Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice

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Granulocyte colony-stimulating factor (G-CSF), the prototypical mobilizing cytokine, induces hematopoietic stem and progenitor cell (HSPC) mobilization from the bone marrow in a cell-nonautonomous fashion. This process is mediated, in part, through suppression of osteoblasts and disruption of CXCR4/CXCL12 signaling. The cellular targets of G-CSF that initiate the mobilization cascade have not been identified. We use mixed G-CSF receptor (G-CSFR)–deficient bone marrow chimeras to show that G-CSF–induced mobilization of HSPCs correlates poorly with the number of wild-type neutrophils. We generated transgenic mice in which expression of the G-CSFR is restricted to cells of the monocytic lineage. G-CSF–induced HSPC mobilization, osteoblast suppression, and inhibition of CXCL12 expression in the bone marrow of these transgenic mice are intact, demonstrating that G-CSFR signals in monocytic cells are sufficient to induce HSPC mobilization. Moreover, G-CSF treatment of wild-type mice is associated with marked loss of monocytic cells in the bone marrow. Finally, we show that bone marrow macrophages produce factors that support the growth and/or survival of osteoblasts in vitro. Together, these data suggest a model in which G-CSF signals in bone marrow monocytic cells inhibit the production of trophic factors required for osteoblast lineage cell maintenance, ultimately leading to HSPC mobilization.

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SUPPLEMENTAL MATERIAL

The Journal of Experimental Medicine

Published January 31, 2011

Supplemental Material can be found at:
/content/suppl/2011/01/31/jem.20101700.DC1.html

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decreased CXCL12 mRNA and protein expression in the bone marrow and decreased CXCR4 expression on HSPCs (Petit et al., 2002; Lévesque et al., 2003a; Semerad et al., 2005; Christopher et al., 2009). A potential mechanism for the decreased CXCL12 expression is provided by the observation that G-CSF treatment is associated with a marked suppression of osteoblasts (Semerad et al., 2005; Katayama et al., 2006; Christopher and Link, 2008; Christopher et al., 2009), which are an important source of CXCL12 in the bone marrow (Ponomaryov et al., 2000; Semerad et al., 2005; Jung et al., 2006; Christopher et al., 2009). The importance of the CXCL12–CXCR4 axis is illustrated by the success of the CXCR4 inhibitor plerixafor (AMD3100) to rapidly mobilize HSPCs in humans and mice (Liles et al., 2003; Broxmeyer et al., 2007).

Through the study of G-CSF receptor (G-CSFR; \(Csf3r^{-/-}\)) bone marrow chimeras, we previously showed that G-CSFR signaling in hematopoietic cells, but not stromal cells, is required for HSPC mobilization by G-CSF (Liu et al., 2000). Within the hematopoietic compartment, the G-CSFR is expressed on neutrophils, monocytes/macrophages, HSPCs, and a subset of B lymphocytes and NK cells. The simplest model suggests that G-CSF directly acts upon HSPCs to induce their mobilization. However, strongly arguing against this model, in mixed bone marrow chimeras containing wild-type and \(Csf3r^{-/-}\) HSPCs, both types of cells were mobilized equally after G-CSF treatment (Liu et al., 2000).

There is some evidence implicating neutrophils in G-CSF–induced HSPC mobilization. During G-CSF treatment, neutrophils release certain proteases into the bone marrow microenvironment (Lévesque et al., 2001, 2002, 2003a,b; Heissig et al., 2002; Petit et al., 2002; Christopherson et al., 2003). Although controversial, these proteases, through cleavage of CXCL12 (Christopherson et al., 2003; Lévesque et al., 2003a), c-kit (Lévesque et al., 2003b), and VCAM-1 (Lévesque et al., 2001), may contribute to HSPC mobilization. A previous study reported that depletion of neutrophils using an antibody against Gr-1 (Ly6C/G) results in reduced HSPC mobilization by G-CSF (Pelus et al., 2004). However, Gr-1 is expressed on both neutrophils and a subset of monocytes; thus, a role for reduced monocytes in this phenotype is possible. In this study, we used a genetic approach to systematically examine the contribution of neutrophils and other hematopoietic cell populations to G-CSF–induced HSPC mobilization. We show that G-CSFR expression on mononuclear cells in the bone marrow is sufficient to initiate HSPC mobilization by G-CSF.

RESULTS

The mobilization response to G-CSF correlates poorly with the number of wild-type neutrophils in the bone marrow

To evaluate the importance of neutrophils to G-CSF-induced mobilization, we generated a series of bone marrow chimeras by transplanting wild-type and \(Csf3r^{-/-}\) bone marrow cells in different ratios into lethally irradiated wild-type mice. Donor chimerism was assessed in peripheral blood leukocytes 6 wk after transplantation. As reported previously (Richards et al., 2003), the contribution of \(Csf3r^{-/-}\) cells to the B cell lineage was near that expected based on the input ratio (Fig. S1). However, because of the marked competitive advantage of \(Csf3r^{+/+}\) cells to the neutrophil lineage, virtually all of the neutrophils in the circulation and bone marrow were derived from \(Csf3r^{+/+}\) cells (Figs. 1, D and E; and not depicted). Moreover, the number of neutrophils in the blood was similar in all of the chimeras (Fig. S2).

We next assessed the mobilization response to G-CSF in these chimeras by quantifying CFUs (CFU-C) in the peripheral blood, bone marrow, and spleen. We predicted that an inverse correlation between \(Csf3r^{-/-}\) donor input and HSPC mobilization would be observed. Indeed, the number of CFU-Cs in blood and spleen after treatment with G-CSF was highest in the 3-to-1 (wild-type-to-\(Csf3r^{-/-}\)) chimeras and progressively decreased with increasing \(Csf3r^{-/-}\) donor input (Fig. 1, A–C). Analysis of donor chimerism in mature neutrophils (Gr-1\(^{+}\) SSC\(^{hi}\)) in the bone marrow after G-CSF revealed that the great majority of neutrophils were derived...
from wild-type (Csf3r+/-) cells in all of the chimeras (Fig. 1 D).
In fact, the correlation between the number of wild-type neutrophils in the bone marrow and CFU-C in the blood was poor (Fig. 1, E and F).

G-CSF–induced HSPC mobilization is normal in lymphocyte-deficient mice

The contribution of B and T lymphocytes to G-CSF–induced HSPC mobilization is controversial. Reca et al. (2007) reported that G-CSF–induced mobilization is impaired in Rag2-/-, SCID, and Jh mice. In contrast, Katayama et al. (2006) reported that Rag1-/- mice and IL-7R-/- mice exhibited a normal mobilization response to G-CSF. In this paper, we characterized HSPC mobilization by G-CSF in two lymphocyte-deficient mouse strains, Rag1-/- and NOD/scid/IL-2-/- null mice. Rag1-/- mice lack mature B and T cells, and NOD/scid/IL-2-/- null mice lack all B, T, and NK cells. Consistent with a previous paper (Katayama et al., 2006), HSPC mobilization in Rag1-/- mice was comparable to that in wild-type mice (Fig. 2 A). In NOD/scid/IL-2-/- null mice, the baseline level of circulating HSPC was increased and marked mobilization in response to G-CSF was observed (Fig. 2 B). These data demonstrate that B, T, and NK cells are not required for G-CSF–induced mobilization.

Generation of transgenic mice with monocyte-restricted expression of the G-CSFR

To evaluate the role of monocytes in G-CSF–induced mobilization, we generated transgenic mice in which the CD68 (macrosialin) promoter drives expression of the G-CSFR (Fig. 3 A). A previous study established that regulatory elements of the CD68 gene are sufficient to direct transgene expression to the monocyte/macrophage lineage (Lang et al., 2002). Two transgenic founder lines were identified that expressed the GFP in circulating monocytes (unpublished data). The transgenic founder mice were crossed with Csf3r-/- mice to obtain CD68:mGCSFR;Csf3r-/- mice, hereafter referred to as CD68:G-CSFR mice. Because the phenotype of CD68 transgenic mice derived from the two founder lines was similar, the data have been pooled. It is of note that the

CD68:G-CSFR mice were generated from B6CDF1 donor oocytes and then backcrossed four generations onto a C57BL/6 background. To minimize the influence of strain effects on phenotype, littermate controls were used in all studies.

Figure 2. G-CSF–induced HSPC mobilization is normal in Rag1-/- and NOD/scid/IL-2-/- null mice. Wild-type, Rag1-/- (A), or NOD/scid/IL-2-/- null mice (B) were treated with G-CSF (250 µg/kg/d for 5 d) or left untreated. The number of CFU-C in the blood, bone marrow, and spleen was measured after 5 d of G-CSF. Data represent the mean ± SEM of four to five mice per genotype per treatment group and is pooled from four independent experiments. *, P < 0.01; **, P < 0.001.

Figure 3. G-CSFR expression is mainly restricted to monocyctic cells in CD68:G-CSFR mice. (A) Schematic of the CD68:G-CSFR transgene. IRES, internal ribosomal entry sequence. (B) Representative histograms showing biotinylated G-CSF binding in the absence (solid line) or presence (dashed line) of a 100-fold molar excess of unlabeled G-CSF in the indicated blood leukocyte population. G-CSFR surface expression is proportional to the difference in median fluorescence intensity (ΔMFI) between the two curves. (C) The mean ΔMFI in the indicated blood leukocyte population is shown. Data represent the mean ± SEM of four to five mice per genotype and is pooled from four independent experiments.
G-CSFR expression in CD68:G-CSFR mice is predicted to be restricted to cells of the monocyte lineage. To test this prediction, we measured surface expression of the G-CSFR on blood leukocytes using a biotinylated G-CSF binding assay (Liu et al., 1996). Consistent with previous results, in wild-type and Csf3r+/− mice, expression of the G-CSFR was highest in neutrophils, detectable but lower in monocytes, and undetectable in B or T lymphocytes (Fig. 3, B and C; and not depicted). As expected, G-CSFR expression was not detected on any blood leukocytes in Csf3r−/− mice. In CD68:G-CSFR mice, the highest expression of the G-CSFR was detected on blood monocytes with levels similar to that seen in wild-type or Csf3r+/− mice. G-CSFR expression was barely detectable on neutrophils, and no expression was detected on B or T lymphocytes. Similar data were observed using bone marrow cells (unpublished data). These data suggest that hematopoietic expression of the G-CSFR in CD68:G-CSFR mice is mainly limited to monocyctic cells.

**CD68:G-CSFR mice are neutropenic**

G-CSFR signals on granulocytic precursors are required for normal cell proliferation and differentiation (Liu et al., 1996).
We have previously shown that G-CSF–induced HSPC mobilization is associated with osteoblast suppression and decreased CXCL12 expression in the bone marrow (Christopher et al., 2009). We assessed osteoblast suppression by measuring mRNA expression of osteocalcin, a specific marker of mature osteoblasts (Semerad et al., 2005). As reported previously (Semerad et al., 2005), G-CSF treatment resulted in a significant decrease in osteocalcin and CXCL12 mRNA expression in the bone marrow of control (Csf3r\textsuperscript{+/−}) mice (Figs. 4, C and D).

A similar decrease was observed in CD68:G-CSFR but not Csf3r\textsuperscript{−/−} mice after G-CSF administration. Together, these data suggest that G-CSFR expression in monocyte lineage cells is sufficient to induce HSPC mobilization and osteoblast suppression.

G-CSF treatment of wild-type mice is associated with a loss of monocytic cells in the bone marrow

We have previously shown that G-CSF–induced HSPC mobilization is associated with osteoblast suppression and decreased CXCL12 expression in the bone marrow (Christopher et al., 2009). We assessed osteoblast suppression by measuring mRNA expression of osteocalcin, a specific marker of mature osteoblasts (Semerad et al., 2005). As reported previously (Semerad et al., 2005), G-CSF treatment resulted in a significant decrease in osteocalcin and CXCL12 mRNA expression in the bone marrow of control (Csf3r\textsuperscript{−/−}) mice (Figs. 4, C and D). A similar decrease was observed in CD68:G-CSFR but not Csf3r\textsuperscript{−/−} mice after G-CSF administration. Together, these data suggest that G-CSF expression in monocyte lineage cells is sufficient to induce HSPC mobilization and osteoblast suppression.

Figure 5. G-CSF treatment leads to a loss of monocytic cells from the bone marrow. CX3CR1\textsuperscript{GERF1} mice were treated with PBS or G-CSF for 5–7 d and the flush and bone fractions harvested as described in Materials and methods. (A) Representative dot plot showing the gating strategy used to identify inflammatory (Gr-1\textsuperscript{high} GFP\textsuperscript{+}) and resident monocytes/macrophages (Gr-1\textsuperscript{low} GFP\textsuperscript{+}). (B) The absolute number of inflammatory and resident monocytes/macrophages in the flushed and bone fractions are shown. Data represent the mean ± SEM of four mice per cohort pooled from two independent experiments. *, P < 0.05, **, P < 0.005, ***, P < 0.001. (C) The percentage of inflammatory and resident monocytes in the flush fraction of bone marrow was determined at the indicated time points. In these experiments, wild-type mice rather than CX3CR1\textsuperscript{GERF1} mice were used, and CD115 was used to identify mononuclear cells. Data represent mean ± SEM of two to six mice per cohort pooled from four independent experiments. **, P < 0.005 compared with untreated mice as determined by one-way ANOVA with Dunnett’s post-test to correct for multiple comparisons.
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Figure 6. Macrophages support the growth of mature osteoblasts in vitro. Unfractionated bone marrow cells from wild-type mice were cultured for 10 d and then sorted by flow cytometry into stromal (CD45− Ter119−) and macrophage (CD45+ CD115+) cell populations. Stromal cells were cultured for an additional 14 d in the absence (No Mø) or presence (Mø) of an equal number of macrophages. Ascorbic acid and calcium were included in all cultures to stimulate mature osteoblast development. (A) Representative photomicrographs of cultures on day 14 that were stained for alkaline phosphatase. (B) Osteocalcin protein concentration in conditioned media was measured by ELISA at the indicated time points. (C and D) CXCL12 protein (C) and Osteocalcin (D) concentrations in conditioned media were measured by ELISA on day 14 of culture. Where indicated, the macrophages were separated from the stromal cells by a semipermeable membrane (transwell). Data represent the mean ± SEM of three independent experiments. *, P < 0.05; **, P < 0.005; ***, P < 0.001.

with G-CSF for 5 d and the number of inflammatory and resident monocytes/macrophages in the bone marrow determined by flow cytometry. A recent study suggested that collagenase treatment of murine long bones after flushing of bone marrow yields a cell population enriched for stromal cells and stromal macrophages (Crocker and Gordon, 1985; Morikawa et al., 2009). Thus, in addition to our standard bone marrow harvesting, we also analyzed cells in the bone fraction obtained after collagenase treatment of flushed femurs. In each fraction, the number of inflammatory monocytes/macrophages was significantly reduced (Fig. 5 B). A smaller decrease in resident monocytes/macrophages in the flushed, but not bone, fractions was also observed. We next examined the kinetics of monocyte/macrophage loss during G-CSF–induced mobilization (Fig. 5 C). Because CX3CR1GFP/+ mice were in limited supply, we performed these experiments using wild-type mice, using CD115 to identify monocyctic cells. Importantly, the decrease in the number of inflammatory and resident monocytes/macrophages in the bone marrow was maximal by 3 d of G-CSF treatment, a time point at which osteoblast suppression first becomes evident (Christopher and Link, 2008).

Macrophages support the growth of osteoblasts in vitro

To further evaluate the role of monocytes/macrophages in osteoblast growth and survival, we cultured bone marrow stromal cells in the presence or absence of bone marrow macrophages. Stromal cells cultured in the presence of macrophages generated many more alkaline phosphatase–positive (osteoblast lineage) cells than those cultured in the absence of macrophages (Fig. 6 A). Moreover, secretion of osteocalcin and CXCL12 into the conditioned media was significantly increased in cultures containing macrophages (Fig. 6, B and C). It is of note that no alkaline phosphatase colonies or osteocalcin or CXCL12 protein secretion were detected in cultures of macrophages alone (unpublished data). These data are consistent with a paper by Chang et al. (2008) showing that macrophages induced osteoblast mineralization in vitro. To determine whether macrophages produce a soluble factor that stimulates osteoblast development, we repeated these experiments, this time including transwell cultures in which the macrophages were separated from the stromal cells by a semipermeable membrane (transwell). Mature osteoblast development, as measured by osteocalcin protein secretion into conditioned media, was partially induced in the transwell cultures. Collectively, these data suggest that bone marrow macrophages produce factors, at least some of which are secreted, that support the growth and/or survival of osteoblasts.

DISCUSSION

The contribution of neutrophils to HSPC mobilization by G-CSF is controversial. Neutrophils are a major source of proteases that have been implicated in HSPC mobilization, including neutrophil elastase, cathepsin G, and MMP-9 (Kjeldsen et al., 1994; Borregaard and Cowland, 1997). However, the importance of these proteases is controversial, as mice lacking these proteases exhibit a normal mobilization response to G-CSF (Robinson et al., 2003; Levesque et al., 2004; Pelus et al., 2004). There is strong evidence showing that HSPC mobilization by the chemokine IL-8 is dependent...
on neutrophils. Gsf3r<sup>-/-</sup> mice, which are profoundly neutropenic, fail to mobilize in response to IL-8 (Liu et al., 1997). Moreover, antibody-mediated depletion of neutrophils (using anti-CD11a or anti-Gr-1 antibodies) abrogated IL-8–induced HSPC mobilization (Pruitt et al., 1998, 2002). With respect to G-CSF, Pelus et al. (2004) reported that neutrophil depletion using anti–Gr-1 antibodies attenuated G-CSF–induced HSPC mobilization. However, Gr-1 is expressed on both neutrophils and a subset of monocytes; thus, a role for reduced monocytes in this phenotype is possible. In the current study, we demonstrate, using G-CSFR–deficient bone marrow chimeras, that the mobilization response to G-CSF is poorly correlated with the number of wild-type neutrophils. Moreover, CD68:G-CSFR mice, which are neutropenic and have barely detectable expression of G-CSFR on neutrophils, exhibit a normal mobilization response to G-CSF. These data, although not excluding a role for neutrophils, strongly suggest that G-CSF signals in neutrophils are not sufficient to induce normal HSPC mobilization.

The contribution of lymphocytes to G-CSF–induced HSPC mobilization is also controversial. Reca et al. (2007) reported that G-CSF–induced mobilization is impaired in Rag<sup>2<sup>-/-</sup>, SCID, and Jh mice and that this defect can be reversed through administration of complement-inducing immunoglobulin. In contrast, Katayama et al. (2006) reported that Rag<sup>1<sup>-/-</sup> mice and IL-7R<sup>-/-</sup> mice exhibited a normal mobilization response to G-CSF. Consistent with the latter findings, we observed that Rag<sup>1<sup>-/-</sup> and NOD/scid/IL-2γ<sub>null</sub> mice exhibit a normal mobilization response to G-CSF. The basis for these discrepancies remains unclear. Although all of these mouse lines share deficits in B and T lymphopoiesis, there are subtle differences. For example, natural killer activity is normal in Rag<sup>1<sup>-/-</sup> and Rag<sup>2<sup>-/-</sup> mice but is absent in NOD/scid/IL-2γ<sub>null</sub> mice. In any case, our data strongly suggest that neither B and T lymphocytes nor NK cells are required for a normal mobilization response to G-CSF.

There is accumulating evidence that monocyte lineage cells in the bone marrow contribute to osteoblast homeostasis and HSPC trafficking. Chang et al. (2008) demonstrated that macrophages are anatomically juxtaposed with endosteal osteoblasts, forming a canopy over the osteoblasts at sites of bone formation. Moreover, they showed that ablation of monocytes using the MAFIA transgenic mouse model resulted in a loss of osteoblasts (Chang et al., 2008). Similarly, Winkler et al. (2010) recently showed that macrophage ablation using the MAFIA transgenic mouse model or through administration of clodronate-loaded liposomes resulted in a loss of osteoblasts and HSPC mobilization. Finally, Chow et al. demonstrated in a companion paper in this issue that depletion of mononuclear lineage cells using a variety of methods is sufficient to induce mobilization of HSCPs. Together, these data strongly suggest that mononuclear cells produce trophic factors required for osteoblast maintenance and HSPC retention. Consistent with this conclusion, we show that macrophages support osteoblast growth in vitro, at least in part, through production of a soluble factor. The identity of these factors is currently unknown.

In this study, we provide novel evidence that G-CSFR signaling in monocytic cells is sufficient to induce HSPC mobilization. We generated transgenic mice in which expression of the G-CSFR is mainly limited to cells of the monocyte lineage and showed that G-CSF–induced HSPC mobilization, osteoblast suppression, and decrease in CXCL12 expression are similar to that control mice. In the bone marrow, there are at least four distinct monocytic cell populations: inflammatory monocytes/macrophages, resident monocytes/macrophages, myeloid dendritic cells, and osteoclasts. Because the CD68 transgene used in our study is expected to direct G-CSFR expression in each of these cell populations, all of them are candidates to mediate HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization.

In summary, we provide evidence that monocytic cells in the bone marrow are sufficient to elicit HSPC mobilization and osteoblast suppression by G-CSF. These data suggest a model in which monocyte lineage cells in the bone marrow produce trophic factors required for the maintenance of osteoblasts. G-CSF–induced suppression of monocytes and/or signaling in these cells results in decreased production of the putative trophic factors, suppression of osteoblast lineage cells (and CXCL12 expression), and ultimately HSPC mobilization. The precise monocytic cell population and factors produced by these cells that regulate osteoblast lineage cells are areas of active investigation.

**MATERIALS AND METHODS**

**Mice.** Sex- and age-matched wild-type, Gsf3r<sup>-/-</sup>, and CX3CR<sup>1GFP</sup> mice on a C57BL/6 background were maintained under SPF conditions according to methods approved by the Washington University Animal Studies Committee (Saint Louis, MO). The Gsf3r<sup>-/-</sup> mice have been previously described (Liu et al., 1996). The CX3CR<sup>1GFP</sup> mice were a gift from D. Littman (Skirball Institute of Biomolecular Medicine, New York School of Medicine, New York, NY).
Generation of mixed chimeras. Wild-type (Ly5.1) or Csf3r-/- (Ly5.2) bone marrow cells were harvested. A total of two million bone marrow cells were mixed at a 3:1, 1:1, 1:3, or 1:9 ratio and injected retroorbitally into lethally irradiated wild-type mice (Ly5.1). Recipient mice were conditioned with 1,000 cGy from a 137Cs source at a rate of ~95 cGy/min before transplantation. Prophylactic antibiotics (trimethoprim-sulfamethoxazole; Alpharma) were given during the initial 2 wk after transplantation. Mice were analyzed 8–10 wk after transplantation.

Generation of CD68/G-CSFR mouse. To express G-CSF receptor under the control of the CD68 promoter, we made use of a variant of the CD68 promoter construct described by Lang et al. (2002), in which the 728 bp of sequence 5’ to the ATG and the 83-bp first intron of the human CD68 gene were subcloned into the pUR19 backbone. A fragment containing bases 93–2694 of the murine G-CSF receptor cDNA, which includes the entire coding region of the murine G-CSF receptor, was subcloned into this backbone 3’ of the first CD68 intron. An IRES-GFP cassette (derived from pIRE2-eGFP; Takara Bio Inc.) was subcloned into the backbone 3’ of the G-CSF receptor fragment. For injection into male nuclei of B6.CDF1 zygotes, the construct was digested with Drai and purified according to standard procedures. Offspring derived from the injection were screened by PCR for GFP. A sample of peripheral blood was taken from those mice positive for GFP by PCR and analyzed for the presence of GFP+ neutrophils and monocytes. Transgene-bearing founder mice were mated with C57BL/6 mice with germine deletion of G-CSF receptor (GFRαc–/–). Two founder lines produced viable offspring. Initial characterization of these mice revealed similar mobilization responses to G-CSF; so the data from the two founder lines were pooled and are collectively referred to as CD68/G-CSF mice.

Macrophage/osteoblast co-culture. Bone marrow cells were recovered from the femurs of wild-type mice by flushing with PBS. The femurs were then infused with PBS containing 50 mg/ml of type II collagenase (Worthington Biochemical) and incubated at 37°C for 30 min. The collagenase-treated femurs were flushed again with PBS. The cells were pooled (containing both hematopoietic and stromal cells) and cultured for 7 d in base media (α-MEM with 10% fetal calf serum). On day 7 of culture, the cells were trypsinized and stained with PE-conjugated anti–mouse CD11b and FITC-conjugated anti–mouse CD45. CD45+ CD115+ (macrophages) and CD45+ stromal cells were sorted separately using a MoFlo high-speed cell sorter (Dako). CD45+ cells were plated at 10,000 cells per well in a 24-well plate in the presence or absence of 10 ng/ml of recombinant murine MCP-1 (StemCell Technologies). In some cases, the bone fraction of the bone marrow was analyzed by flow cytometry. In brief, bone marrow was extracted from femurs either by flushing with PBS or by centrifugation at 3,000 g for 1 min at room temperature. The pellet was set aside for analysis as the flushed fraction. The flushed femurs were then pulverized with a mortar and pestle, and the bone chips were incubated with PBS containing 2 mg/ml of type II collagenase (Worthington Biochemical) at 37°C for 60 min with agitation. After allowing the bone chips to settle by gravity, the cell suspension was harvested and analyzed.

G-CSF mobilization protocol. Recombinant human G-CSF (Amgen) was diluted in phosphate-PBS with 0.1% low endotoxin BSA (Sigma-Aldrich) and administered at a dose of 250 µg/kg/d either by daily subcutaneous injection for 5 d or by continuous infusion via subcutaneous osmotic pump for 7 d. Mice were analyzed 3–4 h after the final cytokine dose.

CFU-C assays. Bone marrow, and spleen cells were harvested from mice using standard techniques, and the number of nucleated cells in these tissues was quantified using a Hemavet (Drew Scientific) automated cell counter. We plated 10 µl of blood, 5 × 10^4 nucleated spleen cells, or 2 × 10^4 nucleated bone marrow cells in 2.5 ml methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; STEMCELL Technologies Inc.). Cultures were plated in duplicate and placed in a humidified chamber with 5% CO2 at 37°C. After 7 d of culture, the number of colonies per dish was counted. The frequency of colonies per femur or spleen was determined based on the number of cells present in the cell suspension immediately after harvest.

Real-time quantitative RT-PCR. Femurs were flushed with a total of 0.75 ml TRizol reagent (Invitrogen). RNA was isolated according to the manufacturer’s instructions and resuspended in 150 µl RNase/DNase-free water. Real-time RT-PCR was performed using the TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems) on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). The reaction mix consisted of 5 µl RNA, 12.5 µl RT-PCR mix, 200 nM of forward primer, 200 nM of reverse primer, 280 nM of internal probe, and 0.625 µl Multiscribe reverse transcription and RNAse inhibitor in a total reaction volume of 25 µl. Reactions were repeated in the absence of reverse transcription to confirm that DNA contamination was not present. RNA content was normalized to murine β-actin. PCR conditions were 48°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers were: CXCL12 forward, 5′-GAGCCAAGCTCAAGTCTC-3′; CXCL12 reverse, 5′-CGGGCTAATGCACATTGTGC-3′; CXCL12 dT-β-actin forward, 5′-ACCAACTTGGAAGCATATGGAGAA-3′; β-actin primer, 5′-TAGCACCAGGACGATACGGGACAA-3′; β-actin dT-β-actin forward probe, 5′-AGCCATGTACGTCGTACCCCTGGCC-3′; osteocalcin forward, 5′-TCTCTGTCATTGCTGCCC-3′; osteocalcin reverse, 5′-TTTGTCAGACTCGGGGCCC-3′; and osteocalcin dT-β-actin probe, 5′-TGCCGTCCTGTCTCCTGCATGGATGCGA-3′. 

Flow cytometry. Red blood cells in peripheral blood and bone marrow mononuclear cell preparations were lysed in Tris-buffered ammonium chloride buffer, pH 7.2, and incubated with the indicated antibody at 4°C for 1 h in PBS containing 0.1% sodium azide, 1 mM EDTA, and 0.2% (wt/vol) BSA to block nonspecific binding. The following directly conjugated monoclonal antibodies were used: allophycocyanin–eFlour 780-conjugated rat anti–mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); allophycocyanin-conjugated rat anti–mouse CD115 (AF589, IgG2a; eBioscience); FITC-conjugated rat anti–mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); allophycocyanin-conjugated rat anti–mouse CD3e (17A2, IgG2b; eBioscience); and allophycocyanin–eFlour 780-conjugated anti–mouse B220 (RA3-6B2, IgG2b; eBioscience). For KLS analysis, we used the following antibodies: PE-conjugated rat anti–mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); PE-conjugated rat ant–mouse B220 (RA3-6B2, IgG2a; eBioscience); PE-conjugated rat ant–mouse B220 (RA3-6B2, IgG2a; eBioscience); and PE-conjugated rat anti–mouse Ter-119 (TER-119, IgG2b; eBioscience). For KLS analysis, we used the following antibodies: PE-conjugated rat anti–mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); PE-conjugated rat ant–mouse B220 (RA3-6B2, IgG2a; eBioscience); and PE-conjugated rat anti–mouse B220 (RA3-6B2, IgG2a; eBioscience). To assess surface G-CSF expression, G-CSF was biotinylated using NHS-LC-biotin (Thermo Fisher Scientific) as previously described (Shimoda et al., 1992). Peripheral blood mononuclear cells were incubated at 4°C for 1 h with biotinylated G-CSF (25 ng/10^6 cells) in the presence or absence of a 100-fold molar excess of nonlabeled G-CSF followed by incubation with PE-conjugated streptavidin. All cells were analyzed on a FACSscan flow cytometer.

In some cases, the bone fraction of the bone marrow was analyzed by flow cytometry. In brief, bone marrow was extracted from femurs either by flushing with PBS or by centrifugation at 3,300 g for 1 min at room temperature. The pellet was set aside for analysis as the flushed fraction. The flushed femurs were then pulverized with a mortar and pestle, and the bone chips were incubated with PBS containing 2 mg/ml of type II collagenase (Worthington Biochemical) at 37°C for 60 min with agitation. After allowing the bone chips to settle by gravity, the cell suspension was harvested and analyzed.

CXCL12 and osteocalcin ELISAs. For ELISAs, 96-well plates were coated with 100 µl CXCL12 capture antibody (2 µg/ml) diluted in PBS and incubated overnight at room temperature. After incubation for 1 h at room temperature with 300 µl of blocking solution (1% BSA, 5% sucrose, and 0.05% NaN3), a 100-µl sample was added to each well and incubated for 2 h at room temperature. After washing, 100 µl polyclonal biotinylated anti–human CXCL12 (250 ng/ml) in ELISA diluent (0.1% BSA, 0.05% Tween 20 in Tris [tris(hydroxymethyl)aminomethane]–buffered saline, pH 7.3) was added to each well and incubated at room temperature for 2 h. The reaction was developed by successive incubations with 1 µg/ml horse-radish peroxidase streptavidin, substrate solution, and 50 µl 2N H2SO4 to stop.
the reaction. A microplate reader set at 450 nm was used to determine optical density with readings at 570 nm subtracted from the results. Recombinant human CXCL12α was used to generate a standard curve. CXCL12 ELISA reagents were purchased from R&D Systems. Osteocalcin ELISA was performed using a kit according to the manufacturer's instructions (Human Osteocalcin Instant ELISA; E Bioscience).

Statistics. Significance was determined using Prism software (GraphPad Software, Inc.). Statistical significance of differences was calculated using two-tailed Student's t tests (assuming equal variance) or, where indicated, one- or two-way ANOVA with Bonferroni's or Dunnelt's post testing. P-values <0.05 were considered significant. All data are presented as mean ± SEM except as noted.

Online supplemental material. Fig. S1 shows that the percentage of CsCr−/− B cells in the mixed chimeras are as predicted from the input ratios of wild-type to CsCr−/− cells. Fig. S2 shows the absolute numbers of circulating neutrophils in the mixed chimeric mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101700/DC1.

We thank Aanger for their generous gift of G-CSF.

This work was supported by a grant from the National Institutes of Health: R01 HL07072 (D.C. Link).

The authors have no conflicting financial interests.

Submitted: 16 August 2010
Accepted: 6 January 2011

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SUPPLEMENTAL MATERIAL

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Figure S1. B lymphocyte chimerism in mixed Csf3r<sup>−/−</sup> chimeras. Mixed bone marrow chimeras were established by transplanting the indicated ratio of wild-type to Csf3r<sup>−/−</sup> bone marrow cells into lethally irradiated recipients. Donor chimerism of peripheral blood B lymphocytes was assessed by flow cytometry 5–6 wk after transplantation. Data represent the mean ± SEM of six to nine mice and is pooled from two independent experiments.

Figure S2. Absolute number of neutrophils in mixed Csf3r<sup>−/−</sup> chimeras. Shown is the absolute number of neutrophils in the peripheral blood before G-CSF treatment. Greater than 95% of all neutrophils were derived from wild-type cells in each case (not depicted). Data represent the mean ± SEM of six to nine mice and is pooled from two independent experiments.