January 2011

Regulation Of Neutrophil Homeostasis By Chemokines Signaling Through The Cxcr2 And Cxcr4 Receptors

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REGULATION OF NEUTROPHIL HOMEOSTASIS BY CHEMOKINES SIGNALING THROUGH THE CXCR2 AND CXCR4 RECEPTORS

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 2011

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

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The number of neutrophils in the blood is tightly regulated to ensure adequate protection against microbial pathogens while minimizing damage to host tissue. Neutrophil homeostasis in the blood is achieved through a balance of neutrophil production, release from the bone marrow, and clearance from the circulation. Accumulating evidence suggests that chemokine signaling in the bone marrow may play a key role in maintaining neutrophil homeostasis. Based on this evidence, we developed a "tug-of-war" model in which opposing chemokine gradients, specifically release-inducing CXCR2 signals and retention-promoting CXCR4 signals, act antagonistically to regulate neutrophil release from the bone marrow. We generated mice with neutrophils genetically deficient for the receptors CXCR2 (*CXCR2*$^{-/-}$), CXCR4 (*CXCR4*$^{-/-}$), or both (double knock-out or DKO) in order to define the mechanisms by which chemokine signals regulate neutrophil homeostasis.

We show that CXCR4 negatively regulates neutrophil release from the bone marrow in a cell autonomous fashion. However, CXCR4 is dispensable for neutrophil clearance from the circulation. Neutrophil mobilization responses to granulocyte colony stimulating factor (G-CSF), CXCL2, or *Listeria monocytogenes* infection are absent or impaired in *CXCR4*$^{-/-}$ neutrophils, suggesting that disruption of CXCR4 signaling may be a common step mediating neutrophil release.

CXCR2$^{-/-}$ neutrophils exhibit abnormal retention in the bone marrow and a peripheral neutropenia, which replicates the phenotype of the human disease myelokathexis and indicates that CXCR2 positively regulates neutrophil release from the bone marrow. *CXCR2*$^{-/-}$ neutrophils do not mobilize in response to an inhibitor of CXCR4 and have an impaired response to G-CSF, suggesting that neutrophil release requires the coordinated regulation of CXCR2 and CXCR4 signals. However, DKO neutrophils exhibit a shift from the bone marrow to the blood that is similar to *CXCR4*$^{-/-}$ cells, indicating that *CXCR4* is dominant to *CXCR2* and that there are likely to be CXCR2-independent mechanisms for directing neutrophil release.

Finally, we show that there is differential production of CXCR2 and CXCR4 ligands by bone marrow osteoblasts and endothelial cells that can be regulated by G-CSF. Taken together, our data suggest that coordinated osteoblast and endothelial production of CXCR2 and CXCR4 ligands is a common mechanism controlling neutrophil release from the bone marrow.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health (NIH) institutional training grants to the Department of Hematology and Medical Scientist Training Program (MSTP) at Washington University and NIH RO1 HL60772 to Daniel C. Link.

Douglas W. White provided invaluable assistance with the *Listeria* infection experiments detailed in section 2.4.7. DWW is supported by the Abbott Scholar Award and NIH K08 AI079011-01. I thank Priya K. Gopalan and Adam M. Greenbaum for their help with the cell sorting and gene expression array experiments in section 3.4.4.

Dan R. Littman (New York University School of Medicine) generously provided the *CXCR4*$^{flox/flox}$ mice and David Rowe (University of Connecticut) supplied the pOBCol2.3-GFP mice. We thank Amgen (Thousand Oaks, CA) for the G-CSF and Genzyme (Cambridge, MA) for the GROβ. We thank the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, Mo., for the use of the High Speed Cell Sorter, Multiplexed Gene Analysis, and Bioinformatics Cores. The Siteman Cancer Center is supported in part by an NCI Cancer Center Support Grant P30 CA91842.

Figure 1-4 used with kind permission from Springer Science+Business Media: Immunologic Research, Neutrophil Homeostasis, 32, 2005, 169-178, Daniel C. Link, Figure 2.

PERSONAL ACKNOWLEDGEMENTS AND DEDICATION

First and foremost, I would like to thank my mentor, Daniel Link, for the extraordinary guidance he has given me as I have worked, grown, struggled, laughed, learned, and discovered in his laboratory. This thesis would not have been possible without him. Thank you Dan for all you have done and will do for me and your present and future trainees. I thank Ram Mohan, my undergraduate mentor, for sparking my interest in science as more than just facts to be read in a textbook, but also those waiting to be discovered. I appreciate the time and effort the members of my thesis committee put in to advise me over the years. I am truly grateful to all my comrades in the Link laboratory over the years for such a professional, collegial, and fun working environment. All your advice and helpful discussion was appreciated. I would also like to personally acknowledge the assistance Jackie Means, an undergraduate student in the lab, provided on some of the experiements in Chapter 2. To my parents, Norm and Cheryl, thank you for your support over the years. Finally, to my wife, Marisa, thank you for everything. You, Preston, and Claire are my greatest inspiration. This thesis is dedicated to you.

BRIEF TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
ANOVA	analysis of variance
APC	allophycocyanin
ARDS	acute respiratory distress syndrome
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C5a	complement factor 5a
C/EBP	CCAAT enhancer binding protein
CFU	colony forming unit
CG	cathepsin G
CMP	common myeloid progenitor
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DARC	duffy antigen receptor for chemokines
DKO	double knockout
DPPI	dipeptidyl peptidase 1
EBV	Epstein-Barr virus
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
ES	embryonic stem cell
FACS	fluorescence activated cell sorting

FITC	fluorescein
G-CSF	granulocyte colony stimulating factor
G-CSFR	G-CSF receptor
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony stimulating factor
GMP	granulocyte/monocyte progenitor
GPCR	G protein coupled receptor
GRK	G protein receptor kinase
GRO	growth regulated oncogene
HPV	human papilloma virus
HSC	hematopoietic stem cell
IL	interleukin
INF	interferon
LAD	leukocyte adhesion deficiency
LKS	lineage^{low} kit^{+} sca^{+} cells
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony stimulating factor
MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
MKO	myeloid-specific knockout
MMP-9	matrix metalloproteinase 9

NDI	neutrophil distribution index
NE	neutrophil elastase
NOD-SCID	non-obese diabetic, severe combined immunodeficiency
PAF	platelet activating factor
PBS	phosphate buffered saline
PE	phycoerythrin
PECAM	platelet endothelial cell adhesion molecule
PMN	polymorphonuclear granulocyte
qRT-PCR	quantitative real time polymerase chain reaction
SAM	significance analysis of microarrays
SDF-1	stromal derived factor 1
SPF	specific pathogen free
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
VCAM	vascular cell adhesion molecule
VLA-4	very late antigen 4
WBC	White blood cell count
WHIM	warts, hypogammaglobulinemia, infections, myelokathexis syndrome
WS	WHIM syndrome

Chapter 1

Introduction

1.1 Overview and goals

The overall goal of this project was to characterize mechanisms that regulate circulating neutrophil counts and thus the ability of neutrophils to traffic to sites of inflammation and perform their various anti-bacterial functions. Particular emphasis was placed on factors governing neutrophil mobilization (the release of neutrophils from the bone marrow, the site of neutrophil production and maturation, into the blood) under either basal or inflammatory conditions.

The number of circulating neutrophils is tightly regulated in order to effectively protect against microbial pathogens while minimizing damage to host tissue. Homeostatic control of neutrophils in the blood is achieved through a balance of neutrophil production, release from the bone marrow, and clearance from the circulation. Accumulating evidence suggests that signaling by the chemokine CXCL12 (SDF-1), through its major receptor CXCR4, plays a key role in controlling neutrophil homeostasis by acting as a bone marrow retention signal. Alteration of CXCR4 signaling by genetic mutations, pharmacologic agents, or an inflammatory stimulus changes the number of circulating neutrophils. The role of CXCR4 in regulating neutrophil homeostasis under basal and stress conditions is investigated using a genetically defined model in **Chapter 2** of this thesis. However, recent genetic data in humans and work detailed in **Chapter 3** of this thesis have implicated additional chemokines and chemokine receptors in the regulation of neutrophil homeostasis. In **Chapter 3**, the role of CXCR2 chemokines in neutrophil trafficking from the bone marrow is investigated using neutrophils genetically deficient for *CXCR2*.

The work in this thesis supports the hypothesis that the degree of neutrophil mobilization is regulated by the balance of retention-promoting CXCL12/CXCR4 signals and release-inducing CXCR2 signals. We show that this may be a shared mechanism of action among diverse types of mobilizing agents. Our experiments delineate the cell-intrinsic and cell-extrinsic effects of CXCR2 and CXCR4 in the regulation of neutrophil trafficking. We investigate the role of these chemokines in neutrophil migration from the circulation into peripheral tissues and subsequent clearance. Finally, we begin to characterize the cellular sources of chemokines within the bone marrow. This thesis provides a more detailed picture of chemokine functions in neutrophil biology. Dysregulated neutrophil counts are implicated in numerous disease states, from bacterial infections in the setting of inherited or iatrogenic neutropenia to inflammatory diseases like atherosclerosis or arthritis that are associated with excessive neutrophils. Therefore, this work also increases our understanding of the pathophysiology and suggests potential treatments of some of these diseases.

1.2 Homeostatic control of circulating neutrophil counts

Neutrophils are leukocytes comprising a key component of the innate immune system, and they play a primary role in establishing an inflammatory response. Their main functions are to phagocytose and kill bacteria while also producing inflammatory mediators that allow resolution of the infection and establishment of long-lasting, adaptive immunity via other cells of the immune system. Patients with congenital or acquired neutropenia are extremely susceptible to severe, life-threatening bacterial

infections, although the advent of antibiotic and cytokine therapy has lessened this risk (1, 2). Conversely, excessive neutrophil accumulation at sites of inflammation can damage host tissue and contributes to disease pathogenesis in a number of acute and chronic human diseases. For example, neutrophils are thought to be key mediators of disease in acute respiratory distress syndrome and rheumatoid arthritis. In murine models of atherosclerosis (3) and autoimmune encephalitis(4), neutrophils have been shown to play a causative role in the disease process. Thus, circulating neutrophil counts must be tightly controlled in order to minimize the risk from both infectious and inflammatory disease.

1.2.1 Normal baseline neutrophil counts and clinical relevance

Neutrophil counts exhibit little variation within an individual during times of health, but there is considerable variation between individuals (Figure 1-1A) (5). This variation is likely to have a genetic basis, as it is dependent on ethnic background. In a large population study, Caucasians had a mean neutrophil count of 4.4 x 10^9/L, while that of African Americans was 3.65 x 10^9/L. Mice also exhibit variation that is dependent on genetic background (Figure 1-1B). Of note, whole genome association studies in model organisms have revealed chromosomal regions associated with neutrophil counts, including a region containing the CXCR2 chemokine CXCL8, but specific polymorphisms and their functional consequences were not identified (6).

In humans, total white blood cell counts (WBC) at baseline are mostly determined by their main component, neutrophils. Underscoring the importance of the regulation of

neutrophil counts, variation in WBCs has been associated with increased mortality. In one study, as WBC and neutrophil, but not lymphocyte, counts moved away from a central number (approximately the mean of the population), they were associated with increased all-cause mortality (Figure 1-1C). Excessively low counts would predispose to infection with common pathogens, while increased counts are independent risk factors for cardiovascular (7) and cancer mortality (8). Notably, mice have much lower circulating neutrophil counts than humans and also do not naturally develop cardiovascular disease (Figure 1-1B,C). These observations are concordant with accumulating research demonstrating that inflammation plays a key role in both cardiovascular disease and cancer. It is unclear whether alterations in neutrophil homeostasis play a causative role in these diseases or simply reflect underlying inflammatory processes.

1.2.2 Kinetics of neutrophil production and clearance

Given their key role in both infectious and inflammatory diseases, precise regulation of circulating neutrophil levels is therefore required. In the average man, over 100 billion mature neutrophils must enter the circulation from the bone marrow each day in order to maintain homeostasis; the blood neutrophil pool is turned over 2.3 times each day with a half-time of disappearance from the blood of only 6.8 hours (2, 9, 10). The bone marrow postmitotic transit time is 4 days in mice (11) and 7 days in humans (12), demonstrating that the vast majority of post-mitotic neutrophil lineage cells are stored in the bone marrow. In comparison to a turnover rate on the order of months for erythrocytes, the rapid kinetics of neutrophils show that complex yet precise regulatory mechanisms are

likely to control neutrophils. At another level of regulatory complexity, circulating neutrophil numbers are rapidly increased in response to infectious stimuli or other stress conditions in a process termed “emergency granulopoiesis”.

Neutrophil homeostasis (5, 13) is determined by regulation of three main processes: neutrophil *production* in the bone marrow, neutrophil *release* from the bone marrow into the circulation, and neutrophil *clearance* from the blood by adhesion, transmigration, apoptosis, and phagocytosis in the tissues. As detailed below, much research has been directed toward describing neutrophil responses in health and disease and determining the processes, cell types, molecules, and signaling pathways that regulate these responses. However, many questions remain incompletely answered.

1.3 Neutrophil production

The production and maturation of neutrophils in the bone marrow, or granulopoiesis, is the process by which a small number of pluripotent, self-renewing hematopoietic stem cells give rise to mature neutrophils through a number of cell division and differentiation steps. Stem cells give rise to increasingly differentiated progenitor cells (14). Granulocyte progenitors undergo characteristic differentiation through myeloblast, promyelocyte, myelocyte, and metamyelocyte forms before becoming mature polymorphonuclear granulocytes (PMNs) that can be described as either band or the more mature segmented form depending on the morphology of the nucleus. It is now known that the development of different lineages of hematopoietic cells (lymphocyte, erythroid, megakaryocyte, myeloid and subsets thereof) is governed by specific cytokines providing

extracellular signals to less differentiated cells and the subsequent activation or repression of transcriptional programs by characteristic transcription factors. To characterize these transcriptional programs, Theilgard-Monch et al undertook a comprehensive microarray analysis of gene expression in cells at various stages of neutrophilic differentiation (15). They showed that neutrophil maturation was associated with a downregulation of proliferative networks, engagement of various anti-apoptotic pathways, expression of host defense effector molecules, and, only upon terminal differentiation, the acquisition of receptors required to activate neutrophil host defense functions.

1.3.1 Transcriptional control

Several recent reviews have covered the transcription factors that regulate neutrophil development and differentiation (16-18). Briefly, the main transcription factors governing myeloid development, in order from earliest to latest, are PU.1, CCAAT enhancer binding proteins alpha and epsilon (C/EBPα and C/EBPε), and GFI-1. Genetic deletion of any of these factors results in blocks at specific stages of differentiation and absence of the appropriate progeny cells. PU.1 is required for the formation of common myeloid progenitors (CMP) and the production of B lymphocytes, monocytes, neutrophils, and eosinophils. C/EBPα is a later transcription factor that is necessary to form granulocyte/monocyte progenitors (GMP) and produce mature cells of those lineages. C/EBPε and GFI-1 are necessary and non-redundant for the final stages of neutrophil maturation, as mice lacking either of these proteins have a differentiation

block at the promyelocyte stage and absent or abnormal neutrophil production. The block is associated with a lack of the secondary and tertiary granule proteins that are characteristic of mature granulocytes. Subsequent studies utilizing conditional expression or more precise assays have shown all of these transcription factors to have additional, overlapping functions in many other hematopoietic processes, particularly in regulating hematopoietic stem cell function and providing a "differentiation block" though to be necessary in the pathogenesis of leukemia. Specifically, PU.1 (19) and C/EBPα (20) mutations or suppression of these genes by the leukemogenic fusion protein PML-RARα (21) are associated with acute myeloid leukemia (AML). A small subset of severe congenital neutropenia cases has been associated with mutations in the transcription factors GFI-1, HAX-1, and LEF-1 (22, 23).

1.3.2 G-CSF: The principle cytokine controlling granulopoiesis

In addition to intrinsic transcription factors governing the production of neutrophils in the bone marrow, extrinsic signals are also required. For hematopoietic cells in general, one of the key mechanisms of extrinsic regulation is by cytokines and growth factors, and the essential cytokine controlling neutrophil production is the granulocyte colony stimulating factor, or G-CSF (reviewed in Basu et al (24) and Panopoulos and Watowich (25)). G-CSF was one of the first cytokines to be isolated, and it has since been shown to have numerous roles in both basal and stress granulopoiesis. G-CSF was purified from human placental (26) or murine lung (27) conditioned medium on the basis of its ability to stimulate the growth of granulocyte colonies from bone marrow cells. The murine (28)

and human (29, 30) cDNA for G-CSF was subsequently cloned based on protein sequence and species homology, and this enabled expression of recombinant protein and other tools to further investigate the function of G-CSF. G-CSF expression is induced in numerous different cell types upon stimulus with appropriate immunogenic factors such as LPS, TNFα, INFγ, or IL-1β, which would be expected given the key role of G-CSF in stimulating neutrophil production to help mount an appropriate immune response. Transcription is initiated by the binding of NF-κB p60 and NF-IL6 at their binding sites in the promoter region (31). G-CSF expressing cells include hematopoietic cells such as monocytes, granulocytes, T lymphocytes, and macrophages as well as stromal cells, fibroblasts, endothelial cells, and mesothelial cells (32). Most cells express very low levels of basal G-CSF expression, although many tumor cells have constituitive expression.

Consistent with this, at baseline there are low but detectable levels of serum G-CSF that increase dramatically in response to infection along with an increase in the circulating neutrophil count (33). G-CSF treatment causes neutrophilia (34-37) resulting from increased proliferation of myeloid precursors and decreased transit time through the post-mitotic compartment in the bone marrow (38-40). As a result, G-CSF is used clinically to treat neutropenia and prevent infections. In vitro, G-CSF treatment stimulates the proliferation of myeloid progenitors (41).

At the molecular level, the effects of G-CSF are mediated by signaling through the G-CSF receptor (G-CSFR). The G-CSFR is a member of the hematopoietic cytokine receptor family and consists of single extracellular, transmembrane, and

cytoplasmic domains. The N-terminal portion of the cytoplasmic domain binds and activates JAK kinases, which phosphorylate tyrosines on the C-terminal portion of the cytoplasmic tail and activate STAT proteins. Other signals are transmitted through the RAS-ERK1/2 pathway and cytoplasmic kinases such as LYN and SYK. The main target cells of G-CSF, hematopoietic stem cells, myeloid progenitor cells, and granulocytes and their precursors, express the G-CSFR, and its expression is upregulated as the cells mature (42). However, other hematopoietic and stromal cell types, including endothelial cells (43), also express the GCSFR .

Genetic evidence that G-CSF signals are required for normal neutrophil production was provided by the identification of rare, dominant negative mutations in the G-CSFR in humans with severe congenital neutropenia (44-46). Conversely, a pedigree with chronic neutrophilia and constitutively active G-CSFR mutations has also been recently reported (47). A definitive role for G-CSF in controlling neutrophil production was shown by studies in transgenic mice. Both G-CSF-deficient (*CSF3*$^{-/-}$) (48) and G-CSFR-deficient (*CSF3R*$^{-/-}$) (49) mice exhibit a profound but not absolute neutropenia along with reduced numbers of myeloid cells in the bone marrow. The evidence suggests that G-CSF controls granulopoiesis through a number of different mechanisms. Early in myeloid development, it is necessary for commitment of primitive progenitors to the myeloid pathway (50). In *CSF3*$^{-/-}$ or *CSF3R*$^{-/-}$ mice, there is decreased proliferation of myeloid progenitors and decreased survival as evidenced by increased susceptibility to apoptosis (11). However, expression of a GCSFR engineered to contain a cytoplasmic tail that was either truncated (D715) or replaced with the cytoplasmic tail of the

erythropoietin receptor (GEPOR) showed that the distal portion of the GCSFR cytoplasmic tail was dispensable for G-CSF induced granulocytic differentiation, but required for other functions of G-CSF as discussed below (51, 52). Conversely, STAT3 activation mediated by JAK kinase attachment at the proximal cytoplasmic portion of the G-CSFR upon ligand binding is required for normal myeloid cell proliferation and neutrophil development (53). G-CSF signals are necessary for appropriate release of neutrophils from the bone marrow into the blood under both basal and stress conditions (discussed below). Lastly, G-CSF is important for neutrophils to function appropriately in arriving at the site of infection and displaying bacteriocidal activity. Neutrophils lacking a complete G-CSFR had impaired chemotaxis and adhesion molecule upregulation (52, 54), while neutrophils from *CSF3*$^{-/-}$ mice had morphological defects, impaired phagocytosis, and defective nitric oxide production (55). Additionally, G-CSF treatment of wild-type neutrophils activates them and enhances phagocytosis, superoxide anion generation, and bacterial killing (56).

1.3.3 Additional mechanisms

Several other factors that regulate neutrophil production deserve mention but will not be covered in detail. Effective granulopoiesis requires proliferative signals from cytokines, but these signals must be dampened by negative regulatory molecules within the cell to prevent excessive production of neutrophils. It is important to note that numerous other cytokines besides G-CSF have been shown to have a role in granulopoiesis, including GM-CSF, M-CSF, IL-3, IL-6, IL-17, and IL-23 (57-62).

Indeed, mice lacking G-CSF, GM-CSF, and M-CSF could still produce neutrophils (63), showing that many compensatory pathways are likely to exist that are non-essential in the presence of G-CSF (e.g. GM-CSF, IL-3, IL-6). Studies of mice with genetic alterations in the signaling molecules STAT3 or SOCS3 showed that they were negative regulators of G-CSF-induced neutrophil production that act by dampening G-CSF signaling (64, 65). Finally, given their rapid production and subsequent destruction and toxic granule components, neutrophils must maintain tight control over apoptosis. The pro-apoptotic molecule BIM and anti-apoptotic MCL-1 seem to be the key regulators of this process in myeloid lineage cells including neutrophils (66, 67).

1.4 Regulation of neutrophil release

After neutrophil production in the bone marrow, a second regulatory checkpoint for neutrophil homeostasis is the control of neutrophil egress from the bone marrow into the circulation.

1.4.1 Bone marrow architecture and composition

Like all other blood cells, neutrophils are produced in the bone marrow hematopoietic compartment, an extravascular space characterized by dense cords of hematopoietic tissue. The bone marrow extracellular matrix (ECM) has a typical composition of various types of collagen, proteoglycans, and glycoproteins (68). In order to enter the circulation, neutrophils must transmigrate across a vascular barrier into venous sinuses dispersed throughout the marrow cavity (69). As demonstrated by ultrastructural studies,

the vascular wall of the venous sinus is physically made up of three layers: endothelial cells, a basement membrane, and adventitial cells (Figure 1-2A-C) (70, 71). In some places, the membranes of the endothelial cells are fused, forming openings known as diaphragmed fenestra through which cells can cross into the circulation (71, 72). Thus, migration across the endothelium occurs via a transcellular route, although passage through the adventitial cell layer occurs via a paracellular route. This process, also known as neutrophil mobilization, must be highly regulated since only mature neutrophils of band or segmented nuclear morphology are released under basal conditions, despite the enormous amount of neutrophils produced in the marrow space daily. In addition to the endothelium-associated cells mentioned above, other stromal cells in the bone marrow include adipocytes, macrophages and various osteoblast lineage cells (68). Together, the ECM and various stromal cells are thought to form niches for hematopoietic cells that regulate their development, function, and survival, but there is limited knowledge about the specific molecules that might mediate these interactions.

Modulation of *adhesive interactions* with the ECM and stromal cells, *chemotaxis* toward the endothelium, and *transmigration* across the vascular barrier must logically occur for neutrophils to exit the bone marrow. The molecules known to be involved in these processes are discussed below, and a major contribution of this thesis is to further define the regulators of neutrophil egress from the bone marrow. Additional avenues of investigation for defining additional molecules that play a role will be discussed in **Chapter 4**.

1.4.2 Neutrophil mobilization by G-CSF

Second, the regulatory mechanism is flexible enough to allow a dramatic and rapid increase in the circulating neutrophil count in response to infection or other bodily stress in a process termed "emergency granulopoiesis". For example, under basal conditions, only 1-2% of the total body neutrophils are present in the circulation, with a large portion of the remainder held as a "ready reserve" in order to respond to infection or other acute demand (73). However, after treatment with G-CSF, the release of neutrophils into the blood increases so that the blood contains ~10% of the total neutrophils. Further experiments showed that the release was dependent upon signals generated by the membrane proximal portion of the G-CSFR acting in a non-cell intrinsic manner (74). G-CSF treatment also acutely increases the circulating neutrophil count (36, 37), in sum showing that G-CSF is a potent regulator of neutrophil release in addition to stimulating neutrophil production.

1.4.3 Emergency granulopoiesis and mobilizing agents

Many diverse compounds can induce neutrophil release (or production) in addition to G-CSF. It is important to note that neutrophil production and release are closely interrelated processes that can be difficult to separate. In general, rapid responses are more attributable to release since additional neutrophils have not been produced yet. Furthermore, neutrophil production and release may be regulated by distinct mechanisms under basal versus stress conditions. Emergency granulopoiesis may utilize multiple pathways with additive or supplementary effects upon the basal regulatory machinery,

while regulation under homeostatic conditions may be relatively less complex. However, even without clinical infection, animals are constantly being exposed to bacteria, indicating that basal versus stress processes likely are a continuum rather than two distinct entities.

A stress granulopoiesis response is characterized by release of neutrophils from the marrow in its early phase and supplemented by increased production of neutrophils via increased myeloid progenitor commitment, increased cell cycling, and increased immature myeloid cells in its later phase. Consistent with their biological function, a neutrophil response can be elicited by bacterial infection. For example, granulopoiesis is enhanced by intratracheal *Escherichia coli* infection (75). *CSF3*$^{-/-}$ mice fail to generate a neutrophilia and have impaired clearance of intravenous *Listeria monocytogenes* compared to wild type controls. However, with intraperitoneal infection *CSF3*$^{-/-}$ mice had a normal neutrophilia but were still susceptible to infection (76). When infected with *Candida albicans*, neutrophils were mobilized in a G-CSF, GM-CSF, and IL-6 independent manner (77). Neutrophil mobilization was impaired in *CSF3R*$^{-/-}$ mice responding to pulmonary *Pseudomonas aeruginosa* infection (78). G-CSF signals seem to be necessary for emergency granulopoiesis in some infections, but there are redundant signals in other cases. In all cases, an increase in circulating neutrophils could be elicited by administration of an infectious agent, and this increase was needed for effective bacterial clearance.

Many other substances can also cause neutrophil release; they are generally in the category of inflammatory mediators produced in response to infection, but there are

examples outside of this paradigm as well. Classes of molecules include cytokines, chemokines, leukotrienes, bacterial products, and complement factors. Specific examples of agents that stimulate emergency granulopoiesis or neutrophil release include the hematopoietic cytokines TNFα (79), TNFβ, G-CSF (36), GM-CSF (80), IL-1 (79), IL-3 (81), and IL-6 (82), the chemokine CXCL8 (IL-8) (83), leukotriene B_4 (84), bacterial endotoxin (LPS) (85), C5a (84), platelet activating factor (PAF) (84), the peptide N-formyl-met-leu-phe (fMLF) (84), prostaglandin analogues, epinephrine, corticosteroids, and even lithium (86). Many of these agents have been documented to increase during infection, and IL-6 (62) and GM-CSF (76) are required for appropriate resolution of infection in experiments utilizing gene-deficient mice. The kinetics of these agents are variable, with peak responses occurring anywhere from one to twelve hours after treatment. This suggests that multiple pathways are involved that have several steps. Indeed, one could envision that agents with delayed responses must induce secondary mediators, while agents with more rapid responses may act more directly upon neutrophils and be downstream steps in the pathway. Some of these agents, such as CXCL8 or C5a, also activate neutrophils and direct their chemotaxis. Mice genetically deficient for GM-CSF (87), IL-3 (88), or IL-6 (62) display normal hematopoiesis, indicating that these cytokines are not required for basal granulopoiesis despite their potential involvement in stress granulopoiesis. Conversely, mice deficient for both the G-CSFR and IL-6 display neutropenia of greater severity than that of the *CSF3R*$^{-/-}$ mouse, suggesting that IL-6 does play a role in basal granulopoiesis (89). Lastly, at least one second messenger has been shown to be important in mediating neutrophil release.

Mice deficient in STAT3, one of the main intracellular tranducers of G-CSF signals, are unable to acutely mobilize neutrophils in response to G-CSF treatment, but have increased basal levels of peripheral neutrophils (90). This suggests an inadequate reserve of immature neutrophils in *STAT3*$^{-/-}$ mice and the possibility of distinct pathways operating in basal versus acute neutrophil release. In sum, many pathways can lead to neutrophilia. However, the steps that lead from elevation of an inflammatory mediator to neutrophil release into the circulation are relatively unknown. The work presented in this thesis supports the idea that chemokine signaling in the bone marrow is a common final step mediating neutrophil trafficking from the bone marrow under both basal and stress conditions. The current knowledge of mechanisms governing neutrophil release from the bone marrow is summarized below.

1.4.4 Adhesion molecules

It is well known that adhesion molecules regulate the egress of leukocytes from the blood into the tissues to arrive at sites of inflammation or immune surveillance (91-93). The major adhesion molecules regulating neutrophils are integrins and selectins. Of note, a considerable portion of the intravascular pool of neutrophils are marginated at the vessel walls and thus not reflected in blood neutrophil counts; the marginated pool can constitute up to one-half of the total intravascular pool (9). This pool can vary depending on neutrophil activation state and be "mobilized" by certain stimuli.

Humans with leukocyte adhesion deficiency (LAD) exhibit neutrophilia and increased susceptibility to infection secondary to impaired neutrophil migration into

tissues caused by genetic deficiency of β_2-integrins (LAD I), selectin signaling (LAD II), or downstream signaling molecules (LAD III) (5). A number of adhesion molecule-deficient mice have been generated, and they all exhibit increased circulating neutrophils, reproducing the phenotype of LAD syndromes (5). These observations are consistent with the requirement for the various classes of molecules at various steps of the leukocyte adhesion cascade that regulates exit from the circulation. The neutrophilia in LAD mice is invariably associated with elevated serum levels of IL-17, the key observation that allowed elucidation of the homeostatic feedback loop described below (94). In β_2-integrin-deficient (*ITGB2*$^{-/-}$) mice, neutrophil counts normalize with the addition of wild-type hematopoietic cells in bone marrow chimeras (94, 95). These studies together showed that LAD neutrophilia is not caused by the passive accumulation of neutrophils in the circulation or altered proliferation. Of note, neutrophil apoptosis is decreased in *ITGB2*$^{-/-}$ mice but not in *ITGB2*$^{-/-}$ neutrophils from bone marrow chimeras that also contain wild-type cells (96).

Recent investigations have focused on whether adhesion molecules could play a role in the movement of neutrophils from the extravascular space of the bone marrow into the circulation. This idea is supported by the fact that adhesion molecules are important in regulating the survival and release of other cell populations in the bone marrow, particularly hematopoietic stem cells (97). Mice carrying genetic deletions of these molecules provide tools to test this hypothesis, but observations must be interpreted with caution because these mice often have elevated white cell counts secondary to excessive granulopoietic cytokine production (described below). Administration of blocking

antibodies for CD18 (β_2-integrins) or L-selectin had no effect on the neutrophilia induced by C5a, TNFα, or fMLF (98). Using an *in situ* perfusion model to precisely measure neutrophils mobilized from one femur in response to the chemokine CXCL2 (MIP-2), Burdon et al assessed the effect of blocking or modulating the adhesion molecules CD18, VLA-4 ($\alpha_4\beta_1$ integrin), or L-selectin on mobilization. Their results suggested that CD18 retains neutrophils in the bone marrow, while VLA-4 plays an opposing role by augmenting neutrophil release and L-selectin is dispensible (99). Consistent with this, irradiated mice reconstituted with a mixture of wild type and *ITGB2*$^{-/-}$ cells had increased percentages of neutrophils in *ITGB2*$^{-/-}$ circulating leukocytes (95). However, another group reports that there was no evidence for altered neutrophil mobilization in selectin-deficient or *ITGB2*$^{-/-}$ neutrophils in mixed bone marrow chimeras (5). No abnormalities in neutrophil release have been described in α_4-integrin (100) or L-selectin deficient mice (101, 102). Finally, in contrast to the results obtained after mobilization by CXCL2, Petty et al showed that blockade of VLA-4 or its receptor VCAM-1 resulted in increased neutrophil mobilization that was augmented by CXCR4 inhibition (103). They also suggested that CXCR4 stimulation increased VLA-4/VCAM-1 adhesion in a pertussis toxin-dependent manner.

Thus, the data on the role of adhesion molecules in neutrophil mobilization are contradictory and likely to be highly dependent on the details of the experimental system employed. Clearly, further experiments must be performed to fully characterize the role of adhesion molecules in the regulation of neutrophil release from the bone marrow. Some adhesion molecules may play redundant roles. The cell-intrinsic role of these

molecules has not been clearly defined in genetically deficient mice. CXCL2 is an inflammatory chemokine important for neutrophil mobilization from the marrow and homing to inflamed tissues, but in general the role of adhesion molecules in stress granulopoiesis has not been characterized.

1.4.5 Neutrophil homeostatic feedback loops

Homeostatic negative feedback loops controlling circulating neutrophil numbers have been suggested in the literature (95, 104), but no data to support them had been presented. Recent work (94, 105, 106) provides evidence that IL-17 production by specialized T-cells stimulates G-CSF production, which in turn positively regulates granulopoiesis (Figure 1-3). IL-17 production is positively regulated by IL-23 produced by macrophages and dendritic cells in the gut. These cells decrease their production of IL-23 upon phagocytosing apoptotic neutrophils, thus providing a way to match neutrophil production with neutrophil clearance. Consistent with such a feedback loop, mice that overexpress IL-17 or IL-23 are neutrophilic (60, 107), while the mice genetically deficient for these cytokines or their receptors are neutropenic and/or have impaired neutrophil responses to stress or infection (59, 106, 108). Bone marrow transplant experiments subsequently confirmed that hematopoietic cells are the source of IL-23 (107), while the cells that respond to IL-17 and produce G-CSF *in vivo* are non-hematopoietic stromal cells (108). An earlier report had demonstrated that IL-17 could stimulate the production of G-CSF, IL-6, CXCL8, and prostaglandin E2 (PGE2) in epithelial, endothelial, and fibroblastic cells *in vitro* (109). It is not yet clear how this

negative feedback loop becomes a *positive* feedback loop during infectious conditions, as the number of neutrophils in the tissues is increased, yet G-CSF production and subsequent granulocyte proliferation and mobilization into the blood remain elevated for the duration of the infectious episode. However, IL-17 signaling is required for an effective innate immune response in murine models of *Toxoplasma gondii* or *Klebsiella pneumoniae* infection, as mice deficient in the receptor for IL-17 (*IL-17RA*$^{-/-}$) mice had decreased survival (59, 110).

1.4.6 Cell biology of neutrophil mobilization by G-CSF

As discussed above, the GCSFR is expressed primarily on hematopoietic cells but also on some stromal cell types (42, 43). These data suggest that G-CSF may act on either stromal cells or hematopoietic cells to induce neutrophil mobilization. Work by Dr. Craig Semerad in our laboratory utilized a series of bone marrow radiation chimeras derived from wild-type and *CSF3R*$^{-/-}$ mice to demonstrate that G-CSFR signaling in hematopoietic but not stromal cells is necessary to induce neutrophil mobilization from the bone marrow to the blood (74) (Figure 1-4A). The G-CSFR is highly expressed on neutrophils; thus, it was expected that G-CSF signals acting directly on neutrophils would lead to their mobilization in a cell-intrinsic manner. However, mice reconstituted with a mixture of wild-type and *G-CSFR*$^{-/-}$ cells showed that this was not the case, as neutrophil release at baseline or in response to G-CSF was equivalent between the two genotypes (Figure 1-4B). Of note, the number of wild-type neutrophils in the bone marrow of the mixed chimeras was nearly equal to the number in control chimeras transplanted with

wild-type cells alone because of the defects in neutrophil production in G-CSFR-deficient cells. These data show that G-CSF signals in neutrophils were neither necessary nor sufficient for their mobilization and suggest the presence of indirect, *trans*-acting signals generated by G-CSF signaling in a non-neutrophilic hematopoietic intermediary. Two such potential signals are discussed below: proteases and CXCL12.

1.4.7 Neutrophil proteases

Neutrophils contain an array of proteases in their granules; these enzymes allow the neutrophil to degrade tissue and cellular debris at a site of inflammation and thereby contribute to the resolution of an infection. A recent hypothesis proposes that these same proteases can mediate neutrophil and/or hematopoietic stem cell mobilization from the bone marrow by cleaving signaling, adhesion, or extracellular matrix proteins. Evidence for this hypothesis shows that G-CSF treatment results in increased expression of the neutrophil serine proteases cathepsin G (CG) and neutrophil elastase (NE) (111). Furthermore, these proteases were able to cleave VCAM-1 (112), c-Kit (113), CXCL12 (SDF-1), and its receptor CXCR4 (114), which are all molecules thought to be important for regulating neutrophil or stem cell release from the marrow. Neutrophil mobilization by CXCL8 (IL-8) was impaired by neutralizing antibodies against MMP-9 (115). However, mice genetically deficient for the proteases MMP-9, both CG and NE, or DPPI all had normal resting neutrophil counts and normal mobilization after G-CSF treatment. This result was confirmed by pharmacologic inhibition of a broad spectrum of metalloproteinases in DPPI-deficient ($CTSC^{-/-}$) mice, which lack the enzyme necessary

for the activation of all neutrophil serine proteases (116). Together, this data shows that proteases are not necessary for neutrophil mobilization, or at least not mobilization by G-CSF. It is possible that different mobilizing agents utilize distinct mechanisms, that redundant mobilization pathways are involved, or that unknown proteases can provide a compensatory function.

1.4.8 The CXCR4/CXCL12 axis provides a key retention signal

Accumulating evidence suggests that signaling through the CXC-chemokine receptor 4 (CXCR4) induced by its ligand, stromal-derived factor-1 (SDF-1 or CXCL12) provides a key retention signal regulating neutrophil release from the bone marrow. The molecular and cellular biology of CXCR4 will be discussed further below. In brief, CXCR4 is broadly expressed on hematopoietic cells, and CXCL12 is constitutively produced in the bone marrow compartment by distinct types of stromal cells as discussed below (Figure 1-2D) (117, 118). Mice reconstituted with *CXCR4*$^{-/-}$ cells have impaired retention of granulocytes in their bone marrow and increased circulating neutrophils (119). AMD3100, specific antagonist of CXCR4 signaling, induces an increase in circulating neutrophils in mice and humans (120, 121). Patients with WHIM syndrome, a disease characterized by mutations in CXCR4 that confer elevated sensitivity to CXCL12, have a severe neutropenia (122, 123). G-CSF induced neutrophil mobilization is accompanied by a decrease in bone marrow CXCR4 and CXCL12 levels (74, 124, 125) (Figure 1-3 and 1-5). Using a series of G-CSFR mutant mice that have varying degrees of neutrophil mobilization in response to G-CSF treatment, our laboratory has shown that the

magnitude of the CXCL12 decrease is inversely correlated with mobilization (74). Although these data convincingly demonstrate that CXCR4 signals play a role in neutrophil homeostasis, the precise manner in which it acts to regulate neutrophils is undefined. The specific role of CXCR4 in neutrophil homeostasis is defined in **Chapter 2** of this thesis using mice with a myeloid-specific deletion of CXCR4.

1.4.9 Regulation of CXCL12 production in the bone marrow by G-CSF

CXCL12 is produced in non-hematopoietic bone marrow stromal cells that include osteoblast lineage cells, endothelial cells, fibroblasts, and other cell types (118). The normal phenotype in irradiated wild-type mice reconstituted with *CXCL12*$^{-/-}$ cells confirmed that non-hematopoietic stromal cells are the source of the biologically important CXCL12 (126). A number of authors have attempted to define the biologically important population of CXCL12-producing cells in the bone marrow (127-130). They show that endothelial cells, osteoblasts, and associated reticular cells all may play a role in regulating CXCL12 (Figure 1-2D). Work by Dr. Semerad and Dr. Matthew Christopher in our laboratory identified osteoblasts as the major producers of CXCL12 within the bone marrow stromal cell compartment and showed that G-CSF or other cytokines downregulate CXCL12 secondary to suppression of osteoblast number (131-133). However, the bone marrow chimera data discussed above suggest that G-CSF does not act directly on osteoblasts. Instead, the sum of the data suggest a model for neutropil mobilization where G-CSF signaling in a subset of hematopoietic cells generates secondary signals that act in *trans* to suppress osteoblasts and CXCL12 expression

(Figure 1-5). Recent, unpublished work by Dr. Christopher and others suggests that monocytes are the key subset of hematopoietic cells upon which G-CSF acts. The nature of the secondary signals, effects of G-CSF on other stromal cell populations, and potential additonal (chemokine or non-chemokine) molecules important for neutrophil mobilization produced by (osteoblast or non-osteoblast) stromal cells need to be determined and are areas of active investigation in our laboratory. Specifically, endothelial cells are capable of producing a wide variety of chemokines, but data on the properties of bone marrow endothelium are limited, as most studies have focused on activated endothelium *in vitro* or in other tissues. Work presented in **Chapter 3** of this thesis will address some of these questions.

1.5 Regulation of neutrophil clearance

A final regulatory step in controlling neutrophil counts is the process of neutrophil margination and extravasation into the tissues. The cellular and molecular signals necessary for the process of neutrophil arrest on the endothelium, adherence, and transmigration across the vascular barrier from the blood to tissue sites of inflammation have been well described (91, 92) and will not be covered here. Another aspect of neutrophil clearance is the investigation of where and how neutrophils are cleared under homeostatic conditions. Neutrophils home to many different tissues, with the most prominent being the bone marrow, spleen, and liver (134). These senescent neutrophils presumably undergo apoptosis and are cleared by tissue macrophages at these sites (135). The kinetics of clearance are rapid, with a calculated blood half life of 3-11 hours

depending upon the method of measurement used. Neutrophil clearance does not depend upon G-CSF signals, as neutrophils in *CSF3*$^{-/-}$ mice have a normal half-life (11), as did mice expressing the chimeric GEPOR (74). Conversely, recent data suggests that in addition to its role regulating neutrophil release, CXCR4 may play a role in neutrophil clearance at bone marrow sites. CXCR4 is upregulated as neutrophils age *in vitro* (121, 136, 137). CXCR4^{high} neutrophils preferentially home to the bone marrow, and this homing is abrogated by blocking antibodies against CXCR4 (121, 138). CXCL12 stimulation of CXCR4 induces TNF related apoptosis inducing ligand (TRAIL) and TRAIL receptors that increase the sensitivity of neutrophils to apoptosis (139). Together, these data suggest that CXCR4 regulates not only neutrophil release, but neutrophil clearance and apoptosis as well. However, these results must be interpreted with caution, as the *ex vivo* manipulation necessary for these experiments can activate neutrophils and alter their homing and apoptosis. In **Chapter 2**, we demonstrate that CXCR4 mediates neutrophil homing to the bone marrow but it is not essential for the clearance of circulating neutrophils.

1.6 Chemokines and regulation of neutrophil release

1.6.1 The Chemokine System

The name chemokine is a combination of the words chemotactic cytokine, and the classically described function of chemokines is indeed to direct the chemotaxis of various leukocyte subsets and thereby regulate their trafficking throughout the body. Chemokines are soluble proteins that form chemotactic gradients, and they are produced

by a variety of cell types. They bind to specific receptors on the cell surface, which are all seven-transmembrane proteins that signal by activating heterotrimeric G proteins through exchange of GDP for GTP. They can be classified into inducible or homeostatic groups, with the former being important for inflammatory responses and the latter being important for immune cell surveillance and developmental functions. The accepted nomenclature for chemokines is based on the presence of a C-C, C-X-C, C-X_3-C, or C-X amino acid motif, and they are numbered in the order of their discovery (140). Chemokine receptors are named similarly according to the class of chemokine they bind (141). Chemokines and their receptors are implicated in the pathogenesis of numerous diseases with an infectious or inflammatory component, including cancer cell survival and metastasis (142), and have therefore been well-studied as drug targets (143).

1.6.2 CXCR4/CXCL12

CXCR4 (reviewed by Busillo and Benovic (144)) was originally discovered for its role as a coreceptor required for HIV-1 entry into cells, and its major ligand CXCL12 (SDF-1) was identified by its ability to block viral entry (145-147). CXCL12 had previously been cloned from a bone marrow stromal cell line and was noted for its role in promoting B lymphocyte development. CXCR4 and CXCL12 are among the most well-studied and unique chemokine ligand-receptor pairs. They have a diverse array of functions in addition to directing leukocyte trafficking. CXCL12 and CXCR4 are expressed in many sites throughout the body, including brain, thymus, heart, lung, liver, lymph node, kidney, spleen, stomach, intestine, and bone marrow. As mentioned above, considerable interest

has been directed at defining the cell type within the bone marrow that expresses the CXCL12 important for regulating leukocyte and stem cell function. Several studies have provided somewhat conflicting results, but in general CXCL12 expression is localized to sites near the endothelium or endosteum (Figure 1-2D) (103, 127-129, 131). CXCR4 is present in every mature leukocyte type in addition to being expressed in more primitive hematopoietic cells (148). Therefore, CXCR4 has been widely studied for its role in directing hematopoietic stem cell migration, a process of critical importance for the treatment of malignancy with stem cell transplant. In addition to its role in HIV infection, CXCR4 has been implicated in the development and metastasis of various cancers of hematopoietic, epithelial, and neural origin (149). Lastly, in an atypical function for chemokines, CXCL12 or CXCR4 is required for the appropriate development of the cerebellum, cardiac septum, gut vasculature, in addition to being necessary for development of B lymphopoiesis and myelopoiesis in the hematopoietic system.

In perhaps another indicator of their distinct properties compared to most chemokines, both *CXCR4* and *CXCL12* are highly evolutionarily conserved. The murine and human orthologues of *CXCL12* have 99% homology, while the *CXCR4* gene is 90% conserved between the two species. This is in contrast to the much lower homology of chemokines *CCL2* (MCP-1) (55%), *CCL3* (MIP-1α) (75%), or the *CXCR2* receptor (71%). Additionally, *CXCL12* has a chromosomal location on 10q distinct from the C-X-C chemokine cluster on 4q. It is constitutively expressed in many tissues, while most other chemokines show inducible expression. *CXCR4* is located on chromosome 2q near

other chemokine receptor genes. CXCL12 is thought to be the major physiologic ligand for CXCR4 because of their nearly identical tissue expression pattern and the fact that *CXCL12*$^{-/-}$ and *CXCR4*$^{-/-}$ mice have nearly identical phenotypes (150). However, additional receptors and ligands have since been described (see below).

The transcriptional expression of CXCR4 is regulated by a number of factors. Importantly for this work, CXCR4 is expressed on neutrophils (136, 138) and downregulated by the cytokiness IFN, GM-CSF, and G-CSF *in vitro* and *in vivo* (124, 136). G-CSF treatment negatively regulates the transcription of CXCR4 by inducing the expression of the late myeloid transcription factor GFI-1(151). If mature neutrophils are shutting off their transcription of CXCR4, this may explain why bone marrow neutrophils have the highest CXCR4 expression and peritoneal neutrophils the lowest, with blood neutrophils intermediate (138). Conversely, other studies have shown that CXCR4 is upregulated as neutrophils are aged *in vitro* (121, 137) Other neutrophil releasing factors such as LPS (152) and lithium(153) have also been shown to downregulate CXCR4 mRNA in neutrophils *in vitro*. Transcriptional downregulation of CXCR4 by neutrophil mobilizing agents or with maturation may contribute to neutrophil release, consistent with the idea of CXCR4 as a retention signal. However, the role of CXCR4 in the clearance of aged, senescent neutrophils is not clear. This question will be addressed in **Chapter 2**.

The mechanisms of CXCR4 trafficking to the cell membrane, signal transduction, desensitization, internalization, and degradation generally follow those described for GPCRs in general and are covered in the review above (Figure 1-6) (144). However, a

few points merit mention. CXCR4 exists and signals as a dimer on the cell membrane, transmitting signals via heterotrimeric G-proteins with a $G_{\alpha i}$ subunit. It has also been shown to signal via JAK proteins and binding of β-arrestin molecules. CXCR4 is degraded by agonist-induced ubiquitination by the E3 ligase AIP4 and sorting to the lysosome. Additionally, its signaling upon CXCL12 binding can be desensitized by phosphorylation of residues on the cytoplasmic tail by G-protein receptor kinases (GRKs). Some of these phosphorylated residues allow binding of β-arrestins, which then mediate receptor internalization. Interestingly, in B and T lymphocytes from mice deficient in GRK6 or β-arrestin-2, membrane GTPase activity was increased but chemotaxis was severely impaired, indicating that chemotaxis in these cells was G-protein independent but dependent on β-arrestin binding to the receptor (154). However, *GRK6*$^{-/-}$ neutrophils had increased chemotaxis toward CXCL12 and impaired G-CSF-induced neutrophil mobilization, but normal basal neutrophil counts (155). This finding was supported by the fact that CXCR4 lacking the entire cytoplasmic tail had impaired desensitization and internalization (156). However, the cytoplasmic tail was required for chemotaxis, indicating that it has important signaling functions in addition to regulating internalization. Desensitization and downregulation of CXCR4 through phosphorylation of the cytoplasmic tail by GRKs are likely to be important in regulating neutrophil release.

CXCR4 signaling has diverse functions in numerous organ systems. Mice genetically deficient in either CXCL12 or CXCR4 die perinatally (embryonic day 18.5 to ~1 hour after birth) and display defective cerebellar granule cell formation, ventricular

septal defects, impaired blood vessel formation, and a failure to establish definitive B lymphopoiesis and myelopoiesis in the bone marrow (150, 157-159). The role of CXCR4 signals in brain development was a novel finding, and it fits nicely with the role of CXCR4 as a proliferative signal in pediatric brain tumors (160). Further studies showed that CXCR4 was the only chemokine receptor expressed during embryogenesis, and that it signaled during gastrulation (161). Studies with radiation chimeras and further analysis of knockout mice confirmed CXCR4/CXCL12 signaling to have a major role in early B lymphopoiesis (162), T lymphocyte repopulation and development in the thymus (163), and retention of B lymphocytes and neutrophils in the bone marrow (119). *CXCR4*$^{-/-}$ but not *CXCL12*$^{-/-}$ hematopoietic stem cells (HSC) exhibit an engraftment defect in repopulating irradiated mice, indicating that CXCL12 is produced by non-hematopoietic cells while CXCR4 is required on hematopoietic cells (126). In agreement with this finding, studies of human HSC treated with blocking antibodies for CXCR4 showed impaired NOD-SCID engraftment (164). Additionally, through studies primarily in human HSC, CXCR4 signals were shown to regulate cell adhesion (165), survival (166), and proliferation (167) in addition to the cell migration defects apparent from studies of knockout mice. Interestingly, the defect in bone-marrow colonization by long term HSCs in *CXCL12*$^{-/-}$ embryos can be rescued by endothelial cell expression of CXCL12, but the reduction in myeloid cells remained (168). This suggests that some other cell type in the bone marrow produces the CXCL12 important for neutrophil retention in the bone marrow, possibly osteoblasts as mentioned above.

Precise control of the level of CXCR4 signaling may be necessary for the appropriate chemotaxis or migration of cells. In a zebrafish model, it has been demonstrated that CXCR4 is essential for appropriate germ cell migration to their target during development (169). However, when a truncated CXCR4 protein that was defective in internalization and thus had an elevated level of signaling was expressed in CXCR4 deficient zebrafish embryos, the germ cells failed to arrive at the gonad despite normal motility and directional sensing (170). This demonstrates that subtle alterations in CXCR4 signaling can affect cell migration *in vivo*.

1.6.3 Additional receptors and ligands: CXCR7 and MIF

Recently, an additional receptor (RDC-1) for CXCL12 has been identified. It was subsequently classified as CXCR7, and it also binds CXCL11 (171). CXCR7 is expressed on a specific subsets of T and B lymphocytes, endothelial cells, in fetal liver, and on many tumor cell lines. Consistent with this, its expression in tumors provides a growth advantage (172). It is a non-signaling receptor, unable to induce chemotaxis or calcium transients in response to SDF-1. However, it can form oligomers with CXCR4, and it augments CXCR4 signaling as demonstrated by additive effects of blocking CXCR4 and CXCR7 in cells co-expressing the receptors (173). *CXCR7*$^{-/-}$ mice have been described, and they have normal hematopoiesis, CNS, and gut vasculature but die perinatally of severe heart valve defects (174). Knockdown of CXCR7 in zebrafish also leads to vascular defects (172). In the zebrafish model of germ cell migration, knockdown of CXCR7 leads to aberrant migration, and the authors conclude that CXCR7

regulates CXCL12 gradients by acting as a sink, thus decreasing CXCL12 levels (175). It is interesting to speculate that CXCR7 may act to regulate CXCL12 levels in the bone marrow or CXCR4 sensitivity on neutrophils, thus fine-tuning CXCL12 gradients and regulating neutrophil release during stress granulopoiesis.

Macrophage migration inhibitory factor (MIF) has recently been identified as a ligand for both CXCR2 and CXCR4, and it played a role in T lymphocyte and monocyte recruitment into atherosclerotic plaques (176). It is unclear what role it might have in neutrophil mobilization.

1.6.4 CXCR2

It is not clear how modulation of neutrophil CXCR4 signaling is linked to their migration toward the vascular endothelium and subsequent entry into the circulation. Therefore, the question of whether neutrophil egress from the bone marrow is a passive, random process or actively directed and what (if any) signals regulate it remains unanswered. Based on their well-characterized role in other aspects of neutrophil biology, we hypothesized that ELR^+ CXC chemokines (CXCL1-3, 5-8), signaling through CXCR1 and CXCR2, oppose CXCL12 and direct neutrophil release from the bone marrow (141, 177). This hypothesis was tested in experiments described in **Chapter 3** of this thesis.

Consistent with this idea, as immature Lin^{lo} Kit^+ Sca^+ (LKS) murine hematopoietic cells were differentiated *in vitro* into mature neutrophils, they upregulated CXCR2 expression (178). CXCR2 ligands are also potent neutrophil mobilizing agents. Treatment of mice with CXCR2 ligands human CXCL2 (GROβ (179), CXCL8 (IL-8)

(83), murine CXCL2 (MIP-2) (99), or CXCL1 (KC) (138) results in a rapid neutrophilia that peaks at ~1 hour and resolves in 2-3 hours. However, it is not clear from these experiments whether CXCR2 signaling plays a role in basal release or how these agents mediate their effects within the bone marrow compartment.

It is important to realize that humans have two receptors, CXCR1 and CXCR2, for CXCL8 (IL-8), the prototypic ELR^+ chemokine. Both receptors are expressed on myeloid cells (177), and the best described functions of CXCR2 and its ligands are granulocyte and monocyte/macrophage specific. ELR^+ chemokines induce neutrophil accumulation at inflammatory sites by mediating a variety of steps in the process, including expression of adhesion molecules, transmigration across the endothelium, and release of proteases (141, 177). Of note, there is only one functional IL-8 receptor in the murine genome, named CXCR2 because it has greater homology to human CXCR2 than CXCR1 (68% vs 71%) (180). However, CXCR2 is also expressed in B lymphocytes, consistent with the B cell expansion observed in *CXCR2*$^{-/-}$ mice (181). Additionally, CXCR2 has been described to play a role in the proliferation of hematopoietic progenitors, mediation of cellular senescence, wound healing, and angiogenesis through its expression on endothelial cells (182-186).

ELR^+ CXC chemokines are classically produced in autocrine fashion by myeloid and endothelial cells to mediate leukocyte infiltration into inflamed tissue, but the expression of ELR^+ chemokines in the bone marrow is poorly characterized. CXCL2 is produced by a subset of Gr-1^+ cells in the bone marrow (187), but we did not observe CXCL2 mRNA in F4/80^+ monocytes under basal or stress conditions (unpublished

observation). Parathyroid hormone-stimulated osteoblasts were recently shown to produce CXCL1 (188).

Surprisingly, *CXCR2*$^{-/-}$ mice were first described to have a profound neutrophilia with a myeloid and B lymphoid expansion in the bone marrow, spleen, and lymph nodes, arguing against CXCR2 as a positive regulator of neutrophil release (189). However, *CXCR2*$^{-/-}$ mice housed in a germ-free facility have normal hematopoiesis with normal to decreased circulating leukocyte counts (183, 190, 191). *CXCR2*$^{-/-}$ neutrophils exhibited defective emigration into sites of inflammation as expected. Taken together, these data suggest that chronic subclinical infection and the systemic release of cytokines that stimulate granulopoiesis are responsible for the unexpected phenotype in *CXCR2*$^{-/-}$ mice, although a negative regulatory role for CXCR2 in myeloid progenitors has also been proposed (183). It is also possible that CXCR2 signals are required for the neutrophil emigration into peripheral tissues and/or clearance by phagocytes necessary to complete the homeostatic feedback loop previously described (Section 1.4.5, Figure 1-3). Of note, the phenotype of *CXCR2*$^{-/-}$ mice is very similar to humans or mice with LAD (190). *CXCR2* mutations have also been recently described in patients with familial myelokathexis (discussed below). A bovine *CXCR2* non-synonymous polymorphism that impaired receptor function was associated with increased mastitis, but neutrophil counts were not altered (192). Despite the common features of increased infection and altered neutrophil homeostasis in the presence of these genetic alterations, the extent to which the mechanism of dysregulation is shared is not clear.

CXCR2 may interact with G-CSF signals to regulate neutrophil mobilization. G-CSF treatment increases CXCR2 expression on neutrophils (193), and G-CSFR-deficient neutrophils treated with CXCL8 have a number of impaired responses including lack of mobilization (89). Neutrophils lacking STAT3, a key transducer of G-CSFR signals, or those with a G-CSFR engineered to transduce EPOR-specific signals both had reduced mobilization after G-CSF administration (74, 90). However, only *STAT3*$^{-/-}$ cells also displayed impaired responses to CXCL2.

CXCR2 may regulate CXCR4 signaling and thereby regulate neutrophil release through heterologous interactions. CXCR4 has been reported to undergo heterologous desensitization in response to CXCR2 stimulation (138), but another report suggests that CXCR1 but not CXCR2 mediates this effect (194). Modulation of CXCR4 signaling by heteroligmerization with other chemokine receptors has been described, although not for CXCR2 in particular (174, 195). Another recent paper showed that G-CSF, acting in a CXCR4 dependent manner, cooperated with CXCL1 and CXCL2 to induce neutrophil release from the bone marrow (196). However, this does not distinguish whether CXCR2 is cooperating by further abrogating CXCR4 signaling or by migrating toward the chemotactic gradient of its own ligand. An alternative but not mutally exclusive mechanism for neutrophil mobilization could act by fine modulation of the chemokine gradients for CXCR4 and CXCR2 in the bone marrow to determine the relative balance of neutrophil retention and release. It remains unknown what chemokines or other mediators, besides CXCL12, would be important for this process and what cell types

within the bone marrow produce these molecules. Bone marrow chemokine production by stromal cells will be investigated in **Chapter 3**.

CXCR2 plays an important role in scavenging ELR^+ chemokines. Scavenger receptors could alter plasma chemokine levels and thus regulate neutrophil mobilization. Accordingly, *CXCR2*$^{-/-}$ mice had elevated levels of CXCL1 (KC) and CXCL2 (MIP-2) (197). Furthermore, in transgenic mice overexpressing CXCL1, CXCR2 signaling and responses actually decreased because of receptor desensitization and downregulation in response to excessive ligand (198).

As mentioned above, African-Americans have significantly lower neutrophil counts, on average, than other ethnic groups (Figure 1-1A). "Benign ethnic neutropenia" is also found in up to 5% of this population and defined as a neutrophil count < 1.5 x 10^9/L (199). Recently, genome wide association studies have implicated an inactivating polymorphism in the duffy antigen receptor (DARC) on red blood cells in this condition (200). DARC is a non-signaling chemokine receptor that acts as a chemokine sink (201). It can bind CXCL8 and has been shown to alter plasma levels of chemokines important in neutrophil homeostasis (202, 203).

It is unclear how CXCR2 would regulate neutrophil release. Does it interact with CXCR4? Does it act in a cell-intrinsic fashion or generate *trans*-acting signals as in the case of G-CSFR? What ELR^+ chemokine ligands for CXCR2 are important for neutrophil mobilization? Does it act during both basal and stress granulopoiesis? These questions will be addressed **Chapter 3**.

1.7 WHIM Syndrome

WHIM (Warts, Hypogammaglobulinemia, Infections, Myelokathexis) syndrome (reviewed by Diaz and Gulino (204)) is a rare disorder characterized by immune dysfunction and neutropenia, first described by Zuelzer (205). Patients with the disorder experience recurrent bacterial infections and extensive verrucosis secondary to chronic human papillomavirus (HPV) infection (122). The number of neutrophils in the peripheral blood increases during the response to infection or with G-CSF treatment, suggesting that the defect in granulopoiesis is not absolute. Despite the peripheral neutropenia, there are bizarre but mature neutrophils found in abundance in the marrow of these patients (termed myelokathexis). Accelerated neutrophil apoptosis has been reported in patients with myelokathexis as a potential mechanism of disease (206-208).

WHIM syndrome is a genetically and clinically heterogenous disease, ranging from isolated myelokathexis to the full phenotype. The majority of reported cases have an autosomal dominant inheritance pattern, while some sporadic (209, 210) or apparently autosomal recessive cases have been reported. Truncating mutations in the cytoplasmic (carboxy-terminal) tail of the *CXCR4* gene located on chromosome 2q21 have been identified the majority of patients tested to date, while 3 patients have a wild-type *CXCR4* open reading frame (123, 210). One patient has been reported with isolated myelokathexis who carries a *CXCR4* mutation (123). As mentioned above, CXCR4 and CXCL12 are essential for the appropriate development of B lymphocytes and myeloid cells, the same cell lineages that are affected in WHIM syndrome. The mutations found in patients with WHIM syndrome truncate 19 (1000C→T, R334X), 17 (1006G>T,

G336X), 15 (1013C→G, S338X),13 (1016-1017delCT, S339fs342X), or 10 (1027G→T, E343X) residues from the 46 amino acids of the cytoplasmic tail of the CXCR4 receptor, with the R334X mutation being the most common (123, 210, 211).

Clinically, patients can also display variable lymphopenia and are subject to a risk of malignancy related to viral infection. Two cases of B cell lymphoma, one of which was Epstein-Barr virus (EBV) related, and several cases of cervical dysplasia related to the common HPV serotypes 16 and 18 have been reported (209, 212-214). Patients have an increased risk of congenital heart defects (7% vs. 0.8%), and there have been two reports of patients with brain tumors, one patient with skeletal abnormalities, one patient with recurrent herpes simplex virus infection (HSV) (204). Again, this spectrum of organ system involvement fits nicely with the known defects in CXCR4-, CXCR7-, or CXCL12- deficient mice. Overall, mortality from WHIM syndrome is low but it carries significant morbidity; in the literature, one patient has died of sepsis, one from EBV-related lymphoma, and one from glioblastoma. For example, one middle aged patient was reported to have been hospitalized for severe infectious episodes over 80 times (215, 216).

CXCR4 constructs lacking the cytoplasmic tail have been shown to enhance production of inositol phosphate, prolong ligand-stimulated release of intracellular calcium, and decrease CXCL12-induced internalization while cell-surface expression and CXCL12 binding remains normal (156). A cell line expressing the R334X mutation showed increased calcium flux and chemotaxis in response to CXCL12, suggesting abnormal, gain-of-function signaling by the mutant receptor (123, 217). The autosomal

dominant inheritance pattern of WHIM syndrome also suggests a gain-of-function mutation. In patient samples normal cell surface expression of CXCR4 and increased leukocyte chemotaxis to CXCL12 was observed. Several reports suggest that impaired CXCL12-induced CXCR4 internalization and desensitization acting in a dominant negative manner could be responsible for the altered response to CXCL12 and the clinical abnormalities (210, 218). However, another group reports no impaired internalization or increased calcium flux in patient cells (211). If WHIM mutations do cause CXCR4 internalization and desensitization defects, presumably it is due to lack of phosphorylation sites for GRKs and subsequently impaired β-arrestin binding. Viral expression of the R334X mutant in human CD34+ cells followed by transplant into NOD-SCID mice led to impaired neutrophil release *in vivo* (219). Because CXCR4 has been implicated in regulation of cell survival, other investigators have suggested that the gain-of-function signaling may lead to increased neutrophil apoptosis (206).

1.7.1 *Wild-type CXCR4 in WHIM syndrome patients*

A puzzling and intriguing aspect of WHIM syndrome is the identification of patients with the full clinical phenotype and impaired desensitization and internalization of CXCR4 who nonetheless have no CXCR4 mutations (210). This suggested the possibility of alterations in molecules that regulate CXCR4 expression and function. This possibility was confirmed in a recent report that demonstrated that impaired GRK3 production or function played a key role in mediating increased CXCR4 signaling in patients with WHIM syndrome (220). However, no mutations in GRK3 were present, suggesting that

alterations in GRK3 a binding partner or other gene silencing mechanisms exist in these patients. In contrast to data in mice indicating that GRK6 and β-Arrestin regulate CXCR4 signaling in neutrophils (155), GRK6 and β-Arrestin function appeared to be normal in these patients. Of note, GRK3 is highly expressed in normal leukocytes including neutrophils (221). *GRK3*$^{-/-}$ mice exist (222), but there is no analysis of their hematopoiesis in the literature.

The potential basis of disease in a second family with autosomal recessive isolated myelokathexis (123, 215) and a wild-type CXCR4 gene has recently been brought to our attention by our collaborator Dr. George A. Diaz (O'Shaughnessy, A.L., Sun, Q., Diaz, G.A., manuscript in preparation). These patients carry homozygous 968delA mutations in *CXCR2* (*IL8Rβ*), a chemokine receptor specifically expressed on neutrophils. The mutation results in H323fs6X truncation of the carboxy terminal cytoplasmic tail of the receptor. However, the consequences of this truncation mutation are quite different than for CXCR4 truncation mutations. The CXCR2 mutation results in the loss of a F-X_6-IL motif that is conserved in many GPCRs and required for export from the endoplasmic reticulum (223). When expressed in cell lines, the mutant CXCR2 appears to be functionally null. There is no cell surface expression, and immunofluorescence experiments showed accumulation of the protein in the endoplasmic reticulum. The effect of this mutation *in vivo* or studies of patient leukocytes were not reported. It is unclear how CXCR2 interacts with CXCR4 to regulate neutrophil release, or how loss of CXCR2 signals could lead to disease in these patients. These questions will be addressed in **Chapter 3**.

1.8 References

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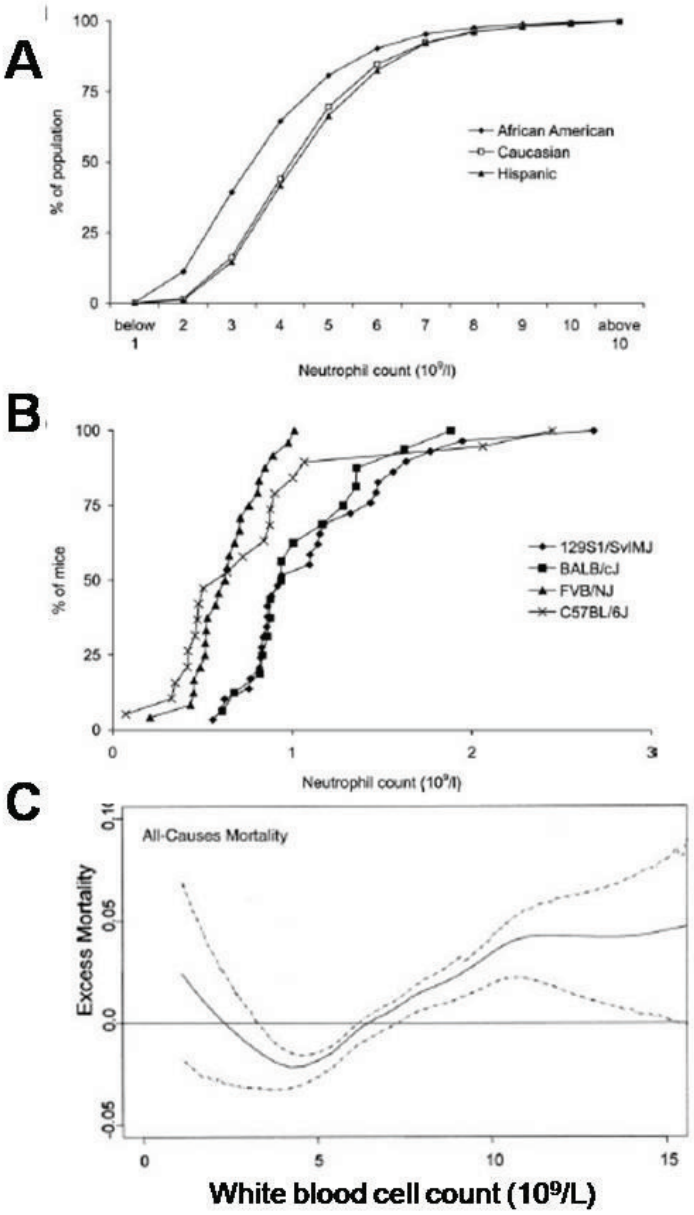

Figure 1-1. Neutrophil counts and mortality. Cumulative incidence of neutrophil counts in a sample of (A) 25,000 US Americans separated by race or (B) different inbred strains of mice (n = 15-30 per strain). (C) Relationship between mortality and white blood cell count (WBC). The dashed lines represent the 95% confidence interval. Modified from von Vietinghoff and Ley (5) and Ruggiero et al (7). Panel A and B: Copyright 2008. The American Association of Immunologists, Inc. Panel C reprinted with permission from the American College of Cardiology. Mouse data originally obtained from The Jackson Laboratory Mouse Phenome Database at www.jax.org/phenome.

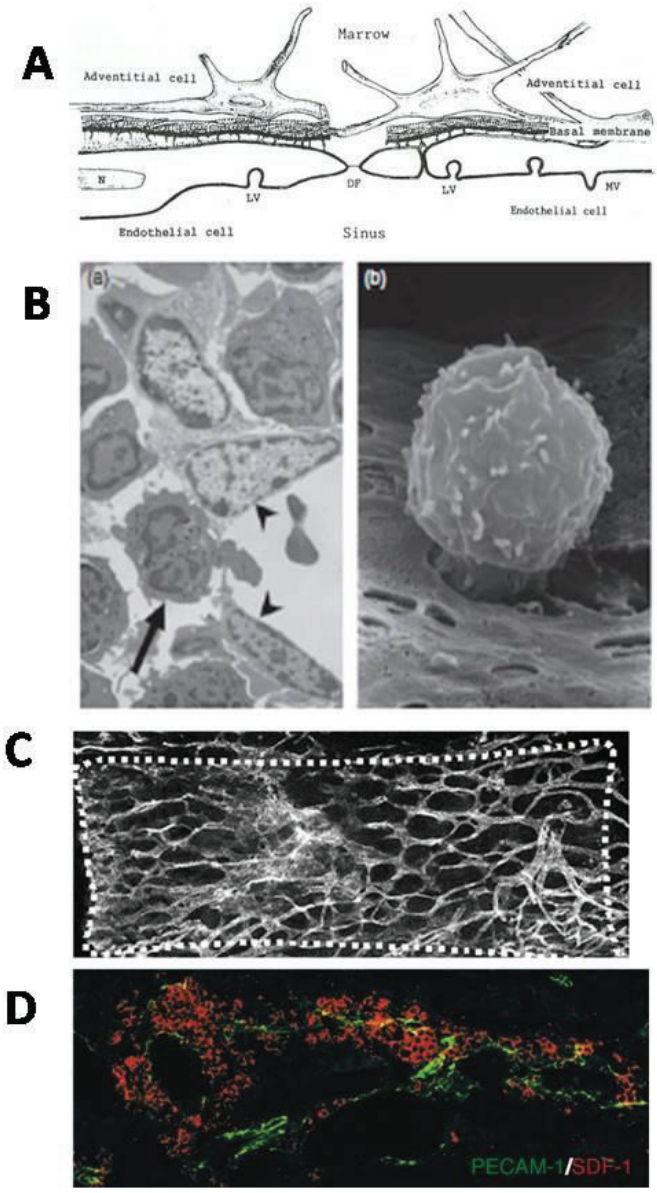

Figure 1-2. Microanatomy of the bone marrow vasculature. (A) Schematic diagram of the trilaminar structure of the bone marrow sinus wall showing the discontinuous adventitial cell and basal membrane layers and the endothelium with openings known as diaphragmed fenestrae (DF). (B) Electron micrographs demonstrating the passage of neutrophils (arrow) though endothelial cells (arrowheads) and into the lumen of the bone marrow venous sinus. (C) Fetal bone marrow endothelium shown by CD31 (PECAM-1) staining demonstrates the normal pattern of blood vessels into which neutrophils produced in the extravascular space must migrate. The dashed line shows the approximate location of the endosteum. (D) Co-localization of some $CD31^{+}$ cells with CXCL12 (SDF-1) producing cells in fetal bone marrow. Modified from Petrides and Dittman (panel A) (68), Furze and Rankin (panel B) (224), and Ara et al (panels C and D) (168). Reprinted with permission from Elsevier.

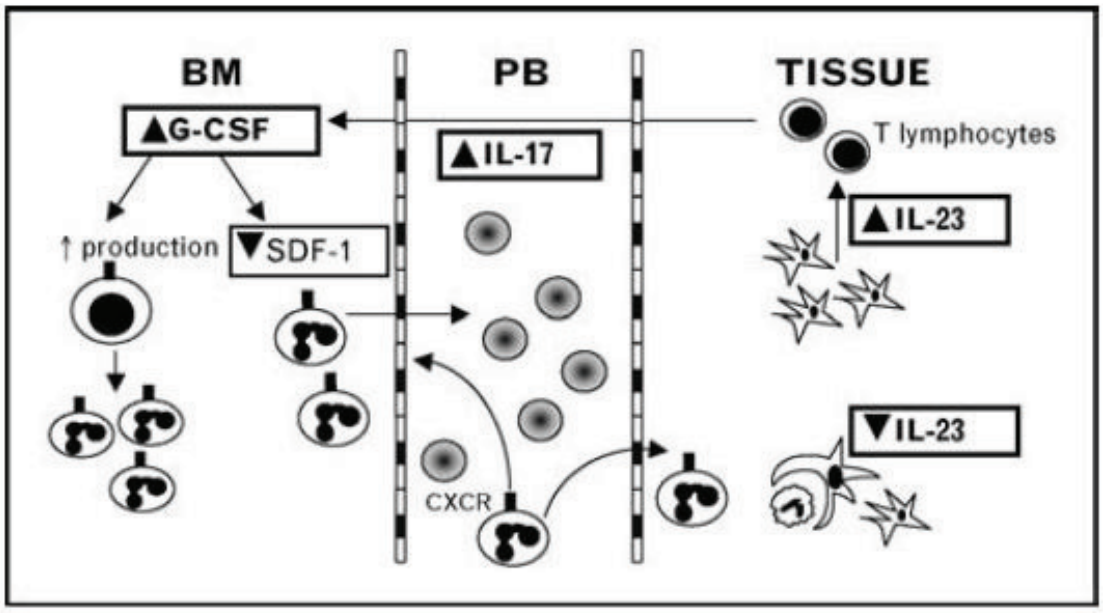

Figure 1-3. Homeostatic control of neutrophils via a feedback loop involving cytokines and chemokines. See text for further details. In brief, tissue macrophages and dendritic cells phagocytose transmigrated neutrophils, which downregulates their constitutive production of IL-23. IL-23 stimulates production of IL-17 by T lymphocyte subsets, which in turn positively regulates serum G-CSF levels. G-CSF induces neutrophil release by down regulating CXCL12/CXCR4 signaling. Data presented in this thesis indicates that CXCR2 also mediates G-CSF induced neutrophil release *and* possibly neutrophil transmigration and subsequent dampening of IL-23 production. Modified with permission from Christopher and Link (13).

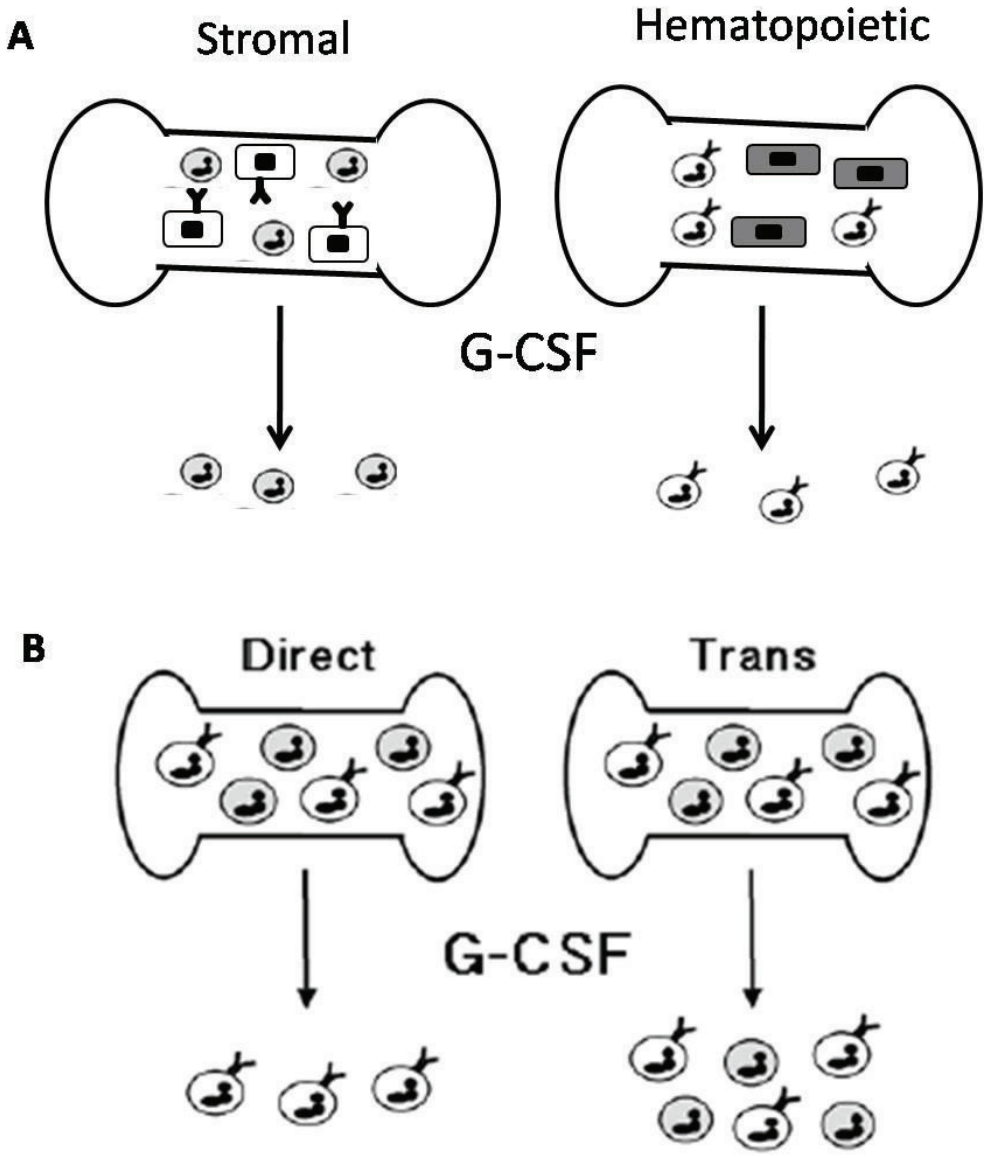

Figure 1-4. Mechanisms of neutrophil mobilization by G-CSF. (A) Irradiated wild-type mice were reconstituted with G-CSFR-deficient hematopoietic cells (left panel) or vice versa (right panel). In the stromal model, G-CSF signaling in non-hematopoietic cells is sufficient for neutrophil mobilization, while in the right panel G-CSF signaling in hematopoietic cells is required for neutrophil release into the blood. (B) Next, chimeric mice were generated with a mixture of wild-type and G-CSFR-deficient hematopoietic cells. On the left, cell-intrinsic G-CSF signals induce neutrophil release, while the *trans* model predicts that G-CSF regulates secondary signals that mobilize neutrophils independently of G-CSF signaling. Experimental data are consistent with the right panels. Shaded cells, G-CSFR-deficient. Figure modified with permission from a review by Link (225).

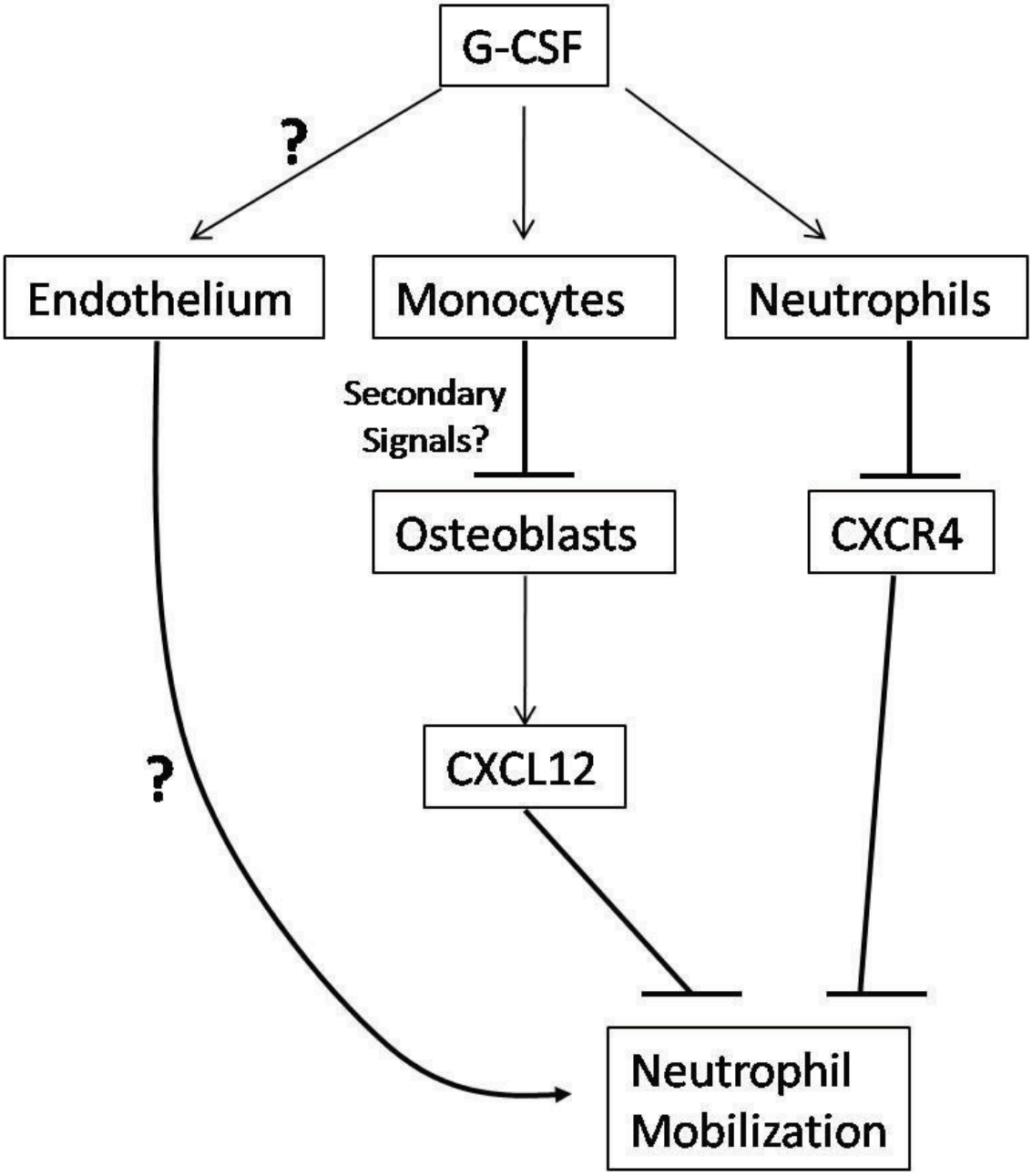

Figure 1-5. Multi-step model of G-CSF-induced neutrophil mobilization. CXCL12 signaling through CXCR4 normally retains neutrophils in the bone marrow, thus inhibiting their mobilization. G-CSF leads to neutrophil mobilization by inhibiting CXCL12/CXCR4 signaling through direct inhibition of CXCR4 expression and indirect suppression of CXCL12 production by osteoblasts. G-CSF signaling in monocytes decreases osteoblast number by yet to be determined pathways. Given their key anatomic position in the physical process of neutrophil mobilization, endothelial cells are likely to play a role that is currently undefined.

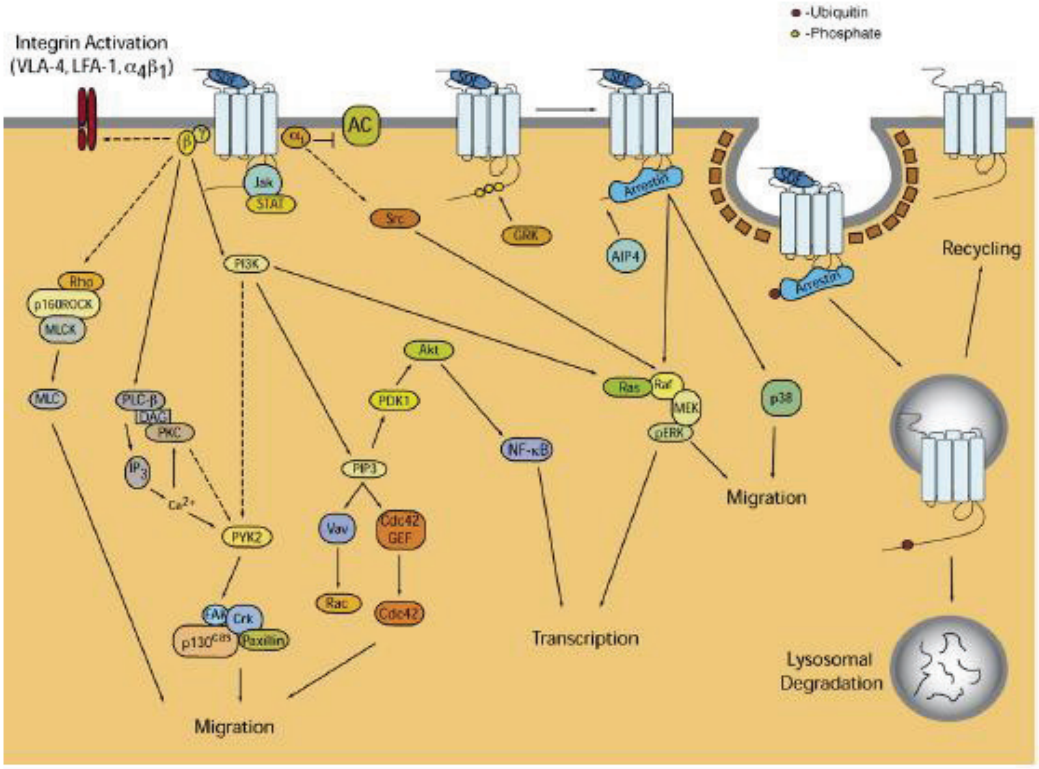

Figure 1-6. Signal transduction pathways and regulation of CXCR4. CXCL12 binding to CXCR4 leads to the activation of multiple G protein-dependent signaling pathways, resulting in diverse biological outcomes such as migration, adhesion, and transcriptional activation. Pathways activated and outcomes elicited may differ between $CXCR4^+$ cell types. Two potential G protein-independent pathways have been described. Tyrosine phosphorylation of CXCR4 results in the recruitment and activation of the JAK/STAT pathway, while p38 and ERK activation has been shown to be partially dependent on arrestin-3 (β-arrestin-2). Following activation, GRK phosphorylation results in the recruitment of arrestin 2/3 (β-arrestin 1/2) and subsequent internalization. CXCR4 is also ubiquitinated by AIP4 at the plasma membrane, which results in its sorting to and degradation in lysosomes. However, a portion of the internalized receptor may also recycle back to the plasma memebrane. Reprinted from a review by Busillo and Benovic (144) with permission from Elsevier.

Chapter 2

CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions

The research in this chapter was originally published in *Blood.*

Eash KJ, Means JM, White DW, and Link DC. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood.* 2009; 113(19): 4711-4719.

2.1 Abstract

The number of neutrophils in the blood is tightly regulated to ensure adequate protection against microbial pathogens while minimizing damage to host tissue. Neutrophil homeostasis in the blood is achieved through a balance of neutrophil production, release from the bone marrow, and clearance from the circulation. Accumulating evidence suggests that signaling by CXCL12, through its major receptor CXCR4, plays a key role in maintaining neutrophil homeostasis. Herein, we generated mice with a myeloid lineage-restricted deletion of *CXCR4* to define the mechanisms by which CXCR4 signals regulate this process. We show that CXCR4 negatively regulates neutrophil release from the bone marrow in a cell autonomous fashion. However, CXCR4 is dispensable for neutrophil clearance from the circulation. Neutrophil mobilization responses to granulocyte colony stimulating factor (G-CSF), CXCL2, or *Listeria monocytogenes* infection are absent or impaired, suggesting that disruption of CXCR4 signaling may be a common step mediating neutrophil release. Collectively, these data suggest that CXCR4 signaling maintains neutrophil homeostasis in the blood under both basal and stress granulopoiesis conditions primarily by regulating neutrophil release from the bone marrow.

2.2 Introduction

Neutrophils play an essential role in the innate immune response, as they are required to effectively protect the host against a variety of bacterial and fungal pathogens. Under basal conditions, the great majority of neutrophils reside in the bone marrow. In response to infection or other stresses, this pool of neutrophils can be mobilized into the blood, providing the host with a mechanism to rapidly increase neutrophil delivery to sites of infection. It is essential that neutrophil number in the blood be tightly regulated. Persistent neutropenia is associated with profound immunodeficiency, while excessive neutrophil infiltration and activation contributes to tissue damage in certain inflammatory disorders, such as rheumatoid arthritis. Neutrophil homeostasis is maintained through a balance of neutrophil production, release from the bone marrow, and clearance from the circulation (1). Contributing to the complexity of this process, neutrophils have the shortest survival of any circulating cell, with reported half-lives of 8-16 hours under basal conditions (2-7). Despite its importance, the mechanisms regulating neutrophil number in the blood are incompletely understood.

Accumulating evidence suggests that the chemokine CXCL12 (stromal derived factor-1, SDF-1), through interaction with its major receptor CXCR4, plays a key role in controlling neutrophil homeostasis (8). Mice deficient for CXCL12 or CXCR4 die perinatally, but the fetal circulation is characterized by elevated numbers of neutrophils and a failure to establish bone marrow myelopoiesis (9-12). In *CXCR4*$^{-/-}$ fetal liver chimeras, mature neutrophils and granulocytic precursors are increased in the blood, while the number of mature neutrophils in the bone marrow is reduced (13, 14). Humans

and mice treated with AMD3100, a selective antagonist of CXCR4, or CXCR4 blocking antibodies display a rapid mobilization of neutrophils into the blood (15-17). Truncation mutations of CXCR4 that cause increased receptor signaling are responsible for most cases of WHIM (Warts, Hypogammaglobulinemia, Infections, Myelokathexis) syndrome, which is characterized by abnormal retention of neutrophils in the bone marrow (18-20). Importantly, CXCR4 is expressed by neutrophils as well as most other hematopoietic cells, and CXCL12 is constitutively expressed at high levels in the bone marrow stroma (21, 22). Together, these data support a model in which CXCL12 signaling through CXCR4 provides a key retention signal for neutrophils in the bone marrow and therefore negatively regulates their release.

As noted previously, peripheral blood neutrophil counts can increase rapidly in response to infection or other stress. A wide variety of infectious agents, bacterial products, cytokines, and chemokines are thought to contribute to this "emergency" or "stress" granulopoiesis response (23, 24). The downstream signals that regulate this response are relatively undefined, but recent evidence suggests that CXCR4 may play a role. Treatment with granulocyte colony-stimulating factor (G-CSF), a key cytokine in the stress granulopoiesis response, results in a decrease in CXCL12 expression in the bone marrow and the downregulation of CXCR4 expression on neutrophils (25-28). These observations suggest the hypothesis that disruption of CXCR4 signaling is a key step mediating neutrophil release by G-CSF. Whether disruption of CXCL12/CXCR4 signaling is a common mechanism by which other mobilizing agents increase neutrophil counts in the blood is unknown.

Neutrophil homeostasis in the blood is determined, in part, by the rate of clearance from the circulation. Neutrophils are cleared primarily in the liver, spleen, or bone marrow, where apoptotic or aged neutrophils are thought to be phagocytosed by macrophages (17, 29, 30). Recent studies suggest that CXCR4 may play a role the clearance of aged, senescent neutrophils, particularly at bone marrow sites. CXCR4 expression increases on neutrophils as they age, and blocking antibodies to CXCR4 impede neutrophil homing to the bone marrow (17, 31). Thus, CXCR4 may have a dual role in regulating neutrophil homeostasis by acting as a signal clearing senescent neutrophils from the blood in addition to regulating neutrophil release.

The broad expression of CXCR4 on hematopoietic cells complicates the analysis of this gene during granulopoiesis. For example, *CXCR4*$^{-/-}$ fetal liver chimeras display defects in multiple hematopoietic lineages, including hematopoietic stem cells (13, 14, 32). Therefore, in the present study we use transgenic mice carrying a myeloid specific deletion of CXCR4 to further define the role of CXCR4 in basal and stress granulopoiesis. Using this system, we show that CXCR4 is dispensable for neutrophil clearance from the blood. In contrast, CXCR4 is required for neutrophil mobilization from the bone marrow in response to G-CSF, CXCL2 (growth regulated gene β, GROβ), or *Listeria monocytogenes* infection. These results suggest that CXCR4 signaling is an essential regulator of neutrophil homeostasis under both basal and stress granulopoiesis conditions.

2.3 Methods

2.3.1 Mice. The *LysM*^{Cre} and *CXCR4*$^{+/-}$ mice (12, 33) were obtained from The Jackson Laboratory (Bar Harbor, ME) and conditional CXCR4 (*CXCR4*$^{flox/flox}$) (34) mice were obtained from Dr. Dan R. Littman (New York University). All mice were inbred onto a C57BL/6 background at least 10 generations. Mice were genotyped by PCR as described (12, 33, 34). Congenic wild-type C57BL/6 mice (B6.SJL-Ptprc* Pep3b BoyJ, The Jackson Laboratory) that have the *Ly 5.1* gene were used to facilitate analysis of chimeric mice. Sex- and age-matched mice between 6 and 16 weeks of age were used in accordance with the guidelines of the Washington University Animal Studies Committee.

2.3.2 CXCR4 genotyping. Peripheral blood neutrophils (Gr-1^{bright}SSC^{hi}) from *CXCR4*$^{flox/-}$ or *LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$ mice were isolated using a MoFlo high-speed cell sorter (Dako, Carpinteria, CA). Genomic DNA was prepared using the ArchivePure DNA kit (5Prime, Gaithersburg, MD). The presence of either a floxed or deleted CXCR4 allele was revealed by PCR amplification using the listed primers. Floxed: 5'-CCACCCAGGACAGTGTGACTC TAA-3' and 5'-GATGGGATTTCTGTATGAGGATTAGC-3' Deleted: 5'-TCTAACGTCCCA GATCCACC-3' and 5'-AACCAAACAAACCATCACACAG-3'.

2.3.3 Blood, bone marrow, or spleen analysis. 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 (CDC Technologies,

Oxford, CT). In some cases, manual leukocyte differentials were performed on Wright-stained blood smears (minimum 100 cells) or bone marrow cytospins (minimum 300 cells). As reported previously,(26) the percentage of total body neutrophils in the blood was estimated using the neutrophil distribution index (NDI), which is calculated by dividing the number of neutrophils in the blood by the number in the blood and bone marrow. Blood and bone marrow neutrophils were calculated assuming a blood volume of 1.8 ml and a whole femur equivalent to 6% of the total bone marrow (35).

2.3.4 Flow cytometry. Cells from bone marrow, blood, or spleen were depleted of red cells by hypotonic lysis, resuspended in phosphate-buffered saline (PBS) supplemented with 0.2% bovine serum albumin (BSA) and 0.1% sodium azide (FACS buffer), incubated for 10 minutes with anti-CD16/32 (Fc-block, BD Biosciences, San Diego, CA) and stained for 20-30 minutes at 4°C with one or more of the following antibodies as described in the text: allophycocyanin (APC)-conjugated anti-Ly6G (Gr-1, Invitrogen/Caltag, Carlsbad, CA), biotinylated anti-CXCR4 (eBioscience, San Diego, CA), biotinylated anti-CD115 (GM-CSFR, eBioscience), PerCP-Cy5.5-conjugated anti-CD3e (BD Biosciences), PerCP-Cy5.5-conjugated anti-Ly5.1 (CD45.1, BD Biosciences), fluorescein (FITC)-conjugated anti-Ly5.2 (CD45.2, eBioscience), Alexa488-conjugated anti-F4/80 (Invitrogen), and APC-Alexa750-conjugated anti-CD45R (B220, Invitrogen). Biotinylated antibodies were detected with phycoerythrin (PE)-conjugated streptavidin (eBioscience). Isotype-matched antibodies and unstained cells were used as negative controls. Mature neutrophils were gated as Gr-$1^{hi}SSC^{hi}$ cells, and in some experiments

F4/80 or CD115 was used to further exclude blood monocytes. Data was collected on a FACScan 5-color, 2-laser flow cytometer (BD Biosciences and Cytek Development, Fremont, CA) using Cellquest software (BD Biosciences) and analyzed with the Flowjo software package (Tree Star, Ashland, OR).

2.3.5 Colony-forming cell assay. Twenty µl of blood, 1 x 10^5 nucleated spleen cells, or 2 x 10^4 bone marrow cells were plated in 2.5 ml methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434, Stemcell Technologies, Vancouver, BC, Canada) or 10 ng/ml recombinant human G-CSF (M3231, Stemcell Technologies). Cultures were plated in duplicate and placed in a humidified chamber with 6% CO_2 at 37°C. Colonies containing at least 50 cells were counted on days 7-10 of culture.

2.3.6 Bone marrow transplantation. Wild type (Ly5.1) or *LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$ (Ly5.2) bone marrow cells were harvested. A total of 2 million bone marrow cells were mixed at a 1:1 ratio and injected retroorbitally into lethally irradiated wild type mice (Ly5.1). Recipient mice were conditioned with 1,000 cGy from a ^{137}Cesium source at a rate of approximately 95 cGy/min prior to transplantation. Prophylactic antibiotics (trimethoprim-sulfamethoxazole, Alpharma, East Bridgewater, NJ) were given during the initial 2 weeks following transplantation. Mice were analyzed 8-10 weeks after transplantation.

2.3.7 BrdU labeling. Bromodeoxyuridine (BrdU, 10 mg/ml solution in 1x Dulbecco's PBS, Sigma, St. Louis, MO) was given by a single intraperitoneal injection at a dose of 1-2 mg/mouse. The percentage of $BrdU^+/Gr\text{-}1^+$ cells was determined by staining with APC-conjugated Gr-1 antibody followed by fixation, permeablization, and intracellular staining with a FITC-conjugated anti-BrdU antibody using reagents from the BrdU Flow kit (BD Biosciences). The half-life ($t_{1/2}$) of neutrophils in the blood was calculated using the formula $N = N_0e^{-\lambda t}$ where N_0 = the peak number of labeled cells, N = the number of cells at time t and λ = the decay constant.

2.3.8 Adoptive transfer experiments. Bone marrow cells were harvested, red blood cells removed by hypotonic lysis at room temperature for 2 minutes, and 8-10 x 10^6 cells injected intravenously into unconditioned wild type (Ly5.1) recipients. Bone marrow cells were harvested 1.5 to 2.5 hours after infusion, and donor neutrophils were identified based on Gr-1 and Ly5.2 expression. The absolute number of donor neutrophils in the bone marrow was calculated assuming a whole femur equivalent to 6% of the total bone marrow (35). This number was divided by the total number of donor neutrophils that were infused to yield the percentage of donor neutrophils that had homed to the bone marrow.

2.3.9 Neutrophil mobilization. G-CSF**:** recombinant human G-CSF, a generous gift from Amgen (Thousand Oaks, CA), was diluted in PBS with 0.1% low endotoxin BSA (Sigma) and administered by twice daily subcutaneous injection at a dose of 125

μg/kg/day for 5 days. Mice were analyzed 3-4 hours after the final injection on day 5. Some cohorts of mice were given a single subcutaneous injection of G-CSF (125 μg/kg), and peripheral blood was analyzed at the indicated times.

GROβ**:** human GROβ, a generous gift from Genzyme (Cambridge, MA), was reconstituted in sterile PBS and administered as a single subcutaneous injection at a dose of 100 μg/kg. Peripheral blood was analyzed at the indicated times.

2.3.10 Listeria monocytogenes infection. Mice were infected intraperitoneally with a dose of 5 x 10^5 colony forming units (c.f.u) of *L. monocytogenes* strain EGD. Peritoneal cells were obtained by lavage with 10 ml of cold PBS, nucleated cells counted, and manual leukocyte differential counts (200 cells) performed on Wright-stained cytospins. At 72 hours, *L. monocytogenes* was quantified by homogenizing the spleens and livers in 10 ml PBS with 0.05% Triton X-100 and plating serial dilutions on LB-agar.

2.3.11 Statistical analysis. Statistical significance was assessed using a two-tailed Student *t* test assuming equal variance for comparison of two groups, one-way ANOVA with Bonferroni post-testing for experiments with more than two groups, or, for time-course experiments, a 2-way ANOVA with Bonferroni post-testing at individual time points. All data are presented as the mean ± SEM.

2.4 Results

2.4.1 CXCR4 is selectively deleted in myeloid cells of LysM$^{Cre/+}$ CXCR4$^{flox/-}$ (MKO) mice

Mice expressing Cre-recombinase under the control of the myeloid *lysozyme M* promoter (*LysM*^{Cre}) were crossed with *CXCR4*$^{+/-}$ and *CXCR4*$^{flox/flox}$ mice to generate myeloid-restricted knockout (MKO) mice with the genotype *LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$. Efficient deletion of CXCR4 in mature neutrophils (Gr-1^{bright}SSC^{hi}) of MKO mice in both the blood and bone marrow was observed by flow cytometry. Whereas CXCR4 expression was easily detectable in wild type neutrophils in the blood, no expression was detected in MKO neutrophils (Figure 2-1 A & B). *CXCR4*$^{+/-}$ mice had intermediate levels of CXCR4 expression on their neutrophils. There was some residual expression of CXCR4 in MKO bone marrow neutrophils, possibly representing less mature myeloid cells that had not yet undergone Cre-mediated excision of *CXCR4*. As expected, expression of CXCR4 was normal to slightly reduced in B lymphocytes and T lymphocytes from MKO mice compared to wild type mice (Figure 2-1A and data not shown). PCR analysis of genomic DNA isolated from MKO blood neutrophils confirmed complete Cre-mediated excision of the coding region of exon 2 of the *CXCR4* gene (Figure 2-1C). These data show that CXCR4 is efficiently and specifically deleted in myeloid cells in MKO mice.

2.4.2 Loss of CXCR4 results in the redistribution of neutrophils from the bone marrow to blood

To assess the effect of the loss of CXCR4 on basal granulopoiesis, MKO or control mice housed in standard pathogen free conditions were analyzed. We initially analyzed 4 separate control groups: wild type mice, $CXCR4^{flox/+}$ mice, $LysM^{Cre/+}$ $CXCR4^{+/+}$ mice, and $CXCR4^{+/-}$ mice. The wild type, $CXCR4^{flox/+}$, and $LysM^{Cre/+}$ $CXCR4^{+/+}$mice had similar phenotypes for all assays and were subsequently pooled as control mice (Figure 2-2 and data not shown). MKO mice displayed a marked isolated neutrophilia in the peripheral blood (Figure 2-2A). The number of Gr-$1^{bright}SSC^{hi}$ cells (neutrophils) in the blood was $1.7 \pm 0.2 \times 10^6$/ml in control mice versus $8.5 \pm 0.4 \times 10^6$/ml in MKO mice ($p < 0.0001$). This increase in circulating neutrophils was confirmed by manual inspection and differential counts of blood smears [absolute neutrophil count: $1.6 \pm 0.4 \times 10^6$/ml (control mice) versus $5.3 \pm 0.7 \times 10^6$/ml (MKO mice); $p < 0.001$]. Interestingly, no increase in immature myeloid cells in the blood was observed (data not shown). MKO mice displayed normal numbers of circulating B lymphocytes and T lymphocytes (data not shown). They also had normal numbers of circulating and splenic hematopoietic progenitor cells as measured by colony forming assays (data not shown).

In the bone marrow of MKO mice, the number of Gr-$1^{bright}SSC^{hi}$ cells was reduced to $71.8 \pm 2.9\%$ of control mice (Figure 2-2B). Although the Gr-$1^{bright}SSC^{hi}$ cell population reliably measures mature neutrophils in the blood, this population is more heterogeneous in the bone marrow with approximately 20% immature myeloid cells (36). Thus, to confirm these findings, we also performed manual leukocyte differentials of

bone marrow cells (Figure 2-3). Consistent with the flow cytometry data, a trend to decreased band and segmented neutrophils in the bone marrow of MKO mice was observed (control, $6.3 \pm 0.9 \times 10^6$/femur versus MKO, $4.2 \pm 1.0 \times 10^6$/femur; $p = 0.14$). Of note, the frequency of granulocytic precursors and number and cytokine responsiveness of myeloid progenitors was comparable in MKO and control mice (Figures 2-3 & 2-4), suggesting that neutrophil differentiation was normal.

Neutrophil trafficking from the bone marrow was estimated by determining the percentage of neutrophils in the blood versus the total number of neutrophils in the bone marrow and blood (26). Consistent with previous reports, in control mice, only $1.9 \pm 0.2\%$ of neutrophils are in the blood. Strikingly, in MKO mice, this percentage increased to $11.9 \pm 1.2\%$ (Figure 2-2C; $p < 0.0001$). There also was a significant increase in splenic neutrophils in MKO mice [number of neutrophils per spleen: $4.3 \pm 1.3 \times 10^6$ (control) versus $13.3 \pm 1.8 \times 10^6$ (MKO); $p < 0.01$]. Collectively, these data suggest that the loss of CXCR4 results in the redistribution of neutrophils from the bone marrow to the blood and spleen.

2.4.3 Loss of CXCR4 results in premature release of neutrophils from the bone marrow but normal clearance from the blood

An increase in circulating neutrophil number could be caused by increased production of neutrophils in the bone marrow, increased release into the blood, decreased clearance from the blood, or some combination thereof. To investigate differences in kinetics of neutrophil release into the blood and the potential difference in neutrophil survival,

control or MKO mice were given a single injection of bromodeoxyuridine (BrdU) to label newly synthesized neutrophils. The fate of blood neutrophils pulse-labeled with BrdU *in vivo* was determined by flow cytometry for Gr-1^{+}BrdU^{+} cells (Figure 2-5A). In the bone marrow, a similar percentage of myeloid cells were labeled with BrdU, suggesting that loss of CXCR4 does not affect granulocytic cell proliferation (data not shown). Consistent with a role for CXCR4 in regulating neutrophil release, the transit time for labeled neutrophils to appear in the circulation was significantly reduced in MKO mice. In control mice, the peak number of labeled neutrophils in the blood occurred 72 hours after BrdU administration, while in the majority of MKO mice, the peak was at 46 hours (Figure 2-5B). However, there was no significant difference in the disappearance of labeled cells from the blood. Consistent with previous studies in mice and humans, the calculated half-life of control neutrophils was 13.7 ± 2.3 hours. A similar calculated half-life was observed for MKO neutrophils (10.0 ± 1.0 hours, Figure 2-5C). Taken together, these data show that neutrophils lacking CXCR4 have accelerated release from the bone marrow but normal clearance from the blood.

2.4.4 CXCR4 directs homing of neutrophils to the bone marrow

Previous studies suggested that aged neutrophil preferentially home to, and are cleared in, the bone marrow in a CXCR4-dependent fashion (17, 31). To test this possibility, we measured the trafficking of neutrophils to the bone marrow after adoptive transfer of control or MKO neutrophils into wild type recipient mice. Donor neutrophils from control mice were clearly detectable in recipient bone marrow 1.5-2.5 hours after

transfer. However, homing of MKO neutrophils to the bone marrow was significantly reduced, although not eliminated completely (Figure 2-6). These data confirm that CXCR4 contributes to neutrophil homing to the bone marrow. However, given the normal circulating half-life of endogenous MKO neutrophils, it appears that the bone marrow is not an essential site of neutrophil clearance.

2.4.5 CXCR4 acts in a cell autonomous fashion to retain neutrophils in the bone marrow

Recent studies suggest that loss of adhesion molecules on neutrophils may result in neutrophilia in a non-cell autonomous fashion through disruption of a homeostatic feedback loop (37, 38). To determine whether CXCR4 acts in a cell autonomous fashion to regulate neutrophil release, mixed bone marrow chimeras were generated in which hematopoiesis was reconstituted with a 1:1 ratio of wild type to MKO cells. The mixed chimeras displayed chronic neutrophilia (absolute neutrophil count of 5.8 ± 0.4 x 10^6/mL). Whereas the percentage of wild type and MKO neutrophils in the bone marrow was similar, the great majority of neutrophils in the blood were derived from MKO cells (Figure 2-7A). Accordingly, the calculated neutrophil distribution index for MKO cells ($23.9 \pm 2.5\%$) was much higher than that for wild type neutrophils ($4.3 \pm 0.6\%$; $p < 0.0001$; Figure 2-7B). As a control, we also assessed the distribution of B lymphocytes in the mixed chimeras. Though a modest increase in MKO-derived B lymphocytes was observed, the ratio of MKO to wild type cells in the bone marrow and blood was similar

(Figure 2-7C). These data show that CXCR4 negatively regulates neutrophil release in a cell autonomous fashion.

2.4.6 Neutrophil mobilization by G-CSF or GROβ is impaired in the absence of CXCR4

A key feature of the innate immune response is the capacity to rapidly increase neutrophil number in the blood in response to infection or other stresses. Certain cytokines, chemokines, and bacterial products are thought to mediate the stress granulopoiesis response. To examine the contribution of CXCR4 signaling in stress granulopoiesis, we first characterized the neutrophil response to G-CSF, the prototypical mobilizing cytokine. Following a single injection of G-CSF, neutrophil number in the blood of control mice increased 3.9 ± 0.7 fold (Figure 2-8A), with peak levels occurring after 6 hours before returning to near-baseline levels at 24 hours. As reported previously (25), this was associated with a marked decrease in surface CXCR4 expression on blood neutrophils (Figure 2-8B). In contrast, no change in neutrophil counts in the blood was observed after G-CSF treatment of MKO mice (Figure 8A). Next, we studied granulopoiesis after 5 days of G-CSF administration. In control mice, an 11.8 ± 1.6 fold increase in circulating neutrophils was observed (Figure 2-8C). This increase was secondary to both increased production (as evidenced by a modest increase in bone marrow neutrophils) and increased neutrophil release (as evidenced by an increase in the NDI). Of note, previous studies showed that G-CSF does not alter the kinetics of neutrophil clearance from the blood (2, 3, 26). In MKO mice, a similar increase in bone

marrow neutrophils after G-CSF treatment was observed, suggesting that G-CSF induced increases in neutrophil production were intact. In contrast, though a modest increase in circulating neutrophils was observed in MKO mice after G-CSF treatment, this appears to be mainly secondary to increased production, rather than release, as no increase in the NDI was seen.

We also examined neutrophil release after treatment with GROβ, a well-characterized mobilizing chemokine. In control mice, GROβ treatment induced a 7.4 ± 2.1 fold increase in circulating neutrophils that peaked 30 minutes after injection and nearly returned to baseline after two hours (Figure 2-8D). In contrast, no significant increase in circulating neutrophils was observed in MKO mice. Surprisingly, CXCR4 expression on neutrophils isolated from control mice treated with GROβ was significantly reduced at the time of peak mobilization compared to pre-treatment levels (Figure 2-8E). These data suggest that G-CSF and GROβ induced neutrophil release from the bone marrow are dependent on CXCR4 signals.

2.4.7 Neutrophil mobilization in response to Listeria monocytogenes infection is impaired in the absence of CXCR4 but homing of neutrophils to the peritoneum and bacterial clearance is normal

To further define the requirement for CXCR4 in emergency granulopoiesis, control and MKO mice were infected intraperitoneally with *Listeria monocytogenes*. Infection with *L. monocytogenes* is an established model of emergency granulopoiesis, and neutrophils are known to play a key role in the clearance of this infection (39). Clearance of bacteria

was similar in MKO and wild type mice; no difference in survival or spleen and liver bacterial load was observed after *L. Monocytogenes* challenge (Figure 2-9 A & B). In control mice, a 3.4 ± 0.6 fold increase in circulating neutrophils was observed 24 hours after bacterial challenge that remained elevated for the duration of the assay. In contrast, MKO mice failed to increase their circulating neutrophils in response to infection with this pathogen (Figure 2-9C). However, neutrophils in MKO mice were able to emigrate into the peritoneal space in similar numbers compared to control mice (Figure 2-9D). These data show that CXCR4, while required for maximal neutrophil mobilization into the blood after *L. monocytogenes* infection, is dispensable for neutrophil emigration to the peritoneum.

2.5 Discussion

There is accumulating evidence suggesting that CXCR4 is a key regulator of neutrophil homeostasis. Perhaps most convincing is the identification of truncation mutations of CXCR4 in most cases of WHIM syndrome. Patients with WHIM syndrome display neutropenia despite normal to increased numbers of neutrophils in the bone marrow. The CXCR4 truncation mutations confer enhanced responsiveness to CXCL12, suggesting a model in which increased CXCR4 signaling in WHIM neutrophils results in their abnormal retention in the bone marrow (18, 19, 40, 41). Consistent with this model, treatment of humans or mice with AMD3100, a specific CXCR4 antagonist, results in the rapid mobilization of neutrophils into the blood (15, 16). However, direct testing of the contribution of CXCR4 to neutrophil homeostasis has been limited by the embryonic lethality of CXCR4 deficient mice and the severe engraftment defect of *CXCR4*$^{-/-}$ hematopoietic stem cells (10, 11, 13, 32). In the present study, we characterized granulopoiesis and neutrophil trafficking in transgenic mice carrying a myeloid-specific deletion of CXCR4. The marked neutrophilia present in these mice confirm a key role for CXCR4 signaling in the regulation of neutrophil homeostasis.

Neutrophil homeostasis in the blood is maintained by balancing neutrophil production, release from the bone marrow, and clearance. There is evidence implicating CXCR4 in the regulation of all three of these processes. In WHIM syndrome, there are reports of dysmorphic neutrophils and increased neutrophil apoptosis, suggesting defective granulopoiesis (42, 43). Moreover, in *CXCR4*$^{-/-}$ fetal liver chimeras, there is a decrease in myeloid progenitors and precursors (13). Our study was not designed to

assess the contribution of CXCR4 to granulopoiesis, since CXCR4 expression in MKO mice was maintained until the final stages of myeloid development. Not surprisingly, granulopoiesis, as measured by leukocyte differentials, BrdU incorporation in myeloid precursors, and number and cytokine responsiveness of myeloid progenitors, was normal in MKO mice. Despite normal granulopoiesis, marked neutrophilia and increased splenic neutrophils were observed in MKO mice, suggesting an alteration in neutrophil release or clearance.

As noted previously, the rapid mobilization of neutrophils into the blood by AMD3100 suggests a role for CXCR4 in regulating neutrophil release. Indeed, the striking redistribution of neutrophils from the bone marrow to blood in MKO mice suggests enhanced neutrophil release. Consistent with this conclusion, BrdU labeled neutrophils appeared in the blood more rapidly in MKO versus control mice. A recent report suggested that CXCR4 may contribute to neutrophil clearance from the blood by directing senescent neutrophils to the bone marrow (31). Consistent with this finding, we observed decreased homing of CXCR4 deficient neutrophils to the bone marrow after adoptive transfer. On the other hand, the half-life of CXCR4 deficient neutrophils in the blood was comparable to wild type neutrophils, suggesting that CXCR4 is not a major regulator of neutrophil clearance from the blood and that the bone marrow represents a non-essential site of neutrophil removal. Collectively, these data suggest that CXCR4 maintains neutrophil homeostasis primarily by regulating neutrophil release from the bone marrow.

Neutrophil homeostasis can be regulated in both a cell autonomous and non-cell autonomous fashion. In a series of elegant studies, Stark and colleagues showed that β2-integrin deficient neutrophils induced neutrophilia in a non-cell autonomous fashion through the suppression of a negative feedback loop that senses the number of neutrophils that have emigrated into the tissue (37, 38). They showed that the presence of even a small number of wild type neutrophils activated the feedback loop and restored normal neutrophil number in the blood. In contrast, we observed persistent neutrophilia and preferential release of CXCR4 deficient neutrophils in mixed bone marrow chimeras reconstituted with both wild type and MKO bone marrow cells. These data show that CXCR4 acts in a cell autonomous fashion to regulate neutrophil release from the bone marrow.

G-CSF, the prototypical neutrophil mobilizing cytokine, is key regulator of both basal and stress granulopoiesis (44). Previous studies have shown that treatment with G-CSF results in a decrease in CXCL12 expression in the bone marrow (26-28). Moreover, G-CSF treatment leads to decreased surface expression of CXCR4 on neutrophils, a finding confirmed in the present study (25). These data suggest the hypothesis that disruption of CXCR4 signaling may contribute to G-CSF induced neutrophil mobilization. However, G-CSF treatment also induces other changes in the bone marrow microenvironment, such as the release of proteases (45), that might contribute to neutrophil mobilization. Thus, the relative importance of CXCR4 signaling in mediating neutrophil mobilization by G-CSF is unclear. In the present study, we show that G-CSF,

though stimulating neutrophil production, did not stimulate neutrophil release from the bone marrow in the absence of CXCR4 signals.

Of note, despite the near normal number of morphologically mature neutrophils in the bone marrow, it is possible that the mobilizable pool of neutrophils in MKO mice is exhausted. Arguing against this possibility, blood neutrophil counts in MKO mice doubled after the administration of a solution of propylene glycol (data not shown). Collectively, these data suggest that disruption of CXCL12/CXCR4 signaling is the dominant pathway by which G-CSF induces neutrophil mobilization.

Besides G-CSF, there are a number of neutrophil mobilizing agents that are thought to contribute to the stress granulopoiesis response. Most notable among these agents are chemokines. The rapidity of neutrophil mobilization by chemokines (minutes to hours) compared with G-CSF (hours to days) suggests distinct mechanisms of mobilization. Consistent with this idea, the CXCR2-chemokine KC (CXCL1) can synergize with G-CSF or AMD3100 to induce neutrophil mobilization (17, 31, 46). Surprisingly, herein we show that GROβ-induced neutrophil mobilization is abrogated in MKO mice. This result suggests two possibilities: 1). The GROβ-mobilizable pool of neutrophils is depleted in MKO mice. 2). GROβ-induced mobilization is dependent on CXCR4 signaling in neutrophils. In support of the latter possibility, several recent reports showed that treatment of wild type neutrophils or monocytes with CXCR2 ligands resulted in impaired CXCR4 signaling, presumably through heterologous desensitization (17, 47). In addition, we observed a significant decrease in cell-surface CXCR4 expression on neutrophils after GROβ treatment. Together, these data suggest that

disruption of CXCL12/CXCR4 signaling is a common mechanism by which chemokines and cytokines induce neutrophil release.

The stress granulopoiesis response to infection requires the coordinated expression of many different cytokines and chemokines. In this study, we utilized a model of *L. monocytogenes* infection to further explore the contribution of CXCR4 signals in the stress granulopoiesis response. Interestingly, the neutrophil mobilization response in MKO mice following *L. monocytogenes* infection was abrogated, suggesting that modulation of CXCR4 signaling may be a common mechanism of neutrophil release.

In summary, our data provide new evidence that CXCR4 is a key regulator of neutrophil homeostasis under basal and stress conditions. CXCR4 signals act primarily to regulate neutrophil trafficking from the bone marrow, and disruption of CXCR4 signals may represent a common mechanism by which cytokines and chemokines induce neutrophil release from the bone marrow. These data suggest that pharmacologic agents that modulate CXCR4 signaling may be effective for controlling neutrophil responses in infectious and inflammatory diseases.

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2.8 Figures and figure legends

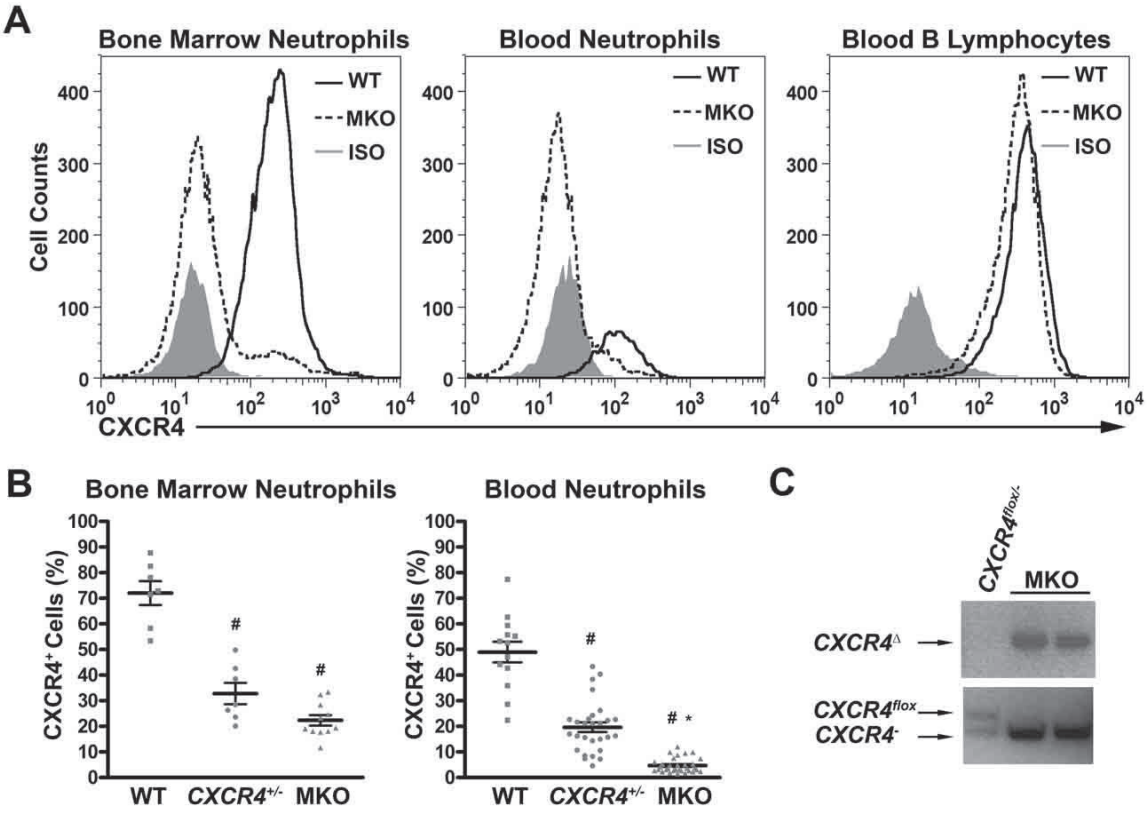


Figure 2-1. CXCR4 is efficiently deleted in neutrophils from LysM $^{Cre/+}$ CXCR4$^{flox/-}$ (MKO) mice. (A) Representative histograms showing cell surface CXCR4 expression in the mature neutrophil (Gr-1^{bright}SSC^{hi}) population from bone marrow or peripheral blood or the peripheral blood B lymphocyte (B-220^{+}) population in wild type (WT) or MKO mice. The isotype control (ISO) is shown in gray. (B) Cell surface CXCR4 expression in the mature neutrophil population in the bone marrow or peripheral blood. Data represent the mean ± SEM. $^{\#}p < 0.05$ compared to wild type mice. $^{*}p < 0.05$ compared to *CXCR4*$^{+/-}$ mice. (C) Genomic DNA was isolated from MKO or control (*CXCR4*$^{flox/-}$ without *LysM*^{Cre}) blood neutrophils and the CXCR4 gene amplified using primers that specifically detected the deleted (*CXCR4*$^{\Delta}$), floxed (*CXCR4*^{flox}), or null (*CXCR4*$^{-}$) *CXCR4* alleles.

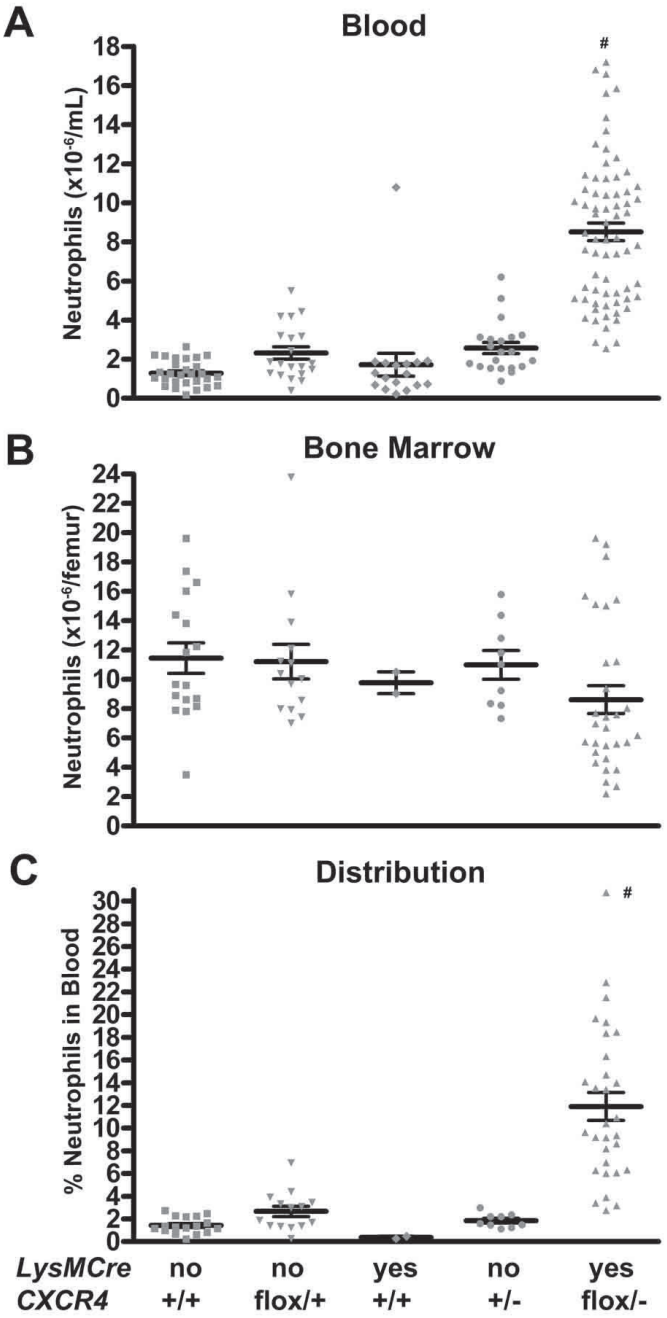
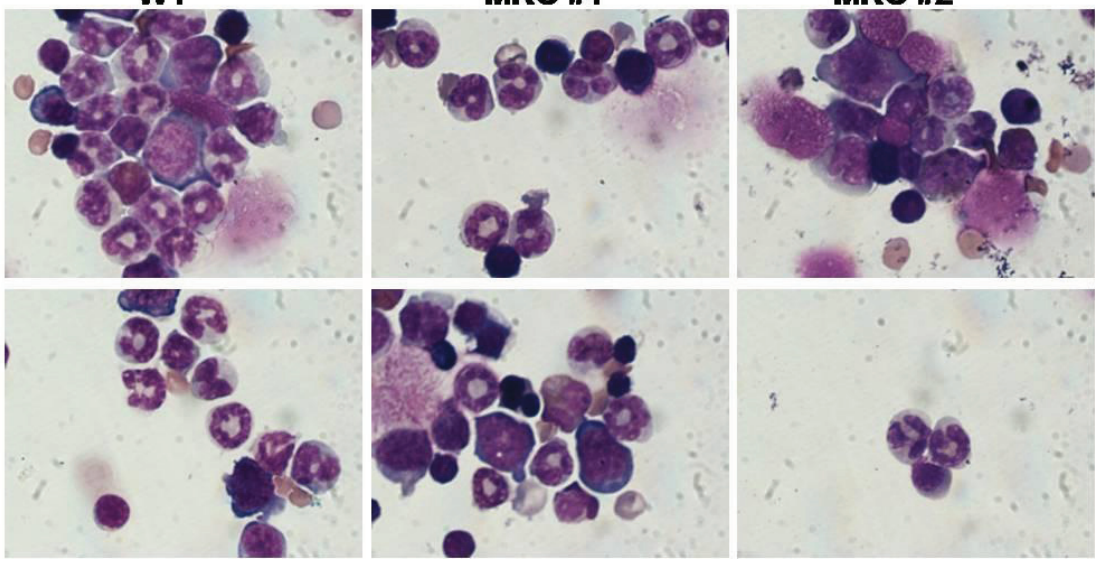


Figure 2-2. Basal granulopoiesis in MKO mice is characterized by a shift of neutrophils from the bone marrow to the blood. (A & B) The number of mature neutrophils (Gr-1^{bright}SSC^{hi}) in the blood and bone marrow was quantified by flow cytometry in mice of the indicated genotype. (C) The neutrophil distribution index (NDI) was calculated to estimate the percentage of total body neutrophils in the blood, using the following formula: NDI = blood neutrophils / (blood + bone marrow neutrophils). Data represent the mean ± SEM. $^{\#}p < 0.05$ compared to all other groups.

A



B

	Blasts and Pro-myelocytes	Myelocytes	Meta-myelocytes	Band and Segmented Neutrophils	Erythroid Cells	Lymphoid Cells	Other
CTL	8.0 ± 1.9%	14.9 ± 1.2%	15.8 ± 3.0%	18.4 ± 2.8%	29.3 ± 4.0%	12.1 ± 1.3%	1.6 ± 0.1%
MKO	6.7 ± 0.2%	15.8 ± 1.5%	15.5 ± 1.4%	10.5 ± 2.5%	37.3 ± 2.0%	12.6 ± 0.7%	1.7 ± 0.5%

Figure 2-3. Morphologically mature neutrophils are present in the bone marrow of MKO mice. (A) Representative micrographs of bone marrow cytospins from two different MKO mice or a wild type (WT) mouse demonstrating the presence of band and segmented neutrophils. Original magnification x1000. Images were acquired using an E plan 100x/1.25 NA oil immersion objective, Microphot SA microscope, Digital Sight DS-Fi1 camera, and NIS-Elements F2.30 software, all from Nikon (Melville, NY). (B) Manual leukocyte differential counts were performed on control (CTL) or MKO mice. Data represent the mean ± SEM (n = 3 mice per group).

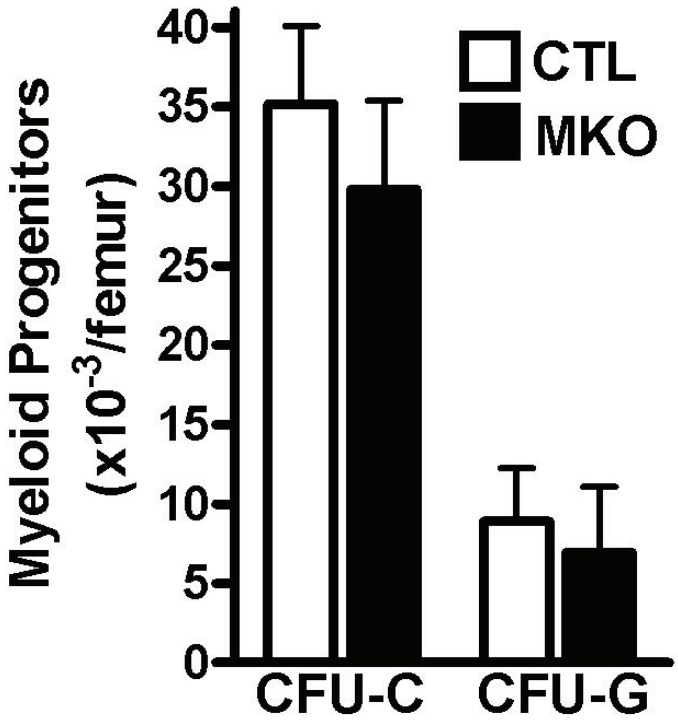


Figure 2-4. MKO mice have normal numbers of myeloid progenitors in the bone marrow. Bone marrow cells from control (CTL) or MKO mice were plated in methylcellulose medium in the presence of stem cell factor, IL-3, IL-6, and EPO (CFU-C) or G-CSF (CFU-G) and colonies enumerated on day 7-10 of culture. Data represent the mean ± SEM (n = 5 mice per group).

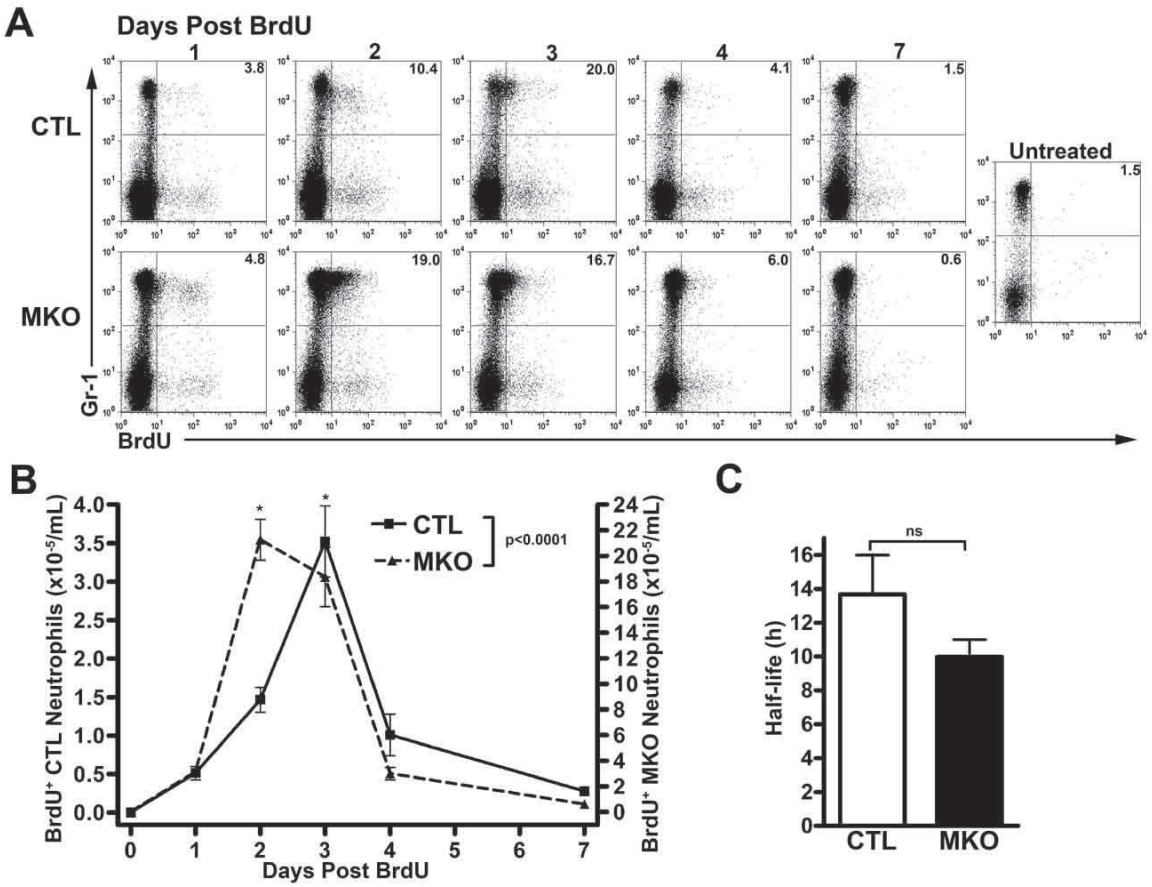


Figure 2-5. Blood neutrophil half-life in MKO mice is normal. BrdU (2 mg) was administered to control (CTL) or MKO mice by a single intraperitoneal injection. Peripheral blood was obtained at the indicated time points and the number of $BrdU^+$ Gr-1^{bright} cells determined by flow cytometry. (A) Representative dot plots showing BrdU staining in the Gr-1^{bright} (mature neutrophil) population. The numbers shown indicate the percentage of Gr-1^{bright} cells that were $BrdU^+$. (B) The absolute number of $BrdU^+$ Gr-1^{bright} neutrophils in the blood is shown. (C) Neutrophil half-life ($t_{1/2}$) in the blood was calculated according to the formulas $t_{1/2} = \ln 2/\lambda$ and $n_t = n_0 e^{-\lambda t}$ where n_0 is the number at a given time, n_t is the number t hours later, and λ is the decay constant. The data shown represent the mean ± SEM of n = 10-11 mice in each group. $^*p < 0.05$ compared to control mice at the same time point. ns, not significant.

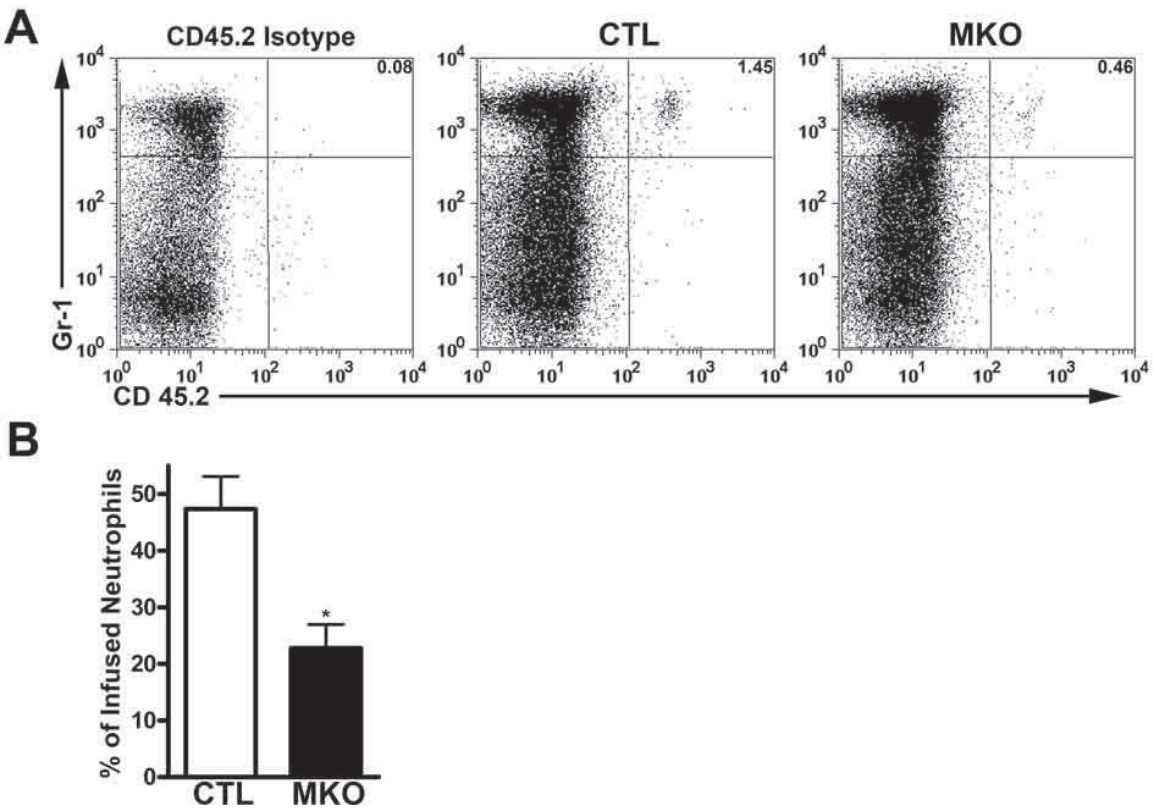


Figure 2-6. MKO neutrophils have impaired homing to the bone marrow. Bone marrow cells (8-10 x 10^6) from control (CTL) or MKO mice carrying the *Ly 5.2* allele were adoptively transferred to WT recipients carrying the *Ly 5.1* allele, enabling detection of infused neutrophils in the bone marrow by flow cytometry using an allele-specific CD 45.2 antibody. (A) Representative dot plots showing donor neutrophils as the percentage of total bone marrow neutrophils 1.5-2.5 hours after infusion. (B) The percentage of transferred control or MKO neutrophils present in the bone marrow of recipient mice. Data represent the mean ± SEM of n = 8-12 recipients for each genotype from 3 separate experiments. $^*p < 0.05$ compared to control neutrophils.

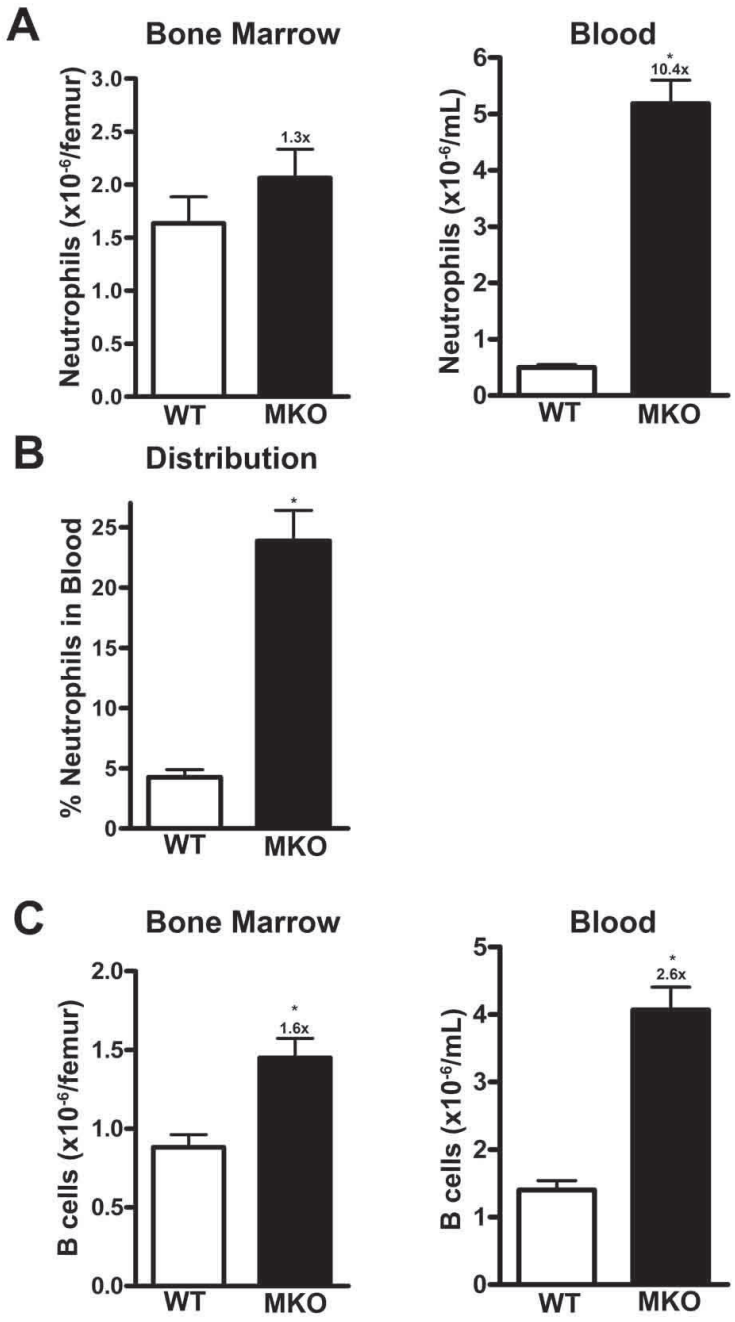


Figure 2-7. Trafficking of MKO neutrophils is altered in mixed chimeras. Whole bone marrow from wild type (WT, *Ly5.1*) and MKO (*Ly5.2*) mice was mixed in a 1:1 ratio and transplanted into lethally irradiated wild type (*Ly5.1*) recipients. After hematopoietic reconstitution (8-10 weeks), the bone marrow and blood were analyzed by flow cytometry. The numbers at the top of the columns indicate the fold increase over the wild type cells. (A) Mature neutrophils (Gr-1^{bright} CD115^{-}). (B) Neutrophil distribution index. (C) B lymphocytes (B220^{+}). The data represent the mean ± SEM of n = 18 recipients from two separate transplants. $^{*}p < 0.05$ compared to wild type cells.

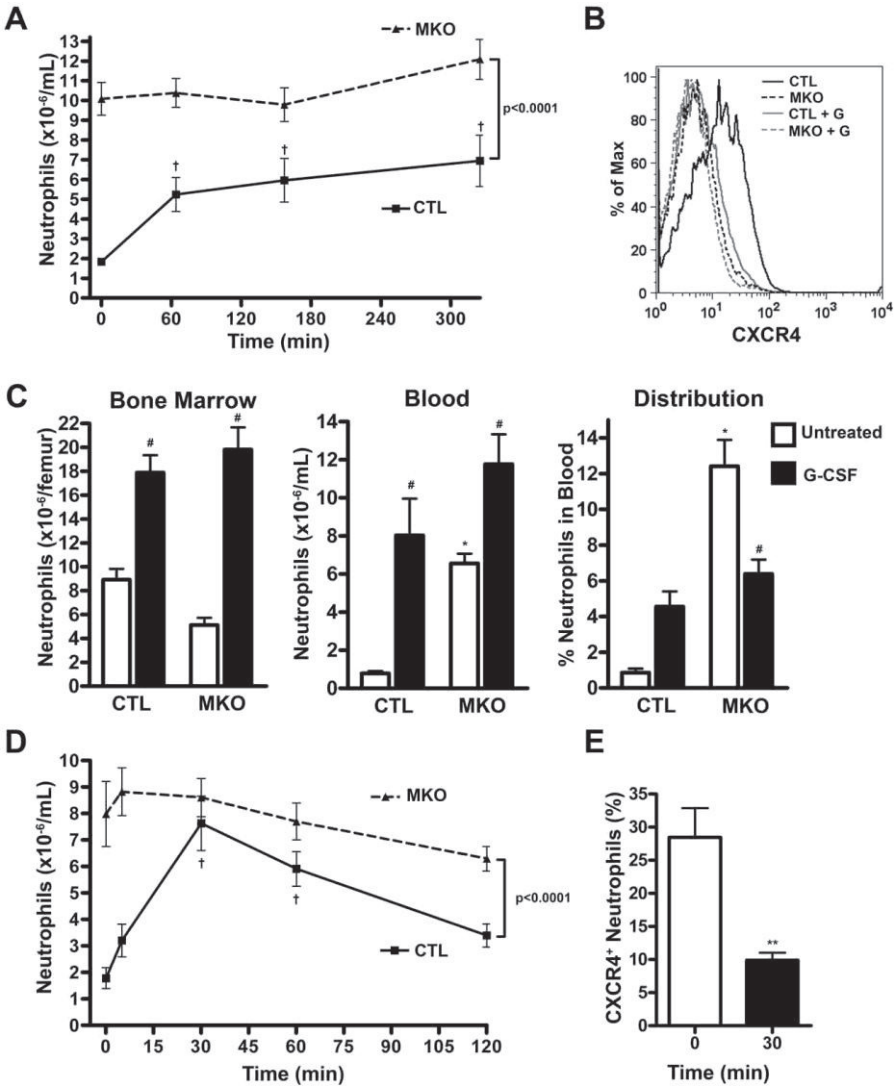


Figure 2-8. Neutrophil mobilization by G-CSF or GROβ is abrogated in MKO mice. (A) Mice (n = 5 per group) were given a single subcutaneous injection of G-CSF (125 μg/kg) and the absolute neutrophil count measured at the indicated times. $^{\dagger}p < 0.05$ compared to time 0. (B) Representative histograms showing cell surface CXCR4 expression on blood neutrophils from control (CTL) or MKO mice at baseline and 65 minutes after a single dose of G-CSF (+G). (C) Mice (n = 8-11 per group) were treated with G-CSF (125 μg/kg/day, twice daily injections) for 5 days, and neutrophils in the bone marrow and blood were quantified. The calculated neutrophil distribution index is shown in the far right panel. $^{*}p < 0.05$ compared with control mice at the same time point. $^{\#}p < 0.05$ compared to untreated mice of the same genotype. (D) Mice (n = 9-12 per group) were given a single subcutaneous injection of GROβ (100μg/kg), and the absolute neutrophil count was determined at the indicated times. $^{\dagger}p < 0.05$ compared to time 0. (E) CXCR4 cell surface expression on peripheral blood neutrophils from control mice was determined by flow cytometry at baseline and at the time of peak mobilization, 30 minutes after GROβ administration. $^{**}p < 0.001$. Data represent the mean ± SEM.

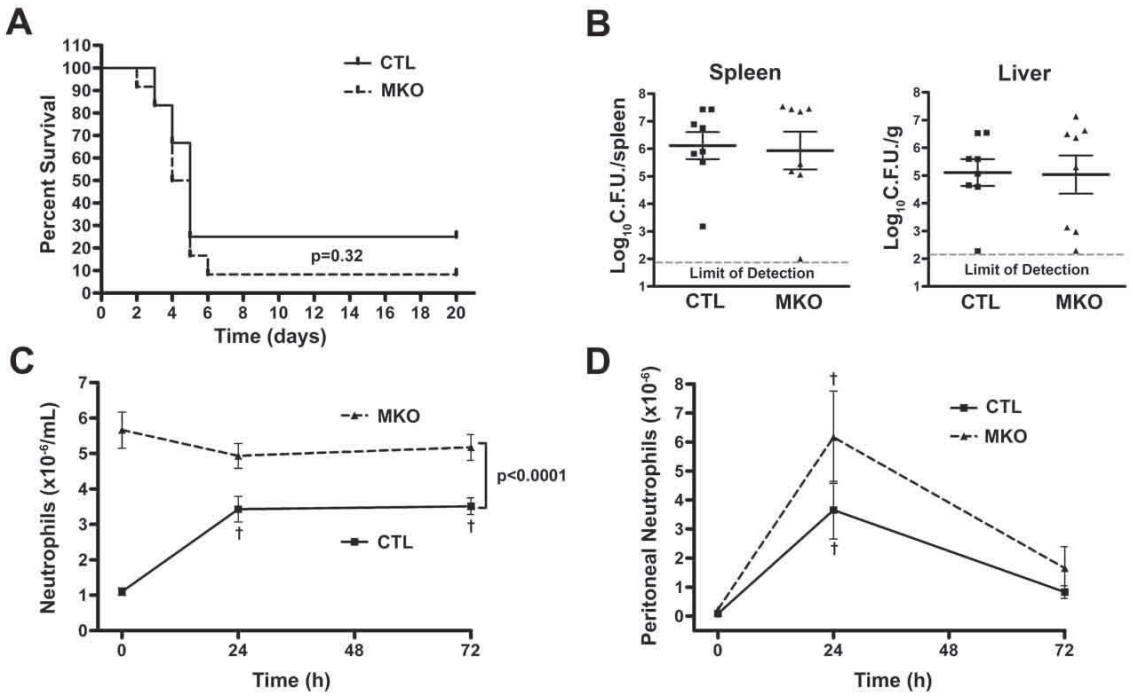


Figure 2-9. MKO mice have impaired blood neutrophil mobilization but normal neutrophil recruitment to the peritoneum in response to Listeria infection. Control (CTL) or MKO mice were infected intraperitoneally with *Listeria monocytogenes.* (A) Survival was assessed in mice (n = 12 per group) from two separate infections with 9.8 to 11.2 x 10^5 C.F.U. of bacteria. (B) The bacterial titer in the spleen and liver of control and MKO mice (n = 8 per group) was determined 72 hours after infection with 2.1 to 7.2 x 10^5 C.F.U. of bacteria. (C) Blood neutrophil counts were assessed by flow cytometry at the indicated times after infection with 2.1 to 9.8 x 10^5 C.F.U. of bacteria (n = 8-19 mice per group depending on the time). (D) Shown is the number of neutrophils in the peritoneum at the indicated times after infection with 2.1 to 7.2 x 10^5 C.F.U. of bacteria (n = 5-8 mice per group depending on the time). Data represent the mean ± SEM. $^{\dagger}p < 0.05$ compared to time 0.

Chapter 3

CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from the bone marrow

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Eash KJ, Greenbaum AM, Gopalan PK, and Link DC. 2010. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from the bone marrow. J. Clin. Invest. 120(7). 2423-2431.

3.1 Abstract

Neutrophil homeostasis is maintained, in part, by the regulated release of neutrophils from the bone marrow. Constitutive expression of the chemokine CXCL12 by bone marrow stromal cells provides a key retention signal for neutrophils in the bone marrow through activation of its receptor CXCR4. Herein, we show that the ELR^+ chemokines CXCL1 and CXCL2 are constitutively expressed by bone marrow endothelial cells and osteoblasts, and CXCL2 expression is induced in endothelial cells during G-CSF-induced neutrophil mobilization. Neutrophils lacking CXCR2, the receptor for CXCL1 and CXCL2, are preferentially retained in the bone marrow, reproducing a myelokathexis phenotype. Transient disruption of CXCR4 failed to mobilize *CXCR2*$^{-/-}$ neutrophils. However, doubly deficient neutrophils (*CXCR2*$^{-/-}$ *CXCR4*$^{-/-}$) displayed constitutive mobilization, showing that CXCR4 plays a dominant role. Collectively, these data suggest that CXCR2 signaling is a second chemokine axis that interacts antagonistically with CXCR4 to regulate neutrophil release from the bone marrow.

3.2 Introduction

Neutrophils are an essential component of the innate immune response and a major contributor to inflammation. Accordingly, the number of neutrophils in the blood is tightly regulated. Neutrophil homeostasis is maintained through a balance of neutrophil production, release from the bone marrow, and clearance from the circulation (1). The bone marrow is the primary site of neutrophil production, requiring that mature neutrophils transmigrate through an endothelial cell barrier to enter the circulation (2-4). Under basal conditions, less than 2% of the total body of mature neutrophils are in the circulation (5). Thus, the bone marrow serves as a reservoir for neutrophils that can be rapidly mobilized in response to infection or other stresses.

Major advances in our understanding of the mechansims regulating neutrophil release from the bone marrow have come from the study of the human disease myelokathexis (kathexis = retention) (6-8). Myelokathexis is characterized by neutropenia despite normal to increased numbers of neutrophils in the bone marrow. It can occur in isolation or as a component of WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) syndrome (WS) (9, 10). Genetic studies have shown that heterozygous mutations of *CXCR4* are the most common cause of WHIM syndrome (11). *CXCR4* encodes for a CXC chemokine receptor whose major ligand is CXCL12 (SDF-1) (12). WHIM-associated mutations of *CXCR4* result in the production of a carboxy-terminal truncated receptor that displays impaired internalization and enhanced signaling, suggesting that excessive CXCR4 signaling may result in abnormal neutrophil retention in the bone marrow (13-16). Conversely, genetic deletion of CXCR4 in murine

hematopoietic cells results in constitutitive mobilization of neutrophils into the blood (17-22). Moreover, treatment of humans or mice with AMD3100, a small molecule antagonist of CXCR4, causes rapid neutrophil mobilization (23, 24). Together with the observation that CXCL12 is constituitively expressed at a high level by bone marrow stromal cells (25), these data indicate that the CXCR4/CXCL12 axis provides a key retention signal for neutrophils in the bone marrow.

Disruption of CXCR4 signaling is an important mechanism by which neutrophils are mobilized into the circulation under stress conditions. Treatment with G-CSF, a major mobilizing cytokine, is associated with decreased CXCL12 expression in the bone marrow and decreased CXCR4 expression on neutrophils (5, 26-28). Yet, the mechanisms by which attenuated CXCR4 signaling leads to migration of neutrophils towards the bone marrow venous sinuses and subsequent entry into the circulation are unclear. Based on their well-characterized role in other aspects of neutrophil biology (29), we hypothesized that ELR^+ CXC chemokines may direct neutrophil migration towards the bone marrow vascular space, thereby opposing the CXCR4/CXCL12 axis. There are 7 ELR^+ CXC chemokines (CXCL1-3, 5-8) that signal through CXCR1 or CXCR2 (12). These chemokines are potent neutrophil chemoattractants and activators and induce neutrophil mobilization from the bone marrow when administered exogenously (4, 30-33). Accordingly, *CXCR2*$^{-/-}$ mice have a profound defect in neutrophil emigration to sites of inflammation (mice lack *CXCR1*) (34). With respect to neutrophil homeostasis, *CXCR2*$^{-/-}$ mice housed under specific pathogen free (SPF) conditions display neutrophilia with a myeloid expansion in the bone marrow and spleen.

In contrast, under gnotobiotic conditions, the level of circulating neutrophils in *CXCR2*$^{-/-}$ mice is normal, raising the possibility that subclinical infections are inducing a stress granulopoiesis response (35-37).

In the present study, we generated mixed bone marrow chimeras reconstituted with wild type and *CXCR2*$^{-/-}$ cells to study the cell-intrinsic role of CXCR2 in neutrophil trafficking. We show that *CXCR2*$^{-/-}$ neutrophils are selectively retained in the bone marrow, reproducing a myelokathexis phenotype. CXCR2 is required for neutrophil mobilization in response to transient CXCR4 inhibition, although the increased release of *CXCR4*$^{-/-}$ neutrophils is not altered in the absence of CXCR2 signals. CXCR2 ligands CXCL1 (KC) and CXCL2 (MIP-2) are constitutively expressed in bone marrow endothelium, and CXCL2 expression is induced during G-CSF-induced neutrophil mobilization. These results suggest that CXCR2 signaling is a second chemokine axis that controls neutrophil release from the bone marrow by opposing CXCR4 signals.

3.3 Methods

3.3.1 Mice. Sex- and age-matched, congenic C57BL/6 *CXCR2*$^{-/-}$ (34, 38) (The Jackson Laboratory, Bar Harbor, ME), *LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$ (22), pOBCol2.3-GFP (39), and wild-type mice that have the *Ly5.1* gene (B6.SJL-Ptprc* Pep3b BoyJ, Jackson Labs) were maintained under SPF conditions according to methods approved by the Washington University Animal Studies Committee. All transgenic strains had been backcrossed at least 10 generations onto a C57BL/6 background.

3.3.2 Bone marow transplantation. Bone marrow cells from wild-type (Ly5.1^{+}) mice and either *CXCR2*$^{-/-}$, *CXCR4*-MKO (*LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$), or DKO (*LysM*$^{Cre/+}$ *CXCR2*$^{-/-}$ *CXCR4*$^{flox/-}$) mice (Ly5.2^{+}) were mixed at a 1:1 ratio, and a total of 2 million cells injected retroorbitally into lethally irradiated (1,000 cGy) wild type (Ly5.1^{+}) recipient mice. Antibiotics (trimethoprim-sulfamethoxazole, Alpharma, East Bridgewater, NJ) were given for 2 weeks post-transplant.

3.3.3 Blood, bone marrow, or spleen analysis. Blood, bone marrow, and spleen cells were quantified using a Hemavet automated cell counter (CDC Technologies, Oxford, CT). Absolute neutrophil numbers were calculated assuming a blood volume of 1.8 ml and a whole femur equivalent to 6% of the total bone marrow (40). Bone marrow extracellular fluid was obtained by sequentially flushing both femurs with 400 μL ice-cold PBS and harvesting the supernatant after pelleting cells by centrifugation at 400 x g for 2 minutes.

3.3.4 Flow cytometry. Cells were stained by standard protocols with the following antibodies (eBiosciences, San Diego, CA unless otherwise noted): Chimerism was assessed using PerCP-Cy5.5-Ly5.1 (CD45.1) and allophycocyanin (APC)-Ly5.2 and one or more of the following lineage markers: FITC-Ly6G (Gr-1, myeloid), PE-CD3e (T lymphocytes), and APC-efluor780-CD45R (B220, B lymphocytes). A lineage cocktail of FITC-conjugated Gr-1, B220, CD3e, and Ter119 (erythroid) was used to sort progenitor cells. CXCR4 expression was assessed by incubating with anti-CD16/32 (Fc-block, BD Biosciences, San Diego, CA) and biotin-conjugated CXCR4 (BD) followed by PE-streptavidin. Isotype-matched antibodies were used as negative controls. Data was collected on a FACScan 5-color, 2-laser flow cytometer (BD Biosciences and Cytek Development, Fremont, CA) using Cellquest software (BD) and analyzed with the FlowJo software package (Tree Star, Ashland, OR).

3.3.5 Bone marrow leukocyte morphology. Gr-1^+ Ly5.1^+ (wild-type) or Ly5.2^+ (*CXCR2*$^{-/-}$) bone marrow cells were isolated using a Reflection high speed cell sorter (i-Cyt, Champaign, IL). Manual leukocyte differentials were performed in a blinded fashion on Wright-stained cytospins prepared by cytocentrifugation of 200,000 sorted cells at 400 rpm for 4 minutes. Photomicrographs were acquired using an E plan 100x/1.25 NA oil immersion objective, Microphot SA microscope, Digital Sight DS-Fi1 camera, and NIS-Elements F2.30 software, all from Nikon (Melville, NY) and

conversion from color to black and white was done using Photoshop CS3 (Adobe Systems, San Jose, CA) .

3.3.6 Colony-forming cell assay. Lineage^{low} Ly5.1^{+} (wild-type) or Ly5.2^{+} (*CXCR2*$^{-/-}$) bone marrow cells were isolated using a Reflection high speed cell sorter, and 350 (CFU-C) or 3,000 (CFU-G) sorted cells were plated in 2.75 mL methylcellulose media supplemented with a cocktail of recombinant cytokines or 10 ng/mL recombinant human G-CSF (MethoCult 3434 or 3231, respectively; Stemcell Technologies, Vancouver, BC). Duplicate cultures were incubated at 37°C for 14 days, after which colonies containing at least 100 cells were counted in a blinded fashion.

3.3.7 Neutrophil mobilization. Recombinant human G-CSF was administered by twice daily subcutaneous injection at a dose of 125 μg/kg/day for 5 days. Mice were analyzed 3-4 hours after the final injection on day 5. AMD3100 (Sigma, Saint Louis, MO) or recombinant human GROβ, was administered as a single subcutaneous injection at doses of 5 mg/kg and 100 μg/kg, respectively. Peripheral blood was obtained within 90 minutes prior to and 1-2 hours after the first injection.

3.3.8 Stromal cell chemokine expression profiling. Groups of 10 Col2.3-GFP or wild-type mice were either left untreated or given G-CSF or vehicle for 7 days as indicated. To isolate stromal cells, femurs were serially digested with collagenase (Worthington Biochemical, Lakewood, NJ) as previously reported (41, 42). Stromal cells were then

incubated with APC-CD45, APC-Ter119, and PE-CD31 (PECAM-1). 7-Amino-Actinomycin D (7AAD) was used to exclude non-viable cells. An average of 20,000 cells was sorted directly into TRIZOL LS reagent (Invitrogen, Carlsbad, CA), and RNA was isolated according to the manufacturer's instructions. RNA was amplified, processed, and hybridized to Affymetrix (Santa Clara, CA) MOE430v2.0 GeneChip microarrays per protocols of the Siteman Cancer Center Multiplexed Gene Analysis Core Facility (Saint Louis, MO, http://pathology.wustl.edu/research/multiplexed.php). After passing quality control, array data was normalized, annotated, and deposited at the Siteman Cancer Center Bioinformatics Core Facility (http://bioinformatics.wustl.edu) according to standard protocols. Expression data for all known chemokines, obtained from at least 4 independent cell sorting experiments, was analyzed and compared using 2-way ANOVA.

3.3.9 CXCL2 ELISA. Quantification of CXCL2 protein in bone marrow extracellular fluid was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit from Peprotech (Rocky Hill, NJ) according to the manufacturer's instructions.

3.3.10 Statistical analysis. Significance was determined using Prism software (Graphpad, La Jolla, CA) to perform two-tailed Student *t* tests assuming equal variance or, where indicated, 1- or 2-way ANOVAs with Bonferroni post-testing. All data are presented as the mean ± SEM.

3.4 Results

3.3.1 Absence of CXCR2 results in abnormal retention of neutrophils in the bone marrow.

Consistent with previous reports (34, 37), we observed marked neutrophilia in *CXCR2*$^{-/-}$ mice maintained under SPF conditions (absolute neutrophil count: $4.63 \pm 1.58 \times 10^9$/L compared with $0.69 \pm 0.06 \times 10^9$/L for congenic wild-type mice, $P = 0.04$). To determine whether this phenotype was due to a cell intrinsic effect of a loss of CXCR2 signaling, mixed bone marrow chimeras were generating by transplanting a 1:1 ratio of wild-type and *CXCR2*$^{-/-}$ bone marrow cells into irradiated congenic mice (Figure 3-1A). Blood neutrophil counts in the *CXCR2*$^{-/-}$ mixed chimeras were reduced in comparison to mice reconstituted with wild-type cells alone ($1.08 \pm 0.08 \times 10^9$/L and $1.81 \pm 0.29 \times 10^9$/L, respectively; $P = 0.003$), suggesting that the neutrophilia in *CXCR2*$^{-/-}$ mice is the result of a cell-extrinsic mechanism.

In the mixed chimeras, the number of *CXCR2*$^{-/-}$ neutrophils in the blood was reduced compared to that in the bone marrow (Figure 3-1B, C). Whereas $65.3 \pm 7.6\%$ of neutrophils in the bone marrow were derived from *CXCR2*$^{-/-}$ cells, only $25.0 \pm 3.5\%$ of neutrophils in the blood were from *CXCR2*$^{-/-}$ cells ($P < 0.0001$). Of note, the number of neutrophils in the spleen, another reservoir for neutrophils, was comparable between *CXCR2*$^{-/-}$ and wild type cells (Figure 3-1C). Neutrophil trafficking from the bone marrow was estimated by calculating the percentage of neutrophils in the blood relative to the total number of neutrophils in the blood, bone marrow, and spleen (neutrophil distribution index or NDI) (5). Consistent with previous studies (22, 43), under basal

conditions 1.84 ± 0.32 % of wild-type neutrophils were present in the blood (Figure 3-1D). In contrast, the percentage of *CXCR2*$^{-/-}$ neutrophils in the blood was only 0.57 ± 0.18% ($P = 0.02$). No perturbation in other hematopoietic lineages was observed (Figure 1E). The number and cytokine responsiveness of myeloid progenitors in the bone marrow were comparable between wild-type and *CXCR2*$^{-/-}$ cells (Figure 3-1F).

Myelokathexis is characterized by the accumulation of mature, often hypersegmented or dysplastic, neutrophils in the bone marrow (10). Consistent with this phenotype, we observed that the percentage of Gr-1^{hi} SSC^{hi} cells, representing the most mature neutrophils (44), relative to the total Gr-1^{+} myeloid cell population was higher for *CXCR2*$^{-/-}$ cells compared with wild-type cells (Figure 3-2A, B). To confirm this finding, *CXCR2*$^{-/-}$ and wild-type Gr-1^{+} myeloid cells were sorted from the bone marrow of the mixed chimeras, and manual leukocyte differentials were performed. *CXCR2*$^{-/-}$ cells showed an increase in the proportion of highly segmented, occasionally dysplastic appearing, neutrophils (Figure 3-2C, D). Collectively, these data show that CXCR2 deficiency results in a myelokathexis-like phenotype with a cell-intrinsic retention of neutrophils in the bone marrow.

3.4.2 Neutrophil mobilization by G-CSF is impaired in the absence of CXCR2.

Since it is the principal cytokine regulating emergency granulopoiesis (45), we next measured the short-term (1-2 hour) and long-term (5 day) neutrophil responses to G-CSF in the *CXCR2*$^{-/-}$ mixed chimeras. Consistent with previous reports (5, 22), administration of G-CSF resulted in a 2.3 ± 0.5-fold increase in the blood of wild-type neutrophils

within two hours (Figure 3-3A). In contrast, there was no significant increase in *CXCR2*$^{-/-}$ neutrophils. After the full 5-day course of G-CSF, a significant increase in both wild-type and *CXCR2*$^{-/-}$ neutrophils in the blood and spleen was observed in the mixed chimeras (Figure 3-3B, C). Neutrophil release, as measured by the NDI, increased in response to 5 day G-CSF treatment in both genotypes (compare Figure 3-1D with Figure 3-3D). However, the percentage of *CXCR2*$^{-/-}$ neutrophils in the blood after G-CSF administration was still significantly lower than that for wild-type cells. These data show that maximal blood neutrophil responses to G-CSF require CXCR2 signaling.

3.4.3 CXCR2 antagonistically regulates CXCR4-mediated neutrophil retention in the bone marrow.

Previous studies have established a dominant role for CXCR4 signals in the retention of neutrophils in the bone marrow (20, 22, 46-50). Since CXCR2 signaling has been shown to regulate CXCR4 cell surface expression through heterologous desensitization and receptor internalization (48, 51), we first assessed CXCR4 expression on *CXCR2*$^{-/-}$ neutrophils. However, cell-surface expression of CXCR4 on bone marrow neutrophils was similar between wild-type and *CXCR2*$^{-/-}$ cells (MFI: 107 ± 7 vs. 100 ± 12, respectively; $P = 0.4$; Figure 3-4A), arguing against a simple mechanism in which the absence of CXCR2 signals results in neutrophil retention through increased CXCR4 expression.

To more directly assess the relationship between CXCR2 and CXCR4 signals in the regulation of neutrophil trafficking, we treated mixed chimeras with AMD3100, a

small molecule CXCR4 antagonist. One hour after administration of AMD3100, a 3.8 ± 1.2-fold increase in wild-type neutrophils in the blood increased was observed (Figure 3-4B). In contrast, no increase in *CXCR2*$^{-/-}$ neutrophils in the blood was observed, despite the fact that the majority of neutrophils in the bone marrow 1 hour after AMD3100 administration were of *CXCR2*$^{-/-}$ origin (Figure 3-4B, C). Accordingly, the NDI for *CXCR2*$^{-/-}$ cells following AMD3100 administration (0.53 ± 0.21%) was dramatically lower than that of wild-type cells (13.0 ± 3.27%; $P = 0.02$; Figure 3-4D), suggesting that neutrophil mobilization in response to transient CXCR4 inhibition is dependent on CXCR2.

We previously reported that mice carrying a myeloid-specific deletion of CXCR4 (MKO; *LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$) displayed marked basal neutrophilia (22). To study the genetic interaction of the *CXCR2*- and *CXCR4*-null alleles, we crossed *CXCR4*-MKO mice with the *CXCR2*$^{-/-}$ mice to generate double knockout mice (DKO; *LysM*$^{Cre/+}$ *CXCR2*$^{-/-}$ *CXCR4*$^{flox/-}$). Similar to the *CXCR2*$^{-/-}$ or *CXCR4*-MKO mice, DKO mice displayed marked neutrophilia at baseline (data not shown). To examine the cell-intrinsic properties of neutrophils lacking both CXCR2 and CXCR4, mixed chimeras were generated as before using either DKO or, as a control, *CXCR4*-MKO bone marrow cells. Recipient mice showed the expected level of donor engraftment in the bone marrow, with 52.1 ± 4.8% (DKO) or 62.2 ± 2.3% (*CXCR4*-MKO) of B lymphocytes derived from mutant cells. As expected, mixed chimeras containing *CXCR4*-MKO cells showed a marked redistribution of *CXCR4*$^{-/-}$ neutrophils into the blood (Figure 3-4E, F). Surprisingly, a similar phenotype was observed in DKO chimeras, showing that loss of

CXCR2 signals cannot rescue the neutrophilic phenotype of CXCR4-deficient neutrophils.

To examine whether CXCR4 signals are required for mobilization by CXCR2 ligands, *CXCR4*-MKO mixed chimeras were given a single injection of the CXCR2 agonist GROβ (CXCL2). Whereas the number of wild-type neutrophils in the blood of *CXCR4*-MKO chimeras increased 4.0 ± 0.4-fold 1 hour after GROβ administration, only a minimal increase in *CXCR4*$^{-/-}$ neutrophils was observed, suggesting that neutrophil release induced by CXCR2 activation is dependent on CXCR4 (Figure 3-4G). Collectively, these data show that CXCR4 and CXCR2 antagonistically regulate neutrophil release from the bone marrow, with CXCR4 playing a dominant role.

3.4.4 Expression of chemokines by osteoblasts and endothelial cells in the bone marrow.

Previous studies have established that bone marrow stromal cells, in particular osteoblasts and endothelial cells, are the major source of CXCL12 in the bone marrow (21, 25, 27, 42, 50, 52-54). However, the expression of other chemokines, specifically ELR^{+} CXCR2 ligands, in bone marrow stromal cells is unknown. To address this issue, we analyzed stromal cells from the bone marrow of transgenic mice expressing GFP in osteoblast lineage cells (Col2.3:GFP) (39, 42). Specifically, CD45^{low} Ter119^{low} stromal cells were sorted into osteoblast (GFP^{+}) and endothelial (CD31^{+}) fractions, which were then subjected to RNA expression profiling (Figure 3-5A). Of note, expression of endothelial- or osteoblast-specific genes was appropriately enriched in the relevant cell fraction,

demonstrating the fidelity of our sorting strategy (Tables 3-1 and 3-2). As reported previously (27, 42), constitutively high expression of CXCL12 was observed in osteoblasts and endothelial cells, with higher expression in osteoblasts (Figure 3-5B). The CXCR2 ligands, CXCL1 (KC) and CXCL2 (MIP-2) also were constitutively expressed in osteoblasts and endothelial cells, but with higher endothelial expression.

To examine the effect of G-CSF on chemokine expression in the bone marrow microenvironment, endothelial cells were isolated from the bone marrow after G-CSF administration. Osteoblasts were not sorted, since their number is markedly reduced by G-CSF (27, 41, 42, 53). Of note, there was no change in bone marrow endothelial cell number (P.K.G., Christopher, M.J., A.M.G., van Os, R., D.C.L., manuscript in preparation). RNA expression profiling showed that CXCL2 expression in bone marrow endothelial cells was induced 2.7 ± 0.3- fold by G-CSF, while CXCL12 mRNA was modestly reduced to 47 ± 3% of its basal level; other chemokines remained unchanged (Figure 3-5C). Consistent with the mRNA data, CXCL2 protein was detected in the bone marrow supernatant at baseline with a trend to increased expression after G-CSF administration (Figure 3-5D). Since osteoblast number is markedly reduced following G-CSF administration, these data suggest that the balance of expression in the bone marrow from pro-retention (CXCL12) to mobilizing chemokines (CXCL1 and CXCL2) may contribute to neutrophil mobilization by G-CSF.

3.5 Discussion

In the present study, we generated *CXCR2*$^{-/-}$ mixed bone marrow chimeras to characterize the cell-intrinsic effect of CXCR2 deletion on neutrophil trafficking from the bone marrow. We show that mature, occasionally hypersegmented, *CXCR2*$^{-/-}$ neutrophils, are selectively retained in the bone marrow, reproducing a myelokathexis phenotype. In contrast, *CXCR2*$^{-/-}$ mice display neutrophilia, suggesting augmented neutrophil production and/or release. Studies of leukocyte adhesion deficiency (LAD) mice provide a potential explanation for these discrepant results. Similar to *CXCR2*$^{-/-}$ mice, β_2-integrin-deficient mice (*ITGB2*$^{-/-}$), which reproduce LAD type I in humans, exhibit impaired emigration of neutrophils from the circulation to peripheral tissues and neutrophilia when housed under SPF conditions (34, 55). In a series of elegant experiments, Forlow et al (56) and Stark et al (57) showed that, in *ITGB2*$^{-/-}$ mixed chimeras, as little as 10% wild-type neutrophils was sufficient to restore normal neutrophil homeostasis. Moreover, they showed that neutrophil emigration into peripheral tissues initiated a negative feedback loop that suppresses IL-17 and G-CSF production. Thus, the emigration defect shared by *CXCR2*$^{-/-}$ and *ITGB2*$^{-/-}$ neutrophils may disrupt this negative feedback loop and lead to the production of stress cytokines that stimulate granulopoiesis. Consistent with this possibility, serum levels of IL-6 are elevated in both *CXCR2*$^{-/-}$ and *ITGB2*$^{-/-}$ mice (34, 55).

Our results indicate that CXCR2 and CXCR4 coordinately regulate neutrophil trafficking from the bone marrow. Previous studies have demonstrated that treatment of neutrophils with CXCR2 ligands results in impaired CXCR4 signaling through

heterologous desensitization and/or internalization (48, 51), suggesting that CXCR2 regulates neutrophil mobilization through modulation of CXCR4 signaling and/or expression. Consistent with this possibility, Martin et al (47) and others (48, 49) previously showed that neutrophil mobilization by the CXCR2 chemokine CXCL1 (KC) was markedly increased by transient CXCR4 inhibition. Conversely, *CXCR2*$^{-/-}$ neutrophils displayed almost no mobilization in response to transient CXCR4 inhibition (Figure 3-4B-D). This surprising result suggests at least two possibilities: 1) loss of CXCR2 may augment basal CXCR4 signaling, thereby rendering cells resistant to AMD3100; 2) CXCR2 signals may function independently of CXCR4 to direct neutrophil release following AMD3100-induced CXCR4 blockade. In support of the first possibility, doubly deficient *CXCR2*$^{-/-}$ *CXCR4*$^{-/-}$ neutrophils display constitutive mobilization (Figure 3-4E, F), demonstrating that CXCR2 signals are not required for neutrophil mobilization in the complete absence of CXCR4 signals and suggesting a dominant role for CXCR4 in the regulation of neutrophil trafficking from the bone marrow. Consistent with this conclusion, the present study (Figure 3-4G) and our previous report (22) show that neutrophil mobilization by the CXCR2 chemokine CXCL2 (GROβ) is impaired in the complete absence of CXCR4 signals (i.e., in *CXCR4*$^{-/-}$ neutrophils). Collectively, these data suggest that CXCR4 and CXCR2 signaling antagonistically regulate neutrophil release from the bone marrow.

Previous studies have established that CXC12 is constitutively expressed by bone marrow stromal cells, including osteoblasts and endothelial cells (21, 25, 27, 42, 50, 52-54). Quantitative analysis of sorted stromal cell populations suggested that osteoblasts

are the major source of CXCL12 in the bone marrow (27). Consistent with these findings, our RNA expression profiling of sorted bone marrow endothelial cells or osteoblasts demonstrated significantly higher expression of CXCL12 mRNA in osteoblasts. The expression profiling data also showed that ELR^+ CXC chemokines CXCL1, CXCL2, and CXCL7 are constitutively expressed in bone marrow endothelial cells and/or osteoblasts. However, in contrast to CXCL12, these chemokines are expressed at higher levels in endothelial cells. Together, these data suggest a "tug-of-war" model wherein endothelial-derived chemokines (primarily CXCR2 ligands) direct neutrophil chemotaxis toward the vasculature for entry into the circulation, while endosteal osteoblasts produce chemokines (primarily CXCL12) that promote neutrophil retention (Figure 6). Under basal conditions, the balance of chemokine production favors neutrophil retention in the bone marrow. Under stress conditions, expression of inflammatory cytokines, most notably G-CSF, is increased (58). We and others previously showed that G-CSF administration is associated with a marked suppression of endosteal osteoblasts (27, 41, 42, 53). In addition, herein we show that CXCL2 expression is increased in bone marrow endothelial cells after G-CSF administration. The net effect is a shift in the balance of chemokine production to the endothelium, thereby promoting neutrophil release from the bone marrow.

In summary, this study provides evidence that ELR^+ CXCR2 ligands are a second chemokine family that, together with CXCL12, controls neutrophil trafficking from the bone marrow. Although most cases of WS are associated with autosomal dominant, gain-of-function mutations in *CXCR4,* several pedigrees have been reported that lack the

characteristic mutations (9, 11, 14). Balabanian and colleagues reported a WS pedigree with decreased expression of GPCR kinase-3 (GRK3), a protein that negatively regulates CXCR4 signaling (59). Recently, homozygous, loss-of-function mutations in *CXCR2* have been identified in a pedigree with isolated myelokathexis (George A. Diaz, personal communication). Our data provide experimental evidence that loss-of-function mutations in *CXCR2* are sufficient to induce a myelokathexis phenotype in mice. Of potential clinical relevance for the treatment of patients with myelokathexis and *CXCR2* mutations, our studies of *CXCR2*$^{-/-}$ neutrophils suggest that mobilization responses to AMD3100 or G-CSF may be impaired.

3.7 References

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3.8 Tables

Table 3-1. Endothelial markers in sorted cells.

Endothelial Genes		Signal Intensity		
Common Name(s)	Gene Symbol	CD31+ Endothelial Cells	GFP+ Osteoblasts	Fold Change
CD34 antigen	Cd34	6,395 ± 1,121	757 ± 447	8.4 ± 1.5
VE-cadherin, CD144	cdh5	15,005 ± 1,561	393 ± 98	38.1 ± 4.0
Endoglin, CD105	Eng	13,849 ± 1,679	1,784 ± 392	7.8 ± 0.9
VEGFR-1	Flt1	11,349 ± 1,542	728 ± 363	15.6 ± 2.1
VEGFR-2	Kdr	16,756 ± 1,864	954 ± 133	17.6 ± 2.0
PECAM-1, CD31	Pecam1	866 ± 325	Abs	NA
MECA-32	Plvap	13,663 ± 1,875	2,821 ± 1,075	4.8 ± 0.7
E-selectin	Sele	753 ± 252	65 ± 28	11.6 ± 3.9
P-selectin	Selp	3,683 ± 1,094	498 ± 121	7.4 ± 2.2
TIE-2	Tek	6,950 ± 792	443 ± 91	15.7 ± 1.8
VCAM-1	Vcam1	11,963 ± 2,782	8,548 ± 1,928	1.4 ± 0.3
VonWillebrand factor	Vwf	11,074 ± 4,690	424 ± 276	26.1 ± 11.1

Abs, gene called absent on chip. NA, not applicable.

Table 3-2. Osteoblast markers in sorted cells.

Osteoblast Genes		Signal Intensity		
Common Name	Gene Symbol	GFP+ Osteoblasts	$CD31^+$ Endothelial Cells	Fold Change
Alkaline phosphatase	Alpl	10,448 ± 1,525	2,109 ± 393	5.0 ± 0.7
Type I collagen	Col1a1	40,881 ± 12,479	26,788 ± 6,605	1.5 ± 0.5
Bone sialoprotein (Bsp)	Ibsp	28,787 ± 12,877	10,966 ± 5,255	2.6 ± 1.2
Osteoglycin, mimecan	Ogn	13,847 ± 1,401	251 ± 78	55.3 ± 5.6
Osteomodulin, osteoadherin	Omd	10,020 ± 2,687	600 ± 357	16.7 ± 4.5
Periostin	Postn	38,346 ± 8,306	1,575 ± 721	24.4 ± 5.3
Runt-related transcription factor 2	Runx2	8,209 ± 3,784	1,482 ± 368	5.5 ± 2.6
Osterix (Osx)	Sp7	3,870 ± 2,083	1,640 ± 877	2.4 ± 1.3

3.9 Figures and figure legends

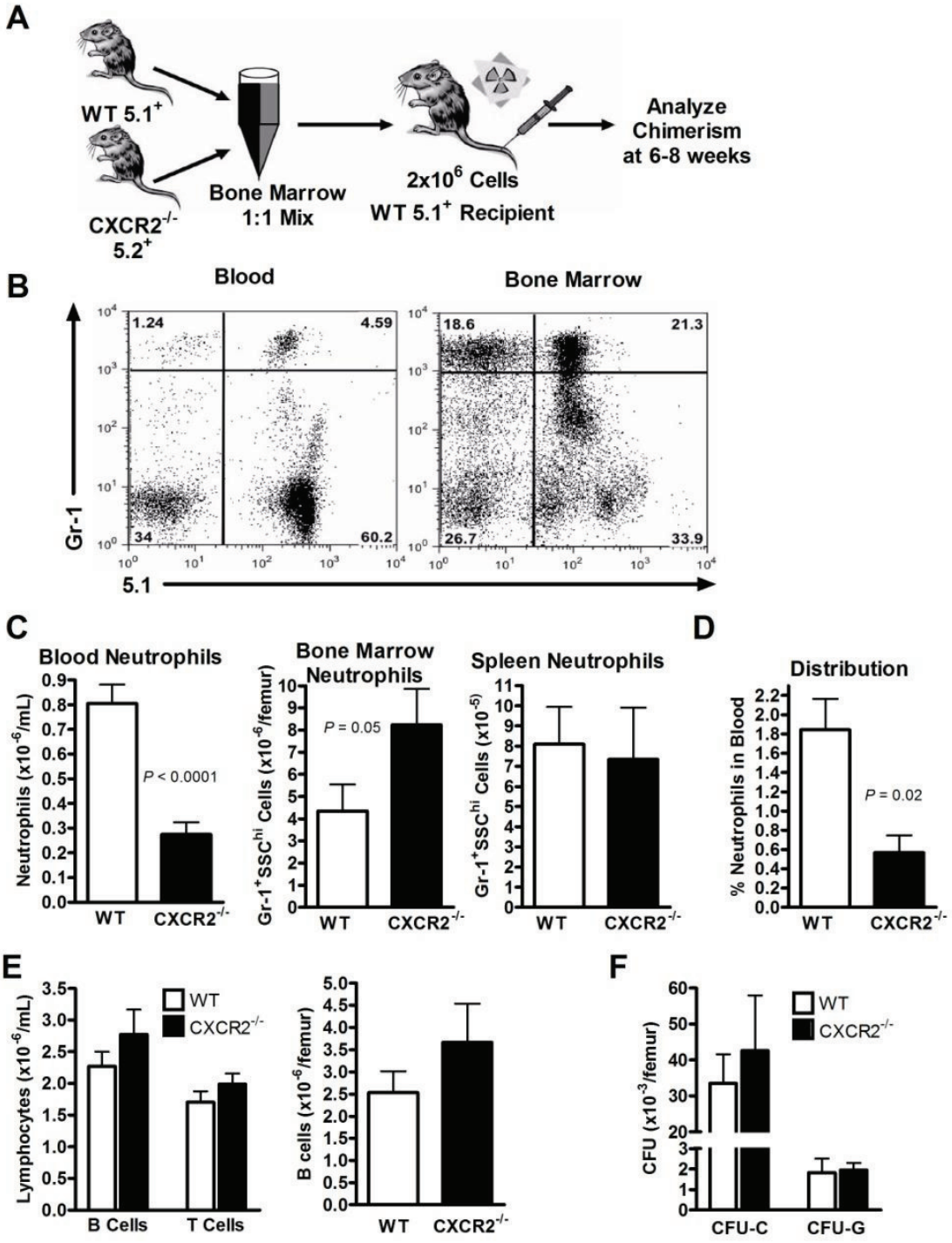

Figure 3-1. Legend on next page.

Figure 3-1. CXCR2$^{-/-}$ neutrophils are selectively retained in the bone marrow of mixed chimeras. (A) Generation of mixed chimeras: bone marrow cells from wild-type (WT) *Ly5.1*$^{+}$ and *CXCR2*$^{-/-}$ *Ly5.2*$^{+}$ mice (1 x 10^{6} cells from each) were mixed in a 1:1 ratio and transplanted into lethally irradiated congenic wild-type (*Ly5.1*$^{+}$) recipients. Mice were analyzed 6-8 weeks after transplantation. (B) Representative dot plots showing the contribution of wild-type (*Ly5.1*$^{+}$) and *CXCR2*$^{-/-}$ (*Ly5.1*$^{-}$) cells to neutrophils (Gr-I^{hi}) in the blood and bone marrow. (C) Quantitation of mature neutrophils (Gr-1^{hi}SSC^{hi}) in the blood, bone marrow, and spleen. (D) The neutrophil distribution index (NDI) was calculated to estimate the percentage of total body neutrophils in the blood using the following formula: NDI = blood neutrophils/(blood + bone marrow + spleen neutrophils). (E) Shown is the number of B lymphocytes (B220^{+}) or T lymphocytes (CD3^{+}) in the blood (left panel) and B lymphocytes in the bone marrow (right panel). T lymphocyte chimerism was assessed 6 months after transplantation (n = 3). (F) Shown is the number of wild-type or *CXCR2*$^{-/-}$ CFU-C or CFU-G in the bone marrow (n = 3). Unless otherwise noted, data represent the mean ± SEM of n = 27 (blood) or n = 6 (bone marrow and spleen) mice from at least 3 independent transplantations.

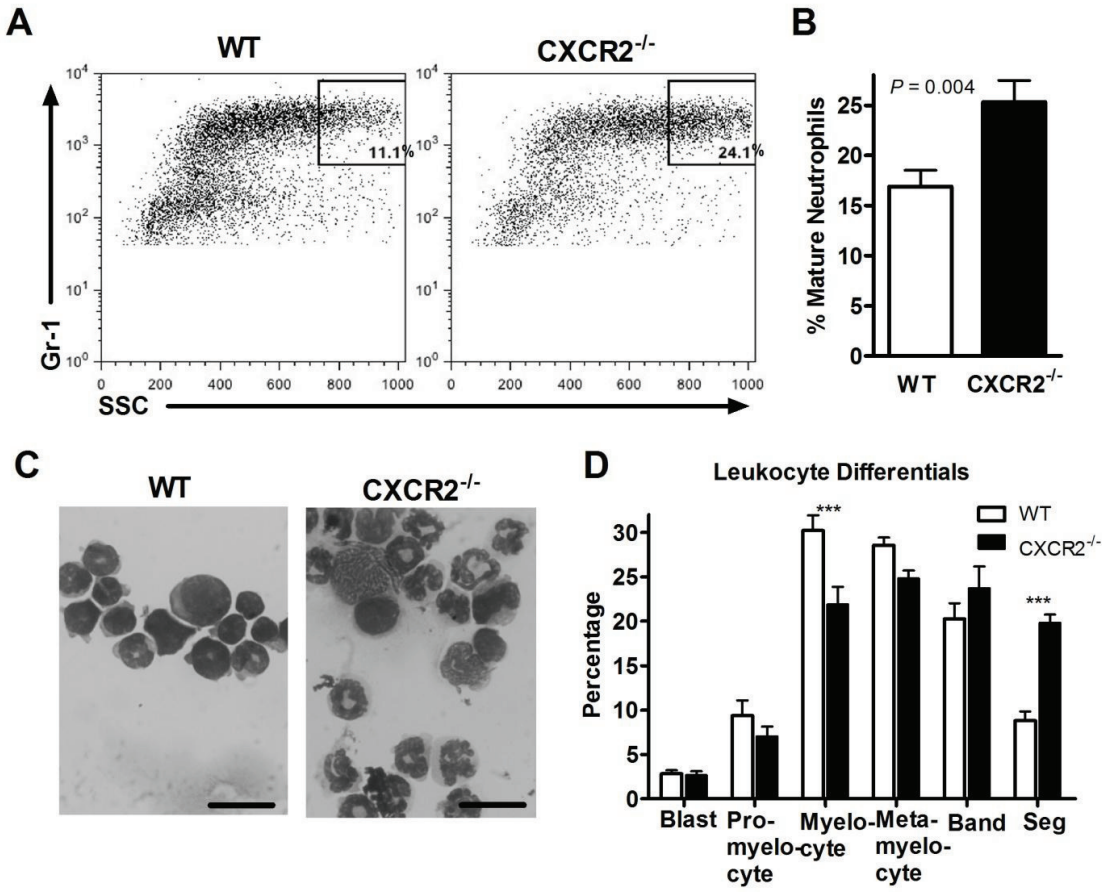

Figure 3-2. CXCR2 deficiency produces a myelokathexis-like phenotype.
(A) Representative dot plots of mixed chimera bone marrow showing the percentage of Gr-1^{hi} SSC^{hi} cells within the total Gr-1^{+} myeloid cell population for wild-type (WT, left panel) or *CXCR2*$^{-/-}$ cells (right panel). (B) Shown is the percentage of Gr-1^{hi} SSC^{hi} cells within the total Gr-1^{+} myeloid cell population for n = 7 chimeric mice from 2 independent transplants. (C) Representative photomicrographs of sorted wild-type (left panel) or *CXCR2*$^{-/-}$ Gr-1^{+} cells (right panel). Scale bar = 20 μm. (D) Manual leukocyte differentials of sorted cells from n = 5 mice from 2 transplants. The data represent the mean ± SEM. ***P < 0.001, 2-way ANOVA.

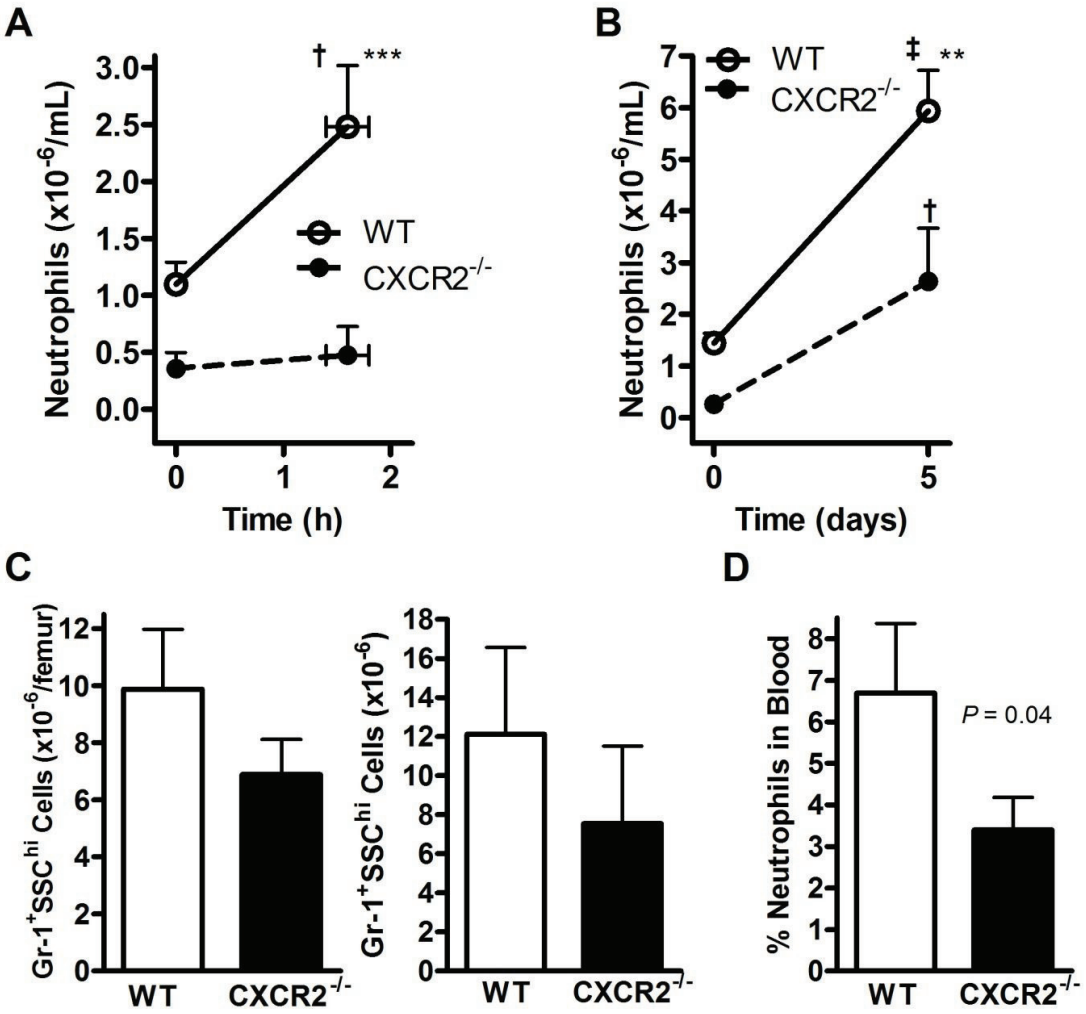

Figure 3-3. Mobilization of CXCR2$^{-/-}$ neutrophils by G-CSF is impaired. (A) Mixed chimeras (n = 5) were given a single injection of G-CSF (125 µg/kg) and the absolute neutrophil count for each genotype was determined 1.5 hours after injection. (B) G-CSF (125 µg/kg per day, twice daily) was administered to a separate cohort of n = 5 chimeric mice for 5 days, and blood neutrophils were quantified. (C) The number of wild-type or *CXCR2*$^{-/-}$ Gr-1^{+}SSC^{hi} cells in the bone marrow (left panel) or spleen (right panel) after 5 days of G-CSF administration. (D) The calculated neutrophil distribution index after 5 days of G-CSF. The data represent the mean ± SEM. †$P < 0.05$, ‡$P < 0.01$ compared with time 0; **$P < 0.01$, ***$P < 0.001$ compared with *CXCR2*$^{-/-}$ cells at the same time point, 2-way ANOVA.

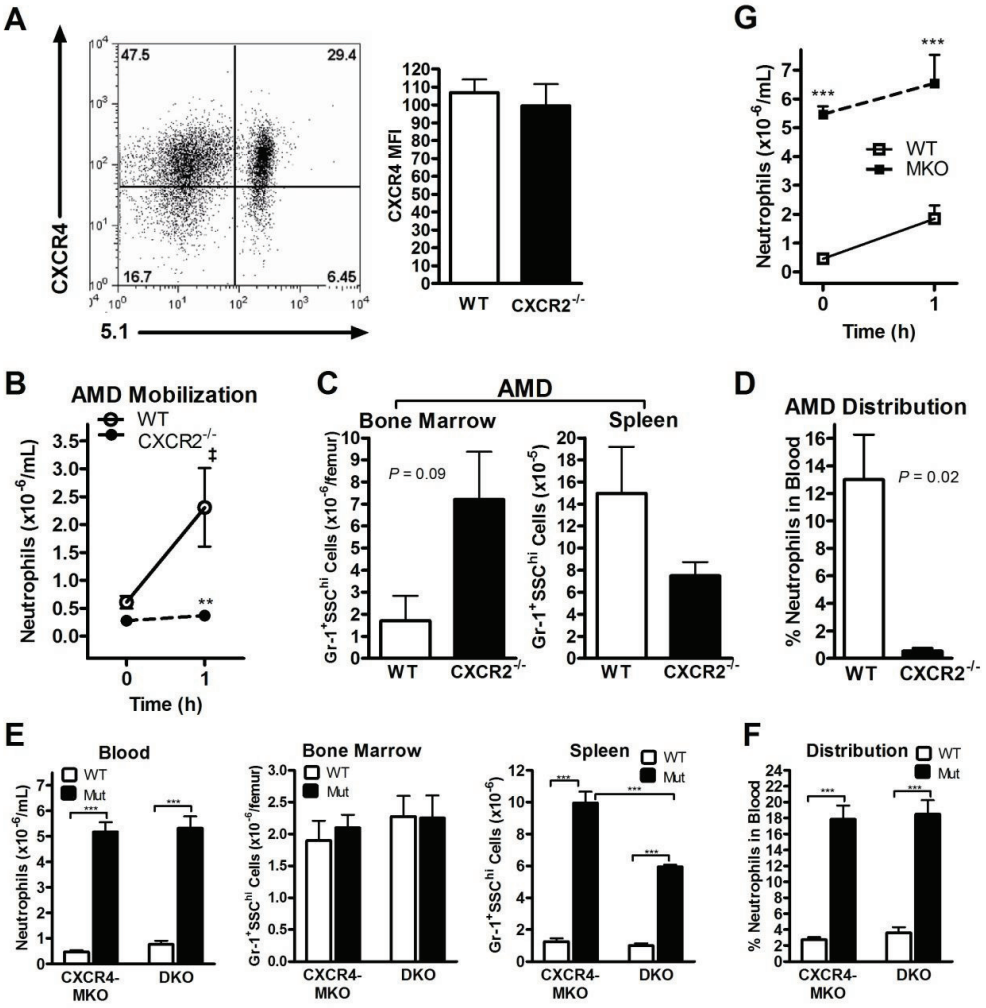

Figure 3-4. CXCR2 and CXCR4 signals interact antagonistically to regulate neutrophil release. (A) Representative dot plot (left panel) or the MFI of n = 5 mixed chimeras (right panel) showing cell-surface CXCR4 expression of wild-type (Ly5.1^{+}) and *CXCR2*$^{-/-}$ (Ly5.1^{-}) bone marrow cells gated on the Gr-1^{+}SSC^{hi} population. (B) *CXCR2*$^{-/-}$ mixed chimeras (n = 5) were given a single subcutaneous injection of AMD3100 (5 mg/kg) and neutrophils quantified at the indicated time. (C,D) Number of neutrophils in the bone marrow and spleen and neutrophil distribution index at 1 hour after AMD3100 administration (n = 3). (E,F) *LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$ (MKO, n = 10) and *LysM*$^{Cre/+}$ *CXCR4*$^{flox/-}$ *CXCR2*$^{-/-}$ double knockout (DKO, n = 4) mixed chimeras were established as described in Figure 1. Blood, bone marrow and spleen neutrophils and the neutrophil distribution index were quantified 7 weeks after transplantation. (G) *CXCR4*-MKO mixed chimeras (n = 3) were given a subcutaneous injection of GROβ (100μg/kg), and the number of wild-type and *CXCR4*$^{-/-}$ neutrophils in the blood measured after one hour. Data represent the mean ± SEM. ***P < 0.001, 1-way ANOVA (panels e and f). ‡P < 0.01 compared with time 0; **P < 0.01, ***P < 0.001 compared with wild-type cells at the same time point, 2-way ANOVA (panels b and g).

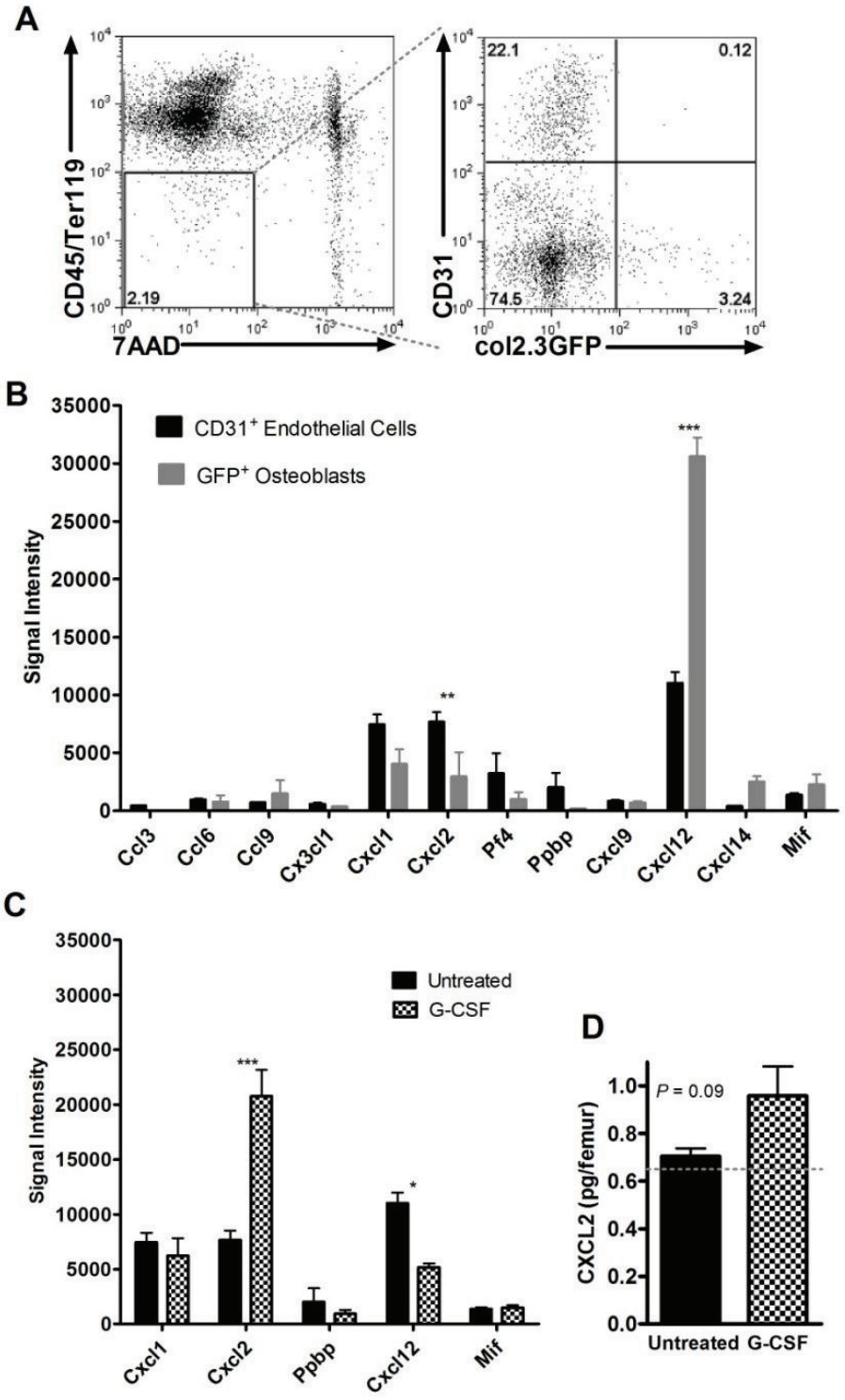

Figure 3-5. Legend on next page.

Figure 3-5. CXCR2 ligands are produced by bone marrow stromal cells and regulated by G-CSF. (A) Bone marrow endothelial cells ($7AAD^{-}$ $CD45^{low}$ $Ter119^{low}$ $CD31^{+}$) or osteoblasts ($7AAD^{-}$ $CD45^{low}$ $Ter119^{low}$ GFP^{+}) were isolated by cell sorting from Col2.3:GFP transgenic mice. Shown are representative dot plots depicting the sorting strategy. (B) Shown is the normalized gene chip signal at baseline for all chemokines with an average signal intensity of greater than 400 in at least one of the cell types. Where more than one probe set existed, the highest signal was selected. *Pf4* encodes for CXCL4, *Ppbp* encodes for CXCL7, and Mif is a non-chemokine ligand for CXCR2 and CXCR4 (60). (C) Expression of CXCR2 and CXCR4 ligands in endothelial cells from wild-type mice at baseline or after G-CSF administration. (D) CXCL2 protein in bone marrow supernatant at baseline or after G-CSF was measured by ELISA (n = 4 mice per group). The dashed line represents the limit of detection for the assay. Data represent the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, 2-way ANOVA.

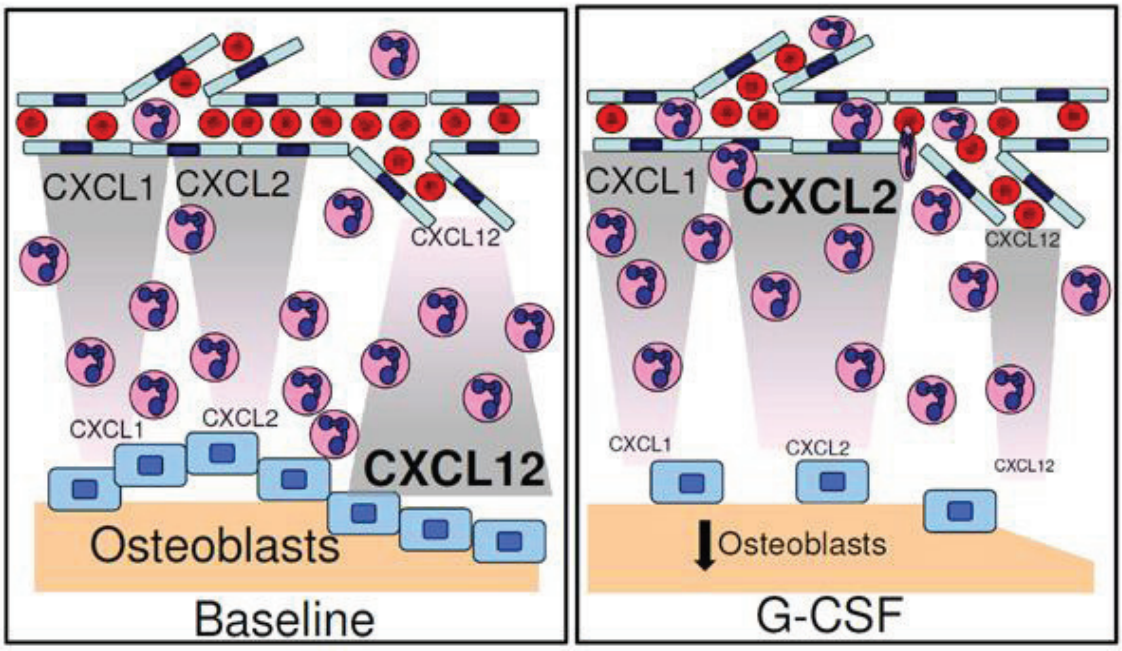

Figure 3-6. Tug-of-war model of neutrophil trafficking from the bone marrow. See text for details.

Chapter 4

Summary and future directions

4.1 Summary

A broad array of diseases are associated with dysregulation of the inflammatory response, and neutrophils are a key initiating component of this response. Therefore, it is vital that organisms maintain precise control of circulating neutrophil numbers to ensure adequate protection against pathogens while minimizing damage to host tissue. Blood neutrophil homeostasis is determined by the integrated control of neutrophil precursor proliferation and differentiation in the bone marrow, release of mature neutrophils from the bone marrow, and clearance of aged, senescent neutrophils from the circulation by margination, transmigration across the endothelium, apoptosis, and phagocytosis. Equally important is the ability to rapidly alter homeostatic levels of these processes in order to mount an effective neutrophil response to infection or other stresses.

This thesis has increased our understanding of the role of the neutrophil chemokine receptors CXCR2 and CXCR4 in the regulation of neutrophil homeostasis. To study chemokine signaling in neutrophils, we used cells genetically deficient for CXCR2, CXCR4, or both receptors. Our goals were: 1) to characterize the role of CXCR2 and CXCR4 signaling in basal and stress granulopoiesis. 2) to characterize the role of CXCR2 and CXCR4 signaling in neutrophil clearance. 3) to define the nature of the interaction (if any) between CXCR2 and CXCR4 in the regulation of neutrophil homeostasis. 4) to identify the chemokines produced in the bone marrow, their cellular source, and how they are regulated.

Our results show that CXCR4 is a dominant, common pathway regulating neutrophil release from the bone marrow under basal and stress conditions, but it is not

essential for neutrophil clearance from the circulation **(Chapter 2)**. CXCR2 opposes CXCR4 in the regulation of neutrophil release from the bone marrow. It is required for egress into the tissues and proper sensing of neutrophils by tissue phagocytes, but cell-intrinsic CXCR2 signals play a minor role, if any, in the regulation of neutrophil production **(Chapter 3)**. CXCR2 and CXCR4 ligands are highly produced by bone marrow endothelial cells and osteoblasts. Their expression pattern and regulation by G-CSF suggests a tug-of-war model where the balance of pro-retention chemokines produced by osteoblasts and mobilizing chemokines produced by the endothelium regulates the level of neutrophil release from the bone marrow **(Chapter 3)**. Our data in genetic knockout mice supports a causative role for CXCR2 and CXCR4 mutations in the pathogenesis of the human disease WHIM syndrome.

4.1.1 CXCR4 myeloid conditional knockout mice

A significant amount of genetic and pharmacologic data in humans and mice suggested that CXCR4 may be a key retention signal for neutrophils in the bone marrow. Even more intriguing, a series of studies by Dr. Sara Rankin and colleagues had suggested that modulation of CXCR2 and CXCR4 signaling may regulate the return of senescent neutrophils to the bone marrow where they are ultimately cleared by apoptosis and phagocytosis (1, 2). However, the interpretation and analysis of all the existing data was complicated by limited samples from patients with WHIM syndrome, perinatal lethality in CXCR4-deficient mice, the potential confounding effects of CXCR4 signaling in precursor cells or other lineages in the bone marrow, *ex vivo* manipulation and aging of

neutrophils, and the inherent limitations, such as off-target effects or incomplete inhibition, of drugs or antibodies. In **Chapter 2**, we utilized mice with a conditional deletion of *CXCR4* restricted to cells of the myeloid lineage to assess the function of CXCR4 in neutrophils and overcome many of these limitations.

Using these mice, we confirmed that CXCR4 acts as a key retention signal that negatively regulates neutrophil trafficking from the bone marrow. We also show that although CXCR4 mediates homing of circulating neutrophils to the bone marrow, this site is not essential for neutrophil clearance, suggesting that other organs (spleen, liver, lung) can compensate for impaired bone marrow clearance without a requirement for CXCR4.

The role of CXCR4 in neutrophil mobilization in response to infection or stress is not clear. In general, there is evidence both for and against the presence of distinct pathways in basal versus stress granulopoiesis, between various sites of infection, and between different bacterial and fungal pathogens. To answer the question of whether CXCR4 is a final common pathway that mediates both basal and stress granulopoiesis, the neutrophil response of the myeloid conditional CXCR4 knockout mice to several important classes of inflammatory mediators (cytokines, G-CSF; chemokines, CXCL2) and a murine model of lethal bacterial infection (*Listeria monocytogenes)* was measured. The results suggested that disruption of CXCR4 signaling is the mechanism of neutrophil mobilization in response to these diverse classes of stimuli. Of note, consistent with the normal homeostatic clearance of CXCR4-deficient neutrophils, CXCR4 was not required for neutrophil emigration into the peritoneum in response to *L monocytogenes* infection.

4.1.2 CXCR2 chemokines mediate neutrophil release

Having shown that CXCR4 seems to be a master retention signal for neutrophils in the bone marrow, we hypothesized that other signals must exist that oppose CXCR4 and direct the key steps of neutrophil mobilization: disruption of adhesive interactions with the bone marrow extracellular matrix, chemotaxis toward the vasculature, and transmigration across the endothelial barrier into the circulation. The alternative hypothesis is that constitutive mobilization is the default program that occurs by mass action of neutrophil production in the bone marrow and random chemokinesis. Under this model, CXCR4 evolved to limit excessive inflammation and tissue damage from circulating neutrophils. Work presented in **Chapter 3** suggests that CXCR2 may be one such opposing signal.

Given the essential role of CXCL12, it seemed logical that other chemokines might play a role in regulating neutrophil mobilization. CXCR2 chemokines were attractive candidates because of the high expression of CXCR2 on mature neutrophils and the diverse neutrophil functions mediated by CXCR2 signals. Furthermore, CXCR2 ligands were already known to be rapid and potent mobilizing agents. However, the mechanism of their action (activation versus chemotaxis, cell-extrinsic versus cell-intrinsic) and whether they had a more general, endogenous role in basal neutrophil release or neutrophil mobilization by other classes of agents was unclear. At about the same time, mutations in CXCR2 associated with myelokathexis were reported. Together, this data supported the idea that CXCR2 positively regulated neutrophil release from the bone marrow.

Stromal cells in the bone marrow are likely to produce a number of molecules, in addition to CXCL12, that are important in regulating neutrophil trafficking. In particular, endothelial cells are highly transcriptionally active and are known to perform a variety of intercellular signaling functions, including mediation of neutrophil extravasation, in the peripheral circulation. Their function as a physical barrier to the circulatiion predicts that endothelial cells play a key role in regulating neutrophil release. To address these possibilities, we performed a survey of gene expression in osteoblasts and endothelial cells and measured endothelial cell changes in response to G-CSF using gene expression arrays as described in **Chapter 3**. Although additional candidate molecules need to be assessed, an unbiased analysis of gene expression changes using SAM and a curated assessment of all chemokines provided further evidence that the CXCR2 was a key positive regulator of neutrophil release. The CXCR2 chemokines CXCL1, CXCL2, and CXCL7 were highly expressed, with higher expression in endothelial cells and increased CXCL2 expression in endothelial cells after G-CSF.

CXCR2-deficient neutrophils in mixed bone marrow chimeras were used to confirm this potential role for CXCR2 in neutrophil release. As mentioned above, the cause of the myeloid expansion in CXCR2-deficient mice is not clear (3-5). Although further experiments need to be done to confirm these conclusions, our data suggest that the neutrophilia in these mice is caused by an interruption of the neutrostat feedback loop involving suppression of IL-23, IL-17, and G-CSF production by phagocytosis of neutrophils in peripheral tissues. Our results argue against excessive cell-intrinsic proliferation or passive accumulation of CXCR2-deficient neutrophils as an explanation

for the neutrophilia. Alternatively, CXCR2 may interact with other, as yet uncharacterized components of this feedback loop. Specifically, CXCR2 may be important for interactions with endogenous bacteria or clearance of subclinical infections that affect cytokine production and neutrophil homeostasis. The environmental conditions of mice or humans with CXCR2 mutations (germ-free, specific pathogen-free, or the normal range of pathogens in the everyday world), must be considered when making generalizations from observed alterations in neutrophil counts. Finally, CXCR2 signaling in non-hematopoietic cells, particularly endothelial cells, may play a role in regulating neutrophil and lymphocyte homeostasis.

Our results suggest that CXCR2 is a second chemokine pathway that positively regulates neutrophil release. We show that CXCR2-deficient neutrophils reproduce a myelokathexis phenotype consistent with the recently described human mutations. CXCR2 appears to play a role in the stress granulopoiesis response to G-CSF. The data indicate that CXCR2 is more important in regulating the immediate (minutes to hours) release of neutrophils after G-CSF treatment. CXCR2 has a more minor role in neutrophil mobilization after prolonged (days) of G-CSF, probably because of the additional effects of G-CSF-induced proliferation, transcription, and microenvironmental changes in the bone marrow.

CXCR2 and CXCR4 interact antagonistically to regulate neutrophil release from the bone marrow. However, CXCR4 appears to play a dominant role, as neutrophils lacking both CXCR2 and CXCR4 still display constitutive mobilization. The importance of CXCR2 signaling in neutrophil trafficking likely decreases with increased magnitude

or duration of CXCR4 inhibition. Our experiments did not definitively answer whether CXCR2 antagonizes CXCR4 signaling through heterologous interactions. On one hand, we did not observe any alteration in CXCR4 expression in the absence of CXCR2, arguing against heterologous regulation of receptor expression. In contrast, CXCR2 had no effect on neutrophil counts in the complete, genetic absence of CXCR4, consistent with a mechanism for CXCR2 that acts through CXCR4. One such mechanism is heterologous desensitization or internalization.

Chemokine receptors, like many GPCRs, can undergo heterologous regulation of their signals at many different levels. They can homo- or hetero-dimerize or even form higher oligomers. Desensitization or internalization signals generated by one receptor can cross-desensitize or –internalize other related receptors. The regulatory mechanisms in play are highly dependent on the cell type, specific receptor, and environmental mileau. Therefore, additional experiments will be required to determine what, if any, interactions there are between CXCR2 and CXCR4 that are important for the phenotype observed in CXCR2-deficient neutrophils. Of note, heterologous desensitization between CXCR4 and CXCR2 has been demonstrated in *ex vivo* cultured monocytes (6) and neutrophils (7). Wild-type or WHIM-mutant CXCR4 can form dimers at the cell surface in cell lines (8). Recently, our collaborator Dr. George Diaz has demonstrated physical interaction between CXCR4 and CXCR2 (unpublished data) in neutrophils.

Our results show that the integration of CXCR2 and CXCR4 signals by neutrophils in the bone marrow is a key pathway regulating their release. Our data and others suggest that this signaling axis may be important in immunodeficiency, infectious

and inflammatory disease. The CXCR2- and CXCR4-deficient mouse models described in this thesis could be tested in additional models of infection to further investigate this possibility. Drugs that activate or inhibit these receptors should be tested in models of acute (sepsis, ARDS) or chronic (rheumatoid arthritis) inflammatory disease. Indeed, our data suggest that prolonged G-CSF treatment but not AMD3100 treatment would be effective in treating patients with myelokathexis and *CXCR2* mutations. The efficacy of chronic AMD3100 treatment in CXCR2-deficient neutrophils needs to be tested. Conversely, either AMD3100 or G-CSF (the currently used therapy) would be effective in other genotypes of WHIM syndrome.

4.2 Future directions

4.2.1 Expression of chemokines and other molecules by bone marrow stromal cells

The production of CXCL1 and CXCL2 in bone marrow endothelial cells and osteoblasts needs to be confirmed. Several methods are currently being employed to accomplish this goal. Bone marrow stromal cells will be sorted as described in **Chapter 3** and expression of CXCL1, CXCL2, CXCL7, and CXCL12 will be measured by qRT-PCR. We are also currently working to develop a flow cytometry assay to measure CXCL1 and CXCL2 in endothelial cells. Finally, as was done with CXCL12, Adam Greenbaum in our laboratory is working to stain bone marrow histologic sections for CXCL1 or CXCL2 and co-localize these proteins with endothelial cell or osteoblast-specific markers. We will also look at these histologic sections to assess any changes in the overall architecture of the bone marrow after G-CSF administration or inhibition of CXCR4 by AMD3100.

Is CXCR4 inhibition associated with increased amounts of neutrophils near the vasculature or absence of neutrophils near the endosteum? When these assays are developed, we will also measure chemokine expression after shorter courses of G-CSF to try to explain the differences observed in CXCR2-deficient neutrophils after short- versus long-term G-CSF. Alternatively, a GFP reporter construct could be knocked in to the CXCL2 locus and GFP expression measured by flow cytometry or histology to confirm the cell types that express CXCL2 and its regulation by G-CSF.

Although the chemokine expression data supports the tug-of-war model for neutrophil release, the requirement for these chemokines has not been demonstrated. To determine the biological significance of chemokine expression in specific stromal cell subsets, Adam Greenbaum is developing a transgenic mouse system with a floxed CXCL12 allele that will allow for osteoblast- (using OSTERIX-CRE mice) or endothelia- (using TIE2-CRE mice) specific deletion of CXCL12. Our model predicts increased neutrophil release with osteoblast deletion and normal or decreased release with endothelial deletion. If this strategy proves successful, we would pursue a conditional deletion model of CXCL2 and possibly even CXCL1. An alternative but less desirable strategy would be to overexpress these chemokines using transgenes specific for osteoblasts or endothelial cells. These types of models are subject to dysregulated expression and leaky expression outside of the target cell population. Furthermore, some of these approaches have already been reported in the literature (9). Actually, this report supports the tug-of-war model since vascular CXCL12 expression in the absence of osteoblast CXCL12 resulted in decreased myeloid cells in the bone marrow. However, it

is clear from the CXCR4 myeloid conditional knockout mice and our CXCL12 expression data that under normal conditions CXCL12 derived from osteoblasts acts as a retention signal that far outweighs any pro-mobilizing effect of CXCL12 produced by bone marrow endothelial cells.

We will also perform further analysis on our existing gene chip data and expression arrays performed in Dr. Tim Ley's laboratory on neutrophils differentiated *in vitro* (10) to search for additional molecules that may regulate basal or G-CSF induced neutrophil release from the bone marrow.

4.2.2 Mouse models of WHIM syndrome

Retroviral overexpression of WHIM-mutant CXCR4 has been reported (11) but is subject to confounding effects of dysregulated expression, insertional mutagenesis, and the xenotransplant setting. A long standing but yet to be accomplished goal of this work has been to develop a "knock in" transgenic mouse model of WHIM syndrome by expressing the CXCR4 R334X mutant under the control of the endogenous CXCR4 promoter. Our preliminary data indicate that this locus is extremely resistant to homologous recombination, as only one out of more than 1,000 neomycin-resistant ES clones displayed targeted recombination. Although human WHIM syndrome is not a highly lethal disease, another possibility is that in the mouse ES cells this mutation is toxic. CXCR4 is expressed throughout development, including in ES cells, and the mutation acts in a dominant fashion. One report demonstrated that merely slightly altered levels of CXCR4 signaling could affect developmental processes (12). In mouse embryos,

expression of mutant CXCR4 impairs viability. In embryos generated from chimeric blastocysts derived from the one correctly targeted ES clone, a high level of chimerism as assessed by coat color resulted in lethality. Using an albino B6 recipient blastocyst, 3/25 mice with black eyes were dead at birth, while the surviving 22 mice were all white. In chimeric pups with chimerism assessed by expression of a *lacZ* transgene in red blood cells, 2/10 mice displayed some *lacZ* activity, but this level of chimerism resulted in infertility and resultant inability to obtain germline transmission of the mutation.

Current efforts by Ryan Day in the lab are focused on generating inducible heterozygous expression of the mutant *CXCR4* so that prior to Cre induction mice will merely be haploinsufficient for *CXCR4*. As described above, $CXCR4^{+/-}$ mice are essentially normal. Such a system for other genes has been reported in the literature, and we are using a construct similar to the one described (13). If these mice can be generated, they would confirm that CXCR4 mutations are responsible for the pathogenesis of WHIM syndrome. The mechanisms of viral infection, brain tumors, and altered B lymphocyte function in addition to the altered neutrophil trafficking could be dissected. The mice would provide a tool to profile the intracellular signaling molecules that mediate neutrophil release. For example, it is unclear whether GRK6, GRK3 or both are the key regulators of CXCR4 desensitization (14, 15).

4.2.3 Importance of stromal CXCR2 expression

In the process of generating chimeras with CXCR2-deficient neutrophils, we predicted that wild-type mice reconstituted with $CXCR2^{-/-}$ hematopoiesis would recapitulate the

neutrophilic phenotype of *CXCR2*$^{-/-}$ mice. However, some chimeras were generated with only *CXCR2*$^{-/-}$ cells, and, to our surprise, these mice were neutropenic with no evidence for myeloid expansion. This suggests two possibilities: 1) Given the data in germ-free *CXCR2*$^{-/-}$ - mice, there may be a critical period during which subclinical infection or endogenous bacteria can become dysregulated and induce excessive cytokine production. This may occur during the neonatal period. Alternatively, the conditioning regimen for bone marrow transplant may interrupt this process. 2) CXCR2 expression on non-hematopoietic, radio-resistant cells (such as endothelial cells) may prevent neutrophilia. To address the role of non-hematopoietic CXCR2 signaling in neutrophil homeostasis, we are generating chimeras derived from CXCR2-/- recipient mice reconstituted with wild-type cells, or CXCR2-/- cells transplanted back into CXCR2-/- recipients as a control. These experiments may demonstrate a novel role for endothelial CXCR2 in neutrophil homeostasis.

4.2.4 Signaling studies

As discussed above, our experiments did not completely determine the role of heterologous interactions between CXCR2 and CXCR4 in the regulation of neutrophil trafficking. We will use CXCR2- or CXCR4- deficient neutrophils in chemotaxis and calcium flux assays to measure any alterations in signaling in the absence of one of these two receptors. If heterologous interactions are responsible for myelokathexis in *CXCR2*$^{-/-}$ mice or humans, we would predict that CXCR4 signaling would be increased in the absence of CXCR2. We will attempt to measure signaling in conditions that mimic either

basal or stress conditions. According to a recent report, β-arrestin transmits CXCR4 signals through ERK phosphorylation (16), so we will also attempt to measure CXCR4 signaling in freshly isolated neutrophils using a phosphoflow assay previously utilized in LKS cells by our lab (17).

4.3 References

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