Peripheral blood stem cell (PBSC) transplantation is successful in improving engraftment without increasing acute graft-versus-host disease (GVHD), despite much larger numbers of T cells in unmanipulated PBSCs than in bone marrow grafts. In mouse models and retrospective human studies, granulocyte colony-stimulating factor (G-CSF) therapy has been associated with less acute GVHD. We studied the effect of G-CSF on interferon (IFN)-γ and IL-4 expression in CD4⁺ lymphocytes. CD4⁺ cells co-cultivated with G-CSF and stimulated with PHA or CD3 monoclonal antibodies showed significant decreases in IFN-γ and increases in IL-4 expression (n = 13; \( P < .01 \)). G-CSF appeared to have a direct effect on CD4⁺ cells independent of monocytes present in the culture because purified CD4⁺ cells exposed to G-CSF, washed, and cocultivated with untreated monocytes demonstrated similar changes in IFN-γ and IL-4 expression, whereas untreated CD4⁺ cells cocultivated with G-CSF-stimulated monocytes behaved as controls. We then studied peripheral blood mononuclear cells (PBMCs) from G-CSF-mobilized PBSC donors. When their PBMCs were cultured with PHA or CD3 monoclonal antibody, the percent of IFN-γ-expressing cells decreased by a mean of 55% and 42%, respectively, whereas the percent of IL-4–containing cells increased by a mean of 39% and 58%, respectively, following G-CSF stimulation. Increased apoptosis of IFN-γ–producing CD4⁺ cells was not responsible for the shift in TH1/TH2 subsets. G-CSF-R mRNA was present in both CD4⁺ and CD8⁺ cells. These results suggest that G-CSF decreases IFN-γ and increases IL-4 production in vitro and in vivo and likely modulates a balance between TH1 and TH2 cells, an effect that may be important in PBSC transplantation. (Blood. 2000;95:2269-2274)

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Materials and methods

Stem cell donor selection and studies

Peripheral blood for in vitro studies was obtained from normal volunteers. For studies involving in vivo G-CSF stimulation of normal donors, samples were obtained from normal stem cell donors before and after 5 days of

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G-CSF treatment (10 µg/kg subcutaneously daily) before apheresis donation. Heparinized blood for cytokine expression studies was obtained on day 1, before the administration of G-CSF, and on day 6. In all cases, informed consent was obtained according to protocols approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute. PBMC from G-CSF–stimulated donors were separated as described below, and cells were cultured for 48 hours with PHA (1 µg/mL) or anti-CD3 monoclonal antibody (mAb) (0.1 µg/mL). Cells were then surface stained with anti-CD4–phycoerythrin (PE) and intracellularly stained with either anti–IFN-γ–fluorescein isothiocyanate (FITC) or anti–IL-4 FITC as described below.

Cocultivation of G-CSF with T lymphocytes

Peripheral blood mononuclear cells (PBMCs) from normal unstimulated donors were separated using density gradient centrifugation with lymphocyte separation media (Organon, Durham, NC). Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in Iscove’s modified medium supplemented with fetal calf serum (FCS; both Life Technologies, Gaithersburg, MD). Cultures of normal unstimulated donors could be performed at a cell density of 0.5 × 10⁶ cells/mL. When appropriate, natural lymphocyte–derived IL-2 (Boehringer Mannheim, Indianapolis, IN) or phytohemagglutinin (PHA; Boehringer Mannheim) were used for stimulation at concentrations of 20 U/mL or 8 µg/mL, respectively. G-CSF was used at a concentration of 100 ng/mL (Amgen, Thousand Oaks, CA). G-CSF was added to cultures; either PHA or CD3 mAb were added to cultures 24 hours later to stimulate cytokine expression. Cells were stained and enzyme-linked immunosorbent assay (ELISA) was performed on supernatants 2 days later.

Isolation of CD4⁺ cells, CD8⁺ cells, and monocytes. For lymphocyte subset separation, after washing with PBS supplemented with 2% human albumin, cells were applied to either a CD4⁺ or a CD8⁺ affinity column (R&D Systems, Minneapolis, MN), and the cell fraction was eluted with PBS according to manufacturer’s instructions. An aliquot of eluted cells was stained with PE-conjugated anti-CD4 or CD8 HPCA-12 mAb (Becton Dickinson, Mountain View, CA) to assess purity. Monocytes were separated by adhesion to 250-mL polystyrene flasks in the presence of 20% FCS for 2 hours; this was followed by the removal of nonadherent cells and by PBS/2% FCS washes. Adherent cells were detached and resuspended by agitation at 4°C in the presence of 1X Versene (BRL; Life Technologies). The usual purity of the adhesion-separated monocytes ranged between 75% and 85% as determined by the expression of CD14⁺ antigens by flow cytometry. Cell viability was measured using a standard trypsin blue exclusion assay (Life Technologies).

Intracellular staining. Intracellular staining was performed on density gradient-separated PBMC. Intracellular staining for ICE expression was performed using the Pharmingen Intracellular Staining Kit (Pharmingen). PBMC were stained with FITC-conjugated CD4 mAb and fixed. After membrane permeabilization, cells were stained with PE-conjugated anti-IFN-γ, anti–IL-4 mAb, or appropriate isotypic control mAb (Pharmingen). Permeabilization of cells was controlled using PermaSure reagents (Biosource). Samples were analyzed using an Epics ELITE flow cytometer (Coulter).

Apoptosis assay. Cultured PBMC were prepared as described above, washed with PBS, and stained with an annexin apoptosis kit (Pharmingen) according to the manufacturer’s specifications and as previously described. Samples were analyzed using flow cytometry.

Cytokine ELISA

Concentrations of IL-4 and IFN-γ in tissue culture supernatants were measured using commercially available ELISA systems (R&D Systems). All determinations were made in duplicate.

Reverse transcriptase–polymerase chain reaction for detection of G-CSF receptor expression

Peripheral blood mononuclear cells were sorted for CD4⁺ and CD8⁺ by flow cytometry. Total RNA was extracted using RNA STAT-60 (Tel-Test, Friendswood, TX). Reverse transcription was performed using an oligo d(T)₁₆ primer (RNA CORE KIT; Perkins-Elmer Cetus, Foster City, CA). After reverse transcription, the cDNA was amplified using 5’ and 3’ primer pairs, 5’-AGTACAGTCCTCACCGTGATG-3’, 5’-AAATGAGCGAGACGCTGGG-3’ specific for G-CSF receptor. The following reverse transcription and amplification conditions were used: 42°C for 30 minutes, 99°C for 2 minutes, at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes for 40 cycles. PCR products were electrophoresed in 1% agarose gel, and the bands were visualized after staining with ethidium bromide and ultraviolet light exposure.

Results

G-CSF stimulated PBSC donors demonstrate decreases in IFN-γ and increases IL-4 expression in CD4⁺ cells

To determine whether G-CSF modulated the cytokine expression pattern of T lymphocytes, we used flow cytometry to examine intracellular cytokine expression in mononuclear cells derived from PBSC of donors receiving G-CSF. Normal donors receiving 10 µg/kg subcutaneously daily for 5 days were studied immediately before and after G-CSF administration for PBSC donation by apheresis. After blood sampling, PBMCs were stimulated for 48 hours with either PHA or CD3 mAb and were subsequently stained for intracellular IL-4 and IFN-γ. Donor CD4⁺ cells stimulated with G-CSF demonstrated significant changes in cytokine expression (Figures 1 and 2). In comparison to pretreatment values, G-CSF increased IL-4 and decreased IFN-γ expression, resulting in a decreased TH1/TH2 ratio (determined by dividing the percentage of cells expressing IL-4 by the percentage of cells expressing IFN-γ). In addition to a decreased proportion of cells expressing IFN-γ and an increased proportion of IL-4–expressing cells, there were characteristic changes in the mean channel fluorescence (MCF) indicating changes in the intracellular cytokine content. IFN-γ and IL-4 MCF was measured on a relative scale (PHA-stimulated normal lymphocytes showed MCF of 5 to 6, whereas isotype controls registered at less than 0.10). CD3 mAb-stimulated CD4⁺ cells from G-CSF–treated patients showed a mean decrease in MCF of −4.2 for IFN-γ, with a decrease in the percent of cells staining by 42% with a concomitant increase in the number of cells staining for IL-4 by 58% with ∆MCF = +6.0 (n = 10; P < .01). Determination of IL-4 and IFN-γ concentrations in culture supernatants by ELISA resulted in similar changes (Table 1A).

G-CSF decreased the proportion of IL-4 and IFN-γ producing lymphocytes in vitro

In a subsequent set of experiments, we asked whether the effects of G-CSF observed in PBSC donors could be replicated in vitro. Normal PBMCs were incubated with varying concentrations of G-CSF, then stimulated with IL-2 and CD3 mAb and stained 2 days later with CD4–PE and (after permeabilization) with either IL-4 or IFN-γ. Flow cytometric analysis showed that the number of CD4⁺ cells expressing IFN-γ decreased, whereas the number of IL-4–producing cells increased in cultures performed in the presence of G-CSF (Figure 3). The effect was less pronounced in cells that were not preincubated with G-CSF before stimulation with either PHA or CD3 mAb (data not shown). The culture supernatants were also tested for IFN-γ and IL-4 by ELISA. G-CSF resulted in changes in the cytokine production pattern that paralleled those detected by flow cytometry (Table 1B). The effects of G-CSF were dose dependent (data not shown).
G-CSF has a direct effect on CD4$^+$ cells

To determine whether the effects of G-CSF on cytokine production by T lymphocytes were directly mediated, we studied the growth factor’s effects on purified cell populations contained in PBMC preparations. We compared IFN-$\gamma$ expression in purified control CD4$^+$ and those treated by G-CSF after PHA stimulation. In the absence of accessory cells, IFN-$\gamma$ production was not detected in PHA-stimulated CD4$^+$ cells (data not shown).

When monocyte preparations, purified CD4$^+$ cells, or CD8$^+$ cells (98% pure by flow cytometry) were cultured in the presence of G-CSF, washed, and then mixed—G-CSF–exposed CD4$^+$ cells with untreated monocytes; G-CSF–exposed monocytes with untreated CD4$^+$ cells not treated with G-CSF; G-CSF–exposed CD4$^+$ cells with G-CSF–exposed monocytes—and stimulated for 48 hours with either CD3 mAb or PHA, only CD4$^+$ cells directly exposed to G-CSF showed changes in IFN-$\gamma$ expression (Figure 4 and Table 2). Neither CD4$^+$ cells cultured with G-CSF–exposed monocytes nor CD8$^+$ cells exposed directly or cultured with G-CSF–treated monocytes had altered IFN-$\gamma$ production.

![Patient 1](image1.png)

![Patient 2](image2.png)

Figure 1. Effects of G-CSF administration on IFN-$\gamma$ and IL-4 production by peripheral blood lymphocytes. Eleven normal stem cell donors received G-CSF (10 µg/kg per day) for 5 days. Blood was obtained before and after the administration of G-CSF. PBMC cells were purified by density-gradient centrifugation and were cultured with PHA for 48 hours. After permeabilization, cells were stained for IFN-$\gamma$ and IL-4 as described in “Materials and methods.” Scattergrams represent log PE (CD4 or CD8) fluorescence activity versus log FITC (IFN-$\gamma$ and IL-4) fluorescence activity for representative donors (A, B).

![Figure 2](image3.png)

Figure 2. Effects of G-CSF on the percentages of CD4$^+$ cells secreting IFN-$\gamma$ and IL-4. Summary of the results obtained from 10 PBSC donors, including those exemplified in Figure 1. Bars represent percentages (mean ± SEM) in the IFN-$\gamma$ and IL-4 expressing CD4$^+$ cells before (black) and after (gray) treatment. Corresponding shifts in the mean fluorescence intensity for IFN-$\gamma$ and IL-4 are described in the text. Statistical analysis (nonparametric Kruskal–Wallis test) for PHA: IL-4, $P < .05$; IFN-$\gamma$, $P < .01$. Statistical analysis for CD3 IL-2: IL-4 $P < .01$; IFN-$\gamma$ $P < .01$.

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<tr>
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<tr>
<td>IFN-$\gamma$</td>
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Table 1. Production of IFN-$\gamma$ and IL-4 in supernatants from cultures of peripheral blood mononuclear cells stimulated with G-CSF in vivo and in vitro

In part A, samples of lymphocytes were obtained before and after the administration of G-CSF. IL-4 and IFN-$\gamma$ concentrations were measured after 24-hour PHA stimulation. In part B, samples of lymphocytes obtained from normal donors were cocultured with G-CSF for 24 hours and subsequently stimulated with anti-CD3 mAb for 48 hours. Concentrations (mean ± SEM of IL-4 and IFN-$\gamma$ concentration in supernatants) were determined using ELISA and expressed as pg/mL. Results represent the summary of 2 experiments for part A and 5 experiments for part B.
G-CSF does not decrease TH1/TH2 ratio by increasing apoptosis

The G-CSF-induced modulation pattern of cytokine expression in CD4+ cells could potentially be mediated by the deletion of TH1 cells secreting IFN-γ, as for example by selective apoptosis. Using the annexin technique, we investigated the effects of G-CSF on apoptosis of CD4+ cells. The presence of G-CSF in culture did not affect the numbers of apoptotic cells, as determined by flow cytometry. After 48 hours of culture, a mean of 48% ± 6% TH1 cells and 54% ± 6% TH2 cells stained with annexin (n = 3).

RT-PCR detects GCSF-R m-RNA expression in CD4 and CD8 cells

Peripheral blood mononuclear cells were sorted for CD4+ and CD8+ by FACS, resulting in a preparation that was 99.6% and 97% pure, respectively. Total RNA was extracted using RNA STAT-60, and RT-PCR was performed using an oligo d(T)16 primer. After reverse transcription, the cDNA was amplified using primer pairs 5'-AGTACAGTCCCTCACCTGATG-3' and 5'-AAAGTATGCA-GATGCCTGGG-3' specific for G-CSF receptor. A band of 900 kbp was present for CD4 and CD8 cells, indicating the molecular presence of G-CSF-R (Figure 5).

Discussion

In this study, we demonstrated that the pretreatment of T cells with G-CSF resulted in diminished IFN-γ and increased IL-4 production when these cells were subsequently subjected to polyclonal stimulation with IL-2, PHA, or CD3 mAb in vitro. In the presence of G-CSF, resting CD4+ cells that expressed low levels of IL-4 produced significant amounts of this cytokine after polyclonal activation. In agreement with in vitro results, we found that when

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Monocytes and CD4+ cells from normal donors were purified to obtain preparations with greater than 98% purity as determined by flow cytometry using CD4 and CD14 mAb. Monocytes and CD4+ cells were separately exposed to G-CSF for 24 hours, washed, and mixed together (ratio 1:1). IFN-γ and IL-4 were measured using ELISA and were expressed as pg/mL.
normal bone marrow (nbm) from a normal control was used as a positive control. Containing ethidium bromide and visualized under ultraviolet light. Unseparated control. mRNA was extracted from the cells, and RT-PCR was performed using

**References**


Pharmacologic doses of granulocyte colony-stimulating factor affect cytokine production by lymphocytes in vitro and in vivo

Elaine M. Sloand, Sonnie Kim, Jaroslaw P. Maciejewski, Fritz Van Rhee, Aniruddho Chaudhuri, John Barrett and Neal S. Young