In the last several years the genes for a number of murine and human hematopoietic growth factors and interleukins have been cloned and their protein products expressed.\(^1\)\(^-\)\(^13\) Some of these proteins play vital roles in the proliferation, differentiation, and function of hematopoietic cells. They include granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), interleukin-1 (IL-1) and IL-4, both alone and in combination. The most effective combination for increasing the circulating absolute neutrophil account (ANC) above the control value at day 7 posttransplant was the combination of G-CSF and IL-1 (mean ANC 2.4 \(\pm\) 1.6 \(\times\) 10\(^7\)/L, as compared with control value of 0.07 \(\pm\) 0.05, \(P<.02\)), followed by G-CSF alone (mean ANC 1.1 \(\pm\) 0.2, \(P<.0001\)), the combination of GM-CSF plus IL-1 (mean ANC 0.8 \(\pm\) 0.3, \(P<.002\)), and the combination of G-CSF plus GM-CSF (mean ANC 0.8 \(\pm\) 0.3, \(P<.005\)). At day 10 posttransplant, the most effective combination in raising the ANC was the combination of G-CSF plus GM-CSF (mean ANC 7.5 \(\pm\) 2.3 as compared with control value of 3.5 \(\pm\) 1.1, \(P<.01\)), followed by G-CSF alone (mean ANC 6.9 \(\pm\) 2.1, \(P<.02\)). At the doses used, neither G-CSF nor GM-CSF had a deleterious effect on the incidence or severity of GVHD; indeed, GM-CSF was associated with improved survival. In contrast, IL-1 at doses \(\geq\) 100 ng twice daily caused marked early mortality, and there was a suggestion that IL-4 at doses of 500 ng twice daily resulted in increased late mortality, possibly owing to exacerbation of GVHD. This model appears to be of value for exploring the use of hematopoietic growth factors before they are used clinically in marrow allograft recipients.\(^\circ\) 1991 by The American Society of Hematology.

MATERIALS AND METHODS

Mice

Mice of the BALB/c ARC (H-2\(^b\)) and C57BL/6 ARC (H-2\(^d\)) strains were bred and housed in the Animal House of the Garvan Institute of Medical Research, St Vincent's Hospital, Sydney, Australia. The mice were housed five to a box and received Milling Chow and water ad libitum. Five days before undergoing transplantation, recipient mice received erythromycin 250 mg/L and gentamicin 320 mg/L in their drinking water. The experimental protocol used in this study was approved by the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee and complied with the code of practice on experimentation in animals of the National Health and Medical Research Council of Australia.

Stem Cell Transplantation

On the day before transplantation, recipient mice received 10 Gy total body irradiation (TBI) from a Theratron 80 \(^{60}\)Co source at a rate of 0.5 Gy/min. On the day of transplantation, donor mice were killed by cervical dislocation, their femora were removed aseptically, and bone marrow (BM) was removed from the femoral shaft by insertion of a 25-gauge needle at the proximal end and displacement of the marrow cells with sterile RPMI 1640 medium/0.5% fetal calf serum (FCS)/1% penicillin-streptomycin. A single-cell suspension was produced. A single-cell suspension of donor spleen cells was obtained by passage through a fine wire mesh before being washed once and resuspended in fresh medium. Recipient mice received a single injection of 0.5 mL medium containing 10\(^7\) nucleated BM cells and 10\(^6\) nucleated spleen cells through a tail vein.

Recombinant Cytokines

Recombinant human G-CSF (rhG-CSF), recombinant murine GM-CSF (rmGM-CSF), rhIL-1a, and rmIL-4 were produced by Immunex, Seattle, Washington. G-CSF provided in RPMI-1640 1% bovine serum albumin (BSA) had a specific activity > 10\(^6\) units/mL. G-CSF and GM-CSF were kindly provided by Immunex Corporation, Seattle, WA. G-CSF provided in RPMI-1640 1% bovine serum albumin (BSA) had a specific activity > 10\(^6\) units/mL.

\(^{1}\) A. Bone Marrow Transplant Foundation, and the Government Employees Assistance to Medical Research Fund.

\(^{2}\) Address reprint requests to Kerry Atkinson, MD, Department of Hematology, St Vincent's Hospital, Sydney NS W2010, Australia.

\(^{3}\) The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

\(^{18}\) Supported in part by grants from the Jenny and Leo Leukemia and Cancer Foundation, New South Wales State Cancer Council, The Australian Bone Marrow Transplant Foundation, and the Government Employees Assistance to Medical Research Fund.

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colony-forming units per milligram (CFU/mg). GM-CSF was provided lyophilized and had a specific activity >4 × 10^7 CFU/mg. IL-1α in phosphate-buffered saline (PBS) had a specific activity >10^8 thymocyte mitogenesis units per milligram of protein. IL-4 in 100 mmol/L Tris, pH 7.4, had a specific activity >10^8 U/mg protein in a standard anti-Ig comitogenesis assay. On receipt, all cytokines were immediately diluted 10-fold in PBS/1% human serum albumin (HSA) and then a further 10-fold in RPMI 1640/1% FCS. They were then stored at −70°C in aliquots of 5 μg/500 μL, or 2 μg/200 μL, or 1 μg/100 μL until the day of use. The lyophilized GM-CSF was initially resuspended in sterile water before dilution as described above. On receipt of each batch of cytokines, bioactivity was assessed in a CFU-GM assay.

**Cytokine administration.** The following cytokines were administered alone to mice receiving allogeneic transplants: G-CSF, GM-CSF, IL-1α, and IL-4. In addition, the following combinations of cytokines were administered: G-CSF and GM-CSF, G-CSF and IL-1β, G-CSF and IL-4; GM-CSF and IL-1β; and GM-CSF and IL-4.

Mice received an intraperitoneal (IP) injection of recombinant cytokine or a combination of cytokines at 9 AM and 5 PM each day for the duration of each experiment. The total volume injected was 200 μL. Control mice received 200 μL normal saline. Mice received these IP injections for 2, 6, 7, 8, 9, or 13 days beginning 1 day preceding the day of transplantation and ending the day before the performance of the blood count. Bleeding was performed at 9 AM on the morning after the preceding day’s 5-PM injection.

**Circulating WBC Count and Absolute Neutrophil Count (ANC)**

Each mouse was bled on a single occasion only to obtain a value for total WBC count and ANC. Up to 1 mL blood was obtained by cardiac puncture under ether anesthesia, after which mice were killed by cervical dislocation before recovery from anesthesia. WBC counts were obtained with a Coulter counter model S 880 (Coulter Electronics, Sydney, NSW, Australia). The ANC was derived from the WBC count by a differential count on a 0.4% Leishman’s stained blood smear.

**Assay for Chimerism Using Fluorescence-Activated Flow Cytometry**

Fluorescence-activated flow cytometry was used to assay the degree of chimerism after transplantation. A single-cell suspension of BM cells was produced as described above and collected in RPMI 1640 medium/0.5% FCS/1% penicillin-streptomycin. An equal volume of Ficoll-Hypaque (Pharmacia, Sydney, Australia) was added, and the preparations were centrifuged for 30 minutes at 20°C. The cells were then washed twice in PBS/1% FCS and were counted before being stained with a murine monoclonal antibody (MoAb) anti-H-2D<sup>b</sup> (Silenus Laboratories, Sydney, Australia) followed by a FITC affinity-isolated F(ab′)<sub>2</sub> fragment of a sheep antimouse IgG antiserum (Silenus). Control samples were incubated with the fluorescein-conjugated sheep antimouse IgG antibody alone or with no added staining reagents. The anti-H-2D<sup>b</sup> antibody was used at a dilution of 1:300, and the FITC reagent was used at a dilution of 1:50. The incubations were performed for 30 minutes at 0°C, after which cells were washed three times and resuspended in 200 μL PBS. Samples were run on a Coulter Epics Elite flow cytometer, using 488 nm/0.1% excitation from a 15 mW argon laser, and gating on light scatter. Analyses were performed on the Coulter Elite work station using Multigraphics analysis software. Each experiment included BM cells from a normal (not transplanted) C57BL/6 mouse (positive control) and from a normal BALB/c mouse (negative control).

**Histologic Studies**

Skin (foot pad, ear, and any clinically affected area), liver, small intestine, spleen, and femur were excised immediately postmortem, fixed in 10% formalin, and processed routinely for histologic examination with hematoxylin and eosin.

**Statistics**

A minimum of five mice were used for generation of data on survival and on WBC count and ANC at each time point. Mean blood counts were compared using the unpaired Student’s t test. Median blood counts were compared using the Mann-Whitney U test. Actuarial survival was compared using the log-rank test.

**RESULTS**

**Allogeneic Hematopoietic Stem Cell Transplant Model**

BALB/c mice given 10 Gy TBI without any subsequent stem cell injection died by 13 days after irradiation (Fig 1). BALB/c mice given 10 Gy TBI followed by injection of 10<sup>7</sup> syngeneic marrow and 10<sup>6</sup> syngeneic spleen cells survived a minimum of 470 days posttransplant (Fig 1). BALB/c mice given 10 Gy TBI followed by injection of 10<sup>7</sup> C57BL/6 marrow and 10<sup>6</sup> C57BL/6 spleen cells survived 40 days, but then began to waste; most were dead by 90 days posttransplant (Fig 1). The principle feature at autopsy was gross atrophy and hypocellularity of spleen and lymph nodes and thymus, and these mice were believed to have died of GVHD. BALB/c mice given 10 Gy TBI followed by injection of 10<sup>7</sup> C57BL/6 marrow and 10<sup>6</sup> C57BL/6 spleen cells together with twice-daily IP injections of 200 μL normal saline from the day of transplant for 14 days showed a survival rate similar to that of allogeneic recipients not so injected (Fig 1). This model thus provides a window during
the first 40 days posttransplant for studying the rate of engraftment and a subsequent window after that time for studying the effects of the administered cytokines on incidence and severity of GVHD and survival.

**In Vivo Effect of Cytokines on Total Number of WBCs at Day 7 Posttransplant**

The data on in vivo effect of cytokines on total number of WBCs are shown in Tables 1 and 2. In control mice given twice-daily IP injections of 0.9% saline, the total WBC count and the ANC increased markedly between days 7 and 8 (Fig 2). Day 7 counts therefore were the key parameter for gauging the effect of the administered cytokines on the rate of recovery of the WBC count and neutrophil count. The most effective approach for increasing both the WBC count and the neutrophil count at day 7 posttransplant was the combination of G-CSF 100 ng together with IL-1 50 ng twice daily. This resulted in an increase in the WBC count from a control value of 0.6 ± 0.4 × 10⁶/L to 6.1 ± 4.0 (P < .02), and an increase in the neutrophil count from 0.07 ± 0.05 to 2.4 ± 1.6 (P < .02). The second most effective agent for increasing the WBC count was GM-CSF 750 ng twice daily (mean WBC count 2.3 ± 1.1). Strangely, GM-CSF 750 ng twice daily did not result in a significant increase in the corresponding neutrophil count. In decreasing order of efficacy, other approaches that resulted in a significant increase in the day 7 WBC count were twice-daily GM-CSF 200 ng plus IL-1 50 ng, G-CSF 100 ng alone, and G-CSF 100 ng in combination with GM-CSF 200 ng.

**In Vivo Effect of Cytokines on ANC at Day 7 Posttransplant**

After the combination of twice-daily G-CSF 100 ng and IL-1 50 ng (Table 2), the next most effective approaches in increasing the day 7 ANC were, in descending order of efficacy: twice-daily G-CSF 100 ng alone, G-CSF 100 ng plus GM-CSF 200 ng, GM-CSF 200 ng plus IL-1 50 ng, GM-CSF 200 ng alone, IL-1 50 ng alone, G-CSF 100 ng together with IL-4 500 ng, GM-CSF 200 ng plus IL-4 50 ng and, finally, IL-4 500 ng (Tables 1 and 2). Thus, three of the four most effective approaches comprised combinations of cytokines.

**Total WBC Count and ANC at Day 10 Posttransplant**

The only approaches that increased the day 10 WBC count or ANC were the combination of twice-daily G-CSF 100 ng plus GM-CSF 200 ng and G-CSF 100 ng alone (Tables 1 and 2).

**Degree of Chimerism**

A majority of BM cells from four normal C57BL/6 mice showed positive staining with an anti-H-2Db MoAb (median 74.5% after subtraction of background) (Table 3), whereas a minority of BM cells from four normal BALB/c mice did so (median of 8.5% positive after subtraction of background). This small positive population may have resulted from Fc receptor binding of the murine MoAb. Similarly, a BALB/c mouse killed at 40 days after TBI and injection of BALB/c spleen and marrow cells (syngeneic transplant) showed only 5% such staining after background subtraction. In contrast, three allogeneic chimeras, killed on days 27, 62, and 55 posttransplant, respectively, and given saline injections, GM-CSF, or IL-4, showed positive staining on the majority of their marrow cells (85%, 89%, and 73%, respectively, after background subtraction).

**Histologic Studies**

BALB/c recipients of syngeneic (BALB/c) marrow and spleen cells showed no pathologic changes in BM, spleen, skin, liver, or small intestine as compared with normal (nontransplanted) BALB/c mice. BALB/c recipients of C57BL/6 marrow and spleen cells, regardless of whether they received saline, GM-CSF, or IL-4 injections posttransplant, showed hydropic degeneration of the basal layer and satellite cell necrosis in the skin, degenerate bile ducts with neutrophil and lymphohytic infiltration around bile ducts in the liver, and in some mice necrotic tips of villi in the small intestine. In addition, all allogeneic recipients showed lymphoid hypocellularity in the spleen.

**Effect on GVHD and Survival**

G-CSF 100 ng twice daily for 14 days had no adverse effect on GVHD; indeed, allogeneic transplant recipients given this regimen of G-CSF appeared, if anything, to survive better than their saline control counterparts, although this did not reach statistical significance (Fig 3A). Similarly, there was no adverse effect of GM-CSF 200 ng twice daily for 14 days and survival was enhanced as compared with that of control mice (P = .05) (Fig 3A). There was a suggestion of enhanced late mortality when IL-1 50 ng was administered twice daily for 14 days (P = .067) (Fig 3B), but when the dose of IL-1 was increased to 100 ng twice daily, there was a marked increase in early mortality (Fig 3B). The cause of this increased early mortality was not clear. The increased early mortality was also observed when a dose of IL-1 200 ng twice daily was given in combination with 200 ng twice daily of GM-CSF (Fig 3B). There was a trend toward increased late mortality in mice given IL-4 500 ng twice daily (P = .06) (Fig 3C), and this appeared to result from an increase in severity of GVHD because these mice showed accelerated wasting.

**DISCUSSION**

Recently, and for the first time, physicians have had the capacity to control the leukocyte count in patients recovering from chemotherapy or marrow transplantation for malignant disease. rhG-CSF was shown to halve the time taken for the ANC to recover in patients who received high-dose chemotherapy for solid tumors. The effect of rhG-CSF on neutropenic period after cytotoxic chemotherapy for solid tumors has been reported. rhG-CSF also decreased the neutropenic period after chemotherapy for solid tumors. There is as yet no published report on use of recombinant IL-1 or IL-4 for this purpose and no reports of combinations of these agents to achieve such goals.
### Table 1. Total WBC Count and Neutrophil Count Posttransplant: Cytokines Administered Singly

<table>
<thead>
<tr>
<th>Injection (cytokine doses in nanograms, IP twice daily)</th>
<th>WBC Count*</th>
<th>Neutrophil Count*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days Posttransplant</td>
<td>Days Posttransplant</td>
</tr>
<tr>
<td></td>
<td>3 7 8 9 10 14 20</td>
<td>3 7 8 9 10 14 20</td>
</tr>
<tr>
<td>Saline 0.9%</td>
<td>0.8 ± 0.9</td>
<td>0.6 (0.4) ± 0.4</td>
</tr>
<tr>
<td>G-CSF 100</td>
<td>1.81 (1.7)</td>
<td>0.4</td>
</tr>
<tr>
<td>GM-CSF 200</td>
<td>1.4 (3.5)</td>
<td>± 0.7</td>
</tr>
<tr>
<td>GM-CSF 750</td>
<td>2.31 (1.8)</td>
<td>± 1.1</td>
</tr>
<tr>
<td>IL-1 50</td>
<td>1.0 (1.0)</td>
<td>± 0.3</td>
</tr>
<tr>
<td>IL-4 50</td>
<td>1.6 (0.9)</td>
<td>± 1.4</td>
</tr>
<tr>
<td>IL-4 500</td>
<td>0.6 (0.6)</td>
<td>± 0.08</td>
</tr>
</tbody>
</table>

*Values are mean ± 1 SD x 10^9/L.

†Values significantly different from control (saline) values: P < .05 to < .0001. Values in parentheses represent medians.

‡Values significantly different from control values: P < .05 to < .0006.

### Table 2. Total WBC Count and Neutrophil Count Posttransplant: Cytokines Administered in Combination

<table>
<thead>
<tr>
<th>Injection (cytokine doses in nanograms, IP twice daily)</th>
<th>WBC Count*</th>
<th>Neutrophil Count*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days Posttransplant</td>
<td>Days Posttransplant</td>
</tr>
<tr>
<td></td>
<td>3 7 8 9 10 14 20</td>
<td>3 7 8 9 10 14 20</td>
</tr>
<tr>
<td>Saline 0.9%</td>
<td>0.8 ± 0.9</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>G-CSF 100 + IL-1 50</td>
<td>6.11 (7.0)</td>
<td>± 4.0</td>
</tr>
<tr>
<td>G-CSF 100 + GM-CSF 200</td>
<td>1.41 (3.1)</td>
<td>± 0.5</td>
</tr>
<tr>
<td>G-CSF 100 + IL-4 500</td>
<td>0.8 (0.8)</td>
<td>± 0.07</td>
</tr>
<tr>
<td>GM-CSF 200 + IL-1 50</td>
<td>2.71 (1.5)</td>
<td>± 1.4</td>
</tr>
<tr>
<td>GM-CSF 200 + IL-4 500</td>
<td>0.7 (0.7)</td>
<td>± 0.3</td>
</tr>
</tbody>
</table>

*Values are mean ± 1 SD x 10^9/L.

†Values significantly different from control (saline) values: P < .05 to < .005. Values in parentheses represent medians.

‡Values significantly different from control values: P < .05 to < .0005.
Injections

0 5 10 15 20 25

Days Post Allogeneic Transplant

Injections

0 5 10 15 20 25

Days Post Allogeneic Transplant

Fig 2. Total WBC count and ANC in BALB/c mice given 10 Gy TBI followed by 10^6 BM and 10^6 spleen cells from C57BL/6 mice together with twice-daily IP injections from the day of transplant to 14 days posttransplant of 0.9% saline. Values are mean ± 1SD.

Of the different single agents and combinations of agents used in this study, the most potent was a combination of G-CSF and IL-1. This same combination was shown by Moore and Warren to be efficacious in increasing the neutrophil count in mice after they were exposed to 5-fluorouracil. Combinations of G-CSF and IL-4, G-CSF and GM-CSF, and GM-CSF and IL-4 administered in vivo after allogeneic transplantation have not previously been reported, and it was noteworthy that three of the four most effective approaches for increasing the day 7 ANC in this study involved combinations of cytokines. We also found in the current study that on a basis of nanogram of protein, G-CSF alone was more active in increasing the circulating ANC than GM-CSF. This was previously demonstrated by Metcalf et al, who noted, however, that although G-CSF was the most efficacious agent in increasing the circulating ANC, GM-CSF was extremely effective in increasing the peritoneal macrophage number. The latter parameter was not measured in the current study. GM-CSF was somewhat disappointing in the study; doses ≤ 750 ng twice daily were relatively ineffective. Thus, there appears to be a difference in responsiveness to GM-CSF (at least in terms of circulating neutrophils) between mice and humans undergoing stem cell transplantation. An additional caveat is the theoretical possibility that combinations of cytokines may not produce similar effects across species barriers.

Of particular concern in allogeneic (as opposed to autologous) transplant is the potential of cytokine administration for exacerbating the incidence or severity of GVHD posttransplant. GVHD is initiated by mature donor T cells present in the donor marrow inoculum, but the detailed mechanism of endstage target damage has not been completely elucidated. One interesting observation, however, has been that MoAbs to tumor necrosis factor (TNF) can decrease the mortality resulting from GVHD in a murine model. Therefore, administration of recombinant murine GM-CSF might have resulted in both increased macrophage numbers and increased macrophage activation, and thus in release of TNF from macrophages. This in turn could have led to increased GVHD damage. This did not occur, and mice that received either GM-CSF or G-CSF showed no evidence of an increase in severity of GVHD. Indeed, both groups of mice survived as well or better than their saline control counterparts. The explanation for this is not immediately clear, although it did not result from autologous reconstitution (Table 3). GM-CSF has been shown to enhance the resistance of mice to bacteria such as Salmonella typhimurium. Perhaps by accelerating neutrophil reconstitution, this growth factor minimized the dura-

Table 3. Assessment of Chimerism Posttransplant

<table>
<thead>
<tr>
<th>Source of Murine Cells/Experimental Protocol</th>
<th>Normal C57BL/6</th>
<th>Normal BALB/c</th>
<th>C57BL/6 BALB/c</th>
<th>C57BL/6 BALB/c</th>
<th>C57BL/6 BALB/c</th>
<th>C57BL/6 BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation Protocol</td>
<td>Normal C57BL/6</td>
<td>Normal BALB/c</td>
<td>BALB/c No Injections</td>
<td>BALB/c Saline Injections</td>
<td>BALB/c GM-CSF Injections</td>
<td>C57BL/6 IL-4 Injections</td>
</tr>
<tr>
<td>Normal C57BL/6 (n = 4)</td>
<td></td>
<td></td>
<td>(n = 1)</td>
<td>(n = 1)</td>
<td>(n = 1)</td>
<td></td>
</tr>
<tr>
<td>No stain</td>
<td>[0, 0, 2, 0]</td>
<td>[1, 2, 1, 0]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sheep anti-mouse Ig-FITC</td>
<td>[14, 8, 14, 4]</td>
<td>[9, 7, 16, 9]</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Anti-H2Db+</td>
<td>[86, 85, 86, 93]</td>
<td>[16, 12, 32, 14]</td>
<td>14</td>
<td>91</td>
<td>96</td>
<td>88</td>
</tr>
<tr>
<td>Sheep anti-mouse Ig-FITC</td>
<td>85.5</td>
<td>17.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bracketed values represent medians. Values are percentages of positively staining cells.
HEMATOPOIETIC GROWTH FACTOR AFTER MURINE BMT

Blazar et al in mice given T-cell–depleted histoincompatible BMT. These findings will encourage early trials of rhGM-CSF currently being initiated in human allogeneic BMT recipients.

IL-1 at a dose $\geq 100$ ng was extremely toxic to the mice in this study and, although IL-1 is likely to be useful in humans in increasing the circulating neutrophil count, there is likely to be dose-limiting toxicity. IL-1 and IL-4 both appeared to increase the late mortality of mice posttransplant, and their increased rate of wasting and earlier death was consistent with an exacerbation of GVHD in these animals. A similar finding was made when IL-2 was used after allogeneic BMT in mice. These preliminary studies with IL-4 in vivo suggest that it will not have a particularly useful role in combination with other cytokines in enhancing hematopoietic recovery.

In summary, some agents and combinations of agents are capable of increasing the circulating neutrophil count in mice that receive allogeneic BMT. Further studies are necessary both to escalate doses of these agents and to introduce other potential candidate cytokines for this purpose, particularly IL-3 and IL-6, to determine the optimum approach to cocktail cytokine therapy after human marrow allografting.

ACKNOWLEDGMENT

We thank Denise Sahu-Khan for expert typing of the manuscript.

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In vivo administration of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF, interleukin-1 (IL-1), and IL-4, alone and in combination, after allogeneic murine hematopoietic stem cell transplantation

K Atkinson, C Matias, A Guiffre, R Seymour, M Cooley, J Biggs, V Munro and S Gillis