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Hematopoietic stem cell mobilization with G-CSF induces innate inflammation yet suppresses adaptive immune gene expression as revealed by microarray analysis

Matthew P. Buzzeo[a], Jie Yang[b], George Casella[b], and Vijay Reddy[a]

[a]*Department of Medicine, Division of Hematology/Oncology, University of Florida, Gainesville, Fla., USA;* [b]*Department of Statistics, University of Florida, Gainesville, Fla., USA*

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Objective. **Granulocyte colony-stimulating factor (G-CSF) is used to boost granulocyte counts in immunocompromised patients, but its effects on the immune system may be counterproductive. We tested the hypothesis that G-CSF–mobilized peripheral blood stem cell (PBSC) products are immunologically downregulated based on gene microarray analysis.**

Methods. **Ten peripheral blood samples from normal donors for allogeneic PBSC transplantation were obtained before and after administration of G-CSF and tested on Affymetrix Human U133 Plus 2.0 GeneChip microarrays and by flow cytometry. Significant changes in gene expression after G-CSF were reported by controlling the false discovery rate at 5%. The quantitative real-time polymerase chain reaction method was used to validate expression of representative genes.**

Results. **All immune cells measured, including neutrophils, monocytes, lymphocytes, and dendritic cells, were significantly increased after G-CSF. In terms of gene expression, inflammatory and neutrophil activation pathways were upregulated after G-CSF. However, adaptive immune-related gene expression, such as antigen presentation, co-stimulation, T-cell activation and cytolytic effector responses, were downregulated.**

Conclusion. **Despite significant increases in lymphocytes and antigen-presenting cells, G-CSF–mobilized PBSC allografts exhibit a suppressive adaptive immune-related gene-expression profile. However, innate and inflammatory responses are elevated. Our data provides an explanation for the potentially immunosuppressive effects observed after G-CSF administration.**

Allogeneic peripheral blood stem cell transplantation (PBSCT) remains the treatment of choice for high-risk hematological malignancies and offers key advantages over conventional bone marrow (BM) transplantation, including efficiency of stem cell harvesting and improved engraftment and immune reconstitution kinetics [1,2]. Hematopoietic stem cell (HSC) mobilization is achieved through administration of granulocyte-colony stimulating factor (G-CSF) to human leukocyte antigen (HLA) compatible allogeneic donors. In addition to being a potent stimulator of HSC mobilization, a growing body of evidence suggests that G-CSF has potent immunomodulatory effects and can influence transplant outcomes [3].

Despite a 1-log greater number of T cells contained in G-CSF–mobilized stem cell product as compared to a BM harvest, the incidence of acute graft-vs-host disease (aGVHD) is not increased [4]; although risk of chronic GVHD may be higher [5]. This effect is generally ascribed to a polarization of donor T cells toward regulatory and T helper-type 2 (Th2) responses that suppress posttransplantation alloreactive events through production of immunosuppressive cytokines, such as interleukin-4 (IL-4), IL-10, and transforming growth factor-β.

Mechanistically, this shift in donor immune responses can be explained by: 1) T cells respond to G-CSF by upregulation of GATA binding protein-3 (GATA-3), which induce polarization of T cells toward a Th2 phenotype [6];

Offprint requests to: Vijay Reddy, M.D. Ph.D, Department of Medicine, Division of Hematology/Oncology, University of Florida, 1600 SW Archer Road, ARB R4-204, PO Box 100277, Gainesville, FL 32610-0277; E-mail: reddyvs@medicine.ufl.edu

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2) G-CSF induces mobilization of tolerogenic dendritic cell (DC) subtypes, specifically plasmacytoid DC, which resemble immature DC and polarize T cells toward Th2 [7]; 3) elevated numbers of monocytes contained in the G-CSF–mobilized allograft suppress T-cell activation through production of IL-10 [8], and 4) G-CSF promotes an increase in regulatory T cells that promote posttransplantation tolerance [9].

The beneficial effects of G-CSF, both in terms of enhanced immune reconstitution kinetics and seeming protection from aGVHD, are mediated by effects on the donor and not on the recipient [10]. Although posttransplantation administration of G-CSF can speed granulocyte recovery, G-CSF is not routinely required for recipients to engraft, nor does it definitively lower the risk of GVHD [11]. In HLA haplotype-mismatched PBSCT, posttransplantation administration of G-CSF can engender complications, such as delayed immune reconstitution and increased susceptibility to viral and fungal infections due to overall immune dysregulation and reduced Th1 cellular responses [12]. Thus, it appears that the role of G-CSF in affecting transplantation outcomes is through its influence on graft composition.

G-CSF is commonly used to mobilize blood stem cells and as a growth factor to promote granulocyte counts in immunocompromised patients. We hypothesized that its effects on the immune system were counterproductive because of overall immune suppression. Our study employed a comprehensive flow cytometric and validated microarray analysis of peripheral blood stem cell products from G-CSF–mobilized donors in order to describe the immunomodulatory effects of G-CSF in a broad context, based on changes in immune cell counts and gene expression.

Materials and methods

Enrollment of subjects and stem cell mobilization

This study was approved by the University of Florida Institutional Review Board. A total of 10 samples from 5 allogeneic (sibling) donors were obtained after they had given informed consent. Approximately 4 mL venous peripheral blood was collected at baseline and then after administration of recombinant human G-CSF (filgrastim, Neupogen; Amgen, Thousand Oaks, CA, USA) for 5 days at 10 μg/kg/day, which coincided with the time of stem cell product harvesting.

Sample collection and processing for gene expression analysis

Peripheral blood for gene expression analysis was processed immediately after collection in order to reduce ex vivo effects of altering the gene-expression profile. The following protocol was adapted from Feezor et al. [13]. In brief, whole blood was centrifuged at 1200 rpm for 10 minutes and plasma was removed. The remaining erythrocyte and buffy coat fractions were added to 40 mL ammonium chloride Buffer EL (Qiagen, Valencia, CA, USA) for 15 minutes at 4°C to lyse erythrocytes. The remaining leukocyte fraction was concentrated by centrifugation, and the cells were washed in 15 mL Buffer EL again to lyse residual erythrocytes.

Leukocyte RNA was purified using the RNeasy mini kits (Qiagen). RNA concentration was determined on a NanoDrop ND-1000 Spectrophotometer and quality was assessed by capillary electrophoresis on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Biotinylated cRNA was generated from 2.5 μg total cellular RNA. For each sample, 15 μg fragmented cRNA was hybridized onto Affymetrix Human U133 Plus 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA, USA) for 16 hours at 45°C. Arrays were washed and stained according to standard Affymetrix protocol, and then scanned on an Affymetrix GeneChip Scanner 3000. Image analysis was performed with Affymetrix GeneChip Operating Software v1.2.

Quantitative RT-PCR validation of gene expression

Four representative genes (ELA2, CD28, CD86, ARTS-1) uncovered by microarray analysis were validated with quantitative real-time polymerase chain reaction (qRT-PCR) using the Taqman Gene Expression Assay system and run on the 7900HT Fast real-time polymerase chain reaction machine (Applied Biosystems, Foster City, CA, USA). For each donor sample, pre– and post–G-CSF, 1 μg total RNA was used for reverse transcription into cDNA. Next, 100 ng cDNA was used to amplify each target gene in triplicate with human β actin as an endogenous control. The fold-change in gene expression was based on average cycling threshold, using the mean of all pre–G-CSF triplicate assays as a calibration reference.

Complete blood cell counts and flow cytometry

Total white blood cell count, absolute neutrophil count, and monocyte counts were generated using a Coulter Ac·T diff analyzer (Beckman-Coulter, Fullerton, CA, USA). Other cell phenotypes (HSC, T cells, B cells, natural killer [NK] cells, DC) were quantified using a BD FACSCalibur four-color flow cytometer (Becton Dickinson, Mountain View, CA, USA). Cell types measured and their corresponding surface markers were as follows: HSC ($CD34^+$); T cell ($CD3^+$); T-cell subsets ($CD4^+$, $CD8^+$, $CD4^+/CD25^+$); B cells ($CD20^+$); NK cell ($CD56^+/CD16^+$). DC counts were analyzed as described previously [14]. DC were negative for lineage markers (CD3, CD14, CD16, CD19, CD20, CD56) and positive for HLA-DR. Myeloid DC were $CD11^+$, and plasmacytoid DC were $CD123^+$. Absolute number of circulating cells ($cells/mm^3$) was calculated by multiplying the percentage of cells with the total number of white blood cells per microliter.

Statistical and pathway analysis of microarray data

We analyzed 54,613 probe sets on each microarray. For each probe set, the signal intensity was first log transformed and then an analysis of covariance adjusting for difference of each donor (e.g, age, gender) was carried out to assess if the expression level significantly changed after administration of G-CSF. Significant changes in gene expression were assessed by controlling the false discovery rate at 5% [15]. Changes in gene expression are reported as the $\log_2$ fold change (FC) of the signal intensity. DChip 2006 software was used to generate an unsupervised hierarchical cluster analysis and corresponding dendrogram.

The NetAffx Analysis Center available through the Affymetrix Web site was used for probe set annotation assistance. In addition,

information gathered from independent literature searches for each significant gene were used to determine cellular, molecular, and immunological significance.

The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, available at: http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE7400.

Additional statistical analyses

To quantitate changes in cell counts after G-CSF administration, the fold change in absolute cell count was calculated based on a post–G-CSF to pre–G-CSF ratio. One-sided paired *t*-tests were performed to generate *p* values with GB-Stat v10.0 software (Dynamic Microsystems, Silver Spring, MD). *p* Values for microarray gene-expression data were generated through the analysis of covariance. The significance threshold for microarray and flow cytometric comparisons was $p < 0.05$. The Cramer's V-coefficient was calculated to determine the strength of association between cell counts and relevant gene expression. Graphs and plots were rendered on Microsoft Excel 2003.

Results

Immune cell count increases after G-CSF mobilization

Table 1 shows mean blood counts and a panel of cell phenotypes that were enumerated via flow cytometry before (pre–G-CSF) and after (post–G-CSF) administration of G-CSF. G-CSF administration for 5 days induced consistently significant increases in total white blood cell count (7.1 vs. 41.4 x 10^3/mm^3, P=0.011), absolute neutrophil count (ANC) (4.4 vs. 36 x 10^3/mm^3, P=0.013) and monocytes (262 vs. 1,672 x 10^3/mm^3, P=0.008). $CD34^+$ counts were not analyzed in pre-G-CSF donors; however, post-G-CSF blood stem cell products contained appreciable amounts of $CD34^+$ stem cells (216±195/mm^3). In measuring cells of the lymphoid lineage, T ($CD3^+$), B ($CD20^+$) and NK ($CD3^-/CD56^+/CD16^+$) cells all significantly increased after G-CSF (P=0.004; P=0.031; P=0.039), with T cells increasing 7.5-fold (1,558 vs. 11,752/mm^3). $CD4^+$, $CD8^+$ and $CD4^+CD25^+$ counts were significantly increased (P=0.005; P=0.013; P=0.019), with $CD4^+$ cells predominating. Finally, myeloid DC and plasmacytoid DC were significantly increased (P=0.014; P=0.015). When comparing the relative increases of plasmacytoid vs. myeloid DC, G-CSF mobilization preferentially increased plasmacytoid DC counts, as the ratio of plasmacytoid-to-myeloid DC was significantly increased (0.46 vs. 1.69, P=0.010).

Microarray analysis reveals the effects of G-CSF on immune response genes

Out of 54, 613 probe sets, 4,501 showed significant changes in expression between the paired pre-G-CSF and post-G-CSF samples, based on a 5% false discovery rate (Fig. 1). Annotations were applied to 459 probe sets, corresponding to 335 unique genes, as relevant to the immune response. Together with probe set annotations, an independent literature search for each gene was carried out in order to assess its known role in the immune response. A representative list of well-characterized genes is reported (Table 2). Among the induced genes (FC>0) were those relevant to inflammatory immune responses, including neutrophil proteases, chemokines, and inflammatory cytokine and chemokine receptors. However, adaptive immune-related gene expression pathways—notably antigen presentation through class I and class II major histocompatibility complex (MHC), T-cell activation and proliferation and cytolytic effector responses, were downregulated. Finally, although G-CSF promoted a predominantly Th2 vs Th1 transcriptional response, GATA-3 was downregulated.

Table 1. Peripheral blood stem cell transplantation donor cell counts before and after mobilization with granulocyte colony-stimulating factor (G-CSF)

Cell type	Pre–G-CSF[a]	Post–G-CSF[a]	Fold-change[b]	*p* Value[c]
WBC ($\times 10^3$)	7.1 ± 2.4	41.4 ± 22.4	+5.8	0.011
ANC ($\times 10^3$)	4.4 ± 1.8	36 ± 22	+8.2	0.013
Monocytes	262 ± 67	1,672 ± 771	+6.4	0.008
$CD34^+$ (HSC)	NA	216 ± 195	N/A	NA
$CD3^+$ (T cell)	1,558 ± 611	11,752 ± 4,860	+7.5	0.004
$CD4^+$	256 ± 147	1,811 ± 1,265	+7.1	0.005
$CD8^+$	338 ± 815	815 ± 764	+2.4	0.013
$CD25^+CD4^+$	64 ± 47	920 ± 658	+14.4	0.019
$CD3^-CD56^+CD16^+$ (NK)	971 ± 240	7,175 ± 3,149	+7.4	0.039
$CD20^+$ (B cell)	529 ± 380	3,883 ± 2,538	+7.3	0.031
Lin^- HLA-DR^+ $CD11^+$ (myeloid DC)	15 ± 3	71 ± 37	+4.7	0.014
Lin^- HLA-DR^+ $CD123^+$ (plasmacytoid DC)	6 ± 5	126 ± 84	+21.0	0.015
Plasmacytoid DC: myeloid DC	0.46 ± 0.31	1.69 ± 0.79	+3.7	0.010

ANC = absolute neutrophil count; DC = dendritic cell; HSC = hematopoietic stem cell; NA = not applicable; WBC = white blood cells.

[a]Absolute cell counts (cells/mm^3) are reported as mean ± standard deviation.

[b]Fold-change generated by computing the ratio of post–G-CSF count to pre–G-CSF count.

[c]Generated from a one-sided paired *t*-test.

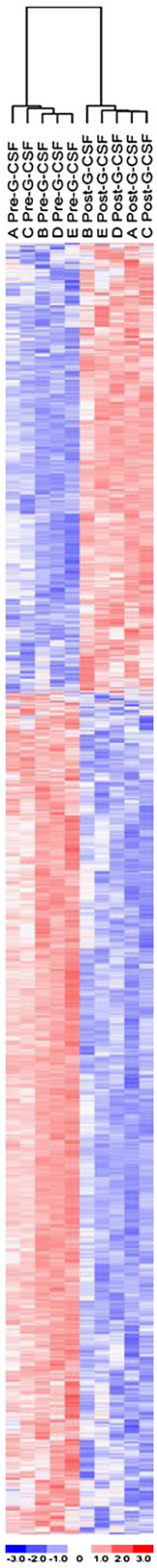

Figure 1. The genome-wide transcriptional response to granulocyte colony-stimulating factor (G-CSF) mobilization. Shown is an unsupervised hierarchical cluster analysis of 4,501 probe sets significantly changed in expression (1,569 increased; 2,932 decreased) after G-CSF mobilization among five healthy donors (**A**–**E**) for allogeneic peripheral blood stem cell transplantation.

qRT-PCR validation of gene expression

Four representative genes (human neutrophil elastase [ELA2], CD28, CD86, and the type 1 TNF receptor shedding aminopeptidase regulator [ARTS1]) were tested by qRT-PCR and successfully validated based on the directional change in gene expression from pre– to post–G-CSF (Fig. 2). Consistent with the microarray analysis, ELA2 (FC = +16.43) was upregulated post–G-CSF, whereas CD28 (FC = −25.10), CD86 (FC = −7.72), and ARTS-1 (FC = −6.43) were downregulated.

G-CSF elicits inverse effects on immune cell counts and costimulatory gene expression

Despite consistent increases in DC and T cells among each of the five donors, costimulatory CD86 and CD28 gene expression was repressed after G-CSF (Fig. 3). The relationship between DC/T-cell count increase and CD86/CD28 costimulatory gene expression decrease was strongly negatively associated, with a Cramer's V coefficient of V = −0.5 for both comparisons (Fig. 4).

Discussion

We demonstrate for the first time a comprehensive gene expression analysis of the G-CSF–mobilized PBSC allograft by utilizing Affymetrix Human Genome U133 Plus 2.0 microarray technology. To strengthen the descriptive power of the study, cell phenotyping by flow cytometry was also used. We found that despite an approximately sixfold increase in white blood cell count content and increased innate inflammatory response gene expression, adaptive immune-related gene transcriptional activity was downregulated, indicating an overall immunosuppressive profile of the PBSC allograft. The advantage of our study is that we report the complete cellular and genomic response to G-CSF, as compared to prior studies employing a less broad approach due to lack of such technology in the past.

Although G-CSF is known to influence immune responses, the precise mechanisms governing its immunomodulatory effects are a matter of debate. Like most growth factors and cytokines, G-CSF results in modulation of immune cell composition, cytokine profiles, and immune cell responses [16]. To describe these effects in a comprehensive fashion, use of high-throughput methods such as DNA microarray analysis is desirable. Yet, gene expression profiling of G-CSF–mobilized allogeneic donor hematopoietic cells has been only minimally investigated [17–19]. In the current study, we utilized the power of DNA microarray technology and flow cytometry to offer descriptive insights into the effects of G-CSF on donor hematopoietic cells.

Table 2. Immune-related gene expression changes after granulocyte colony-stimulating factor mobilization[a]

Probe ID	Gene symbol	Gene title	FC[b]	Function[c]
219669_at	CD177	CD177 molecule	+630.41	Neutrophil marker
207329_at	MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)	+145.65	Proteolysis
206871_at	ELA2	Elastase 2, neutrophil	+60.26	Proteolysis
207269_at	DEFA4	Defensin, α 4, corticostatin	+50.00	Defense response
203948_s_at	MPO	Myeloperoxidase	+22.39	Defense response
205653_at	CTSG	Cathepsin G	+15.36	Proteolysis
209772_s_at	CD24	CD24 molecule	+14.57	Humoral immune response
203936_s_at	MMP9	Matrix metallopeptidase-9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	+9.53	Proteolysis
220034_at	IRAK3	Interleukin-1 receptor-associated kinase 3	+7.24	Cytokine signal transduction
206618_at	IL18R1	Interleukin-18 receptor 1	+6.26	Proinflammatory cytokine
210140_at	CST7	Cystatin F (leukocystatin)	+5.07	Immune response
223454_at	CXCL16	Chemokine (C–X–C motif) ligand 16	+4.74	Chemotaxis
230632_at	IL10RB	Interleukin-10 receptor, β	+4.18	Th2 cell development
236172_at	LTB4R	Leukotriene B4 receptor	+4.13	Inflammatory response
210166_at	TLR5	Toll-like receptor 5	+3.72	Inflammatory response
201743_at	CD14	CD14 molecule	+2.91	Inflammatory response
207890_s_at	MMP25	Matrix metallopeptidase-25	+2.74	Inflammatory response
221058_s_at	CKLF	Chemokine-like factor	+2.68	Chemotaxis
203233_at	IL4R	Interleukin-4 receptor	+2.57	Th2 cell development
205147_x_at	NCF4	Neutrophil cytosolic factor 4, 40 kDa	+2.53	Immune response
203591_s_at	CSF3R	Colony-stimulating factor 3 receptor	+2.44	Signal transduction
225647_s_at	CTSC	Cathepsin C	+2.44	Proteolysis
207113_s_at	TNF	Tumor necrosis factor (TNF superfamily, member 2)	+2.21	Immune response
201502_s_at	NFKBIA	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α	+2.19	Regulation of NF-κB import into nucleus
217930_s_at	TOLLIP	Toll interacting protein	+2.16	Inflammatory response
205707_at	IL17RA	Interleukin-17 receptor A	+2.08	Cell surface receptor linked signal transduction
231779_at	IRAK2	Interleukin-1 receptor-associated kinase 2	+2.02	Inflammatory response
209124_at	MYD88	Myeloid differentiation primary response gene (88)	+1.93	Inflammatory response
230322_at	NFAM1	Nuclear factor of activated T cells–activating protein with immunoreceptor tyrosine-based activation motif 1	+1.91	Inflammatory response
214501_s_at	TLR4 /// H2AFY	Toll-like receptor 4 /// H2A histone family, member Y	+1.89	Inflammatory response
205842_s_at	JAK2	Janus kinase 2 (a protein tyrosine kinase)	+1.89	Cytokine signaling
200709_at	FKBP1A	FK506 binding protein 1A, 12 kDa	+1.89	Inflammatory response
226068_at	SYK	Spleen tyrosine kinase	+1.66	Lymphocyte activation
219618_at	IRAK4	Interleukin-1 receptor-associated kinase 4	+1.59	Cytokine signaling
212549_at	STAT5B	Signal transducer and activator of transcription 5B	+1.44	Cytokine signaling
213074_at	IRAK1BP1	Interleukin-1 receptor-associated kinase 1 binding protein 1	−1.43	I-κB kinase/NF-κB cascade
1559754_at	LTB	Lymphotoxin β (TNF superfamily, member 3)	−1.61	Innate immune response
222062_at	IL27RA	Interleukin-27 receptor, α	−1.79	Th1 cell development
200052_s_at	ILF2	Interleukin-enhancer binding factor 2, 45 kD	−1.85	Regulation of transcription
200887_s_at	STAT1	Signal transducer and activator of transcription 1, 91 kDa	−1.89	Cytokine signaling
1566363_at	DNTT	Deoxynucleotidyltransferase, terminal	−2.00	DNA replication
205685_at	CD86	CD86 molecule	−2.00	T-cell activation
214836_x_at	IGKC /// IGKV1-5	Immunoglobulin κ constant /// immunoglobulin κ variable 1–5	−2.08	Antigen presentation (MHC I)
209619_at	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	−2.08	Antigen presentation
225973_at	TAP2	Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP)	−2.08	Antigen presentation (MHC I)
1552758_at	HDAC9	Histone deacetylase 9	−2.17	B-cell activation
203932_at	HLA-DMB	Major histocompatibility complex, class II, DM β	−2.17	Antigen presentation
209788_s_at	ARTS-1	Type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	−2.22	Antigen presentation (MHC I)
211269_s_at	IL2RA	Interleukin-2 receptor, α	−2.22	Regulation of T-cell proliferation
201762_s_at	PSME2	Proteasome (prosome, macropain) activator subunit 2 (PA28 β)	−2.33	Immune response
232234_at	SLA2	Src-like–adaptor 2	−2.56	T-cell activation
205297_s_at	CD79B	CD79b molecule, immunoglobulin-associated β	−2.56	B-cell receptor signaling
206991_s_at	CCR5	Chemokine (C–C motif) receptor 5	−2.56	Chemotaxis

(*continued*)

Table 2. Continued

Probe ID	Gene symbol	Gene title	FC[b]	Function[c]
210982_s_at	HLA-DRA	Major histocompatibility complex, class II, DR α	−2.63	Antigen presentation
215193_x_at	HLA-DRB1 /// HLA-DRB3	Major histocompatibility complex, class II, DR β 1//DR β 3	−2.63	Antigen presentation
217527_s_at	NFATC2IP	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2 interacting protein	−2.63	Protein modification
217478_s_at	HLA-DMA	Major histocompatibility complex, class II, DM α	−2.70	Antigen presentation
206181_at	SLAMF1	Signaling lymphocytic activation molecule family member 1	−2.78	Lymphocyte activation
214049_x_at	CD7	CD7 molecule	−2.78	T-cell activation
203373_at	SOCS2	Suppressor of cytokine signaling 2	−2.86	Negative regulation of cytokine signaling
213537_at	HLA-DPA1	Major histocompatibility complex, class II, DP α1	−2.94	Antigen presentation
207979_s_at	CD8B	CD8b molecule	−2.94	T-cell activation
1552584_at	IL12RB1	Interleukin-12 receptor, β1	−3.13	Th1 cell development
217422_s_at	CD22 /// MAG	CD22 molecule /// myelin-associated glycoprotein	−3.13	B-cell marker
209602_s_at	GATA3	GATA binding protein 3	−3.13	Th2 cell development
207840_at	CD160	CD160 molecule	−3.23	Cytolytic effector cell marker
201137_s_at	HLA-DPB1	Major histocompatibility complex, class II, DP β1	−3.33	Antigen presentation
205671_s_at	HLA-DOB	Major histocompatibility complex, class II, DO β	−3.33	Antigen presentation
214617_at	PRF1	Perforin 1 (pore-forming protein)	−3.57	Cytolytic effector response
204890_s_at	LCK	Lymphocyte-specific protein tyrosine kinase	−3.57	T-cell activation
216033_s_at	FYN	FYN oncogene related to SRC, FGR, YES	−3.70	T-cell activation
209823_x_at	HLA-DQB1	Major histocompatibility complex, class II, DQ β1	−3.70	Antigen presentation
205267_at	POU2AF1	POU domain, class 2, associating factor 1	−3.70	Regulation of transcription
206366_x_at	XCL2	Chemokine (C motif) ligand 2	−3.85	Chemotaxis
210164_at	GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte–associated serine esterase 1)	−4.00	Cytolytic effector response
210288_at	KLRG1	Killer cell lectin-like receptor subfamily G, member 1	−4.00	Cellular defense response
210865_at	FASLG	Fas ligand (TNF superfamily, member 6)	−4.00	Apoptosis
210321_at	GZMH	Granzyme H (cathepsin G-like 2, protein h-CCPX)	−4.00	Cytolytic effector response
207795_s_at	KLRD1	Killer cell lectin-like receptor subfamily D, member 1	−4.00	Cell surface receptor linked signal transduction
206337_at	CCR7	Chemokine (C–C motif) receptor 7	−4.17	Chemotaxis
206914_at	CRTAM	Cytotoxic and regulatory T-cell molecule	−4.17	Cytolytic effector response
206118_at	STAT4	Signal transducer and activator of transcription 4	−4.17	Th1 cell development
203685_at	BCL2	B-cell chronic lymphocytic leukemia/lymphoma 2	−4.35	Anti-apoptosis
205456_at	CD3E	CD3e molecule, ε (CD3-TCR complex)	−4.55	T-cell activation
205798_at	IL7R	Interleukin-7 receptor	−4.55	T-cell proliferation
213539_at	CD3D	CD3d molecule, δ (CD3-TCR complex)	−4.55	T-cell activation
211005_at	LAT	Linker for activation of T cells	−4.55	T-cell activation
236295_s_at	NOD3	NOD3 protein	−4.76	T-cell activation
214470_at	KLRB1	Killer cell lectin-like receptor subfamily B, member 1	−4.76	Cell surface receptor linked signal transduction
220646_s_at	KLRF1	Killer cell lectin-like receptor subfamily F, member 1	−4.76	Cell surface receptor linked signal transduction
203828_s_at	IL32	Interleukin-32	−4.76	Proinflammatory cytokine
205495_s_at	GNLY	Granulysin	−5.00	Defense response
205831_at	CD2	CD2 molecule	−5.00	T-cell activation
205291_at	IL2RB	Interleukin-2 receptor, β	−5.26	T-cell proliferation
228599_at	MS4A1	Membrane-spanning 4-domains, subfamily A, member 1	−5.26	B-cell activation
205488_at	GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	−5.26	Cytolytic effector response
204891_s_at	LCK	Lymphocyte-specific protein tyrosine kinase	−5.56	T-cell activation
206983_at	CCR6	Chemokine (C–C motif) receptor 6	−5.88	Chemotaxis
205758_at	CD8A	CD8a molecule	−5.88	T-cell activation
214974_x_at	CXCL5	Chemokine (C–X–C motif) ligand 5	−5.88	Chemotaxis
206545_at	CD28	CD28 molecule	−5.88	Regulation of T cell proliferation
206785_s_at	KLRC1 /// KLRC2	Killer cell lectin-like receptor subfamily C, member 1 /// member 2	−5.88	Cellular defense response

(*continued*)

Table 2. Continued

Probe ID	Gene symbol	Gene title	FC[b]	Function[c]
206666_at	GZMK	Granzyme K (granzyme 3; tryptase II)	−6.25	Cytolytic effect or response
206761_at	CD96	CD96 molecule	−6.25	NK/T-cell adhesion
211010_s_at	NCR3	Natural cytotoxicity triggering receptor 3	−7.14	Cytolytic effector response
228518_at	IGH@ /// IGHG1 /// IGHM	Immunoglobulin heavy locus /// immunoglobulin heavy constant γ1 //μ	−12.50	Antigen presentation (MHC I)

NK = natural killer.

[a]A complete and annotated spreadsheet of the immune-related genes is available through the NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and accessible through GEO Series accession number GSE7400.

[b]Expressed as the $\log_2$ fold-change (FC) in signal intensity, from pre– to post–granulocyte colony-stimulating factor (G-CSF) ($FC = 2^{[\log 2\ (\text{avg post–G-CSF} - \text{avg pre–G-CSF})]}$). FC > 0 = upregulated; FC < 0 = downregulated.

[c]From Affymetrix annotations and an independent literature search.

This information is clinically relevant for both the use of G-CSF in the transplantation setting and as a possible adjuvant therapy for certain autoimmune diseases [20].

The ability of G-CSF to consistently induce increases in white blood cell counts confirms its role as a potent hematopoietic growth factor, particularly for cells of the granulocytic lineage [21]. In all donors tested, G-CSF administration yielded high $CD34^+$ counts, and mobilized cells were subsequently collected and used for clinical transplantation. In enumerating immune cell counts in these allografts, a nearly 7.5-fold mean increase in T-cell counts was noted, which is characteristic of G-CSF [1]. It is known that in addition to stimulating myeloid cell differentiation, G-CSF also can simulate T cells that express the G-CSF receptor [22,23].

A large, significant increase in monocytes was observed, suggesting a role for G-CSF in inducing tolerance through increased regulatory activity of monocytes via IL-10 [8,24]. Consistent increases in DC counts were noted after G-CSF mobilization, with a predominance of plasmacytoid DC over myeloid DC. Plasmacytoid DC are known to polarize naïve T cells toward a Th2 phenotype, which is believed to play a role in preventing increased risk of GVHD after PBSCT, without compromising graft-vs-leukemia (GVL) effects [7,25]. Indeed, the potent antigen-presentation activity of DC is central to GVL effects needed to eliminate quiescent cancer cells that may have evaded prior conditioning regimens [26]. This likely accounts for GVL effects seen in our previous findings where patients with high DC counts had lower incidence of relapse and improved survival [14].

An analysis of immune-related gene expression changes occurring after administration of G-CSF revealed that proinflammatory gene expression is active, as evidenced by upregulation of IL-1 receptor signaling components including myeloid differentiation primary response gene (MYD88), toll interacting protein (TOLLIP), and tumor necrosis factor (TNF). High levels of TNF produced by donor cells can enhance both GVH and GVL effects after

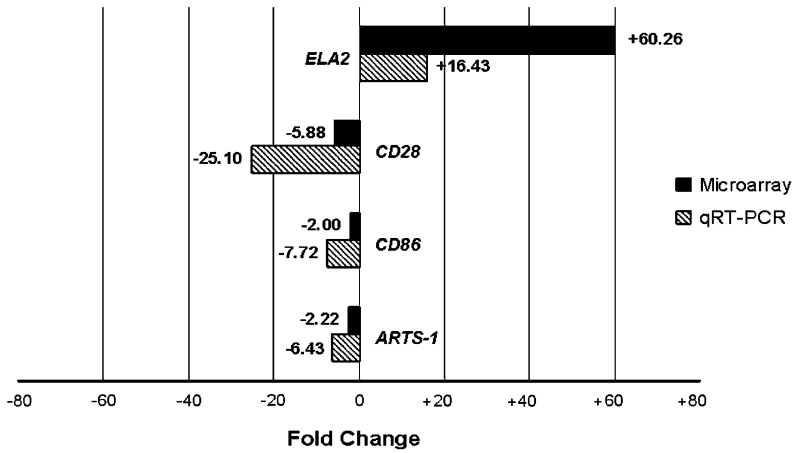

Figure 2. Validation of representative genes uncovered by microarray analysis. Four representative immune-related genes significantly induced or repressed after granulocyte colony-stimulating factor (G-CSF) administration as revealed by microarray analysis were tested using quantitative real-time polymerase chain reaction (qRT-PCR). Comparing the two assays (microarray vs qRT-PCR), the directional change in expression is consistent for each gene analyzed. The fold-change values reported from the microarray and qRT-PCR assay were generated from the probe signal intensity and target gene cycling threshold (ΔCt), respectively.

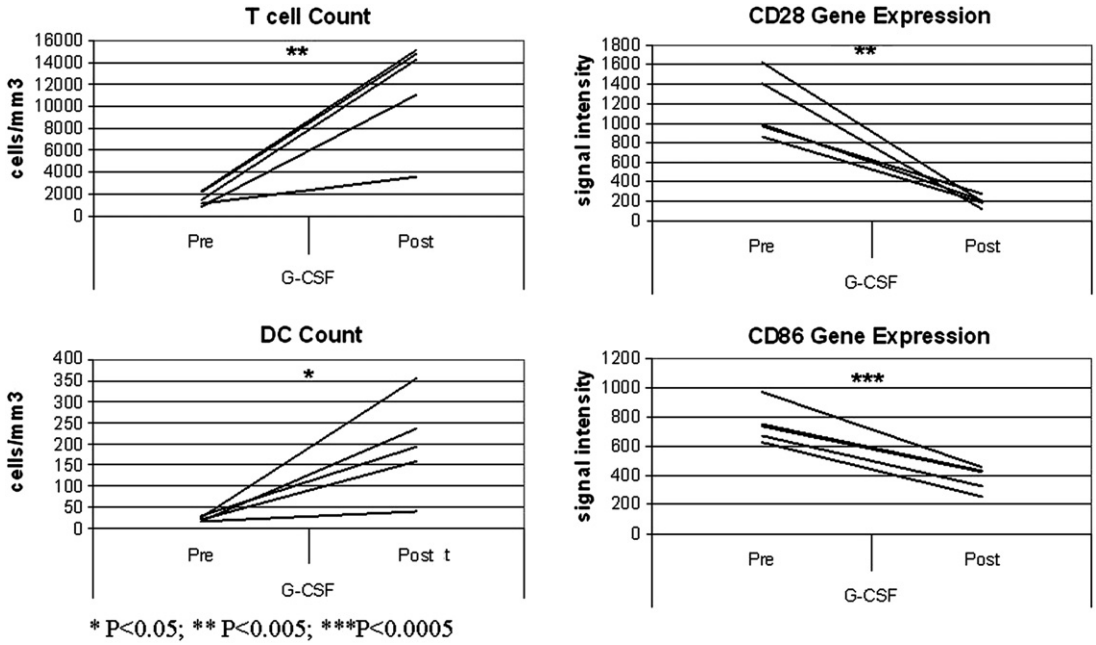

Figure 3. Dendritic (DC) and T-cell counts and costimulatory molecule gene expression. Despite significant increases in T-cell and DC counts, expression levels of their corresponding costimulatory molecules (CD28 and CD86, respectively) were decreased. Lines on each graph correspond to paired samples (Pre/Post) for each donor, with cell counts enumerated by flow cytometry and gene expression measured by microarray analysis.

transplantation [27] and a recent report has shown that donor-derived TNF contributes to the pathogenesis of early aGVHD [28]. In addition to upregulation of TNF, neutrophil activation and protease genes (CD177, ELA2, MMP-9, MMP-25) were upregulated post–G-CSF. The products of these genes are important for creating a highly proteolytic environment in the bone marrow, therefore, facilitating release of stem cells from the stromal microenvironment [29–31]. It has also been demonstrated that G-CSF–mobilized donors show significantly increased plasma levels of matrix metalloproteinase-9 and human neutrophil elastase compared to premobilization states [32]. In rare cases, excessive inflammation may cause vascular or pulmonary complications in otherwise healthy donors [33,34]. Additionally, our finding that G-CSF elicits proinflammatory responses could explain the clinical manifestations of engraftment syndrome that arise in some patients during the immediate postgrafting period after autologous or allogeneic PBSCT [35,36]. Thus, with G-CSF administration there exists a delicate balance between inflammation that is conducive to stem cell mobilization or harmful for the donor and recipient tissues.

Genes and pathways implicated cell-mediated immune responses were suppressed after G-CSF. Several MHC class II genes (HLA-DR, HLA-Q) and other components of the antigen-processing and -presentation pathway were

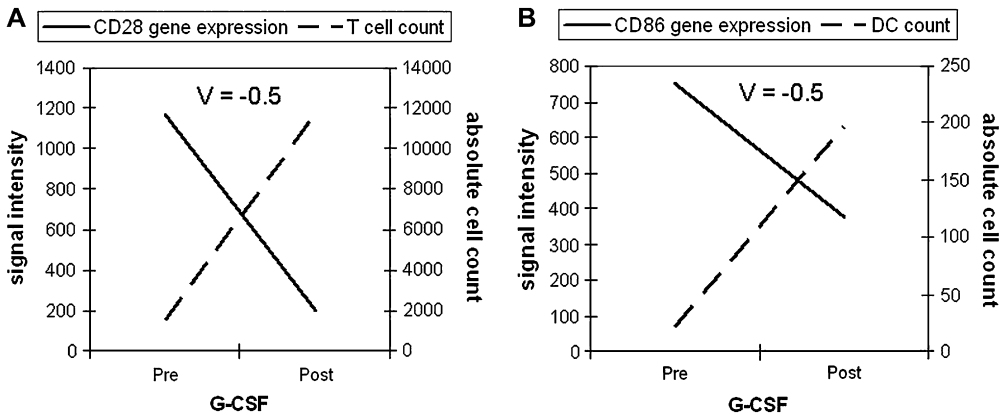

Figure 4. Strong negative association between immune cell counts and costimulatory molecule gene expression. (**A**) T-cell count/CD28 microarray gene expression; (**B**) dendritic cell (DC) count/CD86 microarray gene expression. G-CSF = granulocyte colony-stimulating factor; V = Cramer's V coefficient.

downregulated. In addition, the majority of genes designated as important for T-cell activation were downregulated. Most significantly are components of the T-cell receptor (CD3), accessory molecules (CD8A), and others involved in the DC-to-T–cell dialogue, including costimulatory molecules CD28 and CD86. Finally, α and β subunits of the IL-2 receptor were downregulated, suggesting that, overall, T-cell activation is suppressed after G-CSF administration. Significantly, from the flow cytometry data analysis, both DC and T cells were increased drastically; yet at the transcriptional level, the ability of DC to activate T cells may be compromised. Critical junctions in DC-mediated T-cell activation were suppressed through downregulation of T-cell receptor subunits, MHC II, intracellular kinases (LCK, FYN), costimulatory molecules (CD28, CD86) and the IL-2 receptor (IL2RA, IL2RB). Anti–IL-2rα monoclonal IgG1 drug daclizumab is used clinically as an immunosuppressive prophylaxis for kidney transplantation [37]; however, it has not shown promise as a treatment for aGVHD [38]. Additionally, cytolytic effector response gene expression was downregulated after G-CSF, as reflected in suppression of perforin (PRF1), granzyme B (GZMB), and Fas ligand (FASLG) genes. The products of these genes are central to GVH and GVL reactions imposed by alloreactive donor T and NK cells [39,40]. Our finding that DC counts and antigen-presentation gene expression (e.g., MHC II, CD86) were inversely related suggests that DCs mobilized with G-CSF are functionally immature and may promote host tolerance after transplantation.

With respect to cytokine gene expression, IL-32 was downregulated after G-CSF. IL-32 is a newly discovered, proinflammatory cytokine that drives Th1 responses [41]. Because G-CSF administration is classically associated with a bias toward Th2 responses, downregulation of IL-32 may potentially play a role in reorganization of the cytokine milieu. An unexpected finding, however, was downregulation of GATA-3, a transcription factor noted in the maturation of Th2 cells, as previous studies have reported that GATA-3 is upregulated in T cells after stimulation with G-CSF [23]. The difference in our findings may be explained by the fact that we analyzed a heterogenous mixture of whole blood leukocytes, rather than purified lymphocyte subsets.

In summary, G-CSF initially promotes increased granulocyte counts and innate inflammation, yet suppresses adaptive immunity as reflected by down-regulated expression of genes crucial for antigen presentation, T-cell activation, and cytolytic effector responses. Thus, although G-CSF is greatly beneficial in the transplantation setting as a potent mobilizer of stem cells, it should be used cautiously in those for which cell-mediated immunity is needed.

A microarray analysis of G-CSF–mobilized peripheral blood stem cell product represents a novel way to describe the dichotomous roles of G-CSF in influencing innate and adaptive immune responses. Future studies may be undertaken to further clarify these mechanisms by performing microarray analyses on purified immune cell populations. The ultimate goal will be translating such understanding into treatments that balance innate and adaptive immune responses in disease states.

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