ASBMT American Society for Blood and Marrow Transplantation

BRIEF ARTICLE

Donor Treatment with a Multipegylated G-CSF Maximizes Graft-versus-Leukemia Effects

Tatjana Banovic,[1] Kelli P. A. MacDonald,[1] Kate A. Markey,[1] Edward S. Morris,[1] Rachel D. Kuns,[1] Antiopi Varelias,[1] Geoffrey R. Hill[1,2]

Donor treatment with granulocyle-colony stimulating factor (G-CSF) is known to modulate immune function, characterized by the generation of regulatory myelogenous and T cell populations and Th2 differentiation. Recently, these effects have been shown to be enhanced by pegylation of the G-CSF molecule, which also improves graft-versus-leukemia (GVL) via activation of invariant natural killer (iNK) T cells. We have compared G-CSF bound to a single PEG molecule (monopeg-G-CSF) as used clinically to a G-CSF molecule bound to multiple PEG molecules (multipeg–G-CSF) in major histocompatibility complex (MHC) disparate and matched models of graft-versus-host disease (GVHD) and GVL. We demonstrate that multipeg-G-CSF induces greater levels of progenitor cell, myelogenous, and iNKT cell expansion than monopeg-G-CSF, while inducing similar protection from GVHD. Despite this, multipeg-G-CSF enhanced CTL function in vivo and improved iNKT cell-dependent leukemia clearance. Thus, GVL and GVHD can be further separated after allogeneic stem cell transplantation by mobilization with a multiple-pegylated G-CSF molecule.

Biol Blood Marrow Transplant 15: 126-130 (2009)

KEY WORDS: Graft-versus-host disease, Graft-versus-leukemia

INTRODUCTION

Graft-versus-host disease (GVHD) remains a major complication following allogeneic hemopoietic stem cell transplantation (SCT), with the resultant multiorgan damage and immune deficiency significantly impairing overall transplant survival. The use of recombinant human granulocyte colony-stimulating factor (G-CSF) mobilized stem cell grafts has led to rapid immune and hemopoietic reconstitution, reduced treatment-related mortality (TRM), and improved leukemia eradication in high-risk patients [1]. T cells from donors treated with G-CSF have a reduced capacity to induce GVHD on a per-cell basis relative to those from control treated donors [2] and G-CSF may also reduce GVHD through effects on multiple cells, including invariant natural killer T (iNKT) cells (reviewed in [3,4]). iNKT cells have inhibitory or stimulatory properties, depending on subsets, anatomic location, and the stage of an immune response on which they act [5-9]. We have previously demonstrated that the clinically available mono–pegylated form of G-CSF (Neulasta) is superior to standard G-CSF for the prevention of GVHD, whereas paradoxically improving graft-versus-leukemia (GVL) via iNKT-dependent effects [10,11]. There are potential biologic advantages to cytokine formulations with multiple pegylation conjugates; however, in some cases such modifications can diminish or eliminate function [12]. We, therefore, investigated whether multiple pegylation (at 2 to 4 sites) of the G-CSF molecule resulted in enhanced immune modulation and improved transplant outcome.

MATERIALS AND METHODS

Mice

Female B6 (H-2^b, CD45.2^+ and PTPRC^a, CD45.1^+), B10.D2 (H-2^d), DBA/2 (H-2^d), and B6D2F1 (H-$2^{b/d}$, CD45.2^+) mice were purchased from the Animal Resources Centre (Perth, Western Australia, Australia). J$\alpha18^{-/-}$ B6 (H-2^b, CD45.2^+) mice were supplied by Mark Smyth (Peter MacCullum Cancer Centre, Melbourne, Australia). Mice were housed in sterilized microisolator cages and received acidified autoclaved water (pH 2.5) post-transplantation.

Cytokine Treatment

Recombinant human G-CSF linked to a single PEG molecule (monopeg-G-CSF, or Neulasta, Amgen,

From the [1]The Queensland Institute of Medical Research, 300 Herston Road, QLD 4006, Australia; and [2]Department of Bone Marrow Transplantation, Royal Brisbane Hospital, Brisbane, QLD 4006, Australia.

Financial disclosure: See Acknowledgments on page 130.

Correspondence and reprint requests: Geoffrey Hill, Queensland Institute of Medical Research, 300 Herston Rd, Herston, QLD 4006, Australia (e-mail: geoffh@qimr.edu.au).

Received October 21, 2008; accepted November 7, 2008
1083-8791/09/151-0001$36.00/0
doi:10.1016/j.bbmt.2008.11.019

Thousand Oaks, CA) or multiply pegylated (multipeg-G-CSF, Amgen) was given subcutaneously at 3 μg/animal on day −6. Donor spleens were harvested on day 0.

Stem Cell Transplantation

Mice were transplanted as described previously [10,11,13]. Briefly, on day –1, B6D2F1 or DBA/2 mice received TBI (1100 cGy or 1000 cGy, respectively), split into 2 doses. B6 (10^7) or B10.D2 (2×10^7) donor splenocytes were equilibrated (to deliver the same T cell doses) or T cell depleted, and injected intravenously on day 0. T cell depletion was undertaken as previously described [13]. Transplanted mice were monitored daily, and those with GVHD clinical scores [14] of 6 or greater were sacrificed and the date of death registered as the next day in accordance with institutional animal ethics committee guidelines.

Leukemia Challenge

The mastocytoma cell line P815 (H-2^d, CD45.2^+), which derived from DBA/2 mice, was injected intravenously into recipients on day 0 of transplantation. Survival and clinical scores were monitored daily and the cause of death established as GVHD or leukemia as previously described [10]. In vivo imaging was performed using the IVIS Imaging System (Xenogen, CA) and light emission is presented as photons/second/cm^2/sr.

Flow Cytometry and In Vivo Cytotoxicity Index

Flow cytometry, including the determination of lineage negative (Mac-1, CD19, Gr-1, CD4, CD8, NK1.1, and TER119), *c-kit*, and Sca-1-positive cells was undertaken as previously described [10,15]. In vivo CTL assays were performed as previously

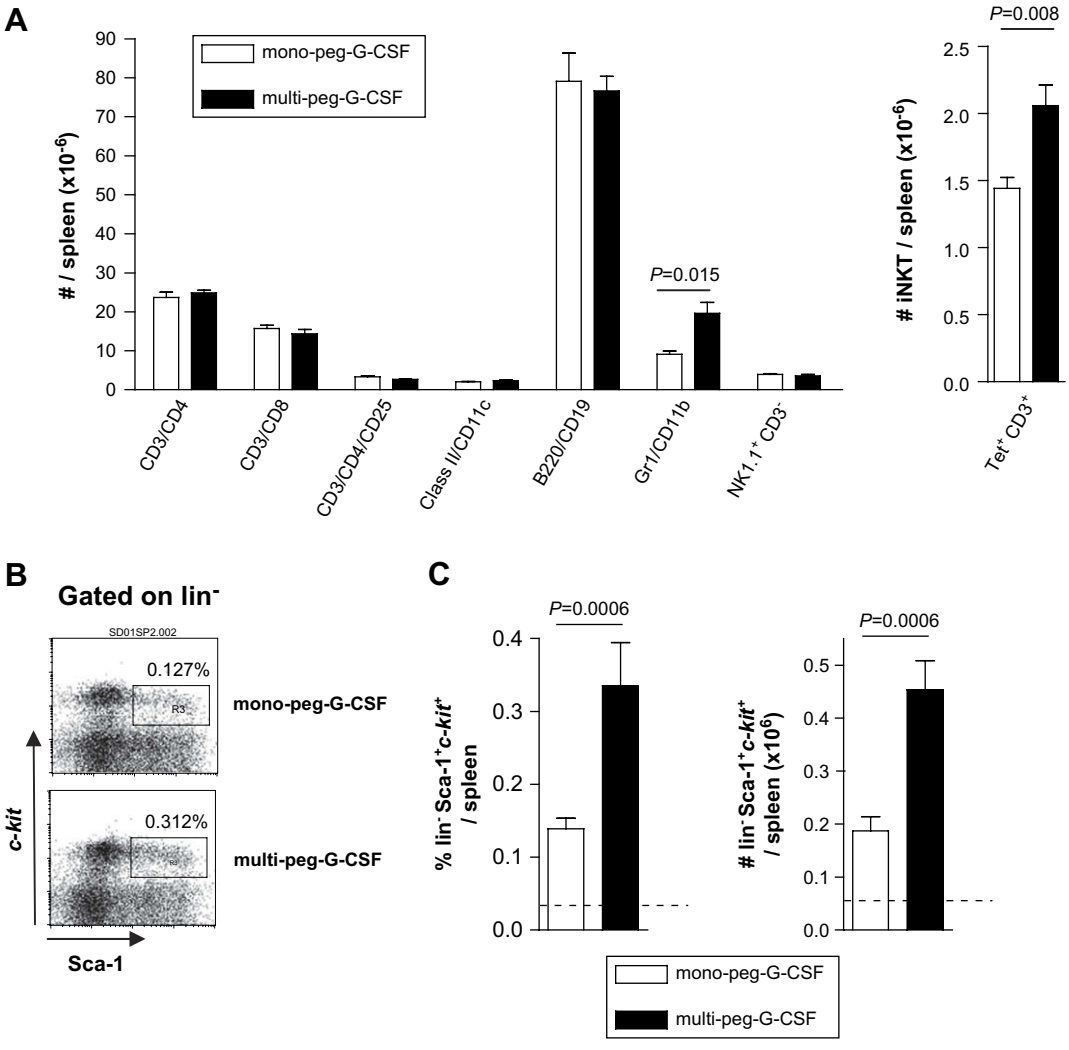

Figure 1. Comparative effects of monopeg-G-CSF and multipeg-G-CSF on donor splenic phenotype. (A) B6 donors received 1 subcutaneous injection of 3 μg of monopeg-G-CSF or multipeg-G-CSF at day −6. Spleens were subsequently phenotyped on day 0, and total numbers of each cell lineage elucidated per spleen (n = 5 or 6 per group). (B) Representative plots of lineage$^-$Sca-1$^+$c-kit$^+$ cells in the spleen 6 days after treatment with monopeg-G-CSF or multipeg-G-CSF. (C) Percentage and absolute numbers of lineage^{neg}*c-kit*$^+$Sca-1$^+$ cells in spleen following monopeg-G-CSF or multipeg-G-CSF treatment (n = 8 per group). Dashed line represents average numbers in naïve animals (n = 3). Data combined from 2 replicate experiments.

described by determining the clearance of adoptively transferred host versus donor splenocytes 12 days after SCT [10,16].

Statistical Analysis

Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank analysis. $P < .05$ was considered statistically significant. Data presented as mean ± SEM.

RESULTS AND DISCUSSION

Multipeg-G-CSF Results in Greater Levels of Progenitor, Myelogenous, and iNKT Cell Expansion, But Similar Protection from GVHD

Phase I clinical studies in normal donors have demonstrated that 12 mg of the singularly pegylated-G-CSF (Neulasta, equivalent to 100-200 μg/kg) results in robust stem cell mobilization with an acceptable side effect profile [17]. Thus, monopeg-G-CSF or multipeg-G-CSF was administered to donor mice at a clinically achievable dose (3 μg/dose, equivalent to 150 μg/kg), and 6 days later spleens were phenotyped. As demonstrated in Figure 1A, the expansion of myelogenous cells (monocytes and granulocytes) and iNKT cells was significantly greater in recipients of multipeg-G–CSF. The absolute numbers of other lineage positive cells were similar. To determine effects on progenitor cell content, lineage negative, *c-kit*$^+$, and sca-1 + stem cells were quantified within the spleen. As shown in Figure 1B and C, multipeg-G-CSF significantly increased the frequency (and number) of progenitor cells in this peripheral site, whereas those in the marrow were mildly reduced (data not shown).

When splenic grafts (equilibrated to T cell dose) were transplanted into major histocompatibility complex (MHC) disparate, lethally irradiated B6D2F1 recipients, both monopeg-G-CSF and multi–peg-G-CSF provided significant protection from GVHD. However, significant differences between monopeg-G-CSF and multipeg-G-CSF were not apparent (Figure 2A), although, in each of 3 experiments, multipeg-G-CSF appeared marginally superior. Because we have previously demonstrated the activation of donor iNKT cells by monopeg-G-CSF with subsequent enhancement of donor CTL function [10], we next determined in vivo CTL generation in SCT recipients 12 days after transplant. As shown in Figure 2B, donor treatment with multipeg-G-CSF resulted in significantly greater CTL activity after SCT than monopeg-G-CSF.

Treatment with Multipeg-G-CSF Improves Overall Survival (OS) and iNKT- Dependent GVL Activity

To study the effect of treatment with multipeg-G-CSF on GVL effects, we utilized an MHC matched (B10.D2 → DBA/2) SCT model of GVHD directed to multiple minor histocompatibility antigens, in

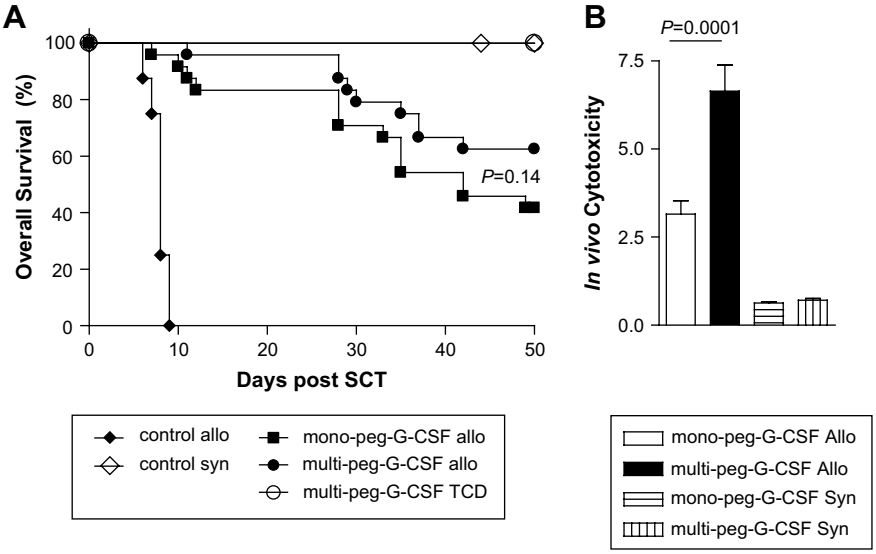

Figure 2. Comparative effects of monopeg-G-CSF and multipeg-G-CSF on GVHD and CTL activity after SCT. (A) Survival by Kaplan-Meier analysis in irradiated B6D2F1 recipients (1100 cGy, day −1) transplanted at day 0 with 10^7 splenocytes from B6 donors treated with monopeg-G-CSF (monopeg-G-CSF allo, n = 24) or multipeg-G-CSF (multipeg-G-CSF allo, n = 24), equilibrated to deliver equal T cell doses. Control B6D2F1 recipients received transplants from saline-treated allogeneic B6 donors (control allo, n = 8) or syngeneic B6D2F1 donors (control syn, n = 9). Additional control recipients were transplanted with T cell-depleted (TCD) allogeneic grafts from multipeg-G-CSF-treated B6 donors (multipeg-G-CSF TCD, n = 4). Results pooled from 3 experiments. *P* values shown compare peg-G-CSF allo groups. (B) Irradiated allogeneic B6D2F1 recipients were transplanted with splenocytes from allogeneic B6 or syngeneic B6D2F1 monopeg-G-CSF (monopeg–G-CSF allo, n = 15, monopeg-G-CSF syn, n = 3) or multipeg-G-CSF (multipeg-G–CSF allo, n = 16, multipeg-G-CSF syn, n = 6) treated donors. At day +12 the in vivo cytotoxicity index was determined as described in Materials and Methods. Data represented as mean ± SE from 3 experiments.

which recipients also received host-type luciferase expressing leukemia (P815) at the time of transplant. The recipients of multipeg-G-CSF mobilized grafts demonstrated significantly improved OS (Figure 3A), relative to recipients of monopeg-G-CSF mobilized grafts because of enhanced leukemia eradication (Figure 3B) that was confirmed by biophotonic imaging post-SCT (Figure 3C and D). To confirm that this was indeed related to effects on iNKT cells, wild-type (WT) and iNKT deficient ($J\alpha 18^{-/-}$) B6 donors were mobilized with multipeg-G-CSF and grafts transplanted into irradiated B6D2F1 recipients in the presence of host-type leukemia and GVL monitored thereafter. As shown in Figure 3E, recipients of T cell-depleted grafts died by day 12 of leukemia, whereas over 60% of recipients of multipeg-G-CSF mobilized T cell-replete grafts survived. In contrast, recipients of multipeg-G-CSF mobilized $J\alpha 18^{-/-}$ grafts all developed progressive leukemia with a median survival of only 23 days.

Consistent with this, the leukemic burden as determined by biophotonic imaging was higher in recipients of $J\alpha\ 8^{-/-}$ multipeg-G-CSF mobilized grafts relative to WT multpeg-G-CSF mobilized grafts (luminescence ($\times 10^5$ photons/second/cm^2/sr, respectively) at day 18: 7.1 ± 4.0 versus 3.7 ± 1.6, $P = .028$). Furthermore, GVHD mortality in recipients of WT multipeg-G-CSF and $J\alpha\ 8^{-/-}$ multipeg-G-CSF grafts was 29% and 0%, respectively ($P = .16$), confirming that GVHD was not increased in the absence of donor NKT cells. This is consistent with our previous data demonstrating that donor NKT cells exacerbated GVHD following mobilization with monopeg-G- CSF.[10]

The ability of pegylated-G-CSF to modulate the immune system to greater levels than standard G-CSF

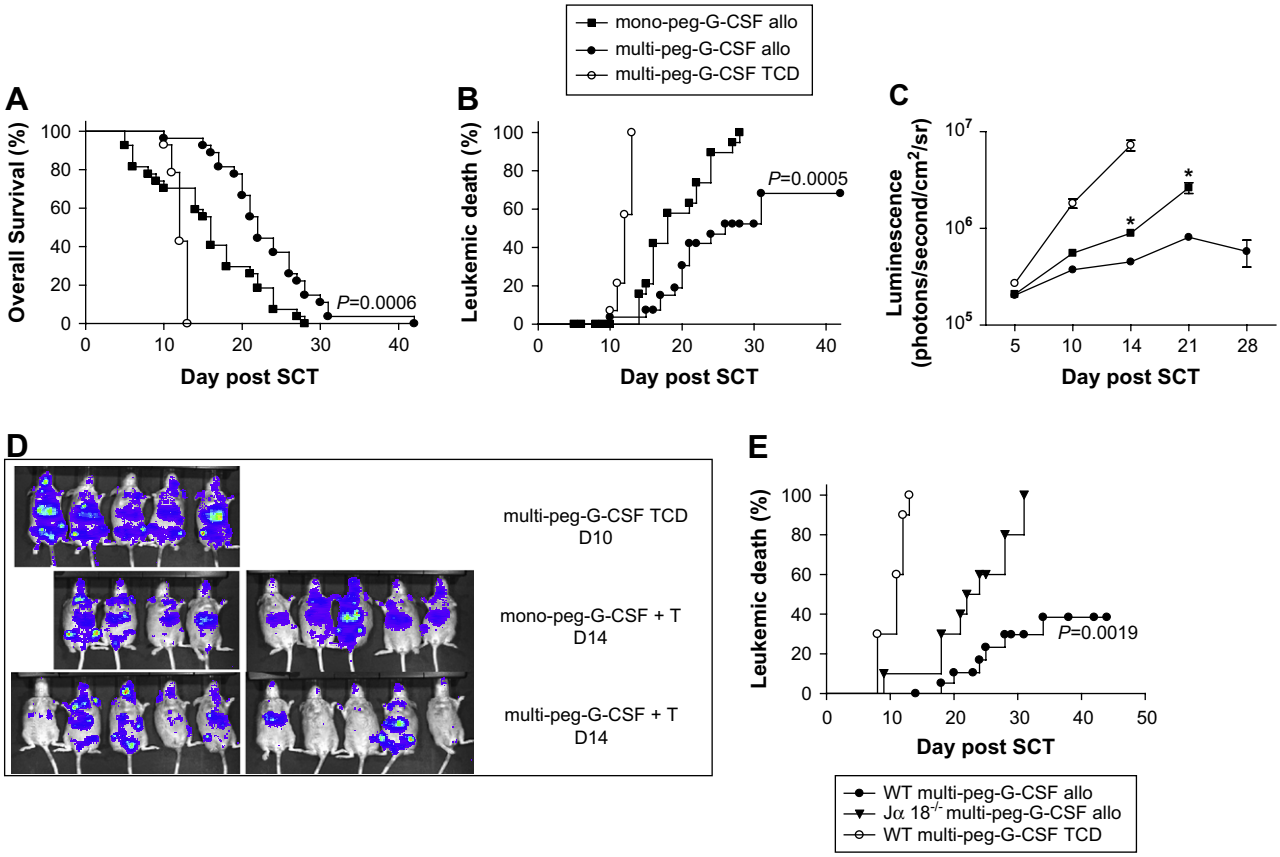

Figure 3. Multipeg-G-CSF mediates GVL effects in an NKT cell-dependent fashion. (A) OS by Kaplan-Meier analysis in irradiated (1000 cGy) DBA/2 recipients transplanted with splenocytes from allogeneic B10.D2 (2×10^7 cells per mouse) donors treated with monopeg-G-CSF or multipeg-G-CSF (n = 27 each), equilibrated to deliver equal T cell doses. Non-GVHD controls received splenocytes from multipeg-G-CSF treated donors that were T cell depleted (n = 15). Leukemia was induced in all recipients by coinjection of 5×10^3 host-type luciferase expressing P815 cells on day 0. Data pooled from 3 experiments. *P* values shown compare peg –G-CSF allo groups. (B) Leukemic mortality in the recipients shown in (A) by Kaplan- Meier analysis. *P* values shown compare peg-G-CSF allo groups. (C) Luminescence (photons/second/cm^2/sr) over time as a determinant of leukemia burden in the recipients shown in (A). Results are mean ± SE from 3 experiments, $*P < .05$, mono–peg-G-CSF allo versus multipeg-G-CSF allo. (D) Xenogen biophotonic imaging of leukemia development at days +10-14 in allogeneic (T cell replete; +T) DBA/2 recipients of monopeg-G-CSF or multipeg-G-CSF-treated B10.D2 grafts or multi–peg-G-CSF TCD grafts. All TCD recipients developed leukemia on day 10 and required sacrifice prior to day 14. (E) Leukemic mortality by Kaplan-Meier analysis in B6D2F1 recipients transplanted with splenocytes from multipeg-G-CSF-treated allogeneic wild-type (WT multipeg-G-CSF allo, n = 20), NKT cell deficient $J\alpha 18^{-/-}$ ($J\alpha\ 18^{-/-}$, multipeg-G-CSF allo, n = 20), or T cell-depleted WT (WT multipeg-G-CSF TCD, n = 10) B6 donors in conjunction with 5×10^4 host-type P815 leukemia cells. *P* values shown compare peg-G-CSF allo groups.

is likely to be the result of the greatly increased half life (from hours to days) in conjunction with an apparent enhancement of receptor-ligand binding and trafficking at the cell surface [3,18]. This appears to allow the molecule to invoke effects in cell subsets that are otherwise not demonstrable following standard G-CSF administration, namely, iNKT cells (reviewed in [3]). The activation of donor $CD4^{neg}CD8^{neg}$ iNKT cells, thereafter, improves CTL priming via effects on host APC [3,10]. The additional increase in biologic activity by multiple-pegylation is likely to be imparted by enhancement of the same mechanisms. However, it is important to note that these effects cannot be reproduced by mobilization with escalating doses of standard G-CSF [10]. These data suggest that mobilizing stem cells with multipegylated versions of G-CSF may be advantageous in the allogeneic SCT setting to further separate GVHD and GVL.

AUTHOR CONTRIBUTION STATEMENT

Financial disclosure: T.B. performed research and wrote the paper; K.P.A.M. performed research and helped design studies; K.M. performed research; E.S.M. performed research; R.D.R. performed research; A.V. performed research; G.R.H, designed the research and helped write the paper.

ACKNOWLEDGMENTS

Financial disclosure: G.R.H. is a National Health and Medical Research Council (NH&MRC) Practitioner Fellow. K.P.A.M. is an NH&MRC RD Wright Fellow. This work was supported by grants from the NH&MRC. The authors have no competing financial interests.

REFERENCES

1. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med*. 2001;344:175-181.
2. Pan L, Delmonte J, Jalonen CK, Ferrara JLM. Pretreatment of donors with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type 2 cytokine production and reduces severity of experimental graft versus host disease. *Blood*. 1995;86:4422-4429.
3. Morris ES, MacDonald KP, Hill GR. Stem cell mobilization with G-CSF analogs: a rational approach to separate GVHD and GVL? *Blood*. 2006;107:3430-3435.
4. Rutella S, Zavala F, Danese S, Kared H, Leone G. Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. *J Immunol*. 2005;175:7085-7091.
5. Morris SZ, Madden KB, Adamovicz JJ, et al. Effects of IL-12 on in vivo cytokine gene expression and Ig isotype selection. *J Immunol*. 1994;152:1047-1056.
6. Hashimoto D, Asakura S, Miyake S, et al. Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells. *J Immunol*. 2005;174:551-556.
7. Zeng D, Lewis D, Dejbakhsh-Jones S, et al. Bone marrow NK1.1(−) and NK1 .1 (+) T cells reciprocally regulate acute graft versus host disease. *J Exp Med*. 1999;189:1073-1081.
8. Pillai AB, George TI, Dutt S, Teo P, Strober S. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol*. 2007; 178:6242-6251.
9. Crowe NY, Coquet JM, Berzins SP, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J Exp Med*. 2005;202:1279-1288.
10. Morris ES, Macdonald KP, Rowe V, et al. NKT cell-dependent leukemia eradication following stem cell mobilization with potent G-CSF analogs. *J Clin Invest*. 2005;115:3093-3103.
11. Morris ES, MacDonald KPA, Rowe V, et al. Donor treatment with pegylated G-CSF augments the generation of IL-10 producing regulatory T cells and promotes transplant tolerance. *Blood*. 2004;103:3573-3581.
12. Bailon P, Berthold W. Polyethylene glycol-conjugated pharmaceutical proteins. *Pharmaceut Sci Technol Today*. 1998;1:352-356.
13. MacDonald KP, Rowe V, Filippich C, et al. Donor pretreatment with progenipoietin-1 is superior to G-CSF in preventing graft-versus-host disease after allogeneic stem cell transplantation. *Blood*. 2003;101:2033-2042.
14. Cooke KR, Kobzik L, Martin TR, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation. I. The roles of minor H antigens and endotoxin. *Blood*. 1996;88:3230-3239.
15. Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, Suda T. In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood*. 1992;80: 3044-3050.
16. Banovic T, MacDonald KP, Morris ES, et al. TGF-beta in allogeneic stem cell transplantation: friend or foe? *Blood*. 2005;106: 2206-2214.
17. Hill GR, Morris ES, Fuery M, et al. Allogeneic stem cell transplantation with peripheral blood stem cells mobilized by pegylated G-CSF. *Biol Blood Marrow Transplant*. 2006;12:603-607.
18. Sarkar CA, Lowenhaupt K, Wang PJ, Horan T, Lauffen burger DA. Parsing the effects of binding, signaling, and trafficking on the mitogenic potencies of granulocyte colony-stimulating factor analogues. *Biotechnol Prog*. 2003;19:955-964.