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Chronic Graft-versus-Host Disease after Granulocyte Colony-Stimulating Factor–Mobilized Allogeneic Stem Cell Transplantation: The Role of Donor T-Cell Dose and Differentiation

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ABSTRACT

The use of granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood as a source of stem cells has resulted in a high incidence of severe chronic graft-versus-host disease (cGVHD), which compromises the outcome of clinical allogeneic stem cell transplantation. We have studied the effect of G-CSF on both immune complex and fibrotic cGVHD directed to major (DBA/2 → B6D2F1) or minor (B10.D2 → BALB/c) histocompatibility antigens. In both models, donor pretreatment with G-CSF reduced cGVHD mortality in association with type 2 differentiation. However, after escalation of the donor T-cell dose, scleroderma occurred in 90% of the recipients of grafts from G-CSF–treated donors. In contrast, only 11% of the recipients of control grafts developed scleroderma, and the severity of hepatic cGVHD was also reduced. Mixing studies confirmed that in the presence of high donor T-cell doses, the severity of scleroderma was determined by the non–T-cell fraction of grafts from G-CSF–treated donors. These data confirm that the induction of cGVHD after donor treatment with G-CSF is dependent on the transfer of large numbers of donor T cells in conjunction with a putatively expanded myeloid lineage, providing a further rationale for the limitation of cell dose in allogeneic stem cell transplantation.

KEY WORDS

G-CSF • Th2 • Scleroderma • Lupus • Fibrosis

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is currently indicated in the treatment of a number of malignant and nonmalignant diseases. However, use of the procedure is limited by its serious complications, the most common of which is graft-versus-host disease (GVHD). Recently, the transplantation of stem cells that are collected from the peripheral blood of granulocyte colony-stimulating factor (G-CSF)–treated donors (peripheral blood stem cell transplantation [PBSCT]) has become widespread. GVHD remains the major limitation of both procedures (hereafter termed stem cell transplantation [SCT]). GVHD in the first 100 days of SCT is termed acute GVHD (aGVHD) and occurs in most SCT recipients, damaging the skin, gastrointestinal (GI) tract, and liver. GVHD more than 100 days after SCT is termed chronic GVHD (cGVHD) and may be limited (to the skin, liver, or both) or extensive (involving the liver, skin, and additional organs, particularly the mouth, eyes, and lung). Extensive cGVHD has a major negative effect on survival and quality of life after PBSCT, necessitating prolonged and poorly effective immunosuppressive therapy. Clinical risk factors for the development of cGVHD include increasing age of donor

and recipient, prior aGVHD, and second transplantation [1-4].

It has recently become clear that extensive cGVHD is more frequent after PBSCT than after traditional BMT [5-7]. Even so, allogeneic PBSCT is associated with improved rates of immune and hematopoietic reconstitution, reduced transplant-related mortality, and improved leukemia eradication relative to BMT [8]. For these reasons, PBSCT has become the procedure of choice for the treatment of advanced hematologic malignancies, and the transplant community must now meet the challenge of improving strategies to both prevent and treat cGVHD. Data from clinical PBSCT have associated the incidence of cGVHD with a number of graft-related factors, the most consistent of which is CD34 number [9]. Studies have also suggested a relationship between type 2 T-cell phenotype, large numbers of type 2 dendritic cells, and protection from cGVHD [10,11]. Previous studies have confirmed that G-CSF alters the capacity of dendritic cells and monocytes to induce inflammatory responses in vitro [12,13], although a causal relationship between these effects and the inhibition of GVHD remains unproven at this time.

Chronic GVHD is characterized by autoantibody generation and scleroderma and has been suggested as a T-helper type 2 (Th2)–dominant disease process [14]. G-CSF is known to alter T-cell function [15], and PBSCT products contain 10 to 20 times the number of T cells relative to bone marrow grafts, both of which may increase cGVHD. Furthermore, G-CSF has been shown to induce Th2 differentiation in donor T cells before SCT, and this been suggested to be a major protective mechanism from aGVHD in this setting [16]. In support of this, administration of other cytokines that induce Th2 differentiation either in vivo [17] or in vitro [18] also protect from aGVHD. However, it is unclear as to whether this change in T-cell differentiation induced by G-CSF before SCT is indicative of T-cell differentiation after SCT or plays any causative role in the high rate of cGVHD seen after PBSCT.

We have examined the ability of stem cell grafts mobilized by G-CSF to induce cGVHD and the mechanisms therein. Our data confirm that donor pretreatment with G-CSF results in donor Th2 differentiation after SCT but that this phenotype is protective from both aGVHD and cGVHD when low numbers of donor T cells are transplanted. In contrast, escalation of the donor T-cell dose results in high rates of cGVHD when the graft also contains a non–T-cell lineage that has been expanded or modified by G-CSF. These data confirm that the development of cGVHD after allogeneic SCT is dependent on the transfer of large numbers of donor T cells in conjunction with a putatively expanded myeloid lineage.

METHODS

Mice

Female C57BL/6 (B6, H-2^b, Ly-5.2^+), B6 *ptprc*^{a} Ly-5a (H-2^b, Ly-5.1^+), B6D2F1 (H-$2^{b/d}$, Ly-5.2^+), B10.D2 (H-2^d), DBA/2, and BALB/c (H-2^d) mice were purchased from the Australian Research Centre (Western Australia, Australia). The age of mice used as BMT recipients ranged between 8 and 14 weeks. Mice were housed in sterilized microisolator cages and received filtered water and normal chow or autoclaved drinking water after BMT.

Cytokine Treatment

Recombinant human G-CSF (Amgen, Thousand Oaks, CA) or control diluent was diluted in 1 μg/mL of murine serum albumin in phosphate-buffered saline (PBS) before injection. Mice were injected subcutaneously with G-CSF (2 μg per animal per day) or diluent once daily from day −6 to day −1.

Bone Marrow Transplantation

Mice underwent transplantation according to a standard protocol that has been described previously [16,19]. Briefly, on day −1, BALB/c recipient mice received 600 cGy of total body irradiation (cesium 137 source at 108 cGy/min), which was split into 2 doses separated by 3 hours to minimize GI toxicity. B6D2F1 recipients were not irradiated. Donor spleens were mashed, and then whole unseparated spleen cells (5×10^7 B6 or 9×10^7 DBA/2) were resuspended in 0.25 mL of Leibovitz L-15 media (Gibco BRL, Gaithersburg MD) and injected intravenously into recipients. Survival was monitored daily, and the recipient's body weight and GVHD clinical score were measured weekly. Donor cell engraftment in the B6 → B6D2F1 model of aGVHD was determined by examining the proportion of Ly-5.1^+/(Ly-5.2^+ + Ly-5.1^+) cells in peripheral blood or spleen after transplantation. Donor cell engraftment in the DBA/2 → B6D2F1 model of cGVHD was determined by examining the proportion of H-2^b-negative, lineage-positive cells.

Assessment of GVHD

The degree of systemic GVHD was assessed by a scoring system that sums changes in 5 clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity (maximum index = 10) [17,20-22]. Individual mice were ear-tagged and graded weekly from 0 to 2 for each criterion without knowledge of treatment group. Animals with severe clinical GVHD (scores >6) were killed according to ethical guidelines, and the day of death was deemed to be the following day. Proteinuria was determined weekly after day 100 by using Multistix (Bayer, Newbury, Berkshire, UK). A cutaneous clinical score was

also generated by summation of the scores for fur and skin from individual animals. The descriptors for scoring were as follows: (1) fur texture—0, normal; 0.5, partial ventral fur ruffling; 1.0, complete ventral fur ruffling; 1.5, complete ventral fur ruffling and partial dorsal fur ruffling; and 2.0, diffuse ventral and dorsal fur ruffling; (2) skin integrity—0, normal; 0.5, scaling of paws, tail, or ears and loss of fur <1 cm^2; 1.0, loss of fur between 1 and 2 cm^2; 1.5, loss of fur >2 cm^2; and 2.0, loss of fur with cutaneous ulceration.

Fluorescence-Activated Cell Sorting Analysis

Fluorescein isothiocyanate–conjugated monoclonal antibodies (mAb) to mouse Ly-5.1 and Ly-5.2 antigens; fluorescein isothiocyanate–conjugated mAb to CD4, CD8, CD11c, class II, CD3, GR-1, CD11b, and B220; and identical phycoerythrin-conjugated mAb were purchased from PharMingen (San Diego, CA). Cells were first blocked with mAb 2.4G2 for 15 minutes at 4°C, followed by the relevant conjugated mAb for 30 minutes at 4°C. Finally, cells were washed twice with PBS/0.2% bovine serum albumin, fixed with PBS/1% paraformaldehyde, and analyzed by FACScalibur (Becton Dickinson, Franklin Lakes, NJ).

Cell Cultures

Culture media additives were purchased from Gibco BRL, and medium was purchased from Sigma (St. Louis, MO). Cell culture was performed in 10% fetal calf serum/Dulbecco modified Eagle medium (day 7 cultures) supplemented with penicillin 50 U/mL, streptomycin 50 μg/mL, L-glutamine 2 mmol/L, sodium pyruvate 1 mmol/L, nonessential amino acid 0.1 mmol/L, β-mercaptoethanol 0.02 mmol/L, and 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.75, at 37°C in a humidified incubator supplemented with 5% CO_2. In experiments of T-cell function ex vivo, splenocytes were removed from animals 7 to 10 days after transplantation, and 3 to 6 spleens were combined from each group. In separation experiments, $CD4^+$ and $CD8^+$ cells were positively selected from splenocyte populations by using fluorescence-activated cell sorting (FACS Vantage; Becton Dickinson) with propidium iodide to exclude nonviable cells. H-2^b was also used to exclude host T cells in the DBA/2 → B6D2F1 system. After selection, positive and negative fractions had $<1\%$ contamination of opposing $CD4^+$ or $CD8^+$ cells. These cells were plated in 96-well flat-bottom plates with platebound anti-CD3 and -CD28 mAbs (both 10 μg/mL).

Cytokine Enzyme-Linked Immunosorbent Assays

The antibodies used in the interferon (IFN)–γ, interleukin (IL)–10, and IL-4 assays were purchased from PharMingen. All assays were performed according to the manufacturer's instructions. Supernatants were collected after 40 hours and stored at −70°C until analysis. Samples were then thawed and diluted, and IFN-γ, IL-10, and IL-4 proteins were captured by the specific primary mAb and detected by biotin-labeled secondary mAb followed by horseradish peroxidase–conjugated streptavidin. The biotin-labeled assays were developed with tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Plates were read at 450 nm by using a microplate reader (Bio-Rad, Hercules, CA). Recombinant cytokines (PharMingen) were used as standards for enzyme-linked immunosorbent assays. Samples and standards were run in duplicate, and the sensitivity of the assays was 0.063 U/mL for IFN-γ and 15 pg/mL for IL-10 and IL-4.

Anti–Single-Stranded DNA Determination

Serum was frozen at −80°C until analysis. Enzyme-linked immunosorbent assay plates were coated with 10 μg/mL of methylated bovine serum albumin for 60 minutes at room temperature and then washed. Plates were then incubated with denatured calf thymus (10 μg/mL), washed, and blocked in 5% fetal calf serum/PBS overnight. Test serum was added at semi–log dilutions for 3 hours the next day after further washing. Specific biotinylated anti-mouse isotype detection antibodies were added (PharMingen), and streptavidin/horseradish peroxidase was added in the final step. Plates were developed with O-phenylenediamine dihydrochloride substrate; reactions were stopped by the addition of HCl 2.5 mol/L and were then read at 490 nm. MRL/lpr serum was used as an internal control to normalize data analyzed at different time points. The optical density of MRL/lpr serum diluted at 1:644 and 1:2120 was used to determine the titer of immunoglobulin (Ig)G1 and IgG2α, respectively, in test samples.

Histology

Formalin-preserved distal small bowel was embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin for histologic examination. Slides were coded and examined in a blinded fashion by 1 individual (A.D.C.), who used a semiquantitative scoring system for abnormalities known to be associated with GVHD [17,21,22]. For the skin, dermal collagen thickness was measured at a minimum of 5 points and averaged. Scleroderma was assigned to the animal when dermal collagen thickness was greater than the average + 3SD of the syngeneic control group. Six parameters were scored from 0 to 4 and summed to assign a dermal inflammatory score (dermal inflammation, keratinocyte apoptosis, cutaneous blistering, epidermal inflammation, dermal fibrosis, and subcutaneous fibrosis). The collagen score was

determined by blinded examination of Masson trichrome staining of skin (graded from 1 to 5). The descriptors were as follows: 1, <50% density collagen without dermal expansion; 2, >50% density collagen without dermal expansion; 3, >80% density collagen with dermal expansion <125% of syngeneic; 4, >80% density collagen, dermal thickness 125% to 200% of syngeneic, or both; and 5, >80% density collagen, dermal thickness >200% of syngeneic, or both. No differences were seen in cutaneous parameters of cGVHD in syngeneic recipients regardless of the donor cell dose or pretreatment (with G-CSF). For the liver, 10 parameters were scored that reflected GVHD of the portal tract (portal tract expansion by inflammatory cell infiltrate, lymphocytic infiltrate of bile ducts, bile duct epithelial cell apoptosis, bile duct epithelial cell sloughing, and vascular endothelialitis) and hepatic parenchyma (parenchymal apoptosis, parenchymal microabscesses, parenchymal mitotic figures, hepatocellular cholestasis, and hepatocellular steatosis). For GI tract analysis, 7 parameters each were scored for small bowel (villous blunting, crypt regeneration, crypt epithelial cell apoptosis, crypt loss, luminal sloughing of cellular debris, lamina propria inflammatory cell infiltrate, and mucosal ulceration). The scoring system for each parameter denoted 0 as normal, 0.5 as focal and rare, 1 as focal and mild, 2 as diffuse and mild, 3 as diffuse and moderate, and 4 as diffuse and severe, as previously published in human [23,24] and experimental [17,21,22] GVHD histology. Scores were added to provide a total score of 24 for the skin, 28 for the small bowel, and 40 for the liver.

Statistical Analysis

Survival curves were plotted by using Kaplan-Meier estimates and were compared by log-rank analysis. The Mann-Whitney *U* test was used for the statistical analysis of cytokine data and clinical scores. $P < .05$ was considered statistically significant.

RESULTS

Donor Pretreatment with G-CSF Reduces Both aGVHD and cGVHD in Nonirradiated Models

We first examined the effects of G-CSF mobilization in well-established murine SCT models of aGVHD (B6 Ly5^a → B6D2F1) or cGVHD (DBA/2 → B6D2F1) that induce GVHD to major and minor histocompatibility antigens (HA) without prior irradiation of the recipient. Although these models use the spleen rather than peripheral blood as a stem cell source, their validity has been established by informative data indicating beneficial effects of G-CSF on both GVHD and graft-versus-leukemia [12,15]; this has since been confirmed clinically [8]. In these studies, allogeneic donor B6 or DBA/2 animals received daily injections of either control diluent or G-CSF (2 μg per animal), administered for 6 days. We have previously shown that this treatment schedule results in similar proportions of T cells in the spleen of B6 donors [15], and this was also true of DBA/2 donors (CD4 T cells, 8.7% ± 0.6% versus 7.2% ± 0.5%; $P = .09$; CD8 T cells, 3.6% ± 0.3% versus 2.9% ± 0.2%; $P = .17$). Splenocytes were harvested on day 7, and B6D2F1 recipient mice underwent transplantation with 5×10^7 or 9×10^7 splenocytes from B6 or DBA/2 donors, respectively. In preliminary experiments, these doses reliably induced GVHD in recipients of control-treated splenocytes. As shown in Figure 1A, aGVHD induced in the acute model was severe; 50% of recipients of control splenocytes died within 8 weeks with characteristic features of GVHD (weight loss, hunching, fur ruffling, and so on), as determined by the clinical score. In contrast, 100% of recipients of non-GVHD controls that underwent transplantation with syngeneic splenocytes survived. Allogeneic SCT recipients of G-CSF splenocytes had a significantly improved survival (95% versus 50%; $P < .01$) and reduced clinical scores compared with recipients of allogeneic control splenocytes. In the cGVHD model, allogeneic SCT recipients of G-CSF–treated DBA/2 splenocytes also had significantly improved survival (85% versus 45%; $P < .05$; Figure 1B). In addition, the incidence of proteinuria was reduced at day 180 in recipients of G-CSF grafts compared with recipients of allogeneic control splenocytes (25% versus 80%, respectively; $P < .01$), although the rate at which disease developed was unchanged (the median time to proteinuria was 90 versus 78 days, respectively).

Donor T-cell engraftment in the acute model at day 28 after SCT was 94.7% ± 1.4% in recipients of control splenocytes and 95.4% ± 0.7% in recipients of G-CSF splenocytes. The cGVHD model is characterized by low-level donor T-cell engraftment, which provides aberrant B-cell help and results in autoantibody generation. Donor CD4 and CD8 engraftment was equivalent in recipients of G-CSF and control-treated splenocytes. The expansion of host B cells in the spleen of animals 14 days after SCT was identical (Table 1), as was host B-cell activation, as determined by major histocompatibility complex class II expression. These data confirm that donor treatment with G-CSF did not induce quantitative differences in donor T-cell engraftment or host B-cell activation and expansion.

Donor Pretreatment with G-CSF Results in Th2 Differentiation after SCT

The preferential differentiation of donor T cells in a type 2 fashion is known to reduce the severity of aGVHD [25]. Donor pretreatment with G-CSF is known to be capable of inducing Th2 differentiation

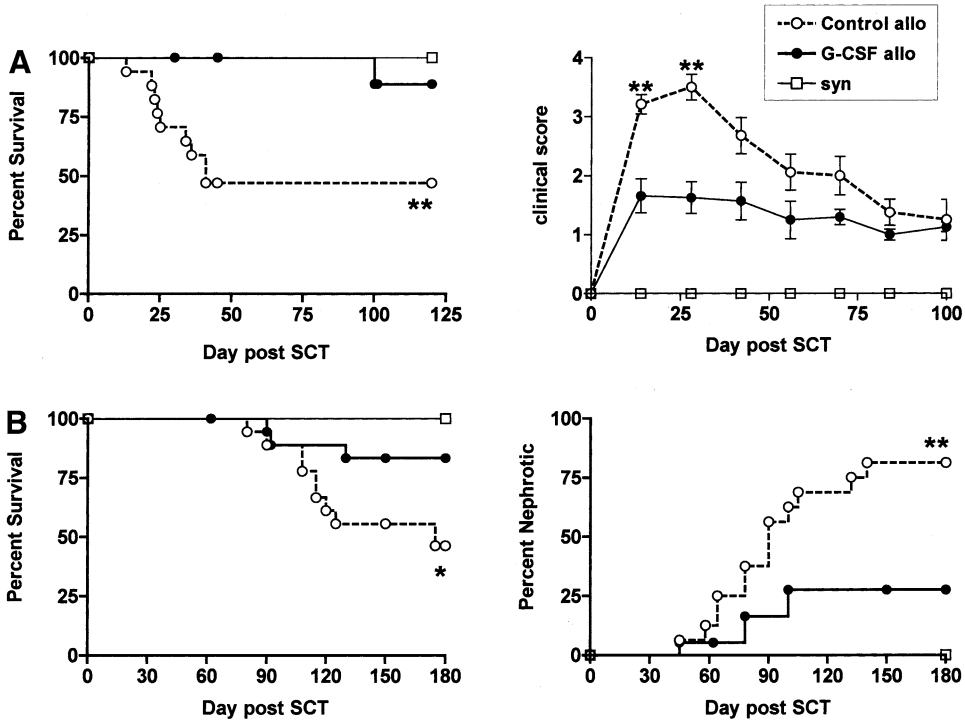

Figure 1. Donor pretreatment with G-CSF prevents acute and chronic GVHD. Survival curves are by Kaplan-Meier analysis pooled from 2 similar experiments. Naive B6 (A) or DBA/2 (B) mice were treated with control diluent (○; n = 17-18) or G-CSF (●; n = 15-19). Nonirradiated B6D2F1 recipients underwent transplantation with unseparated splenocytes, and survival was noted daily. A non-GVHD control group underwent transplantation with syngeneic control-treated B6D2F1 splenocytes (□; n = 8). Animals were monitored daily for survival and weekly for clinical score (A) and proteinuria (B). $^{**}P < .01$ and $^{*}P < .05$, control versus G-CSF.

in response to alloantigen in vitro [16,26], but this has not been demonstrated in vivo, either clinically or experimentally. Because it has been proposed that cGVHD is a Th2-dominant disease process [27], we next studied the relative states of immune deviation in the cGVHD model in vivo. In the first instance, donor CD4 cells (H-2^b negative) were sorted from spleen 14 days after SCT by fluorescence-activated cell sorting and were stimulated in vitro with CD3 and CD28. As shown in Figure 2, donor CD4 T cells from recipients of G-CSF–treated donor cells produced less IFN-γ and more IL-4 than donor CD4 T cells from recipients of control treated donor cells. It is interesting to note that the cytokine profiles of host CD4 T cells (H-2^b positive) were identical (data not shown), consistent with the role of G-CSF in determining T-cell differentiation while within the donor. This model is characterized by high levels of autoantibody that are produced within 2 weeks of transplantation and peak at 6 weeks. We therefore characterized the quantity and quality of autoantibodies produced by the 2 allogeneic groups at these time points. As shown in Figure 2B, the transplantation of splenocytes from G-CSF–treated donors resulted in a significant decrease in the

Table 1. *T-Cell Engraftment and B-Cell Activation*

Variable	Control Allogeneic		G-CSF Allogeneic		Naive B6D2F1
	Donor	Host	Donor	Host	
T cell					
$CD4^+$	**4.7 ± 1.2**	**23.6 ± 1.9**	**6.7 ± 1.4**	**31.4 ± 11.3**	**19.7 ± 6.1***
$CD8^+$	**2.9 ± 1.0**	**20.7 ± 1.2**	**2.4 ± 1.5**	**23.2 ± 6.5**	**11.5 ± 2.1***
B cell					
$B220^+$		**60.7 ± 8.3**		**86.7 ± 26.7**	**47.6 ± 5.0***
Class II expression		**301 ± 43**		**348 ± 26**	**149 ± 32***

Naive DBA (H-2^d) mice received control diluent or G-CSF, and splenocytes were transplanted into B6D2F1 mice as described in Methods. Fourteen days later, splenocytes from transplant recipients (n = 5) were phenotyped by fluorescence-activated cell sorting, and numbers of donor (H-2^b negative) T and B cells were determined per spleen ($\times 10^6$). Host B-cell activation was determined by the mean fluorescence intensity of class II expression on H-2^b positive B cells.

$^{*}P < .05$ versus G-CSF and control allogeneic. Differences between allogeneic groups were not statistically different.

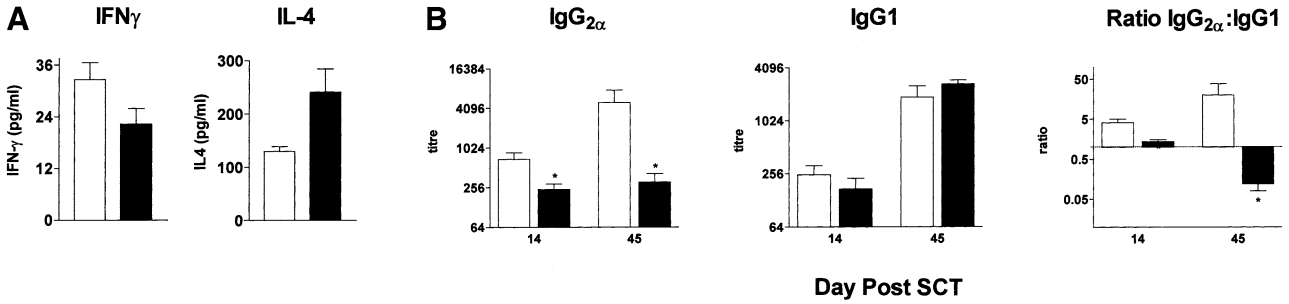

Figure 2. Donor pretreatment with G-CSF induces Th2 differentiation after SCT. Mice underwent transplantation as in Figure 1B. A, Fourteen days after SCT, spleens were removed from control (open bars) or G-CSF (closed bars) allogeneic SCT recipients (n = 4). Donor CD4 T cells were sorted by fluorescence-activated cell sorting and then stimulated with platebound CD3 and CD28. Proliferation and cytokines were determined in culture supernatant as described in Methods. Data represent the mean ± SE of triplicate wells from 1 of 2 experiments. B, The isotype-specific anti-DNA antibody titer was determined in the sera at days 14 and 45 after SCT in control (open bars; n = 4-9) or G-CSF (closed bars; n = 5-9) allogeneic SCT recipients from 2 experiments, as described in Methods, and the ratios for individual animals were determined. The titer in nontransplanted age-matched B6D2F1 controls was <1:64. Results represent mean ± SE; *$P < .05$.

quantity of the type 1 (IgG2α) autoantibody isotype, resulting in a marked reduction in the ratio of IgG2α to IgG1 isotypes, consistent with Th2 differentiation. These data suggest that donor pretreatment with G-CSF does indeed promote Th2 differentiation in vivo during cGVHD, but this is associated with a decrease rather than an increase in the severity of disease in this model.

Donor Pretreatment with G-CSF Reduces cGVHD Mortality Directed against Minor HA

The immune complex nephritis model, although informative, does not reflect the major clinical presentations of cGVHD, which characteristically manifests as hepatic and cutaneous scleroderma. To ensure that the previous findings were predictive of sclerodermatous cGVHD, we next used a model of cGVHD directed to isolated minor HA. B10.D2 donor mice were pretreated with G-CSF or control diluent as described previously. Splenocytes (2.5×10^7) were then transplanted into sublethally irradiated (600 cGy) recipient BALB/c mice, which differ from B10.D2 donors only at minor HA [28,29]. Spleen from G-CSF–treated donors contained equal numbers of T cells but increased numbers of Gr-1 and CD11b cells relative to control-treated donors, as previously described [30]. Syngeneic control and G-CSF–treated grafts from BALB/c donors were transplanted into BALB/c recipients as non-GVHD controls. Donor pretreatment with G-CSF resulted in a 10-fold increase in IL-4 and IL-10 from CD8 T cells and a 2-fold increase from CD4 T cells after SCT in response to mitogen (data not shown). As shown in Figure 3, SCT recipients of splenocytes from G-CSF–treated donors had reduced GVHD mortality (8% versus 24%; $P < .05$) and clinical scores compared with recipients of grafts from control diluent–treated animals. Cutaneous manifestations of cGVHD were

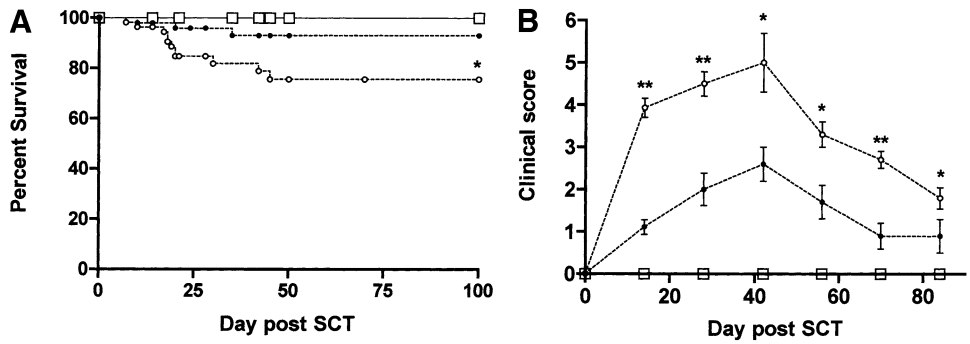

Figure 3. Donor pretreatment with G-CSF prevents cGVHD mortality and morbidity in a scleroderma model. A, Survival curves are by Kaplan-Meier analysis pooled from 6 similar experiments. Donor B10.D2 mice were treated with G-CSF or control diluent. Splenocytes (2.5×10^7) from control (○; n = 55) or G-CSF (●; n = 51) were harvested and transplanted into sublethally irradiated (600 cGy) BALB/c recipient mice. Control-treated syngeneic spleen (□; n = 28) was transplanted as a non-GVHD control. *$P < .04$, G-CSF versus control. B, Clinical scores were assessed by a scoring system that sums changes in 5 clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity (maximum index = 10) as described in Methods. *$P < .05$ and **$P < .01$, G-CSF versus control.

Table 2. *Cutaneous Chronic GVHD Histology*

Variable	Control Allogeneic	G-CSF Allogeneic	Control Syngeneic
Clinical skin score (0-4)	1.07 ± 0.15*	0.68 ± 0.16*	0.00 ± 0.00
Inflammatory score (0-24)	2.75 ± 0.95*	3.64 ± 1.14*	0.21 ± 0.07
Collagen score (1-5)	2.40 ± 0.40*	2.80 ± 0.40*	1.30 ± 0.30
Dermal thickness (mm)	0.22 ± 0.01	0.23 ± 0.02	0.23 ± 0.02
Scleroderma	6% (n = 16)	14% (n = 14)	0% (n = 13)

Mice were transplanted with 2.5×10^7 splenocytes from donor B10.D2 mice treated with diluent or G-CSF as described in Methods. Clinical score was graded before death 6 weeks after SCT. Liver and skin were embedded in paraffin and examined by a histopathologist without knowledge of treatment groups according to the parameters described in Methods. Scleroderma was defined as skin thickness more than average + 3 SD of syngeneic recipients. Data are derived from 2 experiments and are expressed as mean±SE.

*$P < .05$ versus syngeneic recipients. Differences between allogeneic groups were not statistically significant.

assessed by using the inflammatory score (which reflected the degree of cellular infiltrate and secondary inflammatory changes), the density and thickness of dermal collagen deposition, the dermal thickness, and the number of individual animals per cohort that fulfilled criteria for scleroderma of the skin. By using these 4 parameters, cutaneous scleroderma was induced at an equivalent low level in both allogeneic groups, and collagen deposition was moderately increased in both groups relative to syngeneic controls (Table 2). These data suggest that the high incidence of cGVHD seen with the transplantation of grafts from G-CSF–treated donors was not due to the transfer of donor T cells with a type 2 phenotype per se.

Donor Pretreatment with G-CSF Induces Scleroderma Only after the Transfer of High Numbers of Donor T Cells

Because cGVHD after clinical PBSCT is associated with the transfer of very high numbers of G-CSF–modified donor T cells relative to BMT (10-fold increase), we next studied the effects of T-cell dose on the development of cGVHD. In these studies, donor splenocytes were supplemented with 2.5×10^7 purified donor T cells from G-CSF–treated or control-treated donors. Recipients of T cell–escalated grafts from G-CSF–treated donors universally developed clinical features of skin GVHD that were characterized by tail, ear, and paw thickening and desquamation leading to loss of fur (Table 3). These features were not evident or were less severe in recipients of control grafts (1.22 versus 2.09; $P < .05$). To determine whether donor pretreatment with G-CSF altered the development of cGVHD histopathology in SCT recipients, we determined the degree of cGVHD organ damage by semiquantitative histopathology. As shown in Table 3 and Figure 4, the recipients of G-CSF grafts had a >50% increase in dermal thickness relative to recipients of allogeneic and syngeneic control-treated splenocytes ($P < .05$). In conjunction with dermal thickening, the inflammatory score was increased 5-fold in recipients of allogeneic G-CSF–treated grafts relative to control-treated allogeneic grafts. Although recipients of control allogeneic grafts did not develop overt dermal expansion, they had a mild inflammatory cutaneous infiltrate and had increased collagen deposition relative to syngeneic recipients, consistent with mild cGVHD. Surprisingly, 90% of the recipients of grafts containing high numbers of T cells from G-CSF–treated donors had scleroderma, whereas only 11% of the recipient T cells from control donors fulfilled this criterion. Hepatic injury is a major manifestation of cGVHD, and we used semiquantitative histology to analyze injury in transplant recipients 5 weeks after SCT. When high doses of allogeneic T cells were transferred from donors pretreated with G-CSF, hepatic GVHD was increased relative to that in recipients of high numbers of control T cells (Figure 5). The histologic parameters discriminatory between groups were bile duct lymphocytosis (1.3 ± 0.2 versus 0.56 ± 0.32; $P < .03$), hepatocyte apoptosis (1.32 ± 0.25 versus 0.5 ± 0.2; $P < .05$), and lobular inflammation (1.64 ± 0.2 versus 0.4 ± 0.25; $P < .05$).

Table 3. *Cutaneous Chronic GVHD Histology after Escalation of Donor T-Cell Dose*

Variable	Control Allogeneic	G-CSF Allogeneic	G-CSF Syngeneic
Clinical score (0-4)	1.22 ± 0.21*	2.09 ± 0.27*	0.00 ± 0.00
Inflammatory score (0-24)	2.11 ± 0.83	12.18 ± 1.17†	0.50 ± 0.35
Collagen score (1-5)	3.20 ± 0.20*	4.20 ± 0.40*	1.30 ± 0.30
Dermal thickness (mm)	0.25 ± 0.01	0.35 ± 0.03†	0.21 ± 0.01
Scleroderma	11% (n = 9)	90% (n = 10)†	0% (n = 4)

Allogeneic recipients were transplanted with splenocytes (2.5×10^7) supplemented with purified T cells (2.5×10^7) from donor mice treated with diluent or G-CSF as described in Methods. Syngeneic recipients received splenocytes supplemented with T cells from G-CSF–treated BALB/c mice. Clinical score was graded before death 6 weeks after SCT. Skin was embedded in paraffin and examined by a histopathologist without knowledge of treatment groups according to the parameters described in Methods. Scleroderma was defined as skin thickness more than average + 3SD of syngeneic recipients. Data are derived from 2 experiments and are expressed as mean±SE.

*$P < .05$ versus syngeneic recipients;

†$P < .02$ versus control allogeneic and syngeneic recipients.

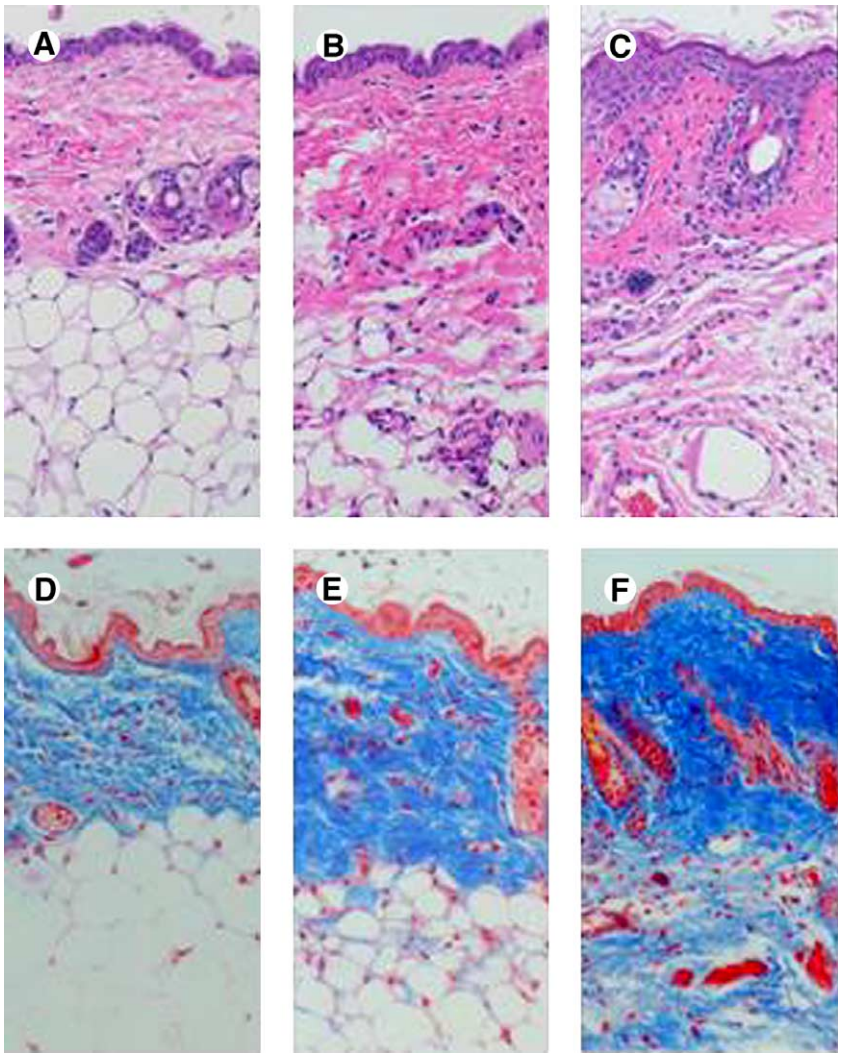

Figure 4. Donor pretreatment with G-CSF induces cutaneous scleroderma after the transfer of high numbers of T cells. Animals underwent transplantation with grafts from syngeneic (A and D), control allogeneic (B and E), or G-CSF allogeneic (C and F) donors. Representative hematoxylin and eosin (A-C) and Masson trichrome (which stains collagen blue; D-F) stains are shown. Skin was harvested 6 weeks after SCT. Animals that received grafts from G-CSF–treated donors had scleroderma characterized by increased density and expansion of dermal collagen, replacement of subdermal fat by collagen, and an intense inflammatory infiltrate.

The Induction of Severe Sclerodermatous cGVHD after Donor Treatment with G-CSF Is Due to Effects on the Non–T-Cell Compartment

We next asked whether the increased severity of cGVHD in recipients of G-CSF–treated grafts was related primarily to the high dose of Th2 cells in isolation or was enhanced by the effect of G-CSF on additional non–T-cell populations (eg, myeloid cells). Recipient mice underwent transplantation with T cell–depleted spleen from control-treated or G-CSF–treated donors with the addition of purified control or G-CSF–treated T cells. Recipients were scored for cutaneous GVHD severity and were monitored for survival and clinical score. Allogeneic recipients developed features of severe GVHD between day 30 and 50, and cutaneous clinical scores continued to increase before death. Because the cutaneous clinical score correlates with the histologic inflammatory score and dermal thickness ($R^2 = 0.52$; $P < .01$), semiquantitative histology was examined after death in allogeneic recipients to allow maximal time for histopathology to develop. As shown in Table 4, the addition of T cells from G-CSF–treated donors to T cell–depleted spleen from control donors accelerated the rate of GVHD mortality relative to recipients of control-treated T cells and control T cell–depleted spleen ($P < .05$). However, the severity of cutaneous and hepatic cGVHD was not increased despite the transfer of high numbers of G-CSF–modified T cells. In contrast, the addition of T cells from control-treated donors to T cell–depleted spleen from G-CSF–treated donors both accelerated mortality and resulted in a significant increase in the severity of cGVHD as manifested by clinical score and histopathology (Table

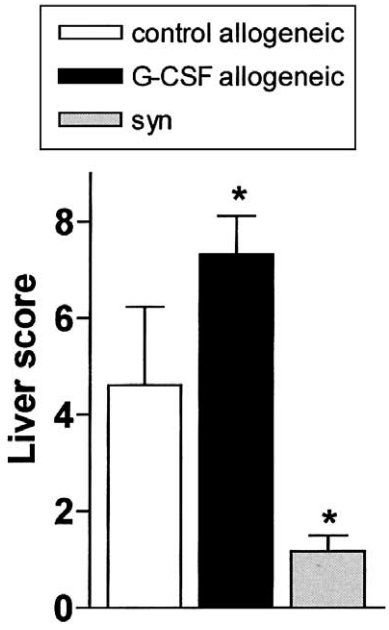

Figure 5. Donor pretreatment with G-CSF augments hepatic cGVHD after the transfer of high T-cell numbers. Animals underwent transplantation with high T-cell doses as in Table 3. Liver was harvested 5 weeks after SCT, and hepatic cGVHD was determined by semiquantitative histology as described in Methods. Open bar, control allogeneic, n = 12; solid bar, G-CSF allogeneic, n = 12; shaded bar, syngeneic, n = 3. *$P < .05$, G-CSF allogeneic and syngeneic versus control allogeneic.

4). The severity of cutaneous cGVHD mirrored that previously seen with both G-CSF T-cell and non–T-cell compartments at this time after SCT (Table 3), suggesting that the non–T-cell fraction is primarily responsible for the induction of scleroderma rather than Th2 differentiation per se. It is interesting to note that neither the G-CSF–modified T-cell nor the non–T-cell compartment in isolation altered the severity of postmortem GI GVHD (Table 4), which is likely to be the cause of universal mortality after the transplantation of the large numbers of donor T cells used in these experiments. Conversely, recipients of large numbers of unseparated splenocytes from G-CSF–pretreated donors (12.5×10^7) had only 20% mortality by day 45 (data not shown), although cutaneous GVHD was increased (skin thickness, 0.33 ± 0.2 mm; inflammatory score, 8.8 ± 1.3). Thus, the transfer of both T-cell and non–T-cell subsets from G-CSF–treated donors is important in combination to limit GVHD mortality, but it results in an increase in cutaneous histopathology. These data suggest that the increase in cGVHD seen after PBSCT occurs when large numbers of donor T cells are transferred in conjunction with a non–T cell that has been expanded or modified by G-CSF.

DISCUSSION

We have shown that donor pretreatment with G-CSF significantly reduces the incidence of aGVHD in an experimental SCT model both in irradiated [16,26] and, now, in nonirradiated hosts. We have further extended these studies to the cGVHD setting and demonstrated that donor pretreatment with G-CSF induces donor Th2 differentiation after SCT and that this is associated with reduced cGVHD mortality and morbidity. However, the increase in cGVHD seen after donor treatment with G-CSF occurs only when large numbers of donor T cells are transferred in conjunction with a non–T cell that has been expanded or modified by G-CSF.

Acute GVHD has been established as a Th1-dominant disease, whereas the production of autoantibodies in both experimental models and clinical practice has suggested that cGVHD is a Th2-dominant process. It has been established that autoantibodies (particularly those of an IgG2α isotype) are pathogenic in the nephritis model of cGVHD [31], as they are in glomerulonephritis in the nontransplant setting. Our data demonstrate that donor pretreatment with G-CSF can prevent glomerulonephritis despite the promotion of Th2 differentiation and that it is consistent

Table 4. *The Effect of the G-CSF–Modified Non–T-Cell Compartment on Chronic GVHD*

Variable	TCD Control + Control T	TCD Control + G-CSF T	TCD G-CSF + Control T	Syngeneic
GVHD mortality (median day)	**52**	**44***	**35***	**NR**
Cutaneous GVHD				
Clinical score (0-4)	**1.90 ± 0.30**	**2.00 ± 0.60**	**3.75 ± 0.25***	**0.00 ± 0.00**
Inflammatory score (0-24)	**6.20 ± 2.40**	**8.50 ± 2.30**	**13 ± 1.60**	**0.17 ± 0.17**
Dermal thickness (mm)	**0.27 ± 0.02**	**0.32 ± 0.03**	**0.37 ± 0.02***	**0.20 ± 0.01**
Hepatic GVHD histology score	**2.80 ± 0.90**	**4.40 ± 1.70**	**7.80 ± 1.40***	**0.70 ± 0.20**
GI tract GVHD histology score	**11.50 ± 1.40**	**8.80 ± 0.90**	**11.50 ± 1.50**	**1.70 ± 0.20**

NR indicates not reached. Mice were transplanted with T-cell–depleted (TCD) splenocytes (2×10^7) and purified T cells (3×10^7) from donor *B*10.D2 mice treated with diluent or G-CSF as described in Methods (n = 4-5 per allogeneic group and n = 3 in syngeneic). The cutaneous clinical score was that before death. Postmortem liver, skin, and GI tract were embedded in paraffin and examined by a histopathologist without knowledge of treatment groups according to the parameters described in Methods. Data are expressed as mean±SE.

*$P < .05$ versus TCD control + control T recipients.

with the promotion of less pathogenic IgG1 autoantibody isotypes (Figure 2) that are known to have a reduced capacity to induce renal disease [31]. This finding is consistent with the prevention of spontaneous nephritis in the MRL/lpr mouse by the administration of G-CSF, although this seemed to be a dose-dependent phenomenon that was related to the anti-inflammatory properties of G-CSF rather than effects on T-cell differentiation [32]. Although autoantibodies develop in association with cGVHD, any role in the pathogenesis of scleroderma in clinical cGVHD remains unproven.

The T-cell cytotoxic pathways (eg, perforin, granzymes, Fas/Fas ligand, and other members of the tumor necrosis factor [TNF]-α superfamily) all play a role in GVHD [33,34]. The induction of cGVHD in the DBA/2 → B6D2F1 lupus nephritis model has been demonstrated to be the result of low antihost cytotoxic T lymphocyte (CTL) frequency (in contrast to the B6 → B6D2F1 model of aGVHD) [35]. Thus, the relative absence of antihost CTL prevents full donor chimerism and eradication of host B cells in experimental models. This promotes the development of low-level donor CD4 T cell-engraftment, which provides aberrant help to host B cells with subsequent autoantibody production. In support of this, the absence of the cytotoxic protein perforin in donor T cells promotes autoimmunity and B-cell hyperactivity [36], and impairment of donor Fas ligand–mediated cytotoxicity has been previously demonstrated as a pathogenic factor in the induction of cGVHD [37]. Similarly, promotion of cytotoxicity by the induction of IL-12 production or the administration of exogenous IL-18 prevents the development of cGVHD [38,39]. Although donor pretreatment with G-CSF seems to have little effect on CTL activity against host antigens, as determined by standard chromium release assays [16,26,30], it is possible that G-CSF alters the frequency of antihost CTL or alters the competency of cytotoxicity pathways that are important in the control of B-cell function in vivo (eg, Fas/Fas ligand). Because such effects would not be demonstrable with the cytotoxicity assay methods described to date (which primarily measure perforin activity), this will require study by more sensitive molecular methods. In the B10.D2 → BALB/c model, the CD4 fraction in isolation is capable of inducing most of the GVHD pathology, and so the effects of G-CSF on cytotoxicity are less likely to be relevant to GVHD in this system [40,41].

The ability of G-CSF to alter T-cell cytokine profiles has been previously documented, although the changes to T-cell differentiation have varied, and this may reflect differences in the dose and schedule of G-CSF used [16,26,42]. We have found that T cells from donors treated with G-CSF produce more IL-4 and IL-10 to alloantigen and mitogen before SCT, although we have seen blunting of all cytokine responses after SCT after high and prolonged doses of G-CSF [16,26,30]. To our knowledge, this is the first experimental study demonstrating that donor pretreatment with G-CSF can induce Th2 differentiation after SCT. Th1 cells produce cytokines (especially IFN-γ) that prime mononuclear cells to produce cytopathic quantities of inflammatory cytokines (TNF-α and IL-1) after stimulation with lipopolysaccharide [43]. This is a major mechanism by which Th1 differentiation results in TNF-α production and gut injury during aGVHD [27]. It is now clear that Th2 cytokines are important in the induction of cutaneous and hepatic GVHD, because the prevention of Th2 cytokine generation after SCT by using STAT-4 knockout donors and eliminating tyrosine kinase–transduced donor Th2 clones after SCT ameliorates experimental GVHD in these organs [44,45]. In addition, the prevention of IL-4 generation after SCT provides protection from GVHD [46]. Therefore, both Th1 and Th2 cytokines are important in the induction of GVHD, and the promotion of Th2 cytokine generation after SCT may therefore be expected to alter the presentation of GVHD rather than prevent its development. Together, these data suggest that Th2 differentiation reduces GVHD mortality because of the limitation of early GI tract injury and inflammatory cytokine production but may be less effective in preventing later hepatic and cutaneous histopathology. Thus, the high numbers of G-CSF–modified donor type 2 T cells transplanted during clinical PBSCT may be expected to induce a similar level of aGVHD as traditional BMT (with 10-fold less type 1 T cells) but to increase the development of cGVHD.

The induction of cutaneous scleroderma and hepatic fibrosis is well described in the B10.D2 → BALB/c model and has been previously prevented by a process of oral tolerance to host splenocytes. This resulted in high levels of IL-10 and reduced IFN-γ in serum; this may reflect Th2 differentiation or the induction of T-cell regulation [28,29]. This is consistent with the reduction in GVHD mortality seen in our studies in association with Th2 differentiation after G-CSF administration. However, the escalation of donor T-cell dose in conjunction with the non–T-cell population of the graft after G-CSF administration overwhelms this protective effect and results in the induction of scleroderma. Given the nature of G-CSF, the non–T cell responsible for this effect is likely to be within an expanded or modified myeloid lineage. G-CSF expands monocytes and basophils up to 10-fold [47], and the development of cutaneous cGVHD is known to involve mast cells [48] that have a similar structure and function to basophils in the induction of allergic disease. Furthermore, the development and degranulation of basophils and mast cells

are in part controlled by the Th2 cytokines IL-4 and IL-10, which are in excess after SCT with G-CSF–mobilized grafts [49]. Degranulation of basophils and mast cells results in the release of numerous fibrogenic mediators, including transforming growth factor-β, basic fibroblast growth factor, and vascular endothelial growth factor. In the scleroderma model of cGVHD reported by Zhang et al. [50] and McCormick et al. [51], low numbers of donor B10.D2 bone marrow and splenocytes were transplanted into irradiated BALB/c recipients, and the subsequent induction of cutaneous and pulmonary fibrosis was associated with the production of transforming growth factor-β by $CD11b^+$ mononuclear cells. It is therefore likely that G-CSF contributes to the severity of cGVHD by expanding donor myeloid populations while providing a Th2 cytokine milieu conducive to ongoing differentiation and degranulation of these cells with subsequent fibrosis. In support of this, inhibitors of IgE binding to FcR1 receptors on mast cells inhibit the development of GVHD [52].

Allogeneic SCT with G-CSF–mobilized stem cells represents a major advance in the management of hematologic malignancies because of improved rates of hematopoietic reconstitution and leukemia clearance relative to traditional BMT [8]. Unfortunately, cGVHD remains a major limitation of allogeneic SCT, and further advances are required to reduce the incidence and severity of this complication. The data presented here suggest that the induction of cGVHD is a consequence of the number of G-CSF–modified donor T cells infused in conjunction with a non–T cell that has been expanded or modified by G-CSF. Thus, the limitation of cell doses during allogeneic SCT may limit both aGVHD and cGVHD while maintaining beneficial effects on graft quality.

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