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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 used 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 can be found at:
http://jem.rupress.org/content/suppl/2011/01/31/jem.20101700.DC1.html
decreased CXCL12 mRNA and protein expression in the bone marrow and decreased CXCR4 expression on HSPCs (Pettit 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; Christopher 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 monocytes 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 (Fig. 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-1hi SSChi) 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-2γnull mice.

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-2γnull 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 influence of strain effects on phenotype, littermate controls were used in all studies.
G-CSFR expression in CD68:G-CSFR mice is predicted to be restricted to cells of the monocytic 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 cells (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 monocytic 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+/−) mice (Figs. 4, C and D). A similar decrease was observed in CD68:G-CSFR but not Csf3r−/− 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+/−) mice (Figs. 4, C and D). A similar decrease was observed in CD68:G-CSFR but not Csf3r−/− 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–induced HSPC mobilization is normal in CD68:G-CSFR mice

We next evaluated the mobilization response of these mice to G-CSF. Specifically, we measured the number of c-kit+ lineage−Sca-1+ (KLS) cells and CFU-C in the blood, bone marrow, and spleen after 7 d of G-CSF. Consistent with previous results (Liu et al., 1996), in Csf3r−/− mice, G-CSF administration resulted in a significant increase in KLS cells and CFU-Cs in the blood marrow (Table I). Interestingly, CD68:G-CSFR mice had a modest but significant increase in circulating B lymphocytes. A nonsignificant trend to increased B lymphocytes also was seen in the Csf3r−/− mice (Liu et al., 1996). All other measured hematopoietic parameters were normal in the CD68:G-CSFR mice.

Figure 5. G-CSF treatment leads to a loss of monocytic cells from the bone marrow. CX3CR1GFP/+ 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-1high GFP+) and resident monocytes/macrophages (Gr-1low GFP+). (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 CX3CR1GFP/+ 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.
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 monocytic 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. Csf3r−/− 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 (Prujit 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 Rag2−/−, SCID, and Jh mice and that this deficit can be reversed through administration of complement-inducing immunoglobulin. In contrast, Katayama et al. (2006) reported that Rag1−/− mice and IL-7R−/− mice exhibited a normal mobilization response to G-CSF. Consistent with the latter findings, we observed that Rag1−/− and NOD/scid/IL-2γnull 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 Rag1−/− mice but is absent in NOD/scid/IL-2γnull 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 monocytic cells 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 monocyctic lineage cells using a variety of methods is sufficient to induce mobilization of HSPCs. Together, these data strongly suggest that monocyctic 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. Kollet et al. (2006) reported that activation of osteoclasts by injection of RANKL (RANK ligand) was associated with moderate HSPC mobilization, and inhibition of osteoclasts, either genetically by knocking out PTPe or by injecting mice with calcitonin, blunts the mobilization response to G-CSF. It is of note that osteoclasts produce the protease cathepsin K, which can cleave CXCL12 in vitro (Drake et al., 1996; Kollet et al., 2006). In contrast, other studies indicate that osteoclasts may actually inhibit mobilization, as mice that were given pamidronate, an osteoclast-inhibiting bisphosphonate, exhibit increased mobilization in response to G-CSF (Takamatsu et al., 1998; Winkler et al., 2010). In contrast to osteoclasts (Takamatsu et al., 1998; Winkler et al., 2010), we show that inflammatory and resident monocytes/macrophages decrease after G-CSF treatment. It is of note that the timing of the decrease in these cell populations during G-CSF treatment is similar to that reported for the decrease in osteoclasts (Christopher and Link, 2008) and precedes HSPC mobilization. Definitive identification of the monocytic cell population that mediates G-CSF–induced HSPC mobilization will require further study.

In summary, we provide evidence that monocyctic cells in the bone marrow are sufficient to elicit HSPC mobilization and osteoblast suppression by G-CSF. These data suggest a model in which monocyctic lineage cells in the bone marrow produce trophic factors required for the maintenance of osteoblasts. G-CSF–induced suppression of monocyctic cells 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 monocyctic 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, Csf3r−/−, and CX3CR1GFP/+ 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 Csf3r−/− mice have been previously described (Liu et al., 1996). The CX3CR1GFP/+ mice were a gift from D. Littman (Skirball Institute of Biomolecular Medicine, New York School of Medicine, New York, NY).
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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 CXCL12a 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; eBioscience).

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 Dunnett’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 Csf3r−/− B cells in the mixed chimeras are as predicted from the input ratios of wild-type to Csf3r−/− 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.

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