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Monocytic Cells Mediate Granulocyte-Colony Stimulating Factor-Induced Mobilization of Hematopoietic Stem and Progenitor Cells

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by

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ABSTRACT OF THE DISSERTATION

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Professor Daniel C. Link, Chairperson

In the normal, healthy adult, hematopoiesis occurs primarily in the bone marrow. As such, at steady state, a majority of hematopoietic stem and progenitor cells (HSPCs) are tightly held within specialized areas of the bone marrow collectively referred to as the 'hematopoietic stem cell niche.' In response to various forms of stress, such as infection or hemorrhage, or following administration of a variety of pharmacologic agents, niche function is altered and HSPCs are released into the peripheral circulation through a process known as mobilization. Granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor, is the most widely-used mobilizing agent clinically. However, the mechanisms by which G-CSF elicits HSPC mobilization are not well-understood. The experiments described herein are directed towards understanding the mechanism by which G-CSF elicits mobilization as a means to understanding regulation of HSPC trafficking between the bone marrow and periphery. We first focused on identifying the hematopoietic lineage directly targeted by G-CSF in the initiation of HSPC mobilization. Using a mouse model in which the G-CSF receptor is selectively expressed on monocytic cells, we demonstrate that G-CSF signaling in monocytic cells is sufficient for a normal mobilization response. Subsequent studies were designed to identify which of the three

major cell types within the monocytic lineage – bone marrow macrophages, osteoclasts, and myeloid dendritic cells – are critical for retention of HSPCs within the bone marrow and for the mobilization response to G-CSF. We demonstrate that loss of myeloid dendritic cells does not abrogate the mobilization response to G-CSF and that pharmacologic ablation of osteoclasts does not lead to HSPC release into the periphery. Lastly, we evaluated the importance of selected factors produced by monocytes in the mobilization response to G-CSF. We demonstrate that hematopoietic loss of osteopontin does not alter the mobilization response to G-CSF. Further work will be required to evaluate the role of bone marrow macrophages and myeloid dendritic cells in HSPC retention as well as the requirement for osteoclasts in G-CSF-induced mobilization. Future studies will be directed at identifying the factors used by monocytic cells to communicate with the stromal components of the HSPC niche during G-CSF-induced mobilization.

CHAPTER 1
INTRODUCTION

In the healthy adult, a majority of hematopoietic stem cells reside within the bone marrow. In response to various forms of physiologic stress, such as infection or hemorrhage, these cells are released into the peripheral circulation, a process termed 'hematopoietic stem cell mobilization.' In the late 1980s, it was observed that large numbers of hematopoietic stem and progenitor cells could be mobilized through administration of a variety of agents, including hematopoietic growth factors (1,2), cytokines, or even cytotoxic agents (3,4). This observation proved to be of great clinical utility, as these mobilized HSPCs could be harvested from the peripheral blood and used for hematopoietic stem cell transplantation (HSCT) (5-8). Granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor, is currently the most commonly used agent in the clinical setting. G-CSF-mobilized stem cells have emerged as the preferred source of stem cells for HSCT, as they are associated with more rapid engraftment, a shorter hospital stay, and, in some circumstances, superior overall survival compared to unmanipulated bone marrow (9-13). However, when used for autologous transplant in the setting of acute myelocytic leukemia, G-CSF-mobilized stem cells are associated with an increased rate of relapse (14,15). Furthermore, nearly 20% of stem cell donors fail to mobilize enough hematopoietic stem cells for successful HSCT in response to either G-CSF alone or in combination with other mobilizing agents (16-18). Thus there is an urgent need to develop novel mobilizing agents; the rational design of such agents requires a detailed understanding of the mechanisms that regulate stem cell trafficking.

Despite its widespread clinical use, the mechanisms underlying G-CSF-induced HSPC mobilization have not been well-characterized. The studies described herein were designed to elucidate the molecular events associated with G-CSF administration as a way to understand regulation of HSPC trafficking. We initially focused on a basic question: what cell type is

directly targeted by G-CSF in the initiation of the mobilization cascade? The G-CSF receptor (G-CSFR) is expressed on a number of different stromal and hematopoietic populations (19-21), and mice lacking G-CSFR (*Csf3r-/-*) do not mobilize HSPCs in response to G-CSF (22). To determine whether the G-CSF target cell type was contained within the stromal or hematopoietic compartments, Liu, et al. constructed a series of chimeric mice using marrow from wild-type and *Csf3r*$^{-/-}$ donors. They observed that wild-type mice reconstituted with *Csf3r*$^{-/-}$ marrow did not mobilize in response to G-CSF, while *Csf3r*$^{-/-}$ mice reconstituted with wild-type marrow exhibited normal mobilization, suggesting that mobilization required G-CSF signaling in the hematopoietic but not the stromal compartment (23).

Within the transplantable hematopoietic compartment there are five major cell types that express G-CSFR: neutrophils, monocytes/macrophages, lymphocytes, NK cells, and HSPCs. The simplest model predicts that G-CSF acts directly on hematopoietic stem and progenitor cells to elicit their mobilization; however, in mixed chimeras of wild-type and *Csf3r*$^{-/-}$ marrow, both cell types mobilize with equal efficiency (23). This suggests that G-CSF does not act directly on HSPCs but rather signals through a 'trans-acting' factor to elicit HSPC mobilization. The nature of this factor is still an area of active investigation.

There is some evidence implicating neutrophils in G-CSF-induced HSPC mobilization. Neutrophils are a major source of proteases, including neutrophil elastase, cathepsin G, and MMP-9 (24,25). During G-CSF treatment, neutrophils release these proteases into the bone marrow microenvironment (26-32). These proteases, through cleavage of CXCL12 (26,29), c-kit (30), and VCAM-1 (31), may contribute to HSPC mobilization. However, their importance is controversial as mice lacking the proteases exhibit a normal mobilization response to G-CSF (33-35).

HSPC mobilization by the chemokine interleukin-8 (IL-8) is dependent on neutrophils. *Csf3r*$^{-/-}$ mice, which are profoundly neutropenic, fail to mobilize in response to IL-8 (22). Moreover, antibody-mediated depletion of neutrophils (using anti-CD11a or anti-Gr-1 antibodies) abrogated IL-8-induced HSPC mobilization (36,37). With respect to G-CSF, Pelus and colleagues reported that neutrophil depletion using anti-Gr-1 antibodies attenuated G-CSF-induced HSPC mobilization (34). However, Gr-1 is expressed on both neutrophils and a subset of monocytes; thus, a role for reduced monocytes in this phenotype is possible.

The contribution of lymphocytes to G-CSF-induced HSPC mobilization is also controversial. Reca, et al. 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 (38). In contrast, Katayama, et al. reported that *Rag1*$^{-/-}$ mice and *IL-7R*$^{-/-}$ mice exhibited a normal mobilization response to G-CSF (21). The basis for these discrepancies remains unclear. Though all of these mouse lines share deficits in B- and T-lymphopoiesis, it is conceivable that subtle differences in the strains could account for the variations observed.

Several lines of evidence indicate that monocyte lineage cells in the bone marrow contribute to osteoblast homeostasis and HSPC trafficking. Chang, et al, demonstrated that macrophages are anatomically juxtaposed with endosteal osteoblasts, forming a “canopy” over the osteoblasts at sites of bone formation (39). Moreover, they showed that ablation of monocytic cells using the MAFIA transgenic mouse model resulted in a loss of osteoblasts (39). Similarly, Winkler et al. 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 (40), although we have observed that when MAFIA mice are crossed to

Csf3r$^{-/-}$ mice, ablation of macrophages does not result in HSPC mobilization (M.J. Christopher and D.C. Link, unpublished data). Finally, Chow, et al. demonstrated that depletion of monocytic-lineage cells using a variety of methods is sufficient to induce mobilization of HSPCs (41). Together, these data strongly suggest that monocytic cells play an important role in retention of HSPCs within the marrow.

There are at least three distinct bone marrow monocytic cell populations: bone marrow resident macrophages, myeloid dendritic cells, and osteoclasts. There is considerable (though conflicting) data on the role of osteoclasts in HSPC mobilization. Kollet and colleagues reported that activation of osteoclasts by injection of RANK ligand (RANKL) was associated with moderate HSPC mobilization, and inhibition of osteoclasts, either genetically by knocking out *PTPε* or by injecting mice with calcitonin, blunts the mobilization response to G-CSF (42). Of note, osteoclasts produce the protease cathepsin K, which can cleave CXCL12 *in vitro* (42,43). On the other hand, 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 (40,44). G-CSF has also been shown to increase the frequency of activated osteoclasts in the marrow, although this occurs several days after the onset of mobilization, perhaps suggesting that the two events are unrelated (40,44).

In contrast to osteoclasts, little is known about the role of bone marrow macrophages and myeloid dendritic cells in regulation of HSPC trafficking or in transduction of a mobilization signal from G-CSF. Chow, et al. report that the selective ablation of CD169+ macrophages using mice expressing diphtheria toxin-receptor under the CD169 promoter is sufficient to elicit HSPC mobilization, suggesting that this population provides a critical retention signal for HSPCs (41). However, our analysis of CD169 expression in bone marrow cells revealed that it is expressed

not only on macrophages but also granulocytic precursors, making it difficult to determine the importance of either population on retention of stem cells within the marrow using this approach. There is currently no data on the role of myeloid dendritic cells in the regulation of HSPC trafficking. The definitive identification of the cell population(s) responsible for HSPC retention or transducing the signal from G-CSF requires further study and is, in fact, the major subject of this thesis.

The regulation of hematopoietic stem cell trafficking by more committed hematopoietic cells, however, is not direct; rather, it proceeds through one or more stromal populations that are part of a specialized structure within the bone marrow known as the 'hematopoietic stem cell niche.' By definition, a 'niche' is a collection of various cell types and secreted soluble factors and extracellular matrix that work in concert to provide a highly specialized microenvironment for a particular cell type. The idea of a hematopoietic stem cell niche was first proposed by Schofeld (45) more than 30 years ago, when it was noted that the distribution of hematopoietic stem cells within the marrow was not random. Instead, it was observed that the most primitive hematopoietic cells could be found along the endosteal surface of the marrow, with successively more committed cells located towards the central axis of the long bone (46-48). These studies raised the possibility that cells near the endosteal surface provided an appropriate environment wherein hematopoietic stem cells could maintain the balance of self-renewal and differentiation required to be able to meet the daily needs of the organism without depleting the stem cell pool. Not surprisingly, over the next several years, there was accumulating evidence that osteoblasts and osteolineage were important for maintenance of the hematopoietic stem cell pool (49-52). Consistent with this idea, induced ablation of mature osteoblasts is sufficient to elicit HSPC mobilization (53).

Osteoblasts produce a number of factors that promote stem cell quiescence and retention within the bone marrow, including VCAM-1, osteopontin, and CXCL12. VCAM-1 is an adhesion molecule that binds to VLA-4 expressed on the surface of hematopoietic stem cells (54,55). Genetic loss of VCAM-1 leads to constitutive mobilization, while antibodies to either VCAM-1 or VLA-4 or administration of a small-molecule inhibitor of VLA-4 all result in mobilization (56-58).

Osteopontin (*Spp1*) is a secreted, multi-domain glycoprotein produced by many different cell types, although the major source of osteopontin in the marrow is osteoblasts. Loss of osteopontin does not appear to have a significant impact on osteoblasts themselves, as *Spp1*-/- mice have normal bone development and normal osteoblast numbers (59). However, several studies have suggested that osteopontin is important for retention of HSPCs within the endosteal niche. For example, osteopontin is expressed only along the endosteal surface, where it binds integrins on the surface of HSPCs as well as fibronectin and collagen, thereby tethering HSPCs to the extracellular matrix of the endosteal niche (60,61). Consistent with the idea of osteopontin serving as a molecular tether, *Spp1*-/- mice do not retain KLS cells in the marrow as well as their wild-type counterparts (60). Several other studies have highlighted a role for osteopontin in the maintenance of stem cell quiescence. For example, exogenously-applied osteopontin suppresses the growth factor-induced proliferation of HSPCs in vitro (60). Similarly, a greater fraction of *Spp1*-/- KLS cells are cycling at baseline compared to wild-type KLS cells (60). Treatment with parathyroid hormone causes a greater increase in HSPC number in *Spp1*-/- than in their wild-type counterparts (61).

There is accumulating evidence that disruption of CXCR4/CXCL12 signaling is a key step in G-CSF-induced HSPC mobilization. CXCL12 (stromal derived growth factor-1, SDF-1)

is a chemokine constitutively expressed at very high levels in the bone marrow and is a potent chemoattractant for HSPCs. In mice lacking CXCL12 (62) or its major receptor CXCR4 (63) there is a failure of the migration of HSPCs from the fetal liver to the bone marrow. Moreover, *CXCR4*-/- bone marrow chimeras exhibit constitutive mobilization and fail to mobilize HSPCs in response to G-CSF (64,65). G-CSF treatment is associated with decreased CXCL12 mRNA and protein expression in the bone marrow and decreased CXCR4 expression on HSPCs (28,29,64,66). 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 (21,64,66,67) which are an important source of CXCL12 in the bone marrow (64,66,68-70). 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 (71,72).

Although the studies discussed above demonstrate an important role for CXCL12 in regulation of HSPC trafficking, the predominant source of CXCL12 in the marrow is not clear. CXCL12 has been reported to be produced by osteoblasts, endothelial cells (68), CXCL12-abundant reticular cells (70), and even bone itself (21). As previously mentioned, we reported that G-CSF treatment was associated with decreased CXCL12 production in osteoblasts (64). However, Mendez-Ferrer, et al. report that changes in total bone marrow CXCL12 levels are mostly the result of changes in population of nestin-expressing mesenchymal stem cells (73); they also report that these nestin-expressing mesenchymal stem cells contribute to formation of a separate niche.

The importance of monocytic cells to survival of niche osteoblasts has been long appreciated, as it was demonstrated that macrophages could support the growth of osteoblasts in an *in vitro* culture system (74,75). More recently, Chow, et al. demonstrated that macrophage-

conditioned media could induce stromal cells to produce CXCL12, thereby promoting retention of stem cells within the marrow. Although the exact identity of this factor – the so-called 'factor x' – remains elusive, it is worth noting that Chow, et al. were able to demonstrate that the ability to support macrophages was lost if the macrophage-conditioned media was pre-treated with proteinase K, suggesting that 'factor X' is indeed a protein (41).

To summarize, there is considerable evidence suggesting a role for monocytic cells in the retention of HSPCs within the bone marrow, although the contributions from other lineages cannot yet be excluded. The model that we propose is that at steady state, monocytic cells provide trophic support for osteoblasts, which, in turn, provide the necessary signals to keep HSPCs within the marrow in a quiescent state. In response to agents such as G-CSF, these monocytic cells withdraw their support for osteoblastic cells, leading to their apoptosis and the subsequent release of HSPCs into the periphery. Further study will be required to refine our concept of which cell type or types participate in the niche and to identify the factor used in monocyte/osteoblast communication.

CHAPTER 2

EXPRESSION OF THE G-CSF RECEPTOR IN MONOCYTIC CELLS IS SUFFICIENT TO MEDIATE HEMATOPOIETIC PROGENITOR MOBILIZATION BY G-CSF IN MICE

*The work in this chapter was a joint effort with Dr. Matthew J. Christopher, a former post-doctoral fellow in the lab, and has been previously published as Christopher, M. J., Rao, M., Liu, F., Woloszynek, J. R., & Link, D. C. (2011). Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. *Journal of Experimental Medicine*, *208*(2), 251–260. doi:10.1084/jem.20101700

INTRODUCTION

In the adult human, the majority of hematopoietic stem and progenitor cells (HSPCs) reside in specialized environments within the bone marrow collectively referred to as the 'hematopoietic stem cell niche.' The number of HSPCs in the circulation can be markedly increased in response to a number of stimuli, including hematopoietic growth factors, myeloablative agents, and environmental stresses such as infection. Recently, mobilized stem cells have become the preferred cellular source for reconstitution of the bone marrow following myeloablative therapy because of their potency, predictability, and safety. Granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor, is the most commonly used mobilizing agent clinically. However, there is considerable variability in the magnitude of HSPCs mobilized by G-CSF, and in about 20% of cases, insufficient cells are mobilized for stem cell transplantation (16-18). An understanding of the mechanisms by which G-CSF elicits mobilization of HSPCs is critical to the development of novel, more effective mobilizing agents.

There is accumulating evidence that disruption of CXCR4/CXCL12 signaling is a key step in G-CSF-induced HSPC mobilization. CXCL12 (stromal derived growth factor-1, SDF-1) is a chemokine constitutively expressed at very high levels in the bone marrow and is a potent chemoattractant for HSPCs. In mice lacking CXCL12 (62) or its major receptor CXCR4 (63) there is a failure of the migration of HSPCs from the fetal liver to the bone marrow. Moreover, CXCR4-/- bone marrow chimeras exhibit constitutive mobilization and fail to mobilize HSPCs in response to G-CSF (64,65). G-CSF treatment is associated with decreased CXCL12 mRNA and protein expression in the bone marrow and decreased CXCR4 expression on HSPCs (28,29,64,66). 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 (21,64,66,67), which are an important source of CXCL12 in the bone marrow (64,66,68,69).

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 (71,72).

Through the study of 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 (23). 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 (23).

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 (26-32). Though controversial, these proteases, through cleavage of CXCL12 (26,29), c-kit (30), and VCAM-1 (31), 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 (34). 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 monocytic 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 weeks after transplantation. As reported previously (76), the contribution of *Csf3r*$^{-/-}$ cells to the B cell lineage was near that expected based on the input ratio (Supplemental Figure 2.1). However, due to 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 (Figure 2.1D-E and data not shown). Moreover, the number of neutrophils in the blood was similar in all of the chimeras (Supplemental Figure 2.2).

We next assessed the mobilization response to G-CSF in these chimeras by quantifying colony-forming units (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 following treatment with G-CSF was highest in the 3-to-1 (wild-type-to-*Csf3r*$^{-/-}$) chimeras and progressively decreased with increasing *Csf3r*$^{-/-}$ donor input (Figure 2.1A-C). Analysis of donor chimerism in mature neutrophils (Gr-1^{hi} SSC^{hi}) in the bone marrow following G-CSF revealed that the great majority of neutrophils were derived from wild-type (*Csf3r*$^{+/+}$) cells in all of the chimeras (Figure 2.1D). In fact, the correlation between the number of wild-type neutrophils in the bone marrow and CFU-C in the blood was poor (Figure 2.1E and 2.1F).

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, reported that G-CSF-induced mobilization is impaired in *Rag2*$^{-/-}$, SCID, and Jh mice (38). In contrast, Katayama et al reported that *Rag1*$^{-/-}$ mice and *IL-7R*$^{-/-}$ mice exhibited a normal mobilization response to G-CSF (21). Herein, we characterized HSPC mobilization by G-CSF in two lymphocyte-deficient mouse strains, *Rag1*$^{-/-}$ and *NOD/scid/IL-2*γ^{null} mice. *Rag1*$^{-/-}$ mice lack mature B and T cells, and *NOD/scid/IL-2*γ^{null} mice lack all B, T, and NK cells. Consistent with a previous report (21), HSPC mobilization in *Rag1*$^{-/-}$ mice was comparable to wild-type mice (Figure 2.2A). In *NOD/scid/IL-2*γ^{null} mice, the baseline level of circulating HSPC was increased and marked mobilization in response to G-CSF was observed (Figure 2.2B). 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 (Figure 2.3A). A previous study established that regulatory elements of the CD68 gene are sufficient to direct transgene expression to the monocyte/macrophage lineage (77). Two transgenic founder lines were identified that expressed the green fluorescent protein (GFP) in circulating monocytes (data not shown). The transgenic founder mice were crossed with *Csf3r*$^{-/-}$ mice to obtain *CD68:mGCSFR;Csf3r*$^{-/-}$ mice, hereafter referred to as 'CD68:G-CSFR mice'. Since the phenotype of CD68 transgenic mice derived from the two founder lines was similar, the data have been pooled. Of note, the CD68:G-CSFR mice were generated from B6CDF1

donor oocytes and then backcrossed 4 generations onto a C57BL/6 background. To minimize 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 monocyte lineage. To test this prediction, we measured surface expression of the G-CSFR on blood leukocytes using a biotinylated G-CSF binding assay (78). Consistent with previous reports, 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 (Figure 2.3B & C and data not shown). 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 (data not shown). 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 (78). Thus, we predicted that the loss of G-CSFR expression on granulocytic cells in CD68:G-CSFR mice would result in neutropenia. Indeed, CD68:G-CSFR mice display basal neutropenia that is similar in magnitude to that seen in *Csf3r*$^{-/-}$ mice (Table 2.1). Interestingly, CD68:G-CSFR mice had a modest but significant increase in circulating B lymphocytes. A non-significant trend to increased B lymphocytes also was seen in the *Csf3r*$^{-/-}$ mice (78). All other measured hematopoietic parameters were normal in the CD68:G-CSFR mice.

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+Lin-Sca-1+ (KLS) cells and CFU-C in the blood, bone marrow, and spleen after 7 days of G-CSF. Consistent with previous reports (78), in *Csf3r*$^{+/-}$ mice, G-CSF administration resulted in a significant increase in KLS cells and CFU-Cs in the blood and spleen (Figure 2.4A & B). As expected, no mobilization was observed in *Csf3r*$^{-/-}$ mice. G-CSF treatment of CD68:G-CSFR mice induced HSPC mobilization that was at least as great as that observed in control mice. In fact, a significantly greater number of KLS cells were mobilized to the spleen in CD68:G-CSFR mice.

We have previously shown that G-CSF-induced HSPC mobilization is associated with osteoblast suppression and decreased CXCL12 expression in the bone marrow (64). We assessed osteoblast suppression by measuring mRNA expression of osteocalcin, a specific marker of mature osteoblasts (66). As reported previously (66), G-CSF treatment resulted in a significant decrease in osteocalcin and CXCL12 mRNA expression in the bone marrow of control (*Csf3r*$^{+/-}$) mice (Figures 2.4C & D). A similar decrease was observed in CD68:G-CSFR but not *Csf3r*$^{-/-}$ mice following 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.

To assess the effect of G-CSF on monocytic cells in the bone marrow, we took advantage of *CX3CR1*$^{GFP/+}$ transgenic mice in which monocytic cells express the green fluorescent protein

(GFP) (79). Based on Gr-1 expression, bone marrow monocytes/macrophages can be divided into two distinct subsets: Gr-1^{high} GFP^{+} inflammatory monocytes/macrophages and Gr-1^{low} GFP^{+} resident monocytes/macrophages (Figure 2.5A) (79). *CX3CR1*$^{GFP/+}$ mice were treated with G-CSF for 5 days 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 following flushing of bone marrow yields a cell population enriched for stromal cells and stromal macrophages (80,81). 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 (Figure 2.5B). A smaller decrease in resident monocytes/macrophages in the flushed but not bone fractions also was observed. We next examined the kinetics of monocyte/macrophage loss during G-CSF-induced mobilization (Figure 2.5C). Since *CX3CR1*$^{GFP/+}$ 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 days of G-CSF treatment, a time point at which osteoblast suppression first becomes evident (67).

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

(Figure 2.6A). Moreover, secretion of osteocalcin and CXCL12 into the conditioned media was significantly increased in cultures containing macrophages (Figure 2.6B & C). Of note, no alkaline phosphatase colonies or osteocalcin or CXCL12 protein secretion were detected in cultures of macrophages alone (data not shown). These data are consistent with a report by Chang and colleagues showing that macrophages induced osteoblast mineralization *in vitro* (39). 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 semi-permeable membrane (Figure 2.6D). 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 factor(s), 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 (24,25). However, the importance of these proteases is controversial, as mice lacking these proteases exhibit a normal mobilization response to G-CSF (34,35,82). There is strong evidence showing that HSPC mobilization by the chemokine interleukin-8 (IL-8) is dependent on neutrophils. *Csf3r*$^{-/-}$ mice, which are profoundly neutropenic, fail to mobilize in response to IL-8 (22). Moreover, antibody-mediated depletion of neutrophils (using anti-CD11a or anti-Gr-1 antibodies) abrogated IL-8-induced HSPC mobilization (36,37). With respect to G-CSF, Pelus and colleagues reported that neutrophil

depletion using anti-Gr-1 antibodies attenuated G-CSF-induced HSPC mobilization (34). 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-CSF receptor (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, while 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 also is controversial. Reca, et al. 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 (38). In contrast, Katayama et al reported that *Rag1*$^{-/-}$ mice and *IL-7R*$^{-/-}$ mice exhibited a normal mobilization response to G-CSF (21). Consistent with the latter findings, we observed that *Rag1*$^{-/-}$ and *NOD/scid/IL-2*γ^{null} mice exhibit a normal mobilization response to G-CSF. The basis for these discrepancies remains unclear. Though 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*$^{-/-}$ and *Rag2*$^{-/-}$ mice, but is absent in *NOD/scid/IL-2*γ^{null} 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, demonstrated that macrophages are anatomically juxtaposed with endosteal osteoblasts, forming a “canopy” over

the osteoblasts at sites of bone formation (39). Moreover, they showed that ablation of monocytic cells using the MAFIA transgenic mouse model resulted in a loss of osteoblasts (39). Similarly, Winkler et al. 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 (40). Finally, Chow, et al. demonstrated in a companion paper in this issue of JEM that depletion of monocytic-lineage cells using a variety of methods is sufficient to induce mobilization of HSPCs. Together, these data strongly suggest that monocytic 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 factor(s) 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 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. Since 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 and colleagues reported that activation of osteoclasts by injection of RANK ligand (RANKL) was associated with moderate HSPC mobilization, and inhibition of osteoclasts, either genetically by knocking out *PTPε* or by injecting mice with calcitonin, blunts the mobilization response to G-CSF (42). Of note, osteoclasts produce the protease cathepsin K,

which can cleave CXCL12 *in vitro* (42,43). On the other hand, 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 (40,44). In contrast to osteoclasts (40,44), we show that inflammatory and resident monocytes/macrophages decrease after G-CSF treatment. Of note, the timing of the decrease in these cell populations during G-CSF treatment is similar to that reported for the decrease in osteoblasts (67) 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 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 monocytic 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 monocytic cell population and factor(s) 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 *CX3CR1*$^{GFP/+}$ 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 (78). The *CX3CR1*$^{GFP/+}$ mice were a generous gift from Dr. Dan

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 2 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 1000 cGy from a ^{137}Cesium source at a rate of approximately 95 cGy/minute before transplantation. Prophylactic antibiotics (trimethoprim-sulfamethoxazole; Alpharma, East Bridgewater, NJ) were given during the initial 2 weeks after transplantation. Mice were analyzed 8 to 10 weeks 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. (83), 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 pIRES2-eGFP, Clontech) was subcloned into the backbone 3' of the G-CSF receptor fragment. For injection into male nuclei of B6CDF1 zygotes, the construct was digested with *Dra I* 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 germline deletion of G-

CSF receptor (*Csf3r*$^{-/-}$). 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-CSFR' 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 minutes. The collagenase-treated femurs were flushed again with PBS. The cells were pooled (containing both hematopoietic and stromal cells) and cultured for 7 days in base media (alpha-MEM with 10% fetal calf serum). On day 7 of culture, cells were trypsinized and stained with PE-conjugated anti-mouse CD115 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 absence or presence of 20,000 $CD115^+$ $CD45^+$ cells. As a control, 20,000 $CD115^+$ $CD45^+$ were plated alone. In each case, after incubation for 1-2 days in base media, cells were cultured for an additional 14 days in base media supplemented with 10 mM beta-glycerophosphate and 50 μg/ml ascorbic acid. Alkaline phosphatase staining was performed on day 14 of culture, per manufacturer's instructions (Vector Laboratories, Burlingame, CA).

G-CSF Mobilization Protocol. Recombinant human G-CSF (Amgen) was diluted in phosphate-buffered saline with 0.1% low endotoxin BSA (Sigma-Aldrich) and administered at a dose of 250 μg/kg/day either by daily subcutaneous injection for 5 days or by continuous infusion via

subcutaneous osmotic pump for 7 days. Mice were analyzed 3 to 4 hours after the final cytokine dose.

CFU-C assays. Blood, 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 blood, 5 x10^4 nucleated spleen cells, or 2.0 x 10^4 nucleated bone marrow cells in 2.5 mL methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; StemCell Technologies). Cultures were plated in duplicate and placed in a humidified chamber with 5% carbon dioxide (CO_2) at 37°C. After 7 days 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, Carlsbad, CA). RNA was isolated according to the manufacturer's instructions and resuspended in 150 μL RNase/DNase-free water. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the TaqMan One-step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA) 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 forward primer, 200 nM reverse primer, 280 nM internal probe, and 0.625 μL Multiscribe reverse transcriptase and RNase inhibitor in a total reaction volume of 25 μL. Reactions were repeated in the absence of reverse transcriptase to confirm that DNA contamination was not present. RNA content was normalized to murine β-actin. PCR conditions

were 48°C for 30 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Primers were: CXCL12 forward primer, 5'-GAGCCAACGTCAAGCATCTG-3'; CXCL12 reverse primer, 5'-CGGGTCAATGCACACTTGTC-3'; CXCL12 dT-FAM/TAMRA probe, 5'-TCCAAACTGTGCCCTTCAGATTGTTGC-3'; β-actin forward primer, 5'-ACCAACTGGGACGATATGGAGAAGA-3'; β-actin reverse primer, 5'-TACGACCAGAGGCATACAGGGACAA-3'; β-actin dT-FAM/TAMRA probe, 5'-AGCCATGTACGTAGCCATCCAGGCTG-3'; osteocalcin forward primer, 5'-TCTCTCTGCTCACTCTGCTGGCC-3'; osteocalcin reverse primer, 5'-TTTGTCAGACTCAGGGCCGC-3'; and osteocalcin dT-FAM/TAMRA probe, 5'-TGCGCTCTGTCTCTCTGACCTCACAGATGCCA-3'.

Flow Cytometry. Red blood cells in peripheral blood and bone marrow mononuclear cell preparations were lysed in Tris-buffered ammonium chloride (pH 7.2) buffer and incubated with the indicated antibody at 4°C for 1 hr in PBS containing 0.1% sodium azide, 1 mM EDTA, and 0.2% (w/v) BSA to block nonspecific binding. The following directly-conjugated monoclonal antibodies were used: allophycocyanin-eFluor 780-conjugated rat anti-mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); allophycocyanin-conjugated rat anti-mouse CD115 (AFS98, IgG2a; eBioscience); fluorescein isothiocyanate (FITC)-conjugated rat anti–mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); allophycocyanin-conjugated rat anti-mouse CD3e (17A2, IgG2b; eBioscience); and allophycocyanin-eFluor 780-conjugated rat anti-mouse B220 (RA3-6B2, IgG2a, eBioscience). For KLS analysis, we used the following antibodies: phycoerythrin (PE)-conjugated rat anti-mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); PE-conjugated rat anti-mouse

B220 (RA3-6B2, IgG2a, eBioscience); PE-conjugated Armenian hamster anti-mouse CD3e (145-2C11, IgG, eBioscience); PE-conjugated rat anti-mouse Ter-119 (TER-119, IgG2b, eBioscience); rat anti-mouse Sca-1 (D7, IgG2a, eBioscience); and rat anti-mouse c-kit (2B8, IgG2b, eBioscience). To assess surface G-CSFR expression, G-CSF was biotinylated using NHS-LC-biotin (Pierce) as previously described (84). Peripheral blood mononuclear cells were incubated at 4°C for 1 h with biotinylated G-CSF (25 ng per 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 FACScan 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 x g for 1 minute 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 type II collagenase (Worthington Biochemical) at 37°C for 60 minutes with agitation. After allowing the bone chips to settle by gravity, the cell suspension was harvested and analyzed.

CXCL12 and osteocalcin ELISAs. For enzyme-linked immunosorbent assays (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 hour at room temperature with 300 µL blocking solution (1% BSA, 5% sucrose, and 0.05% NaN_3), 100 µL sample was added to each well and incubated for 2 hours 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 at pH 7.3) was added to each well

and incubated at room temperature for 2 hours. The reaction was developed by successive incubations with 1 μg/mL horseradish peroxidase streptavidin, substrate solution, and 50 μL 2N H_2SO_4 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 (Minneapolis, MN). Osteocalcin ELISA was performed using a kit according to the manufacturer's instructions (Human Osteocalcin Instant ELISA, Catalog No. BMS2020INST, BenderMed Systems, Vienna, Austria).

Statistics. Significance was determined using Prism software (GraphPad). Statistical significance of differences was calculated using 2-tailed Student's t tests (assuming equal variance) or, where indicated, 1- or 2-way ANOVA with Bonferroni's or Dunnett's post-testing. P values less than 0.05 were considered significant. All data are presented as mean ± SEM except as noted.

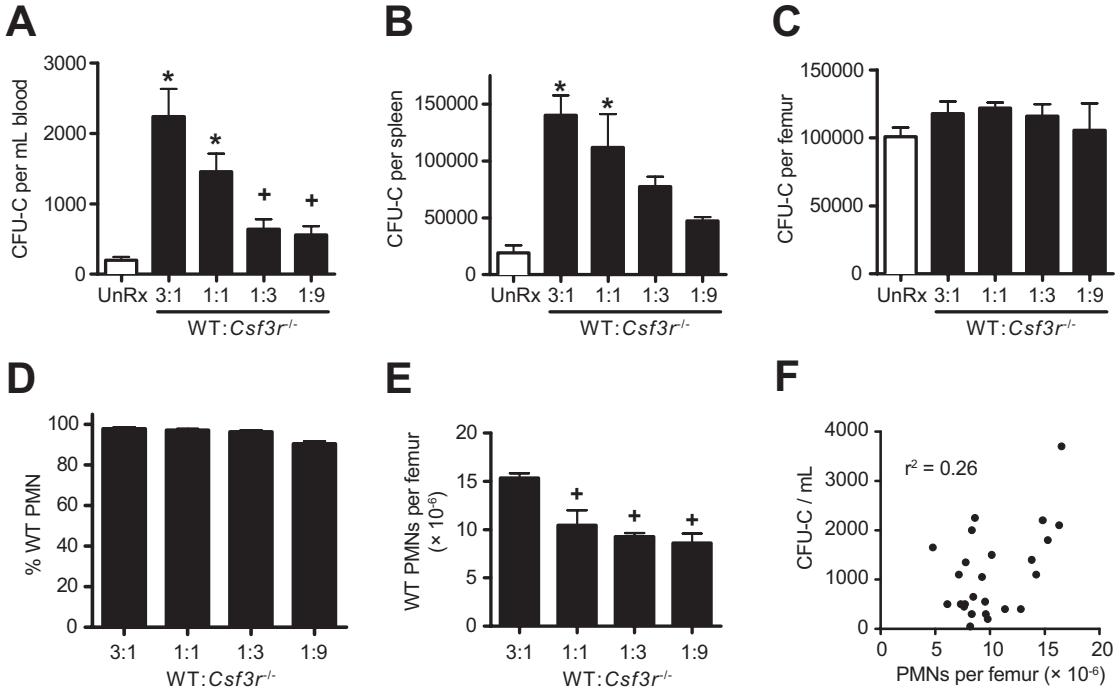
TABLES AND FIGURES

Table 2.1: Peripheral Blood Counts

Parameter	$Csf3r^{+/-}$	$Csf3r^{-/-}$	CD68:G-CSFR	$Csf3r^{+/-}$ vs. $Csf3r^{-/-\dagger}$	Csf3r+/- vs. CD68:G-CSFR†
Hemoglobin (g/dL)	13.80 ± 1.01	15.57 ± 1.06	18.00 ± 2.83	ns	ns
Platelets (K/μL)	820 ± 147	701 ± 90	678 ± 188	ns	ns
Leukocytes (K/μL)	8.76 ± 1.82	8.51 ± 4.10	9.82 ± 2.56	ns	ns
Neutrophils (K/μL)	1.38 ± 0.62	0.16 ± 0.03	0.24 ± 0.05	$p < 0.001$	$p < 0.001$
Monocytes (K/μL)	0.24 ± 0.12	0.12 ± 0.08	0.25 ± 0.06	ns	ns
B cells (K/μL)	2.75 ± 0.57	3.53 ± 1.52	4.92 ± 1.53	ns	$p < 0.05$
T cells (K/μL)	1.95 ± 0.75	2.23 ± 0.82	1.78 ± 0.42	ns	ns

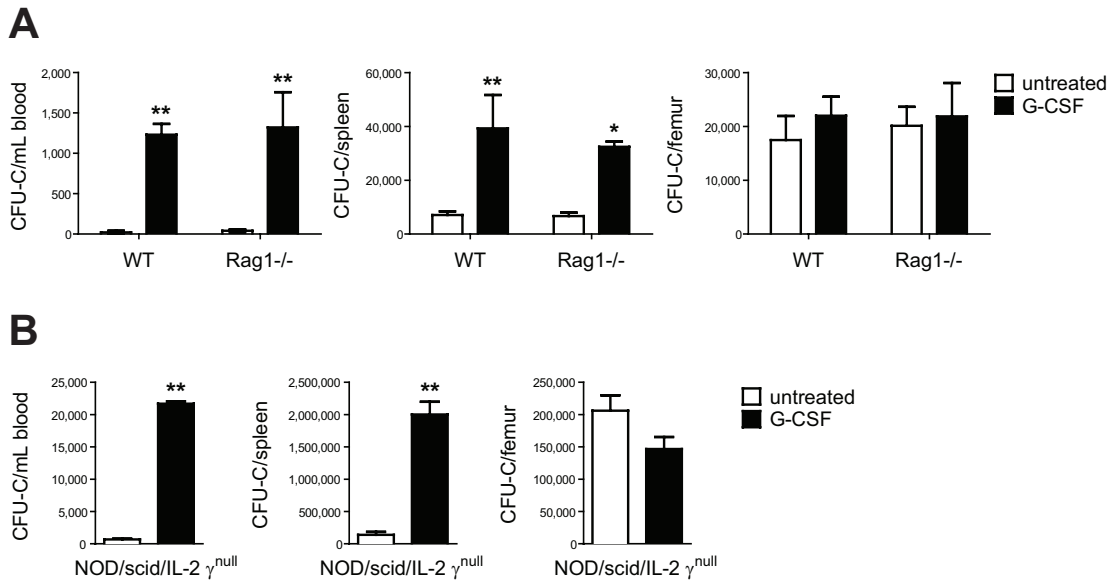
Values represent mean ± standard deviation for 2-5 mice per genotype and is pooled from three independent experiments.
†Comparisons made using a one-way ANOVA with Bonferroni's post test corrected for multiple comparisons

Figure 2.1



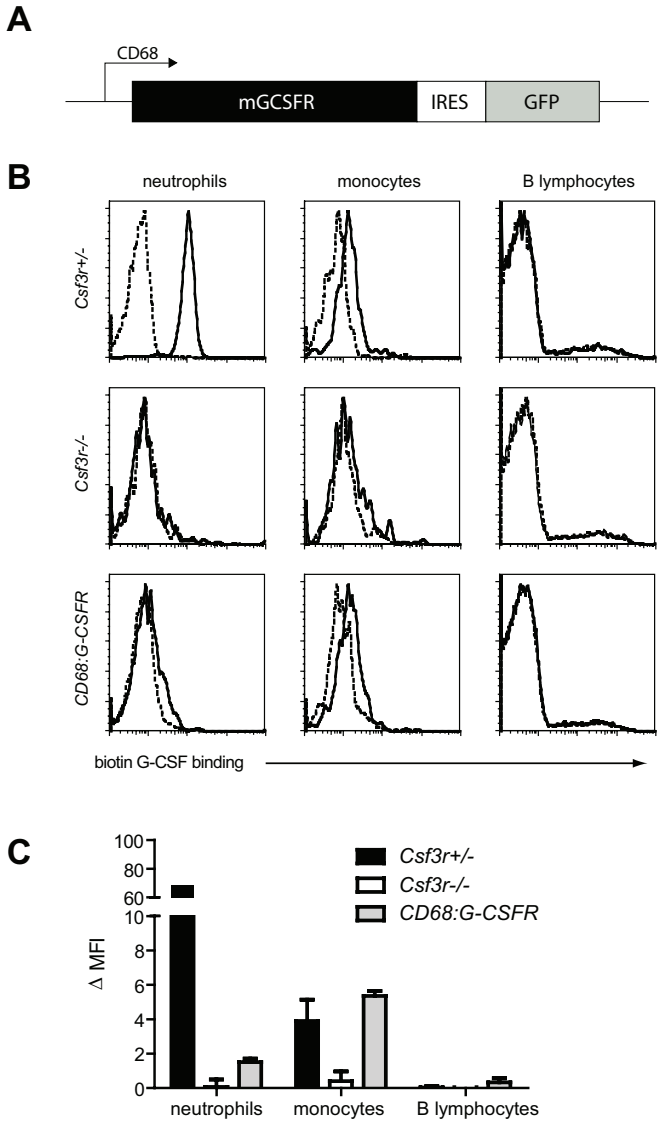
Neutrophil number in the bone marrow correlates poorly with G-CSF-induced HSPC mobilization. Wild-type and *Csf3r*$^{-/-}$ bone marrow cells were mixed at the indicated ratios and transplanted into irradiated recipients. Six weeks later, the mixed bone marrow chimeras were treated with G-CSF (250 μg/kg/day G-CSF for 7 days), and the number of CFU-C in the (A) peripheral blood, (B) spleen, or (C) bone marrow was measured. As a control, a cohort of mixed chimeras was analyzed without G-CSF treatment (UnRx); since similar results were obtained with each type of chimera, the untreated data were pooled. The percentage of neutrophils in the bone marrow derived from wild-type cells (D) and the absolute number of wild-type neutrophils per femur (E) were determined after 7 days of G-CSF administration. (F) The number of CFU-C in the blood versus the number of wild-type neutrophils in bone marrow. The Pearson r^2 value is shown. Data represent the mean ± SEM of 6-9 mice and is pooled from two independent transplantation experiments. *$P < 0.05$ compared with untreated chimeras; $^{+}P < 0.05$ compared with 3-to-1 chimeras.

Figure 2.2



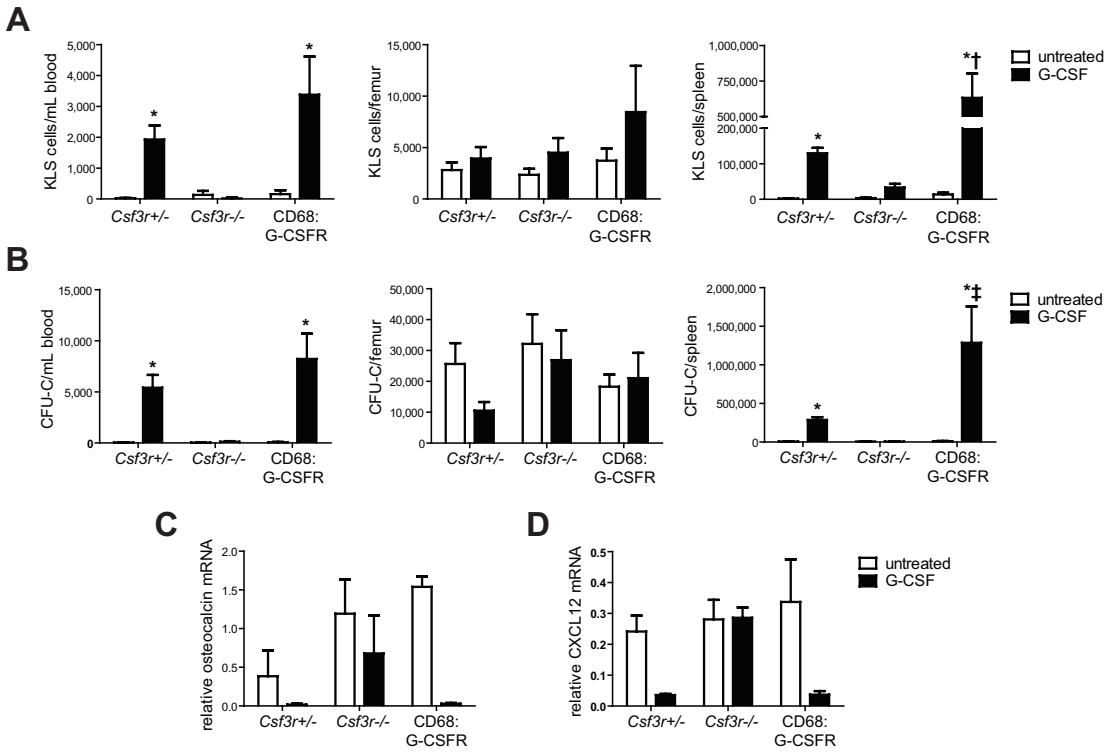
G-CSF-induced HSPC mobilization is normal in Rag1$^{-/-}$ and NOD/scid/IL-2γ^{null} mice. Wild-type, *Rag1*$^{-/-}$ (A), or *NOD/scid/IL-2*γ^{null} mice (B) were treated with G-CSF (250 μg/kg/day for 5 days) or left untreated. The number of CFU-C in the blood, bone marrow, and spleen was measured after 5 days of G-CSF. Data represent the mean ± SEM of 4-5 mice per genotype per treatment group and is pooled from four independent experiments. *P < 0.01; **P <0.001.

Figure 2.3



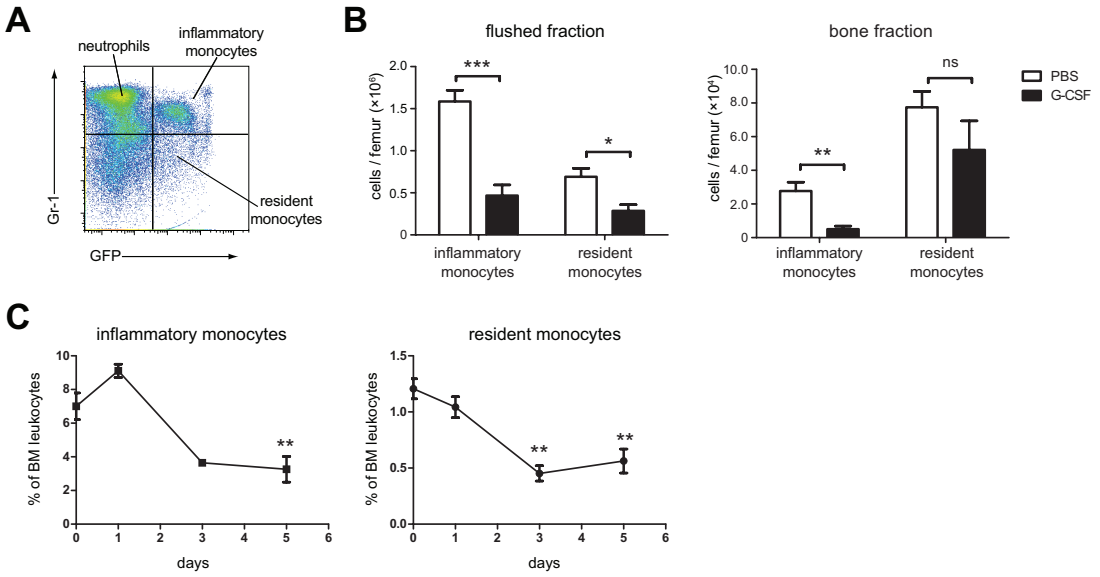
G-CSFR expression is mainly restricted to monocytic cells in CD68:G-CSFR mice. (A) Schematic of the CD68:G-CSFR transgene. IRES: internal ribosomal entry sequence; GFP: green fluorescent protein. (B) Representative histograms showing biotinylated G-CSF binding in the absence (solid line) or presence (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 average ΔMFI in the indicated blood leukocyte population is shown. Data represent the mean ± SEM of 4-5 mice of per genotype and is pooled from four independent experiments.

Figure 2.4



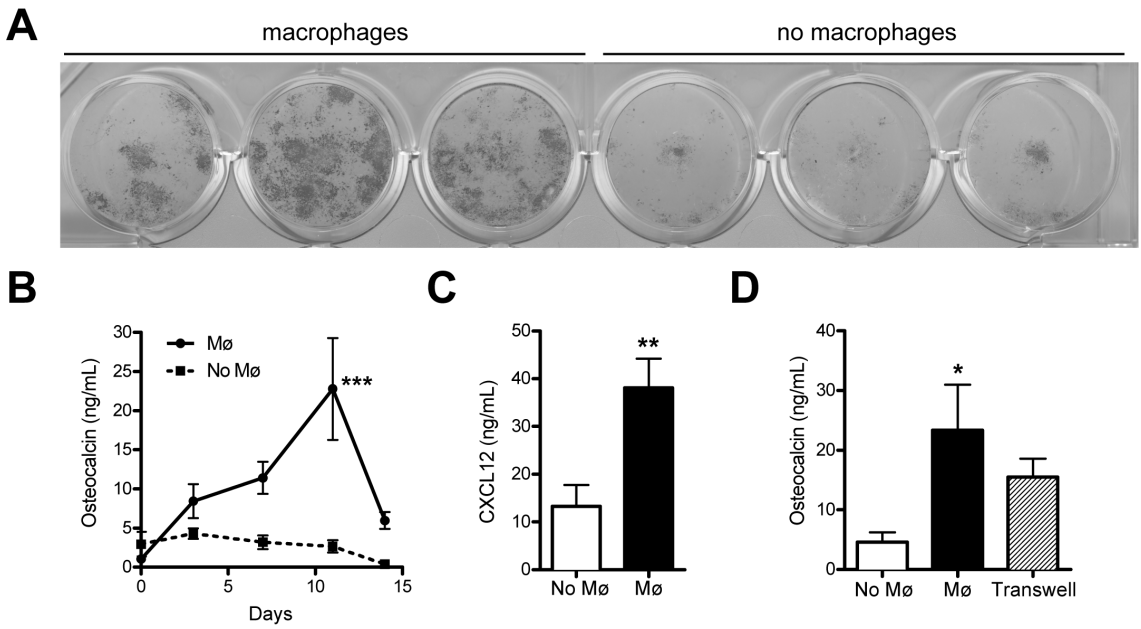
G-CSF-induced HSPC mobilization is normal in CD68:G-CSFR mice. Mice of the indicated genotype were treated with G-CSF for 7 days or left untreated. The number of KLS (A) or CFU-C (B) in the blood, bone marrow, and spleen on day 7 is shown. The mRNA expression in the bone marrow on day 7 of osteocalcin (C) or CXCL12 (D) relative to β-actin is shown. Data represent the mean ± SEM of 3-5 mice per genotype per treatment group and is pooled from three independent experiments. *$P < 0.05$ compared with untreated mice of the same genotype. **$P < 0.01$ compared with untreated mice of the same genotype. $^{\dagger}P < 0.05$ and $^{\ddagger}P = 0.07$ compared with G-CSF-treated *Csf3r*$^{+/-}$ mice.

Figure 2.5



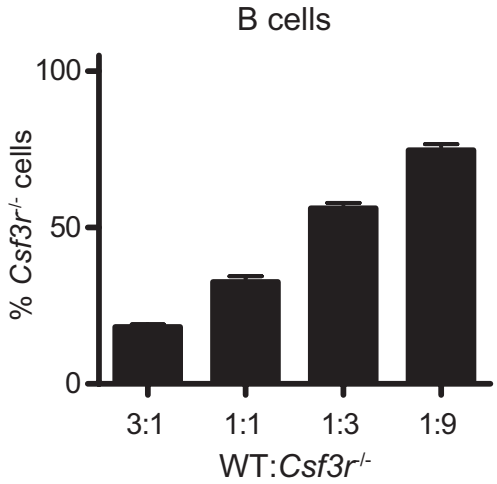
G-CSF treatment leads to a loss of monocytic cells from the bone marrow. *CX3CR1*$^{GFP/+}$ mice were treated with PBS or G-CSF for 5-7 days and the flush and bone fractions harvested as described in Methods. (A) Representative dot-plot showing the gating strategy used to identify inflammatory (Gr-1^{high} GFP^{+}) and resident monocytes/macrophages (Gr-1^{low} 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, and ***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*$^{GFP/+}$ mice were employed, and CD115 was used to identify monocytic cells. Data represent mean ± SEM of 2-6 mice per cohort pooled from 4 independent experiments. **P < 0.005 compared to untreated mice as determined by one-way ANOVA with Dunnett's post-test to correct for multiple comparisons.

Figure 2.6



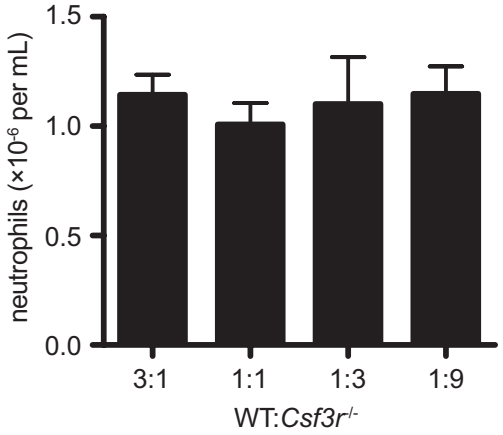
Macrophages support the growth of mature osteoblasts *in vitro*. Unfractionated bone marrow cells from wild-type mice were cultured for 10 days, and then sorted by flow cytometry into stromal ($CD45^-$ $Ter119^-$) and macrophage ($CD45^+$ $CD115^+$) cell populations. Stromal cells were cultured for an additional 14 days 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. 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 semi-permeable membrane (transwell). Data represent the mean ± SEM of 3 independent experiments. *P < 0.05, **P < 0.005, and ***P < 0.001.

Supplemental Figure 2.1



B lymphocyte chimerism in mixed Csf3r$^{-/-}$ chimeras. Mixed bone marrow chimeras were established by transplanting the indicated ratio of wild type-to-*Csf3r*$^{-/-}$ bone marrow cells into lethally irradiated recipients. Donor chimerism of peripheral blood B lymphocytes was assessed by flow cytometry 5-6 weeks after transplantation. Data represent the mean ± SEM of 6-9 mice and is pooled from two independent experiments.

Supplemental Figure 2.2



Absolute number of neutrophils in mixed Csf3r$^{-/-}$ chimeras. Shown is the absolute number of neutrophils in the peripheral blood prior to G-CSF treatment. Greater than 95% of all neutrophils were derived from wild-type cells in each case (data not shown). Data represent the mean ± SEM of 6-9 mice and is pooled from two independent experiments.

CHAPTER 3

OSTEOCLASTS ARE NOT REQUIRED FOR G-CSF-INDUCED HSPC MOBILIZATION

INTRODUCTION

In the healthy adult, a majority of hematopoietic stem cells reside within the bone marrow. In response to various forms of physiologic stress, such as infection or hemorrhage, these cells are released into the peripheral circulation, a process termed 'hematopoietic stem cell mobilization.' In the late 1980s, it was observed that large numbers of hematopoietic stem and progenitor cells could be mobilized through administration of hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF) (1,2). This observation proved to be of great clinical utility, as these mobilized HSCs could be harvested from the peripheral blood and used for hematopoietic stem cell transplantation (HSCT); in fact, several studies have demonstrated that use of mobilized peripheral blood stem cells in HSCT is associated with more rapid engraftment and a lower incidence of graft-versus-host disease (9-13) . Currently, G-CSF is the most commonly used and most effective mobilizing agent. Nearly 20% of stem cell donors, however, fail to mobilize enough hematopoietic stem cells for successful HSCT using standard mobilization regimens (18). Efficient development of novel mobilizing agents requires a detailed understanding of the mechanisms that regulate stem cell trafficking,

We recently reported that G-CSF acts on monocytic cells to elicit HSPC mobilization and that treatment with G-CSF leads to loss of certain monocyte subsets from the bone marrow (85). Similarly, two other groups demonstrated that ablation of monocytic cells is sufficient to cause mobilization of HSPCs (40,41). Together, these data suggest that monocytic cells play an important role in the retention of HSPCs within the bone marrow and that G-CSF may elicit HSPC mobilization, at least in part, through the suppression of monocytic cells. We sought to extend and refine these findings by evaluating the importance of certain monocytic subsets both to the retention of HSPCs within the marrow and the mobilization response to G-CSF.

Within the bone marrow monocytic lineage, there are three major cell types: 1) osteoclasts; 2) myeloid dendritic cells; and 3) bone marrow macrophages. In this study, we evaluated the role of osteoclasts and myeloid dendritic cells in HSPC retention within the bone marrow as well as in the mobilization response to G-CSF using a pair of ablation models. The role of bone marrow macrophages has been difficult to elucidate, largely owing to the lack of tools to selectively ablate them. Use of clodronate-loaded liposomes (40,41) or Mafia transgenic mice (40) have been used in the past, but these agents appear to target multiple monocytic populations, and, at least in the case of the Mafia mice, the ablation paradigm results in a systemic inflammatory response, making it impossible to separate the effects of the cytokine storm from the effects of the G-CSF (Daniel C. Link, unpublished data). A recent study by Chow, et al. demonstrated ablation of CD169+ macrophages without a corresponding loss of inflammatory or resident monocytes using a diphtheria-toxin inducible system, reporting that ablation of these CD169+ macrophages was sufficient to elicit mobilization of HSPCs, suggesting a key role for these cells in HSPC retention within the marrow (41). However, our analysis of CD169 expression in bone marrow suggests that other populations such as granulocytic precursors express high levels of CD169, thereby confounding the interpretation of the data. A definitive assessment of the role of these macrophages will require development of more sophisticated models.

In recent years, the role of osteoclasts in G-CSF-induced mobilization has received considerable attention, but the absence of a model system where osteoclasts can be transiently ablated – thus avoiding the complications of assaying hematologic parameters in the presence of severe osteopetrosis – has made drawing definitive conclusions on their role difficult. In this study, we used osteoprotegerin-Fc, a stabilized form of the RANK ligand decoy receptor

osteoprotegerin, to transiently but completely ablate osteoclasts and measured the effect both on HSPC retention within the marrow as well as in response to G-CSF. We report herein that loss of osteoclasts does not lead to spontaneous mobilization of HSPCs, nor does it alter the mobilization response to G-CSF.

Along with bone marrow macrophages and osteoclasts, monocytic cells give rise to a subset of antigen-presenting cells referred to as myeloid (or bone-marrow resident) dendritic cells. They can be identified immunophenotypically by their high expression of both CD11c and MHC Class II. They represent approximately 0.22% of bone marrow leukocytes and are concentrated in perivascular spaces, where they are believed to participate in immunosurveillance (86). They are also believed to have a role in promoting survival of recirculating B cells, as their ablation results in a profound loss of recirculating B cells (86). In contrast to osteoclasts, however, very little is known about their role in regulation of HSPC trafficking or G-CSF-induced mobilization. Using a diphtheria toxin-based approach to transiently ablate myeloid dendritic cells, we report that their loss is associated with an augmented response to G-CSF and that ablation of myeloid dendritic cells leads to a partial suppression of osteoblasts, suggesting a previously-unidentified role for myeloid dendritic cells in regulation of stromal cell populations.

MATERIALS AND METHODS

Mice. Wild-type 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 CD11c-DTR expressing mice were obtained from Jackson Labs.

Generation of Chimeras. Bone marrow cells from mice carrying the CD11c-DTR transgene or their non-transgenic littermates (both Ly5.2) were harvested. A total of 2 million bone marrow cells were injected retroorbitally into lethally irradiated wild-type mice (Ly5.1). Recipient mice were conditioned with 1000 cGy from a ^{137}Cesium source at a rate of approximately 95 cGy/minute before transplantation. Prophylactic antibiotics (trimethoprim-sulfamethoxazole; Alpharma, East Bridgewater, NJ) were given during the initial 2 weeks after transplantation. Mice were analyzed 8 to 10 weeks after transplantation.

G-CSF Mobilization Protocol. Recombinant human G-CSF (Amgen) was diluted in phosphate-buffered saline with 0.1% low endotoxin BSA (Sigma-Aldrich) and administered at a dose of 250 μg/kg/day either by daily subcutaneous injection for either 5 or 7 days. Mice were analyzed 12-15 hours after the final cytokine dose.

CFU-C assays. Blood, 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 blood, 5 x10^4 nucleated spleen cells, or 2.0 x 10^4 nucleated bone marrow cells in 2.5 mL methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; StemCell Technologies). Cultures were plated in duplicate and placed in a humidified chamber with 5% carbon dioxide (CO_2) at 37°C. After 7 days 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, Carlsbad, CA). RNA was isolated according to the manufacturer's instructions and resuspended in 50 μL RNase/DNase-free water. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the TaqMan One-step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA) on a GeneAmp 7300 Sequence Detection System (Applied Biosystems). The reaction mix consisted of 5 μL RNA, 10 μL RT-PCR mix, 200 nM forward primer, 200 nM reverse primer, 280 nM internal probe, and 0.5 μL Multiscribe reverse transcriptase and RNase inhibitor in a total reaction volume of 20 μL. Reactions were repeated in the absence of reverse transcriptase to confirm that DNA contamination was not present. RNA content was normalized to murine β-actin in a multiplex PCR reaction. PCR conditions were 48°C for 15 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Primers were: CXCL12 forward primer, 5'-GAGCCAACGTCAAGCATCTG-3'; CXCL12 reverse primer, 5'-CGGGTCAATGCACACTTGTC-3'; CXCL12 dT-FAM/TAMRA probe, 5'-TCCAAACTGTGCCCTTCAGATTGTTGC-3'; osteocalcin forward primer, 5'-TCTCTCTGCTCACTCTGCTGGCC-3'; osteocalcin reverse primer, 5'-TTTGTCAGACTCAGGGCCGC-3'; and osteocalcin dT-FAM/TAMRA probe, 5'-TGCGCTCTGTCTCTCTGACCTCACAGATGCCA-3'. Mixtures of primers and FAM-conjugated probes for cathepsin K, tartrate-resistant acid phosphatase and a VIC-conjugated probe for beta-actin were obtained from Applied Biosystems Gene Expression Assay repository.

Flow Cytometry. Red blood cells in peripheral blood and bone marrow mononuclear cell preparations were lysed in Tris-buffered ammonium chloride (pH 7.2) buffer and incubated with

the indicated antibody at 4°C for 1 hr in PBS containing 0.1% sodium azide, 1 mM EDTA, and 0.2% (w/v) BSA to block nonspecific binding. The following directly-conjugated monoclonal antibodies were used: allophycocyanin-eFluor 780-conjugated rat anti-mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); allophycocyanin-conjugated rat anti-mouse CD115 (AFS98, IgG2a; eBioscience); fluorescein isothiocyanate (FITC)-conjugated rat anti–mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); allophycocyanin-conjugated rat anti-mouse CD3e (17A2, IgG2b; eBioscience); and allophycocyanin-eFluor 780-conjugated rat anti-mouse B220 (RA3-6B2, IgG2a, eBioscience). For KLS analysis, we used the following antibodies: phycoerythrin (PE)-conjugated rat anti-mouse Gr-1 (RB6-8C5, IgG2b; eBioscience); PE-conjugated rat anti-mouse B220 (RA3-6B2, IgG2a, eBioscience); PE-conjugated Armenian hamster anti-mouse CD3e (145-2C11, IgG, eBioscience); PE-conjugated rat anti-mouse Ter-119 (TER-119, IgG2b, eBioscience); rat anti-mouse Sca-1 (D7, IgG2a, eBioscience); and rat anti-mouse c-kit (2B8, IgG2b, eBioscience). For identification of myeloid dendritic cells, we used a PE/Cy7-conjugated anti-mouse CD11c antibody and a PerCP/Cy5.5-conjugated anti-mouse MHC Class II antibody (both available from Biolegend). All cells were analyzed either on FACScan or Gallios flow cytometers.

Statistics. Significance was determined using Prism software (GraphPad). Statistical significance of differences was calculated using 2-tailed Student's t tests (assuming equal variance) or, where indicated, 1- or 2-way ANOVA with Bonferroni's or Dunnett's post-testing. P values less than 0.05 were considered significant. All data are presented as mean ± SEM except as noted.

Chemicals. All chemicals were obtained form Sigma unless otherwise noted. Diphtheria toxin was reconstituted to a final concentration of 1 μg/μL in PBS + 0.1% bovine serum albumin and was given via intraperitoneal injection at a final dose of 4 ng diphtheria toxin per gram body weight according to the experimental paradigm. Osteoprotegerin-Fc was a generous gift of Amgen (Thousand Oaks, CA). It was diluted to a final concentration of 1 mg/mL with PBS + 0.1% BSA and administered as a single 100 μg dose via subcutaneous injection.

Immunohistochemistry. Femurs from mice were fixed in 10% neutral buffered formalin overnight. Excess fixative was rinsed off and bones were decalcified in 10% EDTA for two weeks before being rehydrated and embedded in paraffin. 7-μm sections were prepared from each of the blocks and stained for tartrate-resistant acid phosphatase (TRAP) using standard protocols. Sections were counterstained with hematoxylin.

RESULTS

Osteoclasts are not required for retention of HSPCs in the bone marrow.

To evaluate the importance of osteoclasts in the retention of HSPCs within the marrow, we measured HSPC frequency in the peripheral blood, bone marrow, and spleen following osteoclast ablation with osteoprotegrin-Fc (OPG-Fc), a chimera of recombinant human osteoprotegerin (OPG) and the Fc immunoglobulin domain. Murine osteoclast formation is dependent on RANKL/RANK signaling, and mice with targeted deletion of either RANK or RANKL do not form mature osteoclasts (87). *In vivo*, RANKL is secreted primarily by mature osteoblasts, where it binds to RANK on osteoclast progenitors to promote formation of mature osteoclasts. Osteoblasts also produce osteoprotegerin (OPG), which serves as a decoy receptor for RANK ligand. The relative expression of RANKL and OPG is a key determinant of whether

osteoclast formation is supported or inhibited (88). Administration of OPG-Fc reduces the ratio of RANKL to OPG, causing a complete, but transient ablation of osteoclasts in mice (89).

In our experiments, mice were given a single dose of 100 μg OPG-Fc; at various times after receiving OPG-Fc, they were sacrificed and samples of peripheral blood, bone marrow, and spleen were taken. To estimate the number of stem cells, the frequency of c-kit+ Sca-1+ Lin- (KLS) cells was measured by flow cytometry. To estimate the number of progenitor cells, the frequency of colony-forming units (CFU-Cs) was measured. According to the published literature on the pharmacokinetics of OPG-Fc, the treatment regimen used in this experiment should result in complete osteoclast ablation within four days after OPG-Fc administration, and the ablation should last for eight additional days (89). To determine whether osteoclast ablation had occurred in our experimental paradigm, we stained sections from mice seven days after OPG-Fc administration for tartrate-resistant acid phosphatase (TRAP) activity, a marker of mature osteoclasts. Indeed, compared to untreated mice, OPG-Fc-treated mice displayed nearly a complete loss of TRAP-positive cells in the bone marrow (Supplemental Figure 3.1).

We predicted that if osteoclasts promoted retention of HSPCs within the marrow, the loss of osteoclasts should result in an increase in the frequency of hematopoietic stem and progenitor cells in the peripheral blood and/or the spleen. However, we did not detect any significant increase in the frequency of hematopoietic stem or progenitor cells in either the peripheral blood or spleen up to nine days after OPG-Fc administration (Figure 3.1A, B). These data suggest that osteoclasts are not required for retention of HSPCs within the bone marrow.

Osteoclasts are not required for G-CSF-induced mobilization.

The role of osteoclasts in G-CSF-induced mobilization is controversial. Several studies have suggested that osteoclast function is positively correlated with HSPC mobilization. Kollet,

et al. reported that administration of soluble RANK ligand, which activates osteoclasts, induces a mild mobilization response, suggesting that osteoclast activation may promote mobilization (42), a phenomenon that has also been observed by an independent group (90). Kollet, et al. also reported that physiologic stress, such as hemorrhage or lipopolysaccharide (LPS) administration, leads to osteoclast activation that is coincident with mobilization. Similarly, female mice lacking PTP-epsillon, which have a mild osteoclast defect, have a diminished mobilization response to G-CSF. More recently, the same group reported that mice deficient in CD45, which have reduced osteoclast maturation and activity, have a reduced mobilization response to G-CSF (91).

In contrast, several other groups have suggested that osteoclast activity is negatively correlated with mobilization. Takamatsu, et al. reported that pamidronate, one of the first-generation bisphosphonates, augments the mobilization response to G-CSF, suggesting that osteoclasts negatively regulate the mobilization response to G-CSF (44). More recently, Winkler, et al. reported that zoledronate, one of the newer-generation bisphosphonates reported to induce osteoclast apoptosis *in vivo* (92), also augments the mobilization response to G-CSF (40). Using a similar dosing regimen as Winkler, et al., we also observed that zoledronate augmented the mobilization response to G-CSF; however, administration of zoledronate alone was not associated with a significant reduction in numbers of mature osteoclasts, as measured by TRAP staining (Supplemental Figure 3.2), making it difficult to evaluate the role of osteoclasts in G-CSF-induced mobilization based on these data alone.

We sought to definitively determine whether osteoclasts are required for G-CSF-induced mobilization of HSPCs using the OPG-Fc ablation model described above. Wild-type mice were pre-treated with a single dose of 100 μg OPG-Fc or left untreated; four days later they started a five-day course of G-CSF. The frequency of HSPCs in the peripheral blood, bone marrow, and

spleen was measured before and after the G-CSF treatment regimen. We predicted that if osteoclasts were required for G-CSF-induced mobilization, mice pre-treated with OPG-Fc should exhibit a blunted mobilization response to G-CSF. In fact, mice pre-treated with OPG-Fc demonstrated the same mobilization response to G-CSF as mice that had not been pre-treated, as measured by the frequency of KLS cells and CFU-Cs in the peripheral blood and spleen (Figure 3.2). These data suggest that osteoclasts are not required for G-CSF-induced mobilization of HSPCs.

Myeloid dendritic cell ablation augments G-CSF-induced HSPC mobilization.

To determine whether myeloid dendritic cells were required for G-CSF-induced mobilization of HSPCs, we measured the mobilization response to G-CSF after myeloid dendritic cell ablation in CD11c-DTR chimeric mice (93). Administration of diphtheria toxin to these mice elicits a transient ablation of CD11c-expressing cells, including myeloid dendritic cells. A single dose of diphtheria toxin ablates its target cells within eight hours, and the ablation lasts for approximately two days. Sustained ablation can be achieved with multiple doses of diphtheria toxin, although this may cause systemic toxicity. However, if marrow from CD11c-DTR mice is transplanted into lethally-irradiated wild-type mice, sustained ablation of dendritic cells can be achieved through repeated administrations of diphtheria toxin without significant systemic toxicity (94). In contrast to the rapid kinetics associated with cytokine-induced HSPC mobilization, G-CSF-induced mobilization occurs over the course of days, with an increase in peripheral HSPC frequency first evident at 3-4 days after beginning treatment. In order to be able to accurately evaluate the role of myeloid dendritic cells in G-CSF-induced mobilization, therefore, it was necessary to be able to ablate dendritic cells for a period of at least three days. To accomplish this, lethally-irradiated wild-type mice received marrow either from mice

carrying the CD11c-DTR transgene or their non-transgenic littermates. Once hematopoietic reconstitution had occurred, all of the mice received two doses of diphtheria toxin, spaced 48 hours apart. Twenty-four hours after the first dose of diphtheria toxin was administered, half of the mice in each group started a seven-day course of 250 μg/kg/day G-CSF. At the end of the G-CSF treatment regimen, the mobilization response to G-CSF was evaluated by measuring the frequency of KLS cells and CFU-Cs in the peripheral blood, bone marrow, and spleen. A diagram depicting the experimental paradigm is shown in Figure 3.3A. We predicted that if myeloid dendritic cells were required for G-CSF-induced mobilization, the CD11c-DTR chimeras would not mobilize in response to G-CSF. Surprisingly, we observed the opposite phenomenon, wherein the CD11c-DTR chimeras mobilize more HSPCs to the periphery in response to G-CSF than the non-transgenic chimeras (Figure 3.3B, C). This increase was seen with both KLS cell and CFU-C frequency, indicating that it is occurring at both the level of the hematopoietic stem cells and their more committed progenitors. We wondered if the increased mobilization could be the result of generalized inflammation that occurred as a result of dendritic cell ablation or diphtheria toxin administration. We did not, however, observe significant numbers of HSPCs in the periphery of non-transgenic chimeras that received only diphtheria toxin, making it unlikely that the diphtheria toxin itself elicited mobilization. Furthermore, we did not observe significant numbers of HSPCs in the periphery of CD11c-DTR chimeras that received only diphtheria toxin, indicating that the ablation of myeloid dendritic cells did not result in a massive systemic inflammatory response. Consistent with this, all of the mice in this experiment appeared well at the time of sacrifice, exhibiting normal grooming and feeding behavior. Thus, it is unlikely that the increased response to G-CSF observed in the CD11c-DTR

chimeras is solely the result of a systemic inflammatory response, but the basis for this observation remains unclear.

Myeloid dendritic cell ablation augments G-CSF-induced leukocytosis and suppression of mature osteoblasts, but not G-CSF-induced suppression of CXCL12 production.

In addition to mobilizing HSPCs from the bone marrow into the periphery, G-CSF treatment is associated with a number of other hematopoietic and stromal changes, including peripheral leukocytosis (primarily neutrophilia) (95-97), suppression of mature osteoblasts (21,64,66,67), and suppression of bone marrow CXCL12 production (28,29,95). Given that myeloid dendritic cell ablation augmented the HSPC mobilization response to G-CSF, we wondered whether any other aspects of the response to G-CSF were similarly affected. As shown in Figure 3.4A, CD11c-DTR chimeras treated with both diphtheria toxin and G-CSF have a much higher peripheral blood leukocyte concentration than similarly-treated non-transgenic counterparts. A peripheral blood smear performed on blood samples taken from these CD11c-DTR chimeras revealed that the increased leukocyte count in the peripheral blood could be attributed to increased neutrophil counts; no blasts were observed in the sample (data not shown), consistent with an augmented response to G-CSF. Using RNA isolated from tibial flushes, we measured expression of CXCL12 and osteocalcin – a marker of mature osteoblasts – in the bone marrow. We observed that G-CSF-induced suppression of osteocalcin expression was greater in CD11c-DTR chimeras than in non-transgenic chimeras (Figure 3.4B). In contrast, the degree to which G-CSF-induced suppression of CXCL12 was not significantly different between CD11c-DTR chimeras and non-transgenic chimeras. These data raise the possibility that the G-CSF's effects on total CXCL12 production occur through mechanisms distinct from that which causes osteoblast suppression, the latter involving myeloid dendritic cells.

Analysis of osteocalcin expression in the various groups also suggested ablation of myeloid dendritic cells was itself sufficient to reduce osteoblast numbers (Figure 3.4B, comparing DT-only treated non-transgenic chimeras with DT-only treated CD11c-DTR chimeras) without significantly altering total bone marrow CXCL12 expression (Figure 3.4C, comparing the same two groups). These data are consistent with a recent report that suggests that the major source of CXCL12 within the bone marrow is not mature osteoblasts (70).

G-CSF treatment reduces the number of bone marrow myeloid dendritic cells.

We recently reported that G-CSF treatment results in the loss of both inflammatory and resident monocytes from the bone marrow (85). In this study, we observed that G-CSF treatment also results in the loss of myeloid dendritic cells from the bone marrow (Figure 3.5). Whether the loss of these various monocytic subtypes occurs as a result of mobilization into the periphery or cell death in the marrow itself is unclear and remains an area of active investigation.

DISCUSSION

The cell types important for retaining HSPCs within the marrow and which are directly targeted by G-CSF in the initiation of the mobilization cascade are not well-defined. We and others recently identified a key role for monocytic cells in both of these processes (40,41,85); however, the specific cell type(s) within this lineage that regulate these processes have still not been clearly identified. Within the monocytic lineage, there are three major cell types in the bone marrow: 1) bone marrow resident macrophages; 2) myeloid (or bone marrow-derived) dendritic cells; and 3) osteoclasts. The goal of this study was to examine the contribution of osteoclasts and myeloid dendritic cells to the retention of HSPCs in the marrow and initiating the mobilization cascade in response to G-CSF using a series of ablation models.

The role of osteoclasts in HSPC retention and response to G-CSF has been the subject of many studies over the past several years, often with conflicting results. The data which supports a role for osteoclasts in regulation of HSPC mobilization includes the observation that agents which activate osteoclasts (such as soluble RANKL) also elicit HSPC mobilization, while known triggers of HSPC mobilization – such as infection or stress – also activate osteoclasts (42,90). Conversely, several genetic models of osteoclast deficiency, such as PTP-epsillon-deficient females or CD45-deficient mice, also display a reduced mobilization response to G-CSF (42,91). In contrast, the data which suggests that osteoclasts do not play an important role in regulation of G-CSF-induced mobilization include a pair of studies which demonstrated that inhibition of the bone-resorbing capacity of osteoclasts (and perhaps induction of osteoclast apoptosis) leads to an augmented response to G-CSF (40,44). In this study, we report that osteoclast loss following OPG-Fc treatment does not result in spontaneous mobilization of HSPCs nor does it alter the mobilization response to G-CSF. We believe that this approach represents a significant improvement over previous models of osteoclast ablation, as OPG-Fc causes a transient but complete loss of osteoclasts during which measurements into phenomena such as G-CSF-induced mobilization can be made without concern for the effects of adaptation that may have occurred as a result of long-term osteoclast loss. As with any pharmacologic agent, however, there is always concern for off-target effects, so we are currently working on a genetic model of osteoclast ablation involving mice with targeted deletion of RANK (RANK-/-). RANK-/- mice lack the ability to form mature osteoclasts; as a consequence, however, they develop osteopetrosis at a very young age (< 3 weeks) with a resulting disruption in their bone marrow architecture so severe that any studies into hematopoietic stem cell trafficking are compromised. We attempted to create RANK -/- chimeras by transplanting fetal livers from RANK-/- mice into

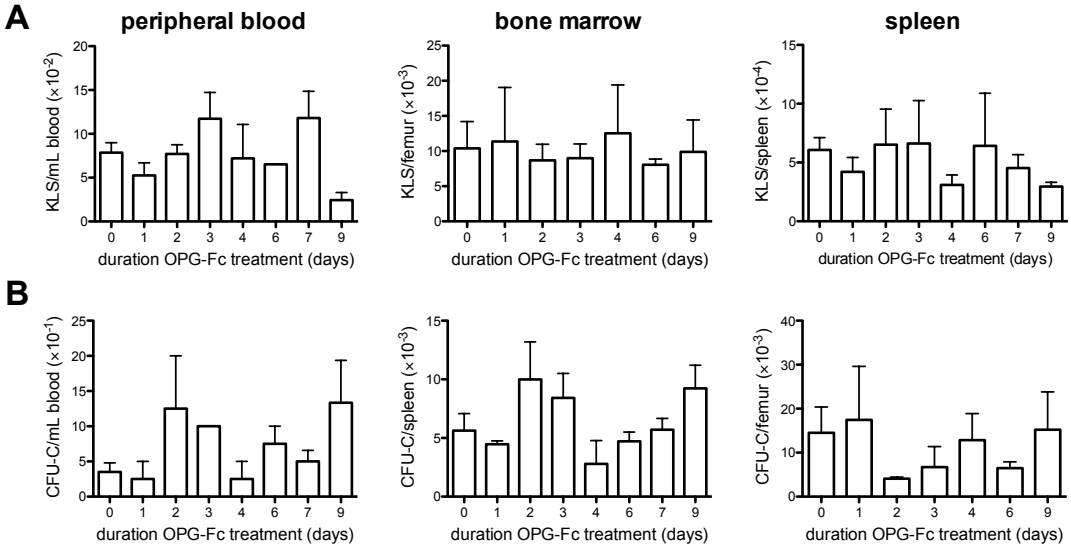
lethally-irradiated wild-type recipients with the expectation that as the hematopoietic reconstitution occurred, recipient-derived osteoclasts would die off and not be replaced. However, even at ten weeks post-transplant, a significant number of osteoclasts remained, making it difficult to interpret any effect on G-CSF-induced mobilization. To circumvent this difficulty, we plan to transplant liver cells from RANK-/- fetuses into lethally-irradiated mice with targeted deletion of G-CSF receptor (G-CSFR-/-). As hematopoietic reconstitution occurs, no new osteoclasts would form because of the lack of RANK expression on donor-derived osteoclast precursors, while any residual recipient osteoclasts would be insensitive to G-CSF. The initial characterization of RANK-/- mice revealed that, in addition to a failure to develop mature osteoclasts, the mice have deficits in lymph node formation, but that hematopoiesis is otherwise unaltered (87). If osteoclasts are not required for G-CSF-induced mobilization, as suggested by the results of the OPG-Fc osteoclast ablation experiments, we would predict that reconstitution of a G-CSFR-/- mouse with RANK-/- marrow will be sufficient to restore a normal mobilization response to G-CSF.

In contrast to osteoclasts, little is known about the role of myeloid dendritic cells in regulation of G-CSF-induced mobilization. In these studies, we observed that induction of myeloid dendritic cell ablation during the first half of the G-CSF treatment regimen actually increases the mobilization response to G-CSF and that myeloid dendritic cell ablation in itself suppresses osteoblasts without a corresponding suppression of CXCL12 production. This is consistent with the hypothesis that myeloid dendritic cells are not required for G-CSF-induced mobilization. Definitive evidence of this, however, requires an experimental paradigm similar to that used for the OPG-Fc osteoclast ablation experiments, wherein both myeloid dendritic cell and HSPC frequency can be measured throughout the course of G-CSF administration.

Although further study is required to elicit the exact mechanisms of the observations reported here, the available data suggest a model in which, at steady state, different cell types provide support to distinct stromal cell populations, such as mature osteoblasts and CXCL12-abundant reticular (CAR) cells, which, in turn, facilitate retention of HSPCs within the marrow. Ablation of a single monocyte population leads to loss of one of the supported stromal cell populations, but there is sufficient redundancy within the system such that release of HSPCs into the periphery is not observed until multiple pathways are targeted. Agents such as G-CSF or clodronate-loaded liposomes target multiple pathways, leading to changes in a number of the important retention molecules and ultimately leading to mobilization of HSPCs. This model is also consistent with a role for sympathetic nervous system neurons in regulation of G-CSF-induced mobilization, as Katayama, et al. reported that G-CSF can act via sympathetic nervous system neurons to regulate osteoblast survival (21). As the number of defined cell populations has increased, it has become clear that a detailed understanding of the mechanisms involved in regulating stem cell trafficking will require a careful dissection of the contributions of each of the identified populations.

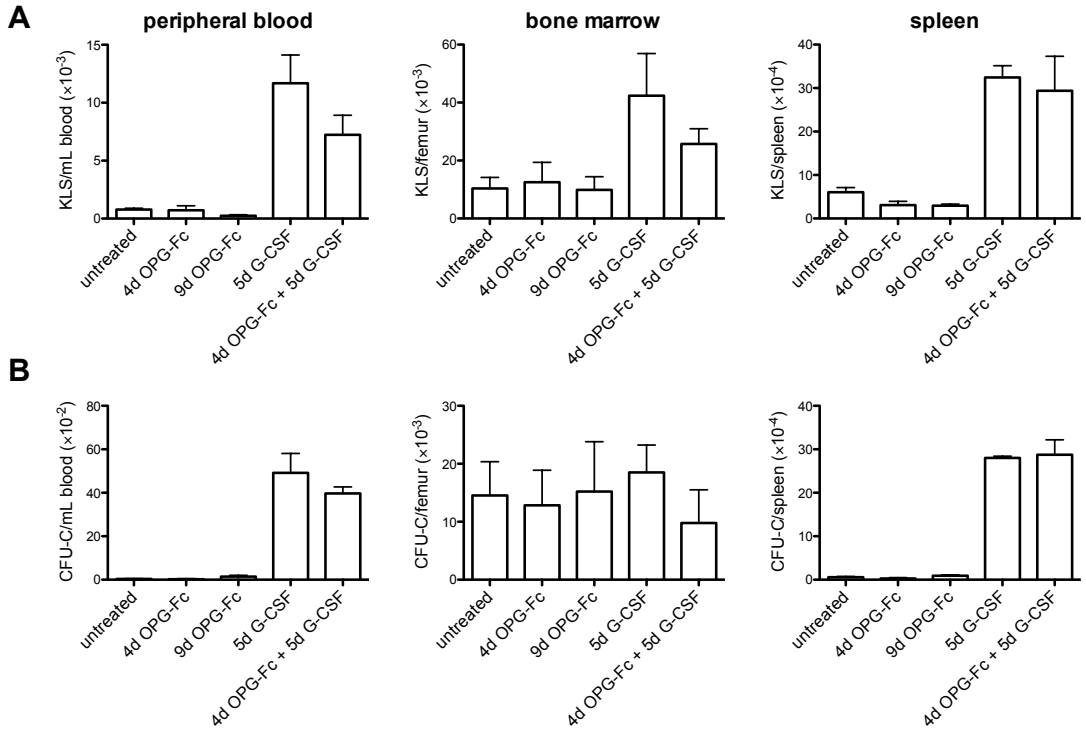
FIGURES

Figure 3.1



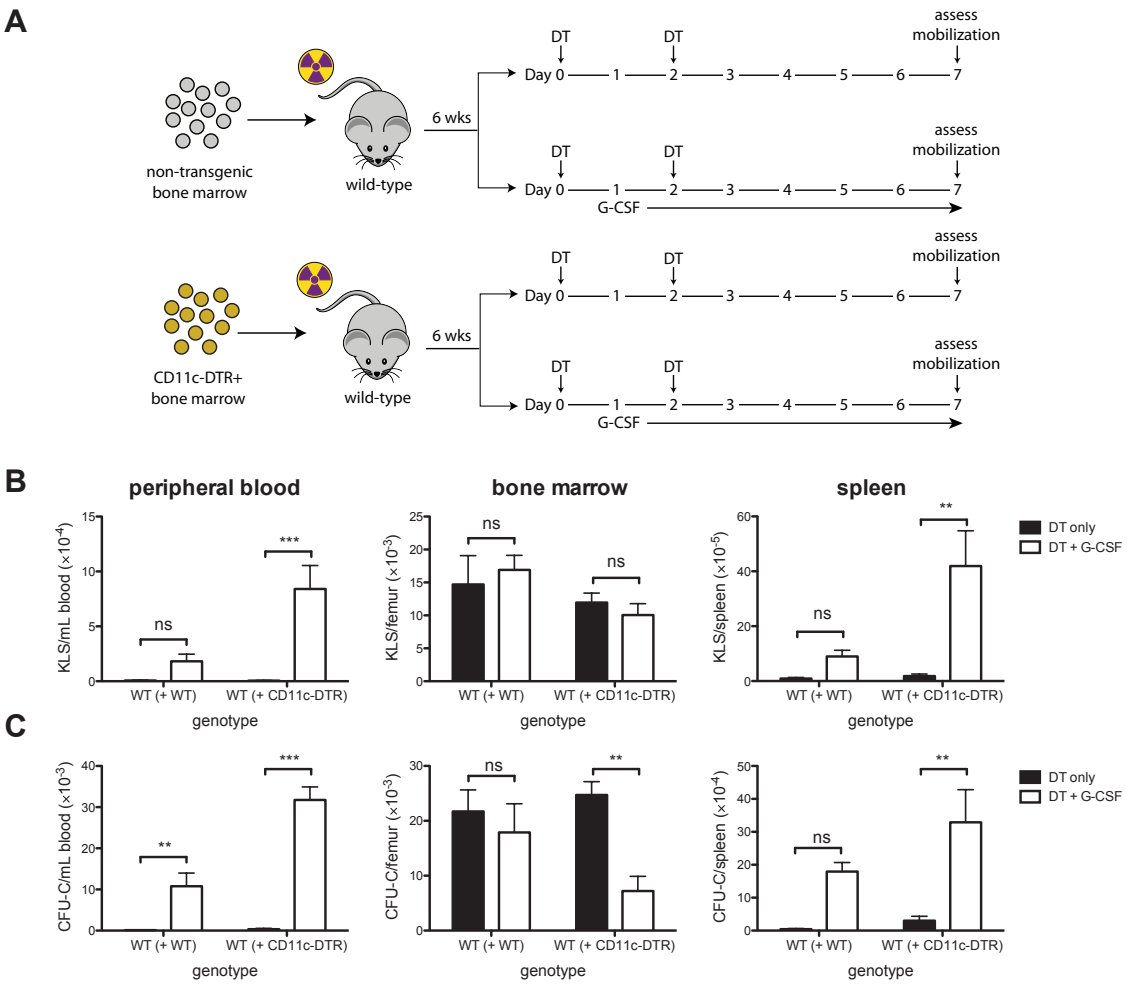
Osteoclast loss does not lead to mobilization of HSPCs. Wild-type mice were given a single dose of 100 μg OPG-Fc on day 0. At the time point indicated, they were sacrificed and the frequency of (A) KLS cells or (B) colony-forming units [CFU-Cs] was measured. None of the peripheral blood or spleen KLS or CFU values were significantly different than the day 0 value using a one-way ANOVA with Dunnett's post-test to correct for multiple comparisons. The data indicate that osteoclast ablation using OPG-Fc is not sufficient to release significant numbers HSPCs into the peripheral circulation n = 2-3 mice per day.

Figure 3.2



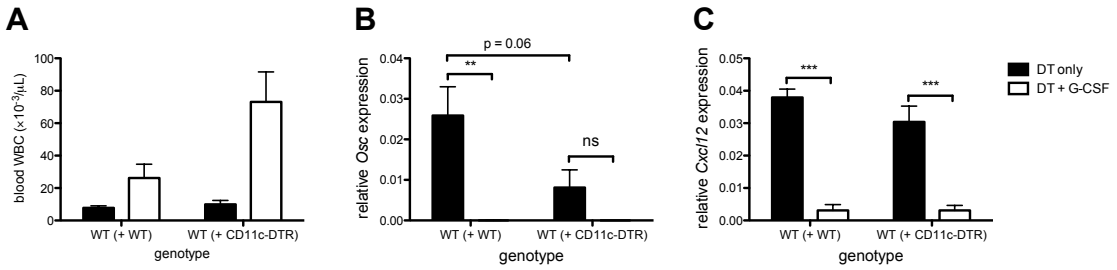
Osteoclasts are not required for G-CSF-induced mobilization of HSPCs. Mice received combinations of G-CSF and OPG-Fc as indicated; at the conclusion of the treatment period, mice were sacrificed and the frequency of KLS cells (A) or colony-forming units [CFU-Cs] (B) was measured. The data indicate that pre-treatment with OPG-Fc for a duration sufficient to ablate osteoclasts does not alter the mobilization response to G-CSF. n = 2-5 animals per group.

Figure 3.3



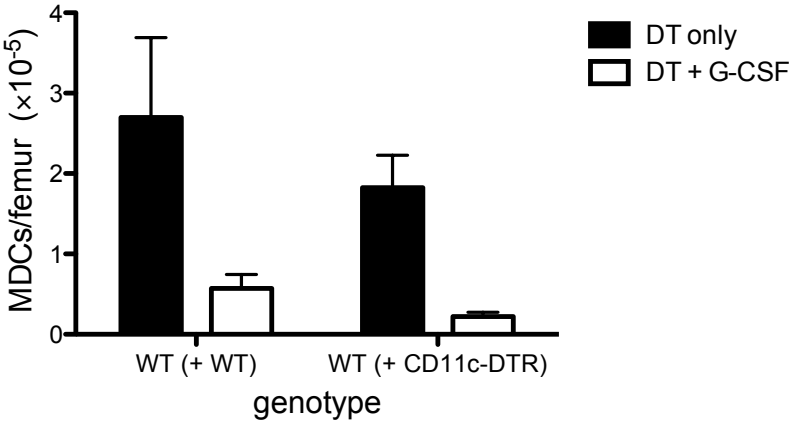
Ablation of myeloid dendritic cells augments the mobilization response to G-CSF. Chimeras of mice carrying the CD11c-DTR transgene or their non-transgenic littermates were constructed. Once reconstitution had occurred, the mice received a combination of diphtheria toxin and G-CSF as indicated in panel (A). At the end of the treatment period, the animals were sacrificed and the frequency of KLS cells (B) and colony-forming units [CFU-C] (C) in the peripheral blood, bone marrow, and spleen were measured. n = 4 animals per donor genotype per treatment group.

Figure 3.4



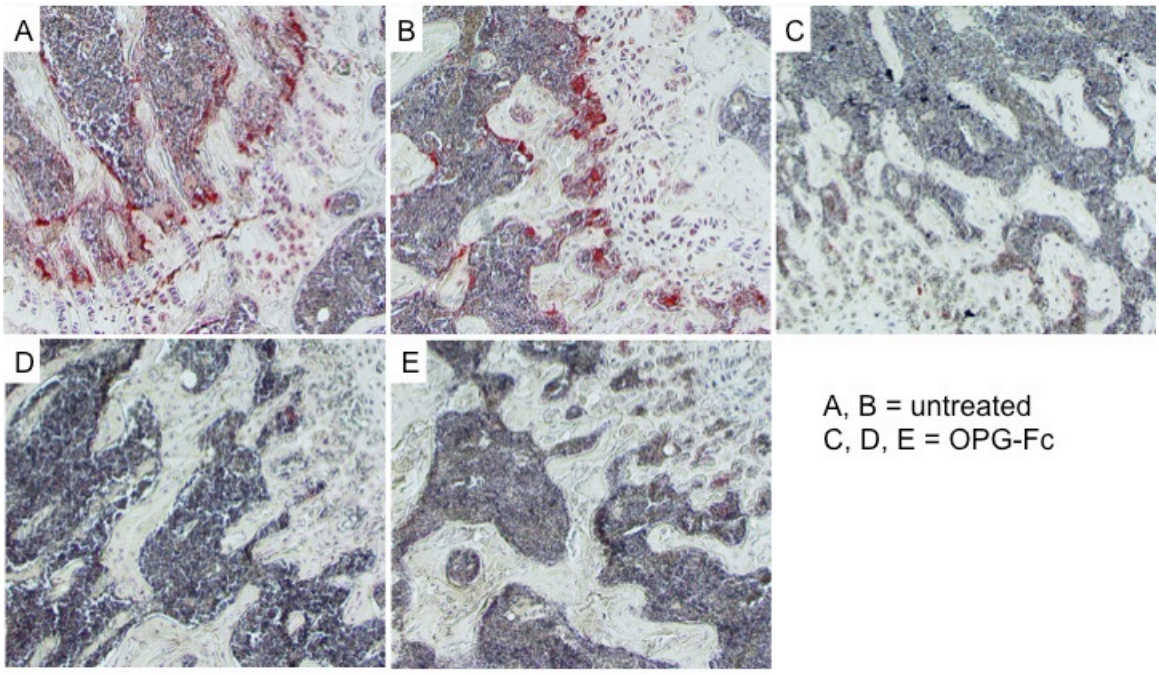
Ablation of myeloid dendritic cells augments the G-CSF-induced peripheral leukocytosis and suppression of mature osteoblasts but not total bone marrow CXCL12 production. Chimeric mice were constructed as described in Figure 3.3. (A) The concentration of white blood cells in the periphery was measured using an automated cell counter. (B,C) RNA was isolated from tibial flushes and assayed for expression of osteocalcin (B) or CXCL12 (C). The effect of G-CSF treatment on each parameter was compared between CD11c-DTR chimeras and non-transgenic chimeras using a two-way ANOVA; $p < 0.05$ for white blood cell counts and osteocalcin expression; $p = 0.31$ for CXCL12 expression. Pairwise comparisions were made using Bonferroni's post-test to correct for multiple comparisons or the Student's t-test where appropriate. $^{**}p < 0.01$, $^{***}p < 0.001$. n = 4 mice per donor genotype per treatment group.

Figure 3.5



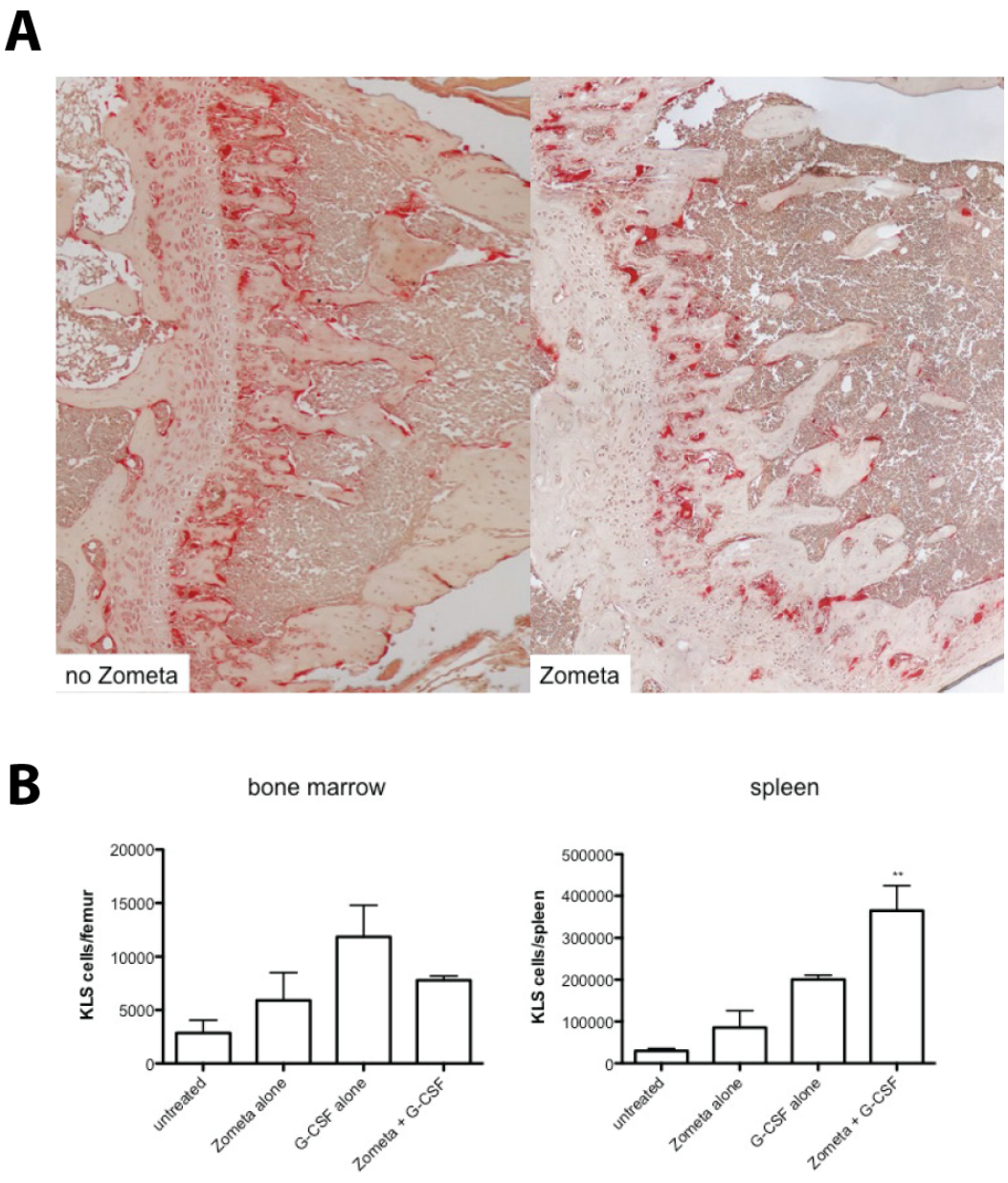
G-CSF administration reduces the frequency of myeloid dendritic cells in the bone marrow. Chimeras constructed as previously described received diphtheria toxin and G-CSF according to the schematic in Figure 3.3A. At the end of the treatment period, the frequency of myeloid dendritic cells ($CD11c^{hi}$MHC Class II^{hi}) was assayed using flow cytometry. n = 4 mice per donor genotype per treatment group.

Supplemental Figure 3.1



Administration of a single dose of 100 µg OPG-Fc is sufficient to ablate osteoclasts. Mice were treated with a single dose of 100 µg OPG-Fc via subcutaneous injection (panels C-E) or left untreated (panels A,B). Seven days later, the animals were sacrificed; sections were prepared and stained for tartrate-resistant acid phosphatase (TRAP) activity and counterstained with hematoxylin. Each panel represents a section from an individual mouse.

Supplemental Figure 3.2



Inhibition of osteoclast activity with zoledronate (Zometa) results in mobilization and augments the mobilization response to G-CSF without a significant reduction in the frequency of TRAP+ cells. (A) TRAP-stained section of femur from untreated and zoledronate-treated animals. (B) Effect of zoledronate on frequency of KLS cells, both alone and in combination with G-CSF. n = 2-3 animals per group.

CHAPTER 4
SUMMARY AND FUTURE DIRECTIONS

Monocytic-lineage cells are directly targeted by G-CSF in the initiation of hematopoietic stem and progenitor cell mobilization.

The long-term goal of these studies was to understand the regulation of hematopoietic stem cell (HSC) trafficking as a prerequisite to the development of novel mobilizing agents. Our approach was to probe the mechanism by which granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor considered to be the best available mobilizing agent, elicits mobilization of hematopoietic stem and progenitor cells (HSPCs). Our studies were focused on a very basic question: what cell type is first targeted by G-CSF in the initiation of HSPC mobilization?

Previously published data from our lab demonstrated that the cell type targeted by G-CSF was contained within the transplantable hematopoietic compartment, as chimeric mice bearing wild-type hematopoietic cells in the background of a G-CSF receptor (G-CSFR)-deficient stroma exhibited a normal mobilization response to G-CSF (23). Within the transplantable hematopoietic compartment, there are four major cell types/lineages with known expression of the G-CSF receptor: neutrophils, monocytes, lymphocytes/NK cells, and HSCs. In Chapter 2, we systematically evaluated the importance of each of these lineages to G-CSF-induced mobilization. We report that in two different mouse models of lymphocyte deficiency, G-CSF-induced mobilization is normal, suggesting that lymphocytes and NK cells are not directly targeted by G-CSF. We also report that in chimeric mice containing various mixtures of wild-type and G-CSFR-deficient neutrophils, the mobilization response to G-CSF is poorly correlated with the frequency of wild-type neutrophils, suggesting that neutrophils are also not directly targeted by G-CSF. Lastly, we engineered a transgenic mouse model in which expression of G-CSF receptor was limited to monocytic cells and demonstrated that the mobilization response to

G-CSF was normal in these animals, suggesting that G-CSF signaling in monocytic cells was sufficient for a normal mobilization response.

We also reported that bone marrow macrophages promoted survival of mature osteoblasts in an *in vitro* co-culture system and that in response to G-CSF there was a loss of monocytic cells – specifically inflammatory and resident monocytes – from the bone marrow. Previously published data from our lab and others had identified osteoblast suppression as part of the response to G-CSF (21,64,66,67), while Visnjic, et al reported that transient ablation of osteoblasts was sufficient to elicit HSPC mobilization (53). Together with data demonstrating that loss of monocytic cells was sufficient to elicit HSPC mobilization (40,41), these data suggested a model in which monocytic cells promoted the retention of HSPCs within the marrow through their support of mature osteoblasts. In response to mobilizing agents such as G-CSF, the monocytic cells are lost from the marrow, thereby reducing support for osteoblasts. These osteoblasts undergo apoptosis, ultimately leading to release of HSPCs into the periphery. Whether the loss of monocytic cells from the marrow is the result of apoptosis or mobilization into the periphery remains an area of active investigation.

Osteoclasts are not required for G-CSF-induced mobilization, while myeloid dendritic cells regulate osteoblast survival without altering bulk CXCL12 levels

With the identification of monocytic cells as critical to both retention of HSPCs within the marrow and the mobilization response to G-CSF, we shifted our focus to identifying the contributions of the various cell types within this lineage. In the bone marrow, there are three major subtypes of monocytic cells: bone marrow macrophages, myeloid dendritic cells, and osteoclasts. Data from Chow, et al. suggested that loss of bone marrow macrophages using a CD169-DTR ablation model resulted in mobilization of HSPCs (41), although the effect of ablation on G-CSF-induced mobilization was not reported. While it is clear that a population of

bone marrow macrophages express CD169 and were therefore likely depleted in Chow, et al.'s paradigm, there appear to be other bone marrow populations such as granulocytic precursors that also express CD169 and were hence also ablated, confounding the data interpretation. The ability to selectively ablate bone marrow macrophages remains a formidable technical challenge.

In Chapter 3, we continued the evaluation of monocytic subtypes, evaluating the role of myeloid dendritic cells and osteoclasts both in HSPC retention and in G-CSF-induced mobilization. As mentioned in Chapter 3, the role of osteoclasts in HSPC trafficking has been debated over the past several years using a variety of different pharmacologic agents and genetic systems to reduce or ablate osteoclast function. As with bone marrow macrophages, the complete ablation of osteoclasts without major disruption of bone marrow architecture has proved to be a technical challenge. We used a recombinant form of osteoprotegerin conjugated to a Fc immunoglobulin fragment (OPG-Fc, a generous gift of Amgen, Inc.) to induce a complete but transient ablation of osteoclasts in mice and measured the effect both on HSC retention and on the mobilization response to G-CSF. We report that the loss of osteoclasts does not result in constitutive mobilization of HSPCs, nor does it alter the mobilization response to G-CSF. We are currently working on a genetic model of osteoclast loss (described in Chapter 3) to complement the data from the OPG-Fc ablation model.

We also evaluated the role of myeloid dendritic cells using a diphtheria toxin-based transient ablation model (93). Our preliminary findings suggest that loss of myeloid dendritic cells is not sufficient to release HSPCs into the periphery, although a more extensive time course of dendritic cell depletion is underway to confirm these findings. Surprisingly, we observed that ablation of myeloid dendritic cells appears to augment the mobilization response to G-CSF. The mechanistic basis for these observations is as yet unclear, although it is unlikely that myeloid

dendritic cells are required for G-CSF-induced mobilization. We also observed that ablation of myeloid dendritic cells leads to a decrease in expression of osteocalcin – a marker of mature osteoblasts – without a corresponding drop in CXCL12 production, consistent with an earlier report suggesting that the mature osteoblasts are not the major source of CXCL12 in the bone marrow (70).

In summary, we have presented data that suggests that G-CSF acts on monocytic cells to initiate the HSPC mobilization cascade and that monocytic cells promote retention of HSPCs within the marrow. Our initial model was that a single member of the monocytic lineage provides trophic support to mature osteoblasts or osteoblastic precursors, which in turn secrete factors (such as CXCL12) that promote retention of HSPCs in the marrow. Agents such as G-CSF direct the monocytic cell population to reduce trophic support for osteoblasts, leading to their apoptosis and release of HSPCs into the periphery. In line with this model, we focused our attention on identifying which cell type within the monocytic lineage fulfilled this role. However, the data from the myeloid dendritic cell ablation studies as well as the data demonstrating that the majority of CXCL12 in the marrow comes from cells other than mature osteoblasts or osteolineage cells suggests that the biology may be more complicated than initially believed. Our current thinking is that multiple monocytic populations provide trophic support to different stromal populations including osteoblasts and CXCL12-abundant reticular (CAR) cells, each of which contributes to regulation of HSPC trafficking. Agents such as G-CSF or clodronate affect multiple monocytic cell types, which, in turn, leads to changes in different stromal populations, the culmination of which is the release of HSPCs into the periphery. The regulation of trafficking is fine-tuned even further by the sympathetic nervous system and/or nestin+ mesenchymal stem cells, as has been suggested by work from the Frenette group

(21,73,98). A detailed understanding of the mechanism is likely to require isolation of individual cell populations and characterization of their function.

Identification and characterization of factors used by monocytic cells to support stromal populations important for HSPC retention

The next major challenge awaiting investigators in this field is the identification of the factor or factors used by monocytic cells to support stromal populations, the so-called 'factor X.' It has been long understood that macrophages can provide trophic support to osteoblasts (74,75), but the importance of such a secreted factor in regulation of HSPC trafficking was not appreciated until 2000, when it was observed that G-CSF mobilized wild-type and G-CSFR-/- HSPCs with equal efficiency in a mixed chimera model (23), but the nature of the factor has remained elusive. This factor is likely to be of great interest in the development of novel mobilizing agents, as disruption of its signaling should be sufficient to suppress the stromal populations critical for HSPC retention within the marrow.

We reported that macrophages provided robust trophic support for osteoblasts in a co-culture system, but that the effect was diminished – although not eliminated – if the osteoblast and macrophage layers were separated by a semi-permeable membrane (85). The trophic effect on osteoblasts could not be sustained using macrophage-conditioned media, however, suggesting that the factor was likely to be labile and hence needed to be continually produced by the macrophages. In a similar experiment, Chow, et al. demonstrated that macrophage-conditioned media could stimulate production of CXCL12 by MS-5 stromal cells, and that pre-incubation of the media with proteinase K abrogated this effect (41). These data suggest that the factor involved is a secreted protein. Using a series of antibodies to neutralize the candidate factor or macrophages isolated from animals with genetic deletion of the candidate factor, Chow, et al evaluated the ability of IGF-1, IL-1, TNF-alpha, and IL-10 to promote CXCL12 production by

MS-5 stromal cells. Unfortunately, individual neutralization of the factors listed did not appear to have any impact on stromal cell production of CXCL12.

Our initial model of the relationship between monocytic cells and osteoblasts predicted that monocytic cells would reduce production of trophic factors for osteoblasts following exposure to G-CSF. To identify candidate trophic factors, therefore, our lab isolated CD115+ from the bone marrow of mice before and after G-CSF treatment. Since osteoblast suppression begins within 48 hours after the onset of G-CSF treatment (66,67), we isolated CD115+ cells at 3 and 16 hours after exposure to G-CSF and subjected them to expression profiling. Three different experiments were performed and the data was analyzed in two different ways: first, the data was analyzed in an unbiased fashion using the significance analysis of microarrays (SAM) algorithm. Using this approach with a false discovery rate of 10%, 15 genes were found to be either upregulated or downregulated. Of these, only one – IGFBP6 – has a described role in regulation of bone metabolism. Next, the data was analyzed in a biased fashion, tracking only the expression of 53 genes with known roles in regulation of osteoblasts. Of these, only six were expressed at a level detectable by the array. These genes are shown in Table 1 (courtesy of D. C. Link).

Table 1. Expression of genes that regulate osteoblast number and/or activation

Gene Symbol	Gene Name	Baseline	G-CSF (3 hours)	G-CSF (16 hours)	2-way Anova
Spp1	Osteopontin	840 ± 80	1,741 ± 316	2,077 ± 182	<0.01
Igf1	Insulin-like growth factor	1,207 ± 322	1,043 ± 247	591 ± 176	ns
Igfbp4	Insulin-like growth factor binding protein 4	8,102 ± 1,207	9,379 ± 790	7,857 ± 152	ns
Igfbp6	Insulin-like growth factor binding protein 6	288 ± 42	826 ± 93	1,769 ± 201	<0.001
Tgfb1	Transforming growth factor, beta 1	13,261 ± 526	11,672 ± 1,709	12,766 ± 629	ns
Jag1	Jagged1	277 ± 38	467 ± 95	165 ± 42	ns

Normalized array signal (mean ± SEM) for genes implicated in the regulation of osteoblasts.
If multiple probes for a gene were present, only the highest signal is shown

We used quantitative PCR analysis to confirm that the changes in expression of each of these genes was not simply the result of probe failure. To determine whether these changes in mRNA levels reflected changes in protein expression, we isolated bone marrow extracellular fluid from mice that had been treated with G-CSF for varying amounts of time[1] and analyzed the concentration of candidate factors using ELISAs. Because the most significant changes in expression were observed for osteopontin and IGFBP6, we focused our attention on those first.

Monocyte-derived osteopontin is not required for G-CSF-induced mobilization

Osteopontin (OPN, gene name *Spp1*) is a cell-surface glycoprotein primarily expressed by osteoblasts. There is no direct evidence implicating osteopontin in osteoblast survival; in fact, *Spp1*-/- mice have normal osteoblast numbers and morphology (59). There are a number of studies, however, suggesting a role for osteopontin in regulation of HSPC trafficking and maintenance of HSPC quiescence, including one study demonstrating that *Spp1*-/- mice exhibit constitutive mobilization (60,61,99-101). In response to G-CSF, the concentration of osteopontin in the bone marrow 3 days after G-CSF treatment was approximately 2.5-fold higher than in untreated mice, consistent with the changes in RNA expression noted on the microarray data (data not shown).

To test the importance of the rise in monocyte-derived osteopontin in G-CSF-induced mobilization, we transplanted marrow from *Spp1*-/- mice into lethally-irradiated wild-type recipients. Once reconstitution had occurred, the mice were treated with a seven-day course of G-CSF, after which the mobilization response was measured. We predicted that if an increase in monocytic osteopontin was important for G-CSF-induced mobilization, the *Spp1*-/- chimeras

[1] Mice were sacrificed by exposure to carbon dioxide and the femurs were dissected out. The patellar cap was removed and the marrow extracted from the femurs by centrifugation at 3,300 x g for 1 minute at room temperature. The resulting mixture of bone marrow cells and extracellular fluid was resuspended in 300 µL PBS. The cellular component was separated out by centrifugation at 3,300 x g. The resulting supernatant was used for ELISA.

would mobilize poorly, if at all. Unlike *Spp1*-/- mice, the *Spp1*-/- chimeras did not display constitutive mobilization, and their mobilization response to G-CSF was normal (data not shown). These data suggest that an increase in monocyte-derived osteopontin is not required for G-CSF-induced mobilization. Based on these data, we decided to abandon further exploration of the role of this protein in regulation of HSPC trafficking.

Monocytes may influence osteoblast survival through modulation of IGF signaling

Insulin-like growth factor binding protein 6 (IGFBP6) is a key regulator of IGF signaling. It is known to bind insulin-like growth factors (IGFs), preventing them from signaling through IGF receptors. The insulin-like growth factors IGF-I and IGF-II are anabolic peptides with described roles in promoting osteoblast survival (reviewed in (102)). The expression profiling of monocytic cells shown above revealed both an increase in IGFBP6 expression along with a decrease in IGF-I expression, suggesting an overall suppression of IGF-I signaling. By ELISA, the concentration of IGFBP6 in bone marrow extracellular fluid increased four-fold over baseline at 24 hours after G-CSF treatment, returning to twice the baseline value by 72 hours and remaining there for the next four days, consistent with the changes observed by microarray.

To evaluate the importance of IGFBP6 levels in regulation of HSPC trafficking, we are preparing a lentiviral construct with tetracycline-inducible expression of IGFBP6. We will infect bone marrow from mice expressing a tetracycline transactivator under the Rosa26 promoter (Rosa-tTA) and transplant these cells into lethally-irradiated mice. Once reconstitution has occurred, we will induce expression of IGFBP6 in the hematopoietic compartment. We predict that if the increase in IGFBP6 levels is an important element of the response to G-CSF, induction of IGFBP6 should result in constitutive mobilization.

We have also obtained a mouse in which the *Igf1* locus is surrounded by loxP sites. We are crossing this mouse with one expressing Cre recombinase under the control of a tamoxifen-inducible promoter (ERT2-Cre). Marrow from offspring homozygous for the floxed *Igf1* allele and carrying the ERT2-Cre transgene will be transplanted into lethally-irradiated wild-type mice. Once reconstitution has occurred, Cre recombinase expression will be induced, thereby diminishing production of IGF-I by hematopoietic cells. We predict that if hematopoietically-derived IGF-I is important for osteoblast survival, the induction of IGF-I deletion will result in osteoblast apoptosis and HSPC mobilization.

Finding the next set of candidates for 'Factor X'

The identification of factors present in the bone marrow extracellular fluid that may regulate survival of osteoblasts is a formidable technical challenge. We are currently exploring new approaches to identifying candidates for 'factor X.' One approach that we are considering is expression profiling of monocytic subpopulations. Preliminary data from our lab suggests that monocytic cells are lost from the bone marrow in response to G-CSF; we are currently tracking the frequency of various monocytic subpopulations using an approach similar to the one described by Chow, et al. (41) to determine if one of the subpopulations changes more dramatically than the others in response to G-CSF. If so, expression profiling of this subpopulation may identify additional candidates for 'factor X.' In addition, we are considering a proteomic analysis of bone marrow extracellular fluid before and after G-CSF treatment; however, only a small fraction of proteins can be analyzed in this fashion.

Conclusion

In this work, we report that monocytic cells are essential both for HSPC retention within the marrow but also for G-CSF-induced HSPC mobilization. Short-term efforts will be focused

on clarifying the roles of the various monocytic subtypes in this process, but the development of novel mobilizing agents will likely require the identification of factors important for communication between monocytic cells and the stromal populations which regulate HSPC retention.

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