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G-CSF Is an Essential Regulator of Neutrophil Trafficking from the Bone Marrow to the Blood

Craig L. Semerad, Fulu Liu, Alyssa D. Gregory, Katherine Stumpf, and Daniel C. Link[1]
Division of Oncology
Department of Internal Medicine
Washington University School of Medicine
St. Louis, Missouri 63110

Summary

Neutrophils are released from the bone marrow in a regulated fashion to maintain homeostatic levels in the blood and to respond to physiological stresses, including infection. We show that under basal conditions granulocyte colony-stimulating factor (G-CSF) is an essential regulator of neutrophil release from the bone marrow. Nonredundant signals generated by the membrane-proximal 87 amino acids of the G-CSF receptor (G-CSFR) are sufficient to mediate this response. Surprisingly, G-CSFR expression on neutrophils is neither necessary nor sufficient for their mobilization from the bone marrow, suggesting that G-CSF induces neutrophil mobilization indirectly through the generation of *trans*-acting signals. Evidence is provided suggesting that downregulation of stromal cell-derived factor 1 expression in the bone marrow may represent such a signal.

Introduction

Neutrophils, under normal conditions, are produced solely in the bone marrow and are released into the blood in a regulated fashion to maintain homeostatic levels of circulating neutrophils. There are two major observations suggesting that neutrophil release from the bone marrow is a highly regulated process. First, in response to inflammatory stimuli, such as infection, the number of neutrophils mobilized from the bone marrow is rapidly increased. Second, under normal conditions, only mature neutrophils and not granulocytic precursors are released in significant numbers into the blood. Though neutrophils play a critical role in innate immune responses, there is increasing evidence that they also may be a major contributor to tissue damage in inflammatory diseases. Thus, regulation of circulating neutrophil levels may be important in controlling both infectious and inflammatory diseases. Moreover, perturbations in the control of leukocyte egress from the bone marrow may contribute to the pathogenesis of leukemias. For example, in chronic myelogenous leukemia, expression of the *bcr-abl* oncogene may, via direct effects on cell adhesion and motility, contribute to the premature release of immature myeloid cells (Salgia et al., 1997).

The mechanisms that regulate neutrophil release from the bone marrow into the blood are largely undefined. To enter the circulation, neutrophils must migrate through the vascular barrier (termed the bone marrow-blood barrier) that separates the hematopoietic compartment from the circulation (Petrides and Dittmann, 1990). Bone marrow venous sinuses are the sites of neutrophil egress from the hematopoietic compartment and represent the only complete barrier to the intravascular space. The sinus wall is a trilaminar structure composed of endothelial cells, a basement membrane, and a layer of adventitial cells (Campbell, 1972; Inoue and Osmond, 2001). A notable feature of neutrophil release from the bone marrow is the diversity of mobilizing agents. Agents with distinct cellular targets and biological activities can induce neutrophil release from the bone marrow including certain chemokines and cytokines, microbial products (e.g., N-formyl-methionyl-leucyl-phenylalanine [fMLP]), and various other inflammatory mediators (e.g., C5a) (Jagels and Hugli, 1992, 1994; Opdenakker et al., 1998). Though many of the mobilizing agents are capable of directly activating neutrophils, it is not a prerequisite for induction of neutrophil mobilization. For example, hematopoietic growth factors that predominantly target T lymphocytes (interleukin-7) and dendritic cells (flt-3 ligand) lead to neutrophil mobilization (Brasel et al., 1996; Grzegorzewski et al., 1994; Molineux et al., 1997).

Granulocyte colony-stimulating factor (G-CSF), the principal hematopoietic cytokine regulating granulopoiesis, is widely used to treat or prevent neutropenia in a variety of clinical settings. In addition to its well-characterized ability to induce neutrophil production, G-CSF is a potent stimulus for inducing neutrophil release from the bone marrow. In mice, a single injection of G-CSF induces a transient neutropenia followed by a 5-fold increase in circulating neutrophils that begins at 30 min and peaks at 12 hr postinjection and is associated with a modest but significant decrease in mature neutrophils in the bone marrow (Ulich et al., 1988). Mature and immature (band) neutrophils but not lymphocytes or eosinophils are mobilized into the circulation, demonstrating that G-CSF-induced mobilization is selective for neutrophils. Similar data have been observed during G-CSF treatment in humans (Cohen et al., 1987). In response to infection, the serum level of G-CSF is often increased, suggesting that G-CSF may play a key role in regulating neutrophil release during stress conditions (Kawakami et al., 1990).

We previously reported the generation of transgenic mice carrying a targeted (knock-in) mutation of their G-CSFR such that the cytoplasmic (signaling) domain of the G-CSFR is replaced with that of the erythropoietin receptor (EpoR) (Semerad et al., 1999). The resulting chimeric receptor (termed GEpoR) is predicted to bind G-CSF but transmit EpoR-specific signals. Mice homozygous for GEpoR are neutropenic, and treatment with G-CSF fails to induce significant mobilization of neutrophils to the blood, despite near normal levels of mature neutrophils in the bone marrow. These data raised the possibility that nonredundant G-CSFR signals may be important regulators of neutrophil trafficking from the bone marrow. In the present study, this hypothesis is confirmed and the nature of these signals is examined.

[1]Correspondence: dlink@im.wustl.edu

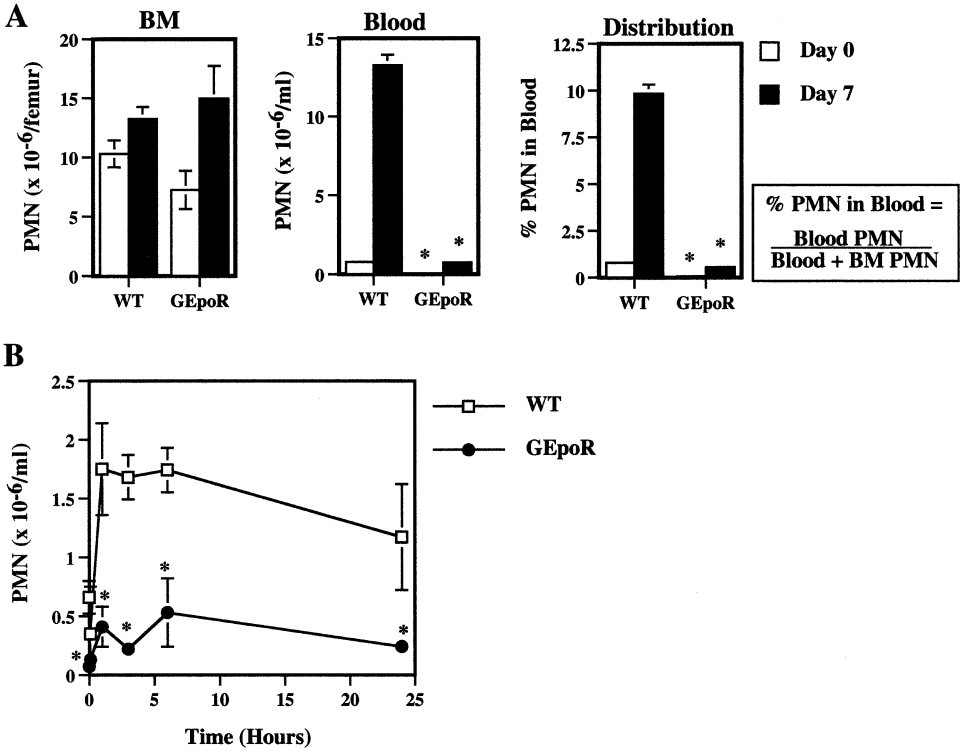

Figure 1. Neutrophil Response to G-CSF Treatment

(A) Long-term neutrophil response to G-CSF treatment. Mice (n = 6) were treated with G-CSF (250 μg/kg/day) for 7 days, and the number of neutrophils (PMN) in the bone marrow (BM) and blood were quantified before (Day 0) and after treatment with G-CSF (Day 7). Neutrophil distribution was estimated using the formula shown.

(B) Short-term neutrophil response to G-CSF treatment. Mice (n = 3) were given a single subcutaneous injection of G-CSF (125 μg/kg), and the absolute neutrophil count was determined at the indicated times. Data represent the mean ± SD. *, $p < 0.05$ compared with similarly treated WT mice.

We show that G-CSFR expression on neutrophils is neither necessary nor sufficient for their mobilization by G-CSF. The data suggest a model in which G-CSF acts, in *trans*, to induce neutrophil mobilization from the bone marrow to the blood.

Results

Neutrophil Mobilization into the Blood by G-CSF Is Impaired in GEpoR Mice

Neutrophil mobilization by G-CSF was characterized in GEpoR mice inbred onto a C57BL/6 background. In GEpoR mice, treatment with G-CSF for 7 days resulted in a 2-fold increase in bone marrow neutrophils resulting in a bone marrow neutrophil count similar to that seen in wild-type mice (Figure 1A). Surprisingly, despite the increase in bone marrow neutrophils to wild-type levels, little increase in circulating neutrophils was observed in GEpoR mice (Figure 1A). In fact, even in untreated GEpoR mice, the severity of neutropenia is out of proportion to the neutrophil content in the bone marrow. Since the great majority of neutrophils reside in the bone marrow and blood (Liu et al., 1997), we approximated the tissue distribution of neutrophils by plotting the percentage of neutrophils in the blood versus the total number of neutrophils in the bone marrow and blood (Figure 1A). In wild-type mice, at baseline 0.79 ± 0.31% of the total body neutrophils are found in the blood. Following G-CSF treatment, this percentage increases to 9.84 ± 2.22%, consistent with G-CSF's ability to induce neutrophil release from the bone marrow. In contrast, only 0.08 ± 0.02% of neutrophils are in the blood of GEpoR mice at baseline, and G-CSF treatment induced a minimal redistribution of neutrophils from the bone marrow to the blood (0.56 ± .24%) (Figure 1A).

To further characterize neutrophil release from the bone marrow, the short-term blood neutrophil response to a single injection of G-CSF was determined. The acute response to G-CSF is thought to primarily reflect neutrophil mobilization from the bone marrow to the blood, since G-CSF treatment has no effect on neutrophil survival in vivo and the time period examined is too brief to reflect G-CSF-driven neutrophil production (Lord, 1992; Lord et al., 1991). In response to a single subcutaneous injection of G-CSF, wild-type mice exhibited a 2.5-fold increase in circulating neutrophils 3 hr postinjection (Figure 1B). In contrast, little neutrophil response to G-CSF was evident in GEpoR mice.

Neutrophil Survival in the Blood Is Normal in GEpoR Mice

There are at least three potential explanations for the peripheral neutropenia observed in GEpoR mice. First, neutrophil survival in the circulation of GEpoR mice may

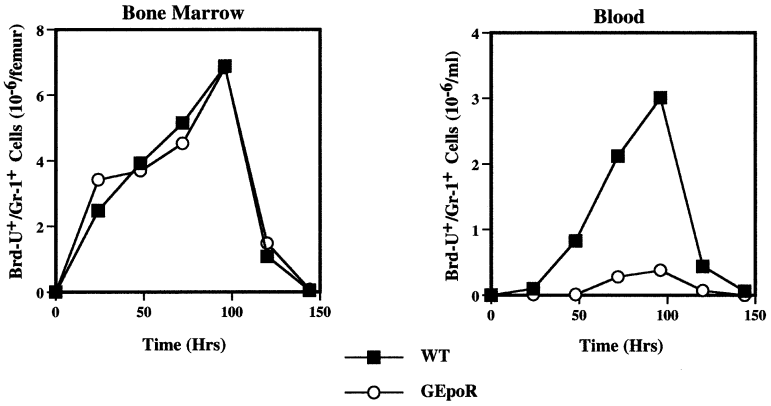

Figure 2. Fate of Brd-U-Labeled Neutrophils

Brd-U was administered to wild-type and GEpoR mice by a single intraperitoneal injection at time zero. Immediately following Brd-U administration, mice were treated with human G-CSF (250 μg/kg/day) for up to 7 days. Blood and bone marrow cells were harvested at the indicated times, and the number of Brd-U^+/Gr-1^+ cells was determined by flow cytometry. Each data point represents the mean of two mice.

be significantly reduced. Second, neutrophil mobilization from the bone marrow to the blood in response to G-CSF may be defective in GEpoR mice. Third, neutrophils mobilized from the bone marrow in GEpoR mice may accumulate in the marginated pool or tissues. The latter possibility seems unlikely, since no evidence of neutrophil accumulation in the spleen or nonhematopoietic tissues has been observed in GEpoR mice (data not shown).

To investigate potential differences in neutrophil survival, wild-type and GEpoR mice were given a single intraperitoneal injection of 5′-Bromo-2′-deoxyuridine (Brd-U) immediately followed by G-CSF treatment for 7 days. The fate of blood and bone marrow neutrophils pulse-labeled with Brd-U in vivo was determined by flow cytometry. Surprisingly, the kinetics of the appearance and subsequent disappearance of Brd-U^+ neutrophils in the bone marrow and blood were similar between wild-type and GEpoR mice (Figure 2). The half-life ($t_{1/2}$) of peripheral neutrophils is calculated from the rate of loss of labeled cells in the blood using the formula $N = N_o e^{-\lambda t}$, in which N_o = peak number of cells, N = number of cells at time t, and λ = the decay constant (Lord et al., 1991). The calculated half-lives for wild-type and GEpoR neutrophils were similar (8.6 and 9.8 hr, respectively) and are consistent with previously published data for wild-type neutrophils in mice and humans (Lord et al., 1989, 1991). A similar neutrophil half-life was observed in mice without G-CSF treatment (data not shown). Collectively, these data suggest that GEpoR signals, while able to support near normal neutrophil production, are not able to induce neutrophil mobilization from the bone marrow.

IL-8-Mediated Neutrophil Release from the Bone Marrow Is Normal in GEpoR Mice

To determine whether the defect in neutrophil mobilization is specific to G-CSF or representative of a more global defect, we characterized neutrophil mobilization by interleukin-8 (IL-8) in GEpoR mice. As reported previously, treatment of wild-type mice with a single intraperitoneal injection of IL-8 induced a rapid increase in the level of circulating neutrophils that peaked within 30 min and subsided by 6 hr postinjection (Figure 3) (Laterveer et al., 1995; Liu et al., 1997). In GEpoR mice, IL-8 induced a significant increase in blood neutrophils, though reduced compared with wild-type mice. However, after accounting for the reduced number of neutrophils in the bone marrow of GEpoR mice (70% that of wild-type mice, Figure 1A), it is clear that mature neutrophils in GEpoR mice are capable of mobilizing in response to IL-8.

The Membrane-Proximal Region of the G-CSFR Is Sufficient to Mediate G-CSF-Induced Neutrophil Mobilization from the Bone Marrow

To define the region(s) of the G-CSFR required for neutrophil mobilization, neutrophil distribution after 7 days of G-CSF treatment was examined in transgenic mice expressing a series of targeted mutations of their G-CSFR (Figure 4A). The d715 G-CSFR mutation introduces a premature stop codon at nucleotide 2403, leading to truncation of the carboxy-terminal 96 amino acids of the G-CSFR (McLemore et al., 1998). It is representative of G-CSFR mutations found in approximately 25% of patients with severe congenital neutropenia. However, mice homozygous for the d715 G-CSFR mutation have normal basal hematopoiesis. In the d715F G-CSFR mutant, the sole remaining tyrosine (Y704) of d715 has

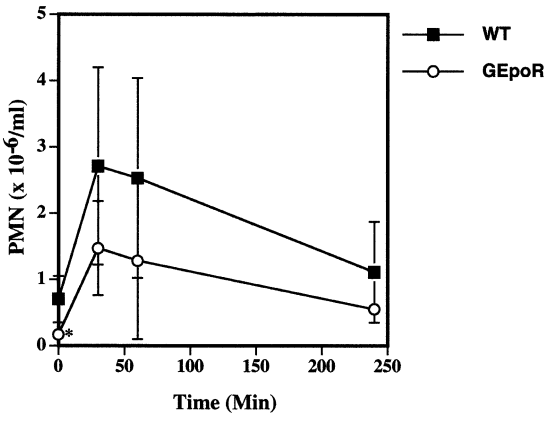

Figure 3. Neutrophil Response to IL-8 Administration

Human recombinant IL-8 (2 μg/mouse) was given to wild-type and GEpoR mice (n = 5) by a single intraperitoneal injection. Peripheral blood was obtained at the indicated times, and the absolute neutrophil count was determined. Data represent the mean ± SD. *, $p < 0.05$ compared with WT mice.

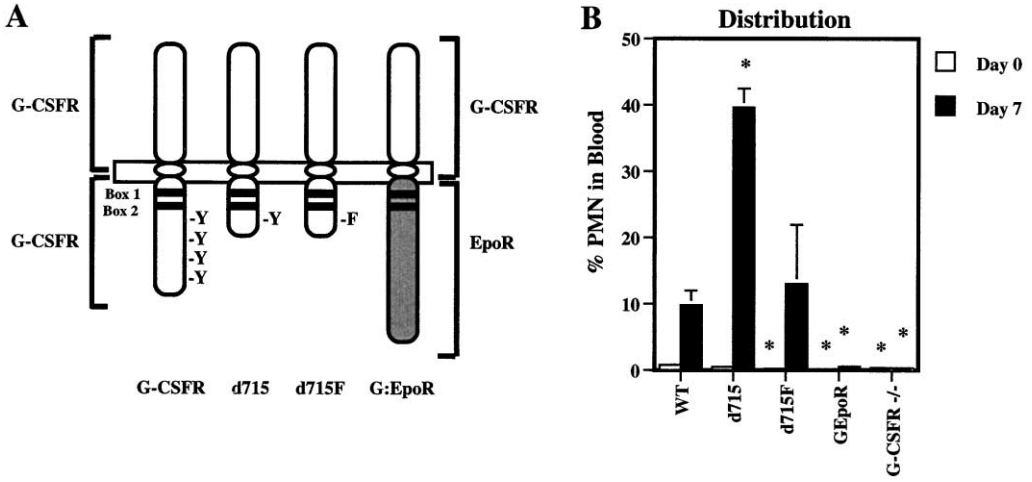

Figure 4. Neutrophil Mobilization by G-CSF in G-CSFR Mutant Mice

(A) Schematic of targeted G-CSFR mutations. Cytoplasmic tyrosines (Y) and the conserved box 1 and box 2 motifs are indicated. In the d715F mutant, tyrosine 704 of the G-CSFR has been mutated to phenylalanine (F).

(B) Neutrophil distribution. Mice (n = 3, each) were treated with G-CSF (250 μg/kg/day) for 7 days, and the neutrophil content of bone marrow and blood was measured on days 0 and 7. Neutrophil distribution was calculated as shown in Figure 1. Data represent the mean ± SD. *, $p < 0.05$ compared with similarly treated WT mice.

been mutated to phenylalanine (McLemore et al., 2001). Importantly, STAT 3 and STAT 5 activation by the d715F G-CSFR is markedly impaired. Mice homozygous for the d715F G-CSFR display an isolated defect in granulopoiesis. All mice were inbred on a C57BL/6 background. In G-CSFR-deficient (−/−) mice, the percentage of neutrophils in the blood at baseline was reduced compared with wild-type mice (WT versus G-CSFR$^{-/-}$, 0.79 ± 0.31% versus 0.25 ± 0.05%), and little change was observed after G-CSF treatment, as expected (Figure 4B). In both d715 and d715F mice, at baseline the percentage of neutrophils in the blood was modestly reduced compared to wild-type mice (d715, 0.45 ± 0.04%; d715F, 0.15 ± 0.03%). In contrast, after G-CSF treatment the redistribution of neutrophils into the blood was comparable in wild-type (9.84 ± 2.22%) and d715F mice (13.0 ± 8.90%) and was greater in d715 mice (39.6 ± 2.92%). These data show that the membrane-proximal 87 amino acids of the G-CSFR are sufficient to mediate G-CSF-dependent neutrophil mobilization from the bone marrow and suggest the presence of an inhibitory domain in the carboxy-terminal tail of the G-CSFR.

Expression of the G-CSFR on Transplantable Hematopoietic Cells but Not Stromal Cells Is Required for Neutrophil Mobilization by G-CSF

The G-CSFR is expressed on subsets of hematopoietic cells and stromal cells. Within the stromal cell compartment, the G-CSFR is expressed on endothelial cells and is capable of inducing endothelial cell proliferation (Bocchietto et al., 1993). To characterize the cell type(s) responsible for G-CSF-dependent neutrophil mobilization, a series of bone marrow chimeras was generated using wild-type and GEpoR mice (Figure 5A). In irradiated wild-type mice reconstituted with GEpoR hematopoiesis (GE > WT), no G-CSF-induced neutrophil mobilization was observed. Conversely, in irradiated GEpoR mice reconstituted with wild-type hematopoiesis (WT > GE), G-CSF-induced neutrophil mobilization into the blood was normal.

The failure of GEpoR neutrophils to mobilize from the bone marrow in response to G-CSF may be due to the loss of nonredundant G-CSFR signals that promote neutrophil mobilization from the bone marrow. Alternatively, it is possible the GEpoR transmits an aberrant signal that interferes with neutrophil mobilization. To address this issue, we generated an additional series of bone marrow chimeras using wild-type and G-CSFR-deficient mice (data not shown). Similar to the GEpoR chimeras, in irradiated wild-type mice reconstituted with G-CSFR-deficient hematopoiesis, no G-CSF-induced neutrophil mobilization was observed. Conversely, in irradiated G-CSFR-deficient mice reconstituted with wild-type hematopoiesis, G-CSF-induced neutrophil mobilization into the blood was normal. Collectively, these data demonstrate that expression of the G-CSFR on transplantable hematopoietic cells—not stromal cells—is required for G-CSF-induced neutrophil mobilization from the bone marrow to the blood.

Expression of the G-CSFR on Neutrophils Is Neither Necessary nor Sufficient for Their Mobilization from the Bone Marrow by G-CSF

Within the transplantable hematopoietic cell compartment, the G-CSFR is expressed on neutrophils, hematopoietic progenitor cells (HPC), monocytes, platelets, and possibly natural killer (NK) and B cells (Demetri and Griffin, 1991; Iizuka et al., 1997; Inukai et al., 1995; Nicola and Metcalf, 1985). G-CSFR signals generated in any or all of these cell types may contribute to neutrophil mobilization from the bone marrow. To determine whether a functional G-CSFR on neutrophils is required for G-CSF-induced neutrophil mobilization, a series of mixed bone marrow chimeras was generated in which mice were reconstituted with both G-CSFR-deficient and wild-type hematopoiesis. If expression of the G-CSFR

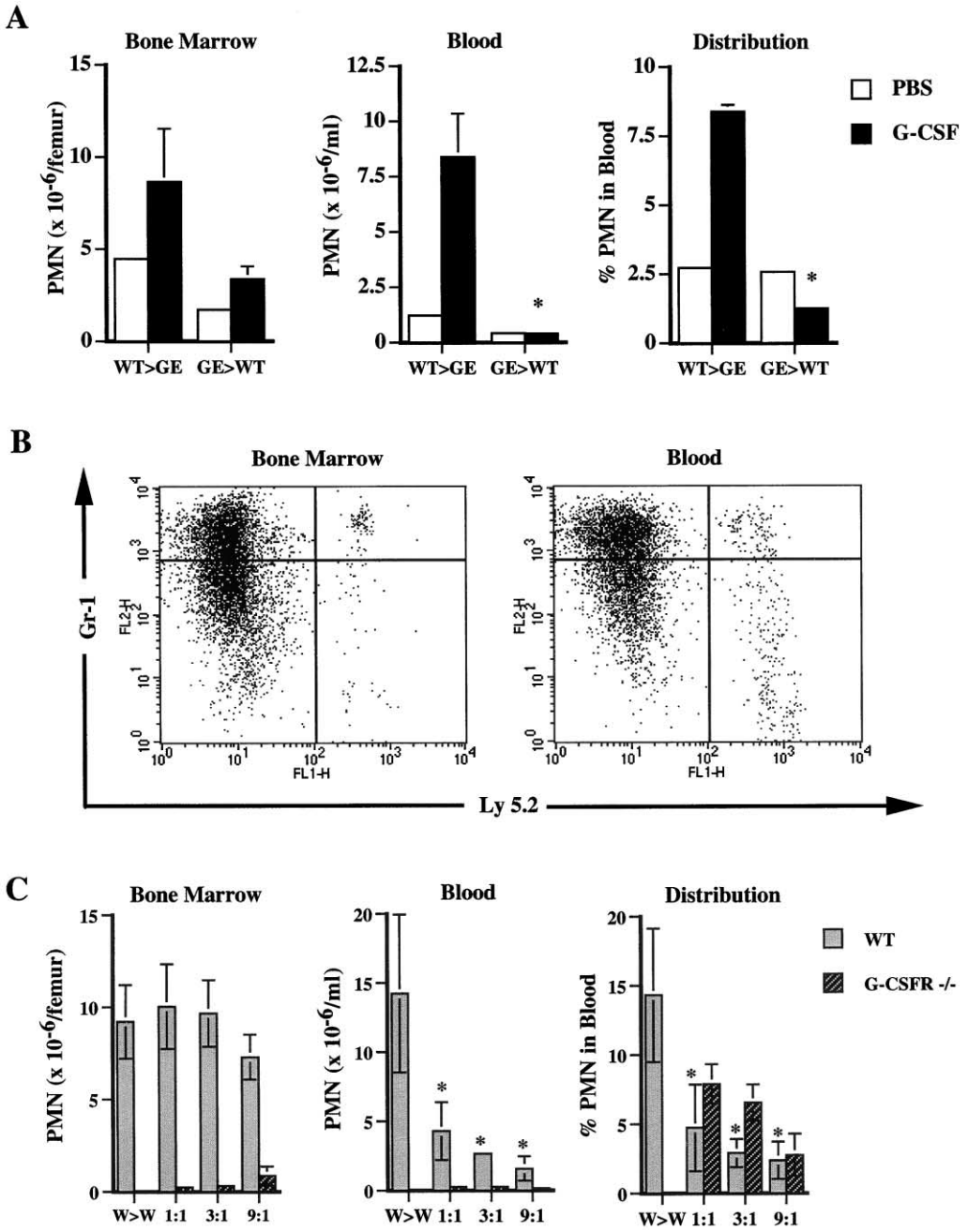

Figure 5. G-CSF-Induced Neutrophil Mobilization in Chimeric Mice

(A) G-CSF-induced neutrophil mobilization in bone marrow chimeras. Irradiated GEpoR mice reconstituted with wild-type hematopoiesis (WT > GE) and irradiated wild-type mice reconstituted with GEpoR hematopoiesis (GE > WT) were treated with PBS alone or G-CSF for 7 days, and the number of neutrophils in the blood and bone marrow was quantified. Data represent the mean ± SD of six mice. *, $p < 0.05$ compared with similarly treated WT > GE chimeras.

(B) Representative flow cytometry histograms of bone marrow and blood from a 3:1 G-CSFR-deficient:WT chimera stained with antibodies against Gr-1 and Ly 5.2. Note the great majority of the Gr-1 bright cells (mature neutrophils) in the bone marrow and blood are Ly 5.2 negative (i.e., of wild-type origin). Sorting of Gr-1 bright cells from the bone marrow, based on the gates shown, established that greater than 90% of the cells were mature neutrophils (data not shown).

(C) G-CSF-induced neutrophil mobilization in the mixed chimeras. Mixed chimeras transplanted with the indicated ratio of G-CSFR-deficient to wild-type bone marrow cells or mice transplanted solely with wild-type cells (W > W) were treated with G-CSF and neutrophil mobilization characterized. The number of wild-type and G-CSFR-deficient neutrophils in the blood and bone marrow of these chimeras is shown separately. Data represent the mean ± SD of six mice. *, $p < 0.05$ compared with W > W chimeras.

on neutrophils is required for their mobilization from the bone marrow, then treatment of these mixed chimeras with G-CSF would be predicted to mobilize only wild-type neutrophils.

Mixed chimeras were generated by mixing G-CSFR-deficient and wild-type bone marrow cells at ratios of 1:1, 3:1, and 9:1 (G-CSFR-deficient:wild-type) and transplanting them into irradiated syngeneic wild-type mice.

Table 1. Donor Origin in Mixed Chimeras

Chimera KO:WT	Blood			Bone Marrow
	B Lymphocytes	T Lymphocytes	Neutrophils	Neutrophils
1:1	53.5 ± 9.3%	55.9 ± 10.7%	96.5 ± 2.0%	96.3 ± 1.4%
3:1	32.2 ± 6.8%	38.9 ± 8.1%	96.0 ± 2.3%	92.9 ± 2.0%
9:1	18.7 ± 6.6%	21.2 ± 5.5%	92.0 ± 5.1%	90.2 ± 4.1%

Percentage of wild-type cells within a given lineage is shown for each group of chimeras 3 months posttransplant (n = 6, each). Data represent the mean ± SD.

In these experiments, congenic wild-type mice carrying the *Ly 5.1* allele were used to facilitate chimerism analysis (G-CSFR-deficient mice are *Ly 5.2*). To control for the bone marrow transplant procedure, a cohort of wild-type mice reconstituted solely with wild-type bone marrow cells also were studied. Mice were analyzed 3 months posttransplantation by flow cytometry to assess the contribution of wild-type (*Ly 5.1*) versus G-CSFR-deficient (*Ly 5.2*) cells to each hematopoietic lineage (Figure 5B and Table 1). Peripheral blood counts, including neutrophils, were similar in all groups of chimeric mice (data not shown). The contribution of G-CSFR-deficient cells to the B- and T-lymphocyte lineages was near the predicted levels. However, the contribution of G-CSFR-deficient cells to the granulocytic lineage was profoundly impaired, such that in the 3:1 mixed chimeras, 96.0% of blood neutrophils and 92.9% of bone marrow neutrophils were of wild-type origin. A detailed description of the effect of the loss of the G-CSFR on granulopoiesis will be presented elsewhere (Richards et al., submitted).

The mixed chimeras were treated with G-CSF for 7 days, and the neutrophil response in the bone marrow and blood was examined (Figure 5C). These studies yielded two important observations. First, though the total number of neutrophils mobilized into the blood was reduced in the mixed chimeras relative to their number in the bone marrow (see below), G-CSFR-deficient neutrophils were mobilized at least as well as wild-type neutrophils. For example, in the 3:1 chimeras, 2.91 ± 1.01% of wild-type and 6.54 ± 1.32% of G-CSFR-deficient neutrophils were distributed into the blood after G-CSF treatment. These data indicate that a functional G-CSFR on neutrophils is not required for their mobilization from the bone marrow to the blood by G-CSF. Second, the presence of G-CSFR-deficient hematopoietic cells inhibited mobilization of wild-type neutrophils in a dose-dependent fashion in the mixed chimeras. For example, comparing the 3:1 chimera to mice reconstituted with only wild-type cells, the absolute neutrophil count in the blood was 2.93 ± .84 × 10^6 versus 14.2 ± 5.7 × 10^6 neutrophils/ml, respectively. Importantly, the number of mature wild-type neutrophils in the bone marrow of these mice was similar (9.67 ± 1.2 × 10^6 versus 9.2 ± 2 × 10^6 neutrophils/femur, respectively). Accordingly, the redistribution of wild-type neutrophils from the bone marrow to the blood was significantly reduced (3:1 chimera versus WT chimera, 2.91 ± 1.01% versus 14.32 ± 4.82%, $p < .05$). Similar results were observed in mixed chimeras generated by transplanting a combination of GEpoR and wild-type bone marrow cells (data not shown). Collectively, these data show that G-CSFR expression on neutrophils is neither necessary nor sufficient for G-CSF-induced neutrophil mobilization from the bone marrow to the blood.

G-CSF-Induced Decrease in Bone Marrow SDF-1 Correlates with Neutrophil Mobilization

Accumulating evidence suggest that the chemokine stromal-derived factor-1 (SDF-1) may be a key retention signal in the bone marrow for hematopoietic cells (Kawabata et al., 1999; Ma et al., 1999; Nagasawa et al., 1996). To determine whether modulation of SDF-1 expression in the bone marrow may regulate neutrophil trafficking, we measured SDF-1α protein levels in the bone marrow and blood of wild-type mice and mice displaying impaired neutrophil mobilization (i.e., GEpoR mice and the 3:1 and 9:1 mixed chimeras). G-CSF treatment induced a significant decrease in SDF-1α protein levels in the bone marrow and blood of wild-type mice (Figure 6A). In contrast, no change in SDF-1α expression was detected in GEpoR mice following G-CSF treatment. Moreover, G-CSF treatment induced a much more modest decrease in SDF-1α in the bone marrow and blood of the mixed chimeras (Figure 6B and data not shown). In fact, a strong correlation was observed between the degree of neutrophil mobilization by G-CSF and the magnitude of the decrease in bone marrow SDF-1α (Figure 6C, $r^2 = .919$).

Discussion

Neutrophils are released from the bone marrow in a regulated fashion to maintain homeostatic levels and to increase their number in response to stresses, including infection. Despite their clinical and biological importance, the mechanisms that regulate neutrophil egress from the bone marrow are poorly understood. In this study, we show that G-CSFR signals are required to maintain basal levels of circulating neutrophils, in large part, through regulation of their release from the bone marrow. In fact, the major mechanism by which G-CSF treatment results in an increased number of neutrophils in the blood is through their augmented release. For example, in wild-type mice at baseline approximately 1% of total body neutrophils are in the blood, whereas after 7 days of G-CSF treatment, this value increases to approximately 10%. Interestingly, GEpoR, while able to transduce near normal proliferative signals in vivo, is not able to transduce the signals generated by the G-CSFR that mediate neutrophil release, raising the possibility that unique G-CSFR signals may mediate this biological response. Of note, all of the mice in this study were maintained in a specific pathogen-free (SPF) envi-

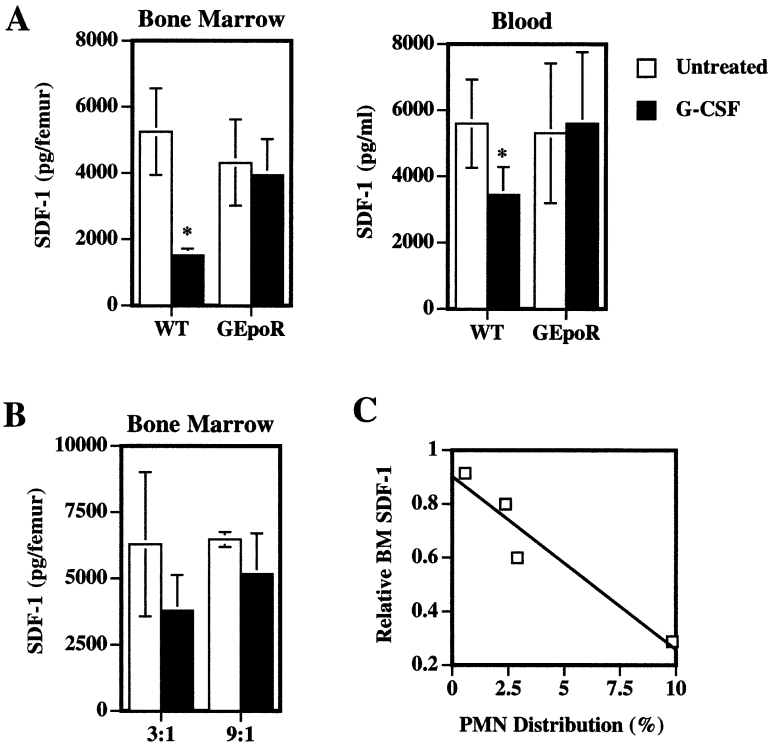

Figure 6. G-CSF-Induced Changes in SDF-1α Protein Expression

(A and B) Wild-type and GEpoR mice (A) (n = 6) and G-CSFR-deficient:wild-type mixed chimeras (B) (n = 3) were treated with G-CSF (250 μg/kg/day) for 5 days, and the amount of SDF-1α protein in the bone marrow extracellular fluid and plasma was measured by ELISA. Data represent the mean ± SD. *, $p < 0.05$ compared with untreated control mice. (C) The magnitude of the decrease in SDF-1α protein in the bone marrow was estimated by dividing the average amount of SDF-1α protein present in G-CSF-treated mice by untreated mice. This value was plotted against neutrophil mobilization, as measured by the percentage of total body neutrophils present in the blood after G-CSF treatment.

ronment. The contribution of G-CSFR signals to the regulation of neutrophil trafficking under a more natural environment in which mice are continuously exposed to pathogens is not clear.

The importance of G-CSF in the regulation of neutrophil release during stress conditions has been assessed in G-CSF-deficient mice in response to infection with *Listeria monocytogenes* or *Candida albicans* (Basu et al., 2000; Lieschke et al., 1994). The acute neutrophilia associated with *Listeria monocytogenes* infection was blunted in G-CSF-deficient mice. Surprisingly however, a similar increase in the level of circulating neutrophils was observed in wild-type and G-CSF-deficient mice after challenge with *Candida albicans*. These studies show that G-CSF may play an important role in regulating neutrophil release in response to some but not all infections. Consistent with this conclusion, we show in the present study that IL-8-induced neutrophil mobilization in GEpoR mice is comparable to wild-type mice. Clearly, G-CSFR signals are not universally required for neutrophil release from the bone marrow.

To begin to define the region(s) of the G-CSFR that mediate neutrophil release from the bone marrow, we characterized neutrophil mobilization by G-CSF in a series of transgenic mice carrying different targeted G-CSFR mutations. These data show that the membrane-proximal 87 amino acids of the G-CSFR are sufficient to induce neutrophil release from the bone marrow. In fact, neutrophil mobilization in d715 G-CSFR mice is significantly increased compared to wild-type mice, suggesting the presence of an inhibitory domain in the carboxy-terminal tail of the G-CSFR. Interestingly, previous studies showed that the d715 G-CSFR also displays enhanced proliferative signaling, possibly through delayed receptor internalization, sustained STAT activation, and/or the failure to recruit the SH2 domain-containing phosphatase-1 (SHP-1) (Dong et al., 2001; Hermans et al., 1999; Hunter and Avalos, 1999). G-CSF also induced significant neutrophil mobilization in d715F G-CSFR mice. These data indicate that STAT 3 and STAT 5 activation by the G-CSFR is not required for the transduction of signals leading to neutrophil release, since their activation by the d715F G-CSFR is markedly impaired (McLemore et al., 2001). The membrane-proximal 87 amino acids of the G-CSFR is sufficient to induce the activation of JAK and src family tyrosine kinases, the PI3kinase/Akt pathway, and some components of the Ras/MAPK pathway (Avalos et al., 1995; Dong and Larner, 2000; Rausch and Marshall, 1997). Studies are under way to assess the contribution of these signal transduction pathways in G-CSF-induced neutrophil mobilization.

The G-CSFR is expressed on a broad range of hematopoietic and bone marrow stromal cells, including endothelial cells, neutrophils, monocytes, progenitors, and possibly natural killer and B cells (Demetri and Griffin, 1991; Iizuka et al., 1997; Inukai et al., 1995; Nicola and Metcalf, 1985). To identify the cell type(s) that mediate G-CSF-induced neutrophil mobilization, a series of bone marrow chimeras between wild-type and G-CSFR mutant mice were generated. Previous studies showed that, following bone marrow transplantation, the great majority of bone marrow stromal cells are of recipient origin (Lennon and Micklem, 1986; Simmons et al., 1987). Thus, our studies of the WT > GE and GE > WT chimeras (Figure 5A) strongly suggest that G-CSFR expression on stromal cells is not required for neutrophil mobilization by G-CSF. Studies of neutrophil mobilization in

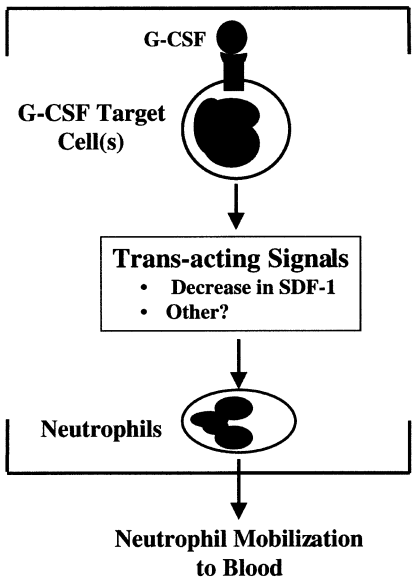

Figure 7. Two-Step Model of G-CSF-Induced Neutrophil Mobilization

In this model, G-CSF engages its receptor on a (as yet undefined) target cell population, resulting in its activation. Following activation, this cell population generates secondary signals that act in *trans* to induce neutrophil mobilization from the bone marrow.

mixed chimeras reconstituted with both wild-type and G-CSFR mutant hematopoiesis yielded several significant observations. First, relative to their number in the bone marrow, wild-type and G-CSFR mutant neutrophils were mobilized equally by G-CSF (Figure 5C). This observation demonstrates that G-CSFR expression on neutrophils is not required for their mobilization by G-CSF and suggests a *trans*-acting mechanism. Second, despite a similar number of wild-type neutrophils in the bone marrow after G-CSF treatment in all of the chimeras, the number of neutrophils mobilized into the blood decreased as the contribution of G-CSFR-deficient cells increased (Table 1 and Figure 5C). This surprising observation demonstrates that expression of the G-CSFR on neutrophils is not sufficient to induce their mobilization by G-CSF. Collectively, our data support a two-step model of G-CSF-induced neutrophil mobilization (Figure 7). In the first step, a (as yet undetermined) subset of hematopoietic cells is activated by nonredundant G-CSFR signals. The second step is the generation of secondary signals by these activated cells that act in *trans* to induce neutrophil release from the bone marrow.

The nature of the secondary signal(s) that mediate neutrophil mobilization by G-CSF is not known; however, there is accumulating evidence suggesting that SDF-1 may play a key role in regulating hematopoietic cell trafficking from the bone marrow. SDF-1 is constitutively produced by bone marrow stromal cells (Aiuti et al., 1997). CXCR4, the receptor for SDF-1, is expressed on a broad range of hematopoietic cells including hematopoietic progenitors and neutrophils (Aiuti et al., 1997; Bruhl et al., 2001; Nagase et al., 2002). Studies of mice lacking SDF-1 or CXCR4 have established that SDF-1 is necessary for the normal migration of hematopoietic progenitors from the fetal liver to the bone marrow (Ma et al., 1999; Nagasawa et al., 1996). Moreover, in mice reconstituted with CXCR4-deficient bone marrow cells, premature release of granulocytic precursors was observed (Kawabata et al., 1999; Ma et al., 1999). Finally, elevation of SDF-1 levels in the blood by administration of SDF-1 or by injection of an adenoviral vector expressing SDF-1 is associated with a significant mobilization of hematopoietic progenitors and mature leukocytes into the blood (Hattori et al., 2001; Moore et al., 2001). These data suggest that SDF-1 may be a key retention signal in the bone marrow for both hematopoietic progenitors and mature leukocytes, including neutrophils. In the present study, we show that G-CSF treatment results in a significant decrease in SDF-1α protein in the bone marrow and blood of wild-type mice. Importantly, the decrease in SDF-1α in the bone marrow was attenuated or absent in mice displaying impaired neutrophil mobilization by G-CSF (i.e., GEpoR mice and the mixed chimeras). In fact, a strong correlation was observed between the degree of neutrophil mobilization by G-CSF and the magnitude of the decrease in bone marrow SDF-1α (Figure 6C). Consistent with our data, Petit and colleagues also reported that SDF-1 protein levels decreased in the bone marrow after G-CSF treatment (Petit et al., 2002). Moreover, they showed that treatment of mice with neutralizing SDF-1 or CXCR4 antibodies partially inhibited hematopoietic cell mobilization by G-CSF (though neutrophils were not specifically measured). Interestingly, they also provided evidence that the decrease in SDF-1 protein in the bone marrow is secondary to its proteolytic degradation by neutrophil elastase. However, we recently observed that G-CSF-induced neutrophil mobilization is normal in neutrophil elastase and cathepsin G-deficient mice, suggesting that multiple mechanisms may regulate SDF-1 expression (Levesque et al., submitted).

In summary, these data provide evidence that nonredundant G-CSFR signals play an important role in regulating neutrophil release from the bone marrow and maintaining homeostatic levels of neutrophils in the blood. Surprisingly, G-CSFR expression on neutrophils is neither necessary nor sufficient for their mobilization from the bone marrow by G-CSF, suggesting that G-CSF induces neutrophil mobilization indirectly through the generation of *trans*-acting signals. Evidence is provided suggesting that downregulation of stromal cell-derived factor 1 expression in the bone marrow may represent such a signal. A better understanding of the mechanisms that regulate neutrophil trafficking may lead to novel pharmacological strategies to modulate neutrophil responses in host defense and inflammation.

Experimental Procedures

Mice

G-CSFR-deficient, GEpoR, d715, and d715F mice were generated in our laboratory, as described previously (Liu et al., 1996; McLemore et al., 1998, 2001; Semerad et al., 1999). Wild-type, G-CSFR-deficient, GEpoR, d715 G-CSFR, and d715F G-CSFR mice were backcrossed ten generations onto a C57BL/6 background. Unless otherwise stated in the text, we used 6- to 10-week-old mice in all studies. Mice were housed in a specific pathogen-free environment. All experiments were approved by the Washington University Animal Studies Committee.

Peripheral Blood and Bone Marrow Analysis

Blood was obtained by retroorbital venous plexus sampling in polypropylene tubes containing EDTA. Complete blood counts were determined using a Hemavet automated cell counter (CDC Technologies, Oxford, CT). Bone marrow was harvested by flushing with Hanks Balanced Salts containing 0.1% low endotoxin bovine serum albumin (Sigma, St. Louis, MO). Manual leukocyte differentials were performed on Wright-stained blood smears (300 cell count) or cytospin preparations of bone marrow mononuclear cells (500 cell count). Neutrophil distribution was estimated by dividing the number of neutrophils in the blood by the number of neutrophils in the blood and bone marrow. Blood and bone marrow neutrophils were calculated assuming a blood volume of 1.8 ml and a whole femur equivalent to 6% of the total bone marrow (Chervenick et al., 1968). Bone marrow extracellular fluid was obtained by flushing each femur with 1 ml of ice-cold PBS without serum, and the supernatant was harvested after centrifugation at 400× g for 5 min.

Mobilization Protocols

Recombinant human G-CSF (Amgen, Thousand Oaks, CA) diluted in PBS with 0.1% low endotoxin bovine serum albumin (Sigma, St. Louis, MO) was administered by daily subcutaneous injection at a dose of 250 μg/kg per day for 7 days. Peripheral blood was obtained before the first and 4 hr after the final injection of G-CSF. In some experiments, mice were treated with a single subcutaneous injection of G-CSF (125 μg/kg), and peripheral blood was analyzed at the indicated times. Recombinant human IL-8, a generous gift from Searle Co. (now part of Pfizer Inc, New York, NY), was given by a single intraperitoneal injection, and peripheral blood was analyzed at the indicated times.

Brd-U Experiments

Brd-U (10 mg/ml solution in 1× Dulbecco's PBS, Sigma, St. Louis, MO) was given to age- and sex-matched wild-type and GEpoR mice by a single intraperitoneal injection at a dose of 240 mg/kg. Immediately following Brd-U injection, recombinant human G-CSF was given by subcutaneous injection at a dose of 250 μg/kg per day for 7 days. The total number of nucleated cells in the blood and bone marrow was quantified at the indicated time points, as described above. The percentage of Brd-U^+/Gr-1^+ cells was determined using the Brd-U Flow kit (PharMingen, San Diego, CA) and staining with phycoerythrin-conjugated (PE-conjugated) anti-mouse Ly-6G (Gr-1, PharMingen, San Diego, CA). Samples were analyzed using a FACScan flow cytometer and CellQuest version 1.2.2 software (Becton Dickinson, Mansfield, MA).

Bone Marrow Transplantation

Recipient mice were conditioned with 1000 cGy given in a single dose from a cesium 137 (^{137}Cs) source at a rate of approximately 95 cGy/min. Bone marrow cells were harvested using standard techniques, and the nonadherent (stromal cell-depleted) fraction was collected after culturing in tissue culture flasks for 4 hr in α-modified Eagle medium (α-MEM) containing 10% heat-inactivated fetal bovine serum, 1 mmol/L L-glutamine, and 10 μg/ml ciprofloxacin (Bayer, Kankakee, IL). 5×10^6 nonadherent bone marrow cells were injected into the tail vein of irradiated sex-matched recipient mice. To generate mixed chimeras, a total of 5×10^6 nonadherent bone marrow cells, containing the indicated ratio of G-CSFR mutant to wild-type cells, were injected into irradiated wild-type mice. Congenic wild-type C57BL/6 mice (B6.SJL-Ptprc* Pep3b BoyJ, Jackson Laboratory, Bar Harbor, ME) that have the *Ly 5.1* gene were used to facilitate analysis of the extent of chimerism. To generate the W > W chimeras, a cohort of wild-type mice (*Ly 5.1*) were reconstituted solely with wild-type (*Ly 5.2*) bone marrow cells. Prophylactic antibiotics (sulfamethoxazole/trimethoprim; Alpharma, Baltimore, MD) were given during the initial 2 weeks after transplantation.

Flow Cytometry

Nucleated cells from blood or bone marrow were incubated with fluorescein isothiocyanate-conjugated rat antimouse *Ly 5.2* (PharMingen) and phycoerythrin-conjugated anti-mouse Ly-6G (Gr-1) at 4°C for 30 min in phosphate-buffered saline (PBS) containing 0.1% sodium azide and 0.2% bovine serum albumin. The wild-type cells used in this study stained positive only for Ly 5.1 while G-CSFR-deficient and GEpoR cells stained positive only for Ly 5.2 (data not shown). All samples were analyzed using a FACScan flow cytometer and CellQuest version 1.2.2 software (Becton-Dickinson, Mansfield, MA).

SDF-1α ELISA

96-well plates were coated with 100 μl of SDF-1 capture antibody at 2 μg/ml, diluted in PBS, and incubated overnight at room temperature. After incubation overnight at 4°C with 300 μl of blocking solution (1% bovine serum albumin [BSA], 5% sucrose, and .05% NaN_3), 100 μl of sample diluted 1:3 (bone marrow) and 1:4 (plasma) in ELISA diluent (0.1% BSA, .05% Tween 20 in Tris-buffered saline at pH 7.3) was loaded and incubated for 2 hr at room temperature. After washing, 100 μl of polyclonal biotinylated anti-human SDF-1 at 250 ng/ml diluted in ELISA diluent was added to each well and incubated at room temperature for 2 hr. The reaction was developed by successive incubations with 1 μg/ml horseradish peroxidase streptavidin, substrate solution, and 50 μl of 2N H_2SO_4 to stop the reaction. A microplate reader set at 450 nm was used to determine optical density with readings at 550 nm subtracted from the results. Recombinant human SDF-1α was used to generate a standard curve. All ELISA reagents were purchased from R&D Systems (Minneapolis, MN).

Statistical Analysis

Statistical significance was assessed using a two-sided Student's t test.

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References

Aiuti, A., Webb, I.J., Bleul, C., Springer, T., and Gutierrez-Ramos, J.C. (1997). The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J. Exp. Med. *185*, 111–120.

Avalos, B.R., Hunter, M.G., Parker, J.M., Ceselski, S.K., Druker, B.J., Corey, S.J., and Mehta, V.B. (1995). Point mutations in the conserved box 1 region inactivate the human granulocyte colony-stimulating factor receptor for growth signal transduction and tyrosine phosphorylation of p75c-rel. Blood *85*, 3117–3126.

Basu, S., Hodgson, G., Zhang, H.H., Katz, M., Quilici, C., and Dunn, A.R. (2000). "Emergency" granulopoiesis in G-CSF-deficient mice in response to Candida albicans infection. Blood *95*, 3725–3733.

Bocchietto, E., Guglielmetti, A., Silvagno, F., Taraboletti, G., Pescarmona, G.P., Mantovani, A., and Bussolino, F. (1993). Proliferative and migratory responses of murine microvascular endothelial cells to granulocyte-colony-stimulating factor. J. Cell. Physiol. *155*, 89–95.

Brasel, K., McKenna, H.J., Morrissey, P.J., Charrier, K., Morris, A.E., Lee, C.C., Williams, D.E., and Lyman, S.D. (1996). Hematologic effects of flt3 ligand in vivo in mice. Blood *88*, 2004–2012.

Bruhl, H., Wagner, K., Kellner, H., Schattenkirchner, M., Schlondorff, D., and Mack, M. (2001). Surface expression of CC- and CXC-chemokine receptors on leucocyte subsets in inflammatory joint diseases. Clin. Exp. Immunol. *126*, 551–559.

Campbell, F.R. (1972). Ultrastructural studies of transmural migration of blood cells in the bone marrow of rats, mice and guinea pigs. Am. J. Anat. *135*, 521–535.

Chervenick, P.A., Boggs, D.R., Marsh, J.C., Cartwright, G.E., and

Wintrobe, M.M. (1968). Quantitative studies of blood and bone marrow neutrophils in normal mice. Am. J. Physiol. *215*, 353–360.

Cohen, A.M., Zsebo, K.M., Inoue, H., Hines, D., Boone, T.C., Chazin, V.R., Tsai, L., Ritch, T., and Souza, L.M. (1987). In vivo stimulation of granulopoiesis by recombinant human granulocyte colony-stimulating factor. Proc. Natl. Acad. Sci. USA *84*, 2484–2488.

Demetri, G.D., and Griffin, J.D. (1991). Granulocyte colony-stimulating factor and its receptor. Blood *78*, 2791–2808.

Dong, F., and Larner, A.C. (2000). Activation of Akt kinase by granulocyte colony-stimulating factor (G-CSF): evidence for the role of a tyrosine kinase activity distinct from the Janus kinases. Blood *95*, 1656–1662.

Dong, F., Qiu, Y., Yi, T., Touw, I.P., and Larner, A.C. (2001). The carboxyl terminus of the granulocyte colony-stimulating factor receptor, truncated in patients with severe congenital neutropenia/acute myeloid leukemia, is required for SH2-containing phosphatase-1 suppression of Stat activation. J. Immunol. *167*, 6447–6452.

Grzegorzewski, K., Komschlies, K.L., Mori, M., Kaneda, K., Usui, N., Faltynek, C.R., Keller, J.R., Ruscetti, F.W., and Wiltrout, R.H. (1994). Administration of recombinant human interleukin-7 to mice induces the exportation of myeloid progenitor cells from the bone marrow to peripheral sites. Blood *83*, 377–385.

Hattori, K., Heissig, B., Tashiro, K., Honjo, T., Tateno, M., Shieh, J.H., Hackett, N.R., Quitoriano, M.S., Crystal, R.G., Rafii, S., and Moore, M.A. (2001). Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. Blood *97*, 3354–3360.

Hermans, M.H., Antonissen, C., Ward, A.C., Mayen, A.E., Ploemacher, R.E., and Touw, I.P. (1999). Sustained receptor activation and hyperproliferation in response to granulocyte colony-stimulating factor (G-CSF) in mice with a severe congenital neutropenia/acute myeloid leukemia-derived mutation in the G-CSF receptor gene. J. Exp. Med. *189*, 683–692.

Hunter, M.G., and Avalos, B.R. (1999). Deletion of a critical internalization domain in the G-CSFR in acute myelogenous leukemia preceded by severe congenital neutropenia. Blood *93*, 440–446.

Iizuka, K., Kaneko, H., Yamada, T., Kimura, H., Kokai, Y., and Fujimoto, J. (1997). Host F1 mice pretreated with granulocyte colony-stimulating factor accept parental bone marrow grafts in hybrid resistance system. Blood *89*, 1446–1451.

Inoue, S., and Osmond, D.G. (2001). Basement membrane of mouse bone marrow sinusoids shows distinctive structure and proteoglycan composition: a high resolution ultrastructural study. Anat. Rec. *264*, 294–304.

Inukai, T., Sugita, K., Iijima, K., Tezuka, T., Goi, K., Kojika, S., Shiraishi, K., Kagami, K., and Nakazawa, S. (1995). Expression of granulocyte colony-stimulating factor receptor on CD10-positive human B-cell precursors. Br. J. Haematol. *89*, 623–626.

Jagels, M.A., and Hugli, T.E. (1992). Neutrophil chemotactic factors promote leukocytosis. A common mechanism for cellular recruitment from bone marrow. J. Immunol. *148*, 1119–1128.

Jagels, M.A., and Hugli, T.E. (1994). Mechanisms and mediators of neutrophilic leukocytosis. Immunopharmacology *28*, 1–18.

Kawabata, K., Ujikawa, M., Egawa, T., Kawamoto, H., Tachibana, K., Iizasa, H., Katsura, Y., Kishimoto, T., and Nagasawa, T. (1999). A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. Proc. Natl. Acad. Sci. USA *96*, 5663–5667.

Kawakami, M., Tsutsumi, H., Kumakawa, T., Abe, H., Hirai, M., Kurosawa, S., Mori, M., and Fukushima, M. (1990). Levels of serum granulocyte colony-stimulating factor in patients with infections. Blood *76*, 1962–1964.

Laterveer, L., Lindley, I.J., Hamilton, M.S., Willemze, R., and Fibbe, W.E. (1995). Interleukin-8 induces rapid mobilization of hematopoietic stem cells with radioprotective capacity and long-term myelolymphoid repopulating ability. Blood *85*, 2269–2275.

Lennon, J.E., and Micklem, H.S. (1986). Stromal cells in long-term murine bone marrow culture: FACS studies and origin of stromal cells in radiation chimeras. Exp. Hematol. *14*, 287–292.

Lieschke, G.J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., Fowler, K.J., Basu, S., Zhan, Y.F., and Dunn, A.R. (1994). Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood *84*, 1737–1746.

Liu, F., Wu, H.Y., Wesselschmidt, R., Kornaga, T., and Link, D.C. (1996). Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice. Immunity *5*, 491–501.

Liu, F., Poursine-Laurent, J., and Link, D.C. (1997). The granulocyte colony-stimulating factor receptor is required for the mobilization of murine hematopoietic progenitors into peripheral blood by cyclophosphamide or interleukin-8 but not flt-3 ligand. Blood *90*, 2522–2528.

Lord, B.I. (1992). Myeloid cell kinetics in response to haemopoietic growth factors. Baillieres Clin. Haematol. *5*, 533–550.

Lord, B.I., Bronchud, M.H., Owens, S., Chang, J., Howell, A., Souza, L., and Dexter, T.M. (1989). The kinetics of human granulopoiesis following treatment with granulocyte colony-stimulating factor in vivo. Proc. Natl. Acad. Sci. USA *86*, 9499–9503.

Lord, B.I., Molineux, G., Pojda, Z., Souza, L.M., Mermod, J.J., and Dexter, T.M. (1991). Myeloid cell kinetics in mice treated with recombinant interleukin-3, granulocyte colony-stimulating factor (CSF), or granulocyte-macrophage CSF in vivo. Blood *77*, 2154–2159.

Ma, Q., Jones, D., and Springer, T.A. (1999). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. Immunity *10*, 463–471.

McLemore, M.L., Poursine-Laurent, J., and Link, D.C. (1998). Increased granulocyte colony-stimulating factor responsiveness but normal resting granulopoiesis in mice carrying a targeted granulocyte colony-stimulating factor receptor mutation derived from a patient with severe congenital neutropenia. J. Clin. Invest. *102*, 483–492.

McLemore, M.L., Grewal, S., Liu, F., Archambault, A., Poursine-Laurent, J., Haug, J., and Link, D.C. (2001). STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation. Immunity *14*, 193–204.

Molineux, G., McCrea, C., Yan, X.Q., Kerzic, P., and McNiece, I. (1997). Flt-3 ligand synergizes with granulocyte colony-stimulating factor to increase neutrophil numbers and to mobilize peripheral blood stem cells with long-term repopulating potential. Blood *89*, 3998–4004.

Moore, M.A., Hattori, K., Heissig, B., Shieh, J.H., Dias, S., Crystal, R.G., and Rafii, S. (2001). Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1, Ann. NY Acad. Sci. *938*, 36–47.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature *382*, 635–638.

Nagase, H., Miyamasu, M., Yamaguchi, M., Imanishi, M., Tsuno, N.H., Matsushima, K., Yamamoto, K., Morita, Y., and Hirai, K. (2002). Cytokine-mediated regulation of CXCR4 expression in human neutrophils. J. Leukoc. Biol. *71*, 711–717.

Nicola, N.A., and Metcalf, D. (1985). Binding of 125I-labeled granulocyte colony-stimulating factor to normal murine hemopoietic cells. J. Cell. Physiol. *124*, 313–321.

Opdenakker, G., Fibbe, W.E., and Van Damme, J. (1998). The molecular basis of leukocytosis. Immunol. Today *19*, 182–189.

Petit, I., Szyper-Kravitz, M., Nagler, A., Lahav, M., Peled, A., Habler, L., Ponomaryov, T., Taichman, R.S., Arenzana-Seisdedos, F., Fujii, N., et al. (2002). G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. Nat. Immunol. *3*, 687–694.

Petrides, P.E., and Dittmann, K.H. (1990). How do normal and leukemic white blood cells egress from the bone marrow? Morphological facts and biochemical riddles. Blut *61*, 3–13.

Rausch, O., and Marshall, C.J. (1997). Tyrosine 763 of the murine granulocyte colony-stimulating factor receptor mediates Ras-dependent activation of the JNK/SAPK mitogen-activated protein kinase pathway. Mol. Cell. Biol. *17*, 1170–1179.

Salgia, R., Li, J.L., Ewaniuk, D.S., Pear, W., Pisick, E., Burky, S.A., Ernst, T., Sattler, M., Chen, L.B., and Griffin, J.D. (1997). BCR/ABL induces multiple abnormalities of cytoskeletal function. J. Clin. Invest. *100*, 46–57.

Semerad, C.L., Poursine-Laurent, J., Liu, F., and Link, D.C. (1999). A role for G-CSF receptor signaling in the regulation of hematopoietic cell function but not lineage commitment or differentiation. Immunity *11*, 153–161.

Simmons, P.J., Przepiorka, D., Thomas, E.D., and Torok-Storb, B. (1987). Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature *328*, 429–432.

Ulich, T.R., del Castillo, J., and Souza, L. (1988). Kinetics and mechanisms of recombinant human granulocyte-colony stimulating factor-induced neutrophilia. Am. J. Pathol. *133*, 630–638.