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STAT3 CONTROLS THE NEUTROPHIL MIGRATORY RESPONSE TO CXCR2 AND ITS LIGAND MIP-2 (CXCL2)

Hoainam Nguyen-Jackson

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STAT3 CONTROLS THE NEUTROPHIL MIGRATORY RESPONSE TO CXCR2
AND ITS LIGAND MIP-2 (CXCL2)

by

Hoainam T. Nguyen-Jackson, B.S.

APPROVED:

Stephanie S. Watowich, Ph.D., Supervisory Professor

Chen Dong, Ph.D.

Gary E. Gallick, Ph.D.

Kimberly Schluns, Ph.D.

Shao-Cong Sun, Ph.D.

APPROVED:

Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

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A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Hoainam T. Nguyen-Jackson, B.S.

Houston, Texas

August, 2011

Dedication

To Anya, Ella, and Tess.

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Publication No. _____

Hoainam Nguyen-Jackson, B.S.

Supervisory Professor: Stephanie S. Watowich, Ph.D.

Abstract

Among the first white blood cells to respond to bacterial and fungal infections, neutrophils are produced in the bone marrow, released into circulating blood, and recruited to inflamed tissue. The cytokine granulocyte colony-stimulating factor (G-CSF) is used clinically to induce neutrophil mobilization from the marrow. This process was previously demonstrated to require the STAT3 transcription factor (signal transducer and activator of transcription 3), the principal signaling molecule activated upon G-CSF-binding of its receptor, but the mechanism was unknown. The chemokines KC (*Cxcl1*) and MIP-2 (*Cxcl2*), and their shared receptor CXCR2 (*Il8rb*), also stimulate neutrophil mobilization, in contrast to SDF-1 (*Cxcl12*), which contributes to neutrophil retention in the bone marrow, requiring downregulation to promote neutrophil release. Using a murine model with conditional STAT3 deletion in bone marrow, we demonstrate that STAT3 regulates G-CSF-dependent changes in bone marrow chemokine and chemokine receptor expression levels. We found that G-CSF/STAT3 signals increase transcription of *Cxcl1*, *Cxcl2*, and *Il8rb*, and concomitantly suppress

Cxcl12. Administration of a MIP-2-neutralizing antibody *in vivo* suppressed G-CSF-stimulated upregulation of circulating neutrophil levels, indicating its critical role in neutrophil mobilization. Consistent with this observation, STAT3-deficient mice were more susceptible than wild type to infection with *Listeria monocytogenes*, a bacterium that initiates a G-CSF-mediated immune response. STAT3-deficient mice failed to upregulate circulating neutrophils after infection, and demonstrated higher levels of bacterial infiltration in the liver, indicating that impaired neutrophil mobilization contributes to an insufficient immune response. Further analysis of molecular events that transpire upon STAT3-binding to the *Cxcl2* and *Il8rb* promoters indicates that G-CSF induces higher levels of activating trimethylated lysine 4 (H3K4me3) modifications relative to repressive trimethylated lysine 27 (H3K27me3) marks, suggesting G-CSF/STAT3 signals stimulate an open chromatin configuration at these promoters. This is further supported by the observation that pharmacologic inhibition of STAT3 phosphorylation blocked G-CSF-stimulated accumulation of H3K4me3 at target promoters, indicating that STAT3 may contribute to accumulation of H3K4me3 marks. Taken together, our study demonstrates that G-CSF-mediated STAT3 regulation of bone marrow chemokine and chemokine receptor expression may regulate neutrophil mobilization and indicates a potential role for STAT3 in opening of the chromatin structure at target genes in neutrophils.

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Chapter 1: Introduction

1.1 Introduction to neutrophils

The neutrophil, the predominant circulating white blood cell type, mediates inflammation as well as anti-bacterial and anti-fungal immunity (1). Hallmark morphological features of neutrophils include the presence of granules, classifying them as a type of granulocyte, and a multi-lobed nucleus, prompting their designation as polymorphonuclear cells. Neutrophils, produced in the bone marrow, are constantly released into peripheral blood under homeostatic conditions, where circulating mature cells can be readily recruited to inflamed tissue. Neutrophil effector functions include phagocytosis, respiratory burst, and degranulation, which is the release of pre-formed molecules such as anti-bacterial peptides and proteases. Recently, neutrophil extracellular traps (NETs) have been characterized in which chromatin and granule components are expelled from the neutrophil, forming sticky bundles that ensnare bacteria (2). Insufficient neutrophil numbers or deficient neutrophil functions have clinical implications in conditions such as severe congenital neutropenia and chronic granulomatous disease, respectively. Both conditions are characterized by increased susceptibility to opportunistic infections that can be life threatening (3). By contrast, unregulated accumulation of neutrophils in the tissue has been implicated in chronic inflammatory conditions such as rheumatoid arthritis (4), asthma (5), and chronic obstructive pulmonary disorder (6). Both extremes

underline the importance of proper regulation of the neutrophil response to avoid pathological conditions.







		Diameter	Nucleus: Cytoplasm	Nuclear shape	Granules present
	Myeloblast	10-20 μm	4:1	round or oval	none
	Promyelocyte	10-20 μm	3:1	round or oval	azurophilic, nonspecific
	Myelocyte	10-18 μm	2:1 or 1:1	oval	azurophilic, specific
	Metamyelocyte	10-18 μm	1:1	indented	specific
	Band Neutrophil	10-16 μm	1:1	elongated narrow band	specific
	Segmented Neutrophil	10-16 μm	1:1	2-5 distinct lobes	specific

Figure 1.1. The stages of neutrophil development. Developing from the hematopoietic stem cell (HSC), the common myeloid progenitor (CMP), and the granulocyte/macrophage progenitor (GMP), the myeloblast differentiates further through the myelocyte stages before becoming a mature segmented neutrophil. Azurophilic, or primary granules, are expressed first in the promyelocyte, before acquisition of the specific, or secondary granules. Adapted from (7, 8).

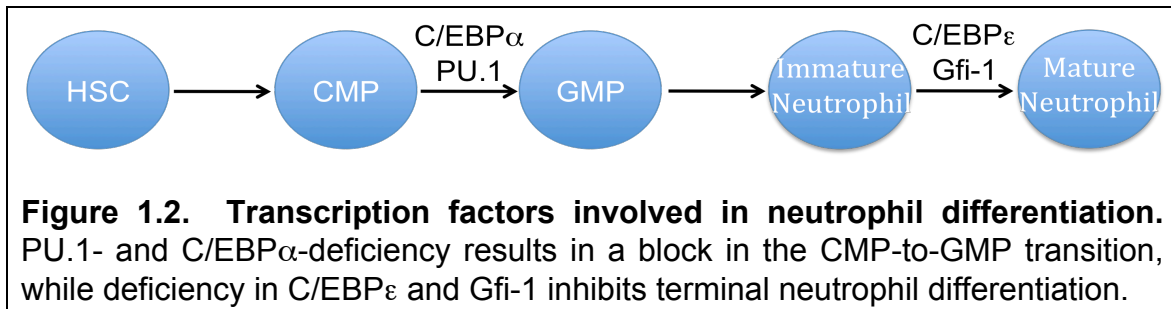
The short half-life of circulating neutrophils, a matter of hours, necessitates their constant replenishment from the bone marrow, where they differentiate from the hematopoietic stem cell (HSC) in a process called granulopoiesis (9). After the HSC, common myeloid progenitor (CMP), and granulocyte/macrophage progenitor (GMP) stages, neutrophils traverse multiple

differentiated states to become a mature segmented cell, as shown in Figure 1.1. Blocks in granulopoiesis resulting in the accumulation of undifferentiated cells are characteristic of myeloid leukemias, such as acute myeloid leukemia and chronic myeloid leukemia (10). Each stage of neutrophil development is distinguished morphologically by nucleus-to-cytoplasm ratio, the presence and type of granules, and nuclear shape (7) (Figure 1.1). Granulopoiesis, as with hematopoiesis in general, is governed by cytokines and transcription factors. In the next two sections, I will introduce the major transcription factors involved in granulopoiesis, and examine the contribution of cytokines to this process.

1.2. Transcription factors involved in granulopoiesis

Several transcription factors are critical for proper neutrophil differentiation, as summarized in Figure 1.2. The ETS domain-containing transcription factor PU.1 promotes the development of the myeloid lineage, and is highly expressed in B cells, granulocytes and monocytes (11). Analysis of PU.1-deficient embryos (due to embryonic lethality of PU.1 deletion) or mice harboring a mutation in the PU.1 DNA-binding domain revealed significant impairment in the development of lymphocytes, monocytes, and granulocytes, while megakaryocytes and erythrocytes were intact (12, 13). These data suggest that PU.1 promotes development of the GMP, while repressing the megakaryocyte-erythrocyte progenitor (MEP) (Figure 1.2). PU.1 is therefore thought to be important during progenitor commitment, although expression of

PU.1 was found in all stages of neutrophil development, and increases with maturation, with the highest expression levels found in the mature cell (14).



The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors is also important for proper myeloid cell development. These family members can form homo- or hetero-dimers with one another. C/EBP alpha (C/EBP α), like PU.1, is a transcription factor predominantly expressed in neutrophil progenitors (Figure 1.2). C/EBP α -deficiency results in a lack of mature neutrophils and eosinophils in embryos and newborn mice (15), while conditional deletion of C/EBP α in adult mice (*Mx-Cre*-mediated) results in a block in the CMP to GMP transition, resembling acute myeloid leukemia (16). Taken together, these data reveal the importance of PU.1 and C/EBP α in the commitment of neutrophil progenitors (Figure 1.2). In confirmation of these findings, C/EBP α is highly expressed in the early stages of neutrophil development, such as in the myeloblast and promyelocyte, and drops significantly in more mature cells (14). Another C/EBP family member, C/EBP epsilon (C/EBP ϵ), is highly expressed during granulopoiesis (17), and its

deficiency results in a lack of functionally mature neutrophils (18) (Figure 1.2). In contrast to the roles of C/EBP α and C/EBP ϵ , C/EBP beta (C/EBP β) does not appear to affect steady-state granulopoiesis, but is instead thought to be involved in emergency, or demand-driven granulopoiesis, which occurs, for example, during infections that coincide with an elevation of G-CSF levels (19, 20). Additional C/EBP family members delta (C/EBP δ), gamma (C/EBP γ), and zeta (C/EBP ζ) are also reportedly expressed in cells of the myeloid lineage, but these molecules have either not been studied extensively, or are thought to play minor and/or redundant roles during granulopoiesis (14, 21).

Recently, the zinc-finger transcription factor Gfi-1 has been implicated in neutrophil development. Initially characterized in the development of lymphocytes, high expression of Gfi-1 was later found in mature neutrophils (22). It is thought that Gfi-1 may promote terminal differentiation of neutrophils while antagonizing the development of monocytes (23). Similar to C/EBP ϵ -deficiency, Gfi-1 deficiency results in a lack of phenotypically mature neutrophils (18, 23) (Figure 1.2).

While the presence of the transcription factors PU.1, C/EBP α , C/EBP ϵ and Gfi-1 is important during granulopoiesis, deficiency of certain cytokines can have a severe impact on neutrophil development. Additionally, cytokines have been shown to alter transcription factor expression levels, thereby affecting cell fate (24). I will next examine the effects of cytokines on neutrophil development.

1.3. Granulocyte colony-stimulating factor (G-CSF) and other cytokines governing granulopoiesis

The cytokine granulocyte colony-stimulating factor (G-CSF) is the major regulator of granulopoiesis, as evidenced by the significant loss of neutrophils in G-CSF- or G-CSF receptor (G-CSFR)-deficient mice (25, 26). However, residual neutrophil levels in these mice, although at low levels, indicate that other cytokines may compensate *in vivo*, such as Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and Interleukin-6 (IL-6) (27, 28). While GM-CSF is important for neutrophil differentiation *in vitro* (29), GM-CSF-deficient mice exhibit normal hematopoiesis (30, 31), indicating the redundant function of GM-CSF *in vivo*. In support of this finding, adult mice deficient in GM-CSF and G-CSF exhibited similar levels of neutrophils as G-CSF-deficient mice (32). Similar to the phenotype of GM-CSF-deficiency, deficiency in IL-6 exhibited normal granulopoiesis under steady state conditions (33). However, mice deficient in G-CSF and IL-6 were more neutropenic than with G-CSF-deficiency alone, indicating the contribution of IL-6 to granulopoiesis under compensatory conditions *in vivo* (34).

G-CSF is present at very low levels in healthy individuals, and is rapidly upregulated in the presence of infection (up to 20-fold increase) (35-37). G-CSF can accelerate the cell cycle, allowing for rapid proliferation of neutrophil progenitors (38, 39). G-CSF also drives the differentiation of neutrophils in the bone marrow, their release into peripheral blood, and promotes pro-survival

signaling (39, 40). These combined functions of G-CSF result in a rapid and sustained elevation in neutrophil counts.

G-CSF is produced by endothelial cells, fibroblasts, monocytes/macrophages and mesenchymal cells (41, 42). Murine and human G-CSF molecules are highly homologous, as recombinant human G-CSF can cross-react with murine G-CSFR (43). The cytokines IL-1 β , IL-3, IL-4, GM-CSF, interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) can strongly promote G-CSF production from these cell types, as does stimulation with lipopolysaccharide (LPS) (42). In addition to these pathways, recent studies indicate that macrophages and dendritic cells initiate a cascade of events that results in G-CSF production. Upon activation of their toll-like receptors, macrophages and dendritic cells produce IL-23 in response to pathogens (44). IL-23, in turn, stimulates IL-17 production from Th17 cells, a subset of CD4 T cells lining mucosal surfaces. IL-17 then drives G-CSF production from cells comprising the stroma, such as fibroblasts, epithelial, and endothelial cells (45-47). In response to bacterial pneumonia infection, IL-17 receptor-deficiency resulted in reduced G-CSF levels, delayed neutrophil recruitment, and higher mortality rates compared to wild type controls, indicating the importance of IL-17-induced granulopoiesis *in vivo* (46).

Because G-CSF is the primary cytokine that drives granulopoiesis, it is administered clinically to treat conditions of reduced levels of circulating neutrophils, or neutropenia, under the trade names Neupogen, Granocyte, or Neulasta; the latter is a pegylated form that is longer lasting in circulation (48).

While congenital forms exist that have been present since birth, neutropenia can also be induced, for example as a side effect of chemotherapeutics. Therefore, recombinant G-CSF is often incorporated into chemotherapy regimens (49). G-CSF is also used to mobilize stem cells into peripheral blood to facilitate collection for transplantation, and is thought to promote survival of cardiomyocytes after myocardial infarction (50, 51). However, because G-CSF treatment has yielded inconsistent results clinically and has been correlated with onset of osteoporosis and malignant transformation of myeloid cells, attention has turned to the study of the molecular pathways activated by G-CSF (52, 53).

1.4. G-CSF receptor (G-CSFR) and downstream signal transduction pathways

As illustrated in Figure 1.3, G-CSFR is a type I cytokine receptor, defined as a single transmembrane protein containing a cytokine receptor homologous (CRH) domain in the extracellular region consisting of four conserved cysteine residues and a Trp-Ser-X-Trp-Ser (WSXWS) motif, and two conserved regions in the intracellular region denoted as Box 1 and Box 2 (54, 55). In addition to elements that define type I cytokine receptors, the G-CSFR also contains an immunoglobulin-like (Ig-like) domain, as well as three fibronectin type III-like (FNIII-like) domains in the extracellular region, and the intracellular domain contains a third element called Box 3 (54, 55) (Figure 1.3). The G-CSFR structure closely resembles gp130, a single transmembrane protein that

transduces downstream signals when hetero-dimerized with ligand-specific alpha subunits for the cytokines IL-6, leukemia inhibitory factor (LIF) and oncostatin M (OSM) (54). G-CSFR and gp130 share 46.3% amino acid homology; like G-CSFR, gp130 contains an Ig-like domain, FNIII-like domains, and Box 3 (56).

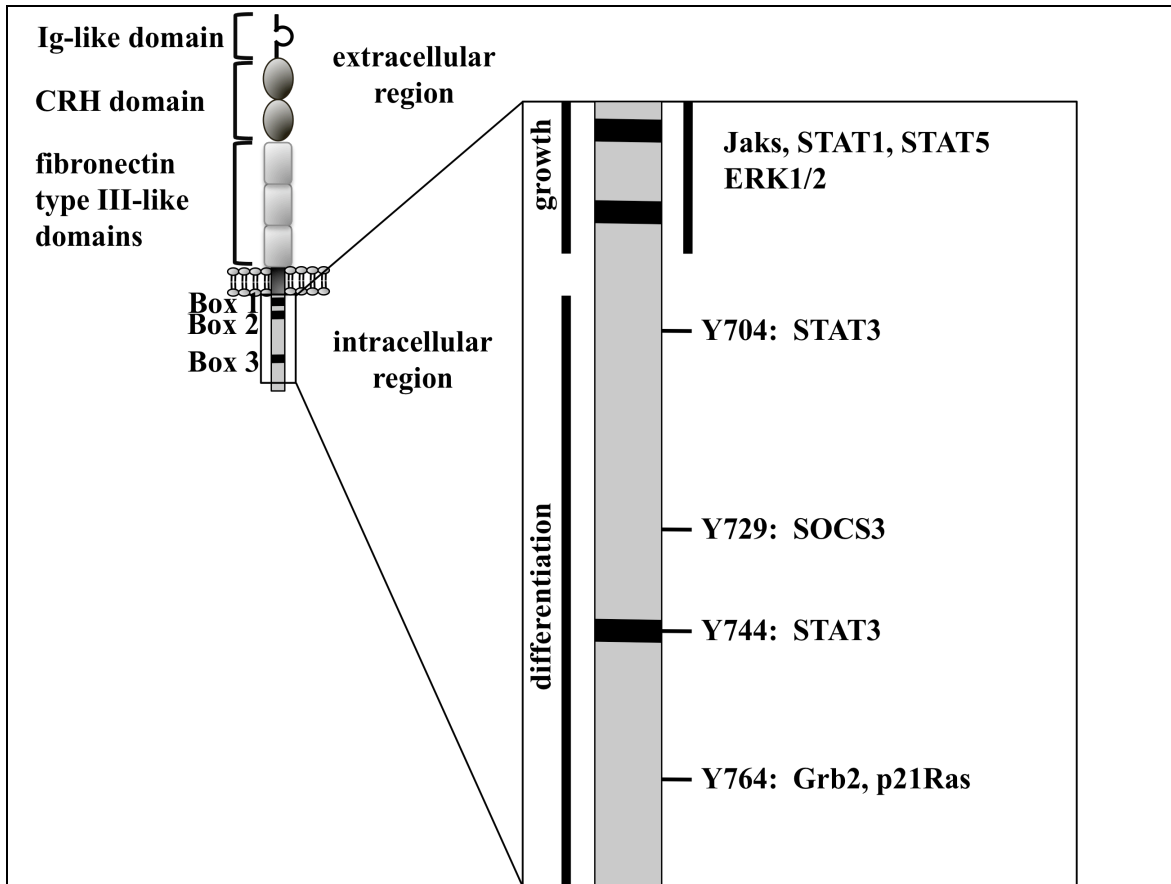
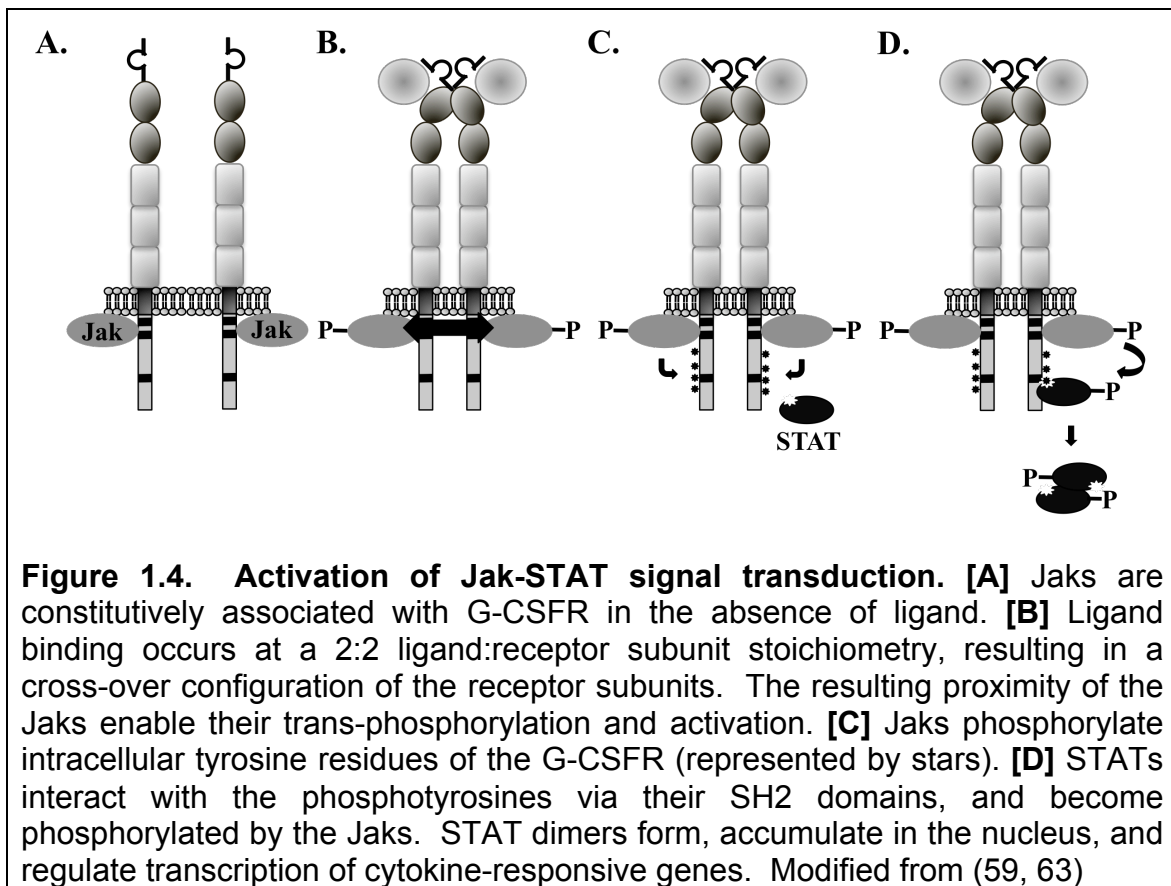


Figure 1.3. G-CSFR structure and downstream signal pathways. The extracellular region of the G-CSFR contains an immunoglobulin-like (Ig-like) domain, a cytokine receptor homologous (CRH) domain with 4 conserved cysteine residues and a conserved WSXWS motif, and 3 fibronectin type III (FNIII)-like domains. The intracellular region, which mediates downstream signal transduction, contains conserved Box 1, Box 2, and Box 3 motifs, and 4 tyrosine (Y) residues. The membrane proximal region of the G-CSFR cytoplasmic domain mediates growth signals, and has been linked to activation of Jaks, STAT1, STAT5, and ERK1/2. The tyrosine residues mediate differentiation signals. Y704 and Y744 recruit STAT3, Y729 is a docking site for SOCS3, a negative regulator of G-CSFR signaling, and Y764 associates with Grb2, and is reported to activate p21Ras, as indicated. Modified from (57, 58).

G-CSF binds with very high affinity to the G-CSFR ($K_d \approx 700$ pM), which homo-dimerizes upon ligation, resulting in a complex of two molecules of G-CSF and two G-CSFR subunits (54, 59) (Figure 1.4[B-D]). X-ray crystallography revealed that each ligand molecule interacts with the CRH domain of one G-CSFR subunit and the Ig-like domain of the second subunit, resulting in a crossover configuration, as shown in Figure 1.4[B-D] (59). The G-CSFR is expressed on HSC, CMP, GMP, mature neutrophils and their progenitors, and the level of expression increases upon maturation (60-62). While other hematopoietic and non-hematopoietic cell types have been demonstrated to express G-CSFR, the profound loss of neutrophils in the G-CSFR knockout indicates that its chief function is in this cell type (26).

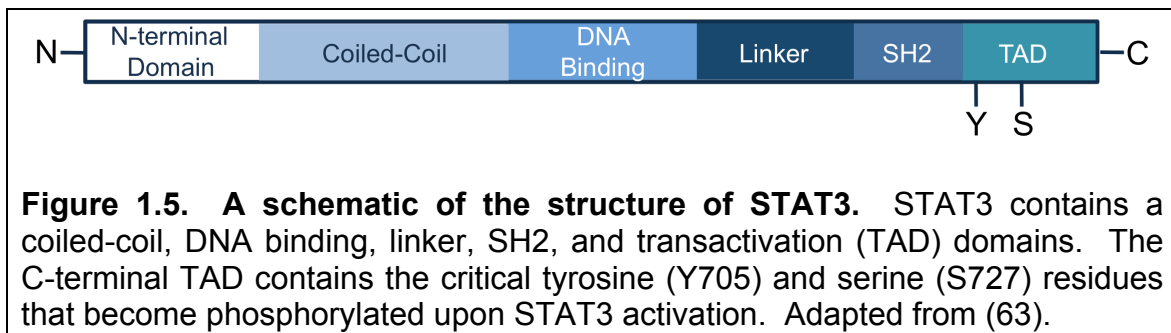


Each G-CSFR subunit lacks intrinsic signaling capability, and signals through constitutive association with Janus tyrosine kinases (Jaks), primarily Jak1 (64-66). Box 1 in the membrane-proximal intracellular region of the G-CSFR contains a proline-rich sequence thought to serve as a docking site for Jaks (67). High affinity interactions between G-CSF and its receptor bring the Jaks into proximity, resulting in their phosphorylation of one another, and ultimately of tyrosine (Y) residues located in the intracellular domain of G-CSFR (Figure 1.4). In humans, these tyrosine residues are Y704, Y729, Y744, and Y764 (corresponding to Y703, Y728, Y743, and Y763 in the mouse), that once phosphorylated, serve as docking sites for downstream signaling molecules, predominantly the signal transducers and activators of transcription (STATs) (68, 69). While there are seven STAT family members, G-CSF stimulation results in robust activation of STAT3, with activation of STAT1 and STAT5 occurring to a lesser extent (65, 70, 71). Although it is widely accepted that G-CSFR signaling chiefly activates Jak/STAT pathways, the G-CSFR has also been linked to multiple components of mitogen-activated protein (MAP) kinase signaling pathways (72-75).

STATs are transcription factors constitutively found in the cytoplasm, and can interact with phosphorylated tyrosines of the G-CSFR through their Src homology 2 (SH2) domains, enabling their phosphorylation by Jaks (Figure 1.4[D]). Upon their activation, STAT family members homo- or hetero-dimerize, as their SH2 domains possess higher affinity for one another's phosphorylated residues when compared to those of the cytokine receptor (76). Dimers of

STATs accumulate in the nucleus, where they bind DNA and activate transcription (76).

STAT3, as with other STAT family members, contains coiled-coil, DNA binding, linker, SH2, and transactivation (TAD) domains, as shown in Figure 1.5 (63). The carboxy-terminal TAD contains the critical tyrosine and serine residues that can become phosphorylated (63). The DNA binding domain of STAT3 binds the consensus sequence TTN₍₃₋₇₎AA (77, 78), although recent computational studies followed by candidate validation approaches indicate that the STAT3 recognition sequence may not be as rigid as once believed (79).



STAT3 is recruited to Y704 and Y744 of the G-CSFR, although receptor truncation studies demonstrated that Y704 was sufficient for STAT3 activation (65, 71, 74). *In vitro*, activation of STAT3 appears to drive neutrophil differentiation, as judged by induction of neutrophil marker genes and morphological changes associated with maturation (80, 81). However, *in vivo* studies revealed a more complex role for STAT3 in regulation of granulopoiesis, which will be discussed in the next section.

1.5. Role for STAT3 in the regulation of granulopoiesis *in vivo*

Previous *in vitro* models indicated that G-CSFR-activated STAT3 is a major regulator of granulopoiesis (65, 71, 74, 80, 81). To assess the role of STAT3 in the G-CSFR signaling pathway *in vivo*, the first studies used G-CSFR deletion mutants expressed in mice which replaced the endogenous gene (Figure 1.6). In one such construct, designated d715, a premature stop codon was introduced in the G-CSFR to mimic the truncated receptor found in some cases of the clinical condition severe congenital neutropenia. In the d715 receptor, the tyrosine residues Y729, Y744 and Y764 are deleted, while the STAT3 docking site Y704 remained intact, as shown in Figure 1.6[B] (82, 83). Steady-state granulopoiesis was normal in d715 mice, as judged by neutrophil numbers in blood and bone marrow (82, 83). However, d715 mutant mice demonstrated an enhanced response to G-CSF as compared to wild type controls, producing an increased absolute number of progenitors and enhanced proliferative responses, resulting in increased peripheral neutrophils (82, 84). These results indicate that Y704 is sufficient in steady state and G-CSF-driven granulopoiesis, and points to the recruitment of negative regulators by the deleted region of the G-CSFR (most likely SOCS3, or suppressor of cytokine signaling).

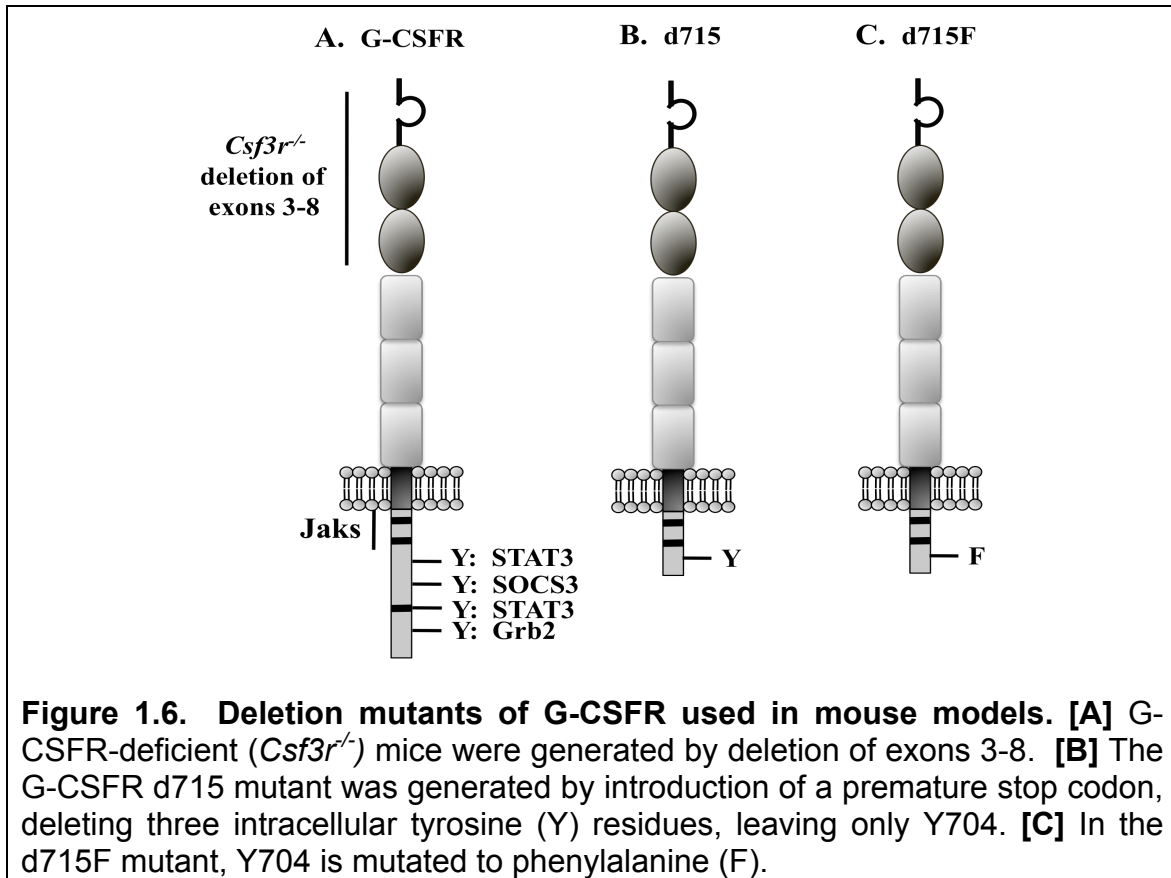


Figure 1.6. Deletion mutants of G-CSFR used in mouse models. [A] G-CSFR-deficient (*Csf3r^{-/-}*) mice were generated by deletion of exons 3-8. **[B]** The G-CSFR d715 mutant was generated by introduction of a premature stop codon, deleting three intracellular tyrosine (Y) residues, leaving only Y704. **[C]** In the d715F mutant, Y704 is mutated to phenylalanine (F).

To understand the role of the remaining STAT3 recruitment site (Y704), this tyrosine residue was mutated to phenylalanine (F), generating the d715F mutation, as shown in Figure 1.6[C] (83). Mice expressing the d715F mutation were severely neutropenic, as circulating neutrophil levels are similar to those in the G-CSFR-deficient mice. In contrast to the findings in d715 mice, G-CSF failed to upregulate circulating neutrophils in d715F mice, and did not induce proliferation of hematopoietic progenitors (83). These proliferative defects were partially restored upon introduction of a constitutively active STAT3 isoform (STAT3C) into d715F progenitor cells *in vitro* (83). These studies agree with results from earlier *in vitro* work, which indicated the importance of STAT3 in

mediating G-CSF-responsive proliferation and differentiation signals (65, 71, 74, 80, 81).

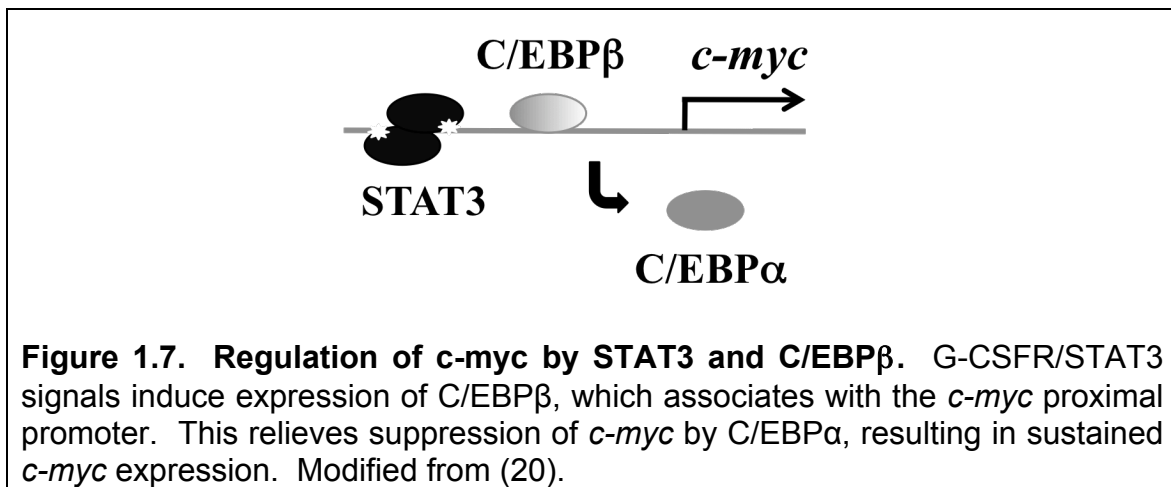
To directly examine the function of STATs in granulopoiesis, STAT-deficient mouse models were generated. Although G-CSFR stimulation results in activation of STAT1, STAT3, and STAT5 (65, 70, 71), deletion of STAT1 or STAT5 has minimal effect on granulopoiesis *in vivo* (85, 86). These results, in context of the results found in the d715F mutant mice, further support the importance of STAT3 in this pathway. Because deletion of STAT3 results in embryonic lethality (87), mice were generated in which STAT3 was conditionally inactivated in the hematopoietic system (88-91). As opposed to the neutropenia observed in d715F mice, a model in which G-CSF-responsive STAT3 activation is suppressed, mice with hematopoietic deletion of STAT3 exhibit generalized inflammation and neutrophilia, or increased circulating neutrophils (83, 88, 91). As compared to wild type animals, mice with conditional STAT3 deletion have increased amounts of morphologically mature neutrophils (band and segmented cells) in the bone marrow, although levels of myeloid progenitor cells and immature granulocytes were similar, suggesting that STAT3 is a negative regulator of terminal neutrophil differentiation (88). Together with the observed neutrophilia in STAT3-deficient mice, these results suggested that STAT3 is required to suppress neutrophil production in homeostatic conditions. This phenotype is similar to that of aged mice with hematopoietic deletion of SOCS3, a STAT3 target gene that negatively regulates G-CSFR signal transduction (92, 93). Therefore, these data indicate that STAT3 and SOCS3 are important

negative regulators of steady-state granulopoiesis. However, recent studies indicate that the role for STAT3 in granulopoiesis may be more complex than these findings initially indicated.

Emergency granulopoiesis occurs upon infection or administration of G-CSF, resulting in enhanced cell cycle progression of granulocytic progenitors in the bone marrow, increased neutrophil production, and the release of mature neutrophils into circulating blood (35, 39). After repeated exposure to G-CSF, *Tg[Tek-cre]12Flv*, *Stat3^{f/Δ}* mice, termed herein as STAT3-deficient mice, fail to increase immature granulocyte amounts in bone marrow, resulting in a skewed ratio of immature:mature cells in bone marrow, blood and spleen (91). This effect was not observed in myeloid-specific SOCS3-deficient mice, which indicates that the role for STAT3 in G-CSF-driven emergency granulopoiesis is independent of SOCS3 (91). In support of these findings, STAT3 was found to promote G-CSF-dependent cell cycle progression and differentiation of immature granulocytes (20). Moreover, the proliferation of multipotent and committed myeloid progenitors in response to G-CSF or following infection by *Listeria monocytogenes*, a bacterial pathogen that is regulated by G-CSF signals *in vivo* (25, 94), is dependent upon STAT3 (20). These data indicate that STAT3 is an important regulator of hematopoietic progenitor proliferation during emergency granulopoiesis driven by G-CSF or bacterial infection (20).

The phenotype of impaired demand-drive neutrophil response in STAT3-deficient mice is similar to *Cebpb^{-/-}* mice, in which C/EBP β , an essential regulator of emergency granulopoiesis, is deleted (19), suggesting that the

functions of STAT3 and C/EBP β are related. Additionally, *Cebpb* was recently described as a STAT3-target gene (20). C/EBP β contributes to the emergency granulopoiesis response, in part, by affecting expression of *c-myc*, an important regulator of the cell cycle (19). The proximal promoter region of *c-myc* contains consensus binding sites for C/EBP α and C/EBP β , which demonstrate opposing roles in *c-myc* regulation (20). Expression of *c-myc* is suppressed when C/EBP α interacts with its promoter, and is relieved when G-CSF/STAT3 signals stimulate expression of C/EBP β , inducing transcription (20). This pathway, illustrated in Figure 1.7, provides a molecular mechanism by which G-CSF signaling via STAT3 and C/EBP β stimulates neutrophil progenitor proliferation during emergency granulopoiesis.



1.6. General mechanisms of transcriptional regulation

The transcription factors discussed thus far, such as STAT3 and the C/EBP family members, are important regulators of gene expression. While it is

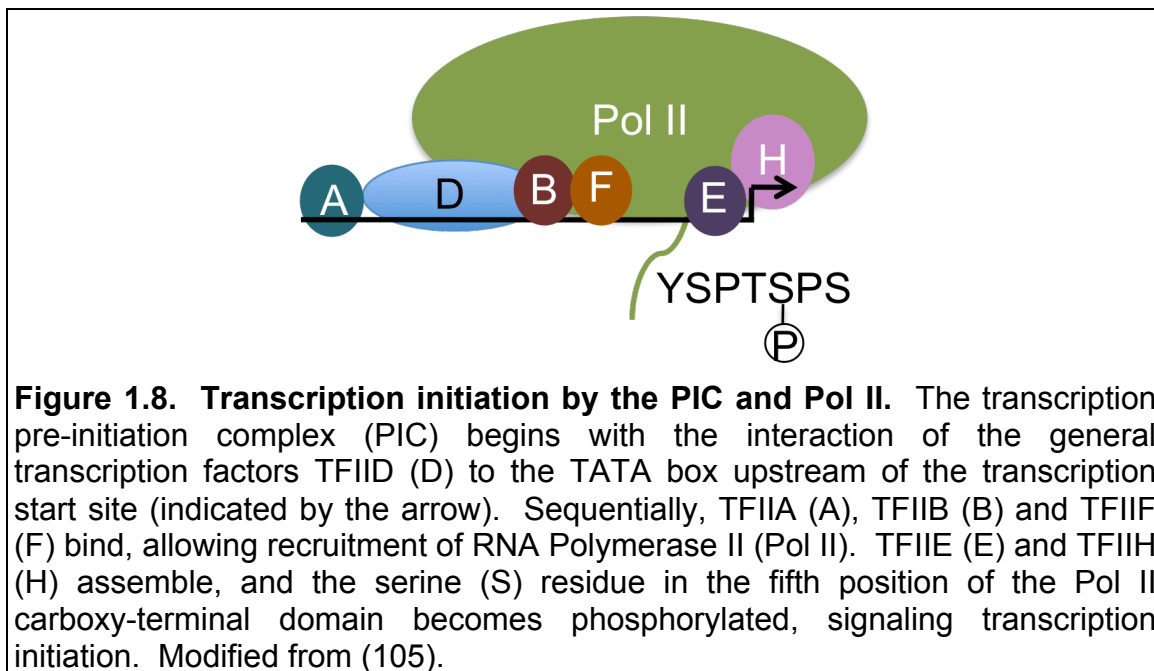
accepted that the binding of these factors regulates transcriptional activity, recent studies have demonstrated that this process is far more complex than initially thought, with much more yet to be revealed. In order to provide a more in-depth view of the transcriptional regulation process, I will discuss the organization of DNA, chromatin dynamics, and the pre-initiation complex in this section, all which coordinate with transcription factors to promote gene expression.

A nucleosome is formed when approximately 150 basepairs of DNA is tightly wrapped around a histone core, an octamer consisting of two of each subunit: H2A, H2B, H3, and H4 (95). These histone subunits have tails at the amino termini that are easily accessed and modified post-translationally, such as by phosphorylation, ubiquitination, acetylation, sumoylation, and methylation, all which affect transcriptional activity (96). The chromatin modification that is best understood is acetylation, which is mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Addition of acetyl groups is thought to neutralize the interactions between DNA and histones, as DNA is negatively charged and histones are positively charged, regulating the accessibility to the transcription machinery (97). Recently, much attention has focused on histone methylation, but it is poorly understood how methylation is regulated, and how it affects transcription. Methylation may affect DNA accessibility in a manner similar to acetylation, however, methyl groups are small and uncharged (96). Instead, it is thought that methylated lysine residues recruit regulators of transcription by direct interaction with their chromodomains (96).

Methylation of histone subunits, catalyzed by histone methyltransferases (HMTs), can occur at two residues. Arginine (R), which can be mono- and dimethylated, has been associated only with transcriptional activity. On the other hand, lysine (K), which can be mono-, di- or tri-methylated, can be associated with transcriptional activation and repression depending on the context (98, 99). The most widely studied modifications are of histone subunit H3, in which trimethylated lysine 4 (denoted as H3K4me3) and lysine 27 (H3K27me3) correlate with regions of active and silent transcription, respectively (96). H3K4me3 tends to accumulate at the promoters of active genes, and has been shown, for example, to recruit chromodomain helicase DNA-binding protein 1 (CHD1), an enzyme with chromatin remodeling activity (100, 101). On the other hand, H3K27me3 is found throughout heterochromatin, and is associated with X chromosome inactivation (102). H3K27me3 has been shown to recruit Polycomb Repressive Complex 2 (PRC2) through its chromodomain, which mediates additional H3K27 tri-methylation and transcription repression (103).

After the chromatin is remodeled and the DNA is more accessible, a pre-initiation complex (PIC) forms at the proximal promoter, which is composed of the general transcription factors TFIID, TFIIB, TFIIF, TFIIIE, and TFIIH. TFIID, which binds the promoter first, contains the TATA-binding protein (TBP), and TBP-associated elements (TAA). Next, TFIIB binds, recruiting RNA polymerase II (Pol II), and allows TFIIF to associate. TFIIIE and TFIIH are required for transcription initiation (104) (Figure 1.8). The carboxy-terminal domain of the largest subunit of Pol II is made of the sequence YSPTSPS, which is repeated

52 times in mammals (Figure 1.8). In a hypophosphorylated state, Pol II can readily bind DNA. Upon its initiation, the serine (Ser) residue in the fifth position of the CTD repeat is phosphorylated (pSer5), and phosphorylation of the Ser in the second position (pSer2) is associated with Pol II elongation. The initiation of transcription by the PIC and Pol II is illustrated in Figure 1.8.



The efficiency of transcription initiation is regulated through the action of transcription regulators, such as activators, repressors, enhancers, and silencers, all of which can have direct or indirect effects on the PIC (106). While some transcription factors have demonstrated direct association with components of the PIC (107), the relationship between transcription regulators and the PIC is not fully understood. Binding of transcriptional regulators requires accessibility to DNA, but it is unknown how this access is gained. Some

transcriptional regulators are thought to bind DNA in its nucleosome state, and are known as pioneer transcription factors (99). On the other hand, it is believed that the majority of transcription factors require the action of chromatin remodeling enzymes to mediate accessibility. While some transcription factors are shown to directly interact with histone modifying enzymes, such as the acetyltransferase p300 (108-112), it is not understood how most transcription factors recruit these enzymes.

It is not fully understood how transcription is regulated in neutrophils, a particularly interesting model to study given the burst of gene expression required for their effector functions (113). Additionally, little is known of the effect of histone modifications during the development of neutrophils, or in neutrophil functions such as mobilization, which will be discussed in the next section.

1.7. Neutrophil mobilization

Neutrophil mobilization, the release of neutrophils from bone marrow to blood, is necessary under steady state conditions to maintain proper circulating levels (114). Under inflammatory conditions, circulating neutrophil amounts need to be rapidly increased, as neutrophils are the first recruited cells to sites of infection (1). Mobilization involves a complicated progression of steps, involving, among other processes, abrogation of retention signals in the bone marrow, migration through hematopoietic niches of the marrow, and entry into the vascular system (114). The molecular events of this pathway are not fully

understood, which is exploited clinically in the use of recombinant G-CSF to treat neutropenias.

The administration of G-CSF is sufficient to induce rapid mobilization of neutrophils out of the bone marrow into circulating blood (115). Kinetics were established in rats, indicating that neutrophilia is induced 30 minutes after a single injection of G-CSF, peaking at 12 hours, and pretreatment levels are restored within 30-36 hours (116). G-CSF had no effect on circulating neutrophil amounts in studies utilizing *Csf3r*^{-/-} mice that are deficient in G-CSFR or G-CSFR/erythropoietin receptor (EpoR) chimeric mice, in which the cytoplasmic tail of the G-CSFR is replaced by the cytoplasmic tail of the EpoR. These findings indicate the necessity of the G-CSFR, as well as its downstream signaling pathways, in the neutrophil mobilization process (115). To map the region of the G-CSFR required for neutrophil mobilization, d715 and d715F mutant mice were used as described in Section 1.4, in which the carboxy-terminal 96 amino acids were truncated, leaving one remaining intracellular tyrosine residue (d715), or its mutation to phenylalanine (d715F) (Figure 1.6[B] and [C]). The d715F mice exhibited similar circulating neutrophil levels as wild type in response to G-CSF, indicating that STAT3 may be dispensable in this pathway, since the recruitment of STAT3 to the G-CSFR is abrogated in these mice (115). By contrast, d715 mice demonstrated approximately four-fold higher levels, revealing the requirement of the membrane-proximal 87 amino acids of the G-CSFR. These findings also suggest the presence of a negative regulator in the deleted segment (115). Bone marrow chimeras utilizing *Csf3r*^{-/-} and wild

type mice revealed that G-CSFR expression is required within the bone marrow compartment, not in stromal cells, to mediate this mobilization response (115). G-CSFR expression was not required on neutrophils to mediate neutrophil mobilization in response to G-CSF, indicating the possible role of *trans*-acting factors, believed to come from monocytes (115, 117). However, these data are from hematopoietic stem and progenitor cell mobilization studies (117) and are not conclusive evidence of the mechanism of neutrophil mobilization.

Another important aspect of neutrophil mobilization that is regulated by G-CSF is the SDF-1/CXCR4 axis. The chemokine SDF-1 (CXCL12) is constitutively expressed in the bone marrow, and contributes to neutrophil retention by interacting with its receptor CXCR4 expressed on the surface of neutrophils (118, 119). G-CSF treatment correlates with downregulation of SDF-1 and CXCR4 (120-122). SDF-1 is thought to be expressed by osteoblasts and endothelial cells (121), and can be cleaved by the proteases neutrophil elastase, cathepsin G, and matrix metalloproteinase 9 (MMP-9), which increase in the serum after G-CSF administration (123-125). G-CSF may also regulate the expression of SDF-1 by regulating osteoblast abundance (121, 126).

STAT3-deficient mice were found to have impaired acute neutrophil mobilization in response to G-CSF (91), indicating that STAT3 regulates important factors mediating neutrophil retention and/or release in the bone marrow, contrary to results from d715F mice (115). These factors are most likely chemokines and chemokine receptors, which will be discussed in the following section.

1.8. Chemokines and their receptors

Chemokines are subset of cytokines that play an important role in the activation and/or chemoattraction of leukocytes, and signal by interacting with seven-transmembrane G protein-coupled receptors (127). A nomenclature system was introduced that grouped chemokines and their receptors based on the position of conserved cysteine residues in the N-terminus (C, CC, CXC, CX3C) (128). CXC chemokines that contain a conserved sequence of glutamic acid (E), leucine (L), and arginine (R) immediately upstream of CXC, are denoted as ELR-CXC chemokines, and are potent neutrophil chemoattractants. In the human, ELR-CXC chemokines are $\text{GRO}\alpha$ (CXCL1), $\text{GRO}\beta$ (CXCL2), $\text{GRO}\gamma$ (CXCL3), ENA-78 (CXCL5), GCP-2 (CXCL6), NAP-2 (CXCL7), and IL-8 (CXCL8) (128).

Chemokines play opposing roles in neutrophil function by mediating retention in the bone marrow, as well as stimulating their release into circulating blood and subsequently inducing trafficking to sites of inflammation in tissues (127). As mentioned in the previous section, the chemokine SDF-1 (CXCL12) is constitutively expressed in the bone marrow, while its receptor CXCR4 is expressed on neutrophils and other hematopoietic cells (118, 119). Mice deficient in SDF-1 or CXCR4 demonstrate impaired myelopoiesis in the fetal liver and bone marrow, and chimeras generated with CXCR4-deficient fetal liver cells similarly have reduced levels of B cell and granulocytic precursor cells in the bone marrow (118, 119). However, these CXCR4-deficient chimeric mice have higher levels of immature circulating B cells and granulocytes, indicating

the importance of the SDF-1/CXCR4 axis in the retention of immature cells in the bone marrow (119). It has been shown that G-CSF treatment correlates with downregulation of SDF-1 and CXCR4, little is known about the molecular regulation of this pathway (120-122). The importance of the SDF-1/CXCR4 axis is demonstrated clinically in warts, hypogammaglobulinemia, infections, and myelokathexis, or WHIM syndrome. These patients exhibit truncation mutations in CXCR4, contributing to the retention of their neutrophils in the bone marrow (129). Lymphocyte numbers are reduced and lymphocyte functions are also affected, resulting in susceptibility to viral and bacterial infections (129).

By contrast, CXCR2, also known as interleukin-8 receptor (IL-8R) in humans, is the major chemokine receptor expressed on the murine neutrophil and mediates neutrophil migration (130). The ligands in the mouse are KC (CXCL1) and MIP-2 (CXCL2), which are potent neutrophil chemoattractants (130). When CXCR2 is knocked out, neutrophil recruitment to infected tissues is severely impaired, thereby rendering the host susceptible to pathogens (130, 131). However, CXCR2 has been implicated in many inflammatory disorders in which unregulated neutrophil recruitment has contributed to tissue damage and even death (132). Furthermore, the role of CXCR2 in angiogenesis, wound healing, and cancer is well documented (132-134). Because STAT3-deficient neutrophils exhibit impaired migration in response to CXCR2 ligands, further study is required to understand the role of STAT3 in this pathway (91).

1.9. Hypothesis

The requirement for STAT3 in the G-CSF mobilization pathway was previously demonstrated by our group; however, the mechanism was not fully understood (91). We hypothesize that G-CSF/STAT3 signals regulate the transcription of chemokines and chemokine receptors, contributing to the neutrophil mobilization response. In Chapter 3, we will examine role of G-CSF and STAT3 in regulating the expression of the chemokines MIP-2 (CXCL2) and SDF-1 (CXCL12), which affect neutrophil release and retention, respectively. Chapter 4 will explore the mechanism by which G-CSF and STAT3 control expression of CXCR2, the receptor for MIP-2. In Chapter 5, we will examine the molecular events that occur upon STAT3 activation at the promoter level. Taken together, our data suggest the mechanisms by which STAT3 controls the neutrophil migratory response to CXCR2 and its ligand MIP-2 (CXCL2).

Chapter 2: Materials and Methods

Bone marrow STAT3-deficient mice, neutrophil isolation

Hematopoietic STAT3-deficient mice were generated by breeding *Tg(Tek-cre)12Flv* (135) and *Stat3f/Δ* (136) mice. For genotyping, genomic DNA was isolated from tails, and analyzed by PCR using previously published primer sequences (135, 137). Littermate or aged-matched control mice, in which both STAT3 alleles were intact, were used between 5-8 weeks of age. C57BL/6NCr mice were obtained from the National Cancer Institute (NCI-Frederick, MD). All mice were maintained in a specific pathogen free (SPF) facility and used in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines at UT M. D. Anderson Cancer Center (UT MDACC).

Peripheral blood samples were collected into ethylenediaminetetraacetic acid (EDTA)-coated Vacutainer® tubes (Becton Dickinson) after retro-orbital puncture, and complete blood counts were determined using automated counting by the UT MDACC Department of Veterinary Medicine & Surgery.

Bone marrow cells were isolated from femurs and tibiae, and red blood cells were lysed using Red Blood Cell Lysing Buffer (Sigma). Cells were then resuspended in PBS containing 2% fetal bovine serum (FBS), before labeling with fluorescein isothiocyanate (FITC)-conjugated anti-Gr-1 (BD Biosciences) and allophycocyanin (APC)-conjugated anti-CD115 (eBioscience); Gr-1

negative, CD115⁺, Gr-1^{lo} CD115⁻ and Gr-1^{hi} CD115⁻ populations were sorted using a BD FACSAria.

To isolate endothelial cells, osteoblasts, and hematopoietic cells, femurs, tibiae, and iliac crests were isolated and minced after removal of the bone marrow. Bone fragments were incubated in PBS containing 3-mg/mL type II collagenase (Worthington) at 37°C for 2 hours with agitation. Cells were labeled with biotinylated antibodies against the lineage markers B220, CD11b, Gr-1, CD3, and Ter119, and then APC-conjugated streptavidin was used to exclude the lineage-positive cells (lin). The following antibodies were also added: Pacific Blue-conjugated anti-CD45, FITC-conjugated anti-CD31, and Phycoerythrin (PE)-conjugated anti-CD51 (all antibodies were from eBioscience). Samples were detected on a BD FACSAria for sorting into the following populations: lin⁺, lin⁻ CD45⁻ CD31⁺ endothelial cells, lin⁻ CD45⁻ CD31⁻ CD51⁺ osteoblasts, and lin⁻ CD45⁺ hematopoietic cells.

Flow cytometry

For CXCR2 staining, cells used were isolated from bone marrow as described above, then resuspended in PBS with 2% FBS and labeled with FITC-conjugated anti-Gr-1 (Pharmingen) and PE-conjugated anti-CXCR2 (R&D Systems). Samples were acquired using a BD FACSCalibur or BD LSR II, and the data were analyzed using FlowJo (Tree Star).

G-CSF and anti-MIP-2 administration *in vivo*

Following dilution in sterile PBS containing 0.1% low endotoxin bovine serum albumin (BSA, Sigma), recombinant human G-CSF (Amgen) was administered by subcutaneous injection (250µg/kg). 20µg of anti-mouse MIP-2 antibody or IgG_{2B} control (R&D Systems) was diluted in sterile PBS, as suggested by the manufacturer, and administered by intravenous injection 30 minutes before administration of G-CSF.

RNA isolation, real-time PCR

Total RNA was isolated from cells as indicated using Tri-Reagent® (Molecular Research Center), and reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR was performed using iQ SYBR Green Supermix, detected on the iQ5 or CFX96 Real-Time PCR Detection Systems and analyzed using iQ5 Optical System Software or CFX Manager Software, respectively (Bio-Rad), following the manufacturer's recommendations. Threshold cycle (C_T) values for each gene were normalized to the *Rpl13a* housekeeping gene to measure relative mRNA expression (ΔC_T). C_T values from chromatin immunoprecipitation samples were normalized to input control. Relative expression was determined using the following formula: $1.8^{\Delta C_T}$, as described in (138). Primers used were *Cxcl1* Forward 5'-CCGAAGTC-ATAGCCACACTCAA-3', *Cxcl1* Reverse 5'-GCAGTCTGTCTTCTTTTC-TCCGTTAC-3'; *Cxcl2* Forward 5'-AGACAGAAGTCATAGCCACTCTCAAG-3', *Cxcl2* Reverse 5'-CCTCCTTTCCAGGTCAGTTAGC-3'; *Cxcl12* Forward 5'-

GAGAGCCACATCGCCAGAG-3', *Cxcl12* Reverse 5'-TTTCGGGTCAA-TGCACACTTG-3'; *Il8rb* Forward 5'-AGCAAACACCTCTACTACCCTCTA-3', *Il8rb* Reverse 5'-GGGCTGCATCAATTCAAATACCA-3'; *Rpl13a* Forward 5'-GAGGTCGGGTGGAAGTACCA-3', *Rpl13a* Reverse 5'-TGCATCTTGGC-CTTTTCCTT-3'.

ELISA

Gr-1^{hi} neutrophils were isolated from the bone marrow, as described above, and cultured in RPMI containing 1% low endotoxin BSA (Sigma) and 1X Antibiotic-Antimycotic (Invitrogen) and stimulated with 25ng/ml rhG-CSF (Amgen), where indicated. Supernatants were isolated and analyzed for MIP-2 expression by ELISA using Mouse CXCL2/MIP-2 DuoSet (R&D Systems) following the manufacturer's instructions.

32D cell culture, retroviral transduction

The retroviral transfer vector pMX-G-CSFR-IRES-GFP was provided by Dr. Huiyuan Zhang. Retroviruses were generated by the introduction of the following plasmids in 293T cells by calcium phosphate transfection: transfer vector pMX-G-CSFR-IRES-GFP, pCMV-Gag-Pol packaging plasmid, and pHCMV-G plasmid encoding VSV.G envelope protein. 12 hours post-transfection, the media were replaced, and the supernatant was harvested 48 hours later. 32D cells expressing human G-CSFR (32D.G-CSFR) were generated by retroviral transduction using viral supernatants, with the addition of

4µg/ml Polybrene® (Hexadimethrine bromide, Sigma), and centrifuged at 2500 rpm for 90 minutes at room temperature. GFP-G-CSFR+ cells were enriched by sorting using a BD FACS Aria. Cells were maintained in RPMI (Invitrogen) supplemented with 10% FBS and 5-10% WEHI-conditioned media, as a source of IL-3. Untreated samples were generated by culturing 32D.G-CSFR in RPMI/10% FBS for at least 4 hours. Where indicated, cells treated with 25ng/ml rhG-CSF (Amgen) diluted in RPMI/10% FBS.

Identification of the *Il8rb* and *Cxcl2* promoters, reporter assays

The putative proximal promoters for *Cxcl2*, *Cxcl12*, and *Il8rb* were identified using Ensembl, and the putative STATx sites were found using TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>). The putative promoter sequences were amplified using AccuPrime™ Pfx DNA Polymerase (Invitrogen) and the following primers: *Cxcl2* Forward 5'-GGAGGTACCCTCAG-ACCCACAACCTATC-3' and *Cxcl2* Reverse 5'-GGAAGATCTGGCT-CTGAGGTCCCGAGA-3'; *Il8rb* Forward 5'-GGAGGTACCGCACAGCA-AGCTGAGAGG-3' and *Il8rb* Reverse 5'-GGAAGATCTGACCTGGGCTA-CCGATGGGGA-3'. Fragments were cloned into the pGL3-Basic plasmid (Promega) using KpnI and BglII restriction sites. The mutation in the STATx site was induced by PCR-based mutagenesis. Sequences were confirmed by the UT MDACC DNA Core Analysis Facility.

32D.G-CSFR cells were electroporated with 2 μ g of the appropriate reporter construct, 4 ng pTK-Renilla, and 0.5-1 μ g of any additional plasmids, as indicated. The Cell Line Nucleofector® Kit V (Lonza) and The Nucleofector® Device (Lonza) were used following the manufacturer's instructions. Cells were then plated in RPMI/10%FBS/5-10% WEHI-conditioned media. After 18 hours, cells were washed, and treated with 25ng/ml rhG-CSF (Amgen) for 6 or 12 hours, as indicated, prior to measurement of luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). In brief, cells were washed with PBS, and lysed with 1X Passive Lysis Buffer. Then, samples were mixed with Luciferase Assay Buffer, and firefly luciferase activity was measured by the Sirius Luminometer (Berthold Detection Systems). After addition of the Stop & Glo reagent, renilla luciferase activity was measured. Data were analyzed as a ratio of firefly light units:renilla light units. Additional plasmids used were: pRc/CMV-STAT3, encoding wild type STAT3 (from Dr. James Darnell); pMX-STAT3-DN-IRES-GFP, encoding a dominant negative form of STAT3, which contains mutations in critical DNA binding residues [derived from pBABE-STAT3-DN from Dr. Curt Horvath (20)]; and pRV-KM C/EBP α (from Dr. Chen Dong).

Chromatin immunoprecipitations (ChIP)

The Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore) was used following the manufacturer's instructions with the following antibodies: normal rabbit-IgG control (SantaCruz), anti-STAT3 c-20 antibody (SantaCruz), anti-

trimethyl-histone H3 (Lys4; Millipore), anti-trimethyl-histone H3 (Lys27; Millipore), anti-histone H3 (Abcam), and anti-RNA pol II CTD pSer5 (Active Motif). Primers used were as follows: *Cxcl2* Forward 5'-GGTCACTTCAGCGCAGAC-3', *Cxcl2* Reverse 5'-TCTGAGGTCCCGAGAGCT-3'; *Cxcl12* Forward 5'-CTCTTGTGTACAGCCTGAGA-3', *Cxcl12* Reverse 5'-GTCATCAGTGGAGACCTA-3'; *Il8rb* Forward 5'-CTCCCAAGTTAGGTAG-CATTTCCAC-3', *Il8rb* Reverse 5'-TACCTGTTTGCCTGTAGGCAGGTA-3'; *Socs3* Forward 5'-CACAGCCTTTCAGTGCAGAG-3', *Socs3* Reverse 5'-GCGAATCAGGCAAAGGAC-3'. For detection of RNA pol II CTD pSer5, the following primers were utilized: *Il8rb* Forward 5'-CCCAGAACAGCCTAGCCA-3', *Il8rb* Reverse 5'-GGCTCCCAACTCTCTGTG-3'; *Cxcl2* Forward 5'-GACCCT-GAGCTCAGGGAA-3', *Cxcl2* Reverse 5'-AGTGTGGCTGGAGTCTGG-3'.

Chemotaxis assays

Bone marrow neutrophils were resuspended in DMEM (Invitrogen) containing 0.1% low endotoxin BSA (Sigma) and plated in the upper chamber of 3µm Transwells® 6.5mm diameter inserts with polycarbonate membranes (Corning). DMEM/BSA in the absence or presence of 250 ng/ml MIP-2 was placed in the lower chamber. After incubation for 3 hours at 37°C, cells that migrated to the lower chamber were counted.

Immunoblotting

Whole cell lysates were generated using Laemmli lysis buffer (80 mM Tris-Cl pH 6.8, 2% SDS, 15% glycerol, 1% β -mercaptoethanol, 0.01% bromophenol blue) and heat-denatured at 100°C. Samples were separated using SDS-PAGE, and transferred to nitrocellulose membranes. After blocking in 5% nonfat dry milk for 1 hour at room temperature, membranes were incubated overnight at 4°C in primary antibody diluted in blocking buffer. Antibodies used were against STAT3 c-20 (SantaCruz), phospho-STAT3 Y705 (Cell Signaling), C/EBP α 14AA (SantaCruz), Histone H3 (Abcam). After washing in PBS/0.1% Tween-20 (Sigma), membranes were incubated with the appropriate horseradish peroxidase (HRP)-linked secondary antibody (Amersham). Samples were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce) before exposure to radiographic film.

Infection with *Listeria monocytogenes*

Experiments were performed with *L. monocytogenes* strain 1043S, generously provided by Dr. Chen Dong and Dr. Hao Shen. The bacteria were first inoculated onto brain heart infusion (BHI) agar plates containing 5 μ g/ml erythromycin. A single colony from the plate was inoculated into BHI broth/erythromycin and grown overnight at 37°C. A portion of the overnight culture was reinoculated and grown to an optical density at OD₆₀₀ = 0.1. The bacteria were suspended in sterile PBS at a final concentration of 1X10⁵/mL. A volume of 200 μ l of bacteria (2x10⁴ cells) was injected intravenously. Peripheral

blood and tissue samples were collected 12 hours post-infection. The levels of circulating neutrophils were determined by CBC, as described above. Tissues were homogenized in sterile PBS containing 0.05% Triton X-100, serially diluted, and plated on BHI agar to determine the number of colony forming units (CFU).

STAT3 inhibition

Before use, Stattic (Sigma) was dissolved in dimethylsulfoxide (DMSO; Sigma), and diluted to a final concentration of 50 μ M in RPMI/10% FBS/1X Antibiotic-Antimycotic (Invitrogen). Cells were pretreated with Stattic/DMSO or DMSO alone for 1 hour at 37°C before treatment with 25ng/ml G-CSF for 6 hours.

Statistical analyses

Shown are mean values \pm standard error mean (SEM). *P* values were determined by unpaired two-tailed Student's *t*-tests using GraphPad Prism version 5 for Mac OS X (<http://www.graphpad.com>). *P* values < 0.05 were considered statistically significant.

Chapter 3: G-CSF alters expression of chemokines in the bone marrow microenvironment affecting neutrophil retention and release in a STAT3-dependent manner

3.1. Background and rationale

G-CSF is administered clinically to boost circulating neutrophil numbers by initiating their release from the bone marrow in a process called mobilization, but its molecular mechanism of action is not fully understood. G-CSF lacks intrinsic chemotactic ability, as its use in migration assays *in vitro* does not induce murine neutrophil migration (139). Furthermore, G-CSF administration into the peritoneal cavity is sufficient to induce neutrophil egress from the bone marrow, but does not stimulate neutrophil migration to the site of injection (139). Taken together, these data indicate that G-CSF likely acts through induction of secondary effector molecules, possibly from generation of *trans*-acting signals (115).

G-CSF administration in humans is reported to induce expression of IL-8 (CXCL8), a potent neutrophil chemoattractant (140). While there is no murine IL-8, the chemokines KC (CXCL1) and MIP-2 (CXCL2) are considered its functional homologues in the mouse, as all three bind the same receptor, CXCR2, the major chemokine receptor expressed on the murine neutrophil (141). KC and MIP-2, like IL-8, are primary mediators of neutrophil migration. In addition to stimulating the release of neutrophils from the bone marrow, they can

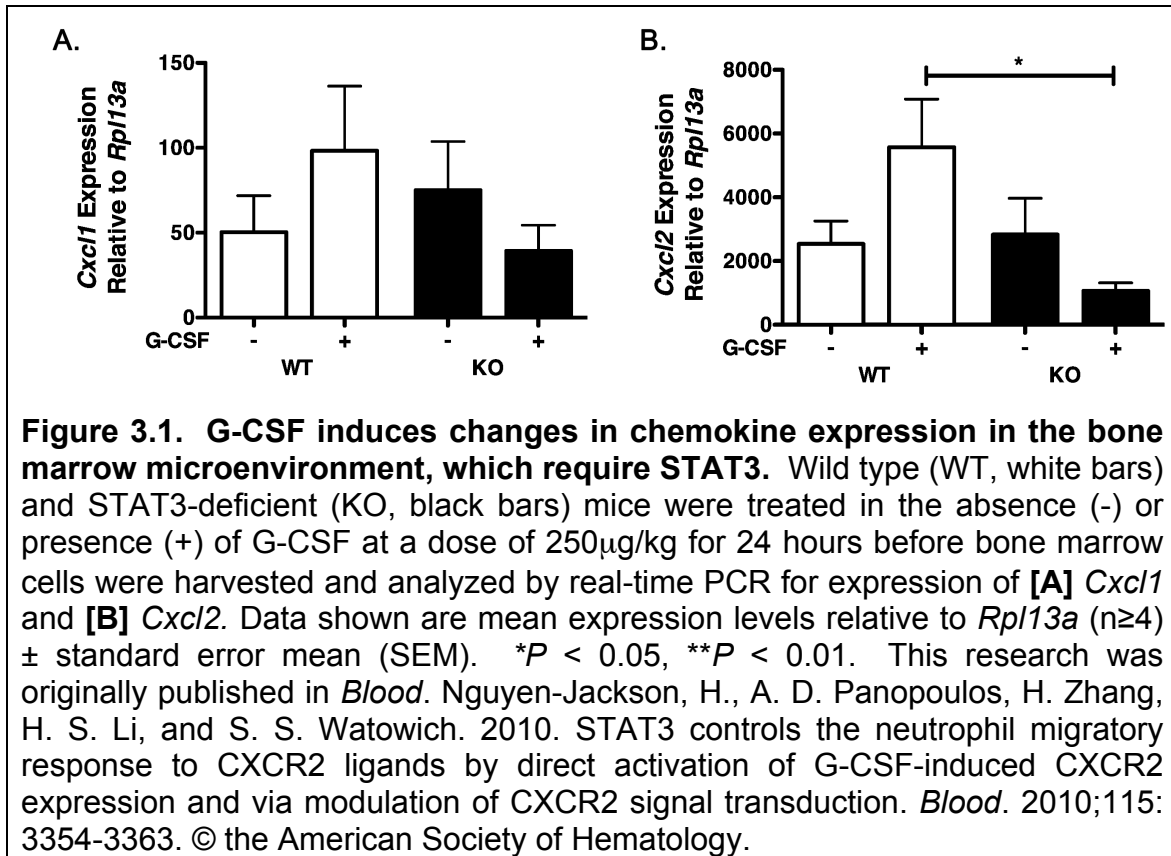
also induce their migration into the tissue (142). *Il8rb*^{-/-} mice that are deficient in CXCR2 exhibit high levels of peripheral neutrophils relative to their wild type counterparts, likely due to the inability of neutrophils to marginate to the tissue, resulting in their accumulation in the blood (130).

In contrast to the function of KC and MIP-2, SDF-1 (CXCL12) contributes to the retention of hematopoietic cells in the bone marrow via interaction with its receptor CXCR4 (118, 119). SDF-1 and CXCR4 are downregulated upon G-CSF treatment, and while this response has been hypothesized to promote neutrophil mobilization, the mechanism is unknown (120-122). Here we test whether G-CSF/STAT3 signals regulate transcription of the chemokines MIP-2, KC, and SDF-1, contributing to neutrophil mobilization.

3.2. Results

G-CSF-induced *Cxcl2* expression in the bone marrow is mediated by Gr-1^{hi} mature neutrophils.

To test the role of STAT3 in regulation of neutrophil chemoattractants, we analyzed the expression of KC and MIP-2 in the bone marrow microenvironment of wild type and STAT3-deficient mice treated with G-CSF.



STAT3-deficient and wild type mice were injected with G-CSF or left untreated, and total bone marrow was harvested 24 hours later, and analyzed by real-time PCR for expression of *Cxcl1* and *Cxcl2*, the genes that encode for KC and MIP-2, respectively. We found that G-CSF induced upregulation of *Cxcl1* and *Cxcl2* in wild type bone marrow, with *Cxcl2* expressed at a much higher level than *Cxcl1* (Figures 3.1[A] and [B]). However, this upregulation was not detected in the STAT3-deficient mice, indicating a role for STAT3 in G-CSF-induced stimulation of *Cxcl1* and *Cxcl2* (Figures 3.1[A] and [B]). These data suggest that STAT3 is required for G-CSF-induced regulation of chemokine

expression in the bone marrow microenvironment, which may contribute to the diminished neutrophil mobilization response of STAT3-deficient animals.

Next, we set out to determine which cells in the bone marrow are the primary producers of *Cxcl2*. Because of the low levels of *Cxcl1* detected in the total bone marrow, and since *Cxcl1* expression is thought to be mediated mostly by fibroblasts, endothelial cells, and megakaryocytes (143-145), we focused on analysis of *Cxcl2* expression. Again, STAT3-deficient and wild type mice were injected with G-CSF or left untreated, and total bone marrow was harvested 24 hours later. Because monocytes/macrophages and neutrophils are thought to be the primary mediators of *Cxcl2* expression under inflammatory conditions (143, 146, 147), bone marrow cells were sorted into Gr-1⁻ cells, monocytes (Gr-1⁺ CD115⁺), immature neutrophils (Gr-1^{lo} CD115⁻), and mature neutrophils (Gr-1^{hi} CD115⁻) (91, 148). Gr-1⁻ cells and CD115⁺ monocytes expressed very low levels of *Cxcl2* (Figure 3.2). This result is consistent with data from bone marrow derived macrophages, which showed no G-CSF-induced expression of *Cxcl2* (data not shown). By contrast, under basal conditions, Gr-1^{hi} mature neutrophils express the highest levels of *Cxcl2* in the bone marrow (Figure 3.2). G-CSF induces a 3-fold increase in *Cxcl2* expression in immature neutrophils, and nearly a 2-fold increase in mature neutrophils, with levels approximately 10-fold higher than in the former (Figure 3.2).

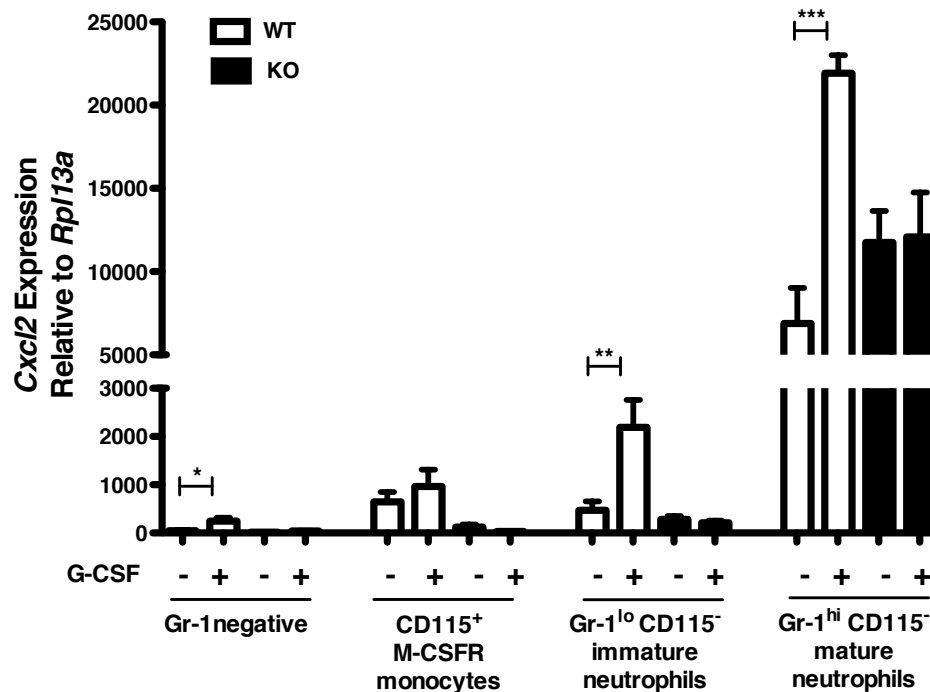
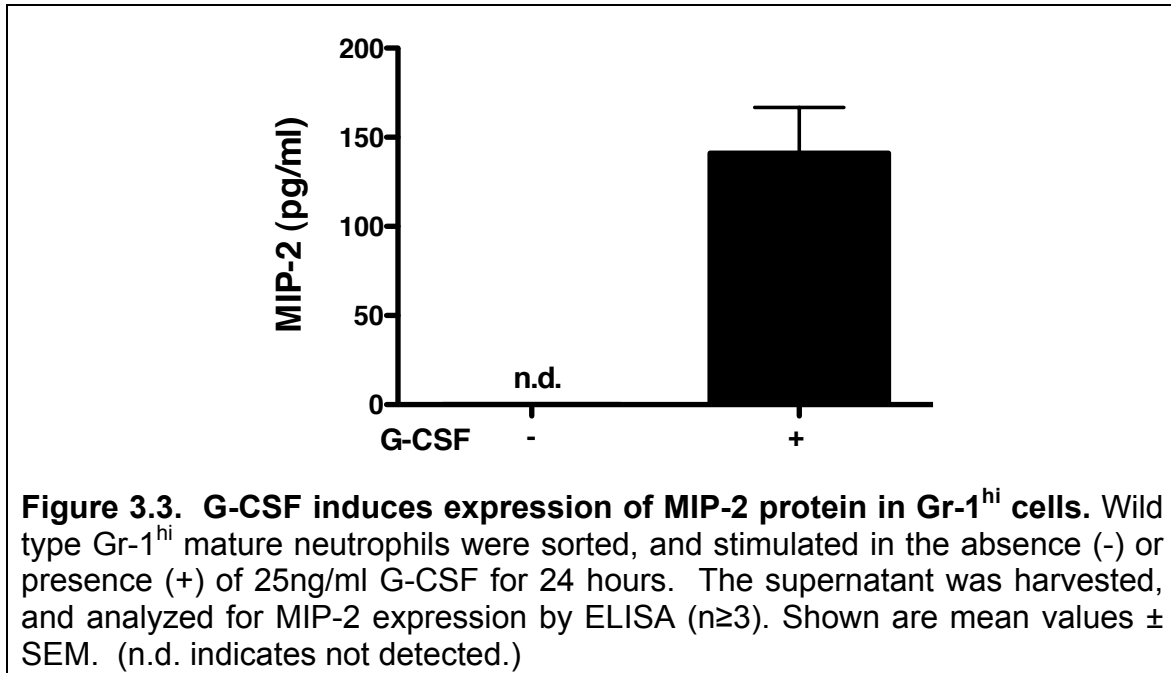


Figure 3.2. G-CSF-induced *Cxcl2* expression in the bone marrow is mediated by mature neutrophils. Wild type (WT, white bars) and STAT3-deficient (KO, black bars) mice were treated in the absence (-) or presence (+) of G-CSF at a dose of 250 μ g/kg for 24 hours before bone marrow cells were harvested, sorted, and analyzed by real-time PCR for expression of *Cxcl2* (n \geq 3). Shown are mean expression levels relative to housekeeping gene *Rpl13a* \pm SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

To verify expression of MIP-2 protein, immature and mature neutrophils were sorted, and treated with G-CSF *in vitro*. Supernatants were collected after 24 hours, and analyzed for MIP-2 protein expression by ELISA. While MIP-2 levels were below the limit of detection in Gr-1^{lo} immature neutrophils (data not shown), approximately 150 pg/ml of MIP-2 was detected in the supernatants of Gr-1^{hi} mature neutrophils stimulated with G-CSF (Figure 3.3). Taken together, these data demonstrate that G-CSF administration *in vivo* results in expression

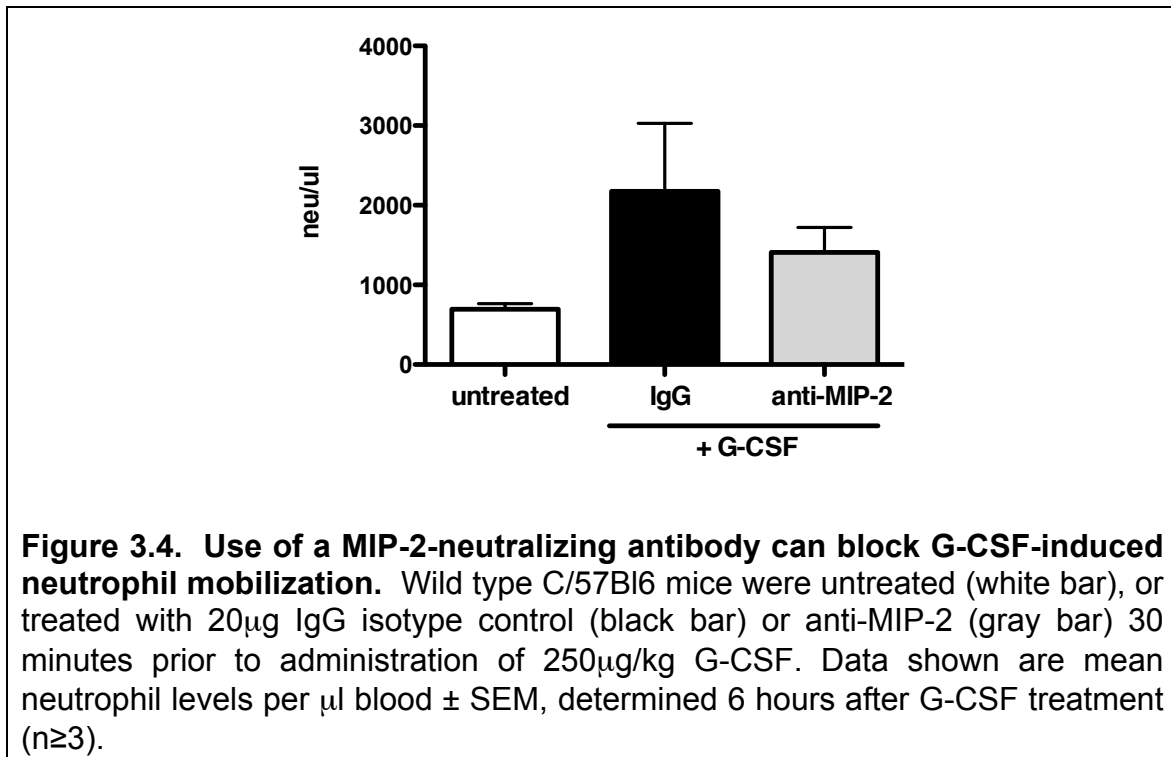
of *Cxcl2* mRNA in the bone marrow, primarily mediated by Gr-1^{hi} mature neutrophils.



G-CSF-stimulated neutrophil mobilization can be inhibited by a MIP-2 neutralizing antibody

To demonstrate that MIP-2 mediates G-CSF-induced neutrophil mobilization, we utilized a MIP-2-neutralizing antibody *in vivo*. We injected anti-MIP2 or isotype control antibody intravenously 30 minutes before administration of G-CSF, and analyzed neutrophil levels in peripheral blood 6 hours later. Mice treated with IgG control antibody and G-CSF demonstrated a 3-4-fold increase in peripheral neutrophils, while mice treated with MIP-2 neutralizing antibody exhibited a 2-fold increase (Figure 3.4). Because MIP-2 neutralization dampens

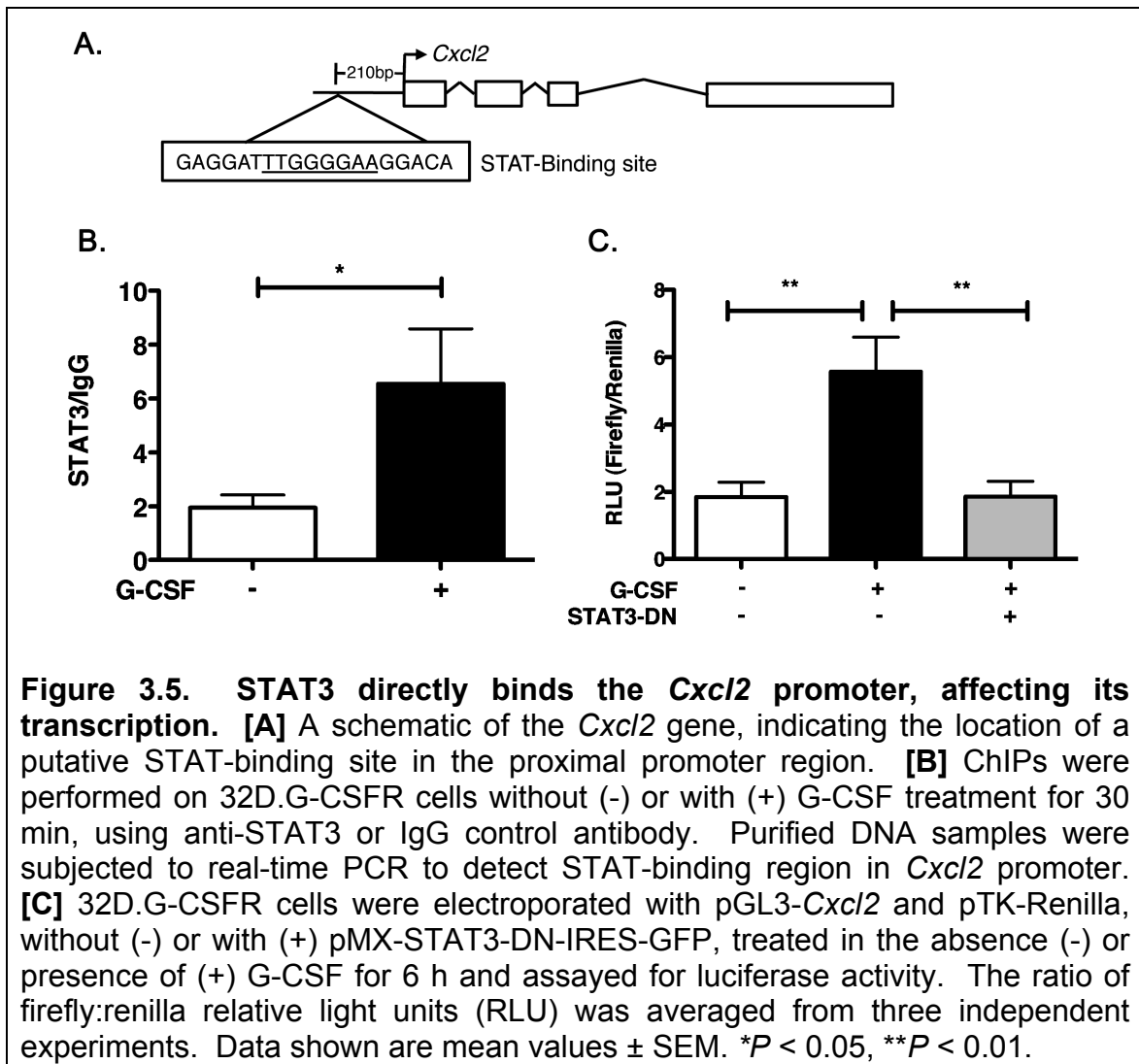
the neutrophil mobilization response to G-CSF, these results suggest that MIP-2 contributes to this pathway.



G-CSF-induced transcription of *Cxcl2* is directly regulated by STAT3

To examine mechanisms of G-CSF-regulated *Cxcl2* expression, we analyzed the *Cxcl2* proximal promoter, which revealed a putative STAT-binding site approximately 200bp from the transcription start site (Figure 3.5[A]). To examine whether STAT3 binds this site, chromatin immunoprecipitations (ChIPs) were performed using 32D.G-CSFR cells, a murine myeloid progenitor cell line (73). We found that G-CSF stimulation induced STAT3 recruitment to the *Cxcl2* promoter within 30 minutes (Figure 3.5[B]). Next, a luciferase reporter construct

was generated containing the proximal promoter sequence encompassing this STAT site. G-CSF induced a 3-fold increase in luciferase activity that was suppressed by the co-expression of a STAT3 DNA-binding mutant that acts as a dominant negative protein (STAT3-DN; Figure 3.5[C]) (77). Altogether, the data support the idea that STAT3 directly binds the *Cxcl2* promoter, controlling its transcription.



G-CSF induces STAT3 binding to the *Cxcl12* promoter; expression of *Cxcl12* in the bone marrow microenvironment is mediated by osteoblasts

In contrast to the roles of KC and MIP-2 in the neutrophil mobilization pathway, SDF-1 is thought to mediate neutrophil retention in the bone marrow, and its expression is downregulated upon G-CSF administration (120, 121). To test the requirement for STAT3 in this pathway, total bone marrow cells were isolated from wild type and STAT3-deficient mice with or without treatment with G-CSF, and assayed for expression of *Cxcl12*, the gene encoding SDF-1. Consistent with previous reports (120, 121), G-CSF administration suppressed *Cxcl12* expression in wild type mice (Figure 3.6). Interestingly, G-CSF treatment resulted in a significant increase in *Cxcl12* expression in STAT3-deficient mice (Figure 3.6).

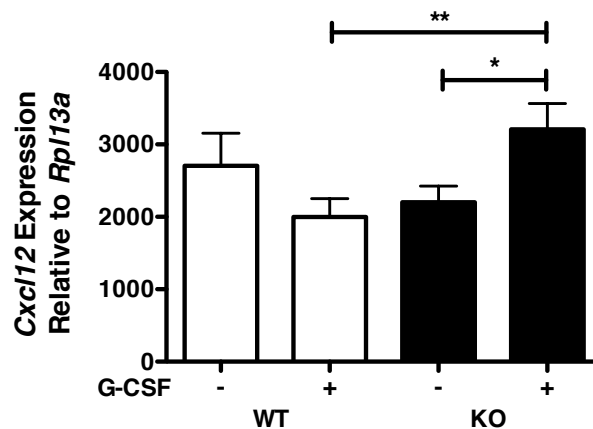
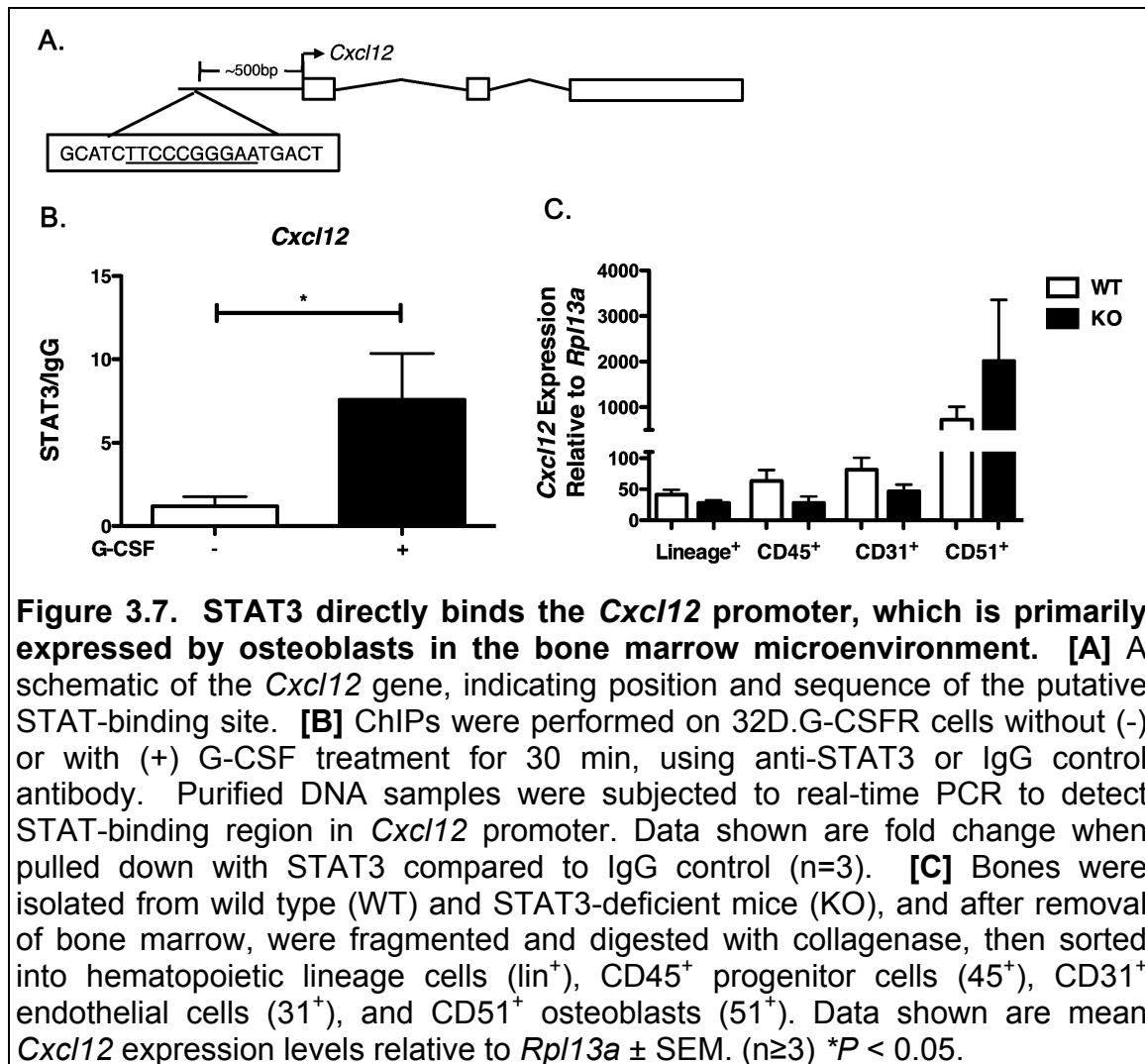


Figure 3.6. G-CSF-induced downregulation of *Cxcl12* is STAT3-dependent. Wild type (WT, white bars) and STAT3-deficient (KO, black bars) mice were treated in the absence (-) or presence (+) of G-CSF at a dose of 250 µg/kg for 24 hours before bone marrow cells were harvested and analyzed by real-time PCR for expression of *Cxcl12*. Data shown are mean expression levels relative to *Rpl13a* ($n \geq 4$) \pm SEM. * $P < 0.05$, ** $P < 0.01$. This research was originally published in *Blood*. Nguyen-Jackson, H., A. D. Panopoulos, H. Zhang, H. S. Li, and S. S. Watowich. 2010. STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. *Blood*. 2010;115: 3354-3363. © the American Society of Hematology.

The proximal promoter of *Cxcl12* contains a putative STAT-binding site approximately 500bp from the transcription start site (Figure 3.7[A]). Chromatin immunoprecipitations were performed, which demonstrated that G-CSF induces STAT3 to bind in the region of the putative STAT site in the *Cxcl12* promoter (Figure 3.7[B]). To identify the cell type that mediates *Cxcl12* expression in the bone marrow, bone fragments were digested with collagenase, and sorted into four populations: lineage-positive cells (B220⁺ CD11b⁺ Gr-1⁺ CD3⁺ Ter119⁺), endothelial cells (lin⁻ CD45⁻ CD31⁺), osteoblasts (lin⁻ CD45⁻ CD31⁻ CD51⁺), and progenitor cells (lin⁻ CD45⁺). Consistent with a previous study (121), we found

that osteoblasts are the primary cell type to express *Cxcl12* (Figure 3.7[C]). Further studies are required to identify the mechanism by which STAT3 regulates expression of *Cxcl12* in osteoblasts.



3.3. Discussion

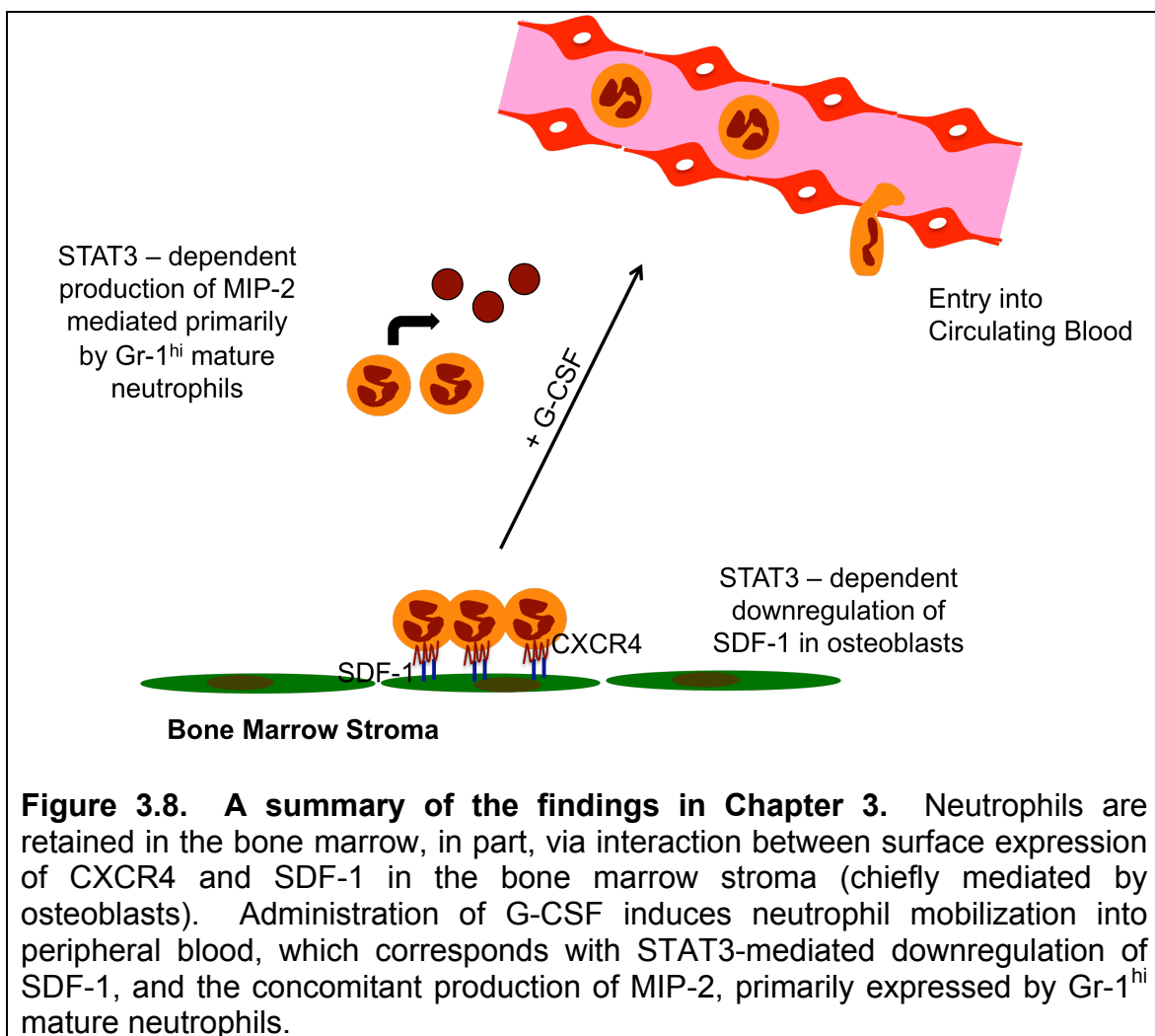
As G-CSF stimulates neutrophil release from the bone marrow, we demonstrate its effects on chemokine expression, likely affecting retention and subsequent mobilization. In total wild type bone marrow, we show that G-CSF upregulates the mRNA expression of the neutrophil chemoattractants *Cxcl1* and *Cxcl2*, while downregulating expression of the retention signal *Cxcl12*, as summarized in Figure 3.8. Further examination of the cellular and molecular mechanisms of *Cxcl2* expression in the bone marrow revealed that it is directly regulated by STAT3 and mediated by mature neutrophils. G-CSF induction of MIP-2 expression is consistent with previous results demonstrating that G-CSF does not directly stimulate neutrophil chemotaxis (139) and G-CSF-induced neutrophil mobilization requires the action of *trans*-acting factors (115). Additionally, patients receiving G-CSF treatments reported an increase in serum levels of IL-8 (140), and knockouts of CXCR2, the IL-8 receptor in mice, do not upregulate circulating neutrophils in response to G-CSF (142, 145). Several recent reports support that KC and MIP-2 are produced in the bone marrow upon G-CSF administration, but conflict as to the mechanism (145, 149). One study demonstrated that G-CSF-induced MIP-2 expression in the bone marrow, and MIP-2 protein was detected in the bone marrow supernatant, but attributed the expression of MIP-2 to endothelial cells (149). In Figure 3.2, we demonstrated that the Gr-1⁻ population also exhibited G-CSF-induced expression of *Cxcl2*, but to a much lesser extent when compared to the Gr-1⁺ populations. Another study described that G-CSF-induced upregulation of

thrombopoietin (TPO) in the bone marrow, which in turn induced production of KC and MIP-2 (145). G-CSF did not directly induce this response in megakaryocytes *in vitro*, but KC was expressed upon treatment with TPO (145). Neutrophil mobilization in response to G-CSF was abrogated in CXCR2-deficient mice, or upon addition of a CXCR2-neutralizing antibody (145). Their demonstration that G-CSF induces expression of KC and MIP-2 in the bone marrow aligns with our results; however, we did not test the effects of direct administration of TPO in our model, nor did we examine the contribution of megakaryocytes. Collectively, our data and published findings support the idea that G-CSF mediates neutrophil release by inducing expression of the chemokines KC and MIP-2. Moreover, it was previously demonstrated in our lab that both MIP-2- and G-CSF-stimulated neutrophil mobilization requires STAT3 (91, 150), further suggesting a functional relationship between these pathways.

In contrast to our MIP-2 findings, we showed that G-CSF suppressed bone marrow *Cxcl12* mRNA expression in wild type mice. Interestingly, G-CSF stimulated an increase in *Cxcl12* mRNA expression in STAT3-deficient bone marrow. Because *Cxcl12* mRNA levels closely follow SDF-1 protein levels, it is thought expression of this gene is regulated transcriptionally (121). Further analysis revealed that STAT3 directly binds the *Cxcl12* promoter, which is consistent with a previous report demonstrating similar ChIP results in vascular smooth muscle cells (151). STAT3 may cause transcriptional repression of *Cxcl12*, though this requires further testing. As basal expression of CXCR4 and CXCR4-mediated chemotaxis are not affected in STAT3-deficient mice [A. D.

Panopoulos and S. S. Watowich, unpublished results, and (150)], we hypothesize that G-CSF/STAT3 signals regulate *Cxcl12* expression and not CXCR4 expression or signaling. We demonstrated that osteoblasts are the primary cells to express *Cxcl12* in the bone marrow, in contrast to a previous report also demonstrating its expression in endothelial cells (121). Others have shown that G-CSF likely regulates SDF-1 expression by affecting the abundance of osteoblasts (121, 126). Because osteoblasts develop from the mesoderm, the contribution of STAT3 to their differentiation can be studied *in vitro* through mesenchymal stem cells (152), which we did not address. Bone maintenance is a dynamic process involving osteoblasts, the bone building cells, and osteoclasts, which are responsible for bone resorption (153). Therefore, osteoblast numbers are regulated by osteoclasts, which share the monocyte lineage (153). This is the more likely scenario in our model, as osteoclastogenesis, or the generation of osteoclasts, is shown to be indirectly regulated by STAT3 (154), although this was not addressed in this study. Another mechanism by which G-CSF may regulate SDF-1 expression is through activation of proteolytic cleavage of SDF-1, as levels of proteases increase in the serum after G-CSF administration (123-125). While hematopoietic progenitor cell mobilization was not affected in protease-deficient mice, neutrophil mobilization was not directly tested, and thus the contribution of proteases to this process is not fully understood (155). Taken together, our findings indicate that G-CSF/STAT3 signals upregulate *Cxcl1* (KC) and *Cxcl2* (MIP-2) expression, while negatively regulating *Cxcl12* (SDF-1), in the bone

marrow of wild type animals, with *Cxcl2* expression attributed to mature Gr-1^{hi} neutrophils and *Cxcl12* expression implicated in osteoblasts. This is consistent with the proposed role for these chemokines in the neutrophil mobilization response. These data suggest that aberrant regulation of chemokine expression in the bone marrow may contribute to the diminished neutrophil mobilization response of STAT3-deficient animals.



Chapter 4: STAT3 controls G-CSF-regulated expression of the chemokine receptor CXCR2 in murine neutrophils

4.1. Background and rationale

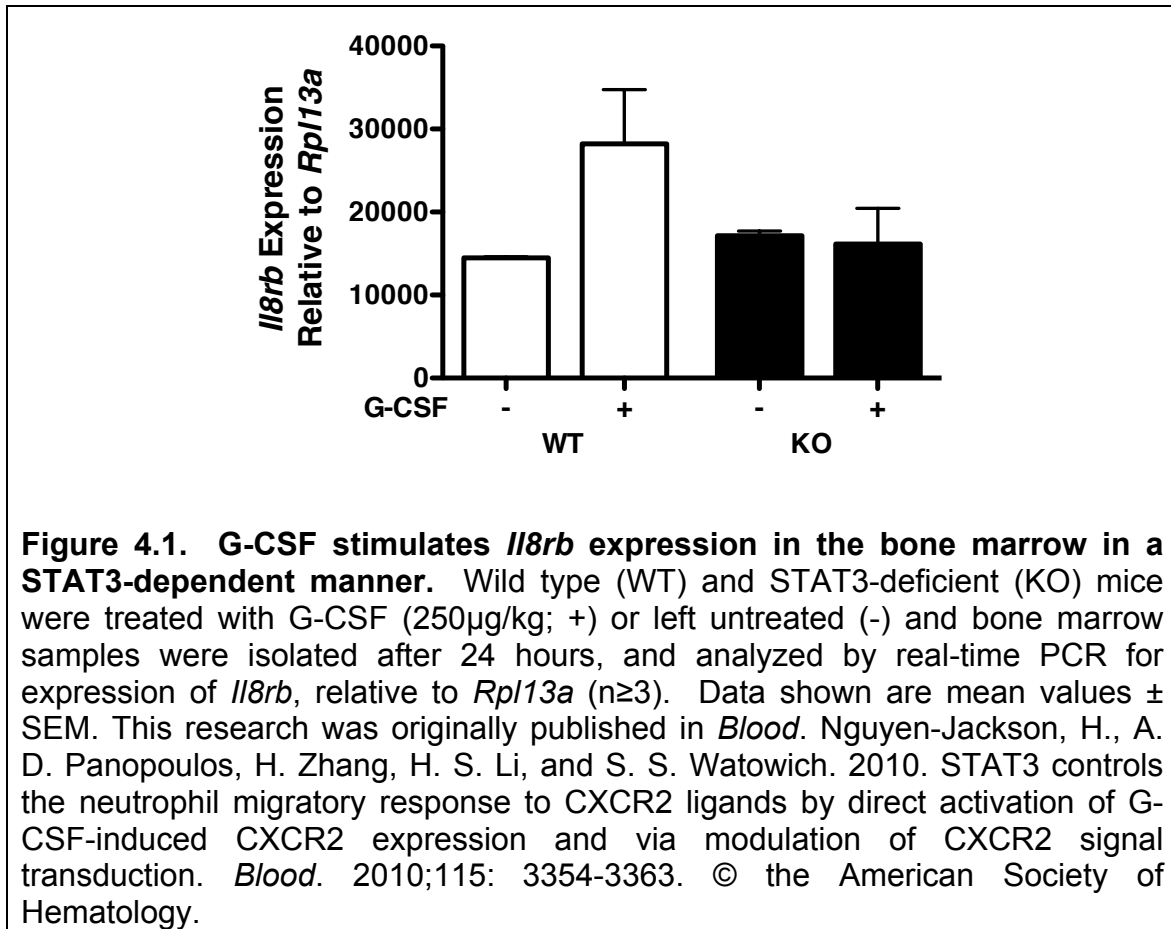
In Chapter 3, we demonstrated the role for STAT3 in regulating G-CSF-induced expression of the chemokine *Cxcl2*; expression of its receptor CXCR2 is also reportedly regulated by G-CSF (156), though the mechanism is not fully understood. It was also demonstrated that neutrophils require STAT3 for efficient migration toward ligands of the chemokine receptor CXCR2 *in vitro* (91), and *Il8rb*^{-/-} mice (deficient in CXCR2) do not demonstrate G-CSF-induced upregulation of circulating neutrophils (142). While there is evidence that STAT3 and G-CSF may regulate the CXCR2 response, such as CXCR2-driven chemotaxis and CXCR2-mediated actin polymerization, the mechanisms are unclear (91). In this chapter, we will examine how G-CSF/STAT3 signals control CXCR2 expression and migratory response in neutrophils.

4.2. Results

G-CSF induces expression of *Il8rb* in a STAT3-dependent pathway

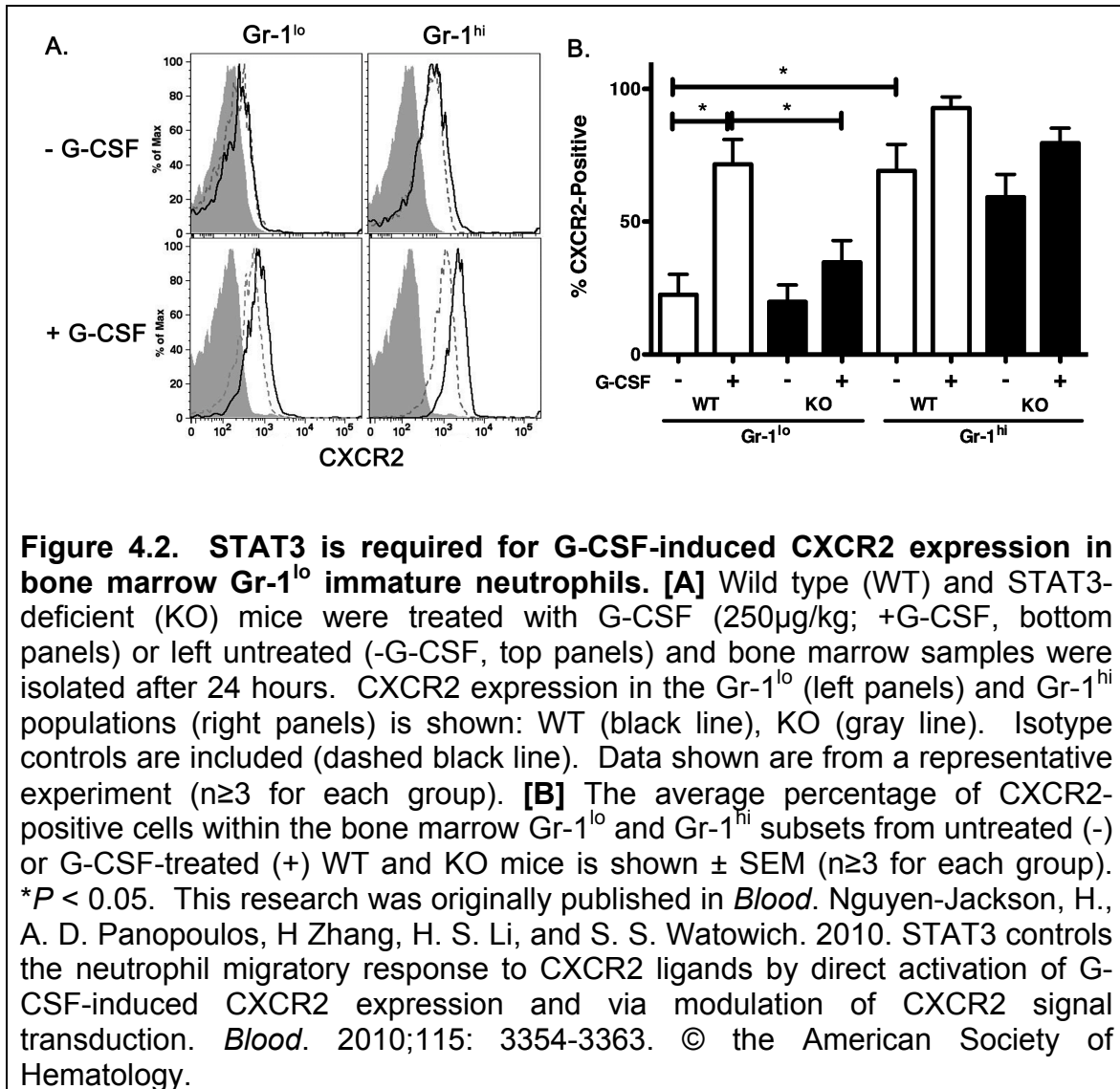
To test the role of STAT3 in regulating expression of *Il8rb*, the gene encoding CXCR2, we administered G-CSF *in vivo*, and measured *Il8rb* levels in

total bone marrow. We found that in wild type cells, G-CSF induced an upregulation in *Il8rb* that was not detected in STAT3-deficient marrow (Figure 4.1).



As neutrophils are the primary cell type to express CXCR2 (130), we tested the effects of G-CSF on CXCR2 expression in immature and mature neutrophil subsets from wild type and STAT3-deficient mice at steady state. We found that almost all mature Gr-1^{hi} neutrophils expressed cell surface CXCR2

and no significant differences were found between wild type and STAT3-deficient cells (Figure 4.2[A] and [B]).



In contrast to mature cells, immature Gr-1^{lo} granulocytes showed lower levels of CXCR2 expression on the cell surface, as judged by differences in mean fluorescence intensity (MFI) (MFI=1007 ± 126 for wild type Gr-1^{lo} vs. MFI=2877 ± 295 for wild type Gr-1^{hi} neutrophils, P<0.001) (Figure 4.2[A]). As

with Gr-1^{hi} cells, there were no differences between wild type and STAT3-deficient Gr-1^{lo} cells. Consistent with cell surface expression, we found that *Il8rb* mRNA levels were similar in wild type and STAT3-deficient immature Gr-1^{lo} granulocytes (Figure 4.3). *Il8rb* mRNA levels were significantly elevated in Gr-1^{hi} neutrophils, relative to immature granulocytes, and no obvious differences were found between wild type and STAT3-deficient cells (Figure 4.3). These data indicate that steady state *Il8rb* mRNA and CXCR2 protein amounts are regulated during neutrophil differentiation, and the pathway that controls this developmental expression is independent of STAT3. Hence, STAT3 appears to be dispensable for regulation of CXCR2 expression during neutrophil development in steady state conditions.

Studies from our lab and others suggest that the mechanisms regulating neutrophils at steady state are distinct from those that operate in emergency, or demand-driven, conditions that require G-CSF (19, 91, 93). Since STAT3 is critical for emergency neutrophil responses (91), we tested whether it was required to regulate CXCR2 in response to G-CSF administration. Wild type and STAT3-deficient mice were treated with a single dose of G-CSF or left untreated, and neutrophils were isolated and examined for CXCR2 expression by flow cytometry or real-time PCR 24 hours later. These experiments showed that cell surface CXCR2 expression was induced by G-CSF treatment in wild type immature Gr-1^{lo} granulocytes (Figure 4.2[A] and [B]). In mature Gr-1^{hi} neutrophils, G-CSF upregulated cell surface CXCR2 expression on wild type cells compared to untreated controls (MFI=2877 ± 295 for untreated Gr-1^{hi} vs.

5212 \pm 675 for Gr-1^{hi} + G-CSF) (Figure 4.2[A] and [B]). In contrast to results in wild type cells, G-CSF did not induce cell surface CXCR2 expression in STAT3-deficient immature or mature neutrophil subsets (Figure 4.2[A] and [B]). Furthermore, STAT3 was required for G-CSF-dependent upregulation of *Il8rb* mRNA in Gr-1^{lo} granulocytes (Figure 4.3).

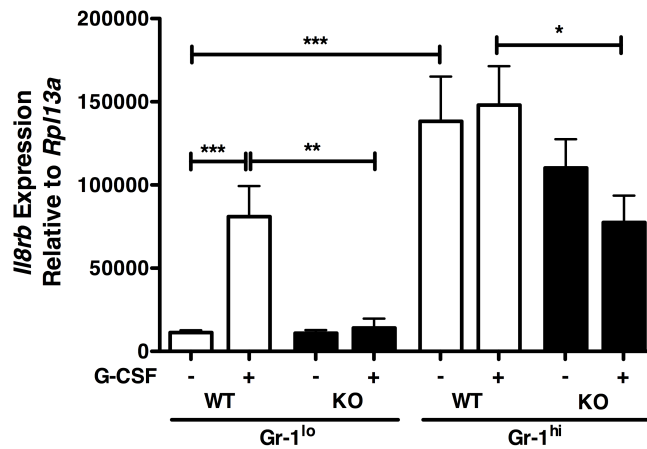


Figure 4.3. STAT3 is required for G-CSF-induced *Il8rb* expression in Gr-1^{lo} immature granulocytes. WT and STAT3-deficient (KO) mice were untreated (-) or treated with G-CSF (+) and bone marrow samples were isolated after 24 hours, and sorted into Gr-1^{lo} and Gr-1^{hi} subsets. *Il8rb* mRNA levels were relative to the housekeeping gene *Rpl13a*. Shown are mean relative expression levels \pm SEM (n \geq 4). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. This research was originally published in *Blood*. Nguyen-Jackson, H., A. D. Panopoulos, H. Zhang, H. S. Li, and S. S. Watowich. 2010. STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. *Blood*. 2010;115: 3354-3363. © the American Society of Hematology.

Because the bone marrow Gr-1^{lo} population may also contain monocytes, we re-analyzed CXCR2 expression in wild type and STAT3-deficient Gr-1^{lo} cells excluding those positive for CD115⁺ (macrophage colony-stimulating factor receptor, or M-CSFR). Gr-1^{lo} CD115⁻ cells comprise >80% of the Gr-1^{lo}

population, and similar patterns of CXCR2 cell surface and mRNA expression relative to the total Gr-1^{lo} subset were found (data not shown). Therefore, our results indicate that STAT3 is necessary to enhance CXCR2 expression in the immature Gr-1^{lo} granulocyte subset during G-CSF administration *in vivo*, at least in part via induction of *Il8rb* mRNA expression. Thus, our data collectively indicate that STAT3 controls the induction of *Il8rb* mRNA and CXCR2 protein amounts during systemic G-CSF administration, while being dispensable for basal expression.

To examine the function of G-CSF-induced CXCR2 expression, immature Gr-1^{lo} and mature Gr-1^{hi} granulocytes were isolated from G-CSF-stimulated or untreated wild type and STAT3-deficient mice and chemotaxis was assayed in response to MIP-2. G-CSF treatment *in vivo* enhanced CXCR2-mediated chemotaxis of immature and mature neutrophil subsets in both groups, although the migratory response was significantly reduced in STAT3-deficient cells relative to wild type (Figure 4.4). Wild type immature Gr-1^{lo} granulocytes from G-CSF-treated animals showed significantly more chemotactic activity toward MIP-2, compared to Gr-1^{lo} cells from untreated animals, while G-CSF-induced chemotactic activity was attenuated in STAT3-deficient Gr-1^{lo} granulocytes (Figure 4.4). These results are consistent with our previous observation of effective mobilization of immature Gr-1^{lo} neutrophils in wild type but not STAT3-deficient mice upon G-CSF treatment (91). Thus, our data collectively demonstrate that STAT3 controls the induction of *Il8rb* mRNA and CXCR2

protein in immature Gr-1^{lo} granulocytes during systemic G-CSF administration, correlating with their enhanced migratory potential.

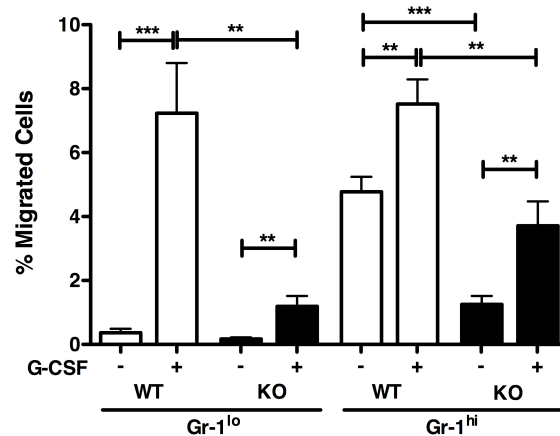
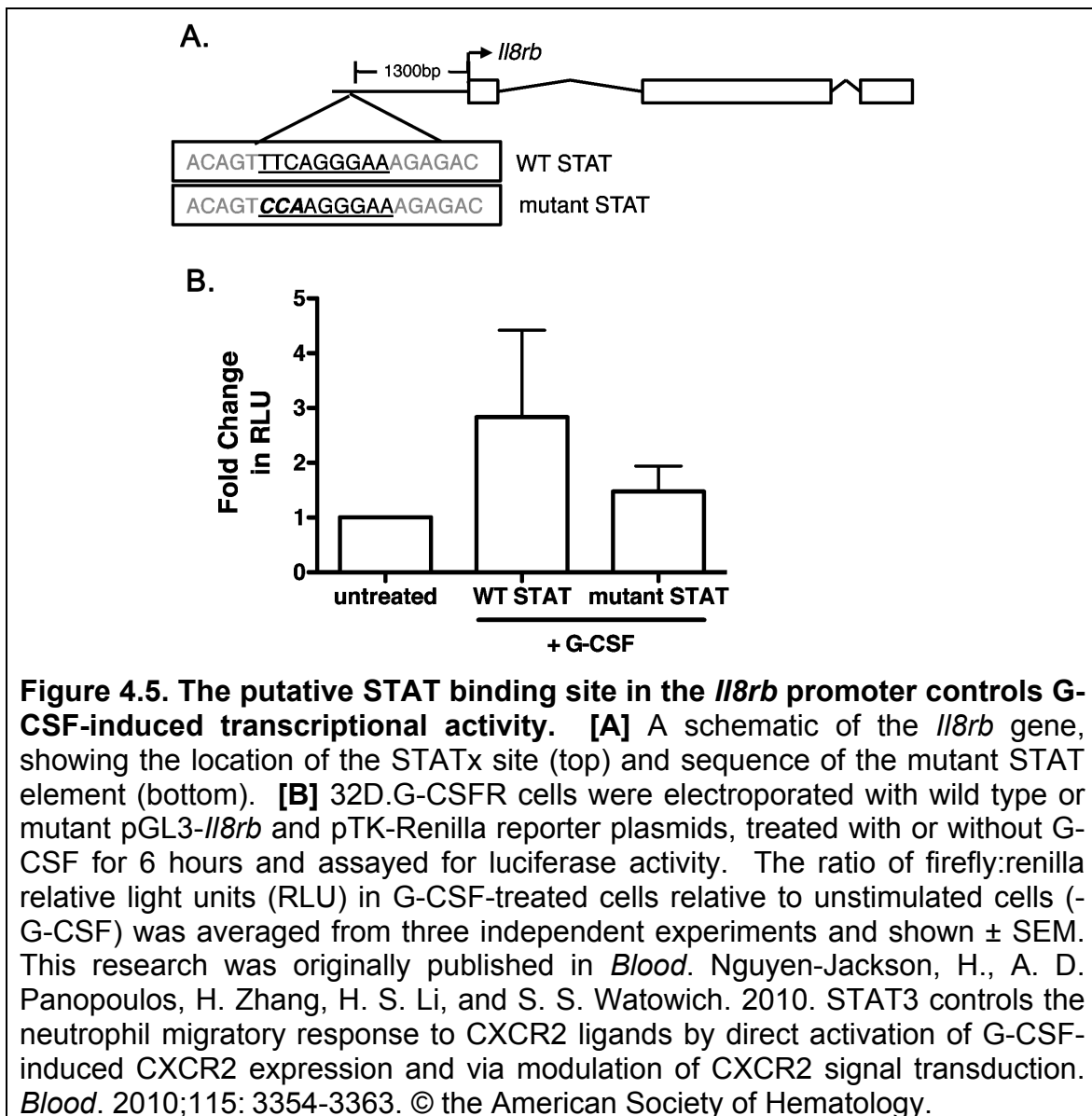


Figure 4.4. G-CSF treatment *in vivo* enhances the neutrophil migratory response to MIP-2. WT and STAT3-deficient (KO) mice were untreated (-) or treated with G-CSF (+) and bone marrow samples were isolated after 24 hours, and sorted into Gr-1^{lo} and Gr-1^{hi} subsets. The chemotaxis response of immature Gr-1^{lo} and mature Gr-1^{hi} neutrophils to MIP-2 was determined by Transwell assays. The average percentage of migrated cells compared to total cells is shown \pm SEM (n=5). ** $P < 0.01$, *** $P < 0.001$. This research was originally published in *Blood*. Nguyen-Jackson, H., A. D. Panopoulos, H. Zhang, H. S. Li, and S. S. Watowich. 2010. STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. *Blood*. 2010;115: 3354-3363. © the American Society of Hematology.

STAT3 controls CXCR2 transcription by directly interacting with the *Il8rb* promoter.

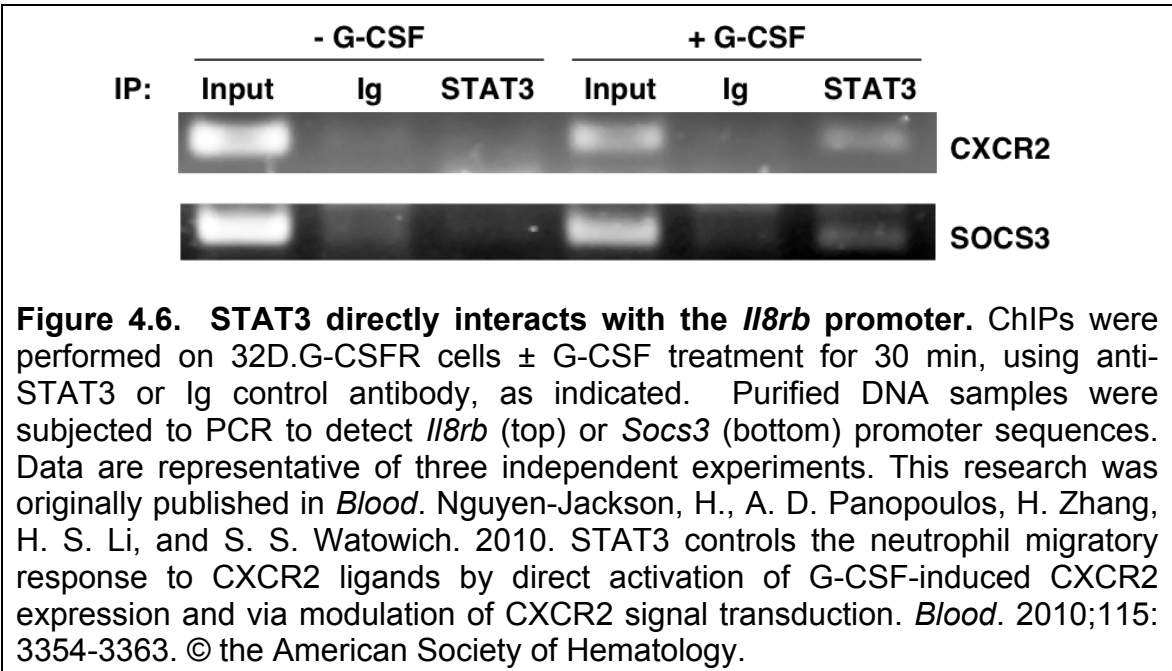
Analysis of the murine *Il8rb* and human *IL8RB* proximal promoters revealed a putative STAT-binding site approximately 1300bp upstream of the transcriptional start site (Figure 4.4[A]), potentially indicating similar regulation in

the two species. The predicted transcriptional start site of *IL8rb* as identified by Ensembl was verified by 5'-rapid amplification of cDNA ends (RACE) (data not shown). Luciferase reporter constructs containing the proximal promoter region encompassing the STAT site or a mutant STAT element (Figure 4.5[A]) were generated. G-CSF treatment induced *IL8rb* reporter activity approximately 3-fold,



relative to non-stimulated conditions (Figure 4.5[B]), indicating G-CSF activates transcription from the *Il8rb* promoter. G-CSF-responsive *Il8rb* reporter activity was suppressed upon mutation of the STAT site (Figure 4.5[B]), demonstrating a role for this element in G-CSF-dependent transcription.

To examine whether STAT3 binds the *Il8rb* promoter *in vivo*, we performed ChIPs in 32D.G-CSFR cells. These assays showed that STAT3 is recruited to the *Il8rb* promoter upon G-CSF stimulation, similar to its inducible interaction with the *Socs3* promoter (Figure 4.6[A]). EMSAs with an oligonucleotide containing the *Il8rb* STAT element revealed induction of a specific protein:oligonucleotide complex after G-CSF stimulation that supershifted upon incubation with a STAT3 antibody (data not shown). Taken together, our data indicate that G-CSF-stimulated *Il8rb* transcription occurs by direct binding of STAT3 to the *Il8rb* promoter.



The neutrophil mobilization response to infection with *L. monocytogenes* is impaired in STAT3-deficient mice, resulting in prolonged infection.

To test whether the role for STAT3 in G-CSF- and MIP-2-responsive neutrophil mobilization is indicative of STAT3 function during bacterial infection, we assessed neutrophil mobilization in wild type and STAT3-deficient mice following infection with *Listeria monocytogenes*. It has been previously demonstrated that clearance of this bacterium *in vivo* requires G-CSF (25, 94).

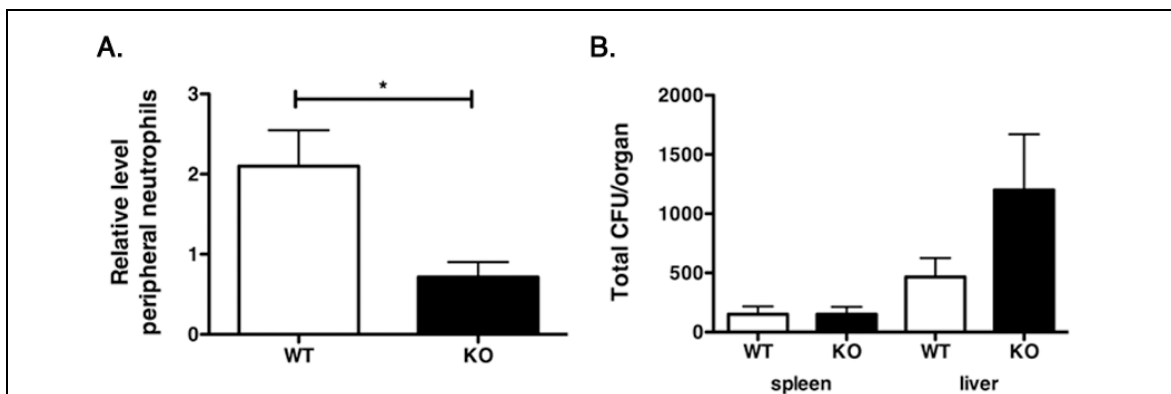


Figure 4.7. Impaired mobilization of STAT3-deficient neutrophils during *L. monocytogenes* infection. Wild type (WT) or STAT3-deficient (KO) mice were infected with *L. monocytogenes* by intravenous injection, as described in the Methods. **[A]** At 12 h post-infection, circulating neutrophil numbers in peripheral blood were determined by automated counting. Data shown are average fold change in peripheral neutrophil numbers in infected vs. uninfected animals (n=5 for WT, n=4 for KO). **[B]** Spleens and livers were isolated 12 h after infection with *L. monocytogenes*, homogenized, and cultured. Colony-forming units (CFU) were enumerated 24 h after culture. Shown are mean CFU/organ (n=6 for WT, n=4 for KO). Error bars represent SEM. *P < 0.05. This research was originally published in *Blood*. Nguyen-Jackson, H., A. D. Panopoulos, H. Zhang, H. S. Li, and S. S. Watowich. 2010. STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. *Blood*. 2010;115: 3354-3363. © the American Society of Hematology.

Wild type mice rapidly induced circulating neutrophil numbers upon infection, while peripheral neutrophil levels remained unchanged in STAT3-deficient mice (Figure 4.7[A]), suggesting a refractory mobilization response to *L. monocytogenes* in the absence of STAT3. Bacterial load was significantly increased in the livers of STAT3-deficient mice relative to wild type animals at 12 hours post-infection, while no apparent differences were detected in spleen (Figure 4.7[B]). These results are consistent with a study that used a granulocyte-depleting antibody during *L. monocytogenes* infection, which demonstrated that neutrophils are important for bacterial clearance from the liver at early stages (157). Furthermore, wild type mice were able to resolve the infection 8 days later, while STAT3-deficient mice retained significant bacterial burden, as assayed by CFU determinations (data not shown). These results indicate that STAT3 is required for neutrophil mobilization in response to *L. monocytogenes*, and suggest that the aberrant neutrophil response contributes to an increased bacterial load in infected STAT3-deficient animals.

4.3. Discussion

Little is known regarding the regulation of chemokine receptor expression, however dysregulated CXCR2-mediated neutrophil migration has been linked to inflammatory conditions such as rheumatoid arthritis or chronic obstructive pulmonary disorder, indicating the clinical relevance of examining CXCR2 (4, 6). Basal expression of CXCR2 is STAT3-independent and controlled

developmentally; however, we found that CXCR2 cell surface expression was enhanced by systemic administration of G-CSF via a STAT3-dependent pathway. Examination of *Il8rb* transcriptional regulation revealed that it is a direct STAT3 target, likely affecting G-CSF-induced expression in immature Gr-1^{lo} granulocytes, and its upregulation correlates with enhanced neutrophil migration *in vitro*. This result is consistent with the observation that G-CSF mobilizes immature neutrophils (91, 158, 159), a mechanism that contributes to upregulation of circulating neutrophil levels. Overall, our results highlight an additional mechanism used by G-CSF to influence neutrophil migratory activity, which is illustrated in Figure 4.8.

Mutations in the DNA binding or transactivation domain of STAT3 are linked to the primary immunodeficiency Hyper IgE Syndrome (HIES), also known as Job's Syndrome (160, 161). In addition to having increased levels of circulating IgE antibodies, these patients are susceptible to recurring bacterial and fungal infections. This aspect of the HIES phenotype is consistent with defective neutrophil function and, in fact, neutrophils isolated from HIES patients have been shown to have impaired chemotaxis (162). In further support of our findings in the mouse model, neutrophils isolated from HIES patients express significantly reduced levels of CXCR2 (163). Additionally, these neutrophils have suppressed amounts of CXCR1 and the fMLP receptor, which recognizes the formylated peptide Met-Leu-Phe (fMLP), a potent chemoattractant (163). Although murine neutrophil migration in response to fMLP was not shown to require STAT3 (91), and mice do not express CXCR1 (130), these findings

suggest that aberrant neutrophil chemotaxis in patients with mutations in STAT3 can contribute to an immunosuppression that increases their susceptibility to infections.

Consistent with the immunodeficiency in HIES patients, STAT3-deficient mice are susceptible to infection with the bacterium *Listeria monocytogenes* (Figure 4.7). While wild type mice are able to clear bacterial infiltrates in the liver at 12 hours and recover from infection after 8 days, STAT3-deficient mice were unable to do so (data not shown). The innate immune response, which is mediated by neutrophils and macrophages, is vital to suppress *L. monocytogenes* at early stages of infection. In line with previously published results, we did not observe a significant difference in circulating monocyte levels 12 hours post-infection in wild type and STAT3-deficient mice, further supporting that neutrophils are the primary responders at this time point [data not shown and (157)]. Collectively, impaired neutrophil migration and reduced bacterial clearance in infected STAT3-deficient mice are indicative of the importance of neutrophil recruitment to suppress *L. monocytogenes* at early stages. STAT3-regulated pathways in granulocytes require additional investigation to understand their potential contribution to HIES as well as other diseases with aberrant neutrophil activity.

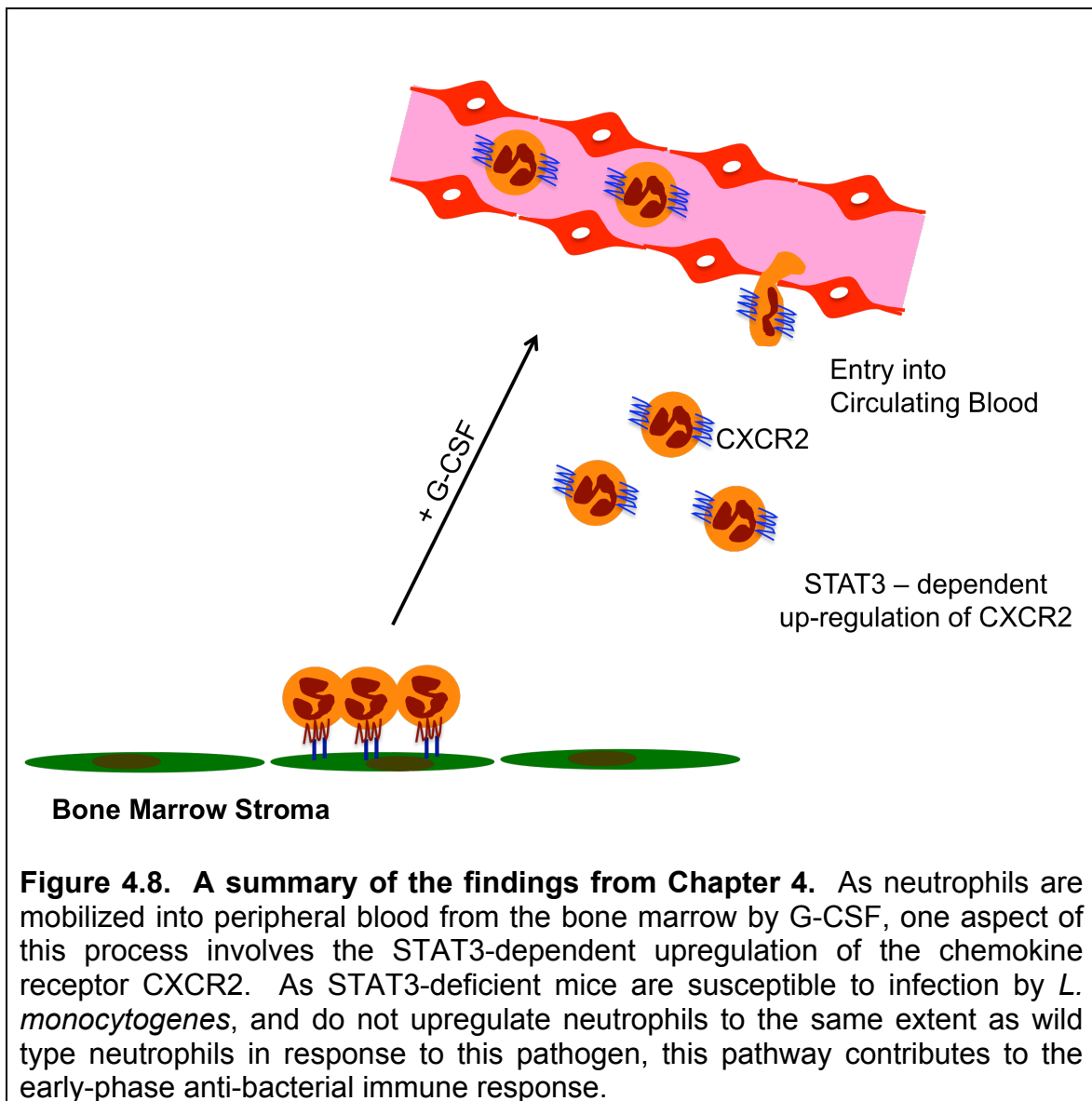


Figure 4.8. A summary of the findings from Chapter 4. As neutrophils are mobilized into peripheral blood from the bone marrow by G-CSF, one aspect of this process involves the STAT3-dependent upregulation of the chemokine receptor CXCR2. As STAT3-deficient mice are susceptible to infection by *L. monocytogenes*, and do not upregulate neutrophils to the same extent as wild type neutrophils in response to this pathogen, this pathway contributes to the early-phase anti-bacterial immune response.

Chapter 5: G-CSF/STAT3 signals control accumulation of H3K4me3 and RNA polymerase II CTD pSer5 at the promoters of *Cxcl2* and *Il8rb*

5.1. Background and rationale

We have established that two molecules responsible for neutrophil migration, the chemokine MIP-2 and its receptor CXCR2, are regulated transcriptionally by G-CSF/STAT3 signals. While it is widely accepted that STAT3 is a transcription factor, the mechanisms of its action at the chromatin level are not fully understood.

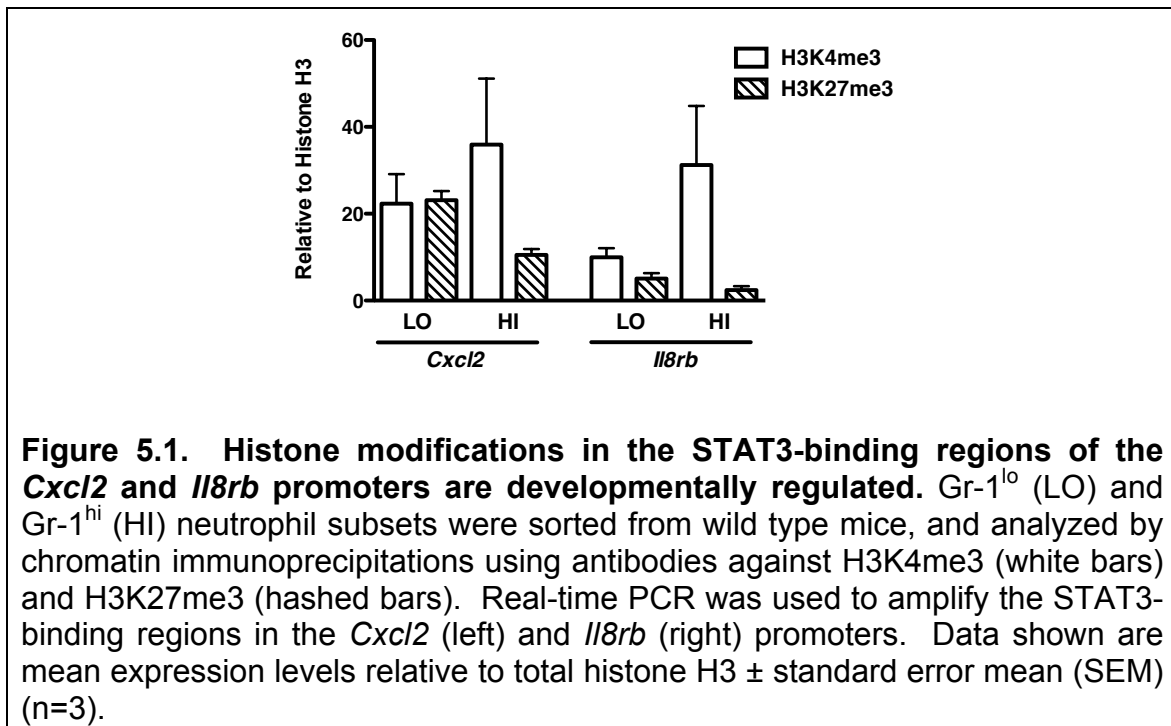
Chromatin is the name given to describe DNA complexed with protein, and is comprised of nucleosomes, which are formed when 146bp segments of DNA is associated with a histone core, an octamer consisting of dimers of each subunit: H2A, H2B, H3, and H4 (96). The tail at the amino terminus of each histone subunit can be modified post-translationally, influencing transcriptional activity of its associated DNA fragment by affecting accessibility of transcriptional regulators. Recent attention has focused on the tri-methylated lysines residues 4 and 27 of the histone subunit H3, denoted as H3K4me3 and H3K27me3, which correlate with regions of active or repressed transcription, respectively (96). To demonstrate the relationship between cytokine treatment, transcription factor binding, histone modifications, and transcriptional activity, we examine H3K4me3 and H3K27me3 levels in the STAT3-binding regions of the *Cxcl2* and *Il8rb* promoters, and test their responses to G-CSF.

5.2. Results

The chromatin modifications of STAT3-binding regions in the promoters of *Cxcl2* and *Il8rb* exhibit different patterns throughout neutrophil development

To examine the state of histone modifications at the STAT3-binding regions of the *Cxcl2* and *Il8rb* promoters, Gr-1^{lo} immature and Gr-1^{hi} mature neutrophils were isolated, and H3 marks were analyzed by chromatin immunoprecipitation utilizing antibodies against total histone H3, H3K4me3, and H3K27me3. Interestingly, we found that levels of tri-methylation of H3K4 and H3K27 varied at different stages of development at the same promoter. Figure 5.1 shows that in the *Cxcl2* promoter, levels of activating H3K4me3 and repressive H3K27me3 are equivalent in Gr-1^{lo} cells, consistent with promoters that are considered poised, in that they are transcriptionally inactive, but can be readily activated (164). By contrast, the level of activating H3K4me3 marks is significantly higher than repressive H3K27me3 marks in Gr-1^{hi} cells, suggesting this region of the promoter is constitutively open in mature neutrophils. Activating H3K4me3 and repressive H3K27me3 marks exhibit a similar pattern at the STAT3-binding region of the *Il8rb* promoter, with Gr-1^{hi} cells possessing significantly higher levels of H3K4me3 than Gr-1^{lo} cells. These results suggest that chromatin marks at the STAT3-binding regions of the *Cxcl2* and *Il8rb* genes are regulated developmentally, favoring an open configuration in mature Gr-1^{hi} cells. This is consistent with our previous results that expression of *Cxcl2* and

Ii8rb is much higher in Gr-1^{hi} cells (Figures 3.2 and 4.2, respectively) under basal conditions, and the observation that neutrophils require maturation to gain effector function (114).



To confirm mRNA abundance of *Cxcl2* and *Ii8rb* under steady-state conditions, Gr-1^{lo} and Gr-1^{hi} cells were isolated. Following the pattern of histone modifications, both genes are more highly expressed in the mature Gr-1^{hi} subset (Figure 5.2). Interestingly, basal levels of *Ii8rb* are much more abundant than *Cxcl2*. These expression data correlate with the histone modification pattern shown in Figure 5.1, and support the idea that high levels of H3K4me3, relative to H3K27me3, are associated with active transcription.

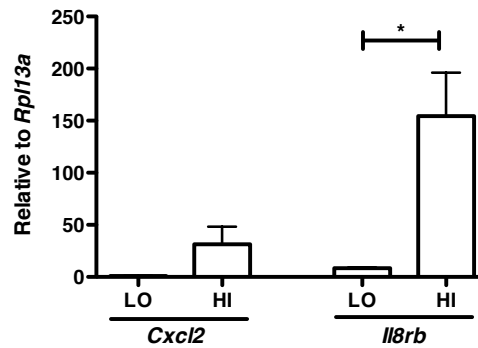
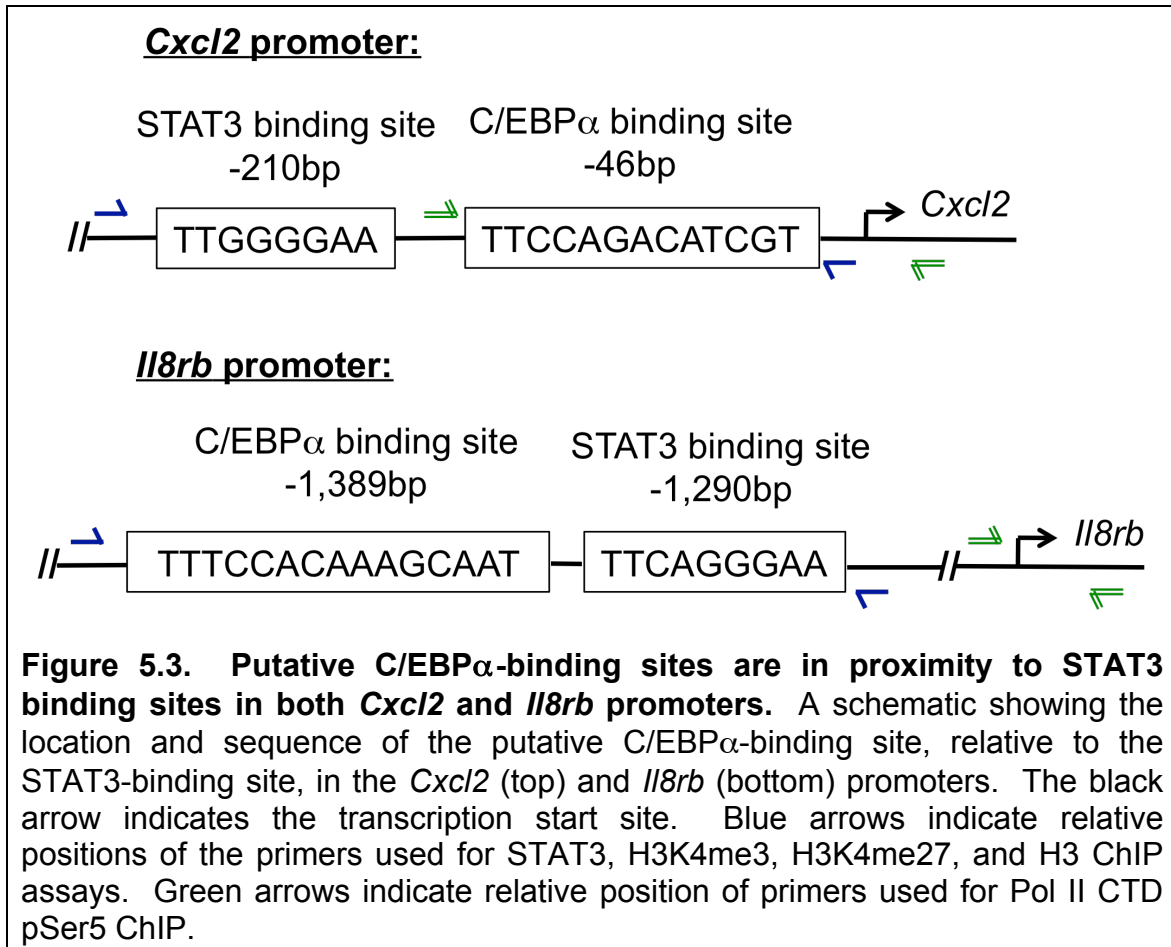


Figure 5.2. mRNA abundance of *Cxcl2* and *Il8rb* is differentially regulated under homeostatic conditions. Gr-1^{lo} (LO) and Gr-1^{hi} (HI) neutrophil subsets were sorted from wild type mice, and analyzed for expression of *Cxcl2* and *Il8rb* mRNA. Data shown are mean *Cxcl2* (left) and *Il8rb* (right) expression relative to the housekeeping gene *Rpl13a* \pm SEM (n=3). **P* < 0.05.

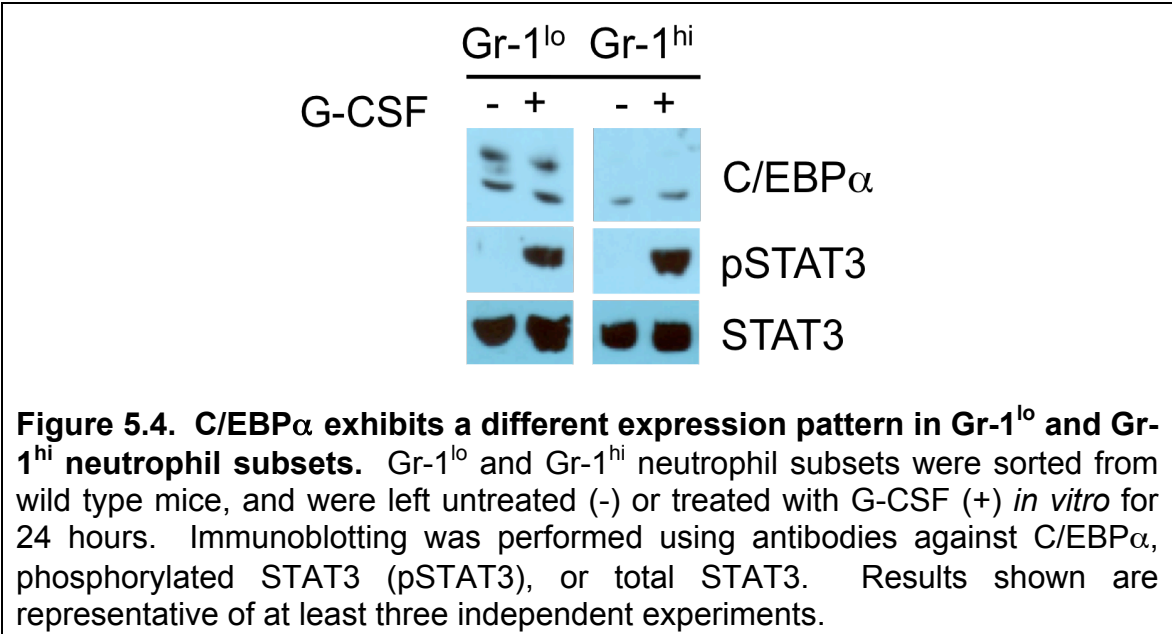
C/EBP α , a transcription factor contributing to neutrophil differentiation, acts as a transcriptional repressor in *Cxcl2* and *Il8rb* reporter assays

We then investigated the possible mechanisms regulating the developmental differences observed in chromatin modifications at *Cxcl2* and *Il8rb* promoters (Figure 5.1). It was previously shown that C/EBP proteins, in particular C/EBP α and C/EBP ϵ , are differentially expressed throughout neutrophil development (14). Further examination of the *Cxcl2* and *Il8rb* promoters revealed that putative C/EBP α binding sites are very close (within 100-200bp) to the STAT3-binding regions (Figure 5.3).



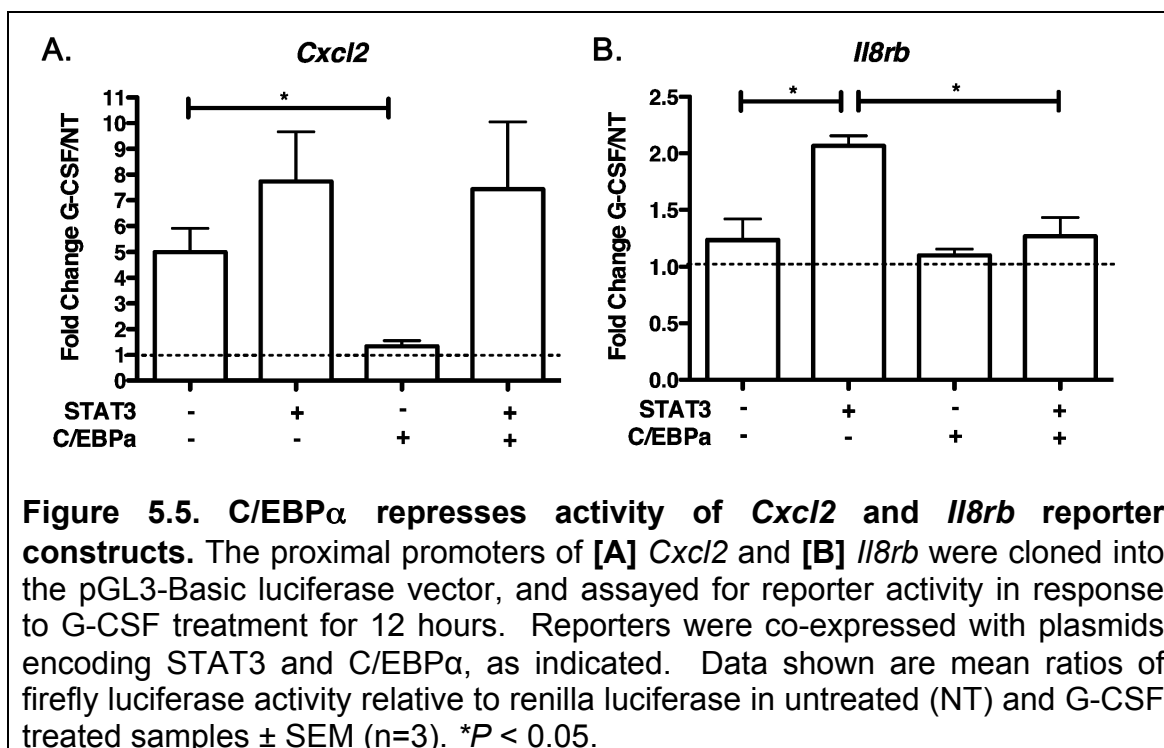
C/EBP α has been shown to regulate transcriptional activity by recruiting histone-modifying enzymes, and interacting with basal transcription factors required for RNA Polymerase II activation (107, 165, 166). Because of the known role for C/EBP α during neutrophil development, and the proximity of its putative binding site to the STAT3-binding site in the *Cxcl2* and *Il8rb* promoters, we examined the contribution of C/EBP α to the observed differences in mRNA abundance. We first determined C/EBP α expression in Gr-1^{lo} and Gr-1^{hi} cells by immunoblotting. While no difference was observed in total STAT3 or STAT3 phosphorylation in Gr-1^{lo} and Gr-1^{hi} cells, there was a striking difference in

C/EBP α expression (Figure 5.4). Two C/EBP α protein sizes were detected in Gr-1^{lo} cells, likely the 30 kD and p42 kD species, in contrast to Gr-1^{hi} cells, in which only the 30 kD protein was detected. Encoded by an intronless gene, C/EBP α can be translated into two products (167, 168). C/EBP α p30 translation is initiated from a downstream AUG, and lacks one transactivation domain when compared to the full-length C/EBP α p42 (167, 168). While C/EBP α p30 was initially thought to function as a dominant negative of p42, there is recent evidence that the two proteins may have distinct functions, as evidenced by differing DNA-binding affinities, as well as regulation of unique sets of genes (169-171).



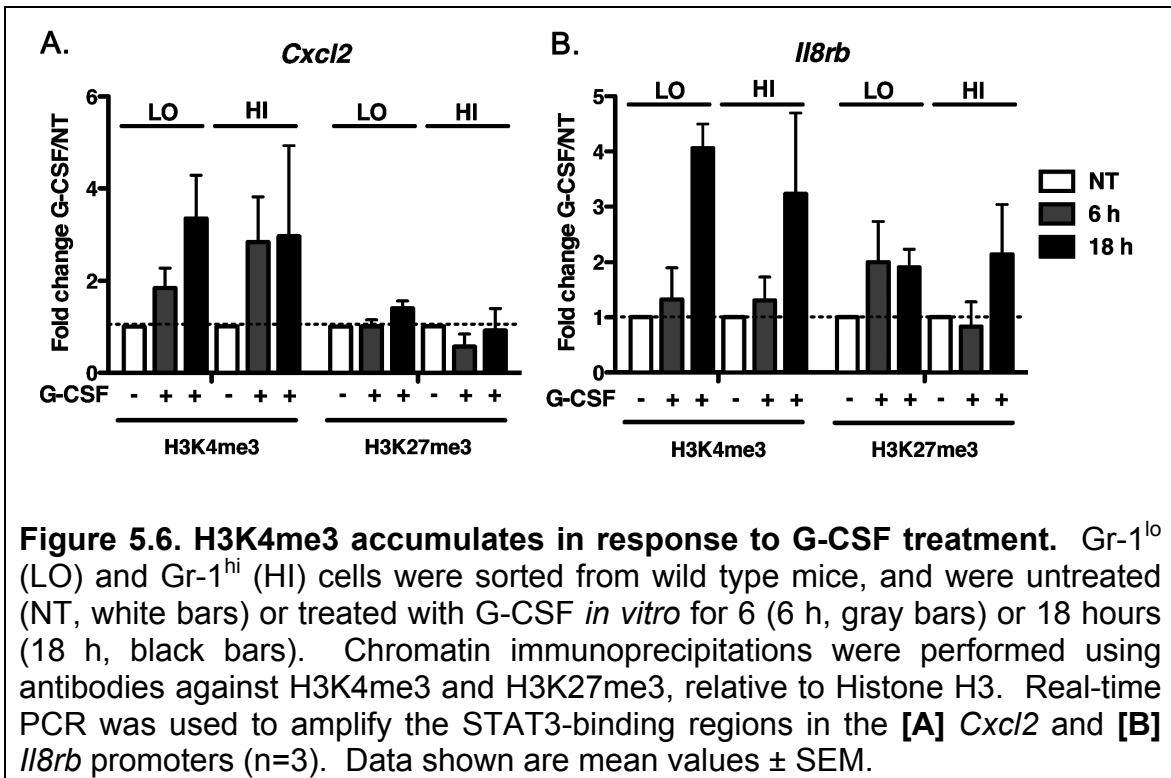
To examine the effect of C/EBP α on transcriptional activity from the *Cxcl2* and *Il8rb* promoters, a plasmid encoding C/EBP α was introduced into luciferase reporter assays. The upstream promoter regions of *Cxcl2* and *Il8rb* were cloned into the pGL3-Basic luciferase vector, and assayed for reporter activity in

response to G-CSF. The *Cxcl2* reporter demonstrated a five-fold increase in reporter activity in response to G-CSF, which was further elevated upon addition of a STAT3 plasmid (Figure 5.5[A]). Interestingly, C/EBP α suppressed this reporter activity, and this suppression was overcome by coexpression with STAT3. In contrast to the *Cxcl2* reporter, the *Il8rb* reporter required addition of STAT3 in order to demonstrate G-CSF-responsiveness (Figure 5.5[B]). Addition of C/EBP α alone did not affect the activity of this reporter, but when coexpressed with STAT3, there was a statistically significant reduction of activity when compared to STAT3 alone. These data suggest that expression of C/EBP α , which is required for neutrophil differentiation and is expressed at higher levels in Gr-1^{lo} neutrophils, acts as a transcriptional repressor in reporter assays. These results may contribute to the difference in basal mRNA abundance of *Cxcl2* and *Il8rb* seen in Figure 5.2.

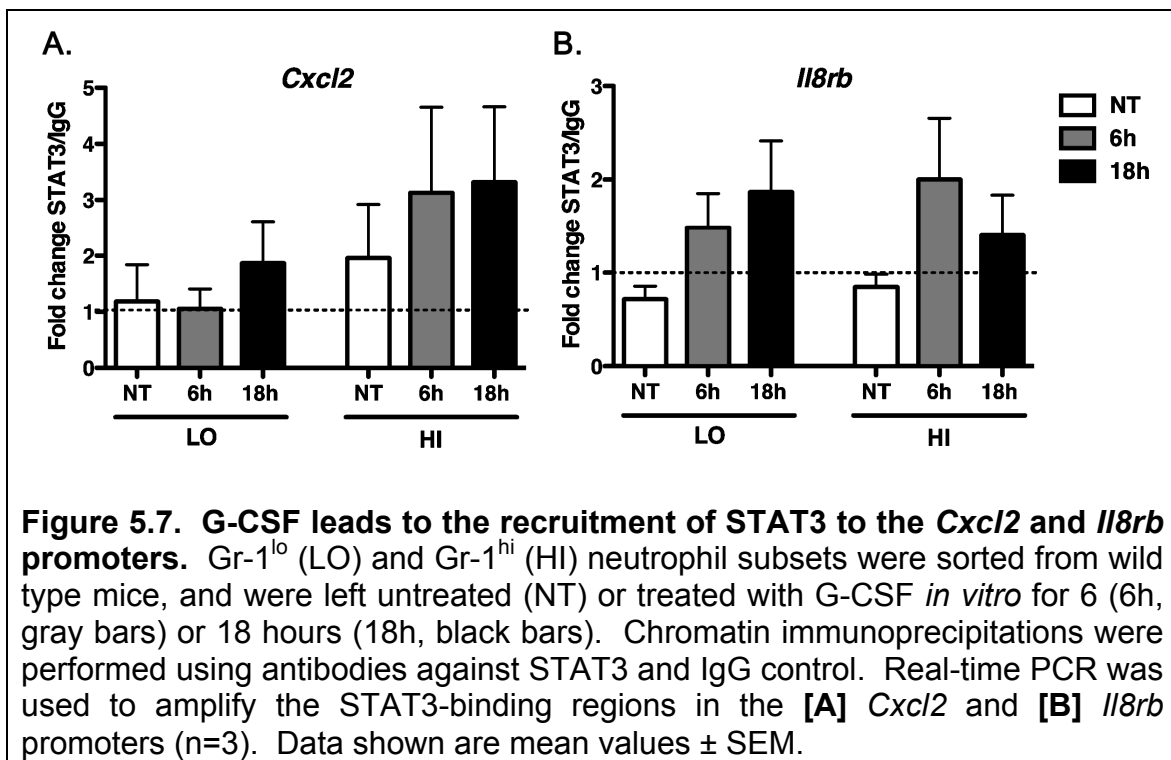


G-CSF upregulates H3K4me3 at the STAT3-binding regions of the *Cxcl2* and *Il8rb* promoters, and promotes accumulation of STAT3 and Pol II CTD pSer5

To examine the effects of G-CSF stimulation on histone modifications in the STAT3-binding regions of the *Cxcl2* and *Il8rb* promoters, Gr-1^{lo} and Gr-1^{hi} cells were isolated and treated with G-CSF *in vitro*, and H3 marks were analyzed by ChIPs. We demonstrate that while G-CSF has relatively little effect on H3K27me3 abundance (two-fold or less), treatment with this cytokine results in two- to four-fold upregulation of H3K4me3, relative to untreated samples, as shown in Figure 5.6[A] and [B]. This result suggests that treatment with cytokine correlates with increased accessibility to the transcription factor binding sites of these promoters.

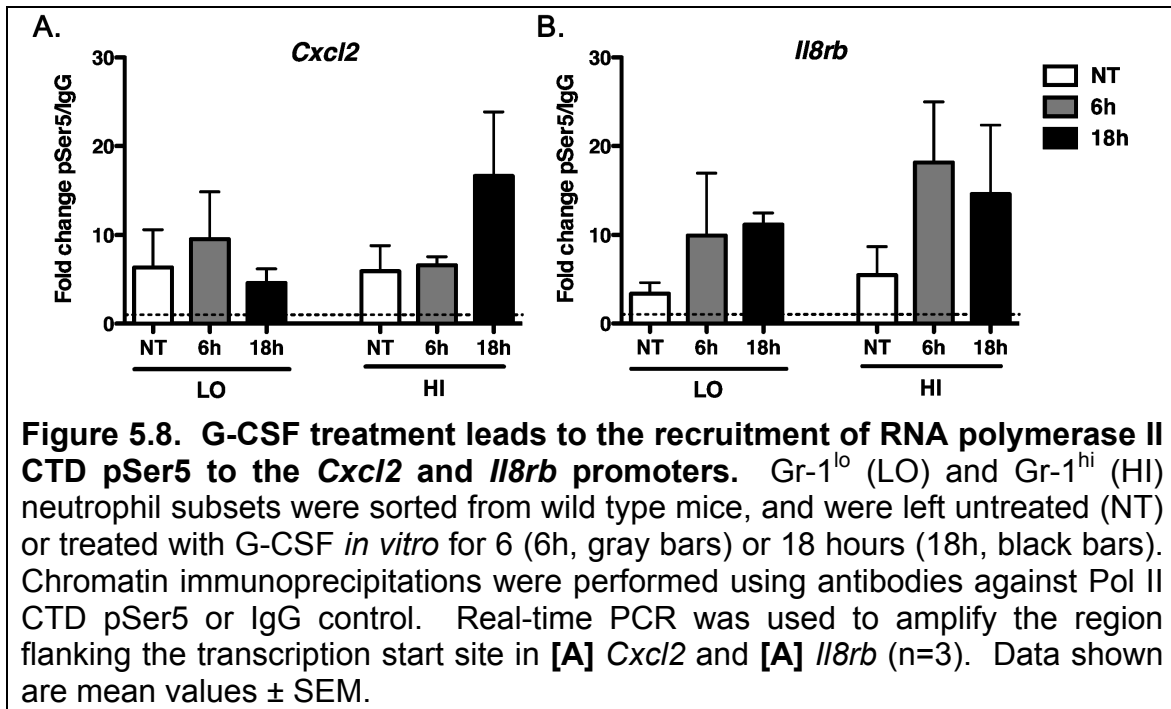


To test the kinetics of STAT3-binding to the *Cxcl2* and *Il8rb* promoters in primary immature and mature neutrophils, ChIPs were performed using a STAT3 antibody. We found that G-CSF stimulation correlates with STAT3 interaction at these promoters in both Gr-1^{lo} and Gr-1^{hi} cells. In the *Cxcl2* promoter, STAT3-binding appears to occur faster in mature Gr-1^{hi} cells compared to Gr-1^{lo} cells (Figure 5.7[A]), whereas in the *Il8rb* promoter, binding kinetics are equivalent in Gr-1^{lo} and Gr-1^{hi} cells (Figure 5.7[B]).



To further characterize the transcriptional activity of the *Cxcl2* and *Il8rb* promoters after G-CSF treatment, ChIPs were performed using an antibody that detects phosphorylated serine 5 (pSer5) of the carboxy-terminal domain (CTD) of RNA Polymerase II (Pol II), which is indicative of transcription initiation. The kinetics of the recruitment of RNA Pol II CTD pSer5 (Figure 5.8[A]) and STAT3

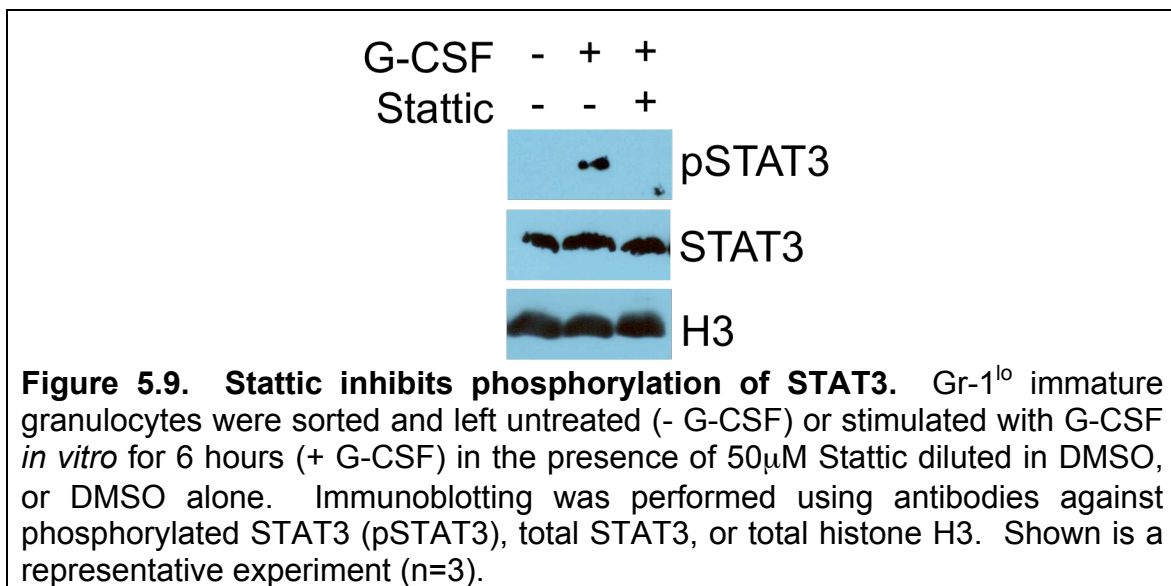
(Figure 5.7[A]) to the *Cxcl2* promoter appears asynchronous, with accumulation of H3K4me3 (Figure 5.6[A]) and pSer5 (Figure 5.8[A]) appearing before STAT3 (Figure 5.7[A]) in Gr-1^{lo} immature cells. By contrast, in Gr-1^{hi} mature neutrophils, H3K4me3 (Figure 5.6[A]) and STAT3 (Figure 5.7[A]) accumulate with similar kinetics, while RNA Pol II CTD pSer5 (Figure 5.8[A]) is recruited later. While these differences may contribute to the developmental differences observed in *Cxcl2* expression, further studies are required. In contrast to the *Cxcl2* promoter, recruitment of STAT3 (Figure 5.7[B]) and RNA Pol II CTD pSer5 (Figures 5.8[B]) to the *Il8rb* promoter follows similar kinetics in both Gr-1^{lo} immature and Gr-1^{hi} mature neutrophils, suggesting a correlation between these two events at this particular promoter. Interestingly, these two molecules are recruited before accumulation of H3K4me3 (Figure 5.6[B]). These results indicate that expression of *Cxcl2* and *Il8rb* may be regulated by distinct mechanisms, and may account for the varying levels of expression that we have observed. Taken together, however, the data demonstrate that G-CSF induces changes in histone modifications by increasing H3K4me3, and results in the recruitment of STAT3 and Pol II CTD pSer5 to the *Cxcl2* and *Il8rb* promoters.



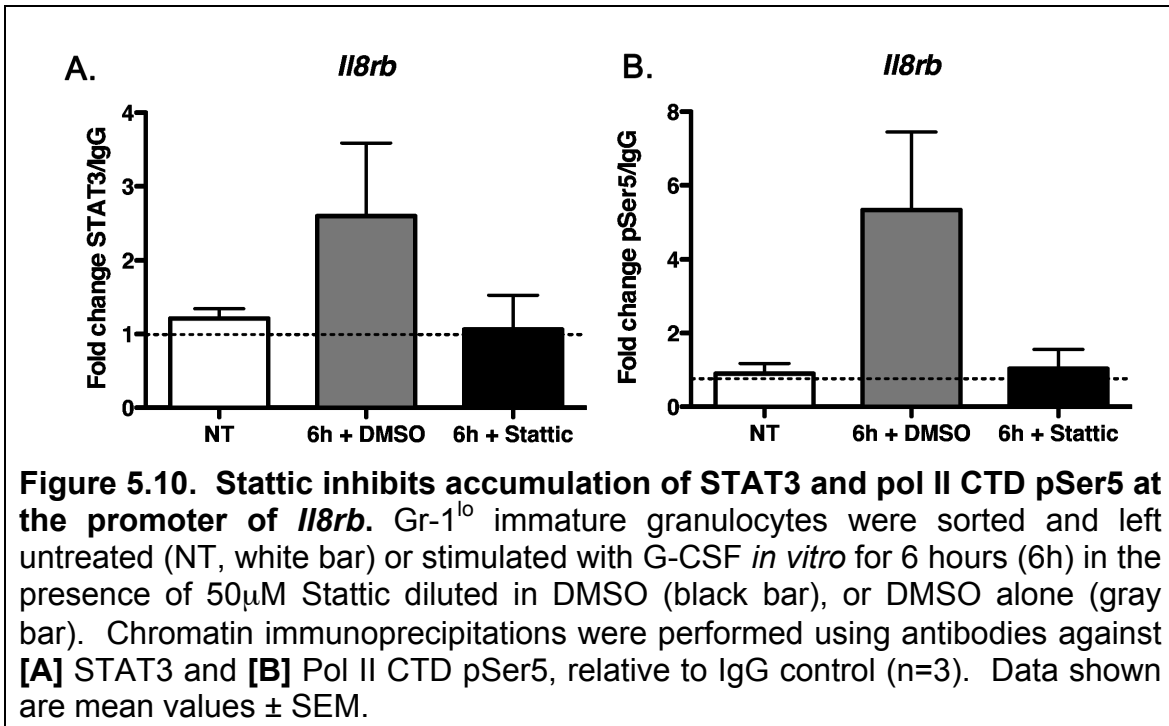
Inhibition of STAT3 phosphorylation blocks accumulation of H3K4me3 and RNA Pol II CTD pSer5

The relationship between the accumulation of STAT3, Pol II CTD pSer5 initiation, and H3K4me3 at promoters is unknown. To examine the role of STAT3 in these events, the experiments above were repeated in the presence of Stattic, a commercially available STAT3 inhibitor. This *in vitro* approach to STAT3 inhibition was used due to the technical limitations in obtaining sufficient numbers of STAT3-deficient cells for these experiments. Stattic specifically inhibits the action of STAT3 by interacting with its SH2 domain, thereby inhibiting its ability to become phosphorylated, dimerize, and bind downstream targets, without affecting activation of other STATs (172). To demonstrate whether

Stattic affects total levels of STAT3 or histone H3, immunoblotting experiments were performed, demonstrating that Stattic inhibited STAT3 phosphorylation while STAT3 and H3 were unchanged (Figure 5.9). Stattic exhibited toxic effects in Gr-1^{hi} cells at all timepoints, and in Gr-1^{lo} cells at 18 hours, therefore, we focused our attention on Gr-1^{lo} cells treated with Stattic for 6 hours.



STAT3 and Pol II CTD pSer5 ChIPs with the *Il8rb* promoter were repeated in the presence of Stattic, as these molecules were recruited by 6 hours (Figures 5.7[B] and 5.8[B]). Figure 5.10 shows that while G-CSF treatment in the presence of DMSO diluent induces STAT3 (Figure 5.10[A]) and Pol II CTD pSer5 (Figure 5.10[B]) to interact with the *Il8rb* promoter, treatment with Stattic inhibits this interaction. These data suggest that STAT3 phosphorylation is required for accumulation of Pol II CTD pSer5, a reflection of Pol II initiation.



Similarly, to examine the role of STAT3 in the acquisition of H3K4me3 in response to G-CSF at the *Cxcl2* promoter, ChIPs using antibodies against H3K4me3 were repeated in the presence of Stattic. This time the *Cxcl2* promoter was assayed because H3K4me3 accumulated at this promoter by 6 hours (Figure 5.6[A]). Again, G-CSF treatment correlated with upregulation of H3K4me3 at the STAT3-binding region of the *Cxcl2* promoter, which is inhibited with the addition of Stattic (Figure 5.11). This result suggests that STAT3 phosphorylation is required for tri-methylation of H3K4, associated with transcriptional activation.

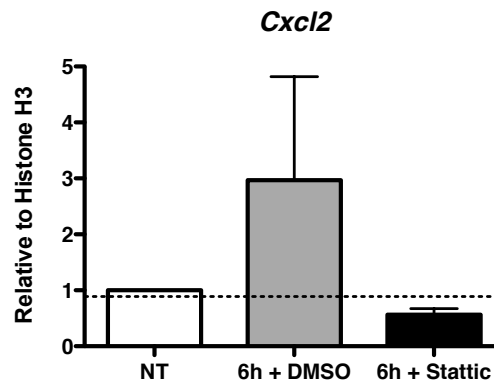


Figure 5.11. Stattic inhibits acquisition of H3K4me3 at the *Cxcl2* promoter. Gr-1^{lo} immature granulocytes were sorted and left untreated (NT, white bar) or stimulated with G-CSF *in vitro* for 6 hours (6h) in the presence of 50μM Stattic diluted in DMSO (black bar), or DMSO alone (gray bar). Chromatin immunoprecipitations were performed using antibodies against H3K4me3 or total histone H3. (n=3) Data shown are mean values ± SEM.

5.3. Discussion

In this chapter, we examine the molecular events that occur as STAT3 binds the promoters of two genes important in the neutrophil migratory pathway, *Cxcl2* and *Il8rb*, which is summarized in Figure 5.12. We found that under basal conditions, the ratio of activating H3K4me3 to suppressive H3K27me3 marks in the STAT3 binding regions of these promoters varied, depending on stage of development. Correspondingly, basal mRNA abundance of *Cxcl2* and *Il8rb* were higher in Gr-1^{hi} mature neutrophils compared to Gr-1^{lo} immature neutrophils, consistent with the idea that higher H3K4me3 relative to H3K27me3 correlates with active transcription.

To examine a potential mechanism contributing to this developmental difference, the candidate transcription factors PU.1, C/EBPα, Gfi-1, and C/EBPε

were evaluated, as these are the major transcription factors involved in granulopoiesis (173). Immunoblotting revealed that expression of PU.1 and Gfi-1 were similar in Gr-1^{hi} and Gr-1^{lo} cells, while expression of C/EBP α was increased in immature cells and C/EBP ϵ was upregulated in mature cells, consistent with previous reports [data not shown, Figure 5.4 and (14)]. Upon further examination of the *Cxcl2* and *Il8rb* proximal promoters, putative C/EBP α -binding sites were found within 100-200bp of the STAT3-binding sites in both promoters. Co-expression of C/EBP α exhibited suppressive effects on the reporter activities of both promoters. This result is consistent with our observation that higher levels of C/EBP α are expressed in Gr-1^{lo} cells, which demonstrate lower mRNA abundance of *Cxcl2* and *Il8rb* compared to Gr-1^{hi} cells. We observed that Gr-1^{lo} cells express a higher ratio of the two known C/EBP α protein products, p42 to p30, relative to Gr-1^{hi} cells. It has been reported that p42 and p30 have different functional activities, as p42, but not p30, inhibited cellular proliferation (167), consistent with our observation that p42 exhibited suppressive effects in reporter studies. While we did not test the function of each protein species in our system, immunoblotting analysis revealed that the C/EBP α plasmid utilized in our reporter assays predominately expressed the p42 protein, consistent with our hypothesis (data not shown).

While C/EBP α p30 and p42 protein products can similarly regulate gene expression in certain contexts (167), there is some evidence of divergent functions, as p30 has only one of the two transactivation domains that p42 contains (167, 168). C/EBP α p30 was first described as the dominant negative

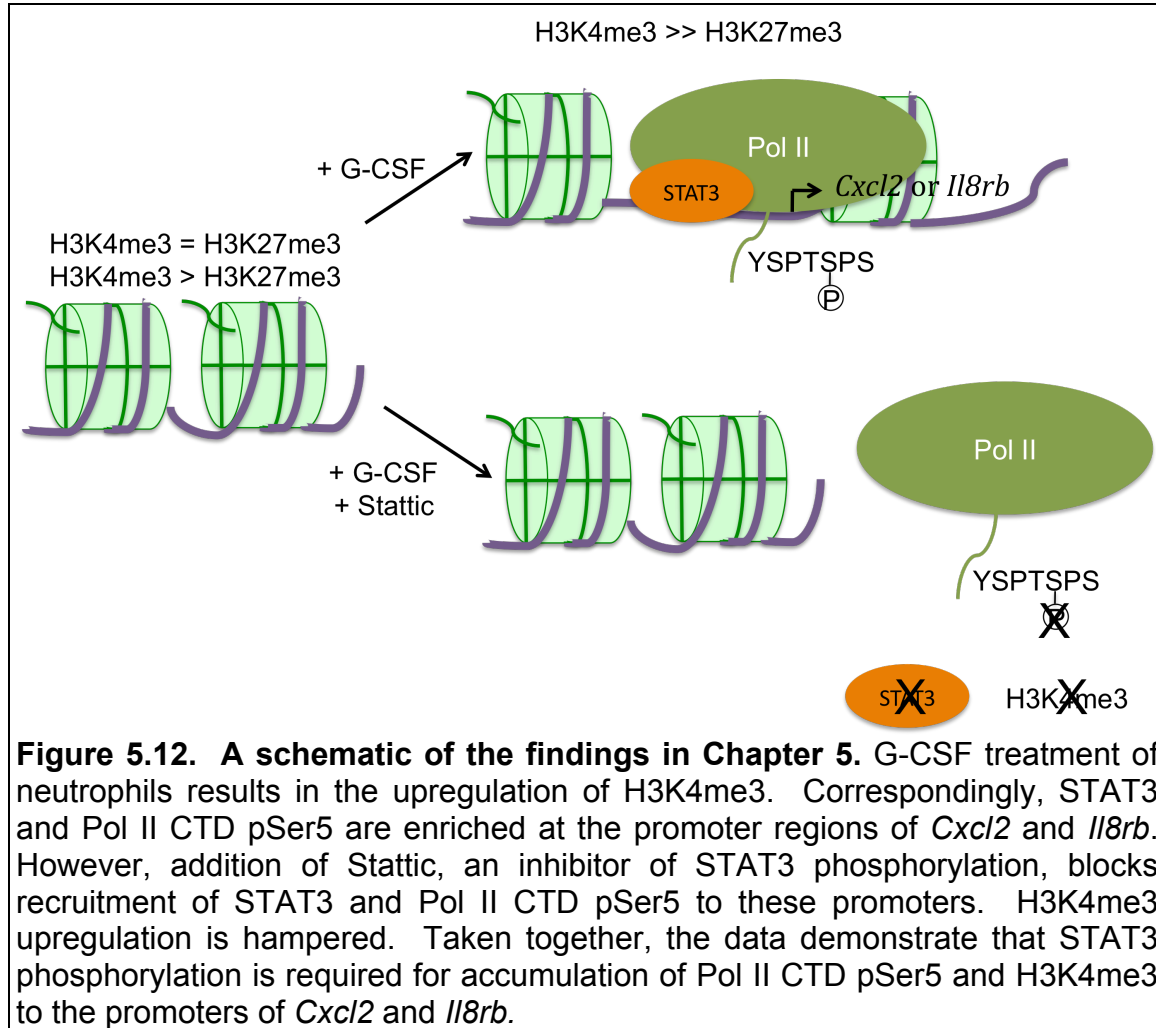
form of p42 in regulation of the gene encoding G-CSFR (170), and demonstrated reduced DNA-binding affinity (169). However, it was later reported that C/EBP α p30, but not p42, binds promoters of a group of genes, indicating that p30 may have distinct functions (171). Recently, it was shown that higher expression of C/EBP α p30 relative to p42 correlated with recruitment of subunits of polycomb repressive complex 2 (PRC2), a histone methyltransferase that primarily mediates tri-methylation of H3K27 (165). While these findings conflict with ours, as we demonstrate increased levels of p30 and H3K4me3 in Gr-1^{hi} cells, the results suggest that the two C/EBP α proteins influence different chromatin modifying functions. While some explanations of the C/EBP α mechanism in our model are consistent with these published findings, they all rely on the premise that C/EBP α acts as a transcriptional repressor, which has also been reported (174). Expression of C/EBP α p30 *in vivo* relieved this repression (175), consistent with our observation that high levels of p30 correlate with higher mRNA expression. While we demonstrate that C/EBP α contributes to repression in our reporter assays, it is thought that C/EBP family members are promiscuous and may have redundant roles (173). Further studies are required to test the contribution of other C/EBP family members in this system.

Examination of the STAT3-binding regions of the *Cxcl2* and *Il8rb* promoters revealed that G-CSF treatment promotes accumulation of H3K4me3, STAT3, and Pol II CTD pSer5, which reflects Pol II initiation. It has been reported that G-CSF affects histone modifications, resulting in a MAP kinase-

dependent increase in the acetylation of histone subunits H3 and H4 (176). G-CSF has also been shown to promote an increase in H3K4me3 and H3K9 acetylation, another mark of transcriptional activation, through a C/EBP α -dependent pathway (177). In this report, small hairpin RNA (shRNA)-mediated knockdown of C/EBP α affected these chromatin modifications, but the mechanism of recruitment was not examined (177). Additionally, our experiments with the STAT3 inhibitor Stattic demonstrate a reduction of H3K4me3 at the *Cxcl2* promoter in response to G-CSF, suggesting that STAT3 may be directly or indirectly involved in the recruitment of methyltransferases.

While the action of the transcription factors STAT3 and C/EBP α in the recruitment of methyltransferases is not fully understood, STAT3 and C/EBP α have both been shown to recruit p300 (108-112), a histone acetyltransferase that may also recruit Pol II and components of the pre-initiation complex (178, 179). Additionally, it has been demonstrated that regions containing H3K4me3 were rapidly acetylated by p300 (180). Taken together, these data suggest that the activity of transcription factors may directly result in histone acetylation, or indirectly by first affecting histone methylation, which promotes activation of transcription.

Ultimately, the goal of these studies is to understand how cytokines relay the message to reorganize chromatin to allow transcription factors to have access to promoters. While the mechanisms are not fully understood, we have established the requirement of the transcription factor STAT3 in the accumulation of H3K4me3 and Pol II CTD pSer5 upon activation by G-CSF.



Chapter 6: General discussion and future directions

It has been previously demonstrated that G-CSF-mediated neutrophil mobilization from bone marrow to peripheral blood is aberrant in mice harboring a STAT3 deficiency in hematopoietic cells (91). However, the molecular mechanisms are still unknown. In this dissertation, we have identified a G-CSF/STAT3-mediated pathway that regulates mobilization by characterizing the expression of the chemokines KC, MIP-2, and SDF-1, and the chemokine receptor CXCR2. Furthermore, we determined the molecular events that occur as STAT3 binds to the promoter regions of such genes.

6.1. G-CSF/STAT3-mediated induction of MIP-2 and CXCR2 contributes to the neutrophil mobilization pathway

Under inflammatory conditions, neutrophils are the first recruited cells, responding to chemokines and cytokines released locally, such as by resident macrophages and dendritic cells (1). To ensure a rapid response, mature neutrophil effector cells are present in the blood under homeostatic conditions, awaiting pro-inflammatory signals, including the chemokines KC and MIP-2, which share the receptor CXCR2 in murine neutrophils (1). Expression of KC, or keratinocyte chemoattractant, is attributed to fibroblasts, epithelial cells and megakaryocytes, and not thought to be a major player in the bone marrow (143-145). On the other hand, MIP-2 or macrophage inflammatory protein-2, was

initially named for the major cell type to secrete it (146, 147). Since then, expression of MIP-2 has been reported in epithelial cells, endothelial cells, mast cells, and neutrophils (147, 181-183). KC and MIP-2 function to draw neutrophils from the bone marrow into circulation, and from peripheral blood to tissues under inflammatory conditions (139). It has been suggested that local induction of chemokines at sites of inflammation is sufficient to induce monocyte mobilization from bone marrow, but this has not been directly examined in neutrophils (139). This possibility can be tested by labeling neutrophils *in vivo*, and visualizing their migration to the tissue using live imaging techniques, where a chemokine, such as KC or MIP-2, is directly administered.

Inflammation also triggers the upregulation of G-CSF, a cytokine present in the serum at very low amounts under steady state conditions (35-37). The ultimate function of G-CSF is to increase neutrophil output, which is achieved at multiple levels, including increased proliferation of neutrophil progenitors, differentiation, and mobilization into blood (38-40). Because G-CSF is a growth factor that lacks direct chemotactic potential (139), it follows that G-CSF may induce the expression of chemokines that would contribute to the neutrophil mobilization response. Indeed, we and others have shown that G-CSF treatment elicits *Cxcl1* (KC) and *Cxcl2* (MIP-2) mRNA expression in bone marrow (145, 149); we show the requirement for STAT3 in this pathway (Chapter 3). Expression of MIP-2 in the bone marrow is primarily attributed to mature Gr-1^{hi} neutrophils, consistent with previous reports [Chapter 3 and (144, 184)]. This is a surprising result, given that the major target of MIP-2 under

inflammatory conditions is also the neutrophil (144, 184). The autocrine effects of MIP-2 production in neutrophils have not been tested, and can be done so *in vitro* by examining the migratory potential (utilizing transwell assays) and the signaling potential (measured by calcium flux), with or without pre-incubation with MIP-2, similar to what has been previously published (185). Additionally, because we demonstrated that G-CSF treatment *in vivo* enhanced the neutrophil migratory response to MIP-2 (Figure 4.4), the inverse can be tested, to demonstrate whether pre-treatment with MIP-2 results in increased sensitivity to G-CSF. These experiments will demonstrate whether MIP-2 has priming effects on neutrophils.

In addition, G-CSF/STAT3 signals are required to upregulate surface expression of CXCR2, the receptor for KC and MIP-2, primarily in immature Gr-1^{lo} neutrophils (Chapter 4). This result is consistent given that G-CSF upregulates circulating immature granulocytes, in addition to mature cells (91, 158, 159). *Il8rb^{-/-}* neutrophils do not respond to KC or MIP-2, suggesting that CXCR2 is the major mediator of neutrophil chemotaxis (186). Because of the redundant functions of KC and MIP-2, blocking one or the other is insufficient to eliminate neutrophil-mediated pathologic inflammation. Rather, targeting CXCR2 would be a more effective means of controlling neutrophil infiltration. Indeed, CXCR2-neutralizing antibodies, CXCR2 antagonists, and CXCR2 inhibitors are being developed to restrain neutrophil migration to the gut and lungs, as well as to prevent tumor progression, since CXCR2 is the receptor for IL-8, a pro-angiogenic factor in humans (187). Because STAT3 is characterized

as a regulator in this pathway, this presents a novel target for therapeutics. Inhibitors of STAT3 are being developed and used clinically, as STAT3 is constitutively activated in many cancers, correlating with poor prognosis (172). These inhibitors can be tested as anti-inflammatory drugs to treat conditions where excessive neutrophil infiltration has been implicated, such as to the lungs and the heart (132). Because STAT3 is ubiquitously expressed, tissue restricted expression of the inhibitor would be ideal. For example, neutrophil elastase 2, which has been used to promote neutrophil-specific expression of *Cre* in conditional deletion models, can be exploited for tissue-specific expression of a STAT3-inhibitor (188).

In a recent publication, Kohler *et al.* utilized intravital two-photon microscopy to visualize neutrophil egress from the bone marrow and entry into the vasculature (145). In this system, EGFP expression was driven by the lysozyme M (LysM) promoter, which enforces expression in myeloid cells. It would be interesting to utilize this model, and examine MIP-2 expression in the bone marrow by Gr-1^{hi} mature neutrophils. As LysM is also expressed in monocytes and dendritic cells, it would be more specific to use a neutrophil-specific promoter, such as the aforementioned construct utilizing neutrophil elastase 2 (*Ela2*) (188). Similarly, an *Ela2*-EGFP/MIP-2-YFP reporter mouse could be generated, and G-CSF mobilization of neutrophils could be visualized in a similar manner, but this time tracking chemokine production. This would enable visualization of MIP-2 itself, and/or the cells that produce it, depending on the intensity of the signal. This is similar to what was previously demonstrated

using CXCR4-EGFP and SDF-1-EGFP (189). As ELR-CXC chemokines are potent neutrophil chemoattractants with redundant functions, it would be interesting to exploit this conserved region to visualize all chemokines that act in this pathway. A fluorescent probe can be generated against this conserved sequence, and antibodies can be used to distinguish individual chemokines for further study, if necessary.

The inability of mice with a STAT3 deficiency in hematopoietic cells to enhance peripheral blood neutrophil numbers upon G-CSF or MIP-2 treatment suggested a defective mobilization response (91, 150). However, STAT3-deficient mice are neutrophilic under basal conditions, which suggests that existing high levels of circulating neutrophils may prohibit further upregulation. Because we previously showed that G-CSF treatment over 5 days induces approximately a ten-fold increase in peripheral blood neutrophils in both wild type and STAT3-deficient mice, this result indicates that circulating neutrophil amounts are not saturated in STAT3-deficient animals under basal conditions (91). An alternative explanation for the neutrophilia is found by examining the ability of STAT3-deficient neutrophils to migrate to the tissue, as blocks in the pathway would result in their accumulation in the blood. We found that MIP-2 treatment caused a significant reduction of bone marrow neutrophils in wild type mice, but these levels were unaffected in STAT3-deficient animals (150). We hypothesized that the drop in bone marrow neutrophils in the wild type mice would result in their accumulation in peripheral blood or spleen, but they did not, indicating that MIP-2 likely induced neutrophils to marginate to other tissues

(150). Because neutrophils were retained in the bone marrow of STAT3-deficient mice upon MIP-2 administration, these results suggest that STAT3 is required for MIP-2-mediated neutrophil mobilization to peripheral blood as well as margination to non-hematopoietic tissues (150). Our findings are consistent with impaired CXCR2-mediated chemotaxis of STAT3-deficient neutrophils *in vitro*, which was previously described by our group (91). Further analysis of this hypothesis can be performed by utilizing *in vivo* imaging techniques to track neutrophil egress from bone marrow to blood, and from circulation to peripheral tissues.

In an attempt to address the neutrophilia in STAT3-deficient mice under steady-state conditions, chimeric mice can be generated by transferring bone marrow from STAT3-deficient mice into wild type mice. This is similar to what was tested in *Il8rb^{-/-}* mice deficient in CXCR2, which also demonstrate basal neutrophilia (149). Analysis of the chimera revealed that *Il8rb^{-/-}* mice exhibited increased neutrophil retention in the bone marrow, indicating a novel function for CXCR2 that had not yet been studied (149). Furthermore, the *Cre*-promoter in our system is driven by TIE-2, which is expressed by bone marrow and endothelial cells. Therefore, to understand the contribution of STAT3-deletion in only the neutrophils, incorporation of neutrophil elastase 2-*Cre* can ensure specific deletion (188).

To explore whether STAT3 is involved in propagation of signals stimulated by MIP-2, these pathways were studied in wild type and STAT3-deficient neutrophils in our laboratory. While STAT3 was dispensable for AKT

signaling and calcium flux in response to MIP-2, STAT3 appeared to control the amplitude of phosphorylated c-Raf, MEK 1/2, and ERK 1/2 [A. D. Panopoulos and S. S. Watowich, unpublished results and (150)]. Addition of the MEK 1/2 inhibitor U0126 significantly affected MIP-2-mediated migration *in vitro* (150). Taken together, these data indicate that STAT3 specifically regulates the Raf/MEK/ERK cascade downstream of MIP-2. As this pathway is required for MIP-2 mediated neutrophil migration, these results may contribute to the defective neutrophil mobilization observed in STAT3-deficient mice. The mechanism by which STAT3 activates the MAP kinase pathway has not yet been defined. However, there is speculation that STAT3 regulates scaffolding proteins and/or regulatory proteins for Rho GTPase family members, which have been implicated in ERK signaling (190, 191). A recent report demonstrated that STAT3 regulates activity of the Rho GTPase Rac1 in mouse embryonic fibroblasts by interacting with β PIX, a guanine nucleotide exchange factor (192). While further studies are required in myeloid cells to determine the mechanism by which STAT3 controls MAP kinase signaling, these results indicate another dimension of neutrophil mobilization that is regulated by STAT3 that could be exploited clinically, as MAP kinase small molecule inhibitors are currently being developed to treat cancers and inflammation (193).

Collectively, the data demonstrate that G-CSF, MIP-2, and KC are potent neutrophil mobilizing agents, which are an important aspect of innate immunity. Further work is needed to understand these mechanisms, enabling the development of targeted therapies against chronic inflammatory conditions due

to excessive neutrophil numbers. Additionally, these mechanisms can be exploited to treat conditions of immunodeficiencies due to insufficient neutrophil numbers.

6.2. Overcoming SDF-1-mediated retention signals in the bone marrow is required for CXCR2-stimulated neutrophil release

It has been shown that G-CSF signals result in downregulation of CXCR4/SDF-1, relieving neutrophil retention in the bone marrow and allowing their release (120-122). We demonstrate that STAT3 is specifically required for G-CSF-induced regulation of SDF-1 in the bone marrow, but the mechanism of this pathway is not fully understood (Chapter 3). First, it is unclear how elimination of a retention signal results in migration out of the bone marrow; most likely a second migratory signal is also required. Although use of the CXCR4 antagonist AMD3100 is sufficient to mobilize neutrophils, its mechanism of action has not been elucidated. It was demonstrated that treatment with AMD3100 resulted in an enhanced response to KC and MIP-2, supporting the idea that the two pathways are distinct, and dependent on one another (139). However, the effects of KC and MIP-2 on SDF-1 expression have not been tested, neither has chemokine production as a result of AMD3100 treatment been examined. Secondly, as previously discussed in Chapter 3, it is not known how CXCR4 and SDF-1 are downregulated by G-CSF. We have demonstrated that the likely target of G-CSF is SDF-1, but not CXCR4 (A.D. Panopoulos and

S. S. Watowich, unpublished results, and Chapter 3). Thus, future studies should be focused on the pathways that regulate SDF-1 expression. It was previously shown that SDF-1 expression in the bone marrow directly correlates with abundance of osteoblasts, which are cells associated with building bone (121, 126). Therefore emphasis should be placed on osteoblasts and their regulatory cells, the osteoclasts (153). Because osteoblasts develop from the mesoderm, differentiation of mesenchymal stem cells should be studied *in vitro* using STAT3-deficient cells to examine the requirement for STAT3 in this pathway (152). Parallel studies should be performed to examine osteoclastogenesis, or the development of osteoclasts from monocyte progenitors, as PIAS3, a negative regulator of STAT3 signaling, has been implicated in this pathway (153, 154). These studies would not only further demonstrate the pathways that contribute to neutrophil mobilization, they would also contribute to our understanding of hematopoietic progenitor stem cell (HPSC) mobilization for transplantations, as AMD3100, G-CSF, and chemokines such as MIP-2 (usually designated in the clinic as GRO- β) are utilized clinically for this purpose (50, 142, 194). While no direct studies comparing neutrophil and HPSC mobilization have been performed, the two pathways are thought to be linked, as they are mediated by the same agents, but exhibit different kinetics, with neutrophil mobilization occurring earlier (50, 142).

There is also the possibility that the CXCR4 and CXCR2 axes exhibit crosstalk. Treatment of neutrophils with CXCR2 ligands desensitizes CXCR4 signaling (185), but the mechanism was not established. Protein kinase C-

epsilon (PKC- ϵ) was implicated in the cross-desensitization of chemokines receptor signaling, but this possibility requires further testing in our model (195). Additionally, neutrophils from *Il8rb*^{-/-} chimeric mice do not mobilize to AMD3100, further indicating a relationship between the two pathways (149).

The baseline neutrophilia exhibited in STAT3-deficient mice has been attributed to impaired expression of the negative regulator SOCS3, a STAT3 target gene; however, the mechanism of the neutrophilia in *Il8rb*^{-/-} has not been demonstrated (88, 130). It was suggested that activation of CXCR2 results in negative feedback signaling to slow neutrophil production, but this has not been tested (130). Future studies examining the effects of CXCR2 activation on neutrophil proliferation and differentiation are required to examine if CXCR2 initiates a negative feedback program. It is known that mobilizing neutrophils upregulate CXCR2 and downregulate CXCR4 [Chapter 4 and (196)]; perhaps uncovering common regulatory or signaling pathways between these two receptors would lead us to their involvement in a negative feedback loop. Additionally, in β_2 -integrin-deficient mice, which also demonstrate basal neutrophilia and impaired neutrophil mobilization responses, excessive neutrophils are released from the bone marrow, and G-CSF and IL-17 levels were elevated in the serum and plasma, respectively (197, 198). However, antibodies blocking G-CSF and IL-17 reduced the observed neutrophilia in these mice (197, 198), indicating to the authors that neutrophil egress from the bone marrow may initiate a negative feedback loop to dampen expression of G-CSF- and IL-17 (197, 198). Further studies are required to establish this mechanism.

To begin with, it may be helpful to perform microarray analysis to compare bone marrow neutrophils and circulating neutrophils under steady-state conditions and mobilization-stimulated conditions (either by G-CSF or chemokines). This would reveal whether the mobilization-stimulated circulating neutrophil expresses anti-neutrophil production factors, such as anti-G-CSF or anti-IL-17.

The actions of KC, MIP-2, and SDF-1 to promote the migration and retention of neutrophils are well established. However, whether the two pathways exhibit crosstalk is not understood, and further analysis will promote our understanding of chemokine function, ultimately adding to our understanding of neutrophil and HPSC mobilization.

6.3. Activation of STAT3 contributes to the recruitment of histone methyltransferases and RNA Polymerase II

The association of DNA with histones results in a highly compacted structure. While some transcriptional regulators are able to bind DNA in this state, others require DNA to be dissociated from histones (99). This involves a dynamic process of DNA-histone association and dissociation as transcription progresses (99). The relationship between recruitment of transcription factors and the transcription pre-initiation complex (PIC), including RNA Polymerase II, is unknown. Furthermore, the relationship between the different types of histone modifications, such as methylation and acetylation, is unknown. It has been demonstrated that transcription initiation “takes a village”, requiring the

coordinated action of a large complex of proteins, but there is much that remains to be understood (199).

We demonstrate that the interaction of STAT3 with the *Il8rb* promoter follows similar kinetics as onset of RNA Polymerase II initiation, demonstrating that the recruitment of these proteins may be linked. As these experiments were performed using an antibody against Pol II CTD pSer5, a residue that becomes phosphorylated upon Pol II initiation, this result does not clarify when Pol II was recruited. These ChIP experiments should be repeated using antibodies against Pol II. Additionally, ChIPs can be performed using antibodies against components of the transcription PIC, such as TFIID, which is the first factor recruited to the PIC (104). Alternatively, studies utilizing deletion mutants of STAT3 can be utilized to map the region of STAT3 required for recruitment of the PIC. For example, the transactivation domain (TAD) of STAT3 may be important in recruitment of co-activators, and deletion of this region and overexpression of this truncated protein would reveal its function in this process.

Our studies further show that inhibition of STAT3 phosphorylation blocked the recruitment of Pol II CTD pSer5, as well as the accumulation of H3K4me3, supporting the idea that activation of STAT3 is required for recruitment of methyltransferases. Further studies are required to examine the relationship between STAT3 phosphorylation and the recruitment of methyltransferases. While there are many methyltransferases, one in particular, mixed-lineage leukemia (MLL), has been implicated in myeloid cells and is responsible for H3K4 methylation, and has also been shown to interact with Pol II (200). STAT3

interaction with MLL should be examined, either by co-immunoprecipitations or re-ChIP (ChIP first with anti-STAT3, then before breaking crosslinks, pull-down with anti-MLL). Similarly, these experiments can be repeated to detect other histone-modifying enzymes that are recruited upon G-CSF stimulation. Alternatively, microscopy techniques can be used to examine whether STAT3 and histone modifying enzymes co-localize in activated cells. These pathways have practical applications, for example 5-azacytidine, a cytidine analogue that inhibits the activity of methyltransferases, is used as a treatment for myelodysplastic syndrome, a condition of aberrant myeloid cell differentiation (201).

While techniques like ChIP-sequencing (ChIP-Seq) or ChIP combined with microarray analysis (ChIP on chip) would be very interesting, the data would indicate the DNA that is associated with the protein (for example, STAT3) for which we are assaying. Another method, such as co-localization studies utilizing detecting STAT3 and histone modifying enzymes, would demonstrate if these proteins are associated with similar regions of DNA. Rather, I would be more interested in exploring the proteins associated with STAT3 as it binds promoters. Co-immunoprecipitations between STAT3 and general transcription factors or other candidate transcription regulators can be tested. Alternatively, ChIP can be combined with protein detection experiments. For example, after stimulating cells to activate STAT3 and associate with DNA, and pulling down with anti-STAT3, this protein complex can be separated on gels, and examined for protein species such as by Coomassie blue staining. Depending on the

protein size, antibodies against candidate proteins can be used for immunoblotting. The Proteomics Core Facility at UT MDACC has the capability to perform in-gel digestion coupled to peptide sequencing to identify unknown proteins. This facility can also perform various types of mass spectrometry to identify proteins complexed with STAT3.

The simplified view that transcription is activated merely upon binding of transcription factors is proving to be outdated, as it is now understood that this process is very complicated, involving many, possibly hundreds, of different proteins (199). Although STAT3 is generally thought of a transcription activator, it has been proposed that a splice variant can act as a repressor (202). Regardless, the prevailing thought is that STAT3 likely coordinates its function with other proteins, forming a major complex that is called an enhanceosome (203, 204). The enhanceosome then, together with co-activators and histone modifying enzymes, increases transcription initiation (203, 204). Thus, further studies are required to understand the basic function of STAT3 as transcription is activated.

6.4. Summary

In this dissertation, we have demonstrated the requirement for STAT3 in the neutrophil mobilization pathway, as STAT3 regulates G-CSF-induced expression of MIP-2 and suppression of SDF-1 (Figure 3.8), as well as stimulation of CXCR2 (Figure 4.8). Further study of the mechanism of

chemokine regulation will reveal novel targets to address the balance between immunodeficiency and pathologic inflammation. Also, understanding neutrophil mobilization may reveal aspects that are common to HPSC mobilization, as both are stimulated by the same agents. We also demonstrate the requirement for phosphorylated STAT3 in the acquisition of G-CSF-induced H3K4me3 and recruitment of Pol II CTD pSer5 (Figure 5.12). Future detailed studies of STAT3 regulation of transcription will add to the understanding of general transcription initiation processes, as epigenetic inhibitors are now being used clinically.

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Vita

Hoai-Nam Tran Nguyen was born in Grand Rapids, Michigan, on April 4, 1976, the daughter of Nhi Nguyen and Lai Tran. After graduating from East Kentwood High School in Kentwood, Michigan, in 1994, she received her B.S. in Cellular and Molecular Biology from the University of Michigan in Ann Arbor, Michigan in 1998. She entered The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences in 2003.