survival, weight loss, and engraftment in a BMT model (B6 Ly-5.1+ B6 D2FlI. B6 Ly-5.2+) were injected with control diluent or G-CSF for 6 days. Total-body–irradiated B6D2Fl recipients (Ly-5.2+) received 5 × 10^5 TCD-BM from control donors (C; n = 8), 2 × 10^5 splenocytes from control donors (D; n = 10), or 2 × 10^5 splenocytes from G-CSF–treated donors (G; n = 10). Survival was measured daily up to 1 month posttransplantation (A); body weights were measured weekly (B); and engraftment was determined on day 28 posttransplantation (C). *, P < .002 versus recipients of control TCD-BM; **, P < .001 versus recipients of control TCD-BM; ***, P < .0001 versus recipients of control splenocytes and recipients of control TCD-BM.
flamatory function of effector cells by increasing expres-
sion of CR1, CR3, FcyRI, and FcyRIII on monocytes.13 The cascade of inflammatory cytokines produced by these effector cells may also be regulated by G-CSF. In a murine model of lipopolysaccharide (LPS)-induced sepsis, G-CSF administration decreased serum TNF-α concentrations, as well as TNF-α released by macrophages on ex vivo LPS challenge.14 Injection of G-CSF into normal volunteers also affected their monocyte responses to LPS challenge by upregulating two important cytokine antagonists, soluble TNF receptors and IL-1 receptor antagonist.15 Such antagonists have been shown to be important in reducing the severity of GVHD, both in experimental models and clinical trials.16,17 Thus, modulation of cytokine production from both T cells and monocytes by G-CSF may help to explain the low incidence of clinical GVHD observed after transplantation of G-CSF–mobilized PBPCs.18,19

Finally, modulation of inflammatory proteins may alter the function of antigen-presenting cells, and such changes may explain the ability of G-CSF to change the patterns of cytokine production. Recent work by two groups has shown that TNF-α increased T-cell proliferation in allogeneic MLRs using human dendritic cells as antigen-presenting cells.18,20 Addition of antiserum specific to TNF-α inhibited this proliferative response.21 TNF-α also increased expression of several critical molecules for antigen presentation such as CD40, ICAM-1, LFA-3, B7.1, and HLA-class I and II molecules.19,22 Thus, G-CSF may affect the antigen-presenting function of monocytes to alloreactive T cells by inhibiting their TNF-α production, which could further favor an anti-inflammatory T-cell cytokine profile. Further analyses of the immunomodulatory activities of G-CSF will be of special interest in the search for alternative sources of hematopoietic stem cells for allogeneic BMT.

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