January 2010

The Hematopoietic Compartment Regulates Osteoblast Differentiation And Apoptosis During Cytokine Treatment

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THE HEMATOPOIETIC COMPARTMENT REGULATES OSTEOBLAST DIFFERENTIATION AND APOPTOSIS DURING CYTOKINE TREATMENT

by

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A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

May 2010

St. Louis, Missouri

Acknowledgements

This work was supported by a grant from the National Institutes of Health (Bethesda, MD) RO1 HL60772

For Gina, Elly, and Ruth

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Biology and Biomedical Sciences
(Molecular Cell Biology)

Washington University in St. Louis, 2010

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Adult hematopoiesis normally occurs in bone marrow, where hematopoietic stem cells (HSC) reside within a specialized microenvironment. At steady state, hematopoiesis state is regulated such that immature hematopoietic stem and progenitor cells (HSPC) are restricted to the bone marrow and are rarely observed in peripheral blood. Under certain circumstances, however, this regulation is loosened and significant numbers of HSPC are released to the circulation, a process termed "mobilization." Mobilization can be induced pharmacologically by a wide range of agents. Of these, the best characterized and most widely used mobilizing agent is Granulocyte-colony stimulating factor (G-CSF). G-CSF is widely used clinically and several molecular mechanisms have been implicated as mediating its mobilizing action. However, many questions remain as to the relationship between these various pathways. This work begins by focusing on one mobilization pathway, the disruption by G-CSF of signaling between CXCL12, a chemokine expressed in the HSC microenvironment, and its receptor CXCR4, broadly expressed on hematopoietic cells. By examining mice

genetically deficient in CXCR4, we show that this mechanism is not only the predominant pathway by which G-CSF induces mobilization, but also a common pathway utilized during treatment with other hematopoietic cytokines. Next, while investigating the mechanism by which G-CSF disrupts CXCL12/CXCR4 signaling, we unexpectedly uncovered a role for osteoblasts in regulating cytokine-induced mobilization. By isolating and sorting different fractions of bone marrow stromal cells we demonstrate that osteoblasts represent a major source of CXCL12 in the bone marrow. In addition, both the number and function of mature osteoblasts declines sharply during cytokine treatment. Subsequent analysis demonstrated that G-CSF both increases the rate of osteoblast apoptosis and blocks osteoblast development. Finally, experiments with G-CSF receptor null chimeras demonstrate that this effect on osteoblasts is not direct but is mediated by the hematopoietic compartment. While the regulation of hematopoiesis by osteoblasts has been well described, the reciprocal regulation of osteoblasts by bone marrow hematopoietic cells has not been widely appreciated. Further work will be required to determine if this regulation occurs not only during the specialized setting of cytokine-induced mobilization but during steady state hematopoiesis in general.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Adult hematopoiesis normally occurs in bone marrow, where hematopoietic stem cells (HSC) reside. This small pool of slow-cycling cells can differentiate into highly proliferating committed progenitors of any hematopoietic lineage according to the needs of the host. As these progenitors differentiate into mature hematopoietic cells, they are released into the circulation to meet the host's need for oxygen transport, hemostasis, and innate and adaptive immunity. The hierarchical organization of hematopoiesis serves to protect the genomic integrity of the stem cell pool while retaining a proliferative potential capable of releasing billions of mature hematopoietic cells into circulation each day. Not surprisingly, then, the localization of HSC within their specialized bone marrow microenvironment and the subsequent release of their mature progeny into circulation are ordinarily tightly regulated.

During periods of stress or injury, however, this strict regulation is loosened, and HSC and other primitive hematopoietic progenitors—collectively termed "HPSC"—are released from the bone marrow, a process termed "mobilization". Conversely, HSC delivered intravenously after myoablative conditioning home from the circulation back to the bone marrow where they reconstitute recipient hematopoiesis.

The discovery in the 1990s that pharmacologic doses of hematopoietic cytokines mobilize HSC to the peripheral blood without harming the subject spurred research into the clinical feasibility of using mobilized peripheral blood HSC in stem cell transplants.[1,2] Mutiple clinical trials showed cytokine-mobilized peripheral blood stem cells to be an effective and cost-efficient source of cells for stem cell transplant.[3-6] Currently, the

majority of therapeutic stem cell transplants worldwide utilize mobilized peripheral blood HSC.[7,8]

Despite the wide clinical use of mobilized HSC, much about the mechanisms underlying HSPC mobilization remains unclear. The purpose of this work is to elucidate mechanisms by which HSPC are retained in the supportive bone marrow environment at steady state and are induced to migrate to the circulation during mobilization. It is hoped that a more precise understanding of these pathways will lead not only to improved protocols for HSC mobilization and collection in the transplant setting, but also to new insights into the manner in which HSPC are maintained in the bone marrow.

1.2 Hematopoietic stem cells reside within specialized "niches" in the bone marrow.

In the strictest sense of the term, a stem cell niche is a specialized microenvironment comprised of supporting cells and extracellular matrix where stem cells reside and receive signals necessary for their maintenance as stem cells. In *Drosophila* ovarioles, for example, a germline stem cell niche is provided by somatic cap cells which provide signals necessary to maintain the critical balance between germ stem cell self renewal and differentiation.[9]

Although the existence of a niche for HSC was first proposed by Schofield in 1978 [10], its anatomical location has remained unknown until relatively recently. Early studies revealed that primitive CFU-C and CFU-S increase in frequency closer to the endosteal surface in long bones.[11,12] Similarly, Nilsson et al. showed that sorted bone marrow populations enriched for stem cell activity (Rho123 low, lineage negative) preferentially homed to endosteal regions.[13] These observations raised the possibility that endosteum-

lining osteoblasts or osteoblast-lineage cells contribute to the stem cell niche. In vitro, osteoblasts abundantly express hematopoietic cytokines G-CSF, GM-CSF, and IL-6. Cultured osteoblasts support the maintenance of primitive hematopoietic cells as determined by CFU-C and LTC-IC assays.[14,15] Recent evidence has suggested that osteoblasts play a key role in supporting the stem cell niche in vivo, as well. Calvi et al reported that transgenic expression of a constitutively active form of the parathyroid hormone receptor (caPRP) under control of a 2.3kb fragment from the *col1a1* locus significantly expanded the osteoblast compartment as measured by standard histomorphometric techniques. This increase in osteoblast number was associated with a roughtly 2-fold increase in number of LTC-IC and in donor chimerism in a competitive repopulation assay, suggesting an increase in HSC number or function.[16] In a similar system, Zhang et al. expanded the osteoblast compartment by deleting the bone morphogenic protein receptor gene *bmpr1a* in non-hematopoietic cells. Similar to the results in Calvi et al., the increase in osteoblast number and trabecular bone was associated with a 2.2-fold increase in number of HSC as determined by limiting dilution assay.[17]

In the converse experiment, Visnjic et al. generated a transgenic mouse expressing the thymidine kinase suicide gene under control of the *col1a1* 2.3kb promoter. Four week treatment with gancyclovir resulted in a striking loss of mature osteoblasts and trabecular bone. Hematopoiesis in bone marrow was severely compromised, with a 5-10-fold decrease in cellularity, a 3-10-fold decrease in primitive c-Kit positive, Sca-1 positive, lineage negative (KSL) cells, and extensive extramedullary hematopoiesis.[18] Collectively, these studies suggest that the size of the HSC pool relates directly to the

osteoblast compartment, consistent with the possibility that osteoblasts play an important role in the HSC niche.

Much study has been given recently to understanding the molecular signals by which osteoblasts might regulate HSC. Osteoblasts express the adhesion molecule VCAM-1 and the chemokine Stromal-derived factor 1 (SDF-1/CXCL12), which play a role in HSC retention in the marrow (see below).[19-21] Calvi et al. observed enhanced osteoblast expression of Notch ligand Jagged-1 in their caPRP transgenic mice, and inhibitors of Notch signaling abrogate the enhanced LTC-IC numbers in their model, suggesting that Notch signaling contributes to HSC support in the osteoblastic niche.[16] In a different study, quiescence in HSC was identified with Tie-2 expression, and Tie-2 positive HSC were identified along endosteal surfaces. Tie-2 ligand angiopoietin 1 (Ang-1), which is expressed by osteoblasts, promoted maintenance of HSC quiescence and preserved long-term repopulating ability in ex vivo cultured HSC.[22] Osteoblasts also express several wingless (Wnt) family members, which have been implicated as important paracrine factors in maintaining the HSC pool.[23,24]

Besides factors expressed by osteoblasts, certain properties of the endosteal niche may contribute to HSC maintenance. Oxygen tension along the endosteal surface is lower than in the bone marrow proper, which might serve to lower the risk of HSC sustaining oxidative damage,[25] and HSC cultured in low oxygen conditions retain their repopulating activity better than HSC cultured in normoxia.[26] Similarly, local calcium ion concentrations along the endosteal surface are much higher than elsewhere in the bone marrow.[27] Lack of CaR, a calcium-sensing G-protein-coupled receptor expressed on HSC, leads to loss of HSC in the bone marrow, suggesting that the local calcium ion

concentration along the endosteal surface might contribute to HSC support.[28] Taken together, these findings suggest an important role for osteoblast-lineage cells in supporting HSC along bone marrow endosteal surfaces.

Despite the above-mentioned findings, the precise characterization of the HSC-supporting osteoblast population remains unclear. Zhang et al. show that slow-cycling, hematopoietic cells found along endosteal surfaces were localized near N-cadherin positive, spindle shaped bone lining cells (SNO cells), raising the possibility that this subset of osteoblastic cells defines the stem cell niche. Likewise, it is possible that other stromal components contribute to the stem cell niche. For example, Sugiyama et al. have identified a population of reticular stromal cells that localize near endosteal surfaces as well as elsewhere in the marrow. These cells, termed "CAR" cells (CXCL12-abundant reticular cells), express high levels of SDF-1, raising the possibility that they contribute to HSC maintenance in the bone marrow.[29]

Besides an endosteal stem cell niche, some evidence suggests that specialized bone marrow endothelium provides an additional niche for HSC. Recent advances in technology have allowed for identification of cell populations highly enriched for HSC. Kiel et al. showed that roughly half of CD150 positive, CD48 negative cells (SLAM cells) are true stem cells.[30] Surprisingly, immunofluorescent imaging localized only a small fraction of this population to the endosteal surface. The majority of SLAM cells were shown in the bone marrow adjacent to bone marrow sinusoids, suggesting that bone marrow endothelial cells may constitute a second HSC niche. In contrast, Sugiyama et al. showed SLAM cells localized to endothelial-associated CAR cells rather than to endothelium itself.[29] Despite these findings, there is currently no data linking

endothelial or CAR cells to HSC function. However, Sacchetti et al. showed that human reticular adventitial cells located adjacent to endothelium and expressing CD146 were necessary and sufficient to form ectopic bone when xenotransplanted into immunocompromise mice. This ectopic bone was colonized by hematopoietic cells, suggesting that these endothelium-associated CD146 positive cells may functionally support stem cells in vivo.[31]

In summary, the support of resident non-hematopoietic cells is critical for the maintenance of HSC in the bone marrow. In particular, osteoblasts fulfill the criteria for stem cell niche-defining cells in that their expansion results in increased HSC numbers and their ablation causes loss of hematopoiesis in the bone marrow. The observation that HSC localize to areas other then endosteal surfaces suggests, however, that HSC may reside in more than one niche and may indeed traffic between niches. Interestingly, despite their need to reside in a niche for long-term functioning, under certain circumstances HSC are observed to leave their bone marrow niche and are released to the circulation.

1.3 Mobilization of HSC from the bone marrow niche.

As described above, the release of hematopoietic cells from the bone marrow is tightly regulated to ensure that immature cells are retained in the bone marrow. Nevertheless, it has long been appreciated that low numbers of hematopoietic stem and progenitor cells (HSPC) can, in fact, be observed in the circulation.[32,33]

Whether these circulating HSPC serve a teleological function is unknown. Experiments in parabiotically joined mice demonstrate that circulating HSPC are able to

colonize distant sites, leading to speculation that circulating progenitors aid recovery from injury or infection.[34] Recent evidence for this hypothesis was provided by Massberg, et al. who showed that circulating HSPC can proliferate and differentiate into dendritic cells within peripheral organs in response to stimulation by Toll-like receptor agonists.[35] At baseline, these circulating HSPC are extremely low in number, estimated 100-400 per mouse.[36] However, a wide range of physiologic and pharmacologic stimuli—including bone marrow stress, hypoxia, infection, and cytotoxic drugs—can significantly increase the number of circulating HSPC, lending further credibility to the hypothesis that circulating HSPC aid recovery from injury (reviewed in [37]).

Mobilization can be induced by a wide variety of pharmacologic agents, including chemokines, cytokines, and cytotoxic drugs (see table 1). Besides these three broad classes, however, a surprisingly diverse list of mobilizing agents is accumulating. These include such disparate molecules as lipopolysachharide (LPS) and fucoidan, a sulfated polysaccharide isolated from seaweed.[38-40] As the molecular underpinnings of mobilization are elucidated, a second generation of mobilizing agents are being designed to activate specific mobilizing pathways. For example, AMD3100, a small molecule inhibitor of CXCR4, has been studied extensively in animal models and has undergone phase I clinical trials in both autologous and allogeneic settings.[41-43]

Mobilizing agents exhibit considerable diversity both in their presumed molecular targets as well as their kinetics, which range from 15-30 minutes to peak mobilization in the case of chemokines to greater than 5 days in the case of hematopoietic cytokines. Given this diversity, it is likely that multiple mechanisms exist for the mobilization of

hematopoietic progenitors. At the same time, the possibility remains that at least certain subsets of mobilizing agents may share common pathways.

Mobilizing Agent	Mobilization	Time to peak response	Reference
G-CSF	20-100-fold increase	7-14 days	[44,45]
GM-CSF	45-fold increase	7 days	[46,47]
Flt-3 ligand	500-fold increase	7 days	[48-50]
Stem Cell Factor	20-fold increase	7-10 days	[47,51]
VEGF	10-fold increase	5 days	[52]
Cyclophosphamide	62-fold increase	8 days	[53,54]
MIP1α	5-10-fold increase	15-30 minutes	[55,56]
Groβ	5-10-fold increase	15-30 minutes	[57]
Fucoidan	7-30-fold increase	30min-2 hr	[38]
LPS	8-fold	5 days	[39,40,58]
Pertussis Toxin	10-20-fold	3-4 days	[59]
AMD3100	5-fold	1 hr	[43]
Intense Exercise	4-fold	n.a.	[60,61]

Table 1.1. Parial list of mobilizing agents [adapted from Thomas et al. *Current opinion in hematology* (2002)]

Although both G-CSF and GM-CSF are currently approved for clinical use, the best studied and most widely used mobilizing agent is G-CSF. As will be discussed below, three general mechanisms have been implicated in mediating G-CSF-induced mobilization: 1) induction of bone marrow proteases, which are believed to cleave ECM and degrade key signaling molecules; 2) downregulation of adhesion molecules that maintain HSPC in the bone marrow at steady state; and 3) disruption of SDF-1/CXCR4 signaling, which is thought to signal to retain HSPC in the bone marrow.

1.4 Induction of proteases is a common motif in HSPC mobilization.

Treatment with G-CSF dramatically increases the myeloid content of the bone marrow along with a corresponding increase in the activity of neutrophil-derived serine proteases neutrophil elastase (NE) and cathepsin G (CG) as well as metalloproteinase 9 (MMP9).[62] Additionally, bone marrow expression of serpin family protease inhibitors decreases, resulting in the induction of a proteolytic bone marrow environment.[63] Proteolytic cleavage of ECM may facilitate HSPC migration from the extramedullary space to the

circulation. Also, enhanced protease activity might promote cleavage of adhesion molecules that tether HSPC to their stromal environment and degrade critical signaling molecules to facilitate HSPC mobilization. In support of this model, bone marrow extracts from treated mice have been shown to cleave the VCAM-1 adhesion molecule as well as chemokine SDF-1 and its receptor CXCR4.[64-66] Furthermore, MMP-9 can cleave in vitro the tyrosine-kinase receptor c-Kit which is expressed on HSPC.[67] Treating mice with inhibitors to NE or both NE and CG inhibited G-CSF-induced HSPC mobilization, and mobilization was blunted 40-50% in mice treated with anti-MMP9 neutralizing antibody.[66,68] These findings, however, contrast with other data showing that G-CSF-induced mobilization is unaffected in NE knockout mice, NE x CG knockout mice, MMP-9 knockout mice, and DPP1 knockout mice which are unable to activate neutrophil serine proteases in general.[69] Possible explanations for this discrepancy include the developmental adaptation of mice deficient from birth in certain proteases or the difference in strains used in these studies. Nevertheless, there currently exists no genetic evidence that neutrophil proteases play a role in G-CSF-induced mobilization.

Similar to G-CSF, there is evidence that protease upregulation plays a role in chemokine-induced mobilization. Treatment with CXCR2 ligands Groβ and IL8 increase plasma and bone marrow levels of MMP9, and treatment with neutralizing antibody against MMP9 or the broad-spectrum protease inhibitor serpin 1a/α1 antitrypsin blocked IL8-induced mobilization.[68,70,71] Curiously, while Groβ treatment mobilizes poorly in MMP9 deficient mice, IL8 mobilization occurs normally.[68,71]

The implication of neutrophil proteases in chemokine-induced mobilization, along with the observation that neutrophils are activated both by chemokines and G-CSF, has

led to the hypothesis that neutrophils play a key role in mobilization.[72,73] Indeed, peripheral neutropenias induced either by clearance with an antibody against Gr-1 or genetically in G-CSF receptor deficient mice prevents mobilization with IL-8.[71,74] The role of peripheral blood neutrophils is less clear in G-CSF-induced mobilization, however, as mixed G-CSFR knockout and wildtype bone marrow chimeras mobilize poorly with G-CSF despite having a normal complement of wild type neutrophils in circulation. (Link DC, unpublished data)

1.5 Adhesion molecules mediate HSPC retention in the bone marrow microenvironment.

The trafficking of HSPC from their local bone marrow microenvironment and release into circulation is thought to involve downregulation of adhesion molecules that normally govern interaction between HSPC and their local stromal and ECM elements. Hematopoietic cells widely express β1 and β2 integrins. β1 integrins VLA-4 and VLA-5 ($\alpha_4\beta_1$ and $\alpha_5\beta_1$) are expressed on bone marrow HSPC and their ligands are expressed on stromal cells and bone marrow ECM. VLA-4 expression and activity are high in bone marrow HSPC and decrease with differentiation. Given that HSPC are preferentially retained in the bone marrow, this observation raises the possibility that VLA-4 in particular may play a role in the release of mature hematopoietic cells during mobilization.[75,76] Indeed, treatment with anti-VLA5 antibody results in a modest HSPC mobilization in mice, and anti-VLA-4 antibody causes robust HSPC mobilization in mice and rhesus monkeys.[77,78] Similarly, mice with an induced deletion of the gene encoding α4 integrin have an elevated level of peripheral blood HSPC.[79] Finally, neutralizing antibody against vascular cell adhesion molecule 1 (VCAM-1), an immunoglobulin

superfamily member and VLA-4 ligand present on stromal cells, mobilizes HSPC to a similar extent as VLA-4 neutralization. Together, these findings suggest that VLA-4/VCAM-1 specifically mediate a HSPC/stromal cell interaction that retains HSPC in the bone marrow. [80]

In contrast to the β1 integrins, whose neutralization leads to HSPC mobilization, β2 integrin neutralization does not result in elevated HSPC numbers in peripheral blood. However, β2 integrin neutralization, either with neutralizing antibody or by genetic deletion, results in enhanced mobilization with VLA-4 neutralization, suggesting that β2 integrins may co-operate with β1 integrins in maintaining HSPC in the bone marrow but do not play a necessary role.[78]

Besides integrins, HSPC adhesion in the bone marrow microenvironment involves CD44, the receptor for hyaluronan, a gycosaminoglycan component of the ECM. Neutralizing antibodies against CD44 disrupt progenitor adhesion to fibronectin *in vitro* and cause a modest mobilization of HSPC *in vivo*.[81,82] On the other hand, CD44 null mice treated with G-CSF have attenuated rather than enhanced mobilization, suggesting that CD44 plays a more complex role than simply mediating HSPC retention to the bone marrow.[82]

1.6 SDF-1/CXCR4 signaling maintains HSPC in their bone marrow niche.

In addition to the role played by proteases and adhesion molecules in mediating HSPC mobilization, signaling between chemokine SDF-1 and its receptor CXCR4 has emerged in recent years as a third major mechanism regulating HSPC trafficking. SDF-1 (CXCL12), a chemokine of the C-X-C family, was initially discovered in a screen of

stromal cell line conditioned media as a chemoattractant for human CD34 positive progenitor cells.[83] Its primary receptor, CXCR4, is a G-protein-coupled seven transmembrane chemokine receptor present on bone marrow hematopoietic cells as well as neuronal, endothelial, and epithelial cells.[84] Several lines of evidence suggest a model wherein SDF-1, expressed by bone marrow stromal cells, signals to hematopoietic cells through CXCR4 to cause their retention in the bone marrow.

First, genetic ablation of CXCR4 signaling impairs retention of HSPC in the bone marrow. Mice deficient in either SDF-1 or CXCR4 die in late gestation with several developmental defects, including a failure of hematopoietic cells to colonize the bone marrow. SDF-1 knockout embryos have a high number of circulating hematopoietic stem cells, however, suggesting either a failure to home or a failure to be retained in the marrow. [85,86] CXCR4 null fetal liver cells do not engraft irradiated recipients efficiently despite homing normally to the bone marrow, again suggesting a defect in retention.[87] Resulting CXCR4 null chimeras have a high number of circulating HSPC.[88] Conversely, hypermorphic mutations in the CXCR4 gene that lead to enhanced signaling result in a human disease characterized by a failure of myeloid cells to be released normally from the bone marrow.[89,90]

Next, antagonism of CXCR4 signaling with AMD3100, a small molecule inhibitor, leads to rapid release of HSPC to the peripheral blood.[91] Continuous administration of AMD3100 leads to sustained and robust HSPC mobilization.[92] Similarly, administration of proteoglycans fucoidan and dextran sulfate both lead to mobilization with an concurrent reduction of bone marrow SDF-1.[93]

Finally, as will be described later, mobilization of HSPC during G-CSF treatment is associated with decrease of bone marrow SDF-1 protein and mRNA that mirrors the kinetics of mobilization.[21,66] At the same time, CXCR4 surface expression and signaling decrease on mobilized progenitor cells compared to bone marrow HSPC, suggesting that G-CSF-induced loss of CXCR4 on a subset of HSPC cells may synergize with loss of bone marrow SDF-1 during mobilization.[64]

The downstream cellular mechanisms by which CXCR4 signaling promotes retention in the bone marrow have not been fully elucidated. SDF-1 binding CXCR4 in human CD34 positive cells activates multiple signaling pathways including phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), and mitogen-activated protein kinases ERK-1 and -2. These events lead to tyrosine phosphorylation of focal adhesion components related adhesion focal tyrosine kinase (RAFTK) and the cytoskeletal protein paxillin. Subsequent cytoskeleton reorganization and cell migration can be blocked with small molecule inhibitors to PI3K and PKC.[94,95] However, it is not fully clear whether hematopoietic progenitors remain in the bone marrow simply via chemotactic signaling toward a gradient of SDF-1 produced by bone marrow stromal cells or whether other cellular processes are involved. Incubation of HSPC with SDF-1 increased VLA-4 affinity for its ligands and enhanced endothelial transmigration in a VLA-4 and VLA-5-dependent fashion in vitro, suggesting that SDF-1 signaling may promote HSPC retention in the niche through regulation of adhesion molecules as well as providing a chemotactic signal.[96,97]

In sum, these data suggest that the SDF-1/CXCR4 interaction is crucial for retention of hematopoietic cells in the bone marrow and that disruption of that interaction leads to

mobilization. How treatment with G-CSF and other mobilizing agents disrupt this interaction, however, is a matter of ongoing investigation.

1.7 Disruption of SDF-1/CXCR4 signaling during mobilization.

During treatment with G-CSF, SDF-1 protein decreases in the bone marrow. However, some controversy exists as to how this occurs. One hypothesis is that SDF-1 protein is degraded by bone marrow proteases. Christophersen *et al.* showed that CD26, a cell-membrane-associated dipeptidyl peptidase, inactivates SDF-1 by cleaving its amino terminal two residues.[98] Indeed, mice deficient in CD26 have a less severe drop in bone marrow SDF-1 and less robust mobilization in response to G-CSF.[99] Expression of CD26, however, does not change with G-CSF treatment, so while this mechanism may function basally to clear SDF-1 from the marrow, it cannot account for the decrease during G-CSF treatment.

In contrast to CD26, bone marrow levels of neutrophil-derived proteases NE, CG, and MMP9 increase during G-CSF treatment. Bone marrow extracts from G-CSF-treated mice cleave SDF-1 in vitro, which has led to the hypothesis that these proteases mediate mobilization in part by degrading SDF-1.[64,66] However, as noted above, mice deficient in neutrophil serine proteases as well as mice treated with broad spectrum metalloproteinase inhibitors mobilized normally,[69] indicating that these specific proteases are not required for SDF-1 degradation. Nevertheless, the great number and overlapping functions of proteases makes it difficult to rule out a role for proteolytic cleavage of SDF-1 based on knockout studies.

Alternatively, it is possible that G-CSF treatment leads to a fall in bone marrow SDF-1 by decreasing its expression. As discussed in Chapter 2 of this thesis, G-CSF treatment leads to a fall in total bone marrow SDF-1 mRNA with similar kinetics to the fall in SDF-1 protein, suggesting that this may be the primary mechanism by which G-CSF targets bone marrow SDF-1.[21,100] Finally, outside the context of hematopoietic mobilization, other studies have shown SDF-1 levels to be transcriptionally regulated.[101-103]

Notably, while downregulation of SDF-1 likely plays an important role in G-CSF-induced mobilization, it is unclear to what extent disruption of SDF-1/CXCR4 signaling plays a role in mobilization induced by other mobilizing agents. No data has been published examining the disruption SDF-1/CXCR4 signaling in bone marrow of mice mobilized with other cytokines or with cytotoxic agents. The kinetics and extent of mobilization induced by these agents, however, are similar to that of G-CSF and are compatible with a role for SDF-1 downregulation. In contrast, chemokine induced mobilization, which peaks within half an hour of chemokine administration, does not likely involve a transcriptional downregulation of SDF-1, and indeed, mobilization induced by the chemokine Groβ does not result in loss of bone marrow SDF-1.[68] Nevertheless, the possibility remains that attenuated CXCR4 signaling may contribute to mobilization induced by chemokine administration. One novel pathway by which this may occur is heterologous desensitization, a phenomenon wherein CXCR4 signaling may be blunted by activation of co-expressed cytokine receptors. A recent study showed that treatment of neutrophils with the CXC chemokine KC led to heterologous desensitization of CXCR4.[104] This effect, however, is not observed with all chemokine receptors, suggesting that downregulation of SDF-1/CXCR4 signaling is not the only mechanism

leading to neutrophil release.[105] Currently, however, there is no evidence for heterologous desensitization of CXCR4 signaling playing a role in HSPC mobilization.

To summarize, G-CSF-induced mobilization is associated with a loss of SDF-1 protein and mRNA in the bone marrow. While proteolytic degradation may play a role in SDF-1 degradation, the loss of SDF-1 mRNA suggests that G-CSF treatment targets SDF-1 transcription in the stromal components of the stem cell niche during HSPC mobilization. To understand how SDF-1 mRNA is downregulated during G-CSF treatment, it is necessary to identify which bone marrow cell types produce SDF-1.

1.8 SDF-1-producing cells in the bone marrow.

The drop in SDF-1 mRNA in the bone marrow suggests that G-CSF targets one or more SDF-1-producing cell populations in the bone marrow, leading to decreased SDF-1 mRNA. Some controversy exists regarding the exact identity of the populations that express SDF-1, and it has not yet been shown if any of these downregulate SDF-1 expression during G-CSF treatment. *In vitro*, the literature reports SDF-1 expression in immortalized stromal cell lines, primary endothelial cells, primary osteoblasts, and cell lines derived from those cell types.[20,86,106,107] Demonstrating SDF-1 expression *in vivo*, however, has proven more difficult. Ponomaryov *et al.* showed human SDF-1 expression in bone-lining osteoblasts, endothelial cells, and scattered, spindle-shaped stromal cells.[20] On the other hand, Ara *et al.*, using a transgenic mouse in which the GFP cDNA was inserted into the SDF-1 locus, showed no colocalization of mouse SDF-1 with endothelial marker PECAM-1.[108] Using this system, little or no SDF-1 expression was detected co-localized with osteocalcin, a specific osteoblast marker, although SDF-1 expression did

co-localize with a subset of VCAM-1 positive cells with reticular morphology termed "CAR" cells that could be found in close proximity to endothelial cells (see above).[29,109] These conflicting results are difficult to interpret, as it is difficult to tell whether replacing exon 2 of the CXCL12 locus with a GFP expression cassette has resulted in disregulated signaling. Further, the lack of co-expression with osteocalcin does not necessarily rule out SDF-1 expression by osteoblasts, as osteocalcin is a late osteoblast marker expressed only in a fraction of morphologically identifiable, functional osteoblasts.[110]

In sum, most studies to date report SDF-1 expression across a spectrum of non-hematopoietic cells in the bone marrow. Even so, further work is needed to resolve whether these populations include endothelial cells or osteoblasts.

1.9 Summary

Under normal circumstances, HSC reside in bone marrow stem cell niches which provide critical support at a molecular level to maintain their function as stem cells. Evidence from mouse models with enhanced osteoblastogenesis suggests that osteoblasts in particular compose a necessary part of the HSC niche. HSPC can be mobilized from the bone marrow in response to treatment with certain mobilizing agents, the best

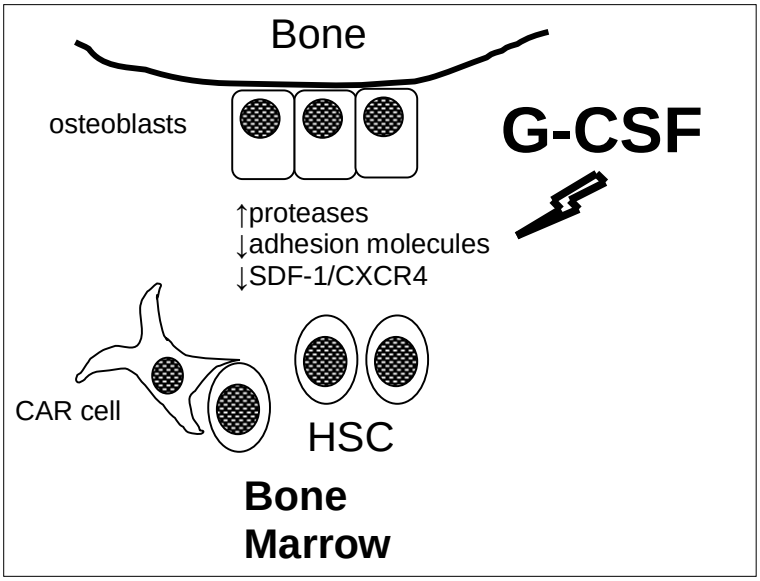

Figure 1.1 G-CSF disrupts the stem cell niche. Hematopoietic stem cells (HSC) are localized at steady state along endosteal surfaces. Their maintenance depends on the presence of osteoblasts and potentially other cell types (CAR cells=CXCL12-abundant reticular cells.) G-CSF treatment leads to upregulation of bone marrow proteases, downregulation of adhesion molecules VCAM-1/VLA-4 and disrupts SDF-1/CXCR4 signaling, leading to stem cell mobilization

characterized and most widely used of which is G-CSF. Several mechanisms likely play a role in G-CSF-induced mobilization, including induction of bone marrow neutrophil-derived proteases, downregulation of critical adhesion molecules, and disruption of SDF-1/CXCR4 signaling due in part to loss of bone marrow SDF-1 (see figure 1).

However, certain questions remain. First, what is the relationship between these several mechanisms in G-CSF-induced mobilization? Do they operate independently of each other or do they work in tandem? Next, to what extent do other mobilizing agents take advantage of these pathways to induce mobilization? Of note, the fall in bone marrow SDF-1 takes place over days during G-CSF treatment, raising the possibility that other cytokine family members—which also mobilize over a period of days—induce mobilization in part by dropping bone marrow SDF-1. Finally, what accounts for the loss of bone marrow SDF-1 during G-CSF treatment? What cell types make SDF-1 normally in the bone marrow, and which of these are targeted during G-CSF treatment?

In **Chapter 2** different subsets of bone marrow stromal cells will be tested for SDF-1 production and a mechanism will be proposed by which G-CSF treatment causes loss of

bone marrow SDF-1 by targeting osteoblasts. **Chapter 3** will extend these findings, investigating whether mobilizing cytokines Flt3 ligand (Flt3L) and stem cell factor (SCF) work in part by disrupting SDF-1/CXCR4 signaling and whether targeting of osteoblasts represents a common motif in cytokine-induced mobilization. Further, the relative importance of disrupted SDF-1/CXCR4 signaling vis-à-vis other pathways is tested by examining G-CSF induced mobilization in the genetic absence of CXCR4. Finally, **Chapter 4** examines the effect of G-CSF on osteoblasts in more detail, focusing on G-CSF effects on osteoblast differentiation and apoptosis. **Chapter 5** synthesizes these findings concerning cytokine effects on the stem cell niche with unpublished data concerning HSC function after G-CSF treatment and summarizes future directions.

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CHAPTER 2

G-CSF POTENTLY INHIBITS OSTEOBLAST ACTIVITY AND CXCL12 mRNA EXPRESSION IN THE BONE MARROW

The experiments presented in Figures 2.1-2.4 are the work of Dr. Craig Semerad, a former post-doctoral fellow in the lab.

2.1 ABSTRACT

There is accumulating evidence that interaction of stromal cell derived factor-1 (SDF-1/CXCL12) with its cognate receptor, CXCR4, generates signals that regulate hematopoietic progenitor cell (HPC) trafficking in the bone marrow. During granulocyte-colony stimulating factor (G-CSF)-induced HPC mobilization, CXCL12 protein expression in the bone marrow decreases. Herein, we show that in a series of transgenic mice carrying targeted mutations of their G-CSF receptor and displaying markedly different G-CSF induced HPC mobilization responses, the decrease in bone marrow CXCL12 protein expression closely correlates with the degree of HPC mobilization. G-CSF treatment induced a decrease in bone marrow CXCL12 mRNA that closely mirrored the fall in CXCL12 protein. Cell sorting experiments showed that osteoblasts and to a lesser degree endothelial cells are the major sources of CXCL12 production in the bone marrow. Interestingly, osteoblast activity, as measured by histomorphometry and osteocalcin expression, is strongly downregulated during G-CSF treatment. However, the G-CSF receptor is not expressed on osteoblasts, accordingly G-CSF had no direct effect on osteoblast function. Collectively, these data suggest a model in which G-CSF, through an indirect mechanism, potently inhibits osteoblast activity resulting in decreased CXCL12 expression in the bone marrow. The consequent attenuation of CXCR4 signaling ultimately leads to HPC mobilization.

2.2 INTRODUCTION

The majority of hematopoietic progenitor cells (HPC) reside in the bone marrow surrounded by a complex, highly organized microenvironment. Under normal conditions, a small number of HPC are released into the peripheral blood. Agents with distinct cellular targets and biological activities can induce the mobilization of HPC into blood, including hematopoietic growth factors, chemotherapeutic agents, and chemokines.[1,2] Recently, mobilized peripheral blood HPC have become the principal cellular source for reconstitution of the hematopoietic system following myeloablative therapy. Currently, granulocyte-colony stimulating factor (G-CSF) is the most widely used agent to induce HPC mobilization due to its potency, predictability and safety.[3] However, the mechanisms responsible for G-CSF-induced HPC mobilization have not been defined.

We previously showed that G-CSF receptor (G-CSFR) expression on HPC is not required for their mobilization by G-CSF, suggesting that G-CSF induces HPC mobilization indirectly through the generation of *trans*-acting signals.[4] The nature of the *trans*-acting signal(s) that mediate G-CSF-induced HPC mobilization is unknown; however, there is accumulating evidence suggesting that interaction of CXCL12 (stromal-derived factor-1) with its cognate receptor, CXCR4, may play an important role in regulating G-CSF-induced HPC mobilization. CXCL12 is a CXC chemokine constitutively produced in the bone marrow by stromal cells.[5] Studies of CXCL12 or CXCR4 deficient mice have established that these genes are necessary for the normal migration of HPC from the fetal liver to the bone marrow and in the efficient retention of myeloid precursors in the adult bone marrow.[6,7] Moreover, treatment with AMD-3100, a

specific antagonist of CXCR4, induces rapid and robust HPC mobilization in both humans and mice. [8,9] Finally, we and others showed that CXCL12 protein expression in the bone marrow is significantly decreased following G-CSF treatment.[10-12]. Collectively, these data suggest a model in which disruption of CXCL12/CXCR4 signaling is a key step in G-CSF induced HPC mobilization.

The mechanism(s) mediating the G-CSF induced decrease in CXCL12 protein expression in the bone marrow have not been defined. Previous reports suggested that neutrophil elastase (NE) and cathepsin G (CG) might regulate CXCL12 protein expression in the bone marrow through proteolytic cleavage of CXCL12.[10,11] However, mice genetically lacking NE and CG display normal G-CSF induced HPC mobilization, and the expected decrease in bone marrow CXCL12 protein was observed.[13] Thus, the G-CSF-induced decrease in CXCL12 protein expression in the bone marrow does not require these proteases. It is possible that other proteases can compensate for the loss of NE and CG. Alternatively, non-proteolytic mechanisms may regulate CXCL12 expression in the bone marrow during G-CSF induced HPC mobilization.

In this study, we characterize G-CSF induced HPC mobilization and CXCL12 expression in the bone marrow in a series of transgenic mice carrying targeted mutations of their G-CSFR. We provide further evidence that disruption of CXCL12/CXCR4 signaling in the bone marrow is a key step in HPC mobilization. G-CSF regulates CXCL12 expression in the bone marrow primarily at the mRNA level. Evidence is provided that G-CSF inhibits osteoblast number and activity through an indirect mechanism leading to decreased CXCL12 expression in the bone marrow.

2.3 METHODS

Mice. GEpoR, d715, and d715F deficient mice were generated, as described previously.[14-16] GEpoR, d715 and d715F mice were backcrossed 10 generations onto a C57BL/6 background. Six to ten week-old mice were used in all studies. Mice were housed in a specific pathogen-free environment. All experiments were approved by the Washington University Animal Studies Committee.

Mobilization protocols. *G-CSF*. Recombinant human G-CSF (Amgen, Thousand Oaks, CA) diluted in phosphate-buffered saline (PBS) with 0.1% low endotoxin bovine serum albumin (Sigma, St. Louis MO) was administered by daily subcutaneous injection at a dose of 250 μg/kg or 100 μg/kg per day for 5 days. Mice were analyzed 3-4 hours after the final G-CSF dose. *AMD3100*. AMD3100, a generous gift from AnorMED Inc. (British Columbia, Canada), was reconstituted in sterile PBS and administered as a single subcutaneous injection at a dose of 5 mg/kg. Mice were analyzed 3 hours post-injection or at the indicated times.

Peripheral Blood and Bone Marrow Analysis. Blood was obtained by retroorbital venous plexus sampling in polypropylene tubes containing EDTA. Complete blood counts were determined using a Hemavet automated cell counter (CDC Technologies, Oxford, CT). Bone marrow was harvested by flushing with α-modification of eagle's medium (α-MEM) containing 10% fetal calf serum. Bone marrow extracellular fluid was obtained by flushing each femur with 1 ml of ice-cold PBS without serum, and the supernatant was harvested after centrifugation at 400 x *g* for 3 minutes.

CXCL12α ELISA. 96-well plates were coated with 100 µl of CXCL12 capture antibody (2 µg/ml) diluted in PBS and incubated overnight at room temperature. After incubation for one hour at room temperature with 300 µl of blocking solution [1% bovine serum albumin (BSA), 5% sucrose, and .05% NaN_3], 100µl of sample was added to each well and incubated for 2 hours at room temperature. After washing, 100 µl of polyclonal biotinylated anti-human CXCL12 (250 ng/mL) in ELISA diluent (0.1% BSA, .05% Tween 20 in Tris-buffered saline at pH7.3) 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 of 2N H_2SO_4 to stop the reaction. A microplate reader set at 450 nm was used to determine optical density with readings at 550 nm subtracted from the results. Recombinant human CXCL12α was used to generate a standard curve. All ELISA reagents were purchased from R&D Systems (Minneapolis, MN).

Colony-forming cell assay. Blood, bone marrow, and spleen cells were harvested from mice using standard techniques and the number of nucleated cells in these tissues quantified using a Hemavet automated cell counter. We plated 10-20 µl blood, 1×10^5 nucleated spleen cells, or 2.0×10^4 nucleated bone marrow cells in 2.5 ml methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; Stem Cell Technologies, Vancouver, British Columbia, Canada). Cultures were plated in duplicate and placed in a humidified chamber with 6% carbon

dioxide (CO_2) at 37° C. Colonies containing at least 50 cells were counted on day 7 of culture.

Real-time quantitative RT-PCR. Femurs were flushed with a total of 2 ml Trizol reagent (Invitrogen, Carlsbad, CA) followed by crushing of the remaining bone in Trizol. 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 reaction mix, 200 nM forward primer, 200 nM reverse primer, 280 nM internal probe, and .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.

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'

Osteocalcin dT-FAM/TAMRA probe: 5'-

TGCGCTCTGTCTCTCTGACCTCACAGATGCCA-3'

Cell Sorting. Bone marrow cells were recovered from the femurs and tibia of mice by extensive flushing with 40 ml of PBS. The femurs were then infused with PBS containing 50mg/ml of type IV collagenase (C5138, Sigma) and incubated at 37^0C for 15 minutes. The collagenase-treated femurs were flushed again with PBS and cells pooled with the first flush fraction. Finally, the "empty" femurs were directly flushed with 1 ml of Trizol, to recover RNA from cells firmly adherent to the bone matrix. The flushed cells were incubated with fluorescein isothiocynate (FITC)-conjugated CD45 antibody and with the following panel of phycoerythrin (PE)-conjugated lineage-restricted antibodies: Gr-1 (granulocytes), B220 (B-lymphocytes), CD3e (T-lymphocytes), and Ter-119 (erythroid cells) (all antibodies from Pharmingen, San Diego, CA). Cells were sorted on a MoFlo high speed flow cytometer (Dako Cytomation, Fort Collins, CO). CXCL12 and β-actin mRNA were measured by quantitative real time RT-PCR. To estimate the total CXCL12 mRNA contribution of each fraction, the number of cells in each fraction was multiplied by the amount of CXCL12 mRNA relative to β-actin mRNA found in that fraction. The number of cells in the Trizol-flushed fraction was estimated using β-actin mRNA expression and was based on a standard curve showing that the level of β-actin mRNA correlated in a linear fashion with cell number (data not shown).

Stromal cell fractionation. Femora, tibiae and iliac crests were cleaned thoroughly to remove associated muscle tissue and then crushed in a mortar and pestle to

release the marrow. Bone fragments were collected by filtration through a 40 μm cell strainer (BD Biosciences, San Jose, CA) and washed extensively in PBS with 2% FCS to remove non-adherent bone marrow cells. The bone fragments were further minced with a scalpel and then incubated at 37^0C with a 3mg/ml solution of Type I collagenase (Worthington, Lakewood NJ) in PBS for 40 minutes in a shaking waterbath. The resulting population of bone derived cells was then depleted of residual hematopoietic cells by incubation with a cocktail of rat anti-mouse antibodies (B220, Mac-1, Gr-1, CD4, CD8, CD3, CD5 and Ter119) followed by incubation with anti-rat Ig coupled Dynabeads (Dynal Biotech, Oslo Norway). Following lineage depletion, the cells were stained with a PE-conjugated anti-CD45, FITC-conjugated anti-CD31, biotinylated anti-CD51 and streptavidin coupled allophycocyanin (all from Pharmingen). The cells were separated using a FACSDiva high speed cell sorter (BD Biosciences) into three fractions: endothelial cells (Lin^- $CD45^-$ $CD31^+$), osteoblasts (Lin^- $CD45^-$ $CD31^-$ $CD51^+$), and progenitor cells (Lin^-, $CD45^+$). The purity of the endothelial and osteoblast fractions was confirmed by staining for von Willebrand factor or alkaline phosphatase, respectively (data not shown). Sorted cells were counted and then lysed in RNAZol (Iso-Tex Diagnostics, Friendswood, TX) or Trizol for RNA isolation and subsequent real time RT-PCR analysis.

Osteoblast Culture. Murine calvarial osteoblasts were obtained using minor modifications of published procedures.[17] In brief, calvariae were removed aseptically from 3-4 day old mice and incubated twice at 37^oC for 10 minutes in PBS containing 4mM EDTA and then subjected to repeated digestion for 10 minutes at 37^oC with

200U/ml type 2 collagenase (Worthington, Lakewood, NJ) in PBS. Products of early digestions were discarded, while later fractions (typically fractions 5-7) were collected by centrifugation and cultured in αMEM containing 10% FCS and 1% Pen/Strep. Cells were cultured until 80% confluent (undifferentiated osteoblasts). In some experiments, cells were then cultured in differentiation medium (αMEM containing 10% FCS, 100 μg/ml ascorbic acid and 5mM β-glycerophosphate) for 1 week (differentiated osteoblasts).

Histomorphometry. Osteoblasts in the bone marrow were quantified by histomorphometry, as previously described.[18] Briefly, femurs and tibiae were harvested, fixed overnight in 10% neutral formalin, decalcified by incubating in 14% EDTA at 4^0C for two weeks, and then embedded in paraffin. To ensure that osteoclasts were excluded from the osteoblast count, deparaffinized sections were stained histochemically for tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin. Osteoblasts were counted in a blinded fashion in 4-6 200X fields per section. In some cases, two sections 75 microns apart were taken from the same sample and osteoblast number averaged. The number of osteoblasts per millimeter bone perimeter (N.Ob/mm) was calculated using the OsteoMeasure Histomorphometry System (OsteoMetrics, Inc., Atlanta, GA).

Statistical analysis. Data are presented as mean +/- SEM or SD, as indicated in the text. Statistical significance was assessed using a two-sided Student's t test.

2.4 RESULTS

2.4.1 The membrane-proximal region of the G-CSFR is sufficient to mediate HPC mobilization.

To define the region(s) of the G-CSFR required for HPC mobilization, G-CSF induced HPC mobilization was characterized in a series of transgenic mice expressing different targeted mutations of their G-CSFR (figure 1A). The d715 G-CSFR mutation introduces a premature stop codon at nucleotide 2403, leading to truncation of the carboxy-terminal 96 amino acids of the G-CSFR. It is representative of G-CSFR mutations found in approximately 35% of patients with severe congenital neutropenia.[19] Mice homozygous for the d715 G-CSFR mutation have normal basal hematopoiesis.[15] In the d715F G-CSFR mutant, the sole remaining tyrosine (Y704) of d715 has been mutated to phenylalanine. STAT-3 and STAT-5 activation by the d715F G-CSFR are markedly impaired.[14] Homozygous d715F G-CSFR mutant mice display an isolated defect in granulopoiesis.[14] In the GEpoR mutation, the entire cytoplasmic (signaling) domain of the G-CSFR is replaced with that of the erythropoietin receptor (EpoR).[16] This chimeric receptor is predicted to bind G-CSF but transmit EpoR-specific signals. Homozygous GEpoR mice display peripheral neutropenia but have normal numbers of neutrophils in their bone marrow.[16]

The G-CSFR mutant mice, all inbred on a C57BL/6 background, were treated with G-CSF (250 μg/kg/day x 5 days) and the number of colony forming cells (CFU-C) in the blood, spleen, and bone marrow measured (figure 1B). A similar number of CFU-C was present in the bone marrow of all mice except for G-CSF treated d715 mice, where a modest, but not statistically significant, increase was observed. Compared with wild type mice, HPC mobilization was significantly enhanced in d715 mice. Whereas a 15-fold increase from baseline in blood CFU-C was observed in wild type mice, a 32-fold

increase was observed in d715 mice. In contrast, HPC mobilization was severely impaired in GEpoR mice (1.7-fold increase in blood CFU-C from baseline), despite a normal number of CFU-C in the bone marrow. d715F mice displayed an intermediate phenotype. Though G-CSF induced a similar rise in blood and spleen CFU-C, the number of CFU-C in the bone marrow of d715F mice was significantly increased compared with wild type mice. Similar results were observed after treating mice with 100 μg/kg/day G-CSF for 5 days (data not shown). These data show that the membrane-proximal 87 amino acids of the G-CSFR are sufficient to mediate G-CSF-induced HPC mobilization. Moreover, these data show that the signals generated by the GEpoR are not able to substitute for those of the G-CSFR to induce HPC mobilization.

2.4.2 Down-regulation of CXCL12α protein expression is a key event in G-CSF induced HPC mobilization. Accumulating evidence suggests that CXCL12/CXCR4 signaling may be a key regulator of HPC trafficking in the bone marrow. We and others previously showed that CXCL12α protein expression in the bone marrow decreases during G-CSF-induced HPC mobilization.[10-12] To extend these findings, we measured CXCL12α protein levels in the bone marrow of the G-CSFR mutant mice following G-CSF treatment (figure 2A). As expected, G-CSF induced a significant decrease in CXCL12α protein expression in the bone marrow of wild type mice. Likewise, a significant decrease in CXCL12α protein expression in the bone marrow of d715 and d715F mice was observed. In contrast, consistent with their impaired HPC mobilization phenotype, no significant change in CXCL12α protein expression was detected in GEpoR mice. In fact, a highly significant correlation was observed between the degree

of HPC mobilization and the level of CXCL12α protein in the bone marrow ($p<.001$, figure 2B).

Recently, AMD3100, a selective CXCR4 antagonist capable of rapidly inducing HPC mobilization, was described.[9] To determine whether disruption of CXCR4 signaling could rescue the HPC mobilization defect in GEpoR mice, mice were treated with AMD3100 and HPC mobilization characterized. As reported previously, in wild type mice, treatment with a single subcutaneous injection of AMD3100 induced a rapid increase in blood CFU-C that peaked 3 hours post-injection (figure 3A).[8] Interestingly, a similar increase in blood CFU-C was observed in GEpoR mice. Moreover, HPC mobilization by AMD3100 was found to be normal in G-CSFR deficient mice (data not shown). These data show that AMD3100-induced HPC mobilization does not require G-CSFR signals. Collectively, these data suggest that down-regulation of CXCL12 protein levels in the bone marrow is a key event in HPC mobilization induced by G-CSF.

2.4.3 G-CSF regulates expression of CXCL12 mRNA in the bone marrow during HPC mobilization. Whereas previous studies have focused on the proteolytic cleavage of CXCL12, we considered an alternative mechanism to account for the decrease in CXCL12 protein in the bone marrow. We measured CXCL12 mRNA expression in the bone marrow during G-CSF treatment by directly flushing isolated femurs with Trizol reagent to ensure that RNA was recovered from all cell types in the bone marrow. Real time RT-PCR was performed for CXCL12 and mouse β-actin, as a control for RNA quality and content. CXCL12 mRNA progressively decreased during G-CSF treatment reaching a nadir on day 5 when HPC mobilization is maximal and returned to normal 2

days after discontinuing G-CSF (figure 5A). The decrease in CXCL12 mRNA closely mirrored the decrease in CXCL12α protein expression in the bone marrow (figure 5A). In fact, a strong correlation between CXCL12 mRNA and protein was observed (figure 5B), suggesting that CXCL12 expression is regulated primarily at an mRNA level by G-CSF.

2.4.4 Osteoblasts are the major source of CXCL12 in the bone marrow. Though controversial, current evidence suggests that CXCL12 is expressed in the bone marrow by osteoblasts, endothelial cells, and scattered stromal cells.[5,20] Moreover, a recent report suggested that stem and progenitor cells may express CXCL12 at a low level.[21] To determine which cell type(s) in the bone marrow express CXCL12 mRNA and are downregulated in response to G-CSF, mice were treated with G-CSF and bone marrow cells sorted into stromal cell (CD45-negative; lineage-negative), progenitor-enriched (CD45-positive; lineage-negative), and mature hematopoietic cell (lineage-positive) fractions (figure 5). In addition, Trizol was directly injected into the flushed femurs to assess the contribution of cells remaining tightly associated with the bone matrix ("bone fraction" in figure 5B). In untreated mice, the great majority of CXCL12 mRNA was found in the stromal cell and bone fractions (figure 5B). Furthermore, CXCL12 expression in these fractions was decreased by G-CSF-treatment. These results suggest that stromal cells are the major source of CXCL12 in the bone marrow and are downregulated by G-CSF treatment.

To define which stromal cell type(s) express CXCL12, the bone-adherent cell population was further fractionated into hematopoietic progenitor, mature osteoblast, and

endothelial cell fractions (see Methods). Low level CXCL12 mRNA was again detected in the hematopoietic cell fraction (figure 5C). Consistent with previous studies showing constitutive CXCL12 expression in bone marrow endothelial cells,[22] a relatively high level of CXCL12 mRNA was detected in the endothelial cell fraction. However, the highest level of CXCL12 mRNA expression was detected in the mature osteoblast fraction. Relative to β-actin mRNA, mature osteoblast express 9.4-fold more CXCL12 mRNA than endothelial cells. These data suggest that the majority of CXCL12 in the BM microenvironment is produced by osteoblasts.

2.4.5 G-CSF treatment potently inhibits osteoblast activity in the bone marrow.

Surprisingly, despite the decrease in total bone marrow CXCL12 mRNA expression (figure 5b), on a per cell basis no significant decrease in CXCL12 mRNA was detected in osteoblasts isolated from mice following G-CSF treatment (figure 5C). These data raised the possibility that, rather than affecting SDF-1 expression per osteoblast, G-CSF regulated the number of osteoblasts in the bone marrow. To explore this possibility, osteoblast number in the bone marrow was measured by histomorphometry. Indeed, after 5 days of G-CSF treatment, a striking reduction in the number of endosteal osteoblast was observed (figure 6 A-C). To confirm this observation, the expression of osteocalcin, a specific marker of mature osteoblasts, in the bone marrow during G-CSF treatment was assessed (figure 6D). Notably, osteocalcin mRNA expression was sharply reduced during G-CSF treatment; a 47 $\pm$ 12 fold reduction in osteocalcin mRNA (relative to β-actin mRNA) was observed in the bone marrow of G-CSF-treated mice compared with untreated mice. Likewise, a significant decrease in serum osteocalcin protein was

detected in G-CSF treated mice (data not shown). This latter finding is consistent with a previous report showing that serum levels of osteocalcin decreased in patients during G-CSF treatment.[23] Collectively, these data provide strong evidence that G-CSF treatment potently suppresses osteoblast activity in the bone marrow.

We next investigated whether G-CSF could directly regulate CXCL12 expression in cultures of primary murine osteoblasts. Osteoblasts were harvested from the calvariae of newborn mice and cultured in the presence or absence of G-CSF for 5 days. In some experiments, the osteoblasts were first cultured for 1 week in the presence of ascorbic acid and β-glycerophosphate to induce osteoblast differentiation. As expected, a high level of CXCL12 protein and mRNA expression was detected in cultures of undifferentiated and differentiated osteoblasts (figure 6E and data not shown). However, G-CSF had no significant effect on CXCL12 expression. Moreover, no G-CSFR mRNA was detected using a sensitive RT-PCR assay (data not shown). These data suggest that G-CSF does not directly regulate CXCL12 expression in osteoblasts.

2.5 DISCUSSION

Clinically, G-CSF is the most widely used agent to mobilize HPC, yet the mechanisms mediating HPC mobilization by G-CSF are poorly understood. To begin to define the region(s) of the G-CSFR that mediate this response, we characterized HPC mobilization by G-CSF in a series of transgenic mice carrying different targeted G-CSFR mutations. HPC mobilization in d715 G-CSFR mice is significantly enhanced compared with wild type mice, suggesting the presence of an inhibitory domain in the carboxy-terminal tail of the G-CSFR. Previous studies have shown that both receptor internalization[24,25] and

activation of negative regulators of signaling (including SOC3,[26] SHP-1,[25] and SHIP[25]) are defective with the d715 G-CSFR. Whether any of these signaling alterations is responsible for the increased mobilization response remains to be answered. Interestingly, the number of CFU-C in the bone marrow of G-CSF treated d715F mice is increased compared with G-CSF treated wild type mice, despite comparable numbers of the CFU-C in the blood and bone marrow. These observations are consistent with a subtle defect in HPC mobilization in d715F mice. Nonetheless, these data suggest that STAT 3 and STAT 5 activation by the G-CSFR is not absolutely required for HPC mobilization, since their activation by the d715F G-CSFR is markedly impaired.[14] Interestingly, G-CSF-induced HPC mobilization is markedly impaired in GEpoR mice, despite a comparable (to wild type mice) expansion in myeloid cells and HPC in the bone marrow. Thus, signals generated by the chimeric GEpoR are able to efficiently transduce proliferative but not mobilization signals, suggesting an element of specificity in the mobilization signaling pathways. Of note, these data clearly demonstrate that increases in bone marrow cellularity and HPC content alone are not sufficient to induce HPC mobilization

Accumulating evidence suggests that CXCL12/CXCR4 signaling plays a key role in regulating HPC trafficking in the bone marrow. Mice with targeted disruptions of CXCL12 or CXCR4 exhibit defective hematopoiesis in the bone marrow, possibly due to the failure of HPC to migrate from the fetal liver to the bone marrow. [7,27] Moreover, mice transplanted with CXCR4 deficient bone marrow cells show reduced engraftment and premature release of immature myeloid cells into the blood.[7,27] Elevation of CXCL12 levels in the blood by administration of CXCL12 or by injection of an

adenoviral vector expressing CXCL12 is associated with a significant mobilization of HPC into the blood. [28,29] Conversely, treatment with AMD3100, a selective antagonist of CXCR4, induces rapid and robust HPC mobilization in mice and humans.[8,9] Finally, we and others previously showed that G-CSF treatment results in a significant decrease in CXCL12 protein levels in the bone marrow of wild type mice. [10-12] In the present study, we show that CXCL12 protein levels in the bone marrow after G-CSF treatment strongly correlate with HPC mobilization in the G-CSFR mutant mice. For example, the greatest decrease in CXCL12 protein expression in the bone marrow was observed in those mice displaying the most robust HPC mobilization, namely the d715 G-CSFR mice. Perhaps most telling is the lack of a significant decrease in CXCL12 protein expression in the mobilization defective GEpoR mice (figure 2). The availability of AMD3100, a selective CXCR4 antagonist, provided the opportunity to determine whether disruption of CXCR4 signaling could rescue the mobilization defect in GEpoR mice. Indeed, AMD3100-induced HPC mobilization in GEpoR mice was comparable to wild type mice. Collectively, these data suggest that CXCL12 is an important retention signal for HPC in the bone marrow, and the data support a model in which disruption of CXCL12/CXCR4 signaling is a key step in G-CSF-induced HPC mobilization.

It is likely that multiple mechanisms contribute to the disruption of this signaling pathway. CD26 (dipeptidylpeptidase IV), a membrane-bound extracellular serine-protease expressed on a subset of HPC, inactivates CXCL12 through proteolytic cleavage.[30,31] Importantly, G-CSF induced HPC mobilization is defective in CD26 deficient mice or in wild type mice treated with a specific CD26 inhibitor.[30,31] However, there is no evidence showing that CD26 activity is modulated during G-CSF treatment.

In contrast, G-CSF treatment induces the release of a number of proteases into the bone marrow microenvironment, including NE, CG and matrix metalloproteinase-9 (MMP-9).[32] These proteases are able to cleave several adhesion molecules thought to play an important role in regulating HPC trafficking in the bone marrow, including c-Kit, VCAM-1, CXCR4, and CXCL12.[10,11,33,34] In particular, NE and CG are able to cleave and inactive CXCL12 *in vitro*.[10,11] However, G-CSF induced HPC mobilization and decrease in bone marrow CXCL12 protein are normal in NE x CG deficient mice.[13] Thus, there must be efficient NE and CG-independent mechanisms to disrupt CXCL12/CXCR4 signaling during G-CSF induced HPC mobilization.

As an alternative mechanism to proteolytic cleavage to regulate CXCL12 expression, we examined the effect of G-CSF treatment on the expression of CXCL12 mRNA in the bone marrow. We show that G-CSF treatment induces a decrease in bone marrow CXCL12 mRNA that mirrors the fall in CXCL12 protein. In fact, a strong correlation between CXCL12 protein and mRNA levels in the bone marrow was observed. This decrease in CXCL12 mRNA is not simply due to the dilution of CXCL12-expressing cells in the bone marrow during G-CSF treatment, since no significant decrease in CXCL12 mRNA was observed in GEpoR mice, despite a similar expansion of myeloid cells in the bone marrow. These data suggest that during G-CSF induced HPC mobilization, CXCL12 expression in the bone marrow is primarily regulated at the mRNA level.

The mechanism by which G-CSF regulates CXCL12 mRNA expression in the bone marrow is an important unanswered question. In particular, the cell type(s) in the bone marrow that express CXCL12 and are regulated during G-CSF treatment are

unknown. One report suggested that CXCL12 is primarily expressed by osteoblasts, endothelial cells, and scattered stromal cells in the mesenchyme.[5] In contrast, Ara and colleagues, using a transgenic mouse in which the green fluorescent protein gene was inserted into the CXCL12 gene locus, reported that endothelial cells and osteoblasts in the bone marrow did not constitutively express CXCL12.[35] Finally, a recent report suggested that a subset of hematopoietic progenitors produce a small amount of CXCL12.[21]

To address this question, we quantified CXCL12 mRNA expression in sorted bone marrow populations of mature hematopoietic cells, progenitor cells, and stromal cells. These data confirm that progenitor cells, defined as CD45-positive lineage-negative cells, express a low level of CXCL12. Given the low level of expression and the relative scarcity of these cells, it is unlikely that hematopoietic progenitor cells contribute significantly to the bulk production of CXCL12 in the bone marrow. Nonetheless, it is possible that CXCL12 expression by progenitor cells may significantly regulate the trafficking of progenitors cells through an autocrine or paracrine mechanism; further study is needed to address this possibility. On the other hand, bone marrow stromal cells appear to be the major source of CXCL12 in the bone marrow. Within the stromal cell fraction, endothelial cells and mature osteoblasts express significant CXCL12 mRNA. Based on the high level of CXCL12 expression per cell and the relative abundance of osteoblasts within the bone marrow stromal cell fraction, we conclude that osteoblasts are the major source of CXCL12 in the bone marrow. Interestingly, CXCL12 mRNA expression per osteoblast did not change during G-CSF treatment. Rather, G-CSF

appears to regulate CXCL12 mRNA expression in the bone marrow by decreasing osteoblast number.

There is accumulating evidence that osteoblasts play a key role in establishing and maintaining the stem cell niche in the bone marrow.[20,36,37] In addition to CXCL12, osteoblasts express several genes thought to be important for stem cell function, including the notch ligand Jagged-1,[37] a number of hematopoietic growth factors (e.g., G-CSF),[38] angiopoietin,[39] and N-cadherin.[36] Herein, we show that G-CSF potently inhibits mature osteoblast activity in the bone marrow. After 5 days of G-CSF treatment, mature osteoblast number in the bone marrow was reduced at least 3-fold. Moreover, osteocalcin mRNA expression in the bone marrow was reduced nearly 50-fold. The magnitude of the change in osteocalcin expression compared with the change in osteoblast number in the bone marrow suggests that G-CSF may regulate both osteoblast number and activity. Intriguingly, patients treated long term with G-CSF develop marked osteopenia.[40] In addition, transgenic mice overexpressing G-CSF develop osteopenia.[41,42] Collectively, these data raise the possibility that G-CSF, by regulating osteoblast function, may have profound effects on the stem cell niche that ultimately contribute to HSC mobilization.

We previously showed by analysis of bone marrow chimeras between G-CSFR deficient and wild type mice that G-CSFR expression on bone marrow stromal cells was neither necessary nor sufficient to mediate G-CSF-induced hematopoietic progenitor cell mobilization.[4] Consistent with this finding, in the present study we show that cultured primary osteoblasts do not express detectable G-CSFR using a sensitive RT-PCR assay. Moreover, G-CSF does not modulate CXCL12 expression in primary osteoblast cultures.

Together, these data provide compelling evidence that G-CSF regulates osteoblast CXCL12 through an indirect mechanism.

In summary, this study provides additional evidence that strongly supports a model in which disruption of CXCL12/CXCR4 signaling is a key event in G-CSF induced HPC mobilization. Osteoblasts appear to be the major source of CXCL12 production in the bone marrow. G-CSF treatment potently inhibits osteoblast activity in the bone marrow, thereby reducing CXCL12 expression. These data suggest a model (figure 7) in which G-CSF initiates the mobilization cascade by stimulating a, as yet unidentified, G-CSFR expressing cell population in the bone marrow. These cells then generate a trans-acting signal that suppresses osteoblast activity and, in particular, CXCL12 expression. The consequent decrease in CXCR4 signaling in hematopoietic progenitor cells then enhances their migration from the bone marrow, through unclear mechanisms. A better understanding of the mechanism by which G-CSF regulates CXCL12 mRNA expression may lead to the development of improved clinical protocols for stem cell mobilization in patients.

ACKNOWLDGEMENTS

The authors thank Jill Mayer and Katherine Stumpf for their expert technical assistance. We also thank Deborah Novack and Pat Kellar for their expert assistance in performing the osteoblast histomorphometry. Finally, the authors thank AnorMed for generously providing the AMD-3100.

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2.7 FIGURE LEGENDS

Figure 2.1 G-CSF-induced HPC mobilization in G-CSFR mutant mice. A. Schematic of targeted G-CSF receptor mutations. Cytoplasmic tyrosines (Y) and the conserved box 1 and box 2 motifs are indicated. In the d715F mutant, the sole remaining tyrosine (Y704) of the G-CSFR has been mutated to phenylalanine (F). B. Tissue distribution of HPC following G-CSF treatment. Wild type (WT) and G-CSFR mutant mice (n=4, each) were treated with G-CSF (250 μg/kg/day) for 5 days and the number of CFU-C in blood, spleen, and bone marrow quantified 4 hours after the final dose of G-CSF. Data represent the mean +/- SD. $^{*}P < 0.05$ compared with G-CSF treated wild type mice.

Figure 2.2 CXCL12α protein expression in the bone marrow following G-CSF treatment. A. G-CSFR mutant mice (n=7, each) were treated with G-CSF (100 μg/kg/day) for 5 days and the amount of CXCL12α protein in the bone marrow extracellular fluid measured by ELISA. Data represent the mean +/- SD. $^{*}p < 0.05$ compared with untreated mice of the same genotype. B. Plot of CXCL12α protein in the bone marrow versus the log of number of CFU-C in the blood on day 5 of G-CSF treatment ($p<.001$).

Figure 2.3 AMD3100 mobilization in GEpoR mice. Mice were treated with a single subcutaneous injection of AMD3100 (5 mg/kg). The number of CFU-C in the blood was measured over a 6 hour period (n=3-4, each time point). Data represent the mean +/- SD.

Figure 2.4 CXCL12 mRNA expression during G-CSF-induced HPC mobilization. A. Wild type mice were treated with G-CSF (100 μg/kg/day) for 5 days followed by a 2-day recovery period. The number of CFU-C in the blood (upper panel) and CXCL12 protein expression in bone marrow extracellular fluid (middle panel) were measured at the indicated time points (n=2, each). CXCL12 mRNA expression in the bone marrow was measured by directly flushing femurs with Trizol and performing real time RT-PCR

on the recovered RNA. Shown is the relative amount of CXCL12 mRNA compared with β-actin mRNA (lower panel). B. Plot of CXCL12α protein versus CXCL12 mRNA (r^2=0.56, p<.02). C. Wild type and GEpoR mice (n=6, each) were treated with G-CSF for 5 days and CXCL12 mRNA quantified. Data represent the mean +/- SD. $^{*}p < 0.05$ compared with day 0 or untreated mice.

Figure 2.5 Regulation of bone marrow stromal cell activity during G-CSF induced HPC mobilization. **A.** Bone marrow cells were recovered from the femurs and tibiae of mice by flushing and collagenase treatment and then sorted into the indicated cell populations based on CD45 and lineage expression. Shown is a representative histogram. **B**. To examine cells firmly adherent to the bone matrix, the flushed femurs were injected with Trizol to obtain the "bone fraction". Total CXCL12 mRNA in each cell population was estimated by multiplying the measured CXCL12 mRNA by the cell number in each cell fraction; the number of cells in the bone fraction was estimated based on β-actin mRNA levels. *p<.05. **C**. Cells harvested from the bone fraction were sorted into the indicated cell populations (see Methods) and CXCL12 mRNA expression relative to β-actin expression measured. Data represent the mean $\pm$ SEM.

Figure 2.6 G-CSF inhibits osteoblast activity in the bone marrow. Wild type mice were treated with G-CSF (125 μg/kg twice daily for 5 days) and osteoblast activity assessed (A-D). A&B. Representative photomicrographs showing endosteal osteoblasts (arrows) in untreated (A) or G-CSF treated mice (B). Original magnification x 400. C. Quantification of osteoblast number by histomorphometry. Shown are the number of osteoblasts (N.Ob) per mm of bone perimeter. D. Bone marrow osteocalcin mRNA expression. Total bone marrow RNA was obtained by directly flushing femurs with Trizol. The expression of osteocalcin mRNA relative to β-actin mRNA is shown. E. Primary osteoblasts were cultured in the presence of 100 ng/ml of G-CSF for the

indicated time and CXCL12 mRNA quantified. Data represent the mean +/- SEM. *p<.05.

Figure 2.7 Model of G-CSF induced HPC mobilization. Osteoblasts constitutively produce large amounts of CXCL12, providing an important retention signal for HPC in the bone marrow. G-CSF initiates the mobilization cascade by stimulating a population of G-CSFR$^+$ cells in the bone marrow. These cells, in turn, negatively regulate osteoblast number and activity, resulting in decreased CXCL12 expression in the bone marrow. The consequent decrease in CXCR4 signaling in HPC leads to their migration from the bone marrow to blood.

2.8 FIGURES

Figure 2.1

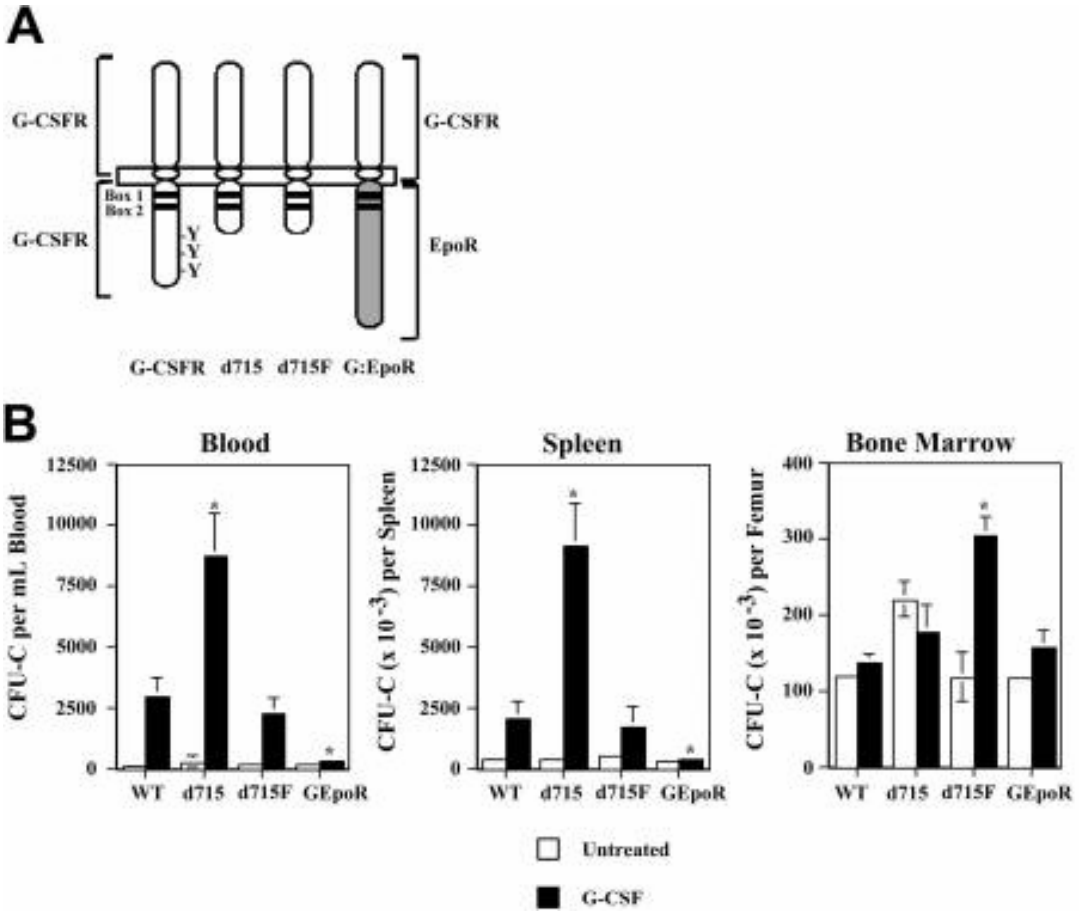


Figure 2.2

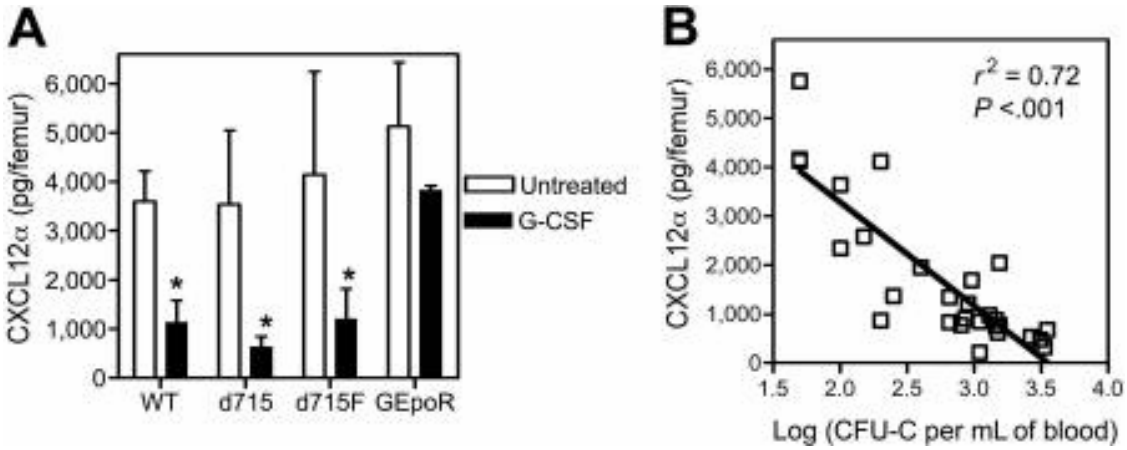
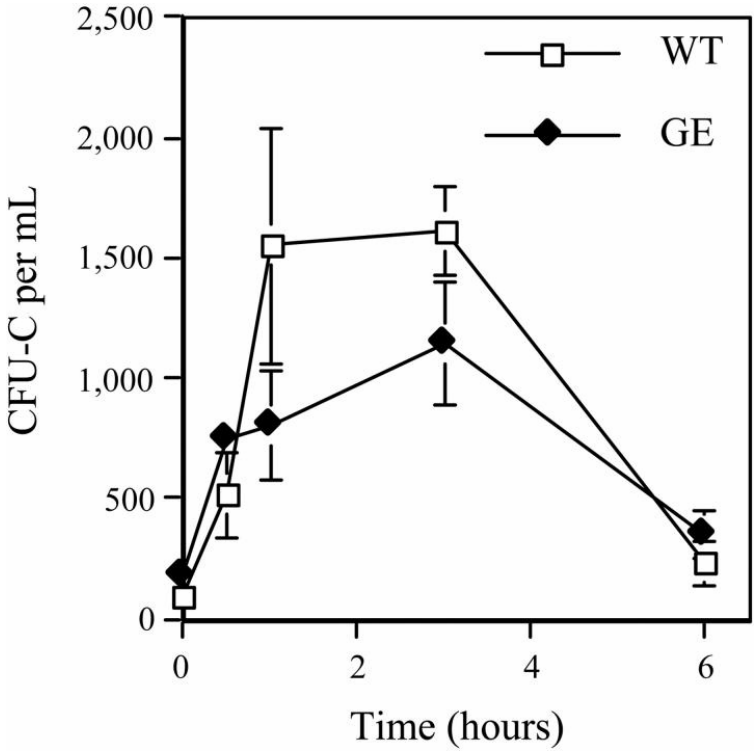

A
CXCL12α (pg/femur)
6,000
5,000
4,000
3,000
2,000
1,000
0
WT
d715
d715F
GEpoR
Untreated
G-CSF
B
CXCL12α (pg/femur)
r^2 = 0.72
P <.001
1.5
2.0
2.5
3.0
3.5
4.0
Log (CFU-C per mL of blood)

Figure 2.3



*

Figure 2.4

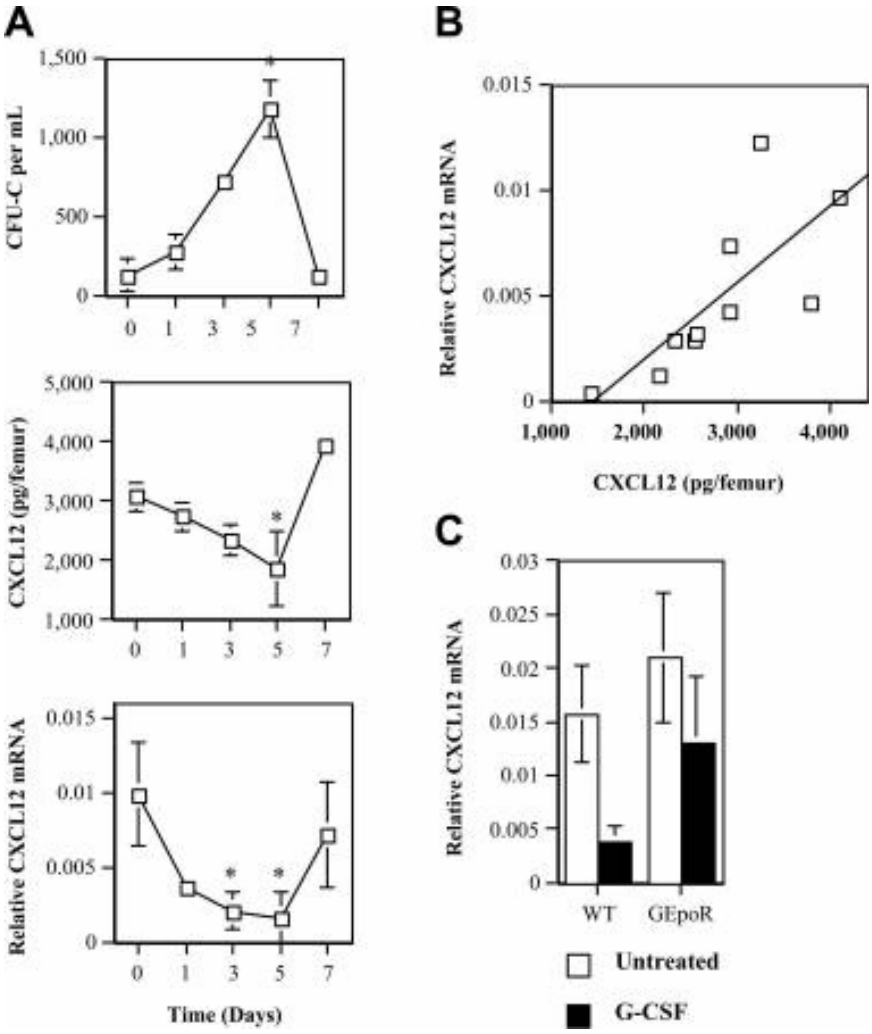

A
CFU-C per mL
1,500
1,000
500
0
0 1 3 5 7
CXCL12 (pg/femur)
5,000
4,000
3,000
2,000
1,000
Relative CXCL12 mRNA
0.015
0.01
0.005
0
Time (Days)
B
Relative CXCL12 mRNA
CXCL12 (pg/femur)
1,000 2,000 3,000 4,000
C
Relative CXCL12 mRNA
0.03
0.025
0.02
0.015
0.01
0.005
WT
GEpoR
Untreated
G-CSF

Figure 2.5

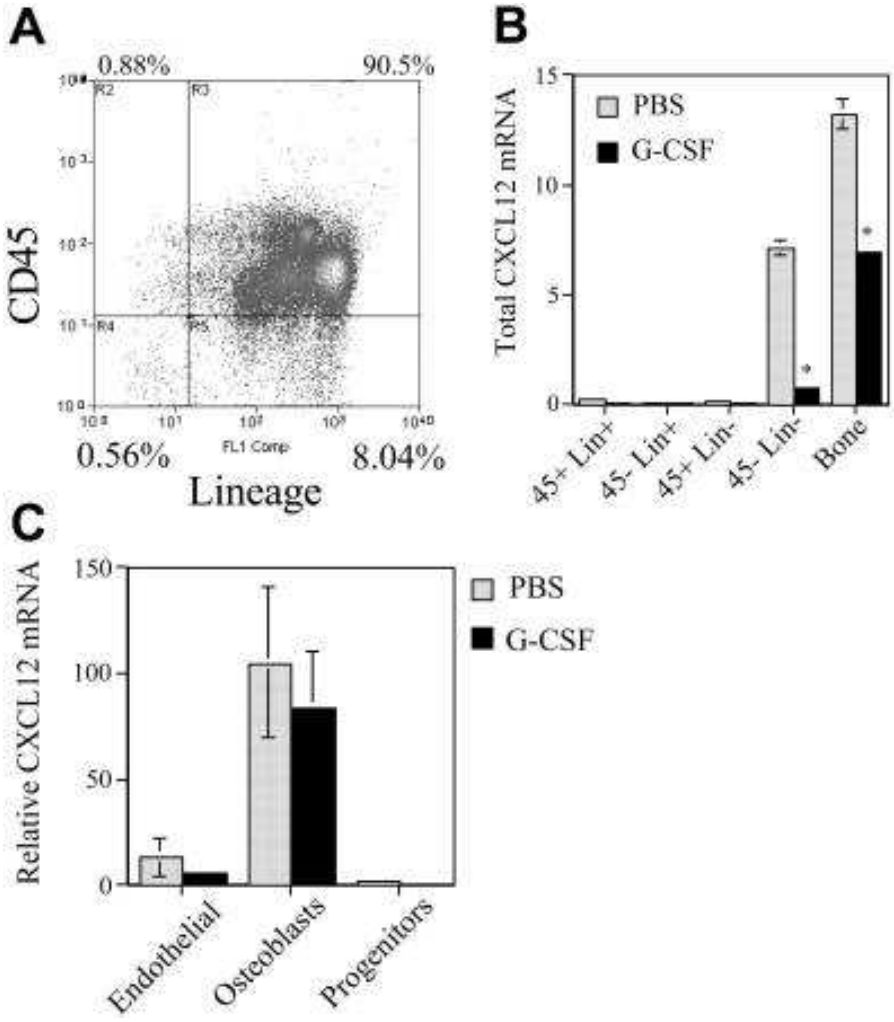

A
0.88%
90.5%
R2
R3
R4
R5
CD45
0.56%
8.04%
FL1 Comp
Lineage
B
Total CXCL12 mRNA
PBS
G-CSF
15
10
5
0
45+ Lin+
45- Lin+
45+ Lin-
45- Lin-
Bone
C
Relative CXCL12 mRNA
150
100
50
0
PBS
G-CSF
Endothelial
Osteoblasts
Progenitors

Figure 2.6

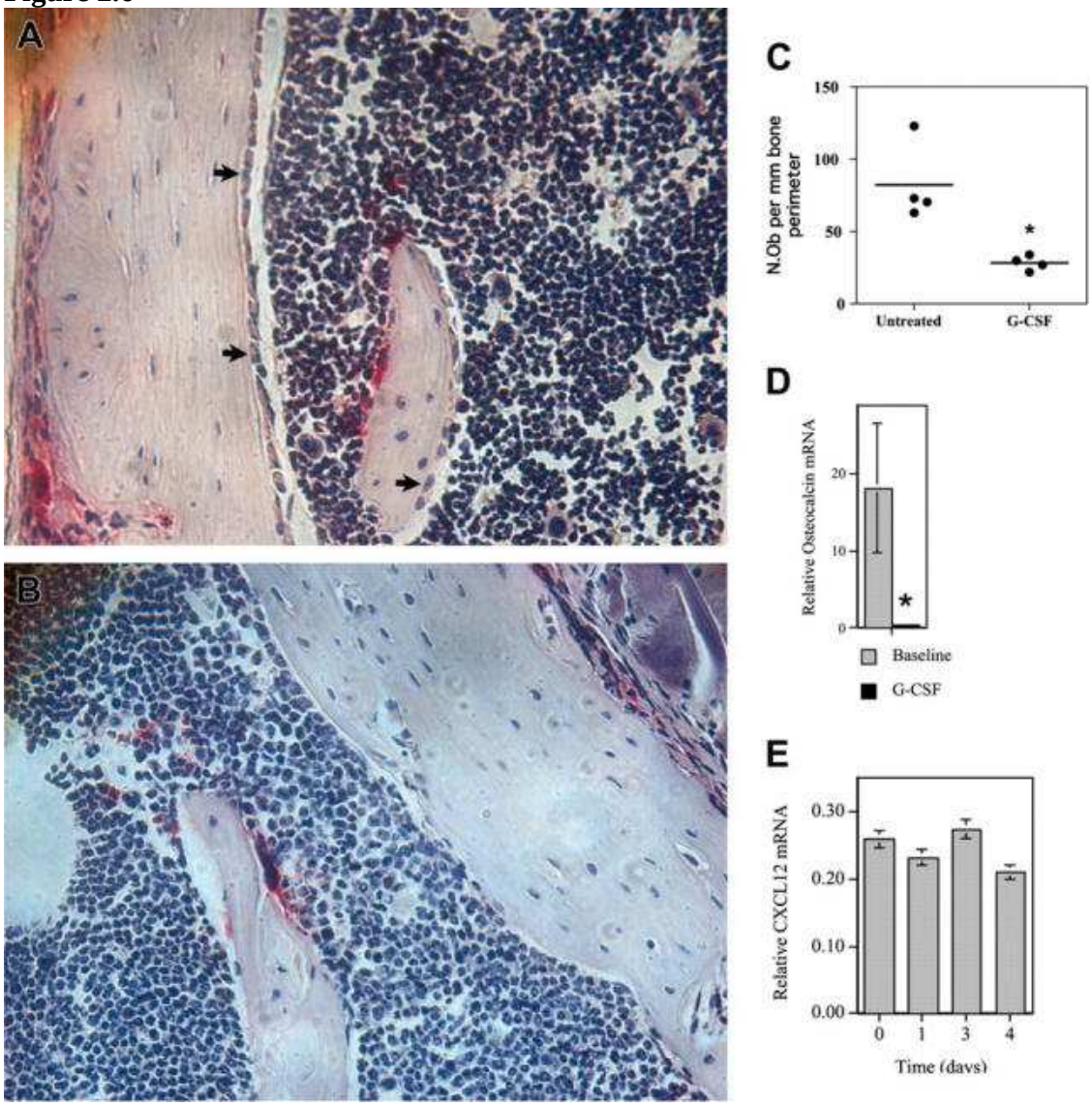
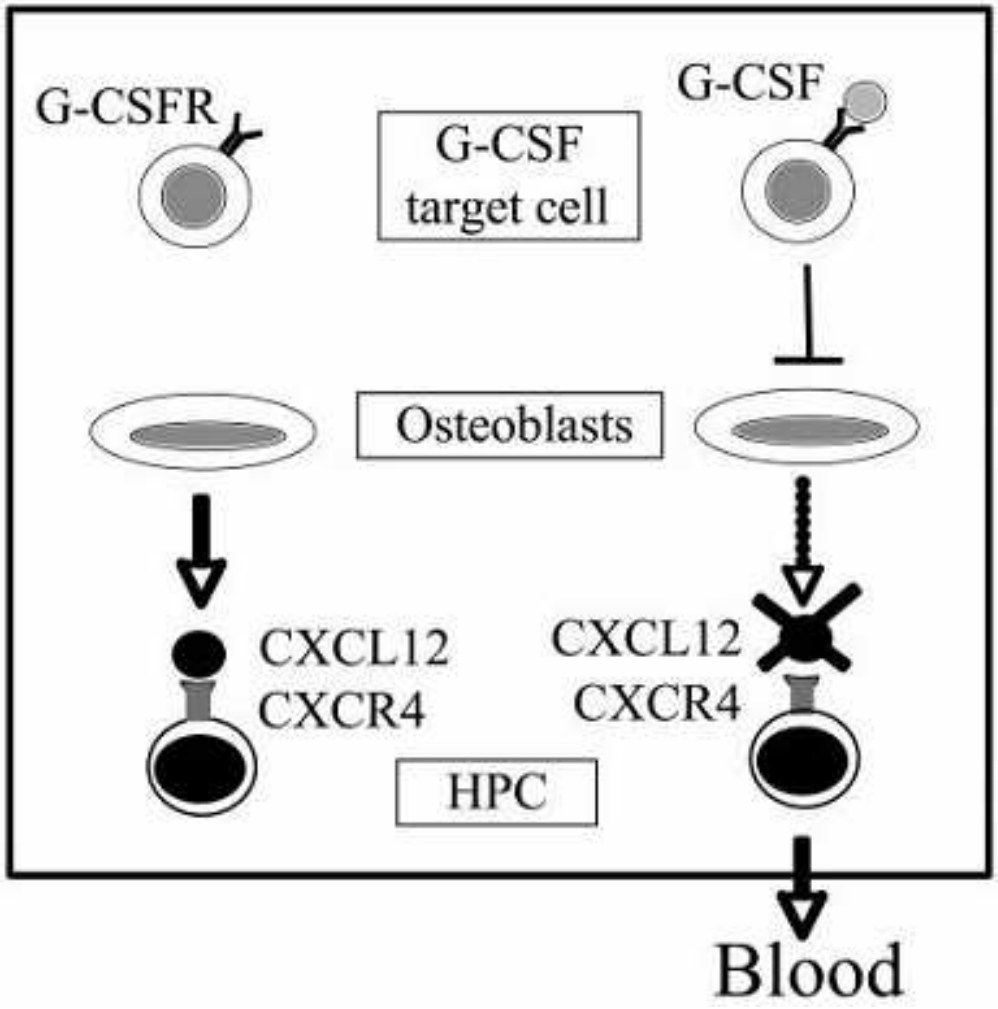

A
B
C
N.Ob per mm bone perimeter
150
100
50
0
Untreated
G-CSF
D
Relative Osteocalcin mRNA
20
10
0
Baseline
G-CSF
E
Relative CXCL12 mRNA
0.30
0.20
0.10
0.00
0
1
3
4
Time (days)

Figure 2.7


G-CSFR
G-CSF target cell
G-CSF
Osteoblasts
CXCL12
CXCR4
CXCL12
CXCR4
HPC
Blood

CHAPTER 3

SUPPRESSION OF CXCL12 PRODUCTION BY BONE MARROW OSTEOBLASTS IS A COMMON AND CRITICAL PATHWAY FOR CYTOKINE-INDUCED MOBILIZATION

3.1 ABSTRACT

Numerous molecular mechanisms have been implicated in G-CSF-induced mobilization, including the induction of bone marrow proteases, attenuation of adhesion molecule function, and disruption of CXCL12/CXCR4 signaling in the bone marrow. In addition, recent reports suggest that a decrease in number of CXCL12-producing osteoblasts may play a role in this latter mechanism. However, little is known about extent to which these mechanisms overlap or function independently of each other. Similarly, it is unclear to what extent mobilizing cytokines besides G-CSF may share common mechanisms. To begin to address these questions we asked whether other mobilizing agents from the hematopoietic cytokine family operate through the CXCL12/CXCR4 pathway. Treatment with G-CSF, Flt3L, and SCF resulted in downregulation of CXCR4 surface expression and function on mobilized c-Kit positive, lineage negative cells and loss of bone marrow CXCL12 protein and mRNA. Isolating and sorting bone marrow stromal cells demonstrated that the loss of CXCL12 expression occurred in the osteoblast—but not non-osteoblast—fraction, suggesting a central role for osteoblasts in mediating mobilization. Next, to investigate the relationship between various mechanisms of mobilization, we studied G-CSF-induced mobilization in mice genetically deficient in CXCR4. G-CSF treatment led to induction of bone marrow metalloproteinases in CXCR4 null bone marrow chimeras, but surprisingly did not increase the number of circulating hematopoietic progenitors. In contrast, treatment with a small molecule antagonist of adhesion molecule Very late antigen 4 (VLA-4/α4β1 integrin) doubled the number of circulating progenitors. Together, these results suggest that CXCL12/CXCR4 signaling—featuring significant loss of CXCL12 expression by

osteoblasts—plays a central role in cytokine-induced mobilization, and that accessory mechanisms such as induction of proteases and downregulation of adhesion molecules must function upstream or downstream of CXCL12/CXCR4 signaling.

3.2 INTRODUCTION

Under normal circumstances, hematopoiesis is regulated such that immature hematopoietic stem and progenitor cells (HSPC) are restricted to the bone marrow and are rarely observed in peripheral blood. Under certain circumstances, however, this regulation is loosened and significant numbers of HSPC are released to the circulation, a process termed "mobilization." Mobilization can be induced pharmacologically by a wide range of agents, including hematopoietic cytokines, chemokines, and cytotoxic drugs. These agents mobilize HSPC to different degrees and with varying kinetics, raising the possibility that several distinct mechanisms may exist by which mobilization may occur. [1,2]. On the other hand, as certain subsets of mobilizing agents work with similar kinetics, the possibility remains that mobilizing agents within a molecular family may share common mechanism of mobilization. For example, virtually all hematopoietic cytokines studied require 5-7 days for maximum mobilization.[3-7] However, common mechanisms in cytokine-induced mobilization have yet to be identified.

The best characterized and most widely used mobilizing agent is Granulocyte-colony stimulating factor (G-CSF).[8] To date, three general mechanisms have been implicated in G-CSF-induced mobilization (reviewed in [2,9]). First, G-CSF treatment leads to downregulation in the bone marrow of serpin family protease inhibitors as well as upregulation of neutrophil-derived proteases neutrophil elastase (NE), cathepsin G (CG), and metalloproteinase 9 (MMP9).[10,11] The net induction of proteolysis in the bone marrow may contribute to cell migration by facilitating degradation of extracelluar matrix (ECM) and enhancing cleavage of key signaling molecules.[12-15]

Second, G-CSF treatment is believed to disrupt mechanisms that cause bone marrow retention of HSPC at steady state, notably the interaction between vascular cell adhesion molecule 1 (VCAM-1) and very late antigen 4 (VLA-4/α4β1 integrin). [16-18]

Finally, accumulating evidence suggests that signaling between CXCL12, a chemokine expressed by bone marrow stroma, and its receptor CXCR4, expressed by HSPC, plays an important role in mobilization. Irradiated mice reconstituted with CXCR4 deficient bone marrow have at baseline a high number of circulating HSPC.[19] Further, bone marrow levels of SDF-1 protein and mRNA fall during G-CSF treatment, and mobilized HSPC express lower levels of functional CXCR4.[12,13,20] Together, these findings suggest that the disruption of CXCL12/CXCR4 signaling leads to the loss of a key retention signal for bone marrow HSPC.

Of note, the three primary mechanisms involved in G-CSF-induced mobilization—induction of proteases, downregulation of adhesion molecules, and CXCL12/CXCR4 disruption—likely interact at several levels. For example, upregulation of bone marrow proteases is believed to contribute to cleavage of bone marrow CXCL12 as well as adhesion molecules VCAM-1 and c-Kit during G-CSF-induced mobilization.[12,21,22] Similarly, CXCL12 signaling contributes to HSPC adhesion to fibronectin coated plates, suggesting that adhesion molecule disruption may occur at least in part downstream of G-CSF-induced CXCL12/CXCR4 disruption in vivo.[23] These findings raise the possibility, therefore, that disruption of CXCL12/CXCR4 signaling represents the primary mechanism by which G-CSF induces mobilization and that other mechanisms—i.e. induction of bone marrow proteases and downregulation of adhesion molecules—operate upstream or downstream within this pathway.

Recently, our lab and others have reported that G-CSF treatment is associated with a decrease in the number of mature osteoblasts in the bone marrow.[20,24] As osteoblasts are thought to be a source of CXCL12 in the bone marrow, this observation suggests that G-CSF-induced loss of osteoblasts plays a role in mobilization. On the other hand, other cell types express CXCL12, notably endothelial cells and CXCL12-abundant reticular (CAR) cells.[25,26] Indeed, several recent reports question whether osteoblasts produce significant amounts of CXCL12, raising the possibility that the loss of osteoblasts represents a coincidental finding unique to G-CSF treatment and does not play a role in mobilization. [26,27]

In this study we undertook to integrate recent findings concerning mechanisms of HSPC mobilization by focusing on one well-described mechanism. Specifically, we hypothesized that loss of osteoblast-produced CXCL12 is a common and critical mechanism in cytokine-induced HSPC mobilization. Accordingly, we investigated whether loss of osteoblasts was a common finding in cytokine-induced mobilization and whether loss of osteoblasts per se could account for the overall decline in bone marrow CXCL12. Next, we sought to determine the relative importance of disruption of CXCR4 signaling compared to other mechanisms known to be involved in mobilization—namely, the induction of bone marrow proteases and downregulation of VCAM-1/VLA-4 interactions—by studying mobilization in mice genetically deficient in CXCR4. In this way, we tried to define whether these separate pathways contribute to mobilization individually or co-operate within the same mobilizing pathway.

3.3 MATERIALS AND METHODS

Mice. Wild type (Ly5.1), *CXCR4* +/- (Ly5.2, provided by K. Weilbaecher, Washington University School of Medicine), and pOBCol2.3-GFP (gift of D. Rowe, University of Connecticut) were on the C57BL/6 strain. Mice were housed in a specific pathogen-free environment in accordance with the Washington University Animal Studies Committee.

Fetal Liver Transplantation. Fetal livers were harvested from embryonic day 14–16 embryos generated by setting up timed pregnancies between CXCR4+/– mice. Single-cell suspensions were frozen in RPMI medium 1640 with 20% FCS and 20% DMSO (Sigma, St Louis, MO). A portion of the embryo was saved to determine genotype by PCR. 6-12 - week-old C57BL/6 Ly5.1 recipients were lethally irradiated by using a ^{137}Cs source with 900 rads. Single-cell suspensions of $5x10^6$ CXCR4–/– or WT thawed fetal liver cells were injected into the lateral tail vein of each recipient mouse to generate CXCR4–/– mice and WT controls.

Mobilization protocols. *G-CSF, Flt-3 ligand (Flt3L), and SCF*. Recombinant human G-CSF, Flt3L, or SCF (Amgen, Thousand Oaks, CA) diluted in phosphate-buffered saline (PBS) with 0.1% low endotoxin bovine serum albumin (Sigma, St. Louis MO) was administered by daily subcutaneous injection at a dose of 250 μg/kg (G-CSF), 10 ug/mouse (Flt3L), or 4 ug/mouse (SCF) for 7 days. Mice were analyzed 3-4 hours after the final cytokine dose. *AMD15057*. AMD15057, a generous gift from AnorMED Inc.

(British Columbia, Canada), was reconstituted to a final concentration of .2mg/ml in sterile 10:36:54 ethanol:propylene glycol:water and administered as a single intravenous injection at a dose of 1 mg/kg. Mice were analyzed 3 hours post-injection.

Peripheral Blood and Bone Marrow Analysis. Blood was obtained by retroorbital venous plexus sampling in polypropylene tubes containing EDTA. Bone marrow cells were isolated by flushing femurs and tibias with 3-5ml cold PBS. Bone marrow extracellular fluid was obtained by flushing each femur with 1 ml of ice-cold PBS without serum, and the supernatant was harvested after centrifugation at 400 x *g* for 3 minutes.

Metalloproteinase Activity. Extracellular fluid was isolated as above. 100ul of each sample was assayed for metalloproteinase activity using the EnzChek Gelatinase kit with DQ Gelatin from pig skin, fluorescein conjugate substrate (Molecular Probes) according to manufacturers' instructions.

CXCL12 ELISA. Quantification of CXCL12 protein in bone marrow extracellular fluid was performed using commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturers' instructions using 50ul undiluted sample.

Colony-forming cell assay. Blood, bone marrow, and spleen cells were harvested from mice using standard techniques and the number of nucleated cells in these tissues quantified using a Hemavet automated cell counter. We plated 7-35 μl blood, 1 x 10^5

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; Stem Cell Technologies, Vancouver, British Columbia, Canada). Cultures were plated in duplicate and placed in a humidified chamber with 6% carbon dioxide (CO_2) at 37° C. Colonies containing at least 50 cells were counted on day 7 of culture.

Real-time quantitative RT-PCR. Femurs were flushed with a total of 2 ml Trizol reagent (Invitrogen, Carlsbad, CA) and RNA was isolated according to the manufacturer's instructions and resuspended in 150 μl RNase/DNase free water. Quantitative Real-time reverse transcriptase-polymerase chain reaction (Q-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, 12.5 μl RT-PCR reaction mix, 200 nM forward primer, 200 nM reverse primer, 280 nM internal probe, and .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.

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′.

CXCR4 cell surface expression. Bone marrow and peripheral blood cells were recovered from control and cytokine treated mice as described above. The flushed cells were incubated with the following panel of fluorescein isothiocynate (FITC)-conjugated lineage-restricted antibodies: Gr-1 (granulocytes), B220 (B-lymphocytes), CD3e (T-lymphocytes), and Ter-119 (erythroid cells), with allophycocyanin (APC)-conjugated anti-c-Kit antibody and a biotinylated anti-CXCR4 antibody followed by incubation with phycoerythrin (PE)-conjugated streptavidin (all antibodies eBiosciences, San Diego, CA, except anti-CXCR4 from BD Pharmingen) Cells were analyze on a FACScan flow cytometer (Becton Dickenson).

Histomorphometry. Osteoblasts in the bone marrow were quantified by histomorphometry, as previously described.[20] Briefly, femurs and tibiae were harvested, fixed overnight in 10% neutral formalin, decalcified by incubating in 14% EDTA at 4^0C for two weeks, and then embedded in paraffin. To ensure that osteoclasts were excluded from the osteoblast count, deparaffinized sections were stained histochemically for tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin. Osteoblasts were counted in a blinded fashion in 4-6 200X fields per section. In some cases, two sections 75 microns apart were taken from the same sample and osteoblast

number averaged. The number of osteoblasts per millimeter bone perimeter (N.Ob/mm) was calculated using the OsteoMeasure Histomorphometry System (OsteoMetrics, Inc., Atlanta, GA).

Isolation of osteoblast lineage cells by flow cytometry. Bone marrow cells were recovered from the femurs of pOBCol2.3-GFP mice by flushing with PBS. The femurs were then infused with PBS containing 50 mg/mL type II collagenase (Worthington Biochemical) and incubated at 37°C for 15 minutes. The collagenase-treated femurs were flushed again with PBS, cells pooled, and the process repeated for a total 6 digests. Pilot experiments demonstrated that virtually all recoverable GFP positive cells were found in these 6 digests (data not shown).

To isolate osteoblast lineage and non-osteoblast cells, pooled fractions were stained with PE-conjugated anti-mouse CD45 and anti-mouse Ter119 antibodies (eBiosciences). $CD45^-$, $Ter119^-$, GFP^+ (osteoblast) and GFP- (non-osteoblast) cells were sorted directly into TRIZOL using a MoFlo high-speed cell sorter (Dako). RNA was subsequently isolated and Q-RT-PCR performed as above

In situ hybridization. In situ hybridization using a probe for CXCL12 was performed on deparaffinized sections from mouse long bones as described previously, using ^{35}S-labeled riboprobes[28] and counterstained with toluidine blue.

Statistical analysis. Data are presented as mean +/- SEM. Statistical significance was assessed using a two-sided Student's t test.

3.4 RESULTS

3.4.1 Loss of osteoblast-produced CXCL12 is a common finding in cytokine-induced mobilization.

The disruption of CXCL12/CXCR4 signaling is known to be one mechanism by which G-CSF treatment induces HSPC mobilization. Bone marrow levels of CXCL12 protein and mRNA fall during treatment and CXCR4 surface expression on mobilized cells is diminished, possibly through proteolytic cleavage of the extracellular portion of the receptor.[13] Of note, this process requires 5 days to reach maximum HSPC mobilization, which mirrors the kinetics of the decline in osteoblast number.([20] and MJC, DCL, unpublished data) As other hematopoietic cytokines—but not chemokines—require this lengthy period to induce mobilization, we reasoned that this mechanism might prove to be a common pathway by which cytokines induce HSPC mobilization. Accordingly, we treated mice with two hematopoietic cytokines, Flt3 ligand (Flt3L) and stem cell factor (SCF) as well as G-CSF. Bone marrow levels of CXCL12 protein and mRNA were measured by ELISA and quantitative real time PCR, respectively. All three cytokines induced a robust mobilization (Figure 1A top). Numbers of HSPC mobilized by each agent was similar when the proliferative effect of Flt3L treatment on progenitors is taken into account. (Figure 1A bottom) Bone marrow CXCL12 protein and mRNA was reduced to a similar extent after mobilization with all three agents (Figure 1B).

Since some reports suggest that CXCR4 function in HSPC is attenuated with G-CSF treatment, both CXCR4 surface expression and function was compared between HSPC from mice treated with G-CSF, Flt3L, and SCF. First, CXCR4 surface expression was measured by flow cytometry, gating on c-Kit positive, lineage negative (KL) cells in mobilized blood and bone marrow. Consistent with previous reports, CXCR4 expression

was decreased in G-CSF-mobilized versus bone marrow KL cells. A similar decrease was noted in KL cells in Flt3L but not SCF treated peripheral blood cells (Figure 1C). Next, CXCR4 function on mobilized peripheral HSPC was measured by comparing migration of peripheral blood versus bone marrow CFU-C toward CXCL12 in a transwell assay. CFU-C derived from Flt3L- or SCF-mobilized peripheral blood failed to migrate as well as bone marrow CFU-C from these mice, suggesting that downregulation of CXCR4 function plays a role in HSPC mobilization (Figure 1D).

Since loss of osteoblasts, a source of CXCL12 in the bone marrow, was observed with G-CSF treatment, we hypothesized that the decrease in CXCL12 mRNA and protein observed after Flt3L and SCF treatment resulted from a similar decrease in osteoblast number. Standard histomorphometry performed on H&E-stained paraffin sections from matched mice treated with G-CSF, Flt3L, and SCF confirmed a decrease in osteoblast surface and number (Figure 1E and data not shown.)

Taken together, these findings suggest that disruption of CXCL12/CXCR4 signaling—featuring a significant loss in CXCL12-expressing osteoblasts—is a common feature of cytokine-mediated HSPC mobilization.

3.4.2 Loss of bone marrow CXCL12 results specifically from loss of bone marrow osteoblasts. As noted above, several cell types in the bone marrow express CXCL12, including osteoblasts, endothelial cells, and CAR cells. Our previous results demonstrate a loss in total bone marrow CXCL12 mRNA and protein with an associated loss of histologically identifiable osteoblasts in mice treated with each of the cytokines tested, G-CSF, Flt3L and SCF. However, as some controversy exists as to the contribution of

osteoblast-expressed CXCL12 to total bone marrow CXCL12, we inquired whether loss of osteoblast CXCL12 specifically was associated with cytokine induced mobilization. First, CXCL12 in situ hybridization was performed on paraffin sections from mice treated with G-CSF or untreated. At baseline, CXCL12 mRNA is detected both on endosteal and trabecular bone surfaces (Figure 2A left panel, arrows) as well as in the bone marrow proper (arrowheads). After G-CSF treatment, abundant scattered cells in the bone marrow continue to express CXCL12, while CXCL12 expression disappears from bone surfaces, suggesting that specifically osteoblast CXCL12 is targeted during G-CSF treatment.

To confirm this finding in a more quantitative manner, we utilized transgenic mice expressing GFP under control of a 2.3 kb fragment of the collagen I promoter. These mice express GFP in osteoblast lineage cells, including mature osteoblasts, bone lining cells, and osteocytes.[29] As shown in representative FACS plots, which are gated on CD45 negative, Ter119 negative cells, the stromal cell compartment can be divided into the GFP positive osteoblast lineage and GFP negative stromal fractions, which would be expected to include both endothelial and CAR cells (Figure 2B). As expected, the GFP positive fraction was highly enriched for osteoblast markers osteocalcin, osteoprotegerin, and Runx2 (data not shown). Mice were treated with G-CSF or Flt3L, osteoblasts and stromal cells were isolated and CXCL12 mRNA was measured in each fraction. Fewer GFP positive cells were observed in cytokine treated mice versus controls, consistent with the loss of osteoblasts observed histologically (data not shown). Within the remaining GFP positive population, CXCL12 mRNA was markedly reduced with respect to beta actin. Importantly, no reduction in CXCL12 mRNA in the stromal fraction was

noted. Together, these results suggest that loss of osteoblast-produced CXCL12 may represent a common pathway in cytokine-induced mobilization.

3.4.3 G-CSF-induced increase in bone marrow metalloproteinase activity does not depend on CXCR4 signaling. Next, to begin to address the relationship of CXCL12/CXCR4 signaling disruption with other known mechanisms involved in mobilization—induction of proteolytic microenvironment, and attenuation of integrin function—we tested whether the upregulation of bone marrow proteolytic activity seen during G-CSF treatment is dependent on disruption of CXCR4 signaling or is activated independently. To this end, protease activation was tested in mice deficient in CXCR4 signaling. CXCR4 null bone marrow chimeras were generated by transplanting Ly5.2 CXCR4 knockout or wild type fetal liver cells into irradiated Ly5.1 recipients. Only recipients that reconstituted with greater than 90% peripheral blood chimerism were analyzed (data not shown). CXCR4 deficient and wild type chimeras were treated with G-CSF, bone marrow plasma was isolated and tested for metalloproteinase activity by measuring fluorescence released by cleavage of labeled gelatin. Consistent with previous reports, G-CSF increased metalloproteinase activity in the bone marrow of treated wild type mice (Figure 3A). A similar trend was seen in CXCR4 null chimeras (Figure 3B), indicating that bone marrow metalloprotease activation does not depend on CXCR4 signaling.

3.4.4 G-CSF does not increase number of circulating HSPC in CXCR4 -/- chimeras. Next we tested the relative importance of disruption of CXCR4 signaling compared to

other mobilization pathways by measuring mobilization in CXCR4 deficient chimeras. As mentioned above, CXCR4 deficiency at baseline leads to an elevated level of HSPC in both peripheral blood and spleen, emphasizing the importance of this pathway in progenitor cell trafficking (Figure 4A-B). We predicted that since G-CSF treatment activates bone marrow proteases and disrupts function of adhesion molecules, treatment of CXCR4 deficient chimeras would increase the number of circulating progenitors. When treated with G-CSF, wild type controls mobilized normally. Surprisingly, however, G-CSF treatment did not increase number of circulating progenitors in CXCR4 null chimeras (Figure 4A-B). This failure to mobilize does not reflect a deficiency in the number of bone marrow resident CFU-C, which is the same as in controls (Figure 4C) and is vast in comparison to the number of mobilized CFU-C.

3.4.5 Treatment with a VLA-4 antagonist mobilizes HSPC in CXCR4 -/- chimeras.

The failure of G-CSF treatment to increase the number of circulating HSPC in CXCR4 -/- chimeras suggests that G-CSF mobilizes primarily through the disruption of this signaling pathway. An alternative possibility, however, is that CXCR4-/- chimeras at baseline already have maximally released their mobilizable pool of HSPC, despite the large progenitor pool still present in the bone marrow.

As a control to ensure that alternative mobilization mechanisms function normally in the CXCR4 null chimeras, we tested if activation of the integrin attenuation pathway could increase the number of circulating progenitors in these mice. Knockout and control chimeras were treated with AMD15057, a small molecule inhibitor of VLA-4. Chimeras transplanted with wild type cells mobilized modestly three hours after AMD15057

administration (Figure 5A). CXCR4 knockout chimeras mobilized to a similar extent, suggesting that this pathway functions normally in CXCR4 deficient chimeras (Figure 5B). Taken together, these observations suggest that disruption of CXCR4 signaling is a necessary component of G-CSF-induced mobilization.

3.5 DISCUSSION

In recent years numerous studies have outlined mechanisms by which G-CSF treatment leads to the mobilization of HSPC. These mechanisms could be integrated in two non-mutally-exclusive models: 1) G-CSF simultaneously activates different mechanisms in parallel, each of which contributes individually to mobilization; and 2) G-CSF activates different mechanisms that lie within the same pathway and depend on each other for action. Combining G-CSF treatment with AMD3100, a specific inhibitor of CXCR4 signaling, leads to increased HSPC mobilization than with G-CSF alone, lending support to the former model.[30] These results, however, are not definitive, since neither AMD3100 nor G-CSF would be expected to completely block CXCR4 signaling. To address this question, therefore, we studied G-CSF-induced mobilization in a model where CXCR4 is genetically deleted from the hematopoietic compartment. Surprisingly, G-CSF does not increase the number of circulating HSPC in this model, suggesting that the pleiotropic mechanisms activated by G-CSF during mobilization—specifically the activation of bone marrow proteases and the downregulation of adhesion molecules—converge in the CXCL12/CXCR4 pathway (Figure 6).

Treatment of mice with protease inhibitors inhibits G-CSF-induced mobilization.[12,13] Protease inhibition could block G-CSF-induced mobilization upstream of

CXCL12/CXCR4 signaling disruption—namely, by decreasing clearance of bone marrow CXCL12 or inhibiting cleavage of CXCR4 from HSPC, as is reported in the literature—or by blocking some parallel pathway, such as inhibiting protease-dependent tissue migration. In CXCR4 -/- chimeras, metalloproteinase activation occurs normally with G-CSF treatment (Figure 3A-B), but fails to enhance mobilization, suggesting that the role of protease activation in G-CSF-induced mobilization is largely upstream of the disruption of CXCL12/CXCR4 signaling (Figure 6). It bears noting that the role of proteases in G-CSF-induced mobilization is controversial, since the finding that protease inhibitors inhibit mobilization contrasts with findings in mice genetically deficient in MMP9 and neutrophil proteases, which have a normal response to G-CSF.

Inhibition of adhesion molecules is another mechanism by which G-CSF may induce HSPC mobilization. Treatment with a VLA-4 antagonist—but not G-CSF—increases the number of circulating HSPC in the CXCR4 -/- chimeras (Figure 4A,5B). This finding suggests that 1) inhibiting VLA-4/VCAM-1 interaction mobilizes HSPC in a CXCR4 independent fashion; and 2) if attenuation of adhesion molecules plays a role in G-CSF-induced mobilization, this would likely occur upstream or downstream of the disruption of CXCR4 signaling (Figure 6). Little is known about what lies downstream of CXCR4 antagonism in mobilization. CXCR4 signaling leads to cytoskeletal reorganization, which may lead to changes in HSPC motility and adhesion to bone marrow stroma.[31-33] Indeed, as noted above, incubation of HSPC with CXCL12 increases adhesion to fibronectin in an ex vivo culture system, suggesting that modulation of adhesion molecules may be one way that disruption of CXCL12/CXCR4 signaling enhances HSPC

release from the bone marrow.[23] Further work will be required to elucidate the role of adhesion molecules downstream of CXCR4 signaling.

Besides activation of bone marrow proteases, one mechanism that likely works upstream of the disruption in CXCL12/CXCR4 signaling is the decrease in osteoblasts observed in cytokine treatment. While the role of other CXCL12-producing cells in maintaining supportive niches for hematopoietic stem cells remains controversial, two findings in this work support the hypothesis that osteoblasts specifically mediate HSPC mobilization. First, loss of osteoblasts is a common finding in G-CSF, Flt3L, and SCF treatment. Second, after sorting bone marrow stromal cells into osteoblastic and non-osteoblast fractions we detected a loss of CXCL12 mRNA in the osteoblastic fraction only. This finding corroborated the RNA in situ hybridization data, where G-CSF treatment resulted in loss of CXCL12 message on endosteal surfaces but not in the bone marrow itself. One intriguing possibility is that CXCL12 loss from the endosteum but not from bone marrow sinusoid-associated cells results in migration of hematopoietic cells toward the vasculature. This possibility will require a great deal more study.

Finally, it is noteworthy that several other mechanisms implicated in G-CSF-induced mobilization may work via osteoblasts upstream of CXCL12/CXCR4 signaling. Levesque and colleagues report that G-CSF treatment increases hypoxia in the bone marrow,[34] a process which may have adverse effects on osteoblasts. [35,36] Katayama et al show that loss of beta adrenergic signaling inhibits G-CSF-induced HSPC mobilization by attenuating loss of bone marrow osteoblasts.[24]

To summarize, our data suggest that disruption of CXCL12/CXCR4 signaling represents a common and critical pathway in cytokine-mediated mobilization and that loss of osteoblast-produced CXCL12 contributes to this effect.

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3.7 FIGURE LEGENDS

Figure 3.1 Disruption of CXCL12/CXCR4 signaling is a common feature in cytokine-induced mobilization. Mice (n=6-8 each group) were treated with G-CSF, Flt3L, or SCF for seven days. (A) Number of CFU-C were measured in the peripheral blood (top) and bone marrow (bottom) by methylcellulose colony-forming assay. (B)Total bone marrow CXCL12 mRNA was measured by Q-RT-PCR (top) and CXCL12 protein in bone marrow plasma was quantified by ELISA (bottom). (C) CXCR4 surface expression was compared between mobilized hematopoietic progenitors (PB) and bone marrow progenitors (BM) by flow cytometry gating on c-Kit positive, lineage negative cells in either compartment. (D) CXCR4 function in mobilized hematopoietic progenitors (PB) and bone marrow progenitors (BM) was compared by measuring the percent of CFU-C that migrated down a gradient of CXCL12 in a transwell assay. (E) Bone marrow osteoblasts were enumerated in H&E stained paraffin sections using standard histomorphometric technique. Data represents mean ± SEM. *p<.05 compared to control, **p<.05 compared to all other groups, ***p<.001 compared to BM.

Figure 3.2 Cytokine-induced mobilization results in specific loss of osteoblast CXCL12. Mice were treated with G-CSF, Flt3L, or SCF. (A) Representative photomicrograph of RNA in situ from G-CSF treated (left) and untreated (right) mouse long bone showing CXCL12 mRNA along endosteal surface (arrows) and within bone marrow (BM, arrowheads). N=2-3 each group (B-C) Transgenic mice (n=4-5 each group) expressing GFP in osteoblast lineage cells (pOBCol2.3-GFP mice) were treated with cytokines, stromal cells were isolated and fractionated by flow cytometry into non-osteoblast and osteoblast fractions, and CXCL12 mRNA was measured in each fraction. (B) representative facsplots gated on CD45 negative, Ter119 negative stromal fraction showing GFP positive osteoblast and GFP negative non-osteoblast fraction. (C) CXCL12 mRNA in GFP positive osteoblast fraction from G-CSF-treated (left) and Flt3L-treated (right) mice. Data represents mean ± SEM. *p<.05

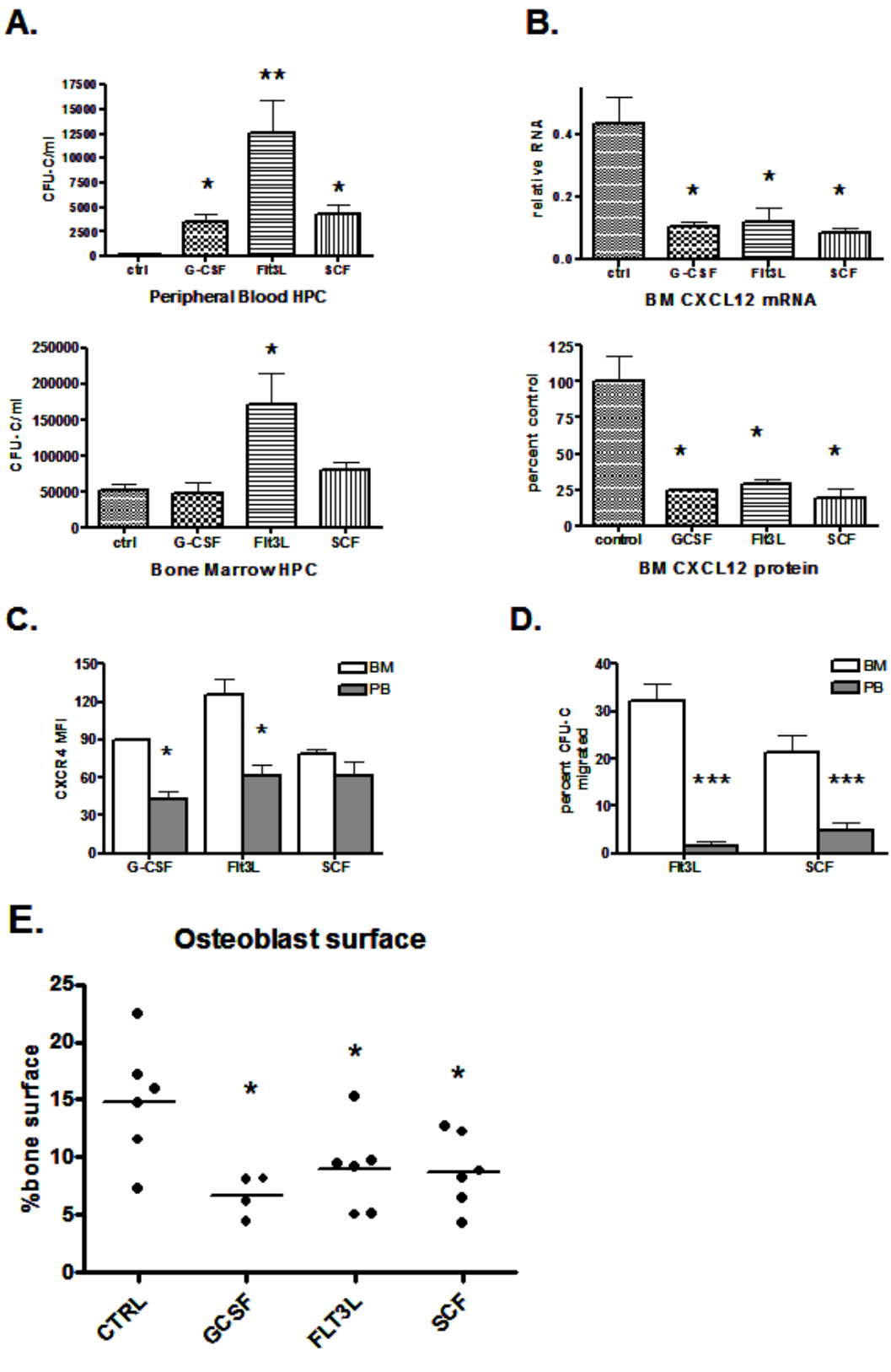
Figure 3.3 G-CSF increases bone marrow metalloproteinase activity in wild type and CXCR4 -/- chimeras. Lethally irradiated chimeras (n=4 each group) reconstituted with CXCR4 +/+ or CXCR4 -/- fetal liver cells were treated with G-CSF and bone marrow plasma was isolated. Metalloproteinase activity in the bone marrow plasma was estimated by measuring cleavage of fluorescently labeled substrate and normalizing for protein content. Shown is metalloproteinase activity for (A) CXCR4 +/+ and (B) CXCR4 -/- chimeras. Data represents mean ± SEM. *p<.05

Figure 3.4 G-CSF treatment does not increase number of circulating progenitors in CXCR4 -/- chimeras. CXCR4 +/+ and -/- chimeras (n=7-10 each group) were treated with G-CSF and CFU-C were measured in (A) peripheral blood, (B) spleen, and (C) bone marrow. Data represents mean ± SEM. *p<.05

Figure 3.5 VLA-4 antagonism increases number of circulating progenitors in CXCR4 +/+ and CXCR4 -/- chimeras. CXCR4 +/+ and -/- chimeras (n=6-9 each group) were treated with AMD15057, a specific VLA-4 antagonist and peripheral blood

CFU-C were measured in (A) CXCR4 +/+ and (B) CXCR4 -/- chimeras. Data represents mean ± SEM. *p<.01

Figure 3.1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SCF
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0.2
0.0
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200000
150000
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50000
Bone Marrow HPC
percent control
125
100
75
50
25
control
GCSF
BM CXCL12 protein
C.
D.
CXCR4 MFI
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120
90
60
30
BM
PB
percent CFU-c migrated
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30
20
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%bone surface
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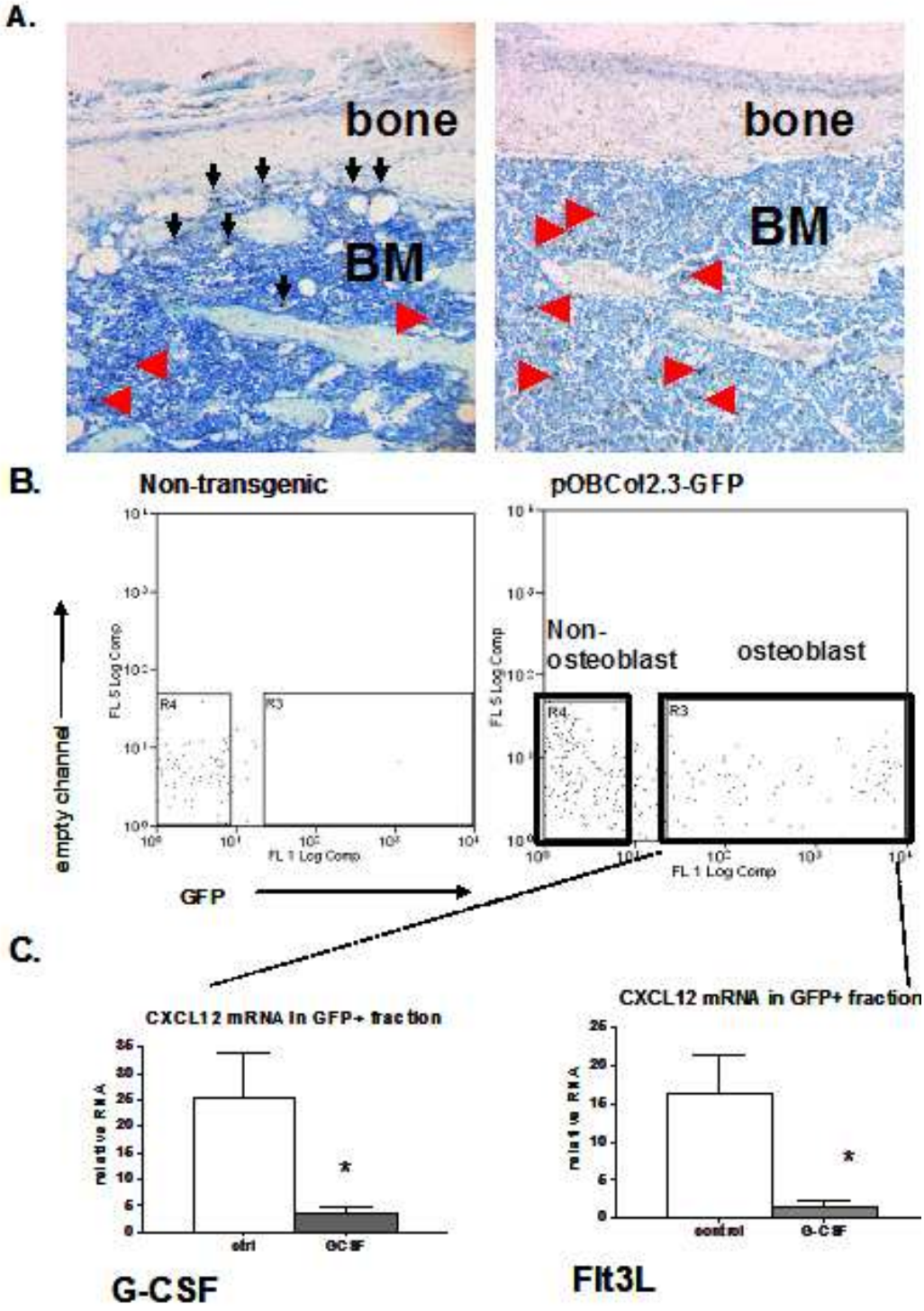
GCSF
FLT3L
SCF

Figure 3.2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FL 1 Log Comp
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R3
Non-
osteoblast
osteoblast
C.
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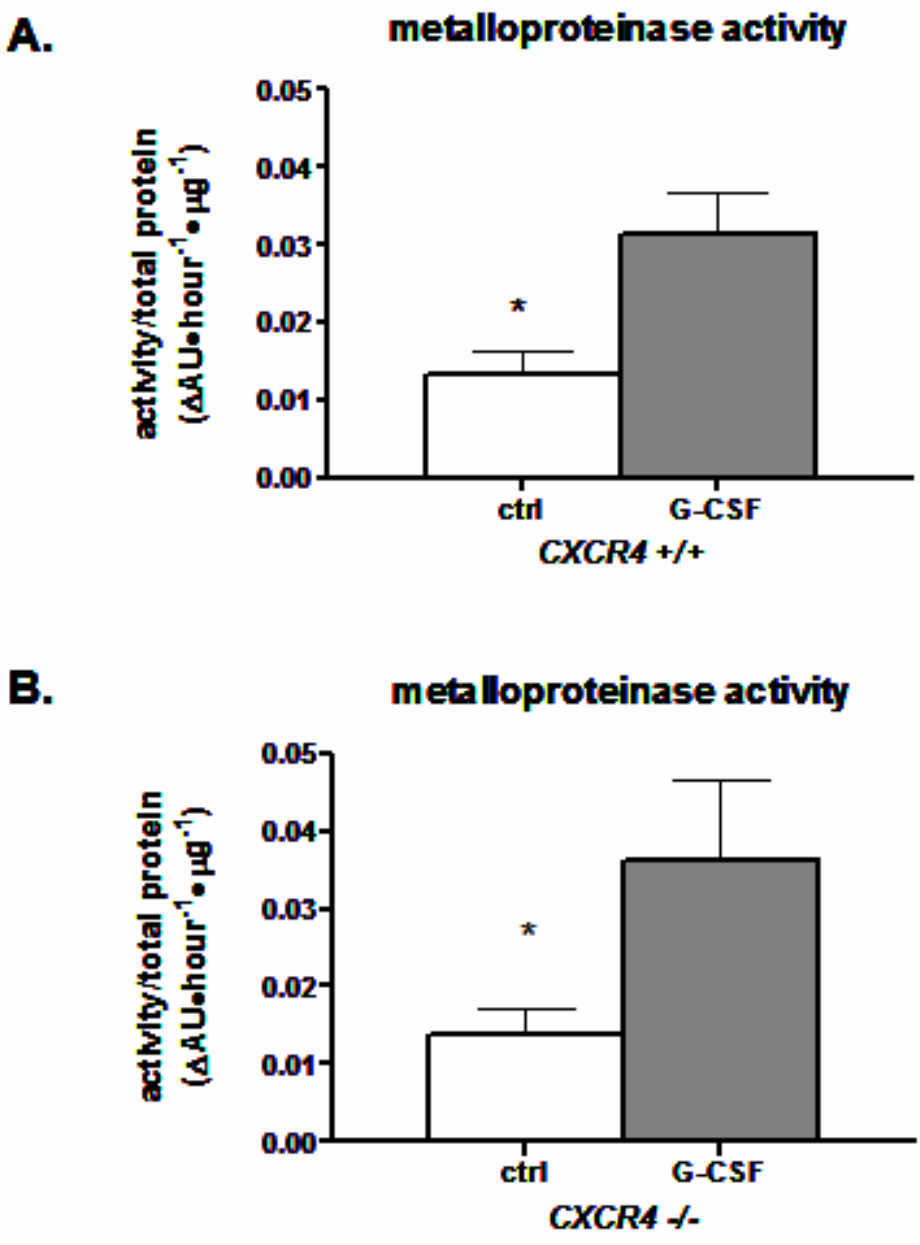
Flt3L

Figure 3.3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*
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G-CSF
CXCR4 +/+
B.
metalloproteinase activity
activity/total protein
(ΔAU•hour-1•µg-1)
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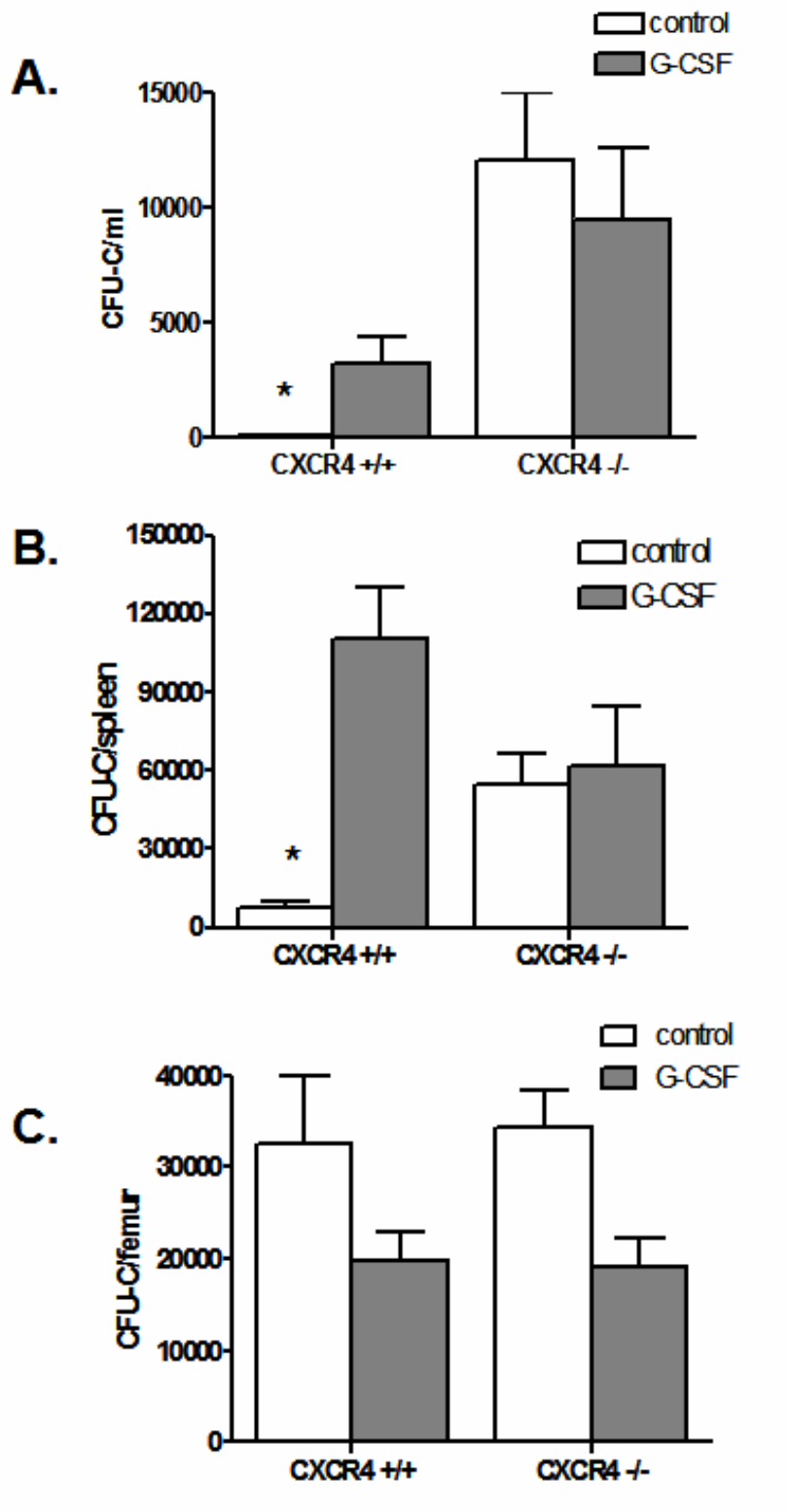
Figure 3.4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C.
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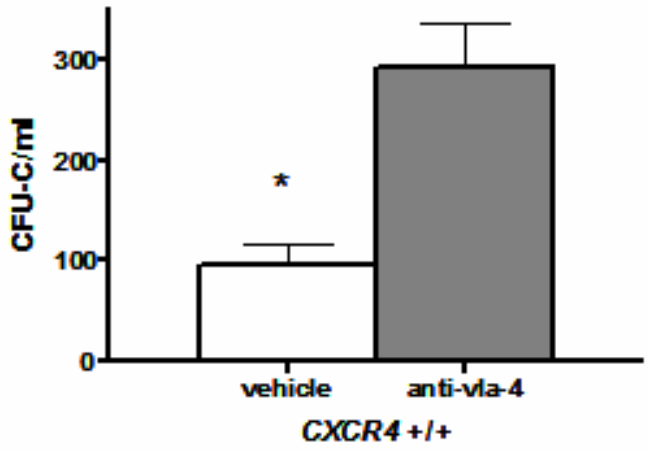
Figure 3.5

A.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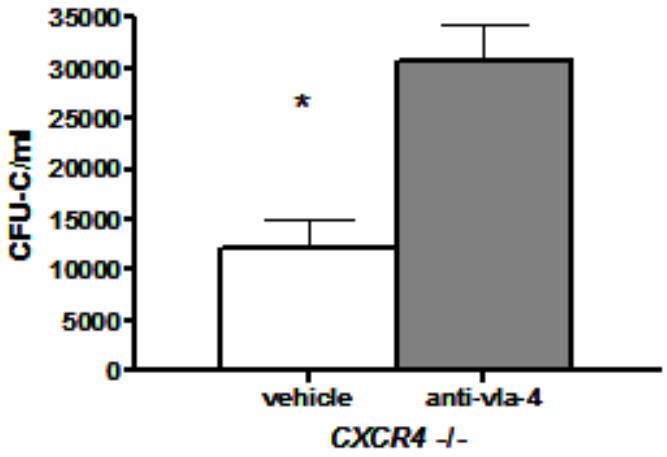
200
100
0
*
vehicle
anti-vla-4
CXCR4 +/+

B.


CFU-C/ml
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10000
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0
*
vehicle
anti-vla-4
CXCR4 -/-

CHAPTER 4

GRANULOCYTE COLONY-STIMULATING FACTOR INDUCES OSTEOBLAST APOPTOSIS AND INHIBITS OSTEOBLAST DIFFERENTIATION

4.1 ABSTRACT

G-CSF administration results in marked decrease in bone mineral density characterized by increased osteoclastogenesis and loss of mature osteoblasts. Herein, we show that the osteoblast decrease results from increased osteoblast apoptosis and inhibited osteoblast differentiation. G-CSF acts indirectly on osteoblasts through a hematopoietic intermediary. Finally, loss of osteoprotegerin (OPG) expression from mature osteoblasts may contribute to the increase in osteoclasts.

Introduction: Long-term treatment with G-CSF leads to a clinically significant osteopenia characterized by increased osteoclast activity and number. In addition, recent reports have observed a decrease in number of mature osteoblasts during G-CSF administration. However, neither the extent of G-CSF's suppressive effect on the osteoblast compartment nor its mechanisms are well understood.

Materials and methods: Transgenic mice expressing the green fluorescent protein (GFP) under control of the rat collagen I promoter (pOBCol2.3-GFP mice) were treated with G-CSF. Osteoblast number and apoptosis were measured by flow cytometry and histology. Osteoblast proliferation and turnover were assessed by labeling with BrdU. Bone marrow chimeras with G-CSF receptor deficient hematopoietic cells were generated to test whether G-CSF acts directly on osteoblast lineage cells.

Results: G-CSF administration leads to a selective loss of endosteal and trabecular osteoblasts; bone lining cells, osteocytes, and periosteal osteoblasts are unaffected.

Osteoblast turnover and apoptosis are increased. G-CSF administration also leads to a significant accumulation of osteoprogenitors in the bone marrow. The effect of G-CSF on osteoblasts was abrogated in wild type mice transplanted with G-CSF receptor deficient hematopoietic cells. Finally, while expression of receptor activator of NFkappaB ligand (RANKL) in the bone marrow is relatively unaffected by G-CSF administration, expression of the RANKL decoy receptor, OPG, is markedly decreased.

Conclusion: G-CSF administration leads to a loss mature osteoblasts in the bone marrow through both an increase in osteoblast turnover and inhibition of osteoblast differentiation. These effects of G-CSF on osteoblasts are mediated via a hematopoietic intermediary. The altered ratio of RANKL to OPG expression provides a novel mechanism by which G-CSF stimulates osteoclastigenesis.

4.2 INTRODUCTION

Bone marrow is the normal site of both hematopoiesis and bone metabolism. As predicted by their proximity, regulation of these tissues is highly integrated. There is strong evidence that osteoblasts play a key role in establishing and maintaining an appropriate microenvironment for hematopoietic stem cells. Conversely, the hematopoietic compartment is also known to regulate bone metabolism, largely through the production of hematopoietic cytokines [reviewed in (1), (2)].

Granulocyte-colony stimulating factor (G-CSF) is the principal cytokine regulating granulopoiesis. Long-term treatment with G-CSF is associated with development of clinically significant osteopenia, characterized by decreased bone mineral density and vertebral compression fractures (3,4). In a recent study, the incidence of osteopenia in patients with severe congenital neutropenia (SCN) treated chronically with G-CSF was 28% (3). Similarly, long-term exposure to G-CSF in mice leads to a decrease in cortical and trabecular bone, suggesting that it is G-CSF and not the underlying disease that is causing osteopenia in patients with SCN. (4,5)

There is evidence that G-CSF induces osteopenia in part by stimulating osteoclast activity. G-CSF treatment increases osteoclast number in the bone marrow of mice and increases the level of urine deoxypyridinoline in humans. (6) Several potential mechanisms by which G-CSF stimulates osteoclastigenesis have been advanced. G-CSF increases the proliferation of myeloid progenitors, potentially increasing the pool of monocytic precursors from which osteoclasts derive. (7) In addition, G-CSF has been shown directly to augment osteoclast formation and activity *in vitro*. (8) Nevertheless, the mechanisms by which G-CSF stimulates osteoclastogenesis *in vivo* remain undefined.

While long-term G-CSF treatment results in increased osteoclastogenesis, recent evidence suggests that short term G-CSF administration decreases osteoblast number and activity (6,9,10). Our lab has shown that administration of G-CSF for 5 days in mice results in a marked decrease in histologically identifiable osteoblasts in the bone marrow and a corresponding decrease in osteocalcin mRNA.(10) Whether this decrease in osteoblast number results from increased osteoblast turnover or from a defect in osteoblast development remains unclear. A third possibility—that G-CSF induces osteoblast quiescence—was raised in a recent report by Katayama et al., who observed a preponderance of bone lining cells in the bone marrow of G-CSF-treated mice.(9)

In this study, we utilize transgenic mice expressing the green fluorescent protein (GFP) under control of an osteoblast-lineage-specific promoter (pOBCol2.3-GFP mice) to measure osteoblast turnover during G-CSF administration. We show that G-CSF administration leads to a selective loss of mature endosteal and trabecular osteoblasts that is secondary to both an increase in osteoblast apoptosis and inhibition of osteoblast differentiation. Bone marrow transplantation studies show that G-CSF regulates osteoblasts indirectly, via a hematopoietic intermediary. Finally, we show that G-CSF treatment markedly decreases the bone marrow expression OPG, providing a novel mechanism by which G-CSF activates osteoclasts.

4.3 MATERIALS AND METHODS.

Mice. The generation and characterization of pOBCol2.3-GFP mice, kindly provided by David Rowe at the University of Connecticut, have been described elsewhere (11). Mice were maintained in a pathogen-free barrier facility in accordance with Washington

University Animal Studies Committee guidelines. 6-12 week old age and sex-matched mice were used in all studies.

G-CSF administration. Recombinant human G-CSF, a generous gift from Amgen, was diluted in phosphate-buffered saline (PBS) with 0.1% low endotoxin bovine serum albumin (Sigma) and administered by twice daily subcutaneous injection at a dose of 250 µg/kg/day for the length of time indicated.

Immunohistochemistry and histomorphometry. Long bones from pOBCol2.3-GFP and wild type mice were processed as previously described (10). Briefly, femurs and tibiae were harvested, fixed overnight in 10% neutral formalin, decalcified by incubating in 14% EDTA at 4°C for 7-10 days, and then embedded in paraffin. Paraffin-embedded sections were deparaffinized, rehydrated, and antigen retrieval was performed by soaking sections in DeCal Retrieval Solution (Biogenex) per manufacturers' instructions. GFP expression was assessed using a rabbit anti-GFP polyclonal antibody (Chemicon International) and positive cells were visualized using Vector Elite ABC kit and DAB substrate (Vector Labs) with Nuclear Fast Red counterstain (Sigma). Slides were analyzed in a blinded fashion to determine the number of osteoblasts per millimeter bone perimeter, osteoblast surface percent, bone lining cell surface percent, and osteocyte number per trabecular area. Bone lining cells and osteoblasts were differentiated based on morphology. Analysis was confined to the trabecular metaphysial region distal to the growth plate. Osteoclasts were identified by staining sections for tartrate-resistant acid phosphatase (TRAP). Osteoclast number and osteoclast surface were calculated based on the presence of TRAP positive cells on trabecular surfaces.

Osteoid surface was determined by analyzing undecalcified, methyl methacrylate-embedded sections stained using the Masson trichrome technique. To determine mineralization rate, mice were injected twice with 0.5mg calcein (Sigma) before and after 7 day G-CSF treatment. 48 hours after the second injection, calvaria were harvested, fixed in 70% ethanol, and embedded in methyl methacrylate. Mineral apposition rate and mineralizing surface were analyzed by fluorescent microscopy, as previously described (12). Images were acquired with Nikon microphot SA microscope using Nikon plan 10x and 20x objectives (Nikon Instruments) and a digital camera from Colorview Soft Imaging System. All parameters were analyzed using OsteoMeasure Histomorphometry System (OsteoMetrics).

Osteocalcin RNA in situ. Osteocalcin sense and antisense ^{33}P-labeled probes for RNA in situ were generated using a SP6/T7 Transcription Kit (Roche) using a plasmid generously provided by David Ornitz (Washington University). RNA in situ hybridization was performed as previously described.(13)

Real-time quantitative RT-PCR. Femurs were flushed with 1 ml of TRIzol reagent (Invitrogen) and RNA was isolated according to the manufacturer's instructions. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed as previously described (10) using the following primers with FAM/TAMRA probes where indicated or using SYBR green (Molecular probes).

Bglap2 (osteocalcin): 5'-TCTCTCTGCTCACTCTGCTGGCC-3' (fwd primer); 5'-TTTGTCAGACTCAGGGCCGC-3' (rev primer); 5'-TGCGCTCTGTCTCTCTGACCTCACAGATGCCA-3' (FAM/TAMRA probe). *Tnfrsf11b* (osteoprotegerin): 5'-TACCTGGAGATCGAATTCTGCTT-3' (fwd primer); 5'-CCATCTGCACATTTTTTGCAAA-3' (rev primer); 5'-ACCGGAGCTGTCCCCCGGG-3'(FAM/TAMRA probe). *Spp1* (bone sialoprotein):

5’-ACGGCGATAGTTCCGAAGAGGAGGG-3’(fwd primer); 5’-GAGTGTGGAAAGTGTGGAGTTCTCTGCC-3’(rev primer). *Akp2* (Alkaline Phosphatase): 5’-TCCATCCTGCGCTGGGCCAAGG-3’(fwd primer); 5’-AGTCCCGATCGGCCGAGTGTGCG-3’(rev primer). *Runx2:* 5’-GCACTGGCGGTGCAACAAGACCC-3’(fwd primer); 5’-CGGAGTAGTTCTCATCATTCCCGGCC-3’(rev primer). *Tnfsf11* (RANKL): 5’-GCAACACATTGTGGGGCCACAGC-3’(fwd primer); 5’-TGGCTGGGCCTCAGGCTTGC-3’(rev primer). *Actb* (β Actin): 5’-ACCAACTGGGACGATATGGAGAAGA-3’(fwd primer); 5’-TACGACCAGAGGCATACAGGGACAA-3’(rev primer); 5’-AGCCATGTACGTAGCCATCCAGGCTG-3’(FAM/TAMRA probe).

Isolation of osteoblast lineage cells by flow cytometry. Bone marrow cells were recovered from the femurs of pOBCol2.3-GFP mice by flushing with PBS. The femurs were then infused with PBS containing 50 mg/mL type II collagenase (Worthington Biochemical) and incubated at 37°C for 15 minutes. The collagenase-treated femurs were flushed again with PBS, cells pooled, and the process repeated for a total 6 digests. Pilot experiments demonstrated that virtually all recoverable GFP positive cells were found in these 6 digests (data not shown).

To quantify osteoblast lineage cells, pooled fractions were stained with allophycocyanin (APC)-conjugated anti-mouse CD45 and phycoerythrin (PE) conjugated anti-mouse Ter119 antibodies (eBiosciences). $CD45^-$, $Ter119^-$, GFP^+ cells were enumerated on a FACScan flow cytometer (Becton Dickenson). In some experiments, $CD45^-$, $Ter119^-$, GFP^+ cells were sorted using a MoFlo high-speed cell sorter (Dako).

BrdU labeling. pOBCOL2.3-GFP mice were treated with 2mg BrdU (Sigma) daily for 14 days before G-CSF treatment. In a separate study, mice were given 2 mg of BrdU twice daily for 5 days after G-CSF treatment. Cell fractions containing osteoblasts were isolated as described and stained with PE-conjugated anti-Ter119 and biotinylated anti-

CD45 coupled with Alexa 750-conjuaged streptavidin (Invitrogen). BrdU positivity was assessed using the BD Pharmingen BrdU Flow Kit (Becton Dickenson) and an Alexa 647-conjugated anti-BrdU antibody (Invitrogen).

Activated Caspase 3 analysis. Bone marrow cells harvested from pOBCOL2.3-GFP mice were stained with APC-conjugated anti-mouse Ter119 and biotinylated anti-mouse CD45 (eBiosciences) coupled with Alexa 750-conjuaged streptavidin (Invitrogen). Cells were then fixed and permeabilized with BD Cytofix/Cytoperm kit and stained with rabbit monoclonal PE-conjugated anti-activated Caspase 3 antibody, per manufacturer's protocol (BD Biosciences Pharmingen).

CFU-F and CFU-Alp culture. Bone marrow was isolated from mice treated 5 days with G-CSF and untreated controls. 3.6 million nucleated cells were plated per well in 6 well plates. Cells were grown for four days in αMEM (Gibco) supplemented with 10% fetal bovine syrum and Pen/Strep. After four days, media was switched to differentiation media containing 50 mg/l ascorbic acid and 2.16mg/l Beta-glycerophosphate (Sigma), and media was changed every 3-4 days thereafter. After 14 days culture, cells were assayed for alkaline phosphatase positivity using a kit (Sigma), and colonies containing more than 20 cells were scored.

Bone marrow transplantation. Wild-type (Ly5.1) and G-CSFR$^{-/-}$ (Ly5.2) bone marrow cells were harvested from strain- and sex-matched mice. Cells were stained with PE-conjugated mouse anti-CD45.1 or anti-CD45.2, APC-conjugated mouse anti-c-Kit, and FITC-conjugated lineage markers anti-CD3, anti-GR1, anti-B220, and anti-Ter119 antibodies. CD45-positive, c-Kit positive, lineage negative hematopoietic progenitors

were purified by high speed cell sorting, and 30,000-50,000 cells were injected into the tail vein of each lethally irradiated wild type (Ly5.1) or G-CSFR$^{-/-}$ (Ly5.2) recipient as previously described.(14) Two independent groups of mice received transplants; mice were analyzed separately, and the results pooled.

Statistical analysis. Data are presented as mean ± SEM. Statistical significance was assessed using 2-sided Student *t* test or two-way ANOVA (BrdU analysis).

4.4 RESULTS

4.4.1 G-CSF treatment results in a loss of osteoblasts but not osteocytes or bone lining cells in the bone marrow.

We and others previously showed that treatment with G-CSF leads to a loss of cuboidal osteoblasts from the bone marrow in mice.(9,10) To further characterize this process, we first determined the kinetics of osteoblast loss during G-CSF treatment. As shown in Figure 1A, loss of mature osteoblasts, as defined by histomorphological criteria, was delayed, with a significant fall only seen after 5 days of G-CSF. This effect was reversible, as osteoblast number recovered within 5 days after stopping G-CSF.

These data were confirmed using transgenic mice expressing GFP driven by a 2.3 kb fragment of the rat type 1 collagen promoter (pOBCol2.3-GFP mice). Consistent with a previous report (11), we observed GFP expression in these mice in mature, cuboidal osteoblasts, morphologically flat bone lining cells, and osteocytes (Figure 1B). G-CSF treatment resulted in a striking loss of GFP+ osteoblasts in trabecular and cortical bone. In contrast, G-CSF had no significant effect on the number of GFP+ bone lining cells or osteocytes (Figures 1B and 1C).

We next developed a method to analyze and sort GFP+ osteoblast-lineage cells by flow cytometry. Briefly, hematopoietic and stromal cells were recovered from long bones by serial collagenase digestion. Immunohistochemistry performed on long bones after harvesting revealed efficient recovery of GFP+ cells from both control and G-CSF treated mice (data not shown). Osteoblast lineage cells were defined as $CD45^-$ $Ter119^-$ GFP^+ cells; $CD45^+$ and $Ter119^+$ cells were excluded to improve specificity. Consistent

with the histomorphometry data, this flow cytometry-based assay showed that the number of osteoblast lineage cells decreased following G-CSF administration (Figure 1D, 1E).

4.4.2 G-CSF treatment selectively suppresses endosteal and trabecular but not periosteal osteoblasts. To directly assess the effect of G-CSF treatment on bone formation, two functional assays of osteoblast activity were measured. Consistent with the loss of mature osteoblasts, a significant decrease in osteoid synthesis was observed in tibias of mice treated with G-CSF (Figure 2A). Similarly, both mineral apposition rate and total surface mineralization were decreased on the endosteal surfaces of calvaria harvested from G-CSF treated mice (Figure 2B). However, G-CSF had no significant effect on either parameter on the periosteal surfaces of calvaria. To test whether G-CSF treatment preferentially targets endosteal and trabecular osteoblasts in mouse long bones as well, we performed RNA in situ hybridization for osteocalcin mRNA. In untreated mice, osteocalcin mRNA was readily detected on endosteal, trabecular, and periosteal surfaces (Figure 2C, left). As expected, G-CSF treatment resulted in a significant reduction in osteocalcin mRNA expression in endosteal and trabecular osteoblasts. In contrast, no significant decrease in osteocalcin expression in periosteal osteoblasts after G-CSF treatment was observed (Figure 2C, center). Collectively, these data suggest that G-CSF selectively suppresses endosteal and trabecular osteoblasts.

4.4.3 G-CSF treatment suppresses osteoblast function through a hematopoietic cell intermediate. The selective targeting of endosteal and trabecular osteoblasts by G-CSF suggested the hypothesis that its effects on osteoblasts are mediated by a hematopoietic

cell intermediary. To test this hypothesis, bone marrow chimeras were generated by transplanting G-CSFR deficient bone marrow cells into wild type mice. Likewise, chimeras were generated in which wild type bone marrow was transplanted in G-CSFR deficient recipients. Since there is evidence suggesting that mesenchymal (stromal) cells can be transplanted to recipient mice(15), we sorted hematopoietic progenitor cells ($CD45^+$ Kit^+ $lineage^-$) to high purity prior to transplantation. Greater than 95% hematopoietic reconstitution with donor cells was confirmed in all chimeras 6-8 weeks after transplantation (data not shown). Chimeric mice were then treated 5 days with G-CSF and the level of bone marrow osteocalcin mRNA was measured to gauge the effect of G-CSF on the osteoblast compartment. In chimeric mice reconstituted with G-CSFR deficient hematopoietic cells, G-CSF treatment had no effect on osteocalcin expression (Figure 3A). In contrast, G-CSF treatment induced a greater than 30 fold decrease in osteocalcin mRNA in G-CSFR deficient mice reconstituted with wild type hematopoietic cells (Figure 3B). These data show that G-CSF does not act directly on osteoblasts or other stromal cells. Instead, G-CSF suppresses osteoblasts through activation of a (presumably G-CSFR-positive) hematopoietic cell intermediate.

4.4.4 G-CSF treatment increases osteoblast turnover by inducing apoptosis. The loss of mature osteoblasts during G-CSF administration could occur through three general mechanisms: increased osteoblast turnover, decreased osteoblast production, or induction of osteoblast quiescence (with attendant loss of GFP expression from the type I collagen promoter). To begin to distinguish between these possibilities, we designed an experiment to measure the turnover rate of labeled osteoblast lineage cells in the bone

marrow. pOBCol2.3-GFP mice were treated with BrdU for 14 days prior to treatment with G-CSF. This treatment resulted in 32% of osteoblasts lineage (GFP^+) cells being labeled with BrdU (Figure 4A). Mice were then treated with G-CSF, and the percentage of BrdU-labeled osteoblast-lineage cells in the bone marrow was determined as a function of time (Figure 4A). In control mice, a gradual loss of $BrdU^+$ GFP^+ cells was observed, with a calculated half-life of 7.7 days. In mice treated with G-CSF, a more rapid turnover of $BrdU^+$ GFP^+ cells was observed, with a half-life of 3.7 days. Of note, after stopping G-CSF, the turnover rate of $BrdU^+$ GFP^+ cells was similar in both groups of mice.

The increased turnover of osteoblasts in the bone marrow following G-CSF administration suggested that G-CSF may induce osteoblast apoptosis. Indeed, regulation of osteoblast survival is thought to be an important mechanism regulating osteoblast number in the bone marrow (16). To test this hypothesis, we determined whether G-CSF treatment induced apoptosis of osteoblasts. Based on the kinetics of osteoblast loss, we focused our analyses on day 3 of G-CSF treatment. GFP+ osteoblast-lineage cells were isolated from mice treated 3 days with G-CSF and the percentage of GFP^+ cells expressing activated caspase 3 was determined by flow cytometry (Figure 4B). In control mice, 4.3±1.1% of GFP^+ cells were apoptotic, as measured by activated caspase 3 expression. Of note, this number is within the range of reported values for osteoblast apoptosis in untreated mice (17-21). In G-CSF treated mice, the percentage of apoptotic cells was significantly increased ($9.2 \pm 0.6\%$, $p = 0.01$). These data suggest that G-CSF treatment suppresses mature osteoblasts, in least in part, by inducing apoptosis.

4.4.5 G-CSF administration is associated with the inhibition of osteoblast differentiation. We next asked whether osteoblast differentiation also was impaired following G-CSF administration. We first measured the effect of G-CSF on the expression of a panel of genes expressed at different stages of osteoblast differentiation (Figure 5A). G-CSF treatment resulted in a significant decrease in all of the osteoblast genes analyzed. However, the greatest decrease in expression was observed with genes expressed late during osteoblast maturation. Whereas a 19 fold decrease in the late osteoblast gene *Bglap2* (osteocalcin, OC) was observed, only a 1.8-fold reduction in the pan-osteoblast lineage transcription factor *Runx2* was noted.

The relative preservation of early osteoblast gene expression prompted us to examine the effect of G-CSF on osteoblast progenitor cells in the bone marrow. Specifically, the number of colony forming unit-fibroblast (CFU-F) and progenitor cells capable of forming alkaline phosphatase positive colonies (CFU-ALP) was measured. G-CSF treatment resulted in a 4.4-fold increase in CFU-F and a 12.6-fold increase in CFU-ALP over untreated controls (Figure 5B). To determine whether this increase in osteoprogenitors resulted in a later increase in mature osteoblasts, we extended the period of G-CSF administration to 22 days and measured osteocalcin mRNA expression in the bone marrow (Figure 5C). The decrease in osteocalcin mRNA expression was maximal by 5 days of G-CSF treatment and remained suppressed throughout the 22 day treatment period. Decreased osteoblast number was confirmed by histology (data not shown). The prolonged loss of osteocalcin-producing osteoblasts, despite the increase in osteoprogenitors, suggests that G-CSF administration leads to a defect in osteoblast maturation in mice.

As noted previously, a prior study suggested that G-CSF treatment might induce osteoblast quiescence. This model predicts that the recovery of mature osteoblasts upon discontinuation of G-CSF results from the reactivation of quiescent osteoblasts, rather than the production of new osteoblasts. To test this prediction, we measured BrdU uptake by osteoblasts during the recovery period after a five day course of G-CSF. In control mice, 11.5± 2.6% of GFP^+ osteoblast-lineage cells were labeled with BrdU at the end of the recovery phase, reflecting the rate of recruitment of new osteoblasts during this five-day period (Figure 5D). In contrast, 31.3± 3.6% of GFP^+ cells were labeled in mice that had received G-CSF, indicating that the rebound in osteoblast number during the recovery phase results from recruitment of new osteoblasts rather than recovery of quiescent osteoblasts.

4.4.6 G-CSF administration results in a decreased OPG/RANKL ratio and is associated with a late increase in osteoclast number. Previous studies have established that chronic treatment with G-CSF leads to increased osteoclast number and activity in the bone marrow. (4-6,22) Though there is evidence that G-CSF can directly activate the osteoclast lineage (8), the potent suppressive effect of G-CSF on osteoblasts suggests another possibility. Namely, since osteoblasts contribute to the regulation of osteoclastogenesis, the loss of osteoblasts during G-CSF treatment may secondarily activate osteoclasts. Indeed, the kinetics of the loss in osteoblasts and increase in osteoclasts is consistent with this possibility. While the decrease in osteoblast number was maximal after 5 days of G-CSF treatment (Figure 1A), no increase in osteoclast number at this time point was noted, a result consistent with previous reports (Figures 6A

and 6B) (6,8). In fact, a significant increase in osteoclasts was not noted until after fourteen days of G-CSF treatment.

A major mechanism by which osteoblasts regulate osteoclast number and activity is by the regulated production of receptor activator of NF-Kappa-B ligand (RANKL, *TNFSF11*) and osteoprotegerin (OPG, *TNFRSF11B*), a decoy receptor for RANKL. RANKL and OPG are positive and negative regulators of osteoclasts, respectively; thus, the relative expression of these genes is a key determinant of osteoclast activation (24). In mice treated with G-CSF for 5 days, no change in RANKL mRNA expression in the bone marrow was detected (Figure 6C). In contrast, a 12-fold decrease in OPG mRNA was observed after 5 days of G-CSF treatment (Figure 6C). The ratio of RANKL to OPG mRNA increased from 0.19 at baseline to 1.98 after 14 days of G-CSF. To verify that the loss of OPG mRNA resulted from the loss of osteoblasts, pOBCol2.3-GFP transgenic mice were treated with G-CSF, GFP^+ osteoblast-lineage cells were isolated, and OPG and RANKL mRNA was measured. While RANKL expression was preserved within this fraction after G-CSF treatment, OPG mRNA was reduced 10-fold, consistent with the loss of GFP^+ mature osteoblasts (Figure 6D).

4.5 DISCUSSION

In this study, we confirm and extend our previous finding that G-CSF treatment suppresses osteoblast number and activity. This effect appears to be specific to mature osteoblasts, as other osteoblast-lineage cells, including osteocytes and bone lining cells, are unperturbed. We provide evidence that G-CSF treatment increases apoptosis of mature osteoblasts while increasing the numbers of osteoprogenitors in the bone marrow.

Transplantation experiments show that G-CSF regulates osteoblasts in an indirect fashion through activation of an undetermined hematopoietic cell intermediary. Finally, we show that G-CSF treatment significantly alters the relative expression of RANKL and OPG in the bone marrow, providing a novel mechanism by which G-CSF treatment results osteoclast activation.

Apoptosis is thought to be one of the primary regulators of osteoblast homeostasis in the bone marrow. There is evidence that glucocorticoid treatment and estrogen withdrawal suppress osteoblast number through the induction of apoptosis (21,25). Conversely, inhibition of osteoblast apoptosis during intermittent parathyroid treatment may contribute to the bone anabolic effect seen with this treatment (20). In the present study, we show that G-CSF treatment results in an approximately two-fold increase in the turnover rate of BrdU-labeled osteoblast lineage cells in the bone marrow. Moreover, the percentage of cleaved caspase 3-positive osteoblasts recovered from G-CSF treated mice was increased two-fold compared with control mice. Together, these data suggest that G-CSF regulates osteoblast number in the bone marrow, in part by, inducing osteoblast apoptosis.

The following observations suggest that G-CSF also inhibits osteoblast differentiation in vivo. 1) G-CSF administration results in a marked increase in osteoprogenitors. 2) The increase in osteoprogenitors does not "rescue" the defect in mature osteoblasts, even after prolonged (22 days) G-CSF administration. In contrast, the increase in osteoprogenitors observed after estrogen withdrawal, which induces a greater degree of osteoblast apoptosis, is able to restore osteoblast number to normal.(26,27) 3). Expression of genes associated with earlier stages of osteoblast differentiation (e.g.,

Runx2) is reduced less than genes associated with later stages (e.g., *Bglap2*). 4). Finally, the rapid recovery of osteoblasts with proliferating (BrdU-labeled) cells following cessation of G-CSF, suggests that G-CSF administration leads to the accumulation of an expanded pool of osteoblasts precursors in the bone marrow. Collectively, these data suggest G-CSF administration leads to a loss mature osteoblasts in the bone marrow through both an increase in osteoblast turnover and inhibition of osteoblast differentiation.

In addition to hematopoietic cells, there is data suggesting that the G-CSFR is expressed on wide range of non-hematopoietic tissues including endothelial cells, neurons, and possibly cardiomyocytes (28,29). Previous studies have shown that the G-CSFR is not expressed on osteoblast cells lines or cultured primary murine calvarial osteoblasts (9,10). Whether the G-CSFR is expressed on osteoblasts in vivo has not been determined, therefore the possibility that G-CSF's effects on osteoblasts are direct cannot be excluded. In this study, we provide definitive evidence through the use of G-CSFR deficient bone marrow chimeras that G-CSF acts indirectly to suppress osteoblasts. Indeed, these data strongly suggest that this phenotype is dependent upon a transplantable hematopoietic cell intermediate. Consistent with this conclusion, G-CSF treatment preferentially targets endosteal and trabecular osteoblasts, with little effect on periosteal osteoblasts.

The hematopoietic cell population(s) that mediate the suppressive effect of G-CSF on osteoblasts are not known. The G-CSFR is expressed at high levels on neutrophils, monocytes, osteoclasts, and hematopoietic progenitors. There also are reports of G-CSFR expression on natural killer cells and a subset of B lymphocytes. However, the

suppressive effect of G-CSF treatment on osteoblasts is preserved in *RAG1* deficient mice, suggesting that lymphocytes are not required for this effect (9). Studies are underway to define the role of neutrophils, monocytes, and osteoclasts in this pathway.

The pathway leading from hematopoietic cell activation by G-CSF to osteoblast apoptosis also remains poorly understood. In a series of elegant studies, Katayama et al recently provided evidence that G-CSF-induced osteoblast suppression is mediated by the sympathetic nervous system (9). Our studies of G-CSFR deficient bone marrow chimeras strongly suggest that G-CSF does not act directly on neurons to suppress osteoblasts. Rather, our data raise the possibility that G-CSF induced activation of hematopoietic cells indirectly leads to activation of the sympathetic nervous system and ultimately osteoblast apoptosis.

A consistent feature of G-CSF-induced osteopenia in both humans and mice is osteoclast activation. Previous studies have demonstrated that G-CSF can act directly on osteoclast precursors stimulating their differentiation *in vitro* into mature osteoclasts (8). In the present study, we provide evidence for a novel mechanism by which G-CSF treatment leads to osteoclast activation. G-CSF treatment leads to a marked decrease in OPG expression in the bone marrow, while levels of RANKL expression remain relatively constant. This altered ratio of OPG to RANKL expression is predicted to increase RANK signaling in osteoclasts precursors, thereby stimulating osteoclast production and activation. In addition to osteoblasts, RANKL and OPG are also expressed by other stromal cells and certain lymphocyte subsets.(24) However, our data suggest that G-CSF specifically targets the osteoblast lineage, as OPG expression was markedly decreased after G-CSF administration in sorted GFP^+ cells from pOBCol2.3-

GFP transgenic mice. Consistent with this conclusion, G-CSF dependent osteoclastogenesis was not observed until at least five days after the beginning of G-CSF treatment, at which time the G-CSF-induced loss of osteoblasts was complete. This late activation of osteoclasts by G-CSF corroborates reports from other groups (6,8) and supports the notion that loss of OPG expression plays an important role in stimulating osteoclastogenesis during G-CSF treatment *in vivo*.

In summary, G-CSF signaling through hematopoietic cells in the bone marrow exerts powerful effects on both osteoblasts and osteoclasts, resulting in imbalance between bone formation and resorption. It is hoped that by continuing to unravel the pathways by which G-CSF targets bone cells, greater insight will be gained into how the hematopoietic compartment interacts with bone cells.

ACKNOWLEDGEMENTS

The authors thank David J. Rowe for providing the pOBCol2.3-GFP mice, Amgen for providing the G-CSF, and Deborah V. Novack for assistance with the histomorphometric analysis. We also thank Deborah V. Novack, Roberto Civitelli, and F. Patrick Ross for their critical review of this manuscript. This work was supported by a National Institutes of Health grant HL60772.

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4.7 FIGURE LEGENDS

Figure 4.1 Loss of osteoblast number and function during G-CSF treatment. (A) Mice were treated with G-CSF (250ug/kg/day) for 5 days, and the number of trabecular osteoblasts per mm of bone perimeter was determined. (B) Immunohistochemistry showing GFP positive (brown) osteoblasts (arrows), bone lining cells (arrowheads), and osteocytes in untreated or day 5 G-CSF treated pOBCol2.3-GFP transgenic mouse femurs. Insets show enlargement of area enclosed by dotted line. Original magnification 100x, scalebar=200μm. (C) Quantification of mature osteoblasts, bone lining cells, and osteocytes in transgenic mice treated with G-CSF for 5 days or untreated (n=4 each group). (D) Representative scatter plots showing GFP expression (lower panels) in the stromal (CD45 negative, Ter119 negative) cell population (upper panels) isolated from non-transgenic and pOBCol2.3-GFP mice (left and right respectively). (E) Shown is the number of GFP^+ cells recovered from the femurs of transgenic mice after treatment with G-CSF (n=2-10 each time point). Data represent the mean ± SEM. *P<0.05, **P<0.01.

Figure 4.2 Loss of endosteal and trabecular, but not periosteal osteoblast activity during G-CSF treatment. Osteoid and mineralization were measured in untreated mice or mice treated for 7 days with G-CSF (n=2-3 each group). (A) Percent osteoid surface was calculated in Masson trichrome stained tibial sections from untreated and treated wild type mice. (B) Mineral apposition rate and percent mineralizing surface were calculated on endosteal and periosteal surfaces from calcein-labeled calvaria. (C) Osteocalcin RNA in situ hybridization of long bones harvested from untreated mice or mice treated for five days with G-CSF. Shown are representative photomicrographs of 3 independent experiments. Periosteal surfaces (arrows), endosteal surfaces (arrow heads), bone (B) and bone marrow

(BM) are indicated. Original magnification 100x, scalebar=100μm. Data represent the mean ± SEM. *P<0.05.

Figure 4.3 G-CSF receptor knockout bone marrow chimeras. (A) G-CSFR$^{-/-}$ CD45^{+} cKit^{+} Lineage^{-} hematopoietic cells (KL) cells were transplanted into wild type recipients (n=4-5 each group). Following hematopoietic reconstitution (6-8 weeks), chimeric mice were treated with G-CSF (or left untreated), and osteocalcin mRNA expression in the bone marrow was measured by real time RT-PCR. (B) Wild type KL cells were transplanted into irradiated G-CSFR$^{-/-}$ recipients (n=6-7, each group) and analyzed in a similar fashion. Data represent the mean ± SEM. *P<0.05

Figure 4.4 Osteoblast turnover during G-CSF treatment. (A) Transgenic pOBCol2.3-GFP mice (n=5-6, each group) were administered BrdU for four fourteen days and then either treated for 5 days with G-CSF or left untreated. Mice were analyzed just prior to G-CSF treatment, after 5 days of G-CSF treatment, or after a 5 day recovery period (arrow heads). Shown is the percent of GFP^{+} cells in the bone marrow that were labeled with BrdU. (B) Representative scatter plots showing activated caspase 3 staining in the GFP^{+} cell population from untreated (left) or G-CSF treated pOBCol2.3-GFP mice (right). (C) Shown is the percentage of GFP^{+} cells that express activated caspase 3 from untreated and day 3 G-CSF-treated mice (n=4 each group). Data represent the mean ± SEM. *P<0.05, **P<0.01.

Figure 4.5 Analysis of early osteoblast lineage cells during G-CSF treatment. (A) Real time RT-PCR for the indicated genes was performed on total bone marrow RNA isolated after 5 days of G-CSF treatment. RNA expression relative to β-actin mRNA was

calculated and compared with untreated bone marrow (assigned a value of 1; n=5-12). (B) Shown is the number of alkaline phosphatase negative (CFU-F, left) and positive (CFU-Alp, right) colonies generated from the bone marrow of untreated or G-CSF treated mice (n=5-6 each group). (C) Mice (n=2-4 each time point) were treated with G-CSF for the indicated period up to 22 days. Mice were sacrificed at time points and analyzed for osteocalcin mRNA by real time RT-PCR. (D) Mice (n=6, each group) were treated 5 days with G-CSF or left untreated and then administered BrdU for five days during the recovery period. Shown is the percent of GFP^+ cells in the bone marrow that were labeled with BrdU. Data represent the mean ± SEM. *P<0.05, **P<0.001.

Figure 4.6 Osteoclastogenesis during G-CSF treatment. (A and B) Wild type mice (n=2-6 each group) were treated with G-CSF for the indicated time or left untreated. Osteoclast number (A) and surface (B) were estimated by enumerating TRAP positive cells in paraffin embedded sections of mouse long bones. (C) RANKL and OPG mRNA expression in the bone marrow of untreated or 5-day G-CSF treated mice (n=5-8 each group) was measured by real time RT-PCR. (D) GFP+ cells were sorted from G-CSF-treated pOBCol2.3-GFP mice. RANKL and OPG mRNA was measured within this fraction. Data represent the mean ± SEM. *P<0.05, **P<.01

Figure 4.1

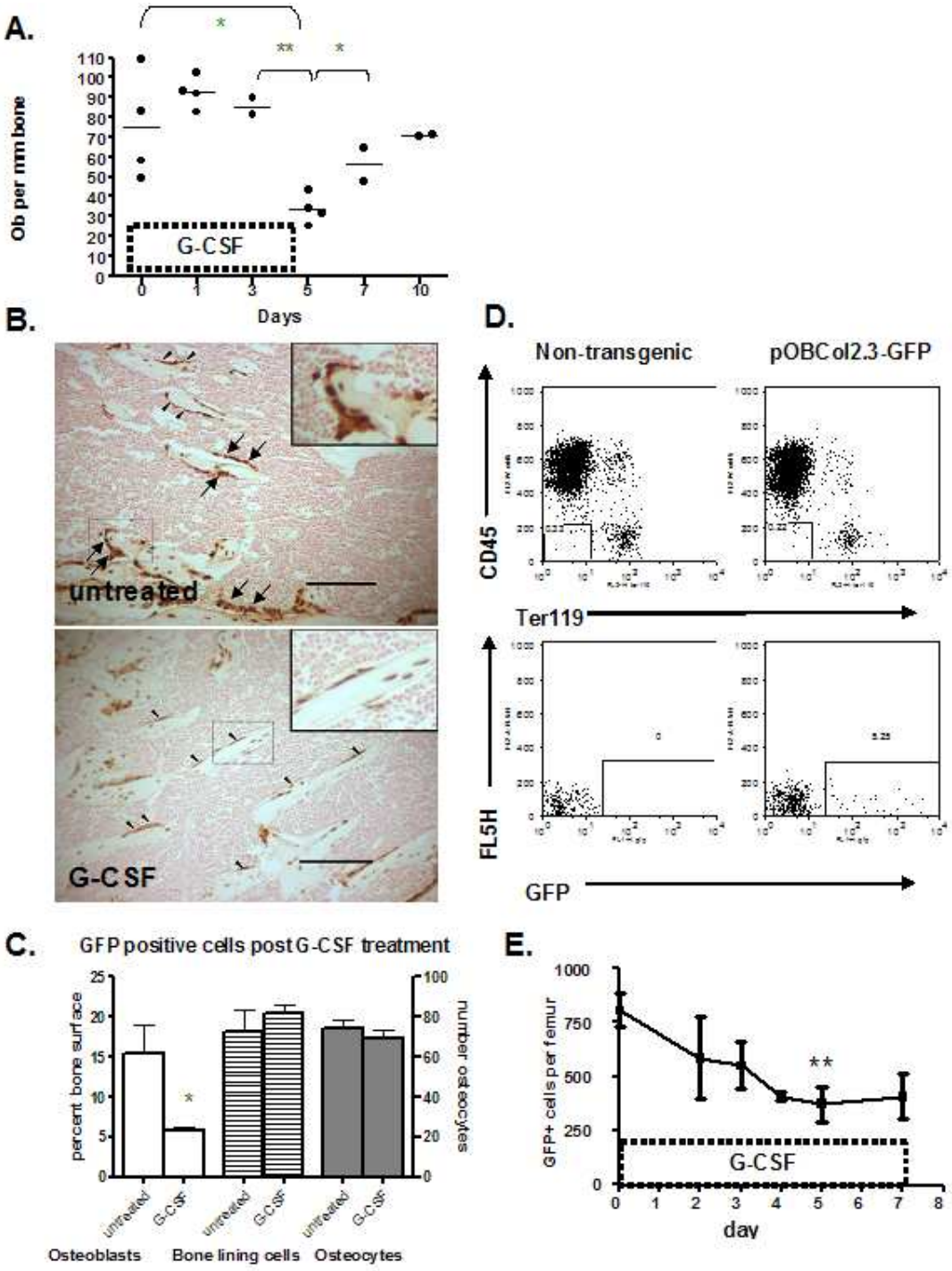

A.
Ob per mm bone
G-CSF
Days
B.
untreated
G-CSF
C.
GFP positive cells post G-CSF treatment
percent bone surface
number osteocytes
untreated
G-CSF
Osteoblasts
Bone lining cells
Osteocytes
D.
Non-transgenic
pOBCol2.3-GFP
CD45
Ter119
FL5H
GFP
E.
GFP+ cells per femur
G-CSF
day

Figure 4.2

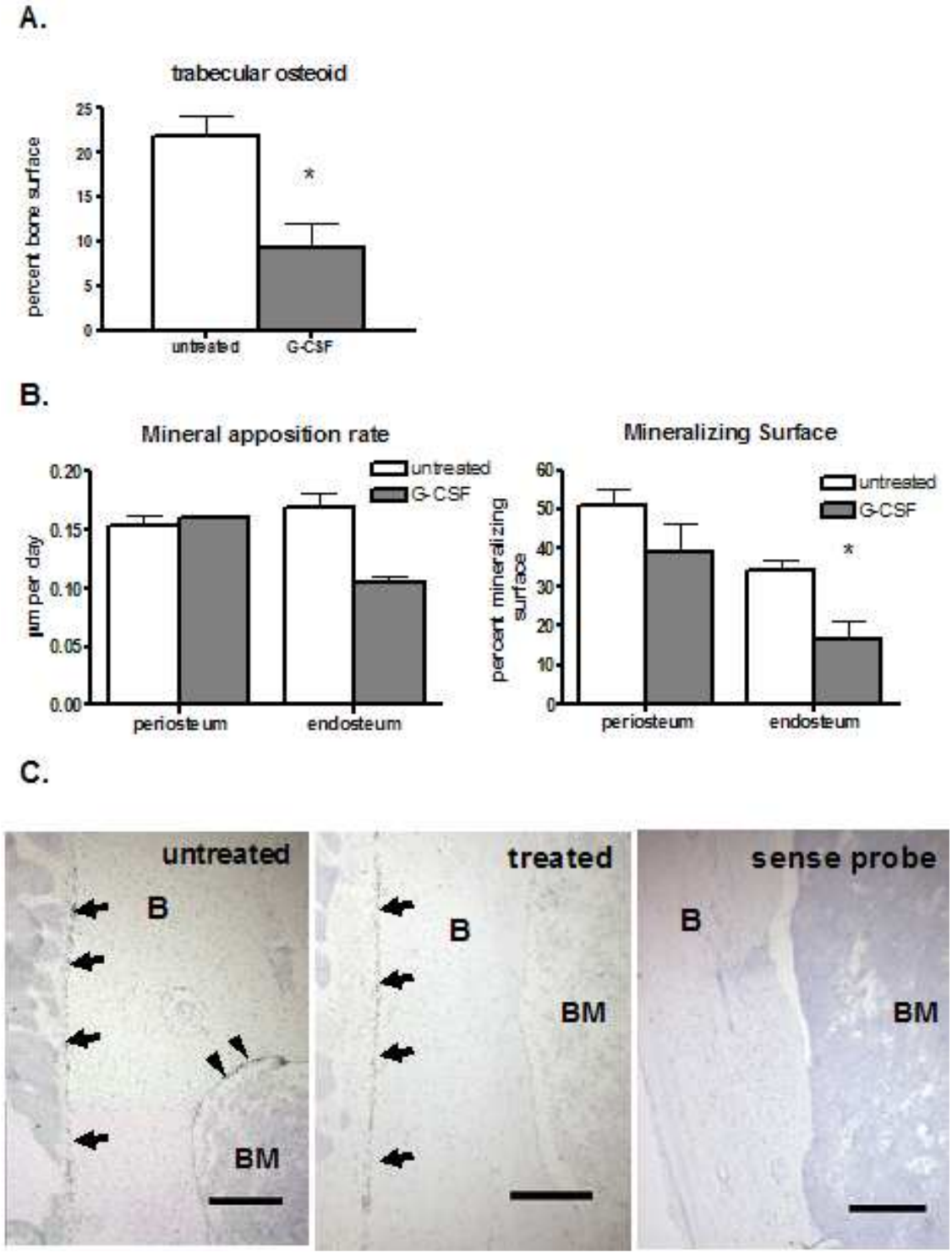

A.
trabecular osteoid
percent bone surface
25
20
15
10
5
0
*
untreated
G-CSF
B.
Mineral apposition rate
μm per day
0.20
0.15
0.10
0.05
0.00
untreated
G-CSF
periosteum
endosteum
Mineralizing Surface
percent mineralizing surface
60
50
40
30
20
10
0
untreated
G-CSF
*
periosteum
endosteum
C.
untreated
B
BM
treated
B
BM
sense probe
B
BM

Figure 4.3

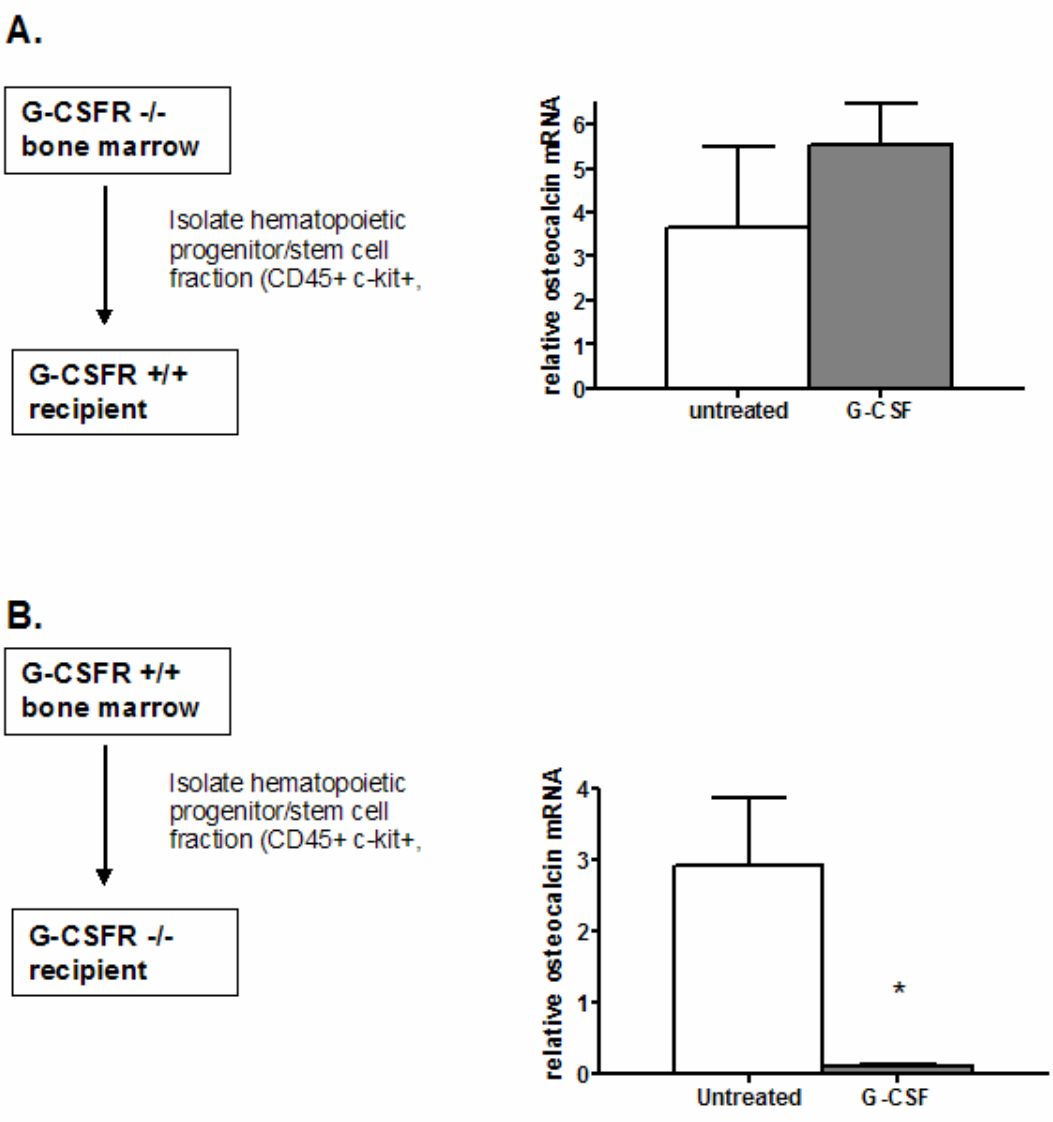

A.
G-CSFR -/- bone marrow
Isolate hematopoietic progenitor/stem cell fraction (CD45+ c-kit+,
G-CSFR +/+ recipient
relative osteocalcin mRNA
0
1
2
3
4
5
6
untreated
G-CSF
B.
G-CSFR +/+ bone marrow
Isolate hematopoietic progenitor/stem cell fraction (CD45+ c-kit+,
G-CSFR -/- recipient
relative osteocalcin mRNA
0
1
2
3
4
*
Untreated
G-CSF

Figure 4.4

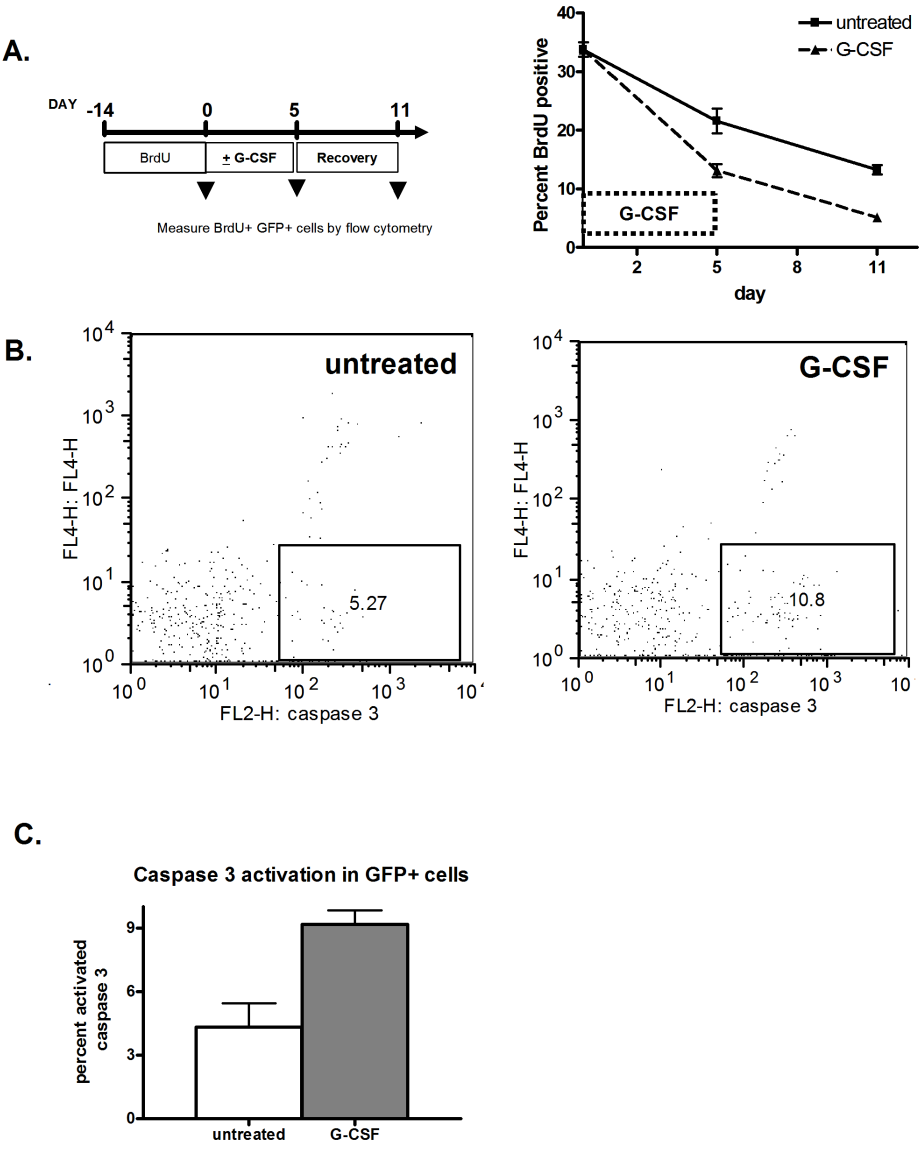

A.
DAY -14
0
5
11
BrdU
± G-CSF
Recovery
Measure BrdU+ GFP+ cells by flow cytometry
untreated
G-CSF
Percent BrdU positive
G-CSF
day
B.
untreated
G-CSF
FL4-H: FL4-H
FL2-H: caspase 3
5.27
10.8
C.
Caspase 3 activation in GFP+ cells
percent activated caspase 3
untreated
G-CSF

Figure 4.5

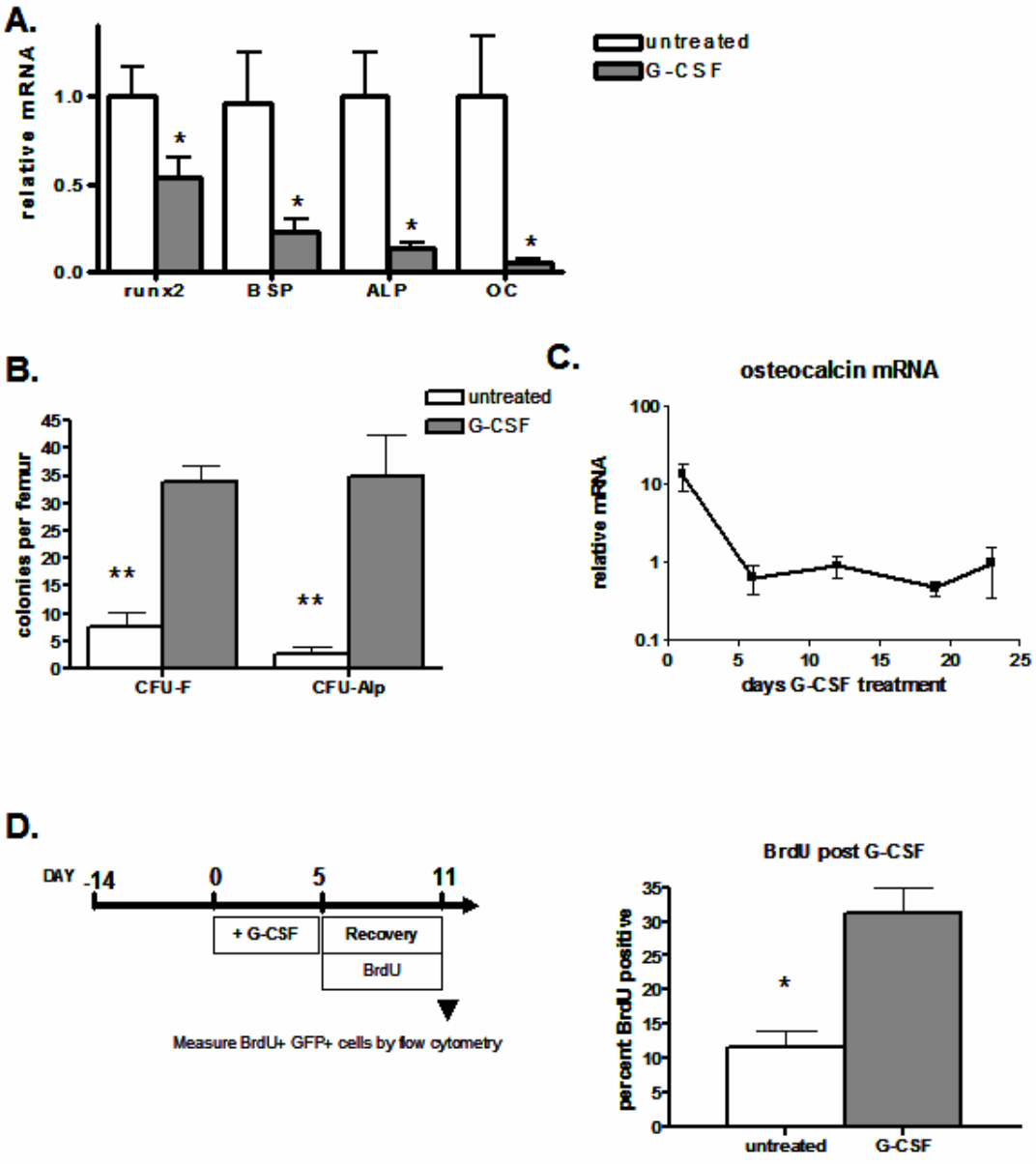
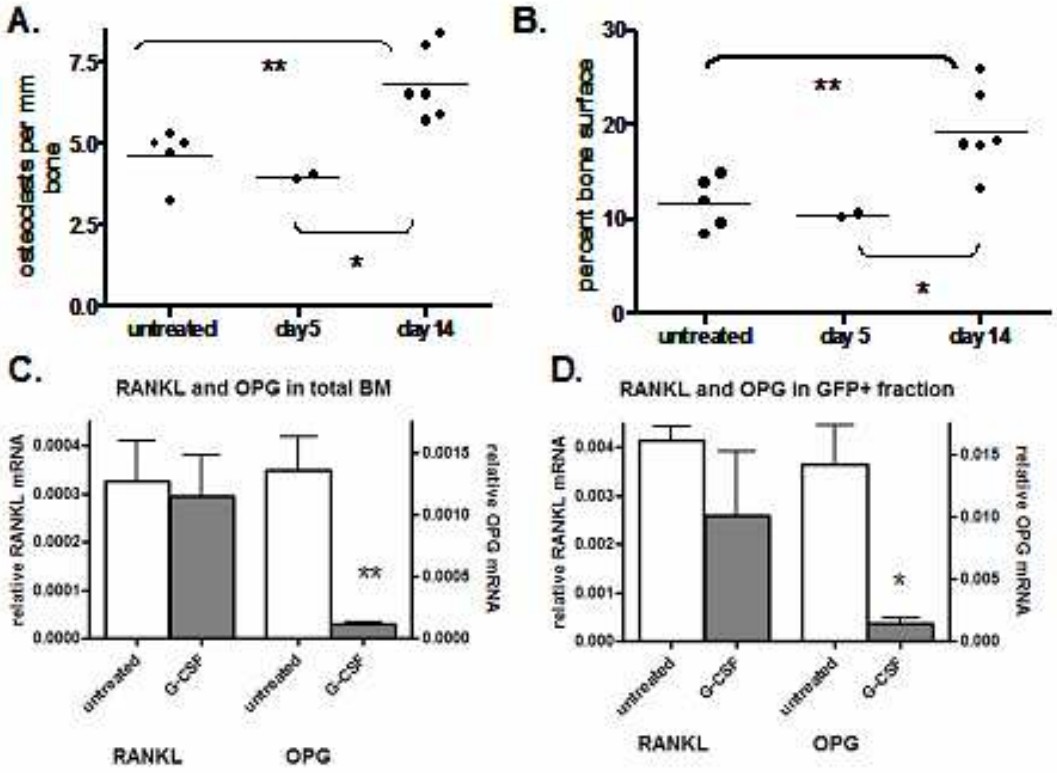

A.
relative mRNA
1.0
0.5
0.0
runx2
BSP
ALP
OC
untreated
G-CSF
B.
colonies per femur
untreated
G-CSF
CFU-F
CFU-Alp
C.
osteocalcin mRNA
relative mRNA
days G-CSF treatment
D.
DAY -14
0
5
11
+ G-CSF
Recovery
BrdU
Measure BrdU+ GFP+ cells by flow cytometry
BrdU post G-CSF
percent BrdU positive
untreated
G-CSF

Figure 4.6


A.
osteoclasts per mm bone
7.5
5.0
2.5
0.0
**
*
untreated
day5
day 14
B.
percent bone surface
30
20
10
0
**
*
untreated
day 5
day 14
C.
RANKL and OPG in total BM
relative RANKL mRNA
0.0004
0.0003
0.0002
0.0001
0.0000
relative OPG mRNA
0.0015
0.0010
0.0005
0.0000
**
untreated
G-CSF
untreated
G-CSF
RANKL
OPG
D.
RANKL and OPG in GFP+ fraction
relative RANKL mRNA
0.004
0.003
0.002
0.001
0.000
relative OPG mRNA
0.015
0.010
0.005
0.000
*
untreated
G-CSF
untreated
G-CSF
RANKL
OPG

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The major goal of this thesis was to clarify the role of CXCL12/CXCR4 signaling in cytokine-induced mobilization and refine our understanding of how CXCL12 expression decreases in the bone marrow during G-CSF treatment. Unexpectedly, we uncovered a role for osteoblasts in regulating HSPC trafficking during cytokine treatment and have demonstrated a role for the hematopoietic compartment in regulating bone homeostasis by mediating osteoblast apoptosis and differentiation. While we have observed this phenomenon only in the rather specialized and non-physiologic setting of cytokine-induced mobilization, it is possible that further study will reveal a critical and reciprocal dependence between the hematopoietic and osteoblast compartments. Finally, recent reports that osteoblasts play a key role in maintaining the hematopoietic stem cell niche raise the possibility that cytokine treatment leads to mobilization in part by disrupting the stem cell niche.

5.1 Osteoblasts are a major source of bone marrow CXCL12 and decrease in number with G-CSF treatment. In Chapter 2 of the thesis, we investigate the decrease in bone marrow expression of CXCL12 observed during G-CSF treatment by comparing CXCL12 expression in different bone marrow populations. To this end, a novel method of isolating stromal cells, including bone-adherent osteoblasts, by subjecting mouse bones to serial collagenase digests was developed. Isolated cells were fractionated into hematopoietic, osteoblast, endothelial, and primitive mesenchymal progenitor fractions by high speed flow cytometry-based cell sorting. It was demonstrated that the osteoblast fraction was highly enriched for CXCL12 expression, suggesting that osteoblasts may play an important role in HSPC mobilization. Therefore, osteoblast number was

compared in mice treated with G-CSF or left untreated, and a roughly 50% reduction in the number of histologically identifiable endosteal and trabecular osteoblasts was observed. This finding corroborates previous data by Takamatsu et al who found that serum osteocalcin decreases in humans treated with G-CSF.[1] The loss of osteoblasts combined with the observation that osteoblasts represent a major source of bone marrow CXCL12 raise the possibility that the decrease in osteoblasts may play an important role in mobilization.

5.2 Loss of osteoblast-derived CXCL12 plays a critical role in cytokine-induced mobilization. Chapter 3 furthers these studies on the role of osteoblasts in mobilization by providing three observations. First, loss of osteoblasts and disruption of CXCL12/CXCR4 signaling is a common finding in mobilization induced by G-CSF, Flt3L, and SCF. Second, the decrease in bone marrow CXCL12 in cytokine-induced mobilization is attributable specifically to the loss of osteoblast-produced CXCL12. Third, disruption of CXCL12/CXCR4 signaling is the principle pathway by which G-CSF induces mobilization. This latter finding was highlighted in experiments that showing that an antagonist of VLA-4 sigaling—but not G-CSF—increased the number of circulating HSPC in the genetic absence of CXCR4 in hematopoietic cells. These data strengthen the association between decreased osteoblast number and HSPC mobilization.

5.3 G-CSF treatment increases osteoblast apoptosis and blocks differentiation through a hematopoietic intermediary. Chapter 4 of the thesis investigates in more detail the effect that G-CSF has on osteoblast number and function. The kinetics of

osteoblast loss were found to mirror mobilization, with no osteoblast loss detected until day 4 of treatment. Both osteoid formation and bone mineralization was reduced by G-CSF treatment, consistent with the loss of osteoblasts, and mRNA expression of osteoblast markers Runx2, bone sialoprotein, alkaline phosphatase, and osteocalcin were sharply decreased. The loss of osteoblasts does not result from failure of osteoprogenitor commitment, as the number of alkaline phosphatase positive CFU-F was strikingly increased with G-CSF treatment. Instead, by making use of transgenic mice expressing GFP in osteoblast lineage cells (pOBCol2.3-GFP mice) we determined that the half life of labeled osteoblasts decreased by half during G-CSF treatment. This decrease may be due to accelerated apoptosis, as there was a roughly 2-fold increase in the percent of activated caspase 3 positive osteoblasts in G-CSF treated mice. Interestingly, despite the increase in osteoprogenitor number, osteoblast number and osteocalcin expression never recover with prolonged G-CSF treatment (up to 22 days) suggesting that G-CSF blocks osteoblast development. Finally, we generated bone marrow chimeras by transplanting G-CSF receptor deficient hematopoietic cells into wild type mice and administered G-CSF. These mice failed to mobilize and had no decrease in osteocalcin expression, showing that the effect of G-CSF on osteoblasts is indirect and requires signaling through the hematopoietic compartment. The nature of these signals and the identification of the hematopoietic cell type(s) involved will be a major goal of our lab in the future.

5.4 Bone marrow monocytes may play a key role in supporting osteoblasts. As a first step toward understanding the molecular pathways by which G-CSF regulates osteoblasts, we hope to identify the hematopoietic cell type or types that are required for

G-CSF-induced suppression of osteoblasts. Hematopoietic cell types likely to regulate osteoblasts include lymphocytes and monocyte/osteoclast lineage cells. However, mice deficient in lymphocytes due to the *Rag2* gene mobilize normally, making it unlikely that lymphocytes mediate this effect. ([2] and D.C.L. unpublished data) Therefore we hypothesize that osteoblast development and apoptosis are mediated by monocyte lineage cells in the bone marrow during G-CSF treatment. The first approach we are using to address this hypothesis is to generate transgenic mice that express the G-CSF receptor under control of the human CD68 promoter.[3,4] This promoter is expected to direct G-CSF receptor expression in monocyte and macrophage lineages only, and when crossed with our G-CSF receptor deficient mice would generate offspring where the G-CSF receptor expression is restricted to monocytes and macrophages. These mice will then be tested for response to G-CSF.

The second approach to testing the hypothesis that bone marrow monocytes mediate G-CSF effects on osteoblasts has been to administer G-CSF to transgenic mice whose monocyte lineage cells have been ablated by a monocyte-expressed suicide gene. So-called Mafia ("Macrophage Fas-induced Apoptosis") mice lose monocyte lineage cells when treated 5 days with a synthetic suicide receptor ligand, referred to as the "dimerizer." [5,6] Mafia mice treated five days with dimerizer mobilize HSPC to peripheral blood, an effect accompanied by a striking loss of osteocalcin expression and identifiable osteoblasts (Figure 5.1B-C) with no discernable effect on osteoclasts or osteoprogenitors (not shown). These results suggested two possibilities. First, the loss of monocytes may result in the loss of a factor that osteoblasts need for survival. Alternately, the loss of monocytes may result in non-specific toxicity in osteoblasts. To help distinguish

between these two possibilities, mixed chimeras were generated by transplanting wild type and Mafia bone marrow mixed 1:1 into lethally irradiated hosts. If loss of monocytes removes a factor that osteoblasts need for survival, it may be supposed that in the absence of one half the normal complement of monocytes, most osteoblasts will survive. On the other hand, if loss of monocytes causes release of a toxic factor, loss of even one half of total monocytes would cause measurable toxicity. In 1:1 mixed wild type to Mafia chimeras, dimerizer treatment resulted in no significant loss in osteocalcin expression, compared to 760-fold reduction in dimerizer-treated Mafia mice (Figure 5.1D). These results suggest that bone marrow monocytes may play a role in steady state maintenance of osteoblasts. Further work will be required both to confirm these findings and to identify a putative monocyte-derived factor that supports osteoblasts.

5.5 Loss of osteoblasts during G-CSF treatment severely compromises hematopoietic stem cell function. As osteoblasts play a critical role in maintaining the hematopoietic stem cell (HSC) pool, we have performed some preliminary experiments assessing the function of HSC after G-CSF treatment. Repopulating function of HSC from G-CSF treated mice is markedly reduced compared to untreated HSC in a competitive repopulation assay, which is the gold standard measure of stem cell function.[7] This effect is seen in both primary and secondary transplants (Figure 2A-B) and is present even when bone marrow from treated animals is injected intrafemorally, suggesting that it does not result from an artifactual failure to home to the bone marrow of irradiated recipients (Figure 2C). Further work on characterizing the precise molecular

defect in HSC from G-CSF treated mice is being continued by Priya Gopalan, an Oncology Fellow in the lab.

5.6 Conclusion. The studies described in this thesis shed light on molecular underpinnings of HSPC both by demonstrating the interdependence of previously described mechanisms and by revealing a novel mechanism involved in mobilization, cytokine-induced loss of osteoblasts. When this work was begun, the prevailing model for HSPC mobilization centered on the role of proteases, upregulated by G-CSF treatment, which work through pathways organized in parallel (Figure 5.3A). Proteolytic cleavage of integrins, hyaluronic acid, c-Kit, and CXCL12 all contribute to mobilization in this model. The work presented here, however, suggests a somewhat different model (Figure 5.3B). The finding that G-CSF treatment leads to loss of bone marrow osteoblasts and CXCL12 mRNA (Chapter 1) raises the possibility that protease activation is not necessary for clearance of CXCL12. That G-CSF does not increase mobilization in the absence of CXCR4 signaling (Chapter 2) suggests that this one pathway is sufficient to mediate HSPC mobilization during G-CSF treatment. In this revised model, loss of osteoblasts—an indirect effect of G-CSF treatment mediated through the hematopoietic system—is upstream of CXCL12/CXCR4 disruption, and future research will focus on what lies upstream and downstream of this signaling axis.

It bears noting that the implications of these findings extend beyond the field of HSPC mobilization. First, the loss of osteoblasts results in a significant, although temporary, loss in HSC repopulation ability. Better understanding G-CSF-induced osteoblast depletion and its downstream effects on HSC may lead to important discoveries about

HSC biology and factors that sustain HSC in their bone marrow niche. Second, identifying pathways by which the hematopoietic compartment regulates osteoblast apoptosis and differentiation may improve our understanding of bone homeostasis and how the hematopoietic compartment and the bone compartment regulate each other's function. Thus G-CSF-induced mobilization may prove to be a useful platform for improving our understanding of wider biological processes.

5.7 BIBLIOGRAPHY

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5.8 FIGURE LEGENDS

Figure 5.1. Monocytes may play a key role in supporting osteoblast survival in vivo. Mafia transgenic mice (n=5 each group) were treated with dimerizer or vehicle to induce monocyte death. (A) HSPC mobilization as measured by number peripheral blood CFU-C. (B) Total bone marrow osteocalcin mRNA as measured by Q-RT-PCR. (C) Representative photomicrograph showing TRAP stained paraffin sections from long bones of vehicle treated (left) or dimerizer treated (right) mice. Osteoblasts are difficult to find in the right panel, although red-staining osteoclasts are abundant. (D) Wile type and Mafia bone marrow was mixed at a 1:1 ratio and transplanted into irradiated recipients. Resulting mixed chimeras were treated 5 days with dimerizer or vehicle, RNA was isolated from long bones and osteocalcin mRNA was measured. The difference was not statistically significant. Data represent mean ± SEM. *p<.05

Figure 5.2. G-CSF treatment causes loss of hematopoietic stem cell function. Ly5.1 mice were treated 7d with G-CSF or left untreated then injected with an equal number of unmanipulated Ly5.2 cells into lethally irradiated Ly5.1/5.2 hosts (n=5 each group). Shown is peripheral blood chimerism of Ly5.1 test cells eight months up to eight months post transplant in mice that received (A) intravenous or (B) intrafemoral transplants. (C) After 8 months, primary recipients were harvested and transplanted into irradiated secondary recipients who were subsequently analyzed 6 weeks later for peripheral blood chimerism in B220, Gr-1, and CD3 lineages. Data represent mean ± SEM. *p<.01

Figure 5.3. Two competing models for G-CSF-induced HSPC mobilization. (A) Previous reports suggested a model where a variety of adhesive or chemotactic interactions worked in parallel to mediate HSC retention in the bone marrow (left). In this model, these interactions are attenuated by proteolytic cleavage upon G-CSF treatment. (B) Model of G-CSF-induced mobilization as suggested by experiments presented here. At steady state monocytes produce trophic factor to maintain osteoblast compartment. G-CSF acts on monocytes to downregulate trophic factor, resulting in loss of osteoblasts. Loss of osteoblast CXCL12 expression in this model is sufficient to lead to mobilization.

5.9 FIGURES
Figure 5.1

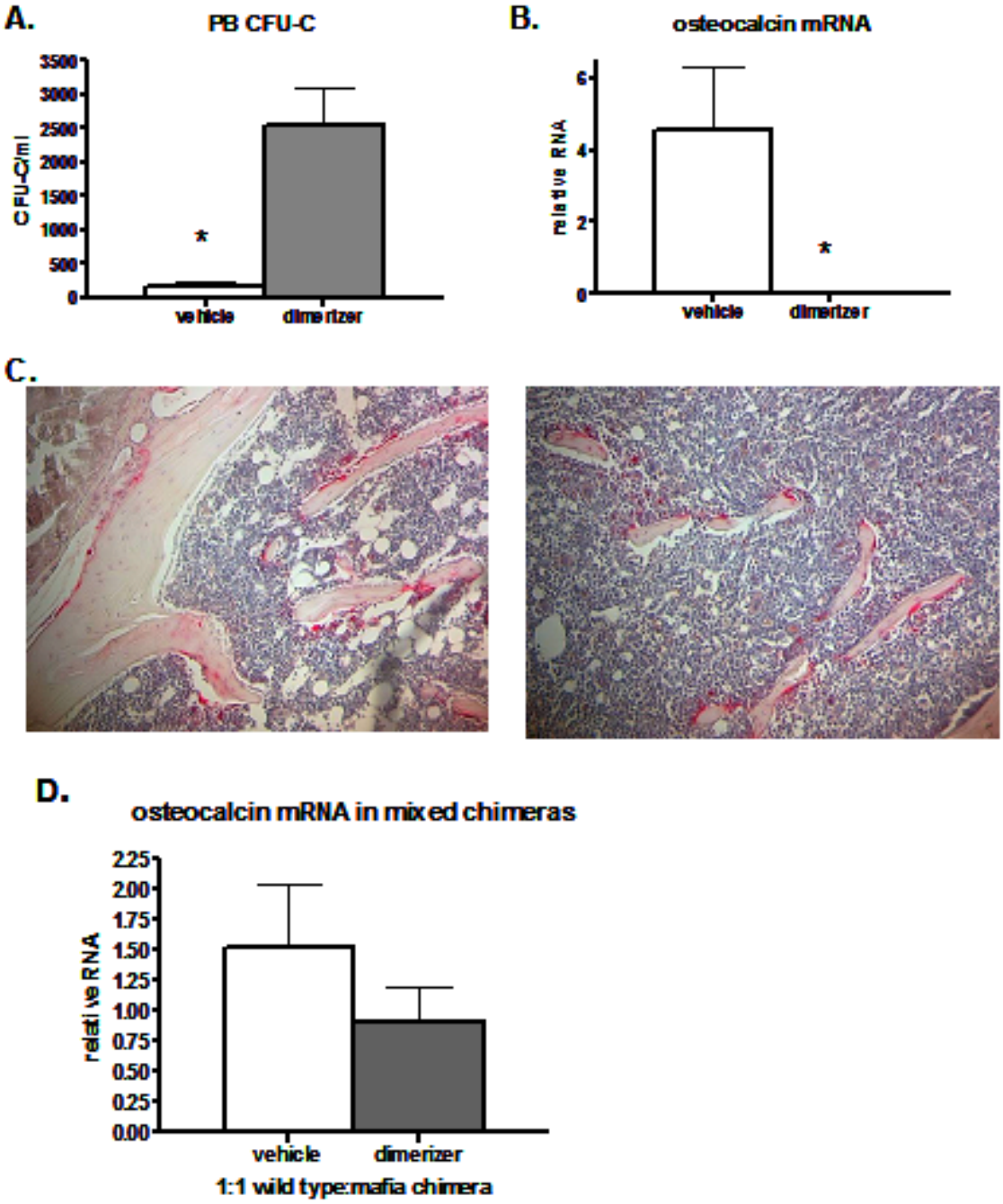


Figure 5.2

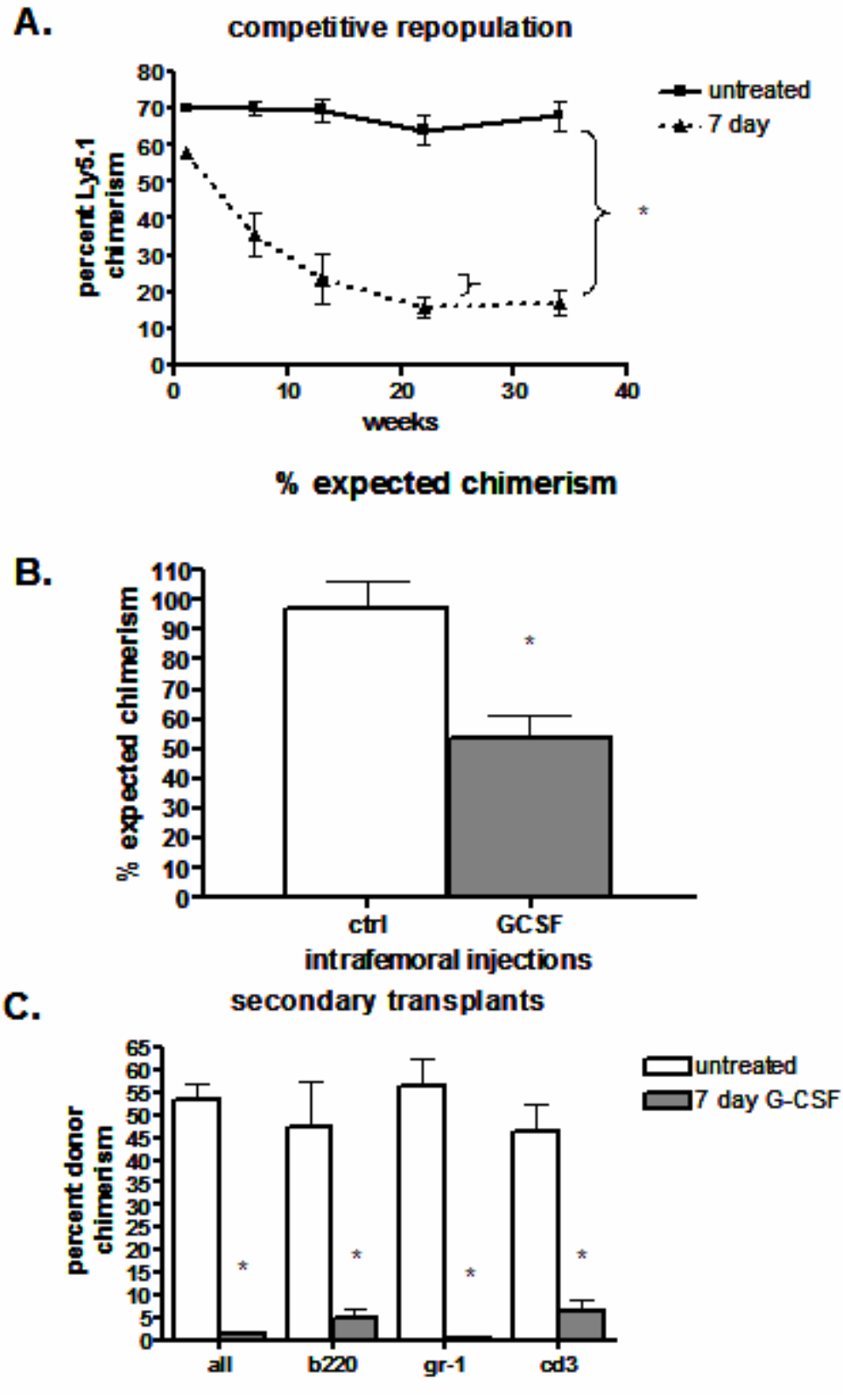
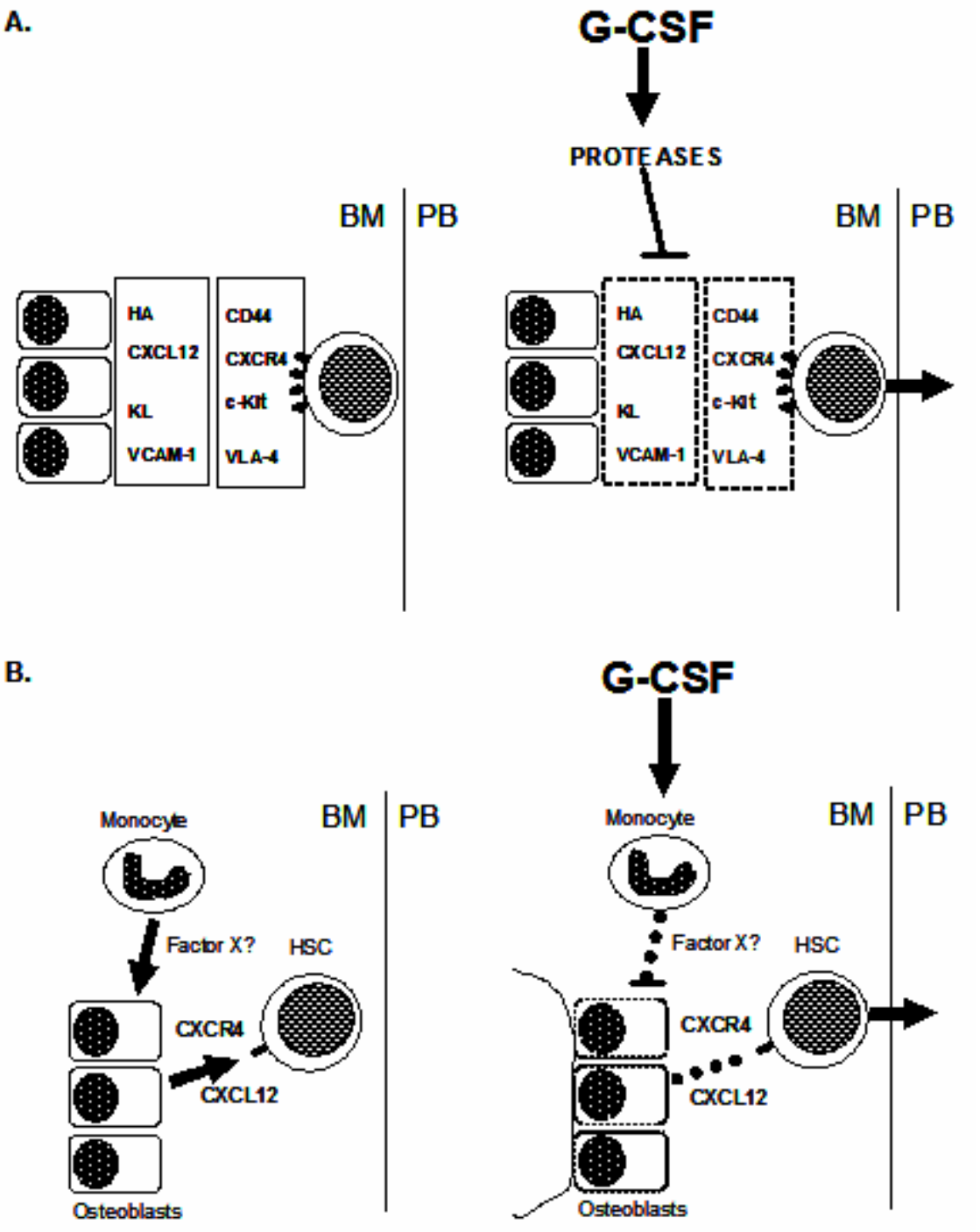

A.
competitive repopulation
percent Ly5.1 chimerism
weeks
untreated
7 day
*
% expected chimerism
B.
% expected chimerism
ctrl
GCSF
intrafemoral injections
*
C.
secondary transplants
percent donor chimerism
untreated
7 day G-CSF
all
b220
gr-1
cd3
*

Figure 5.3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G-CSF
PROTEASES
BM
PB
HA
CXCL12
KL
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BM
PB
HA
CXCL12
KL
VCAM-1
CD44
CXCR4
c-Kit
VLA-4
B.
G-CSF
Monocyte
BM
PB
Factor X?
HSC
CXCR4
CXCL12
Osteoblasts
Monocyte
BM
PB
Factor X?
HSC
CXCR4
CXCL12
Osteoblasts