Regulating Emergency Granulopoiesis

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Normally, neutrophil pools are maintained by “steady-state” granulopoiesis. Infections and inflammation, however, trigger neutrophilias that are supported by a hematopoietic program of accelerated granulopoiesis known as “emergency” granulopoiesis. Steady-state and emergency granulopoiesis are thought to depend on distinct members of the CCAAT enhancer binding protein (C/EBP) family of transcription factors, yet the extracellular cues that determine these developmental pathways are unclear. I hypothesize that inflammation elicits IL-1 which acts directly on hematopoietic progenitor cells for the induction of emergency granulopoiesis. Indeed, IL-1R₁⁻/⁻ mice fail to mount reactive neutrophilias in response to adjuvant-induced inflammation. Analysis of this specific impairment revealed an unanticipated role for IL-1RI in supporting increased proliferation by granulocyte/macrophage progenitors (GMP) and, surprisingly, more primitive multipotent progenitors (MPP) and hematopoietic stem cells (HSC). Whereas IL-1 drives HSC proliferation directly in vitro, inflammation induces comparable rates of proliferation in IL-1RI deficient and -sufficient HSC, MPP, and GMP in mixed chimeric mice. Thus, IL-1RI signals play a necessary, but indirect role in the support of alum-induced neutrophilias by expanding both pluripotent and myeloid progenitor compartments to accelerate granulopoiesis.

The lack of alum-induced neutrophilia in IL-1R₁⁻/⁻ mice is due to defective mobilization of bone marrow (BM) neutrophils and impaired proliferation of hematopoietic stem and progenitor cells (HSPC). Coincident defects in neutrophil mobilization and HSPC proliferation suggest that the trigger for emergency granulopoiesis might be the exhaustion of neutrophil compartments rather than
inflammatory inductions of growth factors. Consistent with this hypothesis, non-inflammatory reductions in BM neutrophil numbers elicit granulopoietic responses similar to those induced by adjuvant. Alum mobilizes BM neutrophils via G-CSF, but increased HSPC proliferation results from a density-dependent mechanism that is only partially dependent on G-CSF. Notably, C/EBPβ, thought to be necessary for enhanced generative capacity of BM, is dispensable for increased proliferation of HSPC, but plays a role in the terminal differentiation of neutrophils. These observations indicate that the draining of BM neutrophil pools is sufficient to activate a latent, homeostatic mechanism of accelerated granulopoiesis. I propose a common model for the regulation of neutrophil production that explains both steady-state and emergency granulopoiesis through negative feedback.
Dedication

To Angie and Sam.
Contents

Abstract ........................................................................................................................................ iv

List of Tables .................................................................................................................................. x

List of Figures .................................................................................................................................... xi

Abbreviations .................................................................................................................................. xii

Acknowledgments ........................................................................................................................... xiv

1. Overview ...................................................................................................................................... 1

  1.1 Effects of inflammation on hematopoiesis ........................................................................... 1

  1.2 Neutrophil development ......................................................................................................... 3

  1.3 Transcriptional control of granulopoiesis by C/EBP transcription factors ......................... 6

  1.4 Extracellular regulators of granulopoiesis .......................................................................... 14

  1.5 IL-1: Master regulator of emergency granulopoiesis? ....................................................... 19

2. IL-1RI dependent HSC proliferation is necessary for inflammatory granulopoiesis and reactive neutrophilia .................................................................................................................. 22

  2.1 Introduction ........................................................................................................................... 22

  2.2 Materials and methods .......................................................................................................... 25

  2.3 Results .................................................................................................................................. 28

    2.3.1 Flow cytometric definitions of myeloid and hematopoietic progenitor compartments .............................................................................................................. 28

    2.3.2 Alum-induced neutrophilias require IL-1RI .................................................................. 32

    2.3.3 Alum-induced inflammatory granulopoiesis is IL-1RI dependent ..................... 34

    2.3.4 Comparable HSC and MPP from naïve and immunized mice ....... 40

    2.3.5 Alum retains robust inflammatory and adjuvant properties in IL-1RI−/− mice ...... 43

    2.3.6 Responsiveness of HSC to IL-1 in vitro ....................................................................... 45
2.3.7 IL-1R\(^{+}\) HSC, MPP, and GMP proliferate in immunized BL/6 hosts...........48

2.4 Discussion ........................................................................................................... 51

3. Inflammation triggers emergency granulopoiesis through a density-dependent feedback mechanism of neutrophil replenishment ..............................................................57

3.1 Introduction ........................................................................................................... 57

3.2 Materials and methods ........................................................................................ 61

3.3 Results ................................................................................................................ 65

3.3.1 Bone marrow neutropenia by antibody depletion ........................................... 65

3.3.2 Bone marrow neutropenia by genetic deletion ............................................... 72

3.3.3 Induced proliferation of hematopoietic progenitors is inversely proportional to BM neutrophil numbers ........................................................... 74

3.3.4 HSC and MPP proliferation rates are uncorrelated to changes in B220\(^{+}\) or Ter119\(^{+}\) BM cell numbers ........................................................................................ 76

3.3.5 Minimal inflammation associated with neutrophil depletion by Gr-1 antibody 79

3.3.6 Cytokines, growth factors, and chemokines elicited by inflammation or neutropenia ............................................................................................................. 82

3.3.7 Dual roles for G-CSF/G-CSF-R signals in the induction of emergency granulopoiesis ......................................................................................................... 87

3.3.8 C/EBP\(\beta\) is dispensable for increased HSPC proliferation during emergency granulopoiesis ......................................................................................... 92

3.4 Discussion ........................................................................................................... 95

4. Conclusions ............................................................................................................... 102

4.1 Neutrophil population density in BM controls emergency granulopoiesis through feedback inhibition ..................................................................................... 103

4.2 HSPC sense BM neutrophil population density via G-CSF-dependent and independent pathways .................................................................................. 105

4.3 Neutrophil mobilization is necessary for the induction of emergency granulopoiesis ................................................................................................. 108
4.4 Redefining the role of C/EBPβ in emergency granulopoiesis......................... 109

4.5 Final remarks..................................................................................................... 111

References.................................................................................................................... 114

Biography...................................................................................................................... 129
List of Tables

Table 1: Factors that promote granulopoiesis ................................................................. 18
Table 2: BM leukocyte numbers (x10^5) in Mcl-1^+ and Mcl-1^- mice .............................. 73
Table 3: Frequencies of BrdU^+ HSPC in Mcl-1^+ and Mcl-1^- mice .................................. 73
Table 4: BM leukocyte numbers (x10^5) in BL/6 and RAG1^-/^- mice ............................. 78
List of Figures

Figure 1: Neutrophil phenotypes of C/EBPα−/− and C/EBPβ−/− mice ........................................... 13
Figure 2: Hypothesis for IL-1 dependent emergency granulopoiesis ...................................... 21
Figure 3: Definitions of granulocyte and monocyte compartments ........................................ 30
Figure 4: Definitions of LSK and myeloid progenitor compartments ...................................... 31
Figure 5: IL-1RI is required for alum-induced neutrophilia ...................................................... 33
Figure 6: IL-1RI is required for alum-induced emergency granulopoiesis .............................. 38
Figure 7: LSK cells from naïve and immunized mice have equivalent lineage potential. 42
Figure 8: Robust inflammatory responses in IL-1RI−/− mice .................................................... 44
Figure 9: HSC express IL-1RI and respond to IL-1 in vitro ................................................... 47
Figure 10: Alum induces IL-1RI-deficient HSC, MPP, and GMP to proliferate in chimeric mice ................................................................. 50
Figure 11: Adjuvant inflammation and experimental neutropenia elicit similar changes in granulopoiesis ........................................................................................................................................... 68
Figure 12: Effects of adjuvant immunization and Gr-1 administration on monocytes, B-lineage cells, eosinophils, and erythroid-lineages cells in BM ....................................................... 71
Figure 13: HSPC proliferation correlates inversely with the number of BM neutrophils. 75
Figure 14: HSC proliferation does not correlate with B-lineage or erythroid-lineage cells numbers after alum or Gr-1. .................................................................................. 77
Figure 15: Neutrophil depletion with Gr-1 mAb elicits emergency granulopoiesis with minimal inflammation. ............................................................... 81
Figure 16: Cytokines elicited by alum-induced inflammation or neutropenia. ................. 85
Figure 17: Dual roles for G-CSF/G-CSF-R signaling in the induction of emergency granulopoiesis. .......................................................... 90
Figure 18: C/EBPβ is dispensable for proliferative responses of HSPC to Gr-1 administration or alum immunization ........................................ 94
Figure 19: Feedback model for the regulation of granulopoiesis ........................................ 113
Abbreviations

BFU-E, burst-forming unit-erythroid
BL/6, C57BL/6
BM, bone marrow
BrdU, bromodeoxyuridine
CBF, core binding factor
C/EBP, CCAAT/enhancer binding protein
CFU, colony forming unit
CFU-G, CFU-granulocyte
CFU-GEMM, CFU-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM, CFU-granulocyte, macrophage
CFU-M, CFU-macrophage
CGG, chicken γ-globulin
CLP, common lymphoid progenitor
CMP, common myeloid progenitor
d, day
ELISA, enzyme-linked immunosorbent assay
FBS, fetal bovine serum
FcγR, Fcγ receptor
FcRγ, Fc receptor common γ chain
G-CSF, granulocyte-colony stimulating factor
G-CSF-R, granulocyte-colony stimulating factor receptor
GFP, green fluorescent protein
GM-CSF, granulocyte/macrophage colony stimulating factor
GM-CSFR, granulocyte/macrophage colony stimulating factor receptor
GMP, granulocyte/macrophage progenitor
HSC, hematopoietic stem cell
HSPC, hematopoietic stem and progenitor cell
IFNγ, Interferon γ
IL, Interleukin
IL-1RI, Interleukin-1 receptor Type I
KC, Keratinocyte-derived chemokine
KO, knockout
LT, long-term
mAb, monoclonal antibody
MCP-1, Monocyte chemotactic protein-1
M-CSF, Macrophage colony-stimulating factor
MEP, megakaryocyte/erythroid progenitor
MIP, Macrophage inflammatory protein
MPP, multipotent progenitor
MyD88, Myeloid differentiation primary response gene(88)
Nalp3, NACHT, LRR and PYD domains-containing protein 3
NP, (4-hydroxy-3-nitrophenyl)acetyl
PBS, phosphate-buffered saline
PI, propidium iodide
RANTES, Regulated upon activation, normal T-cell expressed, and secreted
SA, streptavidin
SCF, Stem cell factor
SDF-1, Stromal cell-derived factor-1
ST, short-term
TLR, Toll-like receptor
TNFα, Tumor Necrosis Factor α
WT, wild-type
Acknowledgments

I am greatly indebted to several people for their help and support in this research project and in my graduate training. Peter Johnson (NCI) provided C/EBPβ−/− mice and Dan Link (Washington University) provided G-CSF-R−/− mice. Within the Department of Immunology, Yiping Yang provided MyD88−/− mice, Tom Tedder provided C3−/− and FcRγ−/− mice, and You-wen He provided Mcl-1− mice. David DiLillo, a graduate student in the Tedder lab, provided technical assistance in the laboratory and thoughtful conversations at conferences. I am thankful for the expertise of Patti McDermott at the Duke Human Vaccine Institute’s Flow Cytometry shared resource facility. Motonari Kondo provided intellectual expertise in my studies of hematopoietic progenitor cells.

In the Kelsoe laboratory, I am thankful for the comradery and support of Pilar B. Snowden, T. Matt Holl, Yoshihiro Ueda, Masayuki Kuraoka, Guang Yang, Alex Reynolds, and Fei Fei Liu. When Pilar, Alex, and Guang first joined the laboratory as rotation students, I had the opportunity to train and mentor them; I only hope that they learned as much from me as I learned from them.

I thank Robert Sackstein, who took a risk and gave me a job in his laboratory. In doing so, he opened my eyes to the remarkable world of experimental science.

I will be eternally grateful to my mentor Garnett Kelsoe. For more than five years, Garnett held me to the highest standards of scientific excellence, and any success that I might have in this field will be a direct result of his training, patience, and wisdom.

My family has played a pivotal role in my education and career choice. I thank my parents for their love and support, and for instilling in me a curiosity for the natural world.
Lastly, I am grateful for my beautiful wife Angie, who attended my talks, edited my writing, propped me up when I struggled, congratulated me when I succeeded, and put up with my late nights on the FACS machine. Of all the events that took place during my time in graduate school, the most memorable was the birth of our son, Sam. I hope that Angie and Sam know how much they have enriched my life.
1. Overview

In 1898, Paul Ehrlich and Adolf Lazarus published “Die Anämien” (“The Anemias”) [translated into English in (Ehrlich and Lazarus, 1900)] in which they discussed the biological purpose of the “polynuclear leucocytosis” observed in infected patients. The authors conjectured that the abundance of neutrophilic leukocytes during inflammatory states represented a protective response of the host organism, and that these cells play important roles in the neutralization of microbial toxins and destruction and/or containment of pathogens (Ehrlich and Lazarus, 1900). In the ensuing century, the importance of neutrophilic leukocytes (polymorphonuclear leukocytes or simply “neutrophils”) to host defense became obvious, as patients rendered neutropenic through radiation exposure, pharmacologic myeloablation, or genetic defect were found to be extremely susceptible to bacterial and fungal infections (Bodey et al., 1966). Therefore, the regulation of neutrophil production, or “granulopoiesis”, has been a subject of intense scientific scrutiny; nevertheless, a consensus model describing the extracellular signals that modulate granulopoiesis has proven elusive.

1.1 Effects of inflammation on hematopoiesis

Under physiologic conditions, hematopoiesis sustains peripheral pools of erythrocytes, plates, and leukocytes. Inflammation, however, skews leukocyte production to favor granulopoiesis at the expense of lymphopoiesis (Nagaoka et al., 2000; Ueda et al., 2005; Ueda et al., 2004). Infections and tissue injury cause large numbers of neutrophils to mobilize from the bone marrow (BM) into the blood, resulting in a process known as reactive neutrophilia. At the same time, myeloid progenitors in the
BM are drawn into cycle, increasing the rate of neutrophil production to sustain blood neutrophilias (Basu et al., 2000; Hirai et al., 2006; Lieschke et al., 1994; Zhan et al., 1998). Indeed, neutrophilia is such a universal response to pathogens that blood neutrophil counts are used as diagnostic indicators of infection severity.

In the absence of inflammation, granulopoiesis occurs at a pace that ensures the routine upkeep of peripheral neutrophil compartments; in humans, \(\approx 10^{11}\) neutrophils are released from the BM daily to maintain circulating pools (Dancey et al., 1976; Price et al., 1996). The condition where neutrophil production equals neutrophil turnover is often referred to as the “steady-state” (Basu et al., 2000; Hirai et al., 2006; Walker and Willemze, 1980). However, the capacity to accelerate neutrophil production is substantial. Inflammation can inflate circulating neutrophil numbers even further and granulopoiesis appears to be particularly sensitive to inflammatory cues. Infections are thought to increase the “demand” for neutrophils by inducing growth factors that drive the proliferation and differentiation of neutrophil precursors [reviewed in (Metcalf, 1998)]. The inflammatory cues that control “demand-driven” or “emergency” granulopoiesis, however, are unknown.

The inflammatory expansion of granulocyte compartments in BM coincides with a reduction in lymphopoiesis. Inflammation mobilizes immature B-lineage cells from the BM to peripheral lymphoid tissues, resulting in extramedullary lymphopoiesis (Nagaoka et al., 2000; Ueda et al., 2004). Inflammation suppresses B lymphopoiesis via TNF\(\alpha\), which reduces retention and growth/survival signals for developing B-lineage cells, thereby increasing the availability of generative niches in BM (Ueda et al., 2005; Ueda et al., 2004). The expansion of granulopoiesis into these newly vacated BM compartments,
however, requires additional inflammatory signals that are independent of TNFα (Ueda et al., 2004).

My dissertation focuses on the mechanisms through which inflammation modifies hematopoietic output, with a special emphasis on the regulation of “emergency” granulopoiesis. Specifically, I seek to answer the following questions:

1. What are the extracellular cues elicited during the inflammatory response that accelerate neutrophil production?
2. What hematopoietic progenitor compartments are sensitive to and altered by inflammatory factors?
3. How do inflammatory signals integrate with the known transcriptional pathways of granulopoiesis?

Since neutrophils participate in a variety of pathologies, both pathogen- and auto-immune related, understanding how inflammation alters granulopoiesis will provide insight into an essential component of innate immunity, and may reveal therapeutic targets for the modulation of inflammatory processes.

1.2 Neutrophil development

As the most common leukocyte in the blood, neutrophils serve as the first-line of immune defense against microbial incursions. Neutrophils passively circulate through the body but rapidly respond to inflammatory signals by adhering to endothelium and migrating into sites of infection or tissue damage [reviewed in (Springer, 1994)]. Within inflamed tissues, neutrophils eliminate pathogens through phagocytosis, the generation of reactive oxygen species, and the release of microbicidal compounds [reviewed in (Nauseef, 2007)]. As pathogens are cleared from the affected tissue, neutrophils
undergo apoptosis; the clearance of these exhausted neutrophils by macrophages is an important step in the resolution of the inflammatory response [reviewed in (Kennedy and DeLeo, 2009)].

The effector functions of the neutrophil are based primarily on the storage of prefabricated compounds in specialized intracellular compartments called granules. These granules develop during neutrophil development. The granule proteins that mediate neutrophil migration, phagocytosis, superoxide generation, and microbicidal activity are produced in sequential “biosynthetic windows” during differentiation, resulting in the segregation of effector molecules into primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules [reviewed in (Faurschou and Borregaard, 2003)]. The mature neutrophil is transcriptionally inactive but is poised to neutralize pathogens; upon neutrophil activation, granules fuse with intra- or extra-cellular membranes to release their contents into pathogen-containing phagosomes or into the extracellular environment (Nauseef, 2007).

The toxic cargo of the neutrophil endows these cells with a formidable capacity to eliminate extracellular pathogens; however, the destructive nature of the neutrophil is a double-edged sword. Whereas neutropenia increases susceptibility to infection (Bodey et al., 1966; Malech and Nauseef, 1997), unrestrained neutrophil activity has the potential to damage healthy tissue. Indeed, neutrophils are chief contributors to the inflammatory pathology of sepsis (Aldridge, 2002), rheumatoid arthritis (Wipke and Allen, 2001), and inflammatory bowel disease (Fiocchi, 1998). Therefore, understanding the extracellular cues that regulate granulopoiesis may expose cellular or molecular targets for the treatment of inflammatory pathologies.
Granulopoiesis takes place in the bone marrow and, like all hematopoietic cells, neutrophils arise from a self-renewing population of hematopoietic stem cells (HSC) (Osawa et al., 1996). Long-term (LT-) HSC give rise to short-term (ST-) HSC by asymmetric division; ST-HSC remain totipotent but have a more limited capacity for self-renewal than LT-HSC (Yang et al., 2005). They, in turn, produce the multipotent progenitors (MPP) that give rise to committed progenitor lineages (Kondo et al., 2003), the common myeloid progenitor (CMP) (Akashi et al., 2000), that subsequently produces granulocyte/macrophage- (GMP) and megakaryocyte-erythroid progenitors (MEP) (Akashi et al., 2000), and the common lymphoid progenitor (CLP) (Kondo et al., 1997). GMP are histologically indistinguishable from the most primitive granulocyte precursors, promyelocytes, which form primary granules containing myeloperoxidase, neutrophil elastase, proteinase-3, and \( \alpha \)-defensins (Bainton et al., 1971; Cowland and Borregaard, 1999). Promyelocytes differentiate into myelocytes, which produce secondary granules containing lactoferrin, collagenase, transcobalamin-I, and cathelicidin (Cowland and Borregaard, 1999). Secondary granules are continuously produced as myelocytes mature into metamyelocytes but, from this stage onward, cells do not proliferate. Metamyelocytes differentiate into band neutrophils, which are characterized by the production of tertiary granules containing gelatinase B [matrix metalloproteinase-9 (MMP-9)]; the production of tertiary granules continues as band neutrophils differentiate into mature, segmented neutrophils (Borregaard et al., 1995; Kjeldsen et al., 1993).

Nascent neutrophils enter a pool of mature neutrophils resident in the BM; this neutrophil reserve comprises approximately 25% of nucleated cells in BM (Chervenick et al., 1968). Neutrophils of the BM reserve are released into the blood to replace
circulating neutrophils lost through turnover. Normally, the number of mature neutrophils in BM is approximately 100 times greater than the total number of neutrophils in the circulation, so the BM neutrophil reserve is thought to serve as a buffer between the peripheral neutrophil pool and the generative capacity of the BM (Semerad et al., 2002). The half-life of a circulating neutrophil is only 7-11 hours (Basu et al., 2002; Cartwright et al., 1964; Price et al., 1996), so the maintenance of the blood neutrophil pool requires the daily release of roughly $10^9$ neutrophils per kg of body weight from the BM (Dancey et al., 1976; Price et al., 1996). In the absence of inflammation, circulating neutrophils undergo spontaneous apoptosis, a form of cell death that does not depend on external stimuli (Akgul et al., 2001; Scheel-Toellner et al., 2004). Senescent neutrophils migrate into tissues or return to the BM for consumption by macrophages and dendritic cells (Martin et al., 2003; Stark et al., 2005).

1.3 Transcriptional control of granulopoiesis by C/EBP transcription factors

Several transcription factors drive the commitment and differentiation of neutrophils from HSC. Factors required for neutrophil differentiation include members of the C/EBP family [reviewed in (Friedman, 2007)], PU.1 (McKercher et al., 1996; Scott et al., 1994), CBF (Okuda et al., 1996), c-Myb (Bies et al., 1995; Mucenski et al., 1991), and GFI-1 (Karsunky et al., 2002). In general, transcription factors involved in granulopoiesis can be categorized by their roles in early vs. late neutrophil differentiation. Transcription factors expressed early in neutrophil development typically activate genes encoding primary granule proteins, whereas those elicited at later stages activate genes that encode secondary or tertiary granule proteins (Ward et al., 2000).
Gene targets for both early and late transcription factors also include cytokine receptors, homeobox genes, and factors involved in cell cycle.

The C/EBP family of transcription factors plays prominent roles in granulopoiesis and, as such, represents a focal point of this dissertation. C/EBPα is the founding member of the basic region-leucine zipper family of transcription factors (Graves et al., 1986) and was originally suspected to regulate genes involved in energy metabolism (McKnight et al., 1989). This hypothesis was confirmed in C/EBPα−/− mice, which die perinatally due to impaired expression of genes involved in glucose synthesis, storage, and export (Wang et al., 1995). C/EBPα was also found to activate the primary granule proteins myeloperoxidase (Nuchprayoon et al., 1994) and neutrophil elastase (Nuchprayoon et al., 1994; Oelgeschlager et al., 1996), suggesting a role in neutrophil differentiation. A crucial role for C/EBPα in granulopoiesis was indicated by the observation that C/EBPα overexpression induces granulocyte differentiation in both human and murine myeloid cell lines (Radomska et al., 1998; Wang et al., 1999). An essential role for C/EBPα in granulopoiesis was revealed by the observation that neonatal C/EBPα−/− mice lack mature neutrophils and eosinophils (Zhang et al., 1997) and, when transferred into irradiated adult hosts, C/EBPα−/− fetal liver cells fail to generate granulocytes (Zhang et al., 1997). In adult mice bearing a conditional deletion of C/EBPα, a severe blockade in myeloid development is evident at the transition from the CMP to GMP stage of development, and these mice exhibit substantially reduced numbers of neutrophils, eosinophils, and monocytes (Zhang et al., 2004). However,
C/EBPα is dispensable for neutrophil differentiation beyond the GMP stage (Zhang et al., 2004).

Mutations in C/EBPα are also thought to contribute to tumorigenesis, as approximately 9% of patients with acute myeloid leukemia bear tumors with mutations in C/EBPα (Pabst et al., 2001). In most of these patients, C/EBPα mutations result in the expression of a dominant-negative form of the transcription factor that impairs granulocyte differentiation while promoting proliferation (Pabst et al., 2001). Together, these observations demonstrate that C/EBPα plays a critical role in the early commitment and differentiation of hematopoietic progenitor cells to the neutrophil lineage, and that mutations in C/EBPα are associated with the developmental block of AML.

Whereas C/EBPα is crucial during early granulocyte differentiation and development (Zhang et al., 2004), C/EBPε plays an important role in terminal stages of neutrophil differentiation. Gene targets for C/EBPε include the G-CSF receptor (G-CSF-R) and lactoferrin (Verbeek et al., 1999; Yamanaka et al., 1997b). C/EBPε−/− mice have reduced numbers of mature neutrophils and eosinophils (Lekstrom-Himes and Xanthopoulos, 1998; Yamanaka et al., 1997a), and the residual neutrophils lack secondary and tertiary granules (Verbeek et al., 1999). Neutrophils in C/EBPε−/− mice also exhibit delayed recruitment into sites of inflammation, likely due to the absence of secondary and tertiary granules, which normally contain intracellular reserves of adhesion molecules (Lekstrom-Himes and Xanthopoulos, 1999). Functional loss of C/EBPε in humans results in a congenital disorder called neutrophil specific granule
deficiency (SGD); neutrophils from these patients exhibit impaired migration and microbicidal activity [reviewed in (Gombart and Koeffler, 2002)].

The general model of granulopoiesis that has emerged from these observations is that C/EBPα is the critical transcription factor for commitment and early differentiation of neutrophil-lineage cells, and that C/EBPε is the subsequent inducer of terminal differentiation. Despite the observation that C/EBPα-/- mice fail to generate granulocytes, two groups found evidence of C/EBPα-independent pathways of neutrophil production. Zhang et al. (Zhang et al., 1998) reported the recovery of mature granulocytes in cultures of C/EBPα-/- fetal liver cells that were supplemented with IL-3 or GM-CSF. Neutrophil-lineage cells were also generated from C/EBPα-/- fetal liver cells in vitro by the addition of IL-6 and soluble IL-6 receptor (Zhang et al., 1998). Collins et al. (Collins et al., 2001) generated multipotent progenitor cell lines deficient for C/EBPα by transducing C/EBPα-/- fetal liver cells with a dominant-negative retinoic acid receptor. Neutrophils arose spontaneously from these lines in culture, and the addition of GM-CSF, especially when combined with G-CSF or retinoic acid, enhanced granulocyte production in vitro (Collins et al., 2001). These observations indicate the existence of alternative pathways of granulopoiesis independent of C/EBPα.

Two studies suggested that C/EBPβ might mediate a second pathway of granulopoiesis (Jones et al., 2002; Wang and Friedman, 2002). Like C/EBPα, C/EBPβ overexpression in a myeloid progenitor cell line induces granulocytic differentiation (Wang and Friedman, 2002) and C/EBPβ expression from the C/EBPα locus rescues granulopoiesis (Jones et al., 2002), indicating that C/EBPα and C/EBPβ share gene
targets necessary for granulocyte differentiation. However, initial observations of C/EBPβ−/− mice did not reveal defects in granulopoiesis (Screpanti et al., 1995; Tanaka et al., 1995). Instead, C/EBPβ was found to play important roles in macrophage function (Tanaka et al., 1995) and gene transcription in response to pathogen products and pro-inflammatory cytokines (Akira et al., 1990; Poli et al., 1990). An important granulopoietic role for C/EBPβ was discovered by Hirai et al. (Hirai et al., 2006), who demonstrated that C/EBPβ−/− mice fail to mount reactive neutrophilias in response to fungal infection or G-CSF treatment, despite having normal granulocyte numbers in the steady-state. These observations suggest that C/EBPβ is dispensable for the routine production of neutrophils but is crucial for the accelerated granulopoiesis that occurs in response to physiologic emergencies, including infections.

C/EBPβ expression normally occurs during late stages of neutrophil development (Bjerregaard et al., 2003), but during infections or in response to cytokines, C/EBPβ transcripts increase in GMP, presumably driving granulopoiesis even in the absence of C/EBPα (Hirai et al., 2006). The current paradigm for the regulation of granulopoiesis is that granulocyte differentiation can occur through two distinct pathways that are differentially dependent on C/EBPα and C/EBPβ (Hirai et al., 2006). Normally, C/EBPα-dependent granulopoiesis produces neutrophils at a rate that maintains steady-state neutrophil pools. Inflammation, however, elicits an accelerated pathway of granulopoiesis that is independent of C/EBPα but requires C/EBPβ. According to this model, infections and other physiologic insults induce the production of growth factors that interact with cognate receptors on GMP and elicit C/EBPβ transcription. C/EBPβ
expression in GMP promotes proliferation, increasing the rate of granulopoiesis to support and sustain neutrophilia. This model explains the cytokine-induced rescue of granulopoiesis from C/EBP<sup>α</sup>-/- hematopoietic progenitor cells (Collins et al., 2001; Hirai et al., 2006; Zhang et al., 1998) by the activation of C/EBPβ-dependent granulopoiesis (Figure 1).

Although neutrophils produced through either C/EBP pathway are functionally identical (Hirai et al., 2006; Jones et al., 2002), the two pathways of granulopoiesis are distinguished by their rates of neutrophil production. Both C/EBP<sup>α</sup> and -β have been implicated as regulators of cell cycle machinery. C/EBP<sup>α</sup> overexpression in several cell lines and primary cells arrests cell growth (Hirai et al., 2006; McKnight, 2001) by directly interfering with the ability of cyclin-dependent kinases, proteins crucial for the transition from G1 to S phase of the cell cycle, to interact with their cyclin substrates (Wang et al., 2001). Furthermore, C/EBP<sup>α</sup> promotes p21, a cyclin-dependent kinase inhibitor, by inducing transcription and prolonging protein half-life via post-translational stabilization (Timchenko et al., 1996). C/EBP<sup>α</sup> also represses E2F, a transcriptional activator of cell cycle genes (Porse et al., 2001), which in turn inhibits c-Myc, a positive regulator of cyclin expression and negative regulator of p21 (Johansen et al., 2001). Cell cycle arrest is necessary for C/EBP<sup>α</sup>-induced granulopoiesis, as mutations that impair C/EBP<sup>α</sup>’s anti-proliferative effects also prohibit its capacity to induce neutrophil differentiation (Wang et al., 2003).

Whereas C/EBP<sup>α</sup> inhibits cell cycle entry, C/EBPβ is thought to permit, and perhaps even promote, proliferation. In the same conditions where C/EBP<sup>α</sup>
overexpression restricts proliferation, C/EBPβ overexpression does not affect proliferation (Wang et al., 2001; Xie et al., 2004). Overexpression of C/EBPβ in multiple myeloma cells actually increases their proliferation, whereas expression of dominant-negative C/EBPβ represses proliferation (Pal et al., 2009). The consensus that has emerged from these observations is that C/EBPα exerts anti-proliferative effects while activating genes for neutrophil differentiation; C/EBPβ, on the other hand, both promotes proliferation and granulocyte development. The diametric effects of C/EBPα and C/EBPβ on cell proliferation represent the fundamental difference between the steady-state and emergency pathways of granulopoiesis (Hirai et al., 2006).
In wild-type mice, infections accelerate granulopoiesis and elicit blood neutrophilia by inducing growth factors. C/EBPα−/− mice, however, fail to produce neutrophils unless treated with inflammatory growth factors. In contrast, C/EBPβ−/− mice generate normal numbers of neutrophils in the steady-state, but inflammation does not elicit neutrophilia.

**Figure 1: Neutrophil phenotypes of C/EBPα−/− and C/EBPβ−/− mice**
1.4 Extracellular regulators of granulopoiesis

Whereas the control of steady-state and emergency granulopoiesis by C/EBPα and C/EBPβ is clear, how that control is manifested by extracellular cues remains obscure. A large number of growth factors have been identified that promote neutrophil production when supplemented into BM cultures or when administered pharmacologically to animals (Table 1). A central issue of my dissertation concerns the integration of the various granulopoietic signals with the known transcriptional programs of granulocyte differentiation. Which growth factors support granulopoiesis in the steady-state and which accelerate granulopoiesis in response to inflammation? If one subscribes to the current paradigm that C/EBPα and C/EBPβ control independent pathways of granulopoiesis, then the following predictions can be made:

I. Normally, peripheral neutrophil pools are maintained through homeostatic mechanisms. Although these mechanisms are not well-defined, there is evidence that neutrophil production rates fluctuate in response to perturbations of granulocyte compartments. Since naïve C/EBPα−/− mice lack neutrophils (Zhang et al., 1997; Zhang et al., 2004), any such homeostatic mechanism is insufficient to elicit C/EBPα-independent pathways of granulopoiesis. Thus, factors that govern neutrophil production in the steady-state (i.e., in the absence of inflammation) cannot be inducers of C/EBPβ-dependent emergency granulopoiesis.

II. If one class of growth factors drives C/EBPα-dependent granulopoiesis for the basal production of neutrophils, then mice deficient for these growth factors will exhibit reduced neutrophil numbers in the unmanipulated state. However, since the
C/EBPβ-dependent pathway of granulopoiesis is still operational in these mice, inflammation will stimulate reactive neutrophilias.

III. Conversely, mice deficient for growth factors that induce emergency granulopoiesis will have normal neutrophil numbers in the naïve state (because of C/EBPα-dependent granulopoiesis), but will fail to generate neutrophilias in response to inflammation.

Using this conceptual framework, I examined the literature surrounding the growth factors listed in Table 1 for evidence of obligatory roles in the inflammatory induction of reactive neutrophilia. Surprisingly, many of the factors postulated to control emergency granulopoiesis, including G-CSF, GM-CSF, IL-3, and IL-6, are capable of eliciting neutrophilia when administered pharmacologically, but are dispensable for accelerations in granulopoiesis during inflammatory responses.

G-CSF is generally thought to be the primary mediator of granulopoiesis, as mice lacking G-CSF or the G-CSF receptor have 80% reductions in blood neutrophil numbers and 50% reductions in BM neutrophil numbers (Lieschke et al., 1994; Liu et al., 1996). These observations are consistent with G-CSF functioning as a regulator of steady-state granulopoiesis. The role for G-CSF in emergency granulopoiesis, however, is less clear. Pharmacologic administration of recombinant G-CSF is a potent stimulator of granulopoiesis (Moore et al., 1987), raising the possibility that G-CSF induction during inflammation might drive emergency granulopoiesis. Indeed, serum G-CSF is commonly elevated during the acute phase of infections (Kawakami et al., 1990).

On the other hand, there is substantial evidence that G-CSF is dispensable for the reactive neutrophilias elicited by infections. Zhan et al. (Zhan et al., 1998) observed
expansions of Gr-1+ granulocyte compartments in BM during *Listeria monocytogenes* infection of wild-type mice, and although the absolute number of granulocytes in infected G-CSF−/− mice was lower than in infected wild-type animals, the fold increase in granulocyte numbers over naïve controls was greater in G-CSF−/− mice than in wild-type animals. This observation demonstrates that infections are capable of accelerating granulopoiesis above the basal production rate through a G-CSF-independent pathway.

A similar observation was made by Basu et al., who observed that G-CSF is dispensable for the inflammatory neutrophilia elicited by *Candida albicans*; emergency granulopoiesis is also intact in mice doubly deficient for G-CSF and GM-CSF or IL-6 (Basu et al., 2000). Furthermore, mice triply deficient for G-CSF, GM-CSF, and M-CSF still mount neutrophilias during thioglycollate-induced peritonitis (Hibbs et al., 2007). These observations implicate G-CSF as a primary factor in the maintenance of steady-state neutrophil pools, but disqualify G-CSF as an essential regulator of emergency granulopoiesis.

Unlike G-CSF, GM-CSF and IL-3 are dispensable for steady-state granulopoiesis as mice deficient for these cytokines or their receptors exhibit normal neutrophil numbers (Lantz et al., 1998; Nishinakamura et al., 1996; Stanley et al., 1994). However, both cytokines are suspected activators of C/EBPβ-dependent emergency granulopoiesis because they rescue neutrophil production from C/EBPα−/− hematopoietic progenitor cells (Collins et al., 2001; Hirai et al., 2006; Zhang et al., 2002). Furthermore, when overexpressed in mice, either GM-CSF or IL-3 induces robust neutrophilia (Hirai et al., 2006). Despite these observations, both factors are dispensable for emergency granulopoiesis elicited by *L. monocytogenes* and/or *C. albicans* infection (Basu et al.,
2000; Nishinakamura et al., 1996), indicating that GM-CSF and IL-3, while sufficient to induce neutrophilia, are not necessary for inflammatory accelerations of granulopoiesis.

IL-6 has also been implicated as an inducer of emergency granulopoiesis because of its ability to support neutrophil production from C/EBPα−/− progenitors in vitro (Zhang et al., 1998). Like GM-CSF and IL-3, IL-6 is not essential for steady-state granulopoiesis (Bernad et al., 1994). Notably, mice doubly deficient for IL-6 and G-CSF-R have fewer neutrophils than mice lacking G-CSF-R alone (Liu et al., 1997), indicating that IL-6 contributes to the residual granulopoiesis in G-CSF-R−/− mice. However, IL-6 injection into mice induces neutrophilia (Pojda and Tsuboi, 1990), providing preliminary evidence for IL-6 as a possible mediator of emergency granulopoiesis. Using an in vitro model of granulopoiesis, Walker et al. (Walker et al., 2008) reported that the conditioned medium of LPS-stimulated embryonic fibroblasts supports neutrophil production via IL-6. However, IL-6 does not appear to play a crucial role in the inflammatory production of neutrophils as mice deficient for both G-CSF and IL-6 retain the ability to mount robust granulopoietic responses to fungal infection (Basu et al., 2000).

The resilience of emergency granulopoiesis to the genetic inactivation of G-CSF, GM-CSF, IL-3, and IL-6 might indicate that multiple factors interact or synergize to induce emergency granulopoiesis. If this notion is correct, C/EBPβ must coordinate the increased and redundant expression of IL-6, IL-3, GM-CSF and/or G-CSF during inflammatory responses. Alternatively, a factor(s) other than G-CSF, GM-CSF, IL-3, and IL-6 could be responsible for triggering emergency granulopoiesis.
Table 1: Factors that promote granulopoiesis

<table>
<thead>
<tr>
<th>Factor</th>
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<tbody>
<tr>
<td>G-CSF</td>
<td>(Nicola et al., 1983)</td>
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<tr>
<td></td>
<td>(Metcalf and Nicola, 1983)</td>
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<tr>
<td></td>
<td>(Souza et al., 1986)</td>
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<td>(Lord et al., 1989)</td>
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<td>GM-CSF</td>
<td>(Burgess et al., 1977)</td>
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<td></td>
<td>(Metcalf et al., 1987b)</td>
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<td></td>
<td>(Bot et al., 1990)</td>
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<td></td>
<td>(Lord et al., 1991)</td>
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<tr>
<td>IL-3</td>
<td>(Metcalf et al., 1986)</td>
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<tr>
<td></td>
<td>(Metcalf et al., 1987a)</td>
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<tr>
<td></td>
<td>(Lord et al., 1991)</td>
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<tr>
<td>IL-6</td>
<td>(Pojda et al., 1990)</td>
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<tr>
<td></td>
<td>(Liu et al., 1997)</td>
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<tr>
<td>Stem Cell Factor (SCF)</td>
<td>(Broxmeyer et al., 1991a)</td>
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<tr>
<td></td>
<td>(Broxmeyer et al., 1991b)</td>
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<tr>
<td>IL-11</td>
<td>(Cairo et al., 1993)</td>
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<tr>
<td></td>
<td>(Cairo et al., 1994)</td>
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<tr>
<td>IL-1</td>
<td>(Moore and Warren, 1987)</td>
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<tr>
<td></td>
<td>(Gasparetto et al., 1989)</td>
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<tr>
<td></td>
<td>(Moore et al., 1990)</td>
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<tr>
<td>Retinoic acid</td>
<td>(Labbaye et al., 1994)</td>
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<td></td>
<td>(Tocci et al., 1996)</td>
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<tr>
<td>M-CSF</td>
<td>(Yanai et al., 1990)</td>
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The factors listed in Table I have the capacity to enhance neutrophil production, either as supplements in BM cultures or by pharmacologic administration into animals.
1.5 IL-1: Master regulator of emergency granulopoiesis?

In my laboratory, studies of inflammation’s effects on hematopoiesis have been conducted using sterile adjuvants, including alum, to stimulate immune responses (Ueda et al., 2005; Ueda et al., 2004). Alum adjuvant has been widely used in human vaccines for almost 70 years because of its ability to enhance specific humoral immunity (Baylor et al., 2002). Despite the global use of alum-based vaccinations, the reasons for its adjuvanticity have remained enigmatic. It has been postulated that alum enhances humoral immunization by inducing leukocyte recruitment to sites of immunization and prolonging exposure of precipitated antigens to immune cells (Goto and Akama, 1982; Kool et al., 2008a; Kool et al., 2008b; Morefield et al., 2005).

Recent studies have demonstrated that alum induces inflammation through an intracellular sensor system called the Nalp3 inflammasome (Eisenbarth et al., 2008; Franchi and Nunez, 2008; Hornung et al., 2008; Kool et al., 2008a). Activation of the Nalp3 inflammasome results in the secretion of IL-1β, IL-18, and IL-33 [reviewed in (Petrilli et al., 2007)]. Since IL-1 treatment has been shown to enhance neutrophil recovery after myeloablative treatment (Moore and Warren, 1987), alum’s capacity to induce IL-1β raises the tantalizing possibility that it is a regulator of emergency granulopoiesis. Mice lacking the Type I IL-1 receptor (IL-1RI) bear no abnormalities in hematopoiesis (Glaccum et al., 1997), indicating that steady-state granulopoiesis is untouched by the absence of IL-1 signaling. The more intriguing observation, however, is that the emergency granulopoiesis elicited by alum immunization can be recapitulated by the co-administration of IL-1β and TNFα, with IL-1β driving the expansion of BM
neutrophil compartments (Ueda et al., 2004). It is also worth noting that IL-1β is already known to induce C/EBPβ expression, raising the possibility of a direct link between an inflammatory signal and the transcription factor that controls emergency granulopoiesis (Akira et al., 1990). These observations provide preliminary evidence for IL-1 as a master regulator of emergency granulopoiesis.

In my dissertation, I hypothesize that inflammation induces the production of IL-1 which stimulates emergency granulopoiesis through direct interaction with IL-1 receptors on hematopoietic progenitor cells (Figure 2). To test this hypothesis, I shall exploit mice with genetic ablation of IL-1RI, the only known signaling molecule for IL-1α/β, and an in vivo model of inflammation based on the injection of alum adjuvant.
Figure 2: Hypothesis for IL-1 dependent emergency granulopoiesis

**Steady-state Granulopoiesis**
- C/EBPα → Homeostatic Factors

**Emergency Granulopoiesis**
- C/EBPβ → IL-1

**Inflammation**

**C/EBPα**

**C/EBPβ**
2. IL-1RI dependent HSC proliferation is necessary for inflammatory granulopoiesis and reactive neutrophilia

In this chapter, I identify IL-1RI signaling as an essential, although indirect, mediator of alum-induced emergency granulopoiesis. This work was conducted in equal collaboration with Yoshihiro Ueda, a post-doctoral fellow in the laboratory. We were assisted by Masayuki Kuraoka, another post-doctoral fellow in the laboratory, who measured humoral responses in wild-type and IL-1R1−/− mice after immunization. Motonari Kondo, professor of immunology in the Department of Immunology at Duke, provided reagents as well as technical and intellectual expertise. The work in this chapter has been published (Ueda et al., 2009).

2.1 Introduction

Neutrophils are vital to innate immunity (Engle and Rosenfeld, 1984; Gessler et al., 1995). Normally, physiologic numbers of mature neutrophils are maintained by a "steady-state" granulopoietic pathway; acute infection or inflammation, however, trigger the mobilization of neutrophil stores from the BM and blood into inflammatory sites (Burg and Pillinger, 2001; Quie, 1980). The result of this mobilization is a reactive neutrophilia immediately followed by accelerated or "emergency" granulopoiesis in the BM (Basu et al., 2000; Hirai et al., 2006).

Neutrophils, like all other leukocytes, originate from self-renewing, LT-HSC. By asymmetrical division, LT-HSC give rise to ST-HSC that possess a limited capacity for self-renewal (Yang et al., 2005) and finally develop into MPP (Adolfsson et al., 2005)]. MPP produce lineage committed progenitors, including CMP that differentiate into MEP or GMP (Akashi et al., 2000). GMP produce neutrophils through a series of
developmental stages, first as promyelocytes, myelocytes, and metamyelocytes, then band neutrophils, and finally, mature, segmented neutrophils (Ueda et al., 2005).

Steady-state and “emergency” granulopoiesis can be distinguished by separate dependencies on the C/EBP$\alpha$ and C/EBP$\beta$ transcription factors (Hirai et al., 2006; Zhang et al., 1997). Genetic disruption of C/EBP$\alpha$ abolishes steady-state granulopoiesis (Zhang et al., 1997) whereas C/EBP$\beta$ deficiency leaves steady-state granulopoiesis intact but abrogates reactive neutrophilias (Hirai et al., 2006).

It is generally thought that emergency granulopoiesis is activated by the increased expression of granulopoietic factors, IL-6, IL-3, G-CSF, and GM-CSF, that induce granulocytic progenitors to proliferate (Hirai et al., 2006; Walker et al., 2008). Indeed, microbial infections increase serum GM-CSF and G-CSF coincidentally with the number of myeloid progenitors in BM (Cheers et al., 1988). In vitro, IL-6, IL-3, GM-CSF, and G-CSF promote proliferation and granulocytic differentiation by myeloid progenitors (Caracciolo et al., 1989; Donahue et al., 1988; Koike et al., 1986; Metcalf, 1978; Souza et al., 1986). Finally, neutrophilias elicited by over-expression of G-CSF, GM-CSF, or IL-3 are associated with increased C/EBP$\beta$ transcription in GMP, suggesting that C/EBP$\beta$ regulates granulopoiesis through GMP (Hirai et al., 2006).

Despite these correlations, mice deficient for G-CSF, GM-CSF and IL-6, or G-CSF and GM-CSF mount reactive neutrophilias (Basu et al., 2000; Hibbs et al., 2007), and both steady-state and emergency granulopoiesis are intact in mice deficient for the common $\beta$ chain of IL-3R/GM-CSFR/IL-5R (Nishinakamura et al., 1996). Thus, IL-6, IL-3, GM-CSF, and G-CSF are dispensable for reactive neutrophilias and emergency
granulopoiesis. To understand emergency granulopoiesis, identification of indispensable factors is required.

We have observed that alum promotes granulopoiesis at the expense of B lymphopoiesis, and have proposed that these lineages compete for developmental resources in the BM (Ueda et al., 2005; Ueda et al., 2004). TNFα initiates these hematopoietic changes by lowering BM CXCL12 expression and mobilizing BM lymphocytes; TNFα alone, however, has little effect on granulopoiesis (Ueda et al., 2004). Instead, TNFα and IL-1 synergize to reproduce alum’s effects on the BM (Ueda et al., 2004) and that synergy implicated IL-1 as a central factor in emergency granulopoiesis.

Here we show that alum does not elicit reactive neutrophilias and emergency granulopoiesis in mice which lack functional receptors for IL-1. These defects are not due to generally diminished responses to alum’s inflammatory or adjuvant properties, as other responses, including eosinophilia and antibody production, remain intact or are enhanced. Alum does not mobilize neutrophils from the BM of IL-1R1−/− mice nor does it elicit emergency granulopoiesis as determined by accelerated granulopoietic output or proliferation by HSC, MPP, and GMP. These effects are indirect, as neutrophil mobilization and emergency granulopoiesis in bone marrow chimeric mice is determined by IL-1RI expression on radiation-resistant, non-hematopoietic cells in the host. We conclude that IL-1RI provides an indispensable signal that induces secondary factors to initiate and sustain reactive neutrophilias and emergency granulopoiesis.
2.2 Materials and methods

**Mice.** C57BL/6 (CD45.2), congenic CD45.1 [B6.SJL-Ptpcr<sup>a</sup> Pep3<sup>b</sup>/BoyJ; (Ueda et al., 2004)], and congenic IL-1R<sup>−/−</sup> mice [B6.129S7-Il1r1<sup>tm1Imx</sup>/J (Glaccum et al., 1997)] were from Jackson Laboratories (Bar Harbor, ME). (C57BL/6 x C57BL/6.CD45.1)F<sub>1</sub> mice were bred locally. Mice were housed in specific pathogen-free conditions at the Duke University Animal Care Facility and with sterile bedding, water, and food. All studies were approved by the Duke University Institutional Animal Care and Use Committee.

**Immunization.** Mice were immunized with one injection of NP<sub>8</sub>-CGG (60 µg) in alum (Ueda et al., 2004).

**Flow Cytometry.** Cell suspensions from blood and BM were stained with FITC-, PE-, PE-Texas-Red, biotin-, allophycocyanin- (APC), APC-Cy7, PE-Cy5-, and PE-Cy7-conjugated mAb specific for mouse B220, IgM, CD11b, CD34, CD4, CD8, Ter119, Gr-1, CD117, Sca-1, IL-7Rα, FcγRII/III, Flt3, or Ly-6G (BD Bioscience or eBioscience); and PE-conjugated Ly-6B mAb (AbD Serotec). Streptavidin (SA) -PE-Texas-Red or SA-Texas-Red (BD Bioscience) were used to identify biotinylated mAb. Propidium iodide (Sigma) labeling identified dead cells. Labeled cells were analyzed or sorted with FACS Vantage SE<sup>TM</sup> or LSRII<sup>TM</sup> flow cytometers (BD Bioscience); data were analyzed with FlowJo software. LSK cells, Flt3<sup>−</sup> LSK cells, and Flt3<sup>+</sup> LSK cells were sorted from BM suspensions following enrichment with CD117 microbeads (Miltenyi Biotec).
**CFU-Complete Assay.** Flt3− LSK cells (100) from naïve or immunized (d 2) mice were cultured in methylcellulose media (StemCell Technologies) with mouse SCF, IL-3, IL-6, and human erythropoietin. Later (8 d), colonies were counted and typed as CFU-GEMM, CFU-GM, BFU-E, CFU-M, and CFU-G by microscopy.

**Limiting Dilution Assays.** Flt3− LSK cells were sorted from BM pools from naïve (n=4) and immunized (d 2; n=4) mice. Cultures containing 1 (n=72), 2 (n=48), or 8 (n=12) Flt3− LSK cells were made by sorting onto OP9 stromal cell layers in 96-well plates with 10 ng/ml IL-7 and 10 ng/ml Flt3 ligand (R&D systems) (Kondo et al., 1997). After 14 d, wells containing CD45−CD11b−B220−CD19+ cells (Kondo et al., 1997) were scored positive for B-cell production.

**BrdU Labeling.** Mice were injected i.p. with 1 mg BrdU; 6 h after injection, BM cells were harvested and labeled to identify specific populations. Labeled BM cells were fixed, permeabilized, and treated with DNase I to expose incorporated BrdU using a commercial kit (BD Bioscience); cells were then stained with FITC-labeled anti-BrdU for flow cytometric analysis. These preparations denatured the CD34 epitope (data not shown), preventing the discrimination of CMP and MEP (Figure 4).

**HSC Culture.** HSC, MPP, and GMP (n=500) were cultured with recombinant IL-1β (1 ng/ml; Peprotech) or GM-CSF (1 ng/ml; Peprotech) in X-VIVO15 serum-free medium (Cambrex) containing SCF (25 ng/ml; R&D systems) to promote HSC survival (Domen and Weissman, 2000). Cultured cells were enumerated and characterized after 4 d.
Quantification of mRNA. mRNA from cells (10^4-10^5) was precipitated in Trizol (Invitrogen) and reverse transcribed with Superscript II (Invitrogen). Quantitative PCR amplifications of cDNA were performed (iCycler thermal cycler, Bio-Rad Laboratories) with SYBR® Green PCR Master Mix™ (Applied Biosystems) using primers specific for IL-1RI, GM-CSFR, and β-actin cDNA: IL-1RI forward, 5’-CTGAGGTCTTGGAGGGACAG-3’, and reverse, 5’-TCCTTCTGGATGAGAGCAT-3’; GM-CSFR forward, 5’-GACACGAGGATGAAGCACTG-3’, and reverse, 5’-GAGGTCCTTCTGAGGGTCT-3’; and β-actin forward, 5’-AGCCATGTACGTAGCCATCC-3’, and reverse, 5’-CTCTCAGCTGTGGTGGTGAA-3’. Amplification parameters: initial denaturation at 94°C for 10 m; amplification cycle, denaturing at 94°C for 10 s, anneal/extension at 60°C for 45 s. Relative gene expression was calculated by the comparative C_T (threshold cycle) method of the manufacturer (Applied Biosystems) normalized to β-actin message; ΔC_T values were determined by subtracting C_T (target) from C_T (β-actin). Expression levels relative to β-actin were defined as: 2^-ΔCT.

Serum Ab measurements. NP-specific serum antibodies were quantified as described (Ueda et al., 2004).

Adoptive reconstitutions. (C57BL/6 x C57BL/6.CD45.1)F_{1} mice were sublethally irradiated [600 rad, (Congdon et al., 1956)] and reconstituted with equal numbers (5x10^6) of congenic C57BL/6.CD45.1 (IL-1RI^+/+) and C57BL/6 (IL-1RI^-/-) BM cells to generate mixed chimeric mice. Reciprocal chimeras were generated similarly [IL-1RI deficient
C57BL/6→irradiated (C57BL/6.CD45.2/CD45.1)F₁ and (C57BL/6.CD45.2/CD45.1)F₁→
into irradiated C57BL/6 (IL-1RI⁻⁻). To control for any effects of hematopoietic
reconstitution in these KO→WT and WT→KO chimeras, homologous [WT→WT and
KO→KO] animals were created as well. Donor- and recipient cells in mixed and
reciprocal chimeras were distinguished by CD45.1 and CD45.2 expression; chimerism
was determined by the CD45.1:CD45.2 ratio of blood leukocytes.

**Statistics.** Paired data were analyzed by Student's t test.

### 2.3 Results

#### 2.3.1 Flow cytometric definitions of myeloid and hematopoietic
progenitor compartments

Previous studies of granulopoiesis in my laboratory defined granulocytes by the
coproxpression of the Gr-1 antigen (Ly-6G/C) and CD11b; surface expression of the Gr-1
antigen increases as granulocytes develop, allowing for the discrimination of immature
neutrophils (myelocytes and metamyelocytes) from mature neutrophils (band and
segmented neutrophils) (Ueda et al., 2005). However, this strategy is not optimal, as
other myeloid cell populations - most notably inflammatory monocytes (Geissmann et al.,
2003) - express Ly-6C, which is recognized by the Gr-1 mAb. To minimize contamination
by inflammatory monocytes, neutrophils were defined by the expression of Ly-6G using
a mAb antibody (clone 1A8) that does not cross-react with Ly-6C (Daley et al., 2008)
(Figure 3). Ly-6G<sup>int</sup>CD11b<sup>+</sup> cells in BM exhibited the histological features of myelocytes
and metamyelocytes, whereas Ly-6G<sup>hi</sup>CD11b<sup>+</sup> BM cells bore characteristics of band and
mature neutrophils (Figure 3). Inflammatory monocytes exhibited the surface phenotype of Ly-6G^−CD11b^+Ly-6B^+ and had low side-scatter properties (Figure 3). Eosinophils were defined as Ly-6G^−CD11b^+Ly-6B^− and exhibited high side-scatter values (Figure 3).

To identify HSPC compartments, BM cells were stained with a panel of mAb that bind lineage-associated surface antigens ("Lin": Gr-1, CD11b, B220, CD4, CD8, and Ter119). Within the Lin^− compartment, cells with the surface phenotype of c-Kit^+Sca-1^+Flt3^− were defined as HSC (Yang et al., 2005). MPP were defined as Lin^−c-Kit^+Sca-1^+Flt3^+ (Adolfsson et al., 2005), CMP as Lin^−c-Kit^+Sca-1^−CD34^+FcγRII/III^ (Akashi et al., 2000), GMP as Lin^−c-Kit^+Sca-1^−CD34^+FcγRII/III^ (Akashi et al., 2000), and MEP as Lin^−c-Kit^+Sca-1^−CD34^−FcγRII/III^ (Akashi et al., 2000) (Figure 4).
Figure 3: Definitions of granulocyte and monocyte compartments

(A) To define granulocyte and monocyte compartments blood and BM cells were stained with mAbs specific for CD11b, Ly-6G, and Ly-6B. Dead cells were excluded by propidium iodide (PI) staining. PI- cells (R1) were divided into three populations: CD11b+Ly-6G- (R2), CD11b+Ly-6Gint (R3, immature neutrophils), and CD11b+Ly-6Ghi (R4, mature neutrophils). In the R2 population, we defined Ly-6B-SSChi cells (R5) as eosinophils whereas the Ly-6B-SSCint population (R6) comprised inflammatory monocytes. (B) Wright-Giemsa staining of granulocytes and monocytes (R3-R6) sorted from BM are shown.
Figure 4: Definitions of LSK and myeloid progenitor compartments

To identify LSK cell and myeloid progenitor compartments, BM cells were stained with mAbs specific for Sca-1, c-Kit, Flt3, CD34, FcγRII/III, and a panel of lineage markers (“Lin” = Gr-1, CD11b, Ter119, B220, CD4, CD8). Dead cells were excluded by PI staining. Lin− cells (R1) were divided into Sca-1−c-Kit+ (R2, myeloid progenitors) and Sca-1+c-Kit+ cells (R3, LSK cells). Myeloid progenitors were separated into three subsets: CD34−FcγRII/III− (R4, MEP), CD34+FcγRII/III− (R5, CMP), and CD34+FcγRII/III+ (R6, GMP). LSK cells were divided into two subsets: CD34−/Flt3− (R7, LT- and ST-HSC) and CD34+Ft3+ (R8, MPP).
2.3.2 Alum-induced neutrophilias require IL-1RI

IL-1 is an important component to inflammatory responses elicited by mineral salts (Hornung et al., 2008) and synergizes with TNFα to increase neutrophil production in BM (Ueda et al., 2004). To determine the role of IL-1RI in reactive neutrophilias, we injected C57BL/6 (BL/6) and congenic IL-1RI−/− mice with alum/antigen (Ueda et al., 2004) and followed changes in blood leukocyte numbers over eight days.

In BL/6 mice, alum elicited a biphasic neutrophilia; neutrophil numbers rose (>2-fold) 1 d after immunization, returned to naïve levels on d 2, and again rose ≈5-fold above controls on d 4- and 8 post-immunization (Figure 5). Alum also modulated the numbers of inflammatory monocytes (Henderson et al., 2003) and eosinophils, with initial decreases 1 d after immunization, followed by steady increases that continued to d 8 (7-fold increase, Figure 5).

The blood of naive BL/6 and IL-1RI−/− mice contain identical numbers of neutrophils, inflammatory monocytes, and eosinophils (Labow et al., 1997) (Figure 5). Alum did not elicit neutrophilia in IL-1RI−/− mice, as blood neutrophil numbers were not significantly changed at any time-point after immunization (Figure 5). Inflammatory monocyte responses were also abrogated, but IL-1RI−/− mice did mount robust eosinophilias that matched BL/6 controls (Figure 5). Thus, alum induces inflammatory neutrophilias and monocytopsies via an IL-1RI dependent pathway, while induction of eosinophilia is IL-1RI independent.
Peripheral blood cells of BL/6 and IL-1RI<sup>−/−</sup> mice were harvested after immunization (1-8 days) with NP<sub>8</sub>-CGG/alum. Neutrophils, inflammatory monocytes, and eosinophils were enumerated by flow cytometry. The mean(+SEM) numbers of cells/ml from BL/6 (•; n=4-7) and IL-1RI<sup>−/−</sup> (○; n=2-5) mice are shown. Significant differences from naive controls are indicated for BL/6 (*, P≤0.05; **, P≤0.01) and IL-1RI<sup>−/−</sup> (†, P≤0.05; ††, P≤0.01) mice.

**Figure 5: IL-1RI is required for alum-induced neutrophilia**
2.3.3 Alum-induced inflammatory granulopoiesis is IL-1RI dependent

The absence of alum-induced neutrophilias in IL-1RI-/- mice implied a defect in emergency granulopoiesis (Basu et al., 2000; Hirai et al., 2006). To determine the role of IL-1RI in emergency granulopoiesis, we immunized BL/6 and IL-1RI-/- mice and followed the dynamics of HSC, MPP, CMP, and GMP populations (Figure 4), as well as the immature and mature neutrophil compartments (Figure 3) in BM. To estimate any changes in proliferation rates, we injected mice i.p. with BrdU 6 hours before sacrifice and compared the frequencies of BrdU+ cells in each cell compartment from naïve and immunized mice (Schittek et al., 1991).

Immunization of BL/6 mice expanded the HSC and MPP compartments 1 d after immunization. HSC numbers rose to 150% of naïve controls, remained elevated through d 4, and then returned to naïve levels by d 6 (Figure 6A). Similarly, MPP numbers increased to 200% of controls 2 d after immunization and returned to normal by d 4 (Figure 6A).

Increased numbers of HSC and MPP were accompanied by increased BrdU uptake. Frequencies of BrdU+ HSC and MPP increased 3.5- and 2.5-fold, respectively, within 1 d of immunization and gradually returned to naïve levels by d 4 (Figure 6B), indicating that alum induces HSC and MPP proliferation.

Alum immunization did not increase CMP numbers in BL/6 mice; rather, CMP numbers fell on d 1 but then recovered and remained at naïve levels (Figure 6A). In contrast to the substantial increases in the frequencies of BrdU+ HSC and MPP, BrdU labeling of CMP/MEP (Lin-c-Kit+Sca-1’FcyRII/III’) rose only modestly and returned to normal by d 4 (Figure 6B).
Unlike CMP, GMP numbers increased significantly 1- and 2 d after immunization and returned to normal by d 4 (Figure 6A). Increased GMP numbers correlated with increased (2-fold) BrdU uptake, 1- and 2 d after immunization, indicating that GMP, like HSC and MPP, are targets of alum-induced proliferative signals (Figure 6B).

Early proliferation by HSC, MPP, and GMP was followed by increased numbers of immature neutrophils. This neutrophil compartment grew >2-fold by d 4 after immunization and remained significantly elevated through d 8 (Figure 6A). The effect of alum on the mature neutrophil compartment in BM was more complex. Initially, mature neutrophils were mobilized from the BM by alum; 1 d after immunization, the numbers of mature, BM neutrophils fell by 85% (Figure 6A), coincident with the first peak of neutrophilia (Figure 5). This loss of mature neutrophils from the BM was soon reversed and followed by significant, 2-fold increases over naïve controls at days 4-8 (Figure 6A). This increase in BM neutrophils coincided with the second and sustained wave of neutrophilia (Figure 5).

We conclude that neutrophilic responses to alum are similar to those elicited by infection (Burg and Pillinger, 2001; Quie, 1980); both begin with the mobilization of BM neutrophil stores and are sustained by inflammatory granulopoiesis. Unexpectedly, GMP were not the most primitive hematopoietic cells to proliferate in response to alum. HSC and MPP proliferation increased coincidentally with that of GMP, suggesting that all three compartments are sensitive to inflammatory signals.

In IL-1RI−/− mice, immunization with alum/antigen neither mobilized mature neutrophils nor expanded the HSC, MPP, or GMP compartments in BM. HSC numbers were not changed by immunization (Figure 6A). The frequency of BrdU+ HSC did not
rise 1 d after immunization, but did exhibit a transient increase on d 2 that was significantly attenuated compared to BL/6 controls (Figure 6B). Likewise, MPP numbers in the BM of IL-1RI−/− mice were not affected by immunization (Figure 6A). Frequencies of BrdU+ MPP transiently increased 1 d after immunization, but returned to naïve levels by day 2 (Figure 6B). These observations indicate that alum-induced expansions of the HSC and MPP compartments in the BM are IL-1RI dependent.

In contrast to BL/6 mice, GMP numbers in IL-1RI−/− mice did not increase after immunization; instead, GMP numbers gradually fell, becoming significantly depleted by d 6 (Figure 6A). This decline was accompanied by reduced BrdU uptake, as BrdU+ GMP were increased only on d 1 and then returned to naïve levels (Figure 6B). Thus, alum coincidentally elicits IL-1RI dependent proliferation in the HSC, MPP, and GMP compartments of BM.

The impaired proliferation by HSC, MPP, and GMP in immunized IL-1RI−/− mice was followed by almost no change in the numbers of immature and mature neutrophils in BM (Figure 6A). Whereas immunization eventually (d 8) produced a modest but significant increase in the numbers of immature neutrophils, the mature neutrophil compartment of BM was neither depleted by mobilization nor expanded by proliferation (Figure 6A). The absence of alum-induced neutrophilias in IL-1RI−/− mice results from defective neutrophil mobilization and abrogation of inflammatory granulopoiesis.

Alum’s failure to elicit reactive monocytoses in IL-1RI−/− mice (Figure 5) suggested defective monocyte output; although immunizations did not significantly alter the numbers of inflammatory monocytes in the BM of BL/6 mice, in IL-1RI−/− mice the numbers of inflammatory monocytes fell to 25% of controls by day 4 before returning to
normal (day 6; Figure 6A). IL-1RI controls, therefore, inflammatory myelopoietic pathways necessary for both emergency granulopoiesis and enhanced production of inflammatory monocytes.
Figure 6: IL-1RI is required for alum-induced emergency granulopoiesis
(Figure 6, continued) (A) BM cells of BL/6 mice and IL-1RI<sup>−/−</sup> mice were harvested after immunization (1-8 d) and labeled to identify HSC, MPP, CMP, GMP, immature neutrophils, mature neutrophils, and inflammatory monocytes. Average cell numbers (+SEM) from BL/6 (●) and IL-1RI<sup>−/−</sup> (○) mice are shown (n=3-11, each point). (B) BL/6 and IL-1RI<sup>−/−</sup> mice were immunized at various times then injected i.p. with 1 mg BrdU 6 h prior to tissue harvest. BM cells were labeled to identify hematopoietic progenitor compartments and then fixed, exposed to DNase, and stained with anti-BrdU mAb. Frequencies of BrdU<sup>+</sup> cells in each progenitor compartment were determined by flow cytometry. Average frequencies (+SEM) of BrdU<sup>+</sup> HSC, MPP, CMP/MEP, and GMP from BL/6 (●; n=3-9) and IL-1RI<sup>−/−</sup> (○; n=3-5) mice are shown. Significant differences from naïve controls are indicated for BL/6 (*, P ≤ 0.05; **, P ≤ 0.01) and IL-1RI<sup>−/−</sup> (†, P ≤ 0.05; ††, P ≤ 0.01) mice.
2.3.4 Comparable HSC and MPP from naïve and immunized mice

IFNγ and TNFα can induce Sca-1 expression by lineage-committed progenitors, causing them to mimic the LSK phenotype (Zhang et al., 2008). Thus, the rapid increases in HSC and MPP numbers after alum immunization could be only apparent, an artifact of proliferation by committed progenitor cells induced to express Sca-1. To exclude this possibility, we analyzed LSK cells from naïve and immunized mice for expression of FcγRII/III and IL-7Rα, molecules not expressed by HSC and MPP but characteristic of GMP and CLP, respectively (Akashi et al., 2000; Kondo et al., 1997). LSK from naïve and immunized (day 2) mice were phenotypically identical and neither expressed FcγRII/III or IL-7Rα (Figure 7A, B), indicating that alum does not cause GMP or CLP to mimic the LSK phenotype.

Next, we compared the ability of HSC (Flt3−LSK cells) from naïve and immunized mice to generate myeloid, erythroid, and multilineage colonies in methylcellulose cultures (Ling et al., 2004). We cultured 100 Flt3−LSK BM cells from naïve and immunized (day 2) mice with SCF, IL-3, IL-6, and erythropoietin (Ling et al., 2004). If inflammation induces myeloid-committed progenitors to mimic the LSK phenotype, then the Flt3−LSK compartment would contain a lower frequency of cells capable of generating multilineage colonies [CFU-granulocyte/erythroblast/macrophage/megakaryocyte (GEMM)] but a higher frequency that would produce myeloid colonies [CFU-granulocyte/macrophage (GM)] compared to naïve controls. After 8 days in culture, both Flt3−LSK cell cohorts produced identical numbers of multilineage colonies (Figure 7C). Furthermore, the frequency of myeloid colonies arising from Flt3−LSK cells of immunized mice was actually lower than naïve
controls (Figure 7C). Flt3‘LSK cells from both naïve and immunized mice produced virtually no single lineage colonies (<1 granulocyte, macrophage, or erythroid colonies/100 Flt3‘LSK) (Figure 7C). These results demonstrate that alum does not enrich the Flt3‘LSK compartment with myeloid-committed progenitors.

We also compared the lymphoid potential of Flt3‘LSK cells from naïve and immunized mice by determining their capacity to generate B-lineage cells in OP9 stromal-cell cultures containing IL-7 and Flt3 ligand (Kondo et al., 1997). The frequencies of Flt3‘LSK cells from immunized (1/3.6) and naïve (1/4.6) mice that supported B-lineage development were virtually identical (Figure 7D); the equivalent lineage potentials of both Flt3‘LSK cohorts identify these cells as HSC.
Figure 7: LSK cells from naïve and immunized mice have equivalent lineage potential

(A) Sca-1 and c-Kit staining of BM Lin− cells from naive and immunized (2 days after injection of NP8-CGG/alum) mice. (B) Analysis of LSK cells from naive (filled histograms) and immunized (open histograms) mice for FcγRII/III and IL-7Rα expression, compared with expression by GMP (PI−Lin−c-Kit+Sca-1−CD34+FcγRII/III−; broken lines, left histogram) and CLP (PI−Lin−IL-7Rα+c-KitlowSca-1low; broken lines, right histogram), respectively. (C) Erythroid and myeloid potential from single Flt3− LSK cells was determined by methylcellulose assay. Flt3− LSK cells (100 cells) were sorted from naive and immunized (day 2) mice and seeded in methylcellulose medium containing SCF, IL-3, IL-6, and erythropoietin. The numbers and types of colonies were determined by optical microscopy 8 days later. The bars show the average numbers of colonies classified as CFU-GEMM (multilineage), CFU-GM (myeloid lineage), and the total number of CFU-G, -M, and -E (CFU-G/M/E; single lineage). The SDs of the total numbers of colonies are shown. (D) B-cell production by Flt3− LSK cells from naive (+) and immunized (○) mice. Multiple wells of 1, 2, and 8 Flt3− LSK cells from naive and immunized (day 2) mice were cultured on OP9 stromal cell layers in the presence of IL-7 and Flt3L. The frequencies of wells with B cell growth were determined by the presence of CD45+CD11b−CD19+B220+ cells after 14 days in culture.
2.3.5 Alum retains robust inflammatory and adjuvant properties in IL-1RI\(-/-\) mice

IL-1 is a potent pro-inflammatory cytokine and IL-1RI is expressed by many cell types (Dinarello, 1996), raising the possibility that the absence of alum-induced neutrophilias in IL-1RI deficient mice is not a specific effect but a general suppression of all inflammatory responses.

We believe this not to be the case. First, alum elicits eosinophilias in IL-1RI\(-/-\) mice (Figure 5) that are equal to or greater than the eosinophilic responses of BL/6 mice (Figure 8A). Second, alum mobilizes pre-B cells from the BM of BL/6 and IL-1RI\(-/-\) mice with equal efficiency (Ueda et al., 2004). In immunized BL/6 and IL-1RI\(-/-\) mice, pre-B-cell numbers in BM fell >50% by day 4 (Figure 8B). Recovery of these inflammatory losses was more rapid in IL-1RI\(-/-\) mice, perhaps due to the lack of competition for growth resources in the absence of expanded granulopoiesis (Ueda et al., 2005) (Figure 8B). Finally, IL-1RI\(-/-\) mice immunized with NP-CGG in alum mounted characteristic and robust serum Ab responses (Figure 8C).

Given that alum induces strong eosinophilic responses, mobilizes BM pre-B cells, and acts as a potent adjuvant in IL-1RI\(-/-\) mice, we conclude that the absence of inflammatory neutrophilias and monocyteoses in IL-1RI\(-/-\) mice represents specific effects on neutrophils and monocytes.
Figure 8: Robust inflammatory responses in IL-1RI−/− mice

BM cells of BL/6 mice and IL-1RI−/− mice were harvested after immunization (1–8 days) and labeled with mAbs to identify eosinophils and pre-B cells. Average numbers (+SEM) of eosinophils (A) and pre-B cells (B) from BL/6 (•) and IL-1RI−/− (○) mice are shown (n = 3–11, each point). Significant differences from naive controls are indicated for BL/6 (*, P<0.05; **, P<0.01) and IL-1RI−/− (†, P<0.05; ††, P<0.01) mice. (C) NP16-BSA binding λ+ and κ+ serum Ab was determined in BL/6 (closed symbols) and IL-1RI−/− (open symbols) mice (n = 2, each point) before and after immunization with 60 µg of NP6-CGG/alum. NP-specific serum λ(circles) and κ(squares) Abs were quantified (mean+SD) by ELISA.
2.3.6 Responsiveness of HSC to IL-1 \textit{in vitro}

If adjuvant elicits emergency granulopoiesis by activating HSC proliferation/differentiation via IL-1RI directly, HSC must express IL-1RI. To determine the patterns of IL-1RI expression in various hematopoietic cell compartments, we used quantitative RT-PCR to compare receptor expression by HSC, CMP, GMP, and MEP from naive and immunized mice (Figure 9A). Indeed, LSK cells expressed IL-1RI message in quantities 6- to 16-fold higher than MEP, CMP, or GMP (Figure 9A). We also measured GM-CSFR expression, which is known to be higher in GMP than in HSC (Akashi et al., 2000). As expected, GM-CSFR mRNA levels were reciprocal to IL-1RI message, with the highest quantities in GMP and lower, comparable amounts in CMP, MEP, and HSC (Figure 9A). The reciprocal patterns of IL-1RI and GM-CSFR expression imply that HSC are more sensitive to IL-1 than are myeloid progenitors, and the effects of GM-CSF are focused on GMP.

To determine if IL-1 drives HSC, MPP, or GMP proliferation, we sorted progenitor cells from BL/6 mice and cultured cells (500 cells/well) in serum free-medium containing stem cell factor (SCF, 25 ng/ml; control medium)(Domen and Weissman, 2000) to which supplements of rIL-1\(\beta\) or rGM-CSF were added (Figure 9B). After 4 d of culture in control medium, viable cell numbers fell to \(\approx40\%\) and \(\approx80\%\) of input HSC and MPP, respectively, although GMP numbers were stable (Figure 9B). Addition of IL-1\(\beta\) (1 ng/ml) resulted in the recovery of \(\geq3\)-fold more cells than originally plated in HSC cultures, and 1.4-fold more cells from MPP cultures; IL-1\(\beta\) did not promote any increase in cell recovery from GMP cultures (Figure 9B). Conversely, GM-CSF did not greatly affect cell recoveries from HSC and MPP cultures, but resulted in \(>60\)-fold increase in
cell numbers recovered from GMP cultures (Figure 9B). Thus, IL-1 responsiveness in HSPC compartments peaks in HSC but decreases with myeloid differentiation, whereas GM-CSF responsiveness is gained upon commitment to the myeloid lineage. These observations suggest that HSC may be direct targets of IL-1 for the emergency production of neutrophils.
Figure 9: HSC express IL-1RI and respond to IL-1 in vitro

(A) To determine if LSK cells, MEP, CMP, and GMP express IL-1RI, cells were isolated by FACS and analyzed for IL-1RI expression by quantitative PCR. The expression (+SD) of IL-1RI and GM-CSFR relative to β-actin is shown for each compartment. (B) HSC, MPP, and GMP from BL/6 mice (500 cells/well) were cultured in serum free-medium containing SCF (25 ng/ml; control medium; white bars) with or without rIL-1β (1 ng/ml; black bars) or rGM-CSF (1 ng/ml; gray bars). Cell numbers (+SD) after 4 d of culture are shown. Significant differences between groups are indicated: *, P ≤ 0.05; **, P ≤ 0.01.
2.3.7 IL-1RI<sup>−/−</sup> HSC, MPP, and GMP proliferate in immunized BL/6 hosts

Although LSK cells express IL-1RI and proliferate in response to IL-1β <em>in vitro</em> (Figure 9), IL-1 could modulate hematopoiesis indirectly (Dinarello, 1996). To determine if IL-1RI expression by HSC, MPP, and GMP is required for their alum-induced proliferation, we generated chimeric mice by reconstituting irradiated (C57BL/6.CD45.2/1)F<sub>1</sub> mice with equal numbers of BM cells from C57BL/6.SJL (WT; CD45.1) and IL-1RI<sup>−/−</sup> (KO; CD45.2) donors. Donor and host hematopoietic cells were identified by CD45 allelism. We determined BrdU incorporation by donor HSC, MPP, and GMP 2 d after immunization, when IL-1RI-dependent proliferative defects are maximal (Figure 6B). If IL-1RI expression by HSC, MPP, or GMP is required for their proliferation in response to alum, then WT progenitor cells should respond to immunization but KO cells should not.

To ensure comparable reconstitution efficiencies, we determined the frequencies of WT and KO hematopoietic cells in the blood of chimeric mice four weeks after reconstitution. All chimeras exhibited a modest excess of KO leukocytes (40 ± 7% WT, 51 ± 6% KO; P≤0.05).

In naïve chimeras, the frequencies of BrdU<sup>+</sup> WT- and KO HSC, MPP, and GMP were equivalent but labeling of HSC and MPP was elevated compared to naive BL/6 and IL-1RI<sup>−/−</sup> controls (P≤0.05; compare Figure 6 and Figure 10). Increased frequencies of BrdU<sup>+</sup> HSC and MPP in chimeras may reflect ongoing hematopoietic replenishment. Immunization of chimeric mice equally increased (∼150%) BrdU labeling of WT- and KO HSC, MPP, and GMP (Figure 10A), and these increases matched those of BL/6 controls (Figure 6B). Robust proliferative responses by IL-1RI deficient hematopoietic cells
demonstrates that an intermediate signal(s) determines HSC, MPP, and GMP proliferation. This intermediate, trans-acting signal could be produced by the IL-1RI⁺ hematopoietic compartment of mixed chimeras or, as IL-1RI is widely expressed (Dinarello, 1996), could come from radiation-resistant host cells of hematopoietic or non-hematopoietic origin.

To determine the origin of this proliferation signal, we generated KO→WT and WT→KO BM chimeras. Four weeks after reconstitution, ~60% of blood leukocytes were derived from donor cells in both chimera types. Chimeric mice were immunized and the frequencies of BrdU⁺ HSC, MPP, and GMP were measured 2 d later. In KO→WT chimeras, immunization significantly increased BrdU labeling of progenitors compared to naïve chimera controls (Figure 10B). In contrast, the frequencies of BrdU⁺ HSC, MPP, and GMP did not increase after immunization of WT→KO chimeras (Figure 10C). We conclude that it is IL-1RI expression/activity in a radiation-resistant, host compartment(s) that determines the reactive proliferation of HSC, MPP, and GMP.
Figure 10: Alum induces IL-1RI-deficient HSC, MPP, and GMP to proliferate in chimeric mice

(A) Irradiated (C57BL/6 x C57BL/6.CD45.1)F₁ mice were reconstituted with equal numbers (5 x 10⁶) of C57BL/6.CD45.1 (WT/CD45.1) and IL-1RI⁻/⁻ (KO/CD45.2) BM cells. Afterward (4 wk), chimeras were immunized with NP₆-CGG/alum and 2 days later were injected i.p. with 1 mg of BrdU. Six hours after BrdU injection, BM cells were harvested and the frequencies of BrdU⁺ HSC, MPP, and GMP were determined by flow cytometry. Average frequencies (+SD) of BrdU⁺ WT (CD45.1) and KO (CD45.2) HSC, MPP, and GMP from naive (open symbols; n = 4) and immunized (closed symbols; n = 3) mice are shown. (B) To generate reciprocal chimeras, irradiated (C57BL/6 x C57BL/6.CD45.1)F₁ mice were reconstituted with KO (C57BL/6.IL-1RI⁻/⁻) BM cells (KO→WT) or (C) irradiated C57BL/6 IL-1RI⁻/⁻ mice were reconstituted with (C57BL/6 x C57BL/6.CD45.1)F₁ BM cells (WT→KO). Four weeks later, chimeric mice were immunized and analyzed as for A. Average (+SD) frequencies of BrdU⁺ HSC, MPP, and GMP from naive (open symbols; n = 4) and immunized (closed symbols; n = 4) mice are shown. Significant differences between groups are indicated. *, P ≤ 0.05; **, P ≤ 0.01
2.4 Discussion

Infections elicit neutrophilias that are sustained by a distinct, emergency granulopoietic program that is activated by ill-defined intrinsic signals (Hirai et al., 2006). We previously noted that reactive neutrophilias induced by alum are mimicked by the co-administration of TNFα and IL-1β (Ueda et al., 2004). More recently, alum has been shown to be a potent inducer of IL-1β secretion by activation of the Nalp3 inflammasome (Eisenbarth et al., 2008). Taken together, these observations suggested that IL-1β might be the intrinsic activating signal for emergency granulopoiesis.

Whereas myeloid cell numbers in naïve IL-1R1−/− and C57BL/6 mice are indistinguishable (Figure 5 and Figure 6), alum/antigen elicited neither the pronounced reactive neutrophilias and inflammatory monocytic responses nor the emergency granulopoiesis in IL-1R1−/− mice (Figure 6) that characterized C57BL/6 controls. To our knowledge, this is the first demonstration of a single, indispensable cytokine/receptor pathway for emergency granulopoiesis (Basu et al., 2000; Nishinakamura et al., 1996).

Alum, a common vaccine adjuvant, promotes inflammation and immunogenicity via the Nalp3 inflammasome and the production of the proinflammatory cytokines IL-1β, IL-18, and IL-33 (Eisenbarth et al., 2008; Li et al., 2007). Nonetheless, abrogation of neutrophilia and emergency granulopoiesis in IL-1R1−/− mice was a specific defect, as other inflammatory responses, reactive eosinophilia (Figure 5 and Figure 8A), mobilization of BM pre-B cells (Figure 8B), and robust antibody production (Figure 8C), remained intact. IL-3 and IL-5 promote eosinophilia (Yamaguchi et al., 1988) and
mobilization of BM B-lineage cells depends on TNFα (Ueda et al., 2004). Thus, other alum-induced inflammatory responses are fully active in IL-1RI−/− mice.

Emergency granulopoiesis is generally thought to represent increased GMP proliferation (Hirai et al., 2006), and we observed significant increases in GMP numbers 1- and 2 d after immunization accompanied by increased BrdU uptake (Figure 6). In addition, however, we found that in C57BL/6 mice, alum simultaneously induced proliferation by HSC and MPP (Figure 6); HSC and MPP are themselves the immediate targets of inflammatory signals.

In contrast to control mice, alum elicited no significant increases in HSC, MPP, and GMP numbers in IL-1RI−/− mice (Figure 6A) and significantly attenuated proliferative responses by these compartments (Figure 6B). The lack of increases in HSC, MPP, and GMP numbers in IL-1RI−/− mice after immunization (Figure 6A), despite modest but significant increases in proliferation (Figure 6B), raises the possibility that IL-1RI-dependent signals act not only to sustain high levels of progenitor proliferation but also to promote progenitor survival. These observations indicate an unexpected role for HSC and MPP proliferation in the induction of emergency granulopoiesis.

Curiously, proliferation and expansion by the HSC and MPP compartments in immunized C57BL/6 mice are associated with increased numbers of GMP but not CMP (Figure 6A). If GMP arise from CMP, why do we not detect an expansion in this compartment as well? One possibility is that inflammation accelerates CMP differentiation, such that the increased CMP production by MPP is obscured by a very rapid CMP to GMP differentiation. Alternatively, inflammation may cause some fraction of MPP to differentiate into GMP directly, a phenomenon suggested by Adolfsson et al.
(Adolfsson et al., 2005). The latter hypothesis is consistent with our failure to observe increased numbers of MEP and CLP after alum immunization (data not shown). Inflammatory signals elicited by alum appear to accelerate myelopoiesis specifically [(Ueda et al., 2005; Ueda et al., 2004), Figure 6 and Figure 8A, B]. Given that alum immunizations do not alter the lineage potential of Flt3-LSK HSC (Figure 7C, D), this hematopoietic specialization likely represents alteration of external cues that influence hematopoietic lineage decisions (Ueda et al., 2005; Ueda et al., 2004).

Although HSC and MPP express IL-1RI and proliferate in response to IL-1 in vitro (Figure 9), IL-1RI expression by HSC, MPP, and GMP is not required for their proliferation in response to alum (Figure 10). In mixed chimeras, proliferation by IL-1RI−/− HSC, MPP, and GMP was identical to that of IL-1RI-bearing cells (Figure 10A). IL-1/IL-1RI signals are, therefore, necessary for the induction of reactive neutrophilias and emergency granulopoiesis by alum, but they must act indirectly to drive proliferation in the HSC, MPP, and GMP compartments.

IL-1 induces the production of many growth factors and inflammatory mediators (Dinarello, 1996); among these, IL-3, IL-6, G-CSF, and GM-CSF have been implicated as agents of emergency granulopoiesis (Hirai et al., 2006; Walker et al., 2008). However, these cytokines are dispensable for robust granulopoietic responses to inflammation (Basu et al., 2000; Nishinakamura et al., 1996). In consequence, these dispensable cytokines can not be the intermediate cues that drive IL-1RI dependent emergency granulopoiesis in alum immunized mice. Given that HSC, MPP, and GMP appear to be the immediate targets of alum-induced, IL-1RI dependent proliferation (Figure 6), IL-1RI signals may act through intermediate hematopoietic factors that induce
proliferation by both HSC and committed progenitors, such as IL-11 (Hangoc et al., 1993; Lemoli et al., 1993) or thrombopoietin (Young et al., 1996). Ancillary growth factors may also be responsible for the modest but significant increase in primitive neutrophils in the BM of IL-1RI<sup>−/−</sup> mice 8 d after immunization (Figure 6A). This late increase suggests the possibility of still another pathway for increased granulopoiesis in response to unresolved inflammatory signals.

Alternatively, the emergency granulopoiesis induced by alum could be driven by a density-dependent feedback between BM neutrophils and their progenitors, i.e., BM neutrophils suppress the proliferation and differentiation of hematopoietic progenitor cells. Inflammatory mobilization of mature neutrophils from the BM would relax this suppression, activating HSC, MPP, and GMP proliferation. A similar hypothesis was offered previously to explain the stability of steady-state neutrophil pools (Layton et al., 1989). If so, abrogation of emergency granulopoiesis in IL-1RI<sup>−/−</sup> mice could be the result of defective mobilization by BM neutrophils and the continuation of suppression (Figure 6A). Consistent with this feedback hypothesis, alum neither mobilized BM neutrophils (data not shown) nor induced HSC, MPP, and GMP proliferation in WT→KO chimeras (Figure 10C). In contrast, all chimeras generated in IL-1RI-sufficient hosts (WT+KO→WT, KO→WT, Figure 10A,B) exhibited robust neutrophil mobilization (data not shown) and progenitor proliferation after immunization with alum.

Earlier, we proposed that developing neutrophils and B cells compete for specific resources in the BM such that the expansion of one lineage results in a compensatory diminution of the other (Ueda et al., 2005). Alum mobilizes B-lineage cells from the BM and effects a central lymphopenia that coincides with the period of emergency
granulopoiesis (Ueda et al., 2005). In IL-1R1−/− mice, alum depleted the BM of pre-B cells but the recovery of this compartment was significantly faster than in control mice (Figure 8B). As alum does not expand granulopoiesis in IL-1R1−/− mice, our competition model predicts that vacated niches/resources normally occupied by GMP and primitive neutrophils might be available to accelerate the restoration of the BM’s B-lineage compartments.

The increased availability of developmental niches in the BM of immunized IL-1R1−/− mice may also explain the enhanced production of eosinophils compared to immunized C57BL/6 mice (days 6 and 8; Figure 8A). On the other hand, neutrophils likely mitigate alum-induced inflammation, and impaired neutrophilic responses in immunized IL-1R1−/− mice may prolong inflammatory responses and enhance eosinophilias. That IL-1R1−/− mice are capable of mounting inflammatory eosinophilias despite attenuated proliferation of HSC, MPP, and GMP implies that a more differentiated progenitor responds to inflammatory signals that increase eosinophil production. This observation is consistent with the work of Iwasaki and colleagues, who showed that eosinophilias elicited by helminth infections are supported by expansions of a committed eosinophil progenitor compartment but not CMP or GMP (Iwasaki et al., 2005).

The requirement for IL-1RI in alum-induced neutrophilias and emergency granulopoiesis emphasizes the central role of IL-1 in innate immune responses (Chen et al., 2007; Chen et al., 2006; Hornung et al., 2008). Alum drives IL-1β and IL-18 secretion by activating the Nalp3 inflammasome (Eisenbarth et al., 2008; Hornung et al., 2008; Li et al., 2007) and these cytokines have been proposed to be the agents of alum’s several
inflammatory properties including adjuvanticity (Eisenbarth et al., 2008). Instead, our work indicates that the effects of secreted IL-1β may be focused on neutrophilic responses, as alum-induced eosinophilia, pre-B cell mobilization, and antibody responses remain intact in IL-1RI−/− mice (Figure 8). Indeed, our findings are consistent with the seemingly paradoxical findings by Eisenbarth et al. (Eisenbarth et al., 2008), showing that the alum’s adjuvanticity is abrogated in mice defective for components of the Nalp3 inflammasome, but intact in animals lacking MyD88, a crucial signaling component of the IL-1 and IL-18 receptors (Adachi et al., 1998). Thus, alum must stimulate humoral responses independently of the cytokines currently associated with the Nalp3 inflammasome.
3. Inflammation triggers emergency granulopoiesis through a density-dependent feedback mechanism of neutrophil replenishment

In Chapter 2, I discovered that IL-1RI signaling operates indirectly to induce emergency granulopoiesis. Interestingly, the defective proliferative responses of HSPC in IL-1RI^− mice were associated with impaired neutrophil mobilization, raising the possibility that emergency granulopoiesis is a response to the loss of BM neutrophils. In this chapter, I test the hypothesis that non-inflammatory reductions BM neutrophils trigger HSPC proliferation and emergency granulopoiesis. In this study, I was assisted by Pilar B. Snowden, a fellow graduate student in the laboratory who provided technical assistance. Gregory Sempowski, a professor at the Human Vaccine Institute at Duke University, provided assistance with the measurement of serum cytokines by Luminex bead array. The work presented in this chapter has been submitted for publication.

3.1 Introduction

Normally, the neutrophil pools of the blood and BM are maintained by a homeostatic process that requires C/EBPα (Hirai et al., 2006; Zhang et al., 1997) and may incorporate density-dependent feedback mechanisms (Haurie et al., 1998). In contrast, inflammation induces neutrophilia by mobilizing neutrophils from BM and accelerating granulopoiesis through a C/EBPβ-dependent pathway (Hirai et al., 2006; Ueda et al., 2009). C/EBPβ is thought to be necessary for increased proliferation of myeloid progenitor cells upon stimulation with inflammatory growth factors (Hirai et al., 2006). However, my observations in Chapter 2 raised the possibility that HSPC proliferation increases in response to perturbations in BM neutrophil numbers,
suggesting that feedback mechanisms operate during inflammatory conditions. To dissect the contributions of inflammation vs. feedback to the “emergency” production of neutrophils, we measured the effects of adjuvant inflammation and experimentally-induced neutropenia on HSPC proliferation and neutrophil output.

C/EBP$\alpha$ and $\beta$ play distinct roles in regulating the tempo of granulopoiesis. Mice with a conditional deletion of C/EBP$\alpha$ fail to produce neutrophils due to defective generation of granulocyte/macrophage progenitors (GMP) from common myeloid progenitors (CMP) (Zhang et al., 2004). Granulopoiesis can be rescued in C/EBP$\alpha^{-/-}$ mice, however, by treatment with GM-CSF and IL-3 (Hirai et al., 2006), or by expression of C/EBP$\beta$ from the C/EBP$\alpha$ locus (Jones et al., 2002). C/EBP$\beta^{-/-}$ mice, in contrast, exhibit normal numbers of neutrophils in the steady-state, but fail to mount neutrophilias in response to infection or cytokine treatment, implicating C/EBP$\beta$ as a key regulator of inflammatory granulopoiesis (Hirai et al., 2006). The paradigm that has emerged from these observations is that growth factors elicited during infections induce GMP to downregulate C/EBP$\alpha$ and upregulate C/EBP$\beta$, which directs neutrophil differentiation while promoting elevated rates of proliferation (Hirai et al., 2006).

While neutrophilias are commonly associated with inflammation, it is generally thought that granulopoiesis is a dynamic process that also responds to non-inflammatory cues elicited through feedback. Evidence for feedback mechanisms of granulopoiesis was first provided in studies showing that animals rendered neutropenic via leukapheresis (Craddock et al., 1955), neutrophil anti-serum (Patt et al., 1957), or irradiation (Morley and Stohlman, 1970) exhibited expansions of myeloid precursor populations in BM prior to the recovery of mature neutrophil compartments. In patients
with cyclic neutropenia, the periodic oscillations in blood neutrophil counts are thought to result from dysfunctional feedback regulation of granulopoiesis (Dale and Hammond, 1988). Numerous models for granulopoietic regulation have been proposed and most contain a feedback component that links the population density of neutrophils to the proliferation and differentiation of neutrophil precursors (King-Smith and Morley, 1970; Ostby and Winther, 2004; Rubinow and Lebowitz, 1975; von Schulthess and Mazer, 1982). The mechanisms of feedback are still debated, however, and the roles of C/EBPα and C/EBPβ in the active regulation of granulopoiesis have not been addressed.

Several cytokines can modulate neutrophil production, but G-CSF is the primary regulator of steady-state and emergency granulopoiesis [reviewed in (Panopoulos and Watowich, 2008)]. In addition to regulating neutrophil output, signaling through G-CSF and G-CSF receptor (G-CSF-R) also controls neutrophil survival, function, and egress from BM (Basu et al., 2002; Lieschke et al., 1994; Liu et al., 1996). Consequently, mice lacking G-CSF or G-CSF-R are severely neutropenic (Lieschke et al., 1994; Liu et al., 1996). G-CSF-independent pathways of granulopoiesis exist, however, as mature neutrophils are present in both G-CSF−/− and G-CSF-R−/− mice, albeit in lower numbers (Lieschke et al., 1994; Liu et al., 1996). Furthermore, G-CSF is dispensable for the inflammatory neutrophilia elicited by fungal infection (Basu et al., 2000), indicative of a compensatory signal(s) that enhances granulopoiesis in response to pathogens. Candidate factors for the inflammatory induction of granulopoiesis include GM-CSF, IL-3, and IL-6 (Arai et al., 1990; Metcalf et al., 1986; Metcalf et al., 1987b; Ulich et al., 1989b).
In Chapter 2, I showed that inflammatory neutrophilias elicited by alum adjuvant require expression of IL-1RI on radiation-resistant cells; the inability of IL-1RI−/− mice to mount neutrophilias in response to alum is due to defective neutrophil mobilization and impaired proliferation of HSC, MPP, and GMP. If emergency granulopoiesis is driven by inflammatory growth factors, then alum must elicit those growth factors via IL-1RI. On the other hand, the coincident defects in neutrophil mobilization and HSPC proliferation in IL-1RI−/− mice raise the possibility that alum-induced emergency granulopoiesis is not a direct result of inflammation, but instead represents a feedback response to the consumption of BM neutrophils.

In this chapter, I test the hypothesis that emergency granulopoiesis is activated by the loss of neutrophil compartments. BM neutropenia induced by three distinct methods, alum immunization, antibody-based depletion (Gr-1 mAb), and genetic ablation, was invariably associated with increased HSPC proliferation and neutrophil output. Significantly, the proliferation of HSPC correlated inversely with the population density of BM neutrophils regardless of how BM neutropenia was induced. Alum elicited G-CSF-dependent neutrophil mobilization through an inflammatory pathway requiring IL-1RI, whereas feedback between BM neutrophils and HSPC was independent of IL-1RI. Notably, the proliferative responses of GMP to neutrophil deficits required G-CSF-R signals, but HSC responsiveness was only partially dependent on G-CSF-R. Surprisingly, C/EBPβ was dispensable for increased proliferation of HSPC following immunization or neutrophil depletion, but was crucial for the terminal differentiation of neutrophils during granulopoietic recovery from neutropenia. These observations reveal the BM neutrophil compartment as the key regulator of neutrophil production; the
exhaustion of this cellular compartment, through inflammatory or non-inflammatory causes, is sufficient to elicit emergency granulopoiesis.

### 3.2 Materials and methods

**Mice.** C57BL/6, congenic IL-1R\(^{-/-}\) mice [B6.129S7-Il1r1\(^{tm1mxy}\)/J (Glaccum et al., 1997)], and RAG1\(^{-/-}\) mice [B6.129S7-Rag1\(^{tm1Mom}\)/J (Mombaerts et al., 1992)] were from Jackson Laboratories. Dr. Y. Yang (Duke University) generously provided MyD88\(^{-/-}\) mice on the BL/6 background (Adachi et al., 1998). C1q\(^{-/-}\) mice (Botto et al., 1998) were obtained from Dr. M. Walport (Imperial College). C3\(^{-/-}\) mice (Wessels et al., 1995), originally developed by Dr. M. Carroll (Harvard University), and FcR common \(\gamma\) chain deficient mice [FcR\(\gamma^{-/-}\) (Takai et al., 1994)] were provided by Dr. T. F. Tedder (Duke University). LysM\(^{Cre/\text{wt}}\) Mcl-1\(^{\text{ff}}\) and LysM\(^{\text{wt/\text{wt}}}\) Mcl-1\(^{\text{ff}}\) mice (Dzhagalov et al., 2007) were kindly provided by Dr. Y. He (Duke University). C/EBP\(\beta\) hemizygous mice on 129/Sv and BL/6 background strains were kindly provided by Dr. Peter Johnson (NCI); C/EBP\(\beta^{-/-}\) mice and control littermates were generated by intercrossing hemizygous 129/Sv and BL/6 parents. Bone marrow cells from CX3CR1\(^{GFP/+}\) (Jung et al., 2000) mice were provided by Dr. M.D. Gunn (Duke University). G-CSF-R\(^{-/-}\) mice (Liu et al., 1996) were kindly provided by Dr. D. Link (Washington University). All mice were housed in specific pathogen-free conditions at the Duke University Animal Care Facility with sterile bedding, water, and food; MyD88\(^{-/-}\) mice were provided with antibiotic in water. All studies were approved by the Duke University Institutional Animal Care and Use committee.
Purification of Gr-1 mAb. The RB6-8C5 hybridoma (Tepper et al., 1992) was adapted to serum-free medium (Hybridoma SFM, Gibco) and cultured in a CELLine CL1000 bioreactor (Wilson Wolf Manufacturing). Gr-1 mAb was purified from supernatants using a HiTrap Protein G column (GE Healthcare) and dialyzed in sterile PBS. Antibody concentration was determined by Bradford assay (Sigma-Aldrich).

Injection of adjuvant and purified proteins. Mice were immunized i.p. with chicken γ-globulin (CGG) conjugated to (4-hydroxy 3-nitrophenyl)acetyl (NP) (NP9-11-CGG) precipitated in alum (Ueda et al., 2004). Purified Gr-1 mAb or recombinant human G-CSF (5 μg, Neupogen, Amgen) was diluted to 200 μl in PBS and injected i.p. To neutralize G-CSF in vivo, mice were injected i.v. with 100 μg rat anti-mouse G-CSF (R&D Systems, clone 67604) 30 minutes prior to immunization or Gr-1 administration.

Flow Cytometry. FITC-, PE-, PE-Cy5-, APC-, APC-Cy7 or APC-eFluor780, PE-Texas Red-, PE-Cy7-, and biotin-conjugated antibodies to Ly-6G (clone 1A8), Gr-1, CD11b, Ter119, CD4, CD8, B220, TCRβ, Ly-6C, CD31, CD34, c-Kit, Sca-1, FcγRII/III, and Flt3 were from BD Pharmingen or eBioscience. PE-conjugated Ly-6B mAb was obtained from AbD Serotec and FITC-conjugated anti-CCR3 was obtained from R&D Systems.

Mice were bled via the retro-orbital sinus and blood was collected in heparin solution. Mice were killed and the femurs and tibiae from both legs were harvested. BM was flushed out with cold IMDM containing 2% FBS. Erythrocytes were lysed in ammonium chloride buffer. Single-cell suspensions were labeled with combinations of
fluorochrome-antibody conjugates; propidium iodide (Sigma-Aldrich) identified dead cells.

As injected Gr-1 mAb reduced levels of ex vivo labeling with fluorochrome-Gr-1 conjugates, Gr-1+ cells were routinely identified by the following procedure. In a first round of cell labeling, cells were exposed to unconjugated Gr-1, washed, and saturated with FITC-conjugated anti-rat IgG (Southern Biotech). This method ensured the identification of all Gr-1+ cells, whether labeled in vivo or ex vivo. After washing, cell labeling with other antibody conjugates was carried out in a second round of staining. Since the Gr-1 antigen is also present on inflammatory monocytes, Ly-6B+SSC<sub>low</sub> cells were excluded prior to gating on Gr-1+ populations to minimize contamination of neutrophil populations by inflammatory monocytes (Figure 3).

Antibody-labeled cells were analyzed on a LSRII flow cytometer (BD Biosciences) or sorted with a FACSVantage (BD Biosciences). Cell sorting was performed in the Duke Human Vaccine Institute Research Flow Cytometry Shared Resource Facility. Flow cytometric data were analyzed with FlowJo software (Treestar). Cytospins of sorted cells were stained using Hema3 (Fisher Scientific) to confirm their identities. For analysis of BrdU uptake by hematopoietic progenitors, HSC were identified as Flt3<sup>Lin</sup>(Gr-1, CD11b, Ter119, CD4, CD8, B220)<sup>c</sup>-Kit<sup>+</sup>Sca-1<sup>+</sup>, MPP were identified as Flt3<sup>+</sup>Lin<sup>c</sup>-Kit<sup>c</sup>Sca-1<sup>+</sup>, and GMP were defined as Lin<sup>c</sup>-Kit<sup>c</sup>Sca-1<sup>+</sup>F<sub>γRII/III</sub>*, as described in Chapter 2 (Figure 4).

**BrdU labeling.** Proliferation rates of HSPC were determined using a BrdU labeling kit (BD Biosciences). Six hours before tissue harvest, mice were injected i.p. with 1 mg
BrdU in 200 µl PBS. After labeling with fluorochrome-antibody conjugates specific for surface antigens, BM cells were fixed/permeabilized, treated with DNase, and incubated with FITC anti-BrdU. Data were acquired on a LSRII flow cytometer (BD Bioscience) and analyzed with FlowJo software (Treestar). HSC were identified as Flt3\(^{−}\)Lin\(^{−}\)c-Kit\(^{+}\)Sca-1\(^{+}\), MPP were identified as Flt3\(^{+}\)Lin\(^{−}\)c-Kit\(^{+}\)Sca-1\(^{+}\), and GMP were identified as Lin\(^{−}\)c-Kit\(^{+}\)Sca-1\(^{−}\)Fc\(_{γ}\)RII/III\(^{+}\), as described in Chapter 2 (Figure 4).

**Quantification of serum cytokines.** Serum cytokines were analyzed in the Duke Human Vaccine Institute Immune Reconstitution & Biomarker Shared Resource Facility. Sera were prepared from naïve BL/6, Mcl-1\(^{+}\), Mcl-1\(^{−}\) mice, and BL/6 mice injected with alum or 100 µg Gr-1 mAb. Cytokine concentrations in undiluted serum samples were determined using a Bio-Plex Pro Group I 23-Plex kit (Bio-Rad). Readings below the lowest point in the standard curve were mathematically extrapolated, but these measurements were imprecise.

Serum G-CSF was quantified by ELISA. Wells of a 96-well assay plate were coated overnight with anti-mouse G-CSF (R&D Systems, clone 67604) then blocked for 1 hour with PBS/1%BSA. Sera from alum- or Gr-1 treated mice were added to wells then incubated for 2 hours at room temperature. Bound G-CSF was detected with biotinylated anti-GCSF (R&D Systems) and SA-horseradish peroxidase (Southern Biotechnology). Horseradish peroxidase activity was revealed with a tetramethylbenzadine peroxidase substrate kit (Bio-Rad). G-CSF concentrations in samples were calculated from a standard curve of recombinant mouse G-CSF (Peprotech); the sensitivity of the assay was 10 pg/ml G-CSF.
Statistics. Significance in paired data was determined by Student’s t-test. Relationships between cell numbers and the frequencies of BrdU$^+$ HSC, MPP, and GMP were evaluated using the Pearson product moment correlation coefficient.

3.3 Results

If mature neutrophil numbers in the BM regulate the pace of granulopoiesis by negative feedback, then mice made neutropenic should exhibit emergency granulopoiesis. To test this hypothesis, we studied two experimental models for induced neutropenia that minimize ancillary inflammatory signals.

3.3.1 Bone marrow neutropenia by antibody depletion

The Gr-1 mAb binds the Ly-6G antigen of murine neutrophils, and is used routinely to deplete neutrophils in vivo (Bliss et al., 2001; Czuprynski et al., 1994). We injected BL/6 mice i.p. with 10- or 100 $\mu$g of Gr-1 mAb and compared the loss and recovery of mature neutrophils in blood and BM to the changes induced by alum immunization. Whereas alum elicited a multiphasic neutrophilia in blood, both doses of Gr-1 induced transient neutropenias, lowering neutrophil numbers >90% ($P \leq 0.01$) for 2 or 4 days (10- or 100 $\mu$g Gr-1, respectively; $P \leq 0.01$; Figure 11A) before recovery on days 6 or 8 (10- or 100 $\mu$g Gr-1, respectively, Figure 11A). A mild neutropenia was evident 12 and 16 days after administration of 10 $\mu$g Gr-1 ($P \leq 0.05$; Figure 11A). The effects of Gr-1 mAb were specific, as injection of 100 $\mu$g purified rat IgG had no effect on blood or BM leukocyte numbers (unpublished data).
Although alum and Gr-1 had dissimilar effects on blood neutrophil counts, the two treatments induced similar changes in BM neutrophil numbers. Both alum and Gr-1 administration transiently reduced BM neutrophil numbers (P<0.01, days 1 and 2; Figure 11B); after two days, BM neutrophil numbers began to recover and eventually surged above normal levels, peaking on day 6 after injection of alum or 10 µg Gr-1 (P<0.01), and on day 8 after 100 µg Gr-1 (P<0.01) before gradually returning to normal (Figure 11B). Notably, the magnitude of the BM neutrophil “rebound” following Gr-1 treatment outpaced that of alum (Figure 11B), suggesting that the myeloablative effects of passive Gr-1 administration are sufficient to induce emergency granulopoiesis.

Given that alum exposure increases proliferation in the HSC (Flt3^Lin^Sca-1^c-Kit^), MPP (Flt3^Lin^Sca-1^c-Kit^), and GMP (Lin^Sca-1^c-Kit^CD34^FcɣRII/III^) compartments (Figure 6), we determined whether neutrophil depletion by Gr-1 mAb also increased proliferation of HSPC. Mice treated with Gr-1 mAb or alum adjuvant were injected i.p. with BrdU 6 hours prior to tissue harvest, and the frequencies of BrdU^+ cells in the HSC, MPP, and GMP compartments were determined by flow cytometry. The frequencies of BrdU^+ HSC, MPP, and GMP increased significantly within one day of Gr-1 administration (P<0.01, all) and then gradually returned to naïve levels by days 4-6 (Figure 11C-E). This observation is consistent with a recent report describing an increase in the number of early hematopoietic progenitors following Gr-1 administration (Scumpia et al.). Notably, administration of 10 µg Gr-1 induced similar proliferative responses in HSPC compartments as alum, and in both cohorts, BM neutrophil numbers peaked simultaneously (day 6; Figure 11B). Injection of 100 µg Gr-1 resulted in a longer period of elevated HSC proliferation (Figure 11C) that was associated with an even
greater rebound in BM neutrophil numbers on day 8 (Figure 11B). Thus, the depletion of Gr-1+ cells elicits a granulopoietic response that closely mimics that induced by alum, suggesting that a reduction in BM neutrophil numbers is sufficient to elicit an increase in HSPC proliferation for the accelerated production of neutrophils.
Figure 11: Adjuvant inflammation and experimental neutropenia elicit similar changes in granulopoiesis
(Figure 11, continued) (A) Treatment of BL/6 mice with Gr-1 mAb [10 μg (open squares), 100 μg (closed diamonds)] transiently reduces concentrations of blood neutrophils [mean(+SD) number/ml neutrophils], whereas immunization with an alum/antigen mixture (shaded triangles) induces neutrophilia. (B) Injection of Gr-1 mAb elicits similar changes in BM neutrophil numbers as alum immunization; a transient reduction in BM neutrophil numbers is followed by a supranormal increase. The mean(+SD) numbers of mature neutrophils in both femurs and tibiae at intervals after treatment are shown. (C-E) Both alum immunization and Gr-1 administration increase proliferation of HSC (C), MPP (D), and GMP (E). To evaluate HSPC proliferation, BrdU was injected into mice 6 hours prior to sacrifice. The mean (+SD) frequencies of BrdU+ cells in the HSC (C), MPP (D), and GMP (E) compartments of alum- or Gr-1- treated BL/6 mice are shown (day 0, n=19; for other intervals, n=3-10; data represent 11 experiments).
Although Gr-1 antibody depleted neutrophils, other hematopoietic lineages were also affected. Expression of the Gr-1 antigen is not restricted to neutrophils and Gr-1 administration also depleted BM monocytes in the BM and, especially at the higher dose, reduced B220⁺ (P ≤ 0.05, days 4-6; 10 μg Gr-1 and P ≤ 0.01, days 1-8; 100 μg) and erythroid lineage cell numbers (P ≤ 0.05, days 2-6; 10 μg and P ≤ 0.01, days 1-8; 100 μg) (Figure 12). Neither B cells nor erythroid cells express detectable levels of the Gr-1 antigen (data not shown) and while we cannot exclude non-specific antibody effects, we suspect that reductions in B- and erythroid cell numbers are secondary to the loss of Gr-1⁺ BM neutrophils. Notably, alum elicited a BM eosinophilia in addition to neutrophilia whereas Gr-1 had little effect on BM eosinophil numbers (Figure 12); inflammation, therefore, more broadly affects hematopoiesis.
Figure 12: Effects of adjuvant immunization and Gr-1 administration on monocytes, B-lineage cells, eosinophils, and erythroid-lineage cells in BM.

BL/6 mice were injected i.p. with 10 μg Gr-1 (open squares), 100 μg Gr-1 (closed diamonds), or an alum/antigen mixture (shaded triangles). BM cells of the hindlimbs were analyzed at different intervals by flow cytometry, and the numbers of monocytes, eosinophils, B-lineage cells (B220⁺), and erythroid lineage cells (Ter119⁺) were determined. The mean(±SD) numbers of cells in the femurs and tibiae at each interval are shown (day 0, n=19; for others, n=3-10; data represent 11 independent experiments). Significant differences from naïve mice are shown for treatment with 10 μg Gr-1 (*, P≤0.05 and **, P≤0.01), treatment with 100 μg Gr-1 (†, P≤0.05 and ††, P≤0.01), and immunization with alum (#, P≤0.05 and ##, P≤0.01).
3.3.2 Bone marrow neutropenia by genetic deletion

To control for any un-specific effects that Gr-1 administration might have on the proliferation of hematopoietic progenitors, we also examined a genetic model of neutropenia. Mice with the myeloid-specific deletion of the anti-apoptotic factor Mcl-1 (LysM\textsuperscript{inw} Mcl-1\textsuperscript{ff} mice; hereafter Mcl-1\textsuperscript{−} mice) exhibit a highly specific defect in neutrophil survival (Dzhagalov et al., 2007). Whereas the numbers of mature neutrophils in the BM of Mcl-1\textsuperscript{−} mice were only 20% of control littermates (P≤0.01; Table 2), eosinophil, monocyte, erythroid and B-lineage cell numbers remained normal or slightly elevated (Table 2). BrdU uptake in neutropenic Mcl-1\textsuperscript{−} mice demonstrated that HSC, MPP, and GMP proliferation was constitutively increased (2.0-, 1.7-, and 1.2-fold, respectively; P≤0.01, all) with respect to littermate control animals (Table 3). These increases in progenitor cell proliferation rates correlated only with reductions in mature neutrophil numbers, as immature neutrophil numbers in the BM of Mcl-1 mice were higher (2.2- and 2.3-fold, respectively; P≤0.05) than in normal littermates (Table 2).
Table 2: BM leukocyte numbers (x10^5) in Mcl-1^+ and Mcl-1^- mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mcl-1^+</th>
<th>Mcl-1^-</th>
<th>t-test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells</td>
<td>330.8 ± 70.0</td>
<td>313.7 ± 66.7</td>
<td>0.663</td>
</tr>
<tr>
<td>Erythroid (Ter119^+)</td>
<td>64.8 ± 16.1</td>
<td>70.7 ± 26.5</td>
<td>0.598</td>
</tr>
<tr>
<td>B-lineage cells (B220^+)</td>
<td>68.6 ± 14.0</td>
<td>56.0 ± 21.2</td>
<td>0.198</td>
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<tr>
<td>Eosinophils</td>
<td>7.5 ± 4.1</td>
<td>10.5 ± 5.8</td>
<td>0.267</td>
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<tr>
<td>Monocytes</td>
<td>16.5 ± 1.8</td>
<td>33.8 ± 17.0</td>
<td><strong>0.010</strong></td>
</tr>
<tr>
<td>Immature Neutrophils</td>
<td>29.5 ± 6.1</td>
<td>66.8 ± 32.1</td>
<td><strong>0.025</strong></td>
</tr>
<tr>
<td>Mature Neutrophils</td>
<td>104.8 ± 28.6</td>
<td>18.7 ± 9.3</td>
<td><strong>0.002</strong></td>
</tr>
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</table>

Table 3: Frequencies of BrdU^+ HSPC in Mcl-1^+ and Mcl-1^- mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mcl-1^+</th>
<th>Mcl-1^-</th>
<th>t-test (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC</td>
<td>12.8 ± 3.7%</td>
<td>25.1 ± 5.5%</td>
<td><strong>0.0003</strong></td>
</tr>
<tr>
<td>MPP</td>
<td>14.1 ± 2.0%</td>
<td>23.8 ± 4.9%</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td>GMP</td>
<td>49.4 ± 4.1%</td>
<td>60.8 ± 4.1%</td>
<td><strong>0.001</strong></td>
</tr>
</tbody>
</table>
3.3.3 Induced proliferation of hematopoietic progenitors is inversely proportional to BM neutrophil numbers

If the population density of mature neutrophils in the BM regulates the proliferation rates of hematopoietic progenitor cells by a physiologic, negative feedback mechanism, then changes in BM neutrophil numbers should elicit reciprocal and proportional changes in proliferation rates. To test this prediction, we injected mice with graded doses of Gr-1 mAb (0.1, 1, 10, or 100 µg) and observed dose-dependent decreases in BM neutrophil numbers on day 2, the nadir of neutrophil losses (Figure 13A). In these mice, increased frequencies of BrdU+ HSC, MPP, and GMP were highly correlated (R = -0.826 to -0.886; P≤0.01, all) with the extent of neutrophil depletion, demonstrating a strong inverse relationship between BM neutrophil population density and HSC, MPP, and GMP proliferation (Figure 13A).

The inverse relationship between BM neutrophil numbers and progenitor proliferation rates was maintained even when BM neutrophil numbers increased above steady-state numbers (Figure 13B). Following the i.p. injection of Gr-1 mAb or alum adjuvant, the kinetics of HSC proliferation and BM neutrophil numbers were virtual mirror images of one another with increased proliferation during the early neutropenic phase (days 1 and 2) and suppressed proliferation when BM neutrophil numbers climbed above normal levels (Figure 13B). The correlation coefficients for these reciprocal relationships (R = -0.842 to -0.941; P≤0.01, all) suggest that the relationship between neutrophil numbers and HSC proliferation is direct and tightly regulated.
**Figure 13: HSPC proliferation correlates inversely with the number of BM neutrophils.**

**(A)** Reductions in BM neutrophil density trigger reciprocal increases in HSC, MPP, and GMP proliferation. C57BL/6 mice were injected with various amounts of Gr-1 mAb (0, 0.1, 1, 10, and 100 μg; n=4-6 mice per treatment) and then BM neutrophil numbers and progenitor proliferation were determined two days later. The mean(±SD) numbers of BM neutrophils (closed diamonds) are shown in each graph, and the mean(±SD) frequencies of BrdU+ HSC, MPP, and GMP are plotted in independent graphs (open squares). Significant differences from naïve mice (0 μg Gr-1) are shown for BM neutrophil numbers (*, P≤0.05 and **, P≤0.01) and for each progenitor compartment (†, P≤0.05 and ††, P≤0.01). Pearson’s correlation coefficients (R) between BM neutrophil numbers and the frequency of BrdU+ cells in each progenitor compartment are shown.

**(B)** HSC proliferation correlates inversely with the density of BM neutrophils following neutrophil depletion or immunization with alum adjuvant. BM neutrophil numbers and frequencies of BrdU+ HSC after injection of 10 μg Gr-1, 100 μg Gr-1, or alum (as shown in Figure 11) were co-plotted; the values at each interval represent the fold change, expressed as logarithm base 2, in BM neutrophil numbers (closed diamonds) or frequency of BrdU+ HSC (open diamonds) from naïve controls. Pearson’s correlation coefficients (R) between BM neutrophil numbers and the frequency of BrdU+ HSC after each treatment are shown.
3.3.4 HSC and MPP proliferation rates are uncorrelated to changes in B220⁺ or Ter119⁺ BM cell numbers

The i.p. injection of Gr-1 antibody or alum adjuvant alters several BM cell populations, including cells of the B lymphocyte and erythroid lineages (Figure 12). Do these confounding effects invalidate our experimental tests of the relationship between HSC proliferation and BM neutrophil numbers? For several reasons, we conclude they do not.

First, there was no significant correlation (P>0.3, all) between the dynamics of B220⁺ (Figure 14A) or Ter119⁺ (Figure 14B) BM cell numbers and HSC proliferation after injections of Gr-1 or alum. The lack of correlation is especially obvious in mice given 10 μg of Gr-1 antibody (B220⁺ vs. BrdU⁺ HSC; R=-0.078 and Ter119⁺ vs. BrdU⁺ HSC; R=0.008), but the correlations are not significant in animals given a higher dose of Gr-1 antibody or alum (compare Figure 13B to Figure 14A and B). Second, in a study analogous to our measurements of HSC, MPP, and GMP proliferation in neutropenic Mcl-1⁻ mice (Table 2 and Table 3), we determined HSC and MPP proliferation rates in lymphopenic RAG1⁻/⁻ mice (Mombaerts et al., 1992). RAG1 deficient animals had similar numbers of BM neutrophils (Table 4) and frequencies of BrdU⁺ HSC and MPP that were no different from RAG1 sufficient controls (Figure 14C). In contrast, HSC proliferation in Mcl-1⁻ mice was significantly higher than in Mcl-1⁺, BL/6, and RAG1⁻/⁻ mice (Figure 14C).
The frequency of BrdU+ HSC (open squares) correlates weakly with the number of B-lineage cells (B220+, closed diamonds) (A) and erythroid lineage cells (Ter119+, closed diamonds) (B) after administration of 10 μg or 100 μg Gr-1 mAb, or immunization with alum adjuvant. Pearson product moment correlation coefficients (R) between the numbers of B-lineage cells or erythroid lineage cells and the frequencies of BrdU+ HSC are shown (day 0, n=19; for others, n=3-10; data represent 11 independent experiments). (C) HSC and MPP are not responsive to constitutive lymphopenia. The frequencies (+SD) of BrdU+ HSC and MPP in C57BL/6 (open, n=19; data represent 11 independent experiments) and lymphopenic RAG1-/- (closed, n=4; data represent 2 independent experiments) are shown (n.s., not significant). For comparison, the frequencies (+SD) of BrdU+ HSC and MPP in control Mcl-1+ (shaded, n=5) and neutropenic Mcl-1- (diagonal lines, n=10) mice are also depicted (**, \( P \leq 0.01 \); data represent two independent experiments).
Table 4: BM leukocyte numbers (x10^5) in BL/6 and RAG1^−/− mice

<table>
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<th>Cell Type</th>
<th>BL/6</th>
<th>RAG1^−/−</th>
<th>t-test (P)</th>
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<tr>
<td>Total cells</td>
<td>614.3 ± 102.2</td>
<td>560.0 ± 114.5</td>
<td>0.429</td>
</tr>
<tr>
<td>Erythroid (Ter119⁺)</td>
<td>162.2 ± 38.9</td>
<td>198.9 ± 40.5</td>
<td>0.168</td>
</tr>
<tr>
<td>B-lineage cells (B220⁺)</td>
<td>184.0 ± 49.7</td>
<td>76.6 ± 33.2</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>10.4 ± 3.1</td>
<td>9.7 ± 3.7</td>
<td>0.745</td>
</tr>
<tr>
<td>Monocytes</td>
<td>9.6 ± 5.2</td>
<td>11.7 ± 3.6</td>
<td>0.371</td>
</tr>
<tr>
<td>Immature Neutrophils</td>
<td>54.0 ± 21.8</td>
<td>66.8 ± 14.3</td>
<td>0.191</td>
</tr>
<tr>
<td>Mature Neutrophils</td>
<td>110.6 ± 31.6</td>
<td>133.7 ± 30.2</td>
<td>0.232</td>
</tr>
</tbody>
</table>
3.3.5 Minimal inflammation associated with neutrophil depletion by Gr-1 antibody

If the depletion of cells by Gr-1 mAb elicits significant inflammatory responses through complement fixation or Fc receptor signaling, then increased proliferation rates in HSPC compartments might not be due to reduced neutrophil population density. However, mice deficient for C1q (Botto et al., 1998) or C3 (Wessels et al., 1995) exhibited increases in HSC proliferation and neutrophil production after Gr-1 administration that were similar to congenic BL/6 mice (Figure 15A). In mice deficient for the common γ chain of the Fc receptor [FcRγ, (Takai et al., 1994)], the neutrophil depleting capacity of Gr-1 mAb was diminished, yet the partial reduction in BM neutrophil numbers was still associated with a significant increase in HSC proliferation (day 1, P≤0.01) and a subsequent rebound in BM neutrophil numbers (day 8, P≤0.05) (Figure 15A). Granulopoietic responses to Gr-1 treatment, therefore, are independent of complement or FcR-mediated inflammation.

In Chapter 2, we demonstrated that IL-1RI expression on radiation resistant cells is necessary for alum-induced emergency granulopoiesis (Figure 6). To determine if granulopoietic responses to neutrophil depletion also depend on IL-1RI signaling, we treated IL-1RI⁻ mice with Gr-1 mAb. As previously observed (Figure 6), granulopoietic responses to alum were severely impaired in IL-1RI⁻ mice (Figure 15B); however, Gr-1 treatment elicited increases in HSC proliferation (day 1, P≤0.01) and BM neutrophil numbers (day 8, P≤0.01) that were identical to congenic BL/6 mice (Figure 15B). Similarly, mice lacking MyD88, an adaptor protein crucial for signaling through many TLR (Takeda and Akira, 2005) and the receptors for IL-1, IL-18 (Adachi et al., 1998), and
IL-33 (Kroeger et al., 2009), failed to elicit emergency granulopoiesis in response to alum, but exhibited significant increases in HSC proliferation (day 1; \( P \leq 0.01 \)) and granulopoietic output (day 8; \( P \leq 0.05 \)) following neutrophil depletion with Gr-1 mAb (Figure 15B).

T cells have been implicated as regulators of granulopoiesis through the production of IL-17 (Ley et al., 2006). However, RAG1\(^{-/-}\) mice, which lack both T and B-lymphocytes (Mombaerts et al., 1992), had normal numbers of developing and mature neutrophils in BM (Table 4), and the frequencies of BrdU\(^{+}\) HSC, MPP, and GMP were identical to BL/6 controls (Figure 14C, unpublished data; \( P > 0.05 \)). Furthermore, granulopoietic responses in RAG1\(^{-/-}\) mice to both alum and Gr-1 were identical to congenic BL/6 mice (Figure 15B), indicating that signals from T- and B lymphocytes are dispensable for proliferative responses by HSPC to these treatments.

Although this survey (Figure 15A, B) of inflammatory defects was not exhaustive, we found no evidence that passive administration of Gr-1 mAb triggers HSPC proliferation via intermediate, inflammatory pathways. Notably, HSC proliferation increased only when BM neutrophil numbers decreased, whether by mobilization in response to alum or following depletion with Gr-1 (Figure 15B). Significantly, IL-1RI\(^{-/-}\) and MyD88\(^{-/-}\) mice that lacked the ability to mobilize BM neutrophils in response to alum retained fully their capacity for increased HSC proliferation and granulopoiesis in response to neutrophil depletion (Figure 15B). Since granulopoietic responses to both alum and Gr-1 were intact in RAG1\(^{-/-}\) mice (Figure 15B), we conclude that innate sensors drive the granulopoietic responses to both adjuvant-induced inflammation and the myeloablative effects of Gr-1 mAb.
Figure 15: Neutrophil depletion with Gr-1 mAb elicits emergency granulopoiesis with minimal inflammation.

(A) Gr-1 administration elicits emergency granulopoiesis in the absence of complement or Fc receptor signals. BL/6 and congenic C3<sup>-/-</sup>, C1q<sup>-/-</sup>, and FcR<sub>γ</sub>-<sup>-/-</sup> mice were treated with 100 µg G-1 mAb; the numbers of BM neutrophils (closed diamonds) and frequencies of BrdU<sup>+</sup> HSC (open diamonds) were assessed 1 and 8 days later. Mean numbers (+SD) of BM neutrophils and frequencies (+SD) of BrdU<sup>+</sup> HSC at days 1 and 8 after treatment are shown. (B) IL-1RI and MyD88, but not T or B cells, are necessary for granulopoietic responses to alum; all of these factors are dispensable for granulopoietic responses to Gr-1 administration. BL/6 mice and congenic IL-1RI<sup>-/-</sup>, MyD88<sup>-/-</sup>, and RAG1<sup>-/-</sup> mice were injected with alum or 100 µg Gr-1 mAb; the numbers of BM neutrophils (closed diamonds) and frequencies of BrdU<sup>+</sup> HSC (open diamonds) were assessed 1 and 8 days later. The mean numbers (+SD) of BM neutrophils and frequencies (+SD) of BrdU<sup>+</sup> HSC are shown (n=19 BL/6 mice, day 0; n=3-8 mice for all other data points; data represent 11 independent experiments for BL/6 mice, and 2-3 independent experiments for each gene deficient mouse strain).
3.3.6 Cytokines, growth factors, and chemokines elicited by inflammation or neutropenia

Several growth factors elicited by inflammatory stimuli have been implicated in the regulation of granulopoiesis, including G-CSF, GM-CSF, IL-3, and IL-6 (Basu et al., 2000; Cheers et al., 1988; Walker et al., 2008; Watari et al., 1989). The inflammatory induction of these factors is thought to elicit a pathway of accelerated granulopoiesis that is dependent on the transcription factor C/EBPβ but is distinct from the steady-state program of neutrophil production (Hirai et al., 2006).

We determined the concentrations of 23 cytokines, growth factors, and chemokines, including G-CSF, GM-CSF, IL-3, and IL-6, in the sera of naïve Mcl-1+, Mcl-1−, and C57BL/6 mice, and in BL/6 animals injected i.p. with alum or Gr-1 (100 μg) antibody (6 h and 1-, 2-, and 8 days after treatment) (Figure 16). The inclusion of naïve Mcl-1− mice and their Mcl-1+ littermate controls allowed us to distinguish patterns of cytokine changes elicited by inflammation from those that arise as a consequence of neutropenia. Serum concentrations of all cytokines were determined in a multiplex bead array (Bio-Rad) against validated murine standards.

Of the 23 cytokines, growth factors, and chemokines surveyed, the levels of 15, IL-1α/β, IL-2, IL-3, IL-4, IL-10, IL-12 p40/p70, IL-13, IL-17, GM-CSF, MIP-1α/β and TNFα, were identical in naïve Mcl-1+, Mcl-1−, and BL/6 mice, and remained unchanged in BL/6 mice after alum immunization or injection of Gr-1 antibody (Figure 16). The serum concentrations of another three cytokines, IL-9, eotaxin, and IFNγ, fell in response to alum and Gr-1; IL-9, eotaxin, and IFNγ levels in naïve Mcl-1+ and Mcl-1− mice were also low and not significantly different (Figure 16).
As expected (McKee et al., 2009), immunization with alum triggered rapid increases in serum G-CSF concentrations; by 6 h after immunization, serum GCSF increased 40-fold (58±34 to 2312±539 pg/ml; P≤0.01) and then fell gradually to naïve levels on day 8 (Figure 16). The injection of Gr-1 antibody also elicited significant increases in serum G-CSF, but with very different kinetics. Instead of the early, 6 h peak of serum G-CSF induced by alum, passive Gr-1 antibody elicited a smaller plateau of elevated G-CSF on days 1 and 2 (605±273 and 560±405 pg/ml, respectively) that returned to control values by day 8 (Figure 16). Mcl-1− mice had constitutively elevated serum G-CSF (244±90 pg/ml vs. 11±4 pg/ml in Mcl-1+, P≤0.01), indicating that the absence of neutrophils is sufficient to elicit G-CSF (Figure 16). The modest increases in G-CSF associated with Gr-1 administration may therefore represent, to some degree, a consequence of neutropenia, rather than a cause (Figure 16).

Alum, but not passive Gr-1 antibody, also induced rapid but transient increases in IL-5 (1891±571 pg/ml; P≤0.01) and IL-6 (41±32 pg/ml; P≤0.05) 6 h after immunization (Figure 16). These increases were brief; IL-5 levels fell some 80% by day 1 and returned to normal levels by day 2 (Figure 16). Serum concentrations of IL-6 returned to normal by day 1 (Figure 16). Given that these changes in serum IL-5 and IL-6 were not elicited by Gr-1 antibody, they cannot be the agents of HSC proliferation elicited by neutrophil depletion.

Remarkably, of the 23 factors surveyed, only two, the chemokines KC and monocyte chemotactic protein (MCP)-1, were similarly elicited by both alum and Gr-1 antibody (Figure 16). KC is a potent neutrophil chemoattractant (Kobayashi, 2006) and MCP-1 recruits monocytes and macrophages to inflammatory sites (Huang et al., 2001).
Alum and Gr-1 antibody triggered biphasic increases of serum KC with maxima at 6 h (773±188 and 980±325 pg/ml, respectively; \( P \leq 0.01 \), both) and on day 2 (Figure 16). Like G-CSF, KC was also constitutively elevated in Mcl-1\(^{-}\) mice (95±58 vs. 35±27 pg/ml in Mcl-1\(^{+}\) littermates; \( P \leq 0.05 \)) (Figure 16), suggesting that, at least in part, elevations in serum KC may be a consequence of reduced neutrophil numbers.

Both alum and Gr-1 antibody increased MCP-1 serum concentrations by 6 h post-injection (1107±508 and 2433±1256 pg/ml, respectively; \( P \leq 0.01 \), both) but alum’s effects were again biphasic with a second, but non-significant, peak on day 2 (730±632 pg/ml; \( P > 0.05 \)) (Figure 16). Serum MCP-1 levels in Mcl-1\(^{-}\) mice were no different than normal littermate controls (Figure 16). We note that in contrast to the changes in serum G-CSF, IL-5, and IL-6, administration of Gr-1 antibody induced greater increases in serum KC and MCP-1 than did alum immunization.

It may also be significant that the Gr-1 antibody depleted some monocyte populations (Figure 12) in addition to neutrophils. Increased expression of serum KC and MCP-1 in mice given Gr-1 antibody may, therefore, reflect neutrophil and monocyte losses rather than inflammatory responses.
Figure 16: Cytokines elicited by alum-induced inflammation or neutropenia.
(Figure 16, continued) (A) Sera from mice immunized with alum or injected with Gr-1 mAb were analyzed for growth factors and chemokines with a multiplex bead array. Serum concentrations of each cytokine are shown at day 0, 0.25 (6 hours), 1, 2, and 8 after treatment with 100 μg Gr-1 (closed diamonds) or alum (shaded triangles) (n=4-5 serum samples per data point; data represent a single experiment). (B) Cytokine concentrations in sera of control Mcl-1+ (closed bars, n=3) and neutropenic Mcl-1− (open bars, n=7) mice were also determined (data represent a single experiment). The horizontal line on each graph represents the lowest point of confident detection; measurements below this are imprecise.
3.3.7 Dual roles for G-CSF/G-CSF-R signals in the induction of emergency granulopoiesis

Although serum G-CSF was elevated during both alum-induced inflammation and experimental neutropenia (Figure 17A), passive Gr-1 administration elicited a more robust granulopoietic response than alum (Figure 11) but with substantially less G-CSF (Figure 17A). Since G-CSF is a potent mobilization signal for BM neutrophils (Lieschke et al., 1994), we hypothesized that the early induction of G-CSF after alum injection (6 h after immunization, Figure 17A) draws neutrophils out of BM as part of the acute inflammatory response. Consistent with this hypothesis, a single injection of recombinant G-CSF into mice recapitulated the effects of alum, significantly reducing BM neutrophil numbers and increasing HSC proliferation on day 1 (P≤0.01, Figure 17B). Notably, single injections of G-CSF failed to reproduce the BM neutrophilia that characterized alum and Gr-1 treatments (Figure 17B, day 8). Furthermore, G-CSF neutralization significantly impaired alum’s capacity to mobilize BM neutrophils (Figure 17C). The blockade in inflammatory mobilization was associated with abrogated proliferative responses of HSC and GMP (Figure 17C).

The capacity of G-CSF to modulate the population density of BM neutrophils by controlling their migration raised the possibility that G-CSF’s effects on HSPC proliferation might be indirect; by mobilizing neutrophils from BM to blood, G-CSF might trigger a G-CSF-independent pathway(s) of accelerated granulopoiesis. To disentangle the mobilizing effects of G-CSF from its potential role as a growth factor for HSPC, we injected mice with G-CSF neutralizing mAb then depleted neutrophils by Gr-1 administration (Figure 17C). In these mice, the frequencies of BrdU⁺ HSC and GMP
were significantly less than in mice treated with Gr-1 alone but were still increased over naïve controls (P ≤ 0.05, Figure 17C), suggesting that a portion of the HSPC response to BM neutropenia is G-CSF-dependent.

We next examined G-CSF-R−/− mice, which are constitutively neutropenic (Liu et al., 1996) (Figure 17D). Despite a 70% reduction in BM neutrophil numbers, the frequencies of BrdU+ HSC and GMP in G-CSF-R−/− mice were identical to congenic BL/6 controls (Figure 17D); for the first time in this study, BM neutropenia was not associated with increased proliferation by HSPC. Interestingly, the number of HSC in BM of G-CSF-R−/− mice was the same as in BL/6 mice (0.62±0.17 x10^5 HSC in G-CSF-R−/− mice vs. 0.63±0.09 x10^5 cells in BL/6 mice, P>0.05), but GMP numbers were reduced by 60% (2.07±0.50 x10^5 GMP in G-CSF-R−/− mice vs. 4.99±0.12 x10^5 cells in BL/6 mice, P ≤ 0.01), suggesting that G-CSF-R is dispensable for the basal proliferation of HSC and GMP, but plays a role in the survival of GMP specifically.

Consistent with our G-CSF neutralization studies (Figure 17C), alum failed to reduce BM neutrophil numbers in G-CSF-R−/− mice (Figure 17D), indicating that the residual BM neutrophils are insensitive to mobilization cues in the absence of G-CSF-R. Furthermore, immunization did not increase proliferation of HSC or GMP, ruling out the activation of G-CSF-R independent pathways (Figure 17D).

To determine if any G-CSF-R-independent pathways contribute to the feedback between the residual BM neutrophils and HSPC, we injected Gr-1 mAb into G-CSF-R−/− mice. Gr-1 administration depleted the residual BM neutrophils in G-CSF-R−/− mice but failed to elicit an increase in GMP proliferation (Figure 17D). Interestingly, the frequency
of BrdU\(^+\) HSC increased modestly but significantly after Gr-1 treatment, indicative of a G-CSF-R independent pathway of feedback (Figure 17D).

Our observations were consistent with a model of emergency granulopoiesis where inflammation rapidly drains the BM neutrophil reserve through the mobilizing properties of G-CSF. In turn, the loss of BM neutrophils activates a latent, feedback mechanism of increased HSPC proliferation mediated partly by the growth-inducing properties of G-CSF. Since alum failed to mobilize BM neutrophils or elicit emergency granulopoiesis in IL-1RI\(^{-}\) mice (Figure 6), IL-1RI must play a role in the rapid induction of G-CSF after immunization. Indeed, alum failed to elicit serum G-CSF in IL-1RI\(^{-}\) mice (Figure 17E). In contrast, neutrophil depletion with Gr-1 mAb induced serum G-CSF similarly in IL-1RI\(^{-}\) and BL/6 mice (Figure 17E). From these observations, we conclude that alum induces G-CSF through an innate, inflammatory pathway dependent on IL-1RI. The resulting reduction in population density of BM neutrophils activates an IL-1RI-independent feedback mechanism of increased HSPC proliferation that is partially mediated by G-CSF/G-CSF-R signals.
Figure 17: Dual roles for G-CSF/G-CSF-R signaling in the induction of emergency granulopoiesis.
(Figure 17, continued) (A) Both alum and Gr-1 injection elicit serum G-CSF. Serum concentrations of G-CSF after treatment of BL/6 mice with 100 μg Gr-1 mAb (closed diamonds) or alum (shaded triangles), and in neutropenic Mcl-1+ (open circle) and control Mcl-1- (closed circle) mice are shown. Significant differences from naive BL/6 mice are shown for Gr-1 treatment (†, P≤0.05 and ††, P≤0.01) and alum immunization (#, P≤0.05 and ##, P≤0.01), and between Mcl-1+ and Mcl-1- mice (**, P≤0.01) (n=3-7 mice per data point, data represent one experiment). (B) Injection of rG-CSF recapitulates the short-term effects of alum on BM neutrophil numbers and HSC proliferation, but fails to induce BM neutrophilia. BL/6 mice were injected with 5 μg rG-CSF and the numbers of BM neutrophils (closed diamonds) and frequencies of BrdU+ HSC (open diamonds) were measured 1 and 8 days later. Significant differences in BM neutrophil numbers (**, P≤0.01) and frequencies of BrdU+ HSC (††, P≤0.01) are shown (n=20 mice for naïve BL/6, and 3 mice for G-CSF treatment; data for G-CSF injection represent one experiment). (C) G-CSF neutralization impairs inflammatory mobilization of BM neutrophils as well as proliferative responses of HPSC to BM neutrophil deficits. BL/6 mice were injected or not with 100 μg anti-G-CSF then administered alum or 10 μg Gr-1 mAb 30 minutes later. The numbers of BM neutrophils and frequencies of BrdU+ HSC were determined 1 day later; each point in the graph represents one animal and horizontal bars depict mean values. Statistical differences between experimental cohorts are denoted by letters; P>0.05 for cohorts sharing the same letter, P≤0.05 for cohorts with different letters (n=4-5 mice per treatment, data represent 3 independent experiments). (D) G-CSF-R is necessary for alum-induced reductions in BM neutrophil numbers and proliferative responses of GMP, but not HSC. BL/6 or congenic G-CSF-R-/- mice were injected with alum or 10 μg Gr-1 mAb, and the numbers of BM neutrophils and frequencies of BrdU+ HSC and GMP were determined one day later; each point in the graph represents one animal and horizontal bars depict mean values. Statistical differences between experimental cohorts are denoted by letters; P>0.05 for cohorts sharing the same letter, P≤0.05 for cohorts with different letters (n=3-9 mice per treatment; data represent 4 independent experiments). (E) IL-1RI is necessary for G-CSF induction by alum, but is dispensable for elevated G-CSF in response to antibody-induced neutropenia. Serum concentrations of G-CSF in BL/6 (closed diamonds) and congenic IL-1RI-/- mice (open diamonds) were determined in naïve mice and 6 hours, 1 day, and 2 days after injection of alum or 100 μg Gr-1 mAb. Serum G-CSF was measured by ELISA. Mean(+SD) concentrations of G-CSF (ng/ml) in serum are shown. Significant differences in G-CSF concentrations in BL/6 and IL-1RI-/- mice at each interval are indicated (**, P≤0.01) (n=3-5 mice per data point, data represent 4 experiments).
3.3.8 C/EBPβ is dispensable for increased HSPC proliferation during emergency granulopoiesis

C/EBPβ is thought to be necessary for a distinct pathway of granulopoiesis elicited by inflammatory signals (Hirai et al., 2006). However, we observed that experimentally-induced neutropenia increased HSPC proliferation and neutrophil production similar to alum (Figure 11), but without evidence of inflammation (Figure 15). To determine if C/EBPβ is necessary for accelerations in granulopoiesis elicited by neutrophil deficits, we depleted neutrophils by injecting 10 μg Gr-1 mAb into C/EBPβ-sufficient and C/EBPβ+/- littermates. C/EBPβ-/- mice and control littermates were generated by crossing hemizygous 129/Sv and BL/6 parents. Hematopoiesis in C/EBPβ+/+ and C/EBPβ+/− mice was identical; data from these mice were pooled and this cohort is referred to hereafter as C/EBPβ+ mice.

As observed in BL/6 mice, Gr-1 treatment of C/EBPβ+ mice transiently depleted BM neutrophils and elicited a >3-fold rebound in BM neutrophil numbers on day 6 (P≤0.01 compared to naïve controls, Figure 18A). C/EBPβ−/− mice had equivalent numbers of BM neutrophils as C/EBPβ+ mice (Figure 18A), consistent with previous reports (Akagi et al., 2008). Neutrophils were similarly depleted by Gr-1 mAb in C/EBPβ+ and C/EBPβ+/− mice (Figure 18A), however the supranormal recovery of BM neutrophils was blunted in mice lacking C/EBPβ (Figure 18A). In Gr-1-treated C/EBPβ−/− animals, BM neutrophil numbers peaked on day 6 only modestly higher than in untreated mice (1.7-fold, P≤0.05), and were only half of that in C/EBPβ+ mice at the same interval (P≤0.01)
(Figure 18A). Thus, C/EBPβ is required for optimal granulopoietic responses to the myeloablative effects of Gr-1 administration.

Since C/EBPβ is thought to allow for increased proliferation of GMP in response to growth factors (Hirai et al., 2006), we next analyzed the effects of Gr-1 administration on the proliferation of HSPC in C/EBPβ+/− mice. Surprisingly, C/EBPβ+/− mice exhibited similar increases in frequencies of BrdU+ HSC, MPP, and GMP after Gr-1 injection as C/EBPβ+ mice (Figure 18A). Moreover, the immature neutrophil population underwent identical changes in C/EBPβ+ and C/EBPβ−/− mice (Figure 18A), indicating equivalent expansions of progenitor compartments and cellular delivery into the neutrophil lineage. These observations dissociate C/EBPβ from the proliferative effects of growth factors on HSPC during emergency granulopoiesis; instead, the lack of rebounding neutrophilias in C/EBPβ−/− mice likely stems from a defect at terminal stages of neutrophil differentiation.

Interestingly, C/EBPβ was dispensable for granulopoietic responses to immunization. Like Gr-1 treatment, alum elicited similar increases in HSPC proliferation in C/EBPβ+ and C/EBPβ−/− mice (Figure 18B). However, the number of mature neutrophils in BM of C/EBPβ−/− mice on day 6, although lower, was not significantly different from control mice (Figure 18B). This observation suggests differential requirements for C/EBPβ in the replenishment of mature neutrophil pools after alum and Gr-1 treatments. Since Gr-1 administration elicits a more robust granulopoietic response than alum (Figure 18), C/EBPβ-independent pathways of terminal neutrophil differentiation must suffice for the less dramatic replenishment of mature neutrophils after immunization.
Figure 18: C/EBPβ is dispensable for proliferative responses of HSPC to Gr-1 administration or alum immunization.

C/EBPβ⁺⁻ mice (open symbols) and control littermates (closed symbols) were injected with 10 μg Gr-1 mAb (A) or alum (B). The numbers of mature and immature neutrophils, and the frequencies of BrdU⁺ HSC, MPP, and GMP were determined at different intervals after treatment. Significant differences between C/EBPβ⁺ and C/EBPβ⁻ mice at different intervals after treatment are indicated (**, P≤0.01) (day 0, n=12 for C/EBPβ⁺ mice and n=7 for C/EBPβ⁻ mice; for all others, n=3-5 mice; data represent 6 experiments).
3.4 Discussion

Recent studies have suggested that inflammatory neutrophilias result from a pathway of accelerated myeloid development that is distinct from hematopoietic processes that sustain neutrophil pools in the steady-state (Hirai et al., 2006). However, in Chapter 2, I observed that failures in adjuvant-induced emergency granulopoiesis coincided with defects in the mobilization of BM neutrophils, raising the possibility that changes in hematopoiesis during the inflammatory response occur through feedback mechanisms that correct for the draining of the BM neutrophil reserve. To dissect the direct contributions of inflammatory mediators to emergency granulopoiesis from feedback responses to neutrophil consumption, we compared the hematopoietic changes evoked by sterile adjuvant to those elicited by experimental neutropenia. Our observations suggest that the emergency granulopoiesis elicited by alum adjuvant can be fully explained as a feedback response to the exhaustion of the BM neutrophil compartment.

To our knowledge, this is the first study to track HSPC proliferation and the population density of neutrophil compartments during inflammatory and neutropenic conditions. In antibody-mediated and genetic models of neutropenia, we found that neutrophil deficits in BM were associated with hematopoietic responses indistinguishable from adjuvant-induced emergency granulopoiesis (Figure 11). Like alum immunization, administration of the neutrophil-depleting Gr-1 mAb induced rapid increases in HSC, MPP, and GMP proliferation followed by supranormal numbers of BM neutrophils (Figure
Mice made chronically neutropenic by myeloid-specific inactivation of Mcl-1 (Dzhagalov et al., 2007) exhibited constitutively elevated rates of HSPC proliferation (Table 3) as well as a surplus of immature neutrophils in BM (Table 2). Significantly, increased HSPC proliferation in Mcl-1− mice was only associated with the absence of mature neutrophils, as the numbers of cells in other hematopoietic lineages were equivalent or increased in Mcl-1− mice compared to Mcl-1+ control littermates (Table 2). Further evidence for a specific link between the mature neutrophil compartment and HSPC was the observation that HSC proliferation in lymphopenic RAG1−/− mice was identical to congenic BL/6 control mice (Figure 14C). A relationship between BM neutrophils and HSPC was solidified by the observation that fluctuations in BM neutrophil numbers after Gr-1 or alum administration were reflected inversely in the proliferation of HSC (Figure 13). An inverse relationship between product (mature neutrophils) and precursor (HSPC) is the hallmark of regulation by negative feedback, where the output of the pathway suppresses input and the system gravitates about an equilibrium point. We conclude that mature neutrophils in BM actively control the pace of granulopoiesis by regulating the proliferation of HSPC.

In both Gr-1 treated and Mcl-1− mice, decreases in BM neutrophil numbers strongly correlated with increased proliferation by HSC and MPP (Figure 13), progenitors for all hematopoietic lineages. Nonetheless, increases in HSC and MPP proliferation appeared to enhance granulopoiesis specifically. Whereas the recovery of BM neutrophil numbers was strongly correlated with HSC and MPP proliferation, numbers of B lymphocyte (B220+ ) and erythroid (Ter119+ ) lineage cells were not; in fact, the numbers of B220+ and Ter119+ BM cells decreased as BM neutrophil numbers recovered after Gr-
This observation suggests that the feedback mechanism activated by neutropenia focuses hematopoiesis for the replenishment of neutrophils, possibly at the expense of other hematopoietic compartments.

The exception to this specificity was the increased production of monocytes. Monocyte numbers closely tracked BM neutrophil numbers after treatment with Gr-1 mAb (Figure 12) and the BM of Mcl-1− mice contained significantly more monocytes than littermate controls (Table 2). We conclude that monocytes and neutrophils either share a common developmental pathway or that their independent development is co-regulated.

Notably, Gr-1 treatment had little effect on BM eosinophil numbers, whereas alum stimulated an expansion of the BM eosinophil compartment (Figure 12). Increases in eosinophil numbers likely resulted from alum-induced IL-5 (Figure 16), a potent inducer of eosinopoiesis (Kopf et al., 1996).

We found no evidence that passive administration of Gr-1 mAb triggers HSPC proliferation via inflammatory pathways (Figure 15). Interestingly, IL-1R1−/− and MyD88−/− mice did not mount granulopoietic responses to alum, but Gr-1 mAb elicited increased HSC proliferation and granulopoietic output in both strains (Figure 15B). These observations distinguish alum-induced inflammation and reactive granulopoiesis from non-inflammatory reductions in BM neutrophil numbers by passive antibody, and provide further support for the BM neutrophil compartment as the source of feedback signals that control HSPC proliferation in both inflammatory and non-inflammatory situations.

In our experimental models of inflammation and neutropenia, we found no evidence for the activity of IL-17-secreting T cells (Ley et al., 2006) as regulators of granulopoiesis. The BM of RAG1−/− and control mice harbored equivalent neutrophil
numbers (Table 4), demonstrating that T cells are dispensable for steady-state granulopoiesis. Furthermore, RAG1−/− mice exhibited normal granulopoietic responses to both alum and Gr-1 mAb (Figure 15). Serum IL-17 levels in BL/6 mice given alum or Gr-1 also matched naïve controls, Mcl-1−, and Mcl-1+ mice (Figure 16). We conclude that both IL-17 and T cells are dispensable for the maintenance of neutrophil pools in the steady-state, and for accelerations in granulopoiesis as a result of neutrophil deficits in BM.

We hypothesize that mature neutrophils in BM suppress HSPC proliferation by regulating the production/availability of growth factors and that the strength of suppression is proportional to the numbers of mature neutrophils in BM. By mobilizing neutrophils from BM, inflammation activates a latent feedback response that increases HSPC proliferation and neutrophil output. As granulopoiesis accelerates, the number of mature neutrophils in BM increases, reestablishing a suppressive environment. Reduced HSPC proliferation curtails neutrophil production and the steady-state equilibrium returns.

Following alum or Gr-1 administration, and in Mcl-1− mice, BM neutropenia was associated with systemic increases in G-CSF (Figure 16), a cytokine with pleiotropic effects (Lieschke et al., 1994; Liu et al., 1996). Whereas alum induced a rapid increase in serum G-CSF, neutropenia resulting from Gr-1 administration or Mcl-1 deficiency was associated with a lower plateau of G-CSF (Figure 16). This difference in kinetics suggested distinct pathways of G-CSF induction, one associated with inflammation and a second associated with neutropenia. G-CSF/G-CSF-R signals were necessary for the mobilization of BM neutrophils in response to immunization (Figure 17C, D), and in IL-
1RI−/− mice, alum failed to induce G-CSF (Figure 17E) or neutrophil mobilization (Figure 6). These observations reveal an acute, inflammatory pathway where alum activates IL-1RI signaling, likely through the Nalp3 inflammasome (Eisenbarth et al., 2008), for the production of G-CSF which, in turn, mobilizes BM neutrophils. In contrast, Gr-1 administration induced G-CSF (Figure 17E) and emergency granulopoiesis similarly in BL/6 and IL-1RI−/− mice (Figure 15), indicative of IL-1RI independent pathways of G-CSF production.

The similar patterns of accelerated granulopoiesis that followed the administration of alum or Gr-1 indicate that (inflammatory) mobilizations of mature neutrophils from BM trigger a common and intrinsic pathway that rapidly replenishes neutrophil pools. Although immunized mice exhibited high serum G-CSF immediately after injection (Figure 17A), G-CSF concentrations fell to the levels observed in neutropenic mice on days 1 and -2 (Figure 17A). The coincident increases in HSPC proliferation (Figure 11C-E) and serum G-CSF (Figure 17A) with decreased BM neutrophil numbers (Figure 11B) suggested that G-CSF might also mediate feedback between BM neutrophils and HSPC compartments. G-CSF neutralization impaired the proliferative responses of HSPC to antibody-mediated neutrophil depletion (Figure 17C). Furthermore, HSC and GMP in G-CSF-R−/− mice were unresponsive to constitutive neutropenia (Figure 17D). Interestingly, depletion of the residual neutrophils in G-CSF-R−/− mice induced a modest, but significant increase in proliferation of HSC but not GMP (Figure 17D). These observations indicate that the G-CSF/G-CSF-R axis is an important mediator of density-dependent feedback between the BM neutrophil compartment and
HSPC, but reveal the involvement of an additional feedback signal(s) that acts specifically on HSC.

How might the mature neutrophils in BM regulate G-CSF and other growth factors that control HSPC proliferation? If HSPC proliferation is dictated by the availability of these factors, then mature neutrophils may reduce their local concentrations in the BM environment. Of BM leukocytes, mature neutrophils express the highest levels of G-CSF-R (Nicola and Metcalf, 1985) and may sequester G-CSF and other growth factors through endocytosis of receptor-ligand complexes. Neutrophils may also diminish cytokine activity through the release of proteases such as neutrophil elastase (El Ouriaghli et al., 2003). Alternatively, a monitoring system might sense neutrophil population density in BM and modulate the production of growth factors in response to changes in neutrophil numbers. In either case, losses of BM neutrophils would promote growth factors that expand HSPC compartments and increase neutrophil output.

Given that HSPC proliferation is inevitably coupled to the population density of BM neutrophils, *in vivo* evaluations of growth factors and/or microbial products on HSPC proliferation and granulopoiesis must consider the indirect effects of neutrophil mobilization. The lack of HSPC proliferation in response to vaccinia virus in MyD88−/− mice (Singh et al., 2008) might be a consequence of impaired neutrophil mobilization. Conversely, agents that drain the BM neutrophil reserve will trigger G-CSF production and increase HSPC proliferation. This prediction is consistent with the observations of Scumpia et al., who observed expansions in HSC compartments coincident with cellular changes in BM composition during *S. aureus* infection (Scumpia et al.).
Recent studies have postulated that the transcription factors C/EBPα and C/EBPβ operate in GMP as developmental switches for independent pathways of neutrophil production, with C/EBPβ playing an indispensable role for inflammatory granulopoiesis (Hirai et al., 2006). Our observations, however, suggest that alum-induced neutrophilias are not the result of a special pathway of granulopoiesis, but occur through the same mechanisms that accelerate granulopoiesis in response to non-inflammatory neutrophil deficits. In C/EBPβ−/− mice, the proliferative responses of GMP to the myeloablative effects of Gr-1 mAb or alum-induced inflammation were the same as control mice (Figure 18A). Rather, C/EBPβ−/− mice exhibited a defect at the immature- to mature neutrophil transition during granulopoietic recovery from Gr-1 treatment, precluding the maximal overshoot in BM neutrophil numbers (Figure 18A). These observations do not exclude the possibility that C/EBPβ expression in GMP triggers C/EBPα-independent neutrophil production (Hirai et al., 2006). However, our results suggest that C/EBPα and -β play roles at distinct stages of neutrophil differentiation, rather than controlling distinct pathways of granulopoiesis.

Over the last 50 years, various mathematical models have been developed to describe the changes in granulopoiesis following the perturbation of neutrophil compartments. Our observations are remarkably consistent with models that posit the BM neutrophil compartment as the primary variable for the regulation of granulopoiesis (Rubinow and Lebowitz, 1975; von Schulthess and Mazer, 1982). Our findings, however, imply that the regulatory effects of BM neutrophil population density extend to inflammatory neutrophilias and emergency granulopoiesis.
4. Conclusions

Neutrophil pools are maintained through homeostatic mechanisms, but infections are thought to induce growth factors that activate an “emergency” pathway of granulopoiesis that is fundamentally distinct from hematopoiesis in the steady-state (Hirai et al., 2006). The steady-state and emergency pathways of granulopoiesis are distinguished by differential dependencies on C/EBP transcription factors (Hirai et al., 2006), but the extracellular cues that determine these differentiation programs have remained elusive.

G-CSF, GM-CSF, IL-3, and IL-6 are suspected inducers of emergency granulopoiesis because of their capacities to elicit neutrophilia when injected into mice or humans (Lord et al., 1989; Lord et al., 1991; Metcalf et al., 1986; Pojda and Tsuboi, 1990). However, these factors are dispensable for granulopoietic responses to infection (Basu et al., 2000; Nishinakamura et al., 1996), suggesting that a distinct inflammatory signal enhances neutrophil production to sustain neutrophilia. Based on the observation that IL-1 administration, when combined with TNFα, recapitulates the neutrophilia elicited by alum (Ueda et al., 2004), I hypothesized that inflammation elicits IL-1 which directly promotes GMP proliferation and emergency granulopoiesis (Figure 2). Indeed, IL-1RI−/− mice fail to mount reactive neutrophilias in response to alum, but the lack of responsiveness is an indirect effect, as IL-1RI deficient HSPC exhibit normal proliferative responses to alum in wild-type hosts (Figure 10).

In IL-1RI−/− mice, alum fails to mobilize neutrophils from BM and HSPC proliferative responses are abrogated (Figure 6). The coincident defects in neutrophil mobilization and emergency granulopoiesis suggested that accelerated granulopoiesis
might be an indirect effect of the loss of BM neutrophil stores. Instead of activating a distinct pathway of granulopoiesis (Hirai et al., 2006), inflammation might trigger a feedback mechanism that corrects for the exhaustion of the BM neutrophil reserve.

4.1 Neutrophil population density in BM controls emergency granulopoiesis through feedback inhibition

Alum immunization increases HSPC proliferation, expands the BM neutrophil compartment, and sustains neutrophilia (Figure 5 and Figure 6), consistent with the paradigm that inflammatory signals enhance the BM’s capacity to produce myeloid cells for host defense (Metcalf, 1998). However, the changes in granulopoiesis elicited by alum adjuvant are recapitulated by treatment with Gr-1 mAb, which transiently depletes circulating and BM neutrophil pools (Figure 11). The granulopoietic response to Gr-1 administration is intact in mouse strains deficient for different inflammatory pathways, including those necessary for alum-induced emergency granulopoiesis (Figure 15). Notably, HSC proliferation correlates inversely with the number of BM neutrophils following alum immunization or Gr-1 administration (Figure 13), suggesting that the population density of BM neutrophils regulates HSPC proliferation in both inflammatory and non-inflammatory conditions.

Thus, I propose that the inflammatory mobilization of BM neutrophils triggers a latent mechanism of neutrophil replenishment mediated by negative feedback. Instead of independent pathways of “steady-state” and “emergency” neutrophil production, a common pathway of granulopoiesis operates in both inflammatory and non-inflammatory conditions.
According to this feedback model (Figure 19), the BM neutrophil reserve functions as the key regulatory component for the tempo of granulopoiesis. The rate of neutrophil production depends on HSPC proliferation which, in turn, is sensitive to the population density of BM neutrophils. Equilibrium is achieved when the neutrophil production rate matches the rate of cellular turnover within the BM neutrophil reserve. Changes in BM neutrophil numbers perturb this equilibrium, generating a negative feedback signal that inversely modulates HSPC proliferation. As changes in HSPC proliferation manifest in the growth or diminution of the BM neutrophil compartment, feedback signals lessen until equilibrium is re-established.

The hypothesis that the BM neutrophil compartment is the source of feedback signals conflicts with models proposed by King-Smith et al. (King-Smith and Morley, 1970), who proposed the neutrophil pool in blood as the source of inhibition, and Stark et al. (Stark et al., 2005), who postulated senescent neutrophils in tissues as the regulators of granulopoiesis. However, HSPC are not sensitive to peripheral neutrophil compartments, as the kinetics of HSPC proliferation after alum immunization or Gr-1 treatment are the same, despite the distinct effects of each treatment on blood neutrophil counts [alum increases neutrophil counts but Gr-1 reduces neutrophil counts (Figure 11)]. Following injection of either alum or Gr-1, HSPC proliferation correlates only with the population density of BM neutrophils (Figure 13B). This finding is consistent with the mathematical models of Rubinow et al. (Rubinow and Lebowitz, 1975) and von Schulthess et al. (von Schulthess and Mazer, 1982), who proposed the BM neutrophil compartment as the source of granulopoiesis-regulating factors.
During the recovery of BM neutrophils after alum or Gr-1 administration, BM neutrophil numbers surpass steady-state levels before gradually returning to normal. Such overshoots are common in biological processes governed by negative feedback where changes in input do not manifest in the output for a substantial period of time (Milsum, 1966). For the regulation of granulopoiesis, this lag is likely due to the 4-6 day transit time of neutrophils through mitotic and postmitotic stages of development (Basu et al., 2002; Terashima et al., 1996). Consistent with this timeline, BM neutrophil numbers peak 5-6 days after surges in HSPC proliferation following alum or Gr-1 treatment (Figure 11). Thus, the sustained neutrophilias that occur during infections may not solely represent a facet of innate immune protection, but might also reflect the intrinsic mechanisms of granulopoietic regulation.

**4.2 HSPC sense BM neutrophil population density via G-CSF-dependent and independent pathways**

G-CSF is a principal regulator of granulopoiesis and neutrophil migration from BM (Lieschke et al., 1994; Liu et al., 1996). My studies revealed distinct pathways of G-CSF induction that control independent aspects of the granulopoietic response to inflammation. Alum induces G-CSF via IL-1RI, and this pathway is responsible for the rapid rise in serum G-CSF following immunization and the early mobilization of BM neutrophils (Figure 17C and E). However, inflammatory G-CSF production appears to be tightly regulated, as high G-CSF concentrations are not sustained beyond 24 hours after immunization (Figure 17A).

G-CSF is also induced in association with neutropenia via an IL-1RI-independent mechanism (Figure 17A and E). How is G-CSF induced by neutropenia? One possibility
is that G-CSF is produced constitutively, but the presence of neutrophils in BM antagonizes its ability to interact with receptors on HSPC. Indeed, G-CSF-R expression by neutrophils plays a role in eliminating G-CSF from the circulation (Ericson et al., 1997), so the large numbers of mature neutrophils in BM might limit the local availability of G-CSF. Furthermore, neutrophil granules contain neutrophil elastase, an enzyme capable of degrading G-CSF (El Ouriaghli et al., 2003). In either case, when the BM neutrophil reserve is intact, the amount of unbound G-CSF in the BM microenvironment is minimal; only when BM neutrophils are removed from the BM can G-CSF accumulate to the point of increasing the proliferation and granulocytic differentiation of HSPC.

Alternatively, cells that support granulopoiesis in BM may monitor neutrophil population density and adjust growth factor production in response to changes in neutrophil numbers. Several cell types are capable of producing G-CSF and other growth factors, but B- and T cells are dispensable for changes in neutrophil output in response to antibody-mediated depletion or alum immunization, eliminating the lymphocytes as mediators of granulopoiesis (Figure 15B).

Although G-CSF/G-CSF-R signals contribute to HSPC proliferative responses to BM neutropenia, these signals appear to be dispensable for the basal proliferation rates of HSC, MPP, and GMP (Figure 17D). These observations indicate that the neutropenia in G-CSF$^{-/-}$ and G-CSF-R$^{-/-}$ mice (Lieschke et al., 1994; Liu et al., 1996) is not due to diminished proliferation of HSPC. Instead, the reduced numbers of GMP and neutrophil-lineage cells may reflect the ability of G-CSF/G-CSF-R signals to promote survival of cells that have committed to the neutrophil lineage.
Interestingly, my neutrophil depletion studies revealed a G-CSF-R-independent mechanism that specifically increases HSC proliferation in response to reduced neutrophil numbers (Figure 17D). Thus, severe neutropenia activates an additional signal(s) that specifically targets HSC for increased proliferation. Factors that have been shown to affect HSC proliferation include SCF (Duarte and Frank, 2000), IL-11 (Hangoc et al., 1993), thrombopoietin (Young et al., 1996), and SDF-1 (Sugiyama et al., 2006). Determining the identity of this supplemental factor(s) will be key to understanding the regulatory mechanisms behind granulopoiesis.

The discovery of a G-CSF-independent pathway for increased HSC proliferation may explain, to some degree, the observations of Basu et al (Basu et al., 2000), who observed inflammatory neutrophilias in G-CSF−/− mice during fungal infection. If *C. albicans* infection mobilizes the BM neutrophil reserve, then G-CSF-independent feedback signals acting on HSC could bolster neutrophil production to support neutrophilia. For this explanation to be valid, *C. albicans* must, unlike alum, mobilize BM neutrophils in a G-CSF-independent manner (see section 4.3 below).

Alternatively, accelerated granulopoiesis during infections in G-CSF−/− mice could result from direct sensing of pathogen products by HSPC. Nagai et al. (Nagai et al., 2006) demonstrated that HSPC express TLR and, at least *in vitro*, TLR ligation induces HSPC proliferation and myeloid differentiation in a MyD88-dependent manner. If TLR signaling directly stimulates HSPC to enhance myelopoiesis, then this pathway may augment or synergize with feedback signals that replenish neutrophil stores. However, the contribution of TLR signaling to HSPC proliferation in animal models of infection does not appear to be substantial, at least not during *S. aureus* infection, as HSPC
compartments expand normally in MyD88<sup>−/−</sup>TRIF<sup>−/−</sup> mice during S. aureus infection (Scumpia et al.). Using the BM chimera strategy employed to evaluate IL-1RI signaling in alum-induced HSPC proliferation (Figure 10), it would be possible to determine the contribution of MyD88 or TLR4 signals to HSPC proliferation during LPS-induced inflammation.

4.3 Neutrophil mobilization is necessary for the induction of emergency granulopoiesis

Normally, the BM contains a large number of mature neutrophils. Inflammation mobilizes these BM neutrophil stores, allowing for the rapid delivery of phagocytes to sites of infection or tissue injury during acute inflammatory responses (Wengner et al., 2008). Neutrophil mobilization can be induced by several factors, including the complement fragment C5a (Kajita and Hugli, 1990), the chemokines KC and MIP-2 (Wengner et al., 2008), and the cytokines G-CSF (Lieschke et al., 1994) and IL-3 (Ulich et al., 1989a). In response to alum, neutrophil mobilization occurs through IL-1RI signals which, in turn, elicit G-CSF. The observation that IL-1RI deficiency impairs alum-induced inflammatory responses is consistent with studies showing alum is a potent stimulator of the Nalp3 inflammasome (Eisenbarth et al., 2008; Kool et al., 2008a; McKee et al., 2009), a cytosolic sensor that activates IL-1β and its related family members, IL-18 and IL-33 (Petrilli et al., 2007).

The IL-1RI-bearing cells that determine neutrophil mobilization are resistant to radiation, suggesting they are of non-hematopoietic origin. Since IL-1β and G-CSF are both rapidly induced at sites of alum injection (McKee et al., 2009), the radiation-resistant cells in or near the inflamed tissue may be the source of G-CSF that acts
systemically to mobilize BM neutrophil stores. Indeed, several types of non-hematopoietic cells, including endothelial cells (Broudy et al., 1987) and fibroblasts (Seelentag et al., 1989), express G-CSF upon IL-1 stimulation.

Despite the absolute requirement for IL-1RI in alum-induced neutrophil mobilization, G-CSF can be induced through other inflammatory pathways. G-CSF production is associated with signaling through pathogen recognition receptors including Toll-like receptor 4 (Li et al., 2009) and Dectin-1 (Yadav and Schorey, 2006), and is also induced by the cytokines IFNγ (Oster et al., 1989), IL-3 (Tsuji et al., 1994), and IL-17 (Fossiez et al., 1996). Thus, inflammatory pathways activated through different sensors may converge at the production of G-CSF for the inflammatory mobilization of BM neutrophils.

Several factors besides G-CSF have been implicated as neutrophil mobilizers. The inflammatory activation of C5a (Kajita and Hugli, 1990), KC, MIP-2 (Wengner et al., 2008), and/or IL-3 (Ulich et al., 1989a) has the potential to mobilize the BM neutrophil reserve. According to my feedback hypothesis, the depopulation of BM neutrophils through any of these pathways would activate HSPC proliferation and emergency granulopoiesis (Figure 19).

4.4 Redefining the role of C/EBPβ in emergency granulopoiesis

Mice lacking C/EBPα fail to generate neutrophils in the absence of pharmacologic doses of GM-CSF or IL-3 (Hirai et al., 2006). In contrast, mice lacking C/EBPβ fail to mount reactive neutrophilias in response to infection (Hirai et al., 2006). These observations, along with a report demonstrating that C/EBPβ can substitute for
C/EBPα in granulopoiesis (Jones et al., 2002), have led to a model for the regulation of granulopoiesis in which C/EBPα and C/EBPβ control independent pathways of steady-state and emergency granulopoiesis, respectively.

According to this model, alum adjuvant should elicit neutrophilia by activating growth factors that induce C/EBPβ-dependent HSPC proliferation and emergency granulopoiesis. Neutropenia, on the other hand, will not elicit increases in HSPC proliferation in the absence of inflammatory signals and C/EBPβ activation. However, my studies clearly show that C/EBPβ is dispensable for the proliferative responses of HSPC to either alum-induced inflammation or antibody-mediated neutropenia (Figure 18). Rather, C/EBPβ promotes the differentiation of mature neutrophils from immature neutrophils during the regeneration of the BM neutrophil reserve after Gr-1 induced neutropenia. A defect in terminal differentiation likely explains the lack of neutrophilia in C/EBPβ−/− mice during fungal infection (Hirai et al., 2006). These observations challenge the hypothesis that C/EBPα and C/EBPβ operate in GMP to regulate distinct pathways of neutrophil production. Instead, they indicate that C/EBPα and C/EBPβ function at distinct stages of neutrophil differentiation, with C/EBPα acting early in neutrophil development (Zhang et al., 2004) and C/EBPβ operating during terminal stages of maturation. This conclusion is consistent with analyses of C/EBPα and C/EBPβ transcription at different stages of neutrophil development (Bjerregaard et al., 2003). If C/EBPβ is dispensable for an alternative pathway of neutrophil differentiation, then another factor must mediate the cytokine-induced rescue of granulopoiesis in C/EBPα−/− mice (Hirai et al., 2006).
Naïve C/EBPβ−/− mice have normal numbers of mature neutrophils, but exhibit a defect in the ability to produce mature neutrophils from immature neutrophils following Gr-1 administration (Figure 18A). This observation suggests that terminal differentiation is different in the steady-state and during granulopoietic recovery from neutrophil deficits. Indeed, G-CSF has been shown to reduce the transit time of developing neutrophils through post-mitotic pools (Price et al., 1996). Therefore, C/EBPβ might play a more prominent role in situations where cytokines hasten the transition from immature to mature neutrophil.

The observation that feedback signals operate on HSC may explain, to some degree, the expanded HSC compartments in neutropenic C/EBPα−/− mice (Zhang et al., 2004). According to my feedback model (Figure 19), the absence of mature neutrophils in C/EBPα−/− mice will be coupled with the constitutive production of growth factors; it stands to reason that C/EBPα−/− mice should exhibit a similar cytokine profile as neutropenic Mcl-1− mice (Figure 16).

4.5 Final remarks

Whereas inflammation was previously thought to stimulate granulopoiesis as a means to cope with infection (Hirai et al., 2006; Metcalf, 1998), the results of this study indicate that the inflammatory mobilization of BM neutrophils activates a latent feedback mechanism of neutrophil replenishment. The corrective actions of feedback are focused on sustaining the BM neutrophil pool. The importance of maintaining neutrophil pools is evident by the extreme susceptibility of neutropenic patients to infection (Bodey et al., 1966). A robust feedback mechanism for neutrophil replenishment likely precludes or
minimizes neutropenia that would otherwise result from the consumption of neutrophil pools during an acute inflammatory response.
Figure 19: Feedback model for the regulation of granulopoiesis
References


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Biography

Derek Wilson Cain was born on June 30, 1975 in Kailua, HI and is the oldest of four children. Derek’s parents, David and Sheila Cain, an engineer and a nurse, respectively, fostered his interest in science from an early age. He studied biology at the College of William and Mary and obtained his Bachelor of Science degree in May 1997.

Prior to graduate school, Derek worked at Mote Marine Laboratory in Sarasota, FL as an eco-tour guide. Here, he met and fell for his future wife, Angie. A chance meeting with a vacationing researcher from Harvard Medical School led to a technician job in Boston, MA, where Derek was introduced to the field of immunology. In 2004, Derek entered the graduate program of the Department of Immunology at Duke University.

Derek is the recipient of a Ruth L. Kirchstein award and has presented his work at the Molecular Targets for Cancer Therapy: Fifth Biennial Meeting, Immunology 2009 and 2010, and FOCIS 2009.

Derek and his wife Angie live in Durham, NC with their wonderful son, Sam.

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* contributed equally