MECHANISM OF NEUTROPHIL HOMEOSTASIS AND IMMUNITY IN PNEUMONIA AND SEPSIS

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MECHANISM OF NEUTROPHIL HOMEOSTASIS AND IMMUNITY IN PNEUMONIA AND SEPSIS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Pathobiological Sciences

by

Sagar Paudel
BVSc. A.H. Tribhuvan University, Nepal 2010
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Dedicated to my wife Shanta Acharya and son Arnav

Their love, motivation and support are responsible for every achievement of my life
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ABSTRACT

Severe bacterial pneumonia and septicemia are pressing health problems. A better understanding of cellular and molecular players of neutrophil immunity and homeostasis in bacterial pneumonia and sepsis is critical for inspiring novel therapeutics. Chemokine CXCL1 is a widely secreted neutrophil attractant and Nod-like receptor (NLRC4) is commonly expressed cytoplasmic pathogen sensor in hematopoietic compartments. How these innate mediators convert pathogen signals into molecular cues of immune response in context of Gram-positive bacterial pneumonia and septicemia largely remain unknown. Utilizing Cxcl1 gene deficient mice, we demonstrate CXCL1 regulates neutrophil influx, bacterial clearance, and host survival in pneumococcal pneumonia-derived sepsis. Furthermore, Cxcl1−/− mice displayed a defect in neutrophil generation in bone marrow and subsequent CD62L and CD49d-dependent release into bloodstream following pneumococcal infections. In stark contrast to CXCL1, NLRC4 exhibits a detrimental role in Methicillin-resistant Staphylococcus aureus (MRSA)-induced pneumonia and polymicrobial sepsis. Nlrc4−/− mice had improved neutrophil recruitment, bacterial clearance, and survival advantage over wild-type mice following MRSA-pneumonia. Mechanistically, the activation of NLRC4 contributes to dampening of IL-17A/neutrophil axis through necroptosis and IL-18 signaling. In polymicrobial sepsis, NLRC4 contributes to sepsis-induced mortality through mediating hyper inflammation, cytokine storm, and cellular dysfunction. Nlrc4−/− mice were protected from sepsis-induced loss and dysfunction of macrophage and lymphocytes. Our results show that CXCL1 and NLRC4 have divergent roles during infections and the modulation of their activity may represent attractive therapeutic targets in treating bacterial pneumonia and sepsis.
CHAPTER 1
INTRODUCTION

1.1. Introduction

Ever since Metchnikoff first observed phagocytosis of bacteria by white blood cells in 1882, the scientific community has been fascinated by polymorphonuclear leukocytes (PMNs), also called neutrophils. Neutrophils are critical players in the innate immune system important for protecting the host from bacterial, viral, and fungal infections. Patients with neutropenia due to clinical disorders, such as severe congenital neutropenia, leukocyte adhesive deficiency and chronic granulomatous diseases, suffer from numerous life-threatening infections. Paradoxically, uncontrolled neutrophil activation may lead to extensive collateral inflammatory tissue damage.

Compared to other cell types PMNs are short-lived and therefore, the human body generates about 0.5-1 x 10^{11} neutrophils per day in the bone marrow (BM) to maintain steady state circulating pools. Neutrophils originate from the dormant, but self-renewing, hematopoietic stem cells (HSCs), which reside in a specialized BM niche and represent the top most cells in the hierarchy of the hematopoietic system. Long-term HSCs initiate the process by differentiating into short-term HSCs (ST-HSCs), which then give rise to multi-potent, but non-renewing, differentiated cells, multi-potent progenitors (MPPs). MPPs can eventually differentiate to oligopotent common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs advance as either granulocyte monocyte progenitors (GMPs), giving rise to neutrophils and macrophages, or megakaryocyte/erythrocyte progenitors (MEPs), giving rise to platelets and erythrocytes. The niche and lineage specifications of HSCs in health and disease has been the subject of several recently published reviews. In addition, a comprehensive review of
neutrophil ontogeny and biology with special emphasis on the differences between neonatal and adult neutrophil populations was recently published by Lawrence et al\textsuperscript{8}.

**Figure 1.1.** Neutrophil granulopoiesis is a hematopoietic hierarchical program. Granulopoiesis, the process of neutrophil generation and maturation, is characterized by sequential development of distinct granules, surface proteins, and nuclear segmentation. Homeostatic granulopoiesis starts with generation of myeloblasts and promyelocytes (rich in primary azurophilic granules), which depends on expression of GATA-1, C/EBP-\(\alpha\), Gfi-1, and c-Myc genes. The development of secondary, specific granules appears in myelocytes (last proliferative stage). C-myc and C/EBP-\(\epsilon\) contribute to formation of secondary granules. The development of metamyelocytes with gelatinase granules marks terminal differentiation of neutrophil granulopoiesis. As expression of C/EBP-\(\beta\), C/EBP-\(\delta\), and C/EBP-\(\zeta\) are upregulated in terminal neutrophil stages, gelatinase granules and secretary vesicles appear and nuclear segmentation intensifies. During emergency granulopoiesis, C/EBP-\(\beta\) takes over the process and excess amounts of neutrophils are generated and released into the bloodstream along with immature neutrophils (band cells).
During hematopoietic stress such as systemic infections or physical insults, neutrophil are used up in larger quantities and the demand for neutrophil in the peripheral blood increases by several folds higher than steady state. Hematopoietic system rapidly senses this neutrophil demand and integrates these pathogen signals to activate dormant HSC, proliferate and differentiate to neutrophil progenitors. This distinct program of accelerated de novo production of neutrophils from amplification of neutrophil progenitors is known as ‘emergency granulopoiesis’, which is usually a protective hematopoietic immune response to fatal infection. This process of differentiation and then amplification of multipotent HSCs into a mature, segmented neutrophil is highly sophisticated and governed by a complex network of molecular, cellular players, and even host-microbiome. Clinical manifestations of patients with heightened emergency granulopoiesis are elevated levels of acute phase proteins, leukocytosis, neutrophilia, and appearance of immature myeloid precursor cells (left shift).

This review focuses on the cellular and molecular players involved in activation of emergency granulopoiesis, including the molecular messengers that integrate pathogen signals into HSC activation, proliferation and differentiation into neutrophils, as well as the regulatory molecular and environmental mechanisms that control emergency granulopoiesis in the context of infectious disease.

1.2. Granulopoiesis: Steady State Versus Emergency

Because neutrophils are mitotically inactive and relatively short-lived, they are constantly replenished in the circulation under physiological conditions through a process termed steady state granulopoiesis. In general, localized mild-grade bacterial infections don’t invoke strong hematopoietic pressure, as the host rapidly resolves these infections
by limiting their dissemination to systemic organs. Consequently, HSCs are not activated and BM granulopoiesis remains unaltered. By contrast, overwhelming systemic infections put much stronger stress on the hematopoietic system, as large quantities of neutrophils are lost in an unsuccessful attempt to limit dissemination. To compensate for depleting neutrophil numbers, the BM hematopoietic system rapidly switches from steady state granulopoiesis to a well-coordinated process of large-scale neutrophil generation that involves activation and proliferation of myeloid progenitors and is known as emergency granulopoiesis. Several elegant studies have suggested the BM niche can be vacated to provide space for the heightened kinetics and magnitude of emergency granulopoiesis at the expense of erythropoiesis and lymphopoiesis. In fact, inflammatory signals have been shown to reduce the expression of lymphoid expansion and retention signals \(^9\)\(^-\)\(^11\). Besides systemic infections, iatrogenic conditions, such as irradiation or chemotherapy-induced myeloablation, result in profound neutropenic conditions\(^12,\)\(^13\) and can also trigger emergency granulopoiesis. Despite the differences in stimuli, granulopoiesis initiated under both conditions is comprised of many of the same molecular and cellular events. Further, accumulating evidence shows infection-induced emergency granulopoiesis may have a major evolutionary impact on the hematopoietic system’s response to stress. This notion of evolution of the hematopoietic system in response to stress became clearer as HSCs were shown to circulate, sense, and respond to pathogens\(^14\)\(^-\)\(^16\). With the discovery of CCAAT/enhancer-binding protein (C/EBP) in the regulation of granulopoiesis\(^9,\)\(^17,\)\(^18\), we have a better understanding that emergency granulopoiesis is differentially regulated at the transcriptional level compared to that occurring at steady state.
1.3. Pathogen Sensing System in Emergency Granulopoiesis

The hematopoietic system usually responds to infection by enhancing cellular output; however, how systemic infections are sensed and this information is messaged to the BM is not understood. Given that the granulopoietic BM niche is spatially disconnected from the site of infection, it was long believed that activation of emergency granulopoiesis is an indirect process relying on pathogen sensing by germ-line encoded pathogen recognition receptors (PRRs) expressed on immune and resident cells. It is well established that toll-like receptor (TLR) expressing cells are the first to detect pathogens or their products. While these cells don’t generate neutrophils themselves, they do secrete hematopoietic cytokines, such as G-CSF and IL-6, which indirectly influence differentiation and proliferation of neutrophil precursors into mature forms. However, recent reports show that HSPCs can also express PRRs, multiple trafficking molecules, and egress and re-enter the BM niche, thereby suggesting their role in immediate and direct activation of emergency granulopoiesis. In the following section, we will delve deeper into how the hematopoietic system has evolved to sense pathogens and rapidly message the bone marrow niche in order to induce emergency granulopoiesis.

1.3.1. Toll-Like Receptors (TLR)

Immature HSCs and early progenitors express PRRs and initiate granulopoiesis directly as they can actively participate in recognition of pathogen-derived products. Nagai et al were the first to demonstrate that HSCs (Lin^-c-Kit^+ subset) express TLR2, TLR4, and their co-receptors (MD-2, CD14). In addition, they showed that GMPs (Lin^-c-Kit^+Sca-1^-CD34^-FcγR^-subset) express TLR2. Furthermore, ex vivo stimulation of HSCs with TLR2 or TLR4 agonists (Pam3CSK4 and LPS, respectively) drives MyD88-
dependent, but cytokine-independent, differentiation to myeloid lineage cells\textsuperscript{20}. Moreover, Massberg \textit{et. al} revealed that lymph-borne HSPCs originate in the BM and possess extensive multi-lineage potential even in peripheral organs when they encounter pathogens\textsuperscript{15}. Specifically, these HSPCs were shown to differentiate into myeloid cells in response to TLR agonists within the kidney when implanted under the kidney capsule\textsuperscript{15}. Human CD34\textsuperscript{+} HSPCs also express functional TLRs whose ligation induces differentiation into myeloid cells without the addition of cytokine, in some cases, at the expense of lymphopoiesis\textsuperscript{23,24,25}. Numerous additional studies have shown TLR ligation is an important step in HSC differentiation into myeloid cells\textsuperscript{11,26-28}. Functional consequences of TLR engagement were initially revealed when an elegant study demonstrated that LPS-stimulated HSPCs secrete IL-6. Further, HSPCs derived from IL-6\textsuperscript{-/-} mice fail to differentiate into myeloid cells under neutropenic conditions caused by treatment with either chemotherapy (5-fluorouracil) or irradiation\textsuperscript{29}. In addition to TLR2 and TLR4, a recent study demonstrated TLR7 signaling drives type I IFN-dependent myeloid differentiation of CMPs \textit{in vitro} and peripheral expansion of monocytes \textit{in vivo}\textsuperscript{30}. In addition, the complex and redundant nature of the \textit{in vivo} response of HSCs to pathogens has been uncovered, as MyD88/Triff\textsuperscript{-/-} or C3H/HeJ (loss of function mutation for TLR4) mice were found to exhibit normal expansion of HSPCs during \textit{S. aureus} infection\textsuperscript{31}. Collectively, these studies reveal that the hematopoietic system has evolved to detect and respond to pathogens directly through TLRs and these interactions lead to HSC differentiation, amplification of progenitors, and generation of progeny cells in the BM or at the site of invading pathogens. Nonetheless, further studies will be required to completely define the mechanisms underlying HSPC activation by pathogens.
A long-standing hypothesis is that emergency granulopoiesis is indirectly activated by both hematopoietic and resident cells through inflammatory mediators. Tissue-resident macrophages and circulating monocytes are equipped with PRRs, sense pathogens at the site of entry, and release ample amounts of granulopoietic cytokines, including G-CSF, GM-CSF and IL-6, during local or systemic infections. However, the discovery of IL-17A-producing innate lymphoid cells and their regulatory role on neutrophils indicated that monocytes and macrophages are not the sole players in this pathway. Although there is a lack of careful in vivo experimentation proving a direct role of these cells in emergency granulopoiesis, the abundance of data suggests their role is largely mediated by cytokines and growth factors. These secondary mediators activate HSC differentiation as well as regulate cell survival and generation of lineage-committed myeloid cells. In this context, experiments using antibody-mediated depletion have broadened our knowledge of HSCs; however, careful interpretation of the data is required, as observed effects be the result of positive feedback loops of granulopoiesis. For instance, anti-ly6G depleted neutropenic mice or LysM<sup>C<sub>re/wt</sub></sup> Mcl<sup>1<sup>f/f</sup></sup> mice (lacking mature neutrophils) elicit elevated levels of G-CSF under steady state conditions resulting in enhanced HPSC proliferation and GMP differentiation. Bugl et al. not only confirmed the enhanced G-CSF and HSC expansion in neutropenic mice, but also showed G-CSF-mediated feedback granulopoiesis is dependent on BM neutrophil mass. In contrast to popular beliefs, using clodronate liposomes or reciprocal TLR4<sup>−/−</sup> chimeras Boettcher et al were the first to reveal that selective TLR4 expression within the hematopoietic compartment cannot induce emergency granulopoiesis and neutrophilia following systemic LPS injection. Moreover, TLR4 signaling in non-hematopoietic
cells, but not in the hematopoietic compartment, was found to be an absolute requirement for LPS-induced, G-CSF-mediated granulopoiesis\textsuperscript{35}. In another elegant study using bone marrow chimeras with endothelial cell (EC) specific MyD88\textsuperscript{-/-} mice, Boettcher et al, demonstrated that EC-intrinsic MyD88 signaling dominantly contributes to G-CSF production and induces GMP expansion and neutrophil generation after \textit{in vivo} LPS or \textit{Escherichia coli} challenge\textsuperscript{36}. Moreover, the highest G-CSF expression was noted in BM EC after LPS challenge. Thus, it is probable that ECs in the lining of the vasculature could be important for local signaling to the BM to initiate granulopoiesis.

1.3.2. NOD-Like Receptors (NLR)

The role of intracellular NLRs in hematopoiesis has been characterized in recent studies. Burberry et al. showed that NOD1 and NOD2 cooperate with TLR4 receptors to decrease CXCL12 expression in the BM and induce G-CSF, thereby mobilizing HSCs to give rise to neutrophils and monocytes during \textit{E. coli} infection\textsuperscript{37}. Furthermore, the NLRP3 inflammasome regulates HSC mobilization to the peripheral blood through IL-1\beta and IL-18 production following administration of G-CSF and AMD3100\textsuperscript{38}. However, genetic ablation of caspase-1/11 in neonatal mice resulted into enhanced emergency myelopoiesis and elevated level of G-CSF, M-CSF thereby augmenting bacterial clearance and host survival\textsuperscript{39}. Moreover, mice deficient in NLRC4 and NLRP6 have enhanced neutrophil recruitment and elevated level of hematopoietic cytokines during pulmonary and systemic infection\textsuperscript{40-42} although whether these mice also present granulopoietic defect in BM was not explored. Therefore, future studies are needed to identify if these intracellular sensors are actually a class of negative regulators of TLR-mediated granulopoiesis.
Figure 1.2. Pathogen sensing system of emergency granulopoiesis. During infection and inflammation, the hematopoietic cells, resident cells, and circulating HSCs sense disseminating pathogens and danger signals. Pathogen-induced granulopoiesis depends heavily upon TLR-signaling, which translates pathogen signals into molecular cues of granulopoiesis. Engagement of TLRs on these cells results into the secretion of several granulopoietic growth factors (such as G-CSF, IL-6, CXCL1), which initiate signaling cascades on HSCs and neutrophil progenitors, thereby activating emergency granulopoiesis. Expression of TLRs by HSCs and endothelial cells facilitates more direct initiation of granulopoiesis compared to the indirect activation of immune cells at the site of infection/inflammation.

1.4. Molecular Translation of Pathogen Signals into Granulopoiesis

The hematopoietic system must translate pathogen sensing into the process of emergency granulopoiesis. Although a few studies suggest HSPCs can respond to direct pathogen sensing, a vast number of studies have unequivocally demonstrated that a coordinated action of myeloid growth factors, cytokines, chemokines, and transcription
factors mediate emergency granulopoiesis. Moreover, recent evidence revealed these mediators can instruct lineage choice for uncommitted HSPCs, rather than just enhancing proliferation and survival of precursor. In this section, we dissect how these myeloid growth factors mediate HSPC differentiation, lineage choices, proliferation, and survival of myeloid progenitors during emergency granulopoiesis.

1.4.1. Colony Stimulating Factors

In the context of granulopoiesis, myeloid growth factors such as G-CSF (and its receptor G-CSFR), GM-CSF, M-CSF are the best-studied colony stimulating factors. Hematopoietic cells, including HSPCs, are known to express receptors for these cytokines\(^\text{11}\). Moreover, \textit{in vivo} daily administration of G-CSF or GM-CSF along with stem cell factor results in neutrophilia and synergistic GMP expansion in bone marrow\(^\text{43}\), while GM-CSF stimulates the expansion of myeloid lineage cells both \textit{in vivo} and \textit{in vitro}\(^\text{44, 45}\). Daily \textit{in vivo} administration of M-CSF expands the number of blood monocytes and resident macrophages in spleen and liver\(^\text{46}\), and mice deficient in G-CSF (\textit{Gcsf}\(^{-/-}\)) and G-CSFR (\textit{Gcsfr}\(^{-/-}\)) display diminished levels of peripheral neutrophils and a corresponding decrease in neutrophil progenitors in BM during steady state\(^\text{47, 48}\). Further, exogenous administration of G-CSF into uninfected \textit{Gcsf}\(^{-/-}\) mice augments the level of circulating neutrophils\(^\text{47}\). However, the role of GM-CSF at baseline appears to be redundant as mice deficient in GM-CSF (\textit{Gmcsf}\(^{-/-}\)) show no major defect in steady state granulopoiesis and maintain normal pools of myeloid cells and their precursors in blood, marrow, and spleen\(^\text{49}\). However, the usage of these mice in infection-induced granulopoiesis has yielded some discrepant results. Earlier reports demonstrated that both \textit{Gcsf}\(^{-/-}\) and \textit{Gmcsf}\(^{-/-}\) mice have impaired emergency granulopoiesis and host defense
against *Listeria monocytogenes* infection. In contrast, emergency granulopoiesis in *Gcsf*<sup>−/−</sup> mice was not found to be dampened and was indistinguishable from that of control mice when challenged with *Candida albicans*. In addition, *Gcsf*<sup>−/−</sup> mice have strikingly high levels of IL-6, while mice deficient in both G-CSF and IL-6 also develop neutrophilia following BM expansion of GMPs. Furthermore, mice deficient in GM-CSF (*Gmcsf*<sup>−/−</sup>) have reduced circulating pools of hematopoietic cells; however, surprisingly, display only subtle changes in BM precursors during *Mycobacterium avium* infection. Additionally, mice deficient in all three colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) have reduced circulating myeloid cells at resting state, although unexpectedly, they are able to mount emergency granulopoiesis during thioglycollate-induced peritonitis.

Accumulating evidence shows intriguing functional consequences of stimulation of cellular targets by these cytokines. Mechanistically, while G-CSF acts as proliferative signal for HSCs it also impairs their renewal ability, which is dependent on TLR signaling and the host microbiome. Additionally, G-CSF disrupts the HSC niche resulting in their mobilization, suggesting G-CSF induced defects in repopulation could in fact be due to increased HSC mobilization from the BM. Using long-term bio-imaging at the single-cell level, Riegar *et al.* demonstrated that bipotent GMPs respond with generation of exclusively mature neutrophilic granulocytic or monocytic cells in response to G-CSF and GM-CSF, respectively. More insight into the instructive actions of cytokines were gained when Mossadegh-Keller *et al.* utilized time-lapse imaging and single cell gene expression analysis to reveal that M-CSF directly stimulates HSCs to activate the PU.1 transcription factor, which promotes their differentiation into myeloid...
cells. It will be of great interest to delineate which of the many intracellular signaling pathways activated by these cell-extrinsic cytokines stimulate the lineage commitment machinery.

1.4.2. CXC and CC-Chemokines

Although conventional wisdom is that chemokines and their receptors recruit leukocytes, recent reports have revealed that chemokine systems are also involved in the differentiation, proliferation, and mobilization of hematopoietic progenitors. CXCL12 (or SDF1) is produced in copious amounts by various cell types in the BM, and together with its receptor, CXCR4, is a representative homeostatic chemokine system primarily regulating HSC retention. Mice deficient in CXCL12 or CXCR4 display a virtual absence of myelopoiesis in the BM, a reduced number of myeloid cells in fetal liver, and impaired B-lymphopoiesis. Recently, CXCL12 has been shown to regulate mitochondrial respiration in early HSCs, which is critical for maintaining their undifferentiated state. CCL3, a CC-chemokine long implicated in hematologic malignancies, has been shown to enhance the myelopoietic activity of mature progenitors and to suppress the myelopoietic activity of more immature progenitors. Using CCR1 deficient mice, CCL3 signaling through CCR1 was shown to play a dominant role in the proliferation, but not in the suppression, of lineage-committed myeloid progenitors. In contrast, mice lacking CCL3 (Ccl3−/−) were found to have profound increases in HSPCs and immature myeloid progenitors, but reduced numbers of committed myeloid progenitors without alteration of populations in the BM. In addition to CXCL12/CXCR4 and CCL3/CCR1, CCR2 and its ligands (CCL2/12/13) have long been known to have myelosuppressive activity. Using RFP-CCR2 reporter mice, marked
expression of CCR2 was observed in neutrophils\textsuperscript{70}. Additionally, mice lacking CCR2 display increased sequestration of all subsets of myeloid cells in BM and corresponding reduced numbers of these cells in blood and spleen\textsuperscript{70}. Recently, another CC-chemokine, CCL5/Rantes, has been demonstrated to influence BM cell subtypes and lineage skewing phenotypes\textsuperscript{71}. Indeed, mice lacking CCL5 (Ccl5\textsuperscript{−/−}) displayed a reduced level of myeloid-biased HSCs and myeloid progenitors with a concomitant increase in lymphoid-biased HSCs and T cell progeny\textsuperscript{71}. However, retroviral expression of CCL5 in BM progenitors or a brief \textit{ex vivo} stimulation of HSCs with CCL5 resulted in T cell deficiency and expansion of myeloid progenitors\textsuperscript{71}.

Among CXC-chemokines, CXCL1 and CXCL2 are also known to suppress the \textit{ex vivo} proliferation of myeloid progenitors through activation of CXCR2\textsuperscript{72, 73}. The myelosuppressive activity of CXCR2 signaling was confirmed in mice lacking CXCR2 (Cxcr2\textsuperscript{−/−}), which have enhanced expansion of myeloid progenitors in BM and neutrophilia in peripheral blood\textsuperscript{73-75}. Although Cxcr2\textsuperscript{−/−} stem/progenitor cells showed initial expansion capacity \textit{in vivo}, their self-renewal capacity was found to be exhausted in a serial transplantation assay\textsuperscript{75}. However, Cxcr2\textsuperscript{−/−} mice displayed comparable numbers and cytokine responsiveness of myeloid progenitors in BM, although there was abnormal retention of mature neutrophils in these mice\textsuperscript{76}. In another elegant study, Mei, J. et al.\textsuperscript{77} showed that both Cxcr2\textsuperscript{−/−} and Cxcl5\textsuperscript{−/−} mice exhibit IL-17A/G-CSF and commensal bacteria dependent-neutrophil hyperplasia in BM and mild neutrophilia in peripheral blood during steady state. Using chimeric mice, the authors delineated that the loss of CXCR2 in the hematopoietic compartment contributes to enhanced granulopoiesis while enterocytes and BM resident cells supply CXCL5 under basal conditions\textsuperscript{77}. 
Recently, we examined the role of the CXC chemokine in the context of emergency granulopoiesis. Contrastingly, we showed that mice deficient in CXCL1 (Cxl1\(^{-/-}\)) display reduced blood neutrophilia and recruitment to lungs with a corresponding defect in generation of neutrophils in BM\(^{78}\). Using multi-parametric flow cytometry, Cxl1\(^{-/-}\) mice were found to have reduced numbers of hematopoietic stem cells and granulocyte-monocyte progenitors, which was corrected by exogenous administration of other CXCL2 ligands such as CXCL2 and CXCL5 \(^{78}\).

1.4.3. Interleukins

IL-6 is among the most well studied of the interleukins in the context of hematopoiesis. Although mice deficient in IL-6 (Il-6\(^{-/-}\)) have no apparent deficit in neutrophil numbers at the basal state\(^{79}\), these mice displayed reduced neutrophilia and neutrophil responses during *Listeria* and *Candida* infections\(^{80, 81}\). Enhanced granulopoiesis in Gcsf\(^{-/-}\) and Gmcsf\(^{-/-}\) mice is associated with elevated IL-6 expression\(^{51}\), and the usage of mice with a triple deficiency in IL-6, G-CSF, and GM-CSF implicated IL-6 as a rescue cytokine that can take over granulopoiesis in the absence of the classical granulopoietic cytokines, G-CSF and GM-CSF\(^{82}\). Since these triple knockout mice die shortly after birth, an *in vitro* granulopoiesis assay using embryonic fibroblasts was used to identify the additive effect of IL-6\(^{82}\). To this end, the neutrophil promoting activity of medium conditioned by Gcsf\(^{-/-}\)/Gmcsf\(^{-/-}\)/Il-6\(^{-/-}\) fibroblasts was reduced by 40% compared to that of Gcsf\(^{-/-}\)/Gmcsf\(^{-/-}\) fibroblasts\(^{82}\). Recently, Zhao *et al.*\(^{29}\) and Schurch *et al.*\(^{83}\) demonstrated that HSPCs and MSCs produce IL-6 in response to inflammatory signals that stimulate HSC mobilization and myeloid differentiation during neutropenia or to promote clearance of infections. In addition to HSPCs and MSCs, IL-6 is markedly
expressed in response to LPS stimulation of CXCL12-abundant reticular cells residing in the BM. Thus, this suggests IL-6 is a critical mediator of HSPC and non-hematopoietic cell initiated granulopoiesis.

Discrepancies exist regarding the role of IL-3 in steady state hematopoiesis. Innate response activator (IRA) B cells are the main producers of IL-3 during polymicrobial sepsis and are essential for emergency hematopoiesis, which dampens host survival benefit. In contrast, the specific depletion of IRA B cells in sepsis is detrimental as it leads to loss of GM-CSF signaling, increase in bacterial burden, and elicitation of a cytokine storm. Unlike IL-6 and IL-3, the involvement of the IL-1 family and IL-1R in hematopoiesis have not been explored extensively until recently. Mice deficient in IL-1R (Il1r−/−) have reduced proliferation of HSCs, MPPs, and GMPs during alum-induced inflammatory granulopoiesis. Furthermore, Pietras et al. demonstrated that acute exposure to IL-1 enhances HSC proliferation and differentiation along the myeloid lineage through activation of transcription factor PU.1. In contrast, chronic exposure to IL-1 impairs HSC function and self-renewal capacity. IL-27, an IL-6/IL-12 family cytokine, was recently shown to specifically in work in synergy with stem cell factor to promote the expansion and differentiation of LSK cells, particularly MPPs, into myeloid lineages. Similarly, IL-27 Tg mice display enhanced myelopoiesis and impaired development of B cell lineages. These results suggest that IL-27 is one of a limited number of cytokines that play a role in HSC regulation.

1.4.4. Interferons

Type I (IFNα/β) and type II (IFNγ) IFNs are pro-inflammatory cytokines that regulate early hematopoiesis. Early reports using models of virus-induced transient
pancytopenia revealed that mice lacking $\alpha/\beta$ (IFN-$\alpha/\beta R^{-/-}$) or $\gamma$ (IFN-$\gamma R^{-/-}$) receptors have different degrees of BM cellularity$^{91}$. Indeed, during virus-induced pancytopenia, BM cells (specifically pluripotent and committed progenitors) are lost in substantial numbers from WT and IFN-$\gamma R^{-/-}$ mice, but not from IFN-$\alpha/\beta R^{-/-}$ mice$^{91}$. Later, two elegant reports conducted in different models confirmed that IFN-$\alpha$ stimulates the exit from the BM and proliferation of HSCs$^{92,93}$. Essers et al.$^{92}$ reported that in vivo treatment with IFN-$\alpha$ enhances HSC proliferation in mice while HSCs deficient in IFN-$\alpha/\beta R$ are unresponsive to this treatment. Similarly, Sato et al.$^{93}$ discovered mice deficient in interferon response factor 2 ($Irf2^{-/-}$), a transcriptional suppressor of IFN-$\alpha$ signaling, have an enhanced proportion of proliferating HSCs, while $Irf2^{-/-}$ HSCs exhibit accelerated cell division and fail to generate hematopoietic cells. In contrast to that of type I IFNs, the role of IFN$\gamma$ in HSC proliferation remains controversial. IFN$\gamma$ is sufficient to induce HSC proliferation in vivo and HSCs from $Ifng^{-/-}$ mice have a low proliferative rate$^{94}$. In stark contrast, IFN$\gamma$ has been shown to impair HSC proliferation and restoration during viral infection through induction of SOCS1 expression, which results in suppression of STAT5 phosphorylation$^{95}$. Moreover, the anti-proliferative activity of IFN$\gamma$ was shown to be dependent on integrin $\beta3$ signaling as it promotes serine phosphorylation of STAT1 in HSCs$^{96}$. Additionally, de Bruin et al. demonstrated that IFN$\gamma$ directs GMP differentiation into monocytes over neutrophils during viral infection as IFN$\gamma$ enhances the expression of PU.1 and IRF8 while dampening STAT3 phosphorylation$^{97}$.

1.4.5. Reactive Oxygen Species

The presence of ROS in HSCs has been shown to regulate the self-renewal, migration, and development of these cells$^{98-102}$. While ROS-low HSCs are long-term
repopulating and low cycling, ROS-high HSCs are short-term repopulating, cycling and differentiating\textsuperscript{99,100}. Recently, cell extrinsic effects of ROS on hematopoiesis have been explored in thioglycollate and infection-elicited granulopoiesis\textsuperscript{103,104}. BM myeloid (GR-1\textsuperscript{+}) cells produce NADPH oxidase-dependent ROS, which regulates the expansion of GMPs in a PTEN oxidation-dependent manner during \textit{E.coli}-induced emergency granulopoiesis\textsuperscript{104}. Another study by same group demonstrated that ROS-producing BM GR-1\textsuperscript{+} cells are also critical for neutrophil homeostasis in sterile inflammation-induced granulopoiesis\textsuperscript{103}. However, the contribution of pathogen sensing to ROS production by the BM GR-1\textsuperscript{+} niche and its ability to induce cell-specific proliferation of GMPs, and not MEPs or CEPs, still requires further investigation.

1.5. Regulation of Emergency Granulopoiesis

1.5.1. CCAAT/enhancer-binding proteins (C/EBPs)

Numerous studies have shown that the transcription factors CCAAT/enhancer-binding proteins (C/EBPs) -\(\alpha\), -\(\beta\), and -\(\epsilon\) have a major regulatory role in neutrophil granulopoiesis\textsuperscript{8,105,106}. C/EBP-\(\alpha\), a prototypical basic-region leucine zipper (bZIP) transcription factor, is predominantly expressed in myeloid cells within the hematopoietic system and is known to regulate steady state granulopoiesis\textsuperscript{17,18}. Mice deficient in C/EBP-\(\alpha\) (\(C/EBP-\alpha^{-/-}\)) display impaired steady state granulopoiesis, as they selectively lack mature granulocytes but retain monocytes\textsuperscript{18}. Furthermore, these mice are unresponsive to exogenous IL-6 and G-CSF since expression of IL-6 and G-CSF receptors is compromised with loss of C/EBP-\(\alpha\)\textsuperscript{18,107}. \(C/EBP-\alpha^{-/-}\) fetal liver progenitors display a high plating capacity for HSPCs; however, these cells also exhibit a striking block in differentiation of MPPP into bipotent GMPs both \textit{in vivo} and \textit{in vitro}\textsuperscript{108}.
Mechanistically, C/EBP-α negatively regulates expression of c-Myc, a cell proliferative factor, through the E2F binding site, thereby altering the differentiation of myoblasts into CMPs. Moreover, C/EBP-α directly causes proliferation arrest by inhibiting cyclin-dependent kinase 2 (Cdk2) and Cdk4. C/EBP-α also decides cell fate as it directs the myeloid gene expression program through interactions with factors involved in the expression of Il-6r, Csf3r, Gfi-1, Irf-8, and Klf5. For instance, expression of C/EBPα in HSCs or in vivo activation of C/EBPα in mice results in an increase in myeloid progenitors and granulocytes along with a concomitant decrease in erythroid and/or non-myeloid progenitors. Further, the conditional silencing of C/EBPα in HSPCs leads to loss of expression of myeloid genes and aberrant expression of T cell-related genes such as Cd7 and Lck.

C/EBP-ε regulates terminal differentiation of neutrophil development. Although C/EBP-ε−/− mice have no defects in generation of any other blood cell lineages, they fail to develop functional neutrophils. C/EBP-β is upregulated in hematopoietic subpopulations under infectious or inflammatory states and orchestrates emergency granulopoiesis. C/EBP-β can be induced in C/EBP-α−/− cells in vitro with IL-3, GM-CSF stimulation or transduction with G-CSF to generates neutrophils, suggesting the existence of C/EBPα-independent pathways for granulocyte generation. Hirai et al were the first to show conclusively that a large number of mature granulocytes can be generated from C/EBP-α−/− progenitors following cytokine (G-CSF, GM-CSF, and IL-3) treatment. Following C. albicans or cytokine administration, the sustained upregulation of C/EBP-β, but not C/EBPs-α, -δ, or -ε, was observed in granulocyte progenitors. Further, C/EBP-β−/− progenitors exhibit impaired proliferation and differentiation during...
cytokine or fungal-induced emergency granulopoiesis\textsuperscript{9, 120}. Unlike C/EBP\textalpha, C/EBP-\textbeta mediated emergency granulopoiesis may be dependent on prolonged expression of cMyc in progenitor cells\textsuperscript{9}. However, how pathogen sensing during systemic infection leads to the switch from C/EBP\textalpha to C/EBP-\textbeta to promote emergency granulopoiesis is unknown.

1.5.2. STATs proteins

The exact mechanism of the switch from C/EBP\textalpha-dependent steady state granulopoiesis to C/EBP-\textbeta-mediated emergency granulopoiesis during infections is poorly understood. However, the heightened level of granulocytic cytokines (G-CSF, GM-CSF, IL-6) and their interactions with receptors and cellular targets suggests that several other transcription factors may act as a bridge between pathogen sensing and molecular translation during emergency granulopoiesis. For example, the engagement of G-CSF signaling induces JAK-STAT pathways that mostly involve activation of STAT3, but also STAT1 and STAT5, albeit to a lesser extent. \textit{Gcsfr} \textsuperscript{−/−} mice display impaired expression of STAT3 and STAT5 and a point defect in G-CSF induces granulocytic differentiation \textit{in vivo} and \textit{in vitro}\textsuperscript{122}. Furthermore, STAT3 directly regulates expression of C/EBP-\textbeta in Gr1\textsuperscript{+} granulocytes in \textit{L. monocytogenes}-induced granulopoiesis, and STAT3 favors an increase in occupancy of C/EBP-\textbeta over C/EBP-\textalpha by the cMyc promoter during G-CSF-induced granulopoiesis\textsuperscript{123}. However, mice harboring deletion of STAT3 in hematopoietic progenitors exhibited enhanced output of granulocyte precursors and functional neutrophils in G-CSF-induced granulopoiesis, providing direct evidence of a STAT3-dependent negative feedback loop\textsuperscript{124}. Mechanistically, STAT3 deficiency results in impaired induction of suppressor of cytokine signaling (SOCS3), which is a negative regulator of JAK catalytic activity during G-CSF-induced
granulopoiesis. Similarly, Socs3−/− neutrophilic granulocytes display enhanced STAT3 activity in vitro and Socs3−/− mice develop neutrophilia when treated with G-CSF. The GM-CSF receptor pathways also participate in emergency granulopoiesis mediated through STAT5A and STAT5B since Stat5a/b−/− mice exhibit defective GMP generation and neutrophil survival during GM-CSF-induced granulopoiesis and 5FU-mediated myelosuppression.

1.5.3. Other myeloid transcription factors

Besides C/EBPs and STATs, the Ets transcription factors, PU.1, Runt-related transcription factor 1 (Runx1), zinc finger transcription factor (Gfi-1), lymphoid enhancer-binding factor 1 (LEF1), interferon regulatory factor 8 (IRF8) and homeobox gene-A10 (HOXA10), are also important for regulation of granulopoiesis. PU.1 is critical for development of all hematopoietic lineages and the expression level of PU.1 determines lineage choice. A low concentration of PU.1 allows C/EBP-α to induce myoblasts toward granulocyte generation over monocyte generation during steady state granulopoiesis. One study reported that Runx1−/− mice exhibit a mild myeloproliferative phenotype with enhanced neutrophilia and expanded myeloid progenitors, while other studies demonstrated that deletion or dominant inhibition of Runx1 reduces C/EBP-α and favors monopoiesis over granulopoiesis. Gfi1 antagonizes PU.1 and is essential for steady state and emergency granulopoiesis. Consequently, Gfi1−/− mice are highly susceptible to bacterial infections. LEF1 directly controls C/EBPα expression and mediates generation and differentiation of neutrophils, while IRF8 influences the expression of Fanconi DNA repair proteins (FNCC and FNCF), which are critical for genomic stability during S phase.
mice display decreased FAS sensitivity in myeloid progenitors and sustained granulocyte generation during alum-induced emergency granulopoiesis\textsuperscript{137}. Mice deficient in Fanconi-C are neutropenic and exhibit impaired HSC survival and function in alum-induced granulopoiesis\textsuperscript{135}. Like SOCS3 and IRF8, HOXA10 is negative regulator of emergency granulopoiesis as \textit{Hoxa10}\textsuperscript{−/−} mice develop uncontrolled neutrophilia associated with heightened activity of Triad1, an anti-proliferative E3 ubiquitin ligase\textsuperscript{138, 139}. More recently, secretary leukocyte protease inhibitor (SLPI), a natural repressor of neutrophil elastase, has been shown to regulate the expression of several transcription factors in G-CSF-triggered emergency granulopoiesis and is severely reduced in congenital neutropenic patients\textsuperscript{140}. Further, by transducing SLPI-shRNA into human BM CD34\textsuperscript{+} progenitors and leukemic NB4 cells, Klimenkova \textit{et al} demonstrated that SLPI regulates G-CSF-induced granulopoiesis through regulation of NFκB, ERK1/2:LEF-1, and c-myc activation\textsuperscript{140}.

1.5.4. Microbiome

Crosstalk between the host microbiome and the immunity system is well known to occur. In addition, a large body of evidence suggests that gut microbiota can directly regulate neutrophil homeostasis and contribute to priming host immune responses. Antibiotic-treated and germ-free mice exhibit reduced numbers of granulocyte progenitors in BM and spleen, and a decline in blood neutrophils\textsuperscript{77, 141-145}. Mice replenished and enriched with gut microbiota exhibit restored granulopoiesis\textsuperscript{141, 146, 147}. Microbiota-derived products such as peptidoglycan or lipoprotein can make their way into the BM niche, thereby allowing HSPCs and myeloid precursors to detect them and initiate hematopoiesis directly\textsuperscript{144, 147-149}. Another possibility is that HSPCs can exit the BM,
patrol peripheral organs or even reside within them for a few days, and may encounter
gut microbiota-derived pathogens/products before re-entering the BM\textsuperscript{14, 15}. On the other
hand, gut microbial products such as LPS can activate TLR4/MyD88 pathways to induce
IL-17A and G-CSF, thereby indirectly activating granulopoiesis\textsuperscript{14}. Granulopoiesis in GF
mice is also dependent on TLR signaling; as Myd88\textsuperscript{-/-}/Ticam\textsuperscript{-/-} GF mice fail to restore
microbiota-driven granulopoiesis following transfer of microbiota or serum from
colonized mice\textsuperscript{147}. Malnourished hosts also exhibit impaired hematopoiesis, and
supplementation with immunobiotics such as \textit{Lactobacillus rhamnosus} CRL1505 can
restore granulopoiesis and improved immune responses in these patients\textsuperscript{150}. Thus, it is
tempting to speculate that microbiota educate the hematopoietic system to respond to
peripheral demands along a continuum, and manipulation of microbiota or
immunobiotics could improve host immunity in neutropenic patients. transfered with
microbiota or serum from colonized mice\textsuperscript{147}. Malnourished host has impaired
haematopoiesis, and supplementation of immunobiotic such as \textit{Lactobacillus rhamnosus} CRL1505 in host restored the granulopoiesis and improved immune
responses\textsuperscript{150}. Thus, it is tempting to speculate that microbiota may have educated
hematopoietic system to respond to peripheral demands along a continuum, and
manipulation of microbiota or immunobiotic could improve host immunity in neutropenic
patients.
Figure 1.3. Molecular and cellular regulators of emergency and homeostatic granulopoiesis. Granulopoiesis is a process tightly regulated by several molecular and cellular players. At steady state, C/EBPα is a primary transcription factor that regulates proliferation of neutrophil progenitors by limiting the expression of cell cycle genes such as c-Myc, CDK2, and CDK4. However, during emergency granulopoiesis, C/EBPβ directly replaces C/EBPα on the MYC promoter and expedites c-Myc expression to enhance the generation of neutrophils. Robust C/EBPβ activity relies on strong activation of JAK/STAT pathways by G-CSF, which is released at substantial levels during infection/inflammation. At steady state, clearance of apoptotic neutrophils by macrophages activates LXR expression, which downregulates IL-23 secretion, thereby limiting the activity of G-CSF/IL-17A. Bone marrow stromal fibroblasts and CXCL12 abundant reticular (CAR) cells also secrete IL-6, which regulate granulopoiesis. Additionally, ROS release by BM GR+1 cells also regulates proliferation and differentiation of GMPs into neutrophil lineages. The gut microbiome provides PAMPs to activate and regulate steady state and emergency granulopoiesis though the IL-23/G-CSF/IL-17A axis.
1.5.5. Macrophages

The macrophage’s inability to clear apoptotic neutrophils regulates steady state granulopoiesis\textsuperscript{151-153}. Once phagocytized, aged neutrophils and macrophages secrete less IL-23 thereby curbing the IL-17/G-CSF axis and steady state granulopoiesis\textsuperscript{151}. Mice harboring a conditional deletion of the anti-apoptotic gene, cellular FLICE-like inhibitory protein (C-FLIP), in myeloid cells (\textit{c-FLIP}\textsuperscript{f/f}\textit{LysM-Cre}) lack macrophages specifically in the marginal zone and BM stroma\textsuperscript{152}. Moreover, \textit{c-FLIP}\textsuperscript{f/f}\textit{LysM-Cre} mice exhibit delayed clearance of circulating neutrophils and develop severe neutrophilia in a G-CSF, but not IL-17, dependent manner \textsuperscript{152}. Mechanistically, the engulfment of senescent neutrophils activates liver x receptors (LXR) in macrophages, which directly suppress the IL-23/IL-17/G-CSF granulopoietic cascade\textsuperscript{153}. Recently, mast cells were shown to enhance macrophage phagocytosis, and mice lacking mast cells (Kit\textsuperscript{W-sh}) exhibited elevated IL-17/G-SCF activity, aberrant intramedullary myelopoiesis, and peripheral blood neutrophilia\textsuperscript{154}.

1.5.6. Non-hematopoietic BM stromal cells

With recent advances in reporter mice, BM chimeras, imaging, and flow cytometry sorting techniques, the components of the non-hematopoietic cellular compartment have been mapped out and are now appreciated as critical regulators of granulopoiesis and immunity\textsuperscript{155}. BM endothelial cells express TLR4 and MyD88 at the basal state and respond with heightened G-CSF expression following LPS stimulation\textsuperscript{36}. However, TLR4 signaling confined to the non-hematopoietic compartment is sufficient to promote LPS-induced granulopoiesis\textsuperscript{35}. In particular, VCAM\textsuperscript{CD146\textsuperscript{low}} BM stromal fibroblasts produce IL-6, which contributes to expansion of GMPs at the expense of
erythropoiesis during *Toxoplasma gondii* infection\textsuperscript{156}. Similarly, BM CXCL12-abundant reticular (CAR) cells secrete IL-6 in response to IFN\(\gamma\) produced by CD8 T cells during viral infections. IL-6, in turn, favors HSPC differentiation toward myeloid lineages\textsuperscript{83}.

### 1.6. Special Notes on Neutrophil Circadian Rhythms

Circadian control of the immune system is an emerging concept for the regulation of immune homeostasis\textsuperscript{157}. A recent study in mice showed that like HSCs\textsuperscript{158} and monocytes\textsuperscript{159}, circulating neutrophils also follow daily circadian fluctuations as they age in peripheral blood and re-enter the BM for clearance\textsuperscript{160}. Mechanistically, daily aged circulating neutrophils are characterized by low levels of L-selectin (CD62L) and high levels of CXCR4, which are used to guide their way back to the BM within 12 hours\textsuperscript{160}. An elegant study utilized an organism-wide circadian screening approach to identify circadian expression of trafficking molecules (ICAM-1, VCAM-1, CD11a, CD49d, CXCR4) for neutrophils and other leukocytes responsible for regulating rhythmic emigration of leukocytes from blood\textsuperscript{161}. More specifically, chronopharmacological targeting of these migratory molecules revealed that CXCR4, L-selectin, and ICAM-1 govern the rhythmic neutrophil migration to bone marrow, spleen and liver, respectively\textsuperscript{161}. Following acute LPS exposure, blood counts of neutrophils and other leukocytes drop dramatically, although the difference in their numbers between day and night are preserved, extending the notion of rhythmic oscillation of neutrophils even under inflammatory conditions\textsuperscript{161}. Although study of the circadian control of neutrophil oscillation is still in its infancy, we can speculate that it is a critical mechanism of immune regulation that results in maintenance of neutrophil homeostasis and controls the strength of the immune response during infection and inflammation.
Figure 1.4. Circadian rhythm regulates neutrophil homeostasis and function. Neutrophils numbers oscillate in blood and tissues in a manner that coincides with daily circadian rhythms. Neutrophil numbers in the blood peak a few hours after initiation of light and then decrease thereafter. Under the influence of clock genes (such as Bma1) and granulopoietic cytokines (mainly G-CSF), CXCL12/CXCR4 regulates daily release and retention of neutrophils. Furthermore, a circadian fluctuation in the expression of CD62L and CXCR4 on the surface of neutrophils dictates their counts in the blood at steady state. Further, CD62L$^{hi}$CXCR4$^{lo}$ neutrophils enter the circulation during the light phase. After 8-12 hours, aged neutrophils (CD62L$^{lo}$CXCR4$^{hi}$) enter the marrow for clearance, which coincides with daily circadian rhythms. Additionally, oscillatory expression of pro-inflammatory and adhesive molecules on the endothelium and by neutrophils exhibit a robust circadian rhythmicity coinciding with neutrophil release and egress.
1.7. Concluding Remarks and Future Directions

It is now abundantly clear that neutrophil homeostasis during infection is a complex and tightly regulated process. Neutrophil homeostasis involves the sensing of pathogens or other stimuli by hematopoietic and non-hematopoietic cells (TLR/MyD88 pathways), the release of growth factors to activate several transcriptional machineries (G-CSFs, IL-6, CXCs) and ultimately, instructions to activate dormant HSCs and generate neutrophils in BM (C/EBP-β, STATs). Together this is all geared toward the generation of a protective hematopoietic immune response.

Accumulating evidence from preclinical models has deepened our knowledge regarding mechanisms of neutrophil homeostasis. However, conflicting data has caused authors to sometimes postulate contradictory mechanisms of neutrophil homeostasis. These discrepancies may be explained by differences in experimental models (infection or cytokine-induced), types and inoculum of pathogens (bacterial vs. fungal), route of infection, timing of analysis, endpoints used to measure granulopoiesis (BM vs. peripheral blood output, host survival vs. recruitment), and even composition of microbiota.

It is now obvious that the hematopoietic system has emerged to detect invading pathogens in a very fast and versatile way. As a result, a diverse spectrum of microbial messaging to the marrow is in place. For instance, direct pathogen sensing by HSCs and myeloid progenitors allows the hematopoietic system to rapidly respond with early granulopoiesis to control imminent infection. However, the mechanisms by which these pathways of direct activation differ from indirect activation of HSPCs, in terms of conversion of pathogen signals to molecular cues during granulopoiesis, are largely
unknown. It is unclear if HSCs with TLR agonists or PAMPs can be used clinically. Future studies also should investigate the relative contributions and differences of direct versus indirect HSPC activation. The discovery that ECs regulate the emergency granulopoietic response suggests that ECs in the vascular lining are a critical component of the hematopoietic system that convert signals from pathogens spreading systemically into molecular cues. In addition, diverse types of bone marrow stromal cells, macrophages, and aged neutrophils appear to control granulopoiesis. Future studies are also needed to define the crosstalk between hematopoietic and resident cells during granulopoiesis. Specifically, to determine what the contributions of each are and if each cell population initiates distinct signaling mechanisms. An emerging challenge is to solve how diverse pathogen signals picked up by different cells are translated to signals that stimulate HSCs to launch emergency granulopoiesis.

Growth factors, cytokines, and chemokines activate HSCs, instruct lineage choice, and regulate generation of neutrophils, thereby making them excellent molecular targets for therapeutics. G-CSF and GM-CSF are already in use for clinical applications to regenerate myeloid cells. However, decades of preclinical studies have demonstrated that these signals are extremely redundant and greatly differ between the steady state and emergency state. For example, IL-6 can act as a surrogate for the function of all three colony-stimulating factors during emergency granulopoiesis. Generation of experimental models with gain- or loss-of-function of these molecules are warranted to further understand their specific roles. Moreover, defining common cellular targets of these molecular messengers in order to improve therapeutic efficiency would be of great interest.
In addition, there is limited knowledge of the key cellular, molecular, and environmental players that regulate emergency granulopoiesis. Several outstanding questions remain unanswered including: 1) What are the mechanisms in place to control overexuberant granulopoietic responses? 2) Could dysregulation of granulopoietic responses or direct HSC activation contribute to BM failure and development of hematologic diseases?, 3) How do we translate the insights gained during preclinical studies into human infectious and hematologic diseases?, and 4) How do daily oscillations in neutrophil numbers determine the strength of the immune response under infectious conditions? Both systemic biology and single cell approaches may be needed to understand how the hematopoietic system integrates molecular signals into a single and unique granulopoietic response. In addition, the evolving role of the host microbiome in shaping and priming hematopoietic responses is also a promising area to explore.

A better understanding of the sensors, mediators, and regulators of emergency granulopoiesis may inspire the development of novel therapeutic approaches for neonatal, pediatric, and adult infectious diseases. Furthermore, operational pathways used during emergency granulopoiesis could be examined and exploited for development of therapeutic targets for treatment of hematologic and malignant diseases.

1.8. References


CHAPTER 2
CXCL1 REGULATES NEUTROPHIL HOMEOSTASIS IN PNEUMONIA-DERIVED SEPSIS CAUSED BY STREPTOCOCCUS PNEUMONIAE SEROTYPE 3

2.1. Introduction

The accumulation of neutrophils is crucial for the innate immune response against a broad array of infectious agents\textsuperscript{1,2}. While neutrophils are critical for clearance of pathogens, excessive recruitment can induce substantial tissue/organ damage\textsuperscript{2,3}. Therefore, neutrophil homeostasis is critical and two separate programs for generation of neutrophils occur in the bone marrow. The maintenance of a stable basal level of mature neutrophils (granulocytes) in bone marrow occurs through \textit{steady-state granulopoiesis}, while large numbers of neutrophils are generated through \textit{emergency granulopoiesis} following infection or injury of the host\textsuperscript{4}. These two programs of granulopoiesis are differentially regulated at the transcriptional level\textsuperscript{4,5}. Mature neutrophils from bone marrow are rapidly mobilized into the blood in order to migrate to infected tissues. Neutrophil recruitment to tissues is a complex, multistep process tightly regulated by cytokines/chemokines\textsuperscript{6,7}, and transcription factors\textsuperscript{8,9} as well as through interactions between lectins and integrins\textsuperscript{10}.

ELR (Glu-Leu-Arg motif)$^+$ CXC chemokines are produced locally and initiate neutrophil recruitment during inflammation. CXCL1/Keratinocyte-derived chemokine (KC), also known as GRO-$\alpha$ in humans, is a prototypic ELR$^+$CXC chemokine and mediates neutrophil recruitment through the CXCR2 receptor. ELR (Glu-Leu-Arg motif)$^+$

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CXC chemokines are produced locally and initiate neutrophil recruitment during inflammation. CXCL1/Keratinocyte-derived chemokine (KC), also known as GRO-α in humans, is a prototypic ELR⁺CXC chemokine and mediates neutrophil recruitment through the CXCR2 receptor. CXCL2/Macrophage Inhibitory protein-2 (MIP2) and CXCL5/LPS-induced chemokine (LIX) are two other ELR⁺CXC chemokines that act through CXCR2. Mice overexpressing CXCL1/KC in lungs have augmented neutrophil influx, whereas blockade of this chemokine with neutralizing antibodies markedly blunts neutrophil recruitment. We and others have shown that CXCL1 orchestrates neutrophil-dependent immunity in Klebsiella pneumoniae and Aspergillus fumigatus-induced lung inflammation, sepsis, and colitis. In addition to infectious inflammation, CXCL1 also plays an important role in angiogenesis, tumorigenesis, and wound healing. These studies shed light on the role of CXCL1 in host defense and homeostasis.

*Streptococcus pneumoniae* is a significant human pathogen, causing a wide range of diseases including pneumonia, meningitis and septicemia. Among several reported pneumococcal serotypes (STs), pneumonia caused by ST3 strains are the most common and associated with higher risk of deaths in adults. Similar to ST3 strains, ST2 frequently causes pneumococcal diseases, but these infections are associated with lower mortality. Using a model of Gram-positive bacterial pulmonary infection, we investigated the role of CXCL1 signaling in neutrophil influx, bacterial clearance, and host survival in pneumococcal pneumonia-derived sepsis. The results indicate that CXCL1 is involved in controlling bacterial infections in the lungs as well as proliferation and differentiation of neutrophilic granulocytes and their mobilization from bone marrow. Moreover, CXCL1-mediated neutrophil mobilization is CD62L and CD49d-
dependent. Our results reveal a previously unrecognized link through CXCL1 between host defense, granulopoiesis, and neutrophil mobilization from the bone marrow following bacterial lung infection. Furthermore, these findings are of potentially high clinical relevance for a broad spectrum of infection-related pulmonary diseases.

2.2. Methods

2.2.1. Mice

*Cxcl1<sup>−/−</sup>, Myd88/Trif<sup>−/−</sup>,* and *Nlrc4<sup>−/−</sup> mice* were generated as described previously<sup>14,21,22</sup>. *Asc<sup>−/−</sup>, Nlrp3<sup>−/−</sup>,* and *Nlpr6<sup>−/−</sup> mice* were obtained from Millennium Pharmaceuticals. All mice were backcrossed 8-10 times on the C57BL/6J genetic background. Age and gender-matched C57BL/6J mice were used as controls. A/J and BALB/cJ, were purchased from Jackson Laboratory. The Institutional Animal Care and Use Committee of the Louisiana State University approved *in vivo* experiments.

2.2.2. Pneumonia-derived sepsis model

*Streptococcus pneumoniae* 6303 (ATCC), WU2, A66.1, and D39 strains (gifts from David E. Briles, University of Alabama at Birmingham, AL) were used to induce pneumonia as described<sup>12</sup>. All strains were grown overnight at 37°C and 5% CO<sub>2</sub> in Todd-Hewitt broth plus 0.5% yeast extract and then subcultured for 6-8 hours to reach the mid-logarithmic phase in order to prepare inoculum. Mice were anesthetized and inoculated intratracheally with *S. pneumoniae* as follows; 5 x10<sup>4</sup> CFU (6303), 5 x10<sup>7</sup> CFU (WU2), 2 x10<sup>5</sup> CFUs (A66.1), and 5 x10<sup>4</sup> CFU (D39) to induce pneumonia or survival studies.
2.2.3. BALF collection, cell counts and bacterial burden

BALF and organs were collected as described earlier. In brief, mice were sacrificed, trachea was exposed and cannulated with a 20-gauge catheter, and the lungs were flushed 4 times with 0.8 mL PBS containing heparin and dextrose. Total and differential cell counts were performed on BALF using light microscopy. The lungs were excised, homogenized in PBS, and plated in serial dilutions on TSA plates (with 5% sheep’s blood) to enumerate the bacterial burden.

2.2.4. Antibodies and reagents

Mice were treated with 50 µg of anti-CXCL1 mAb (clone 48415) (ThermoFisher Scientific), anti-CXCL2 polyclonal Ab, or anti-CXCL5 mAb (clone 61905) (R&D Systems) i.p. at 24 and 2 hours prior to infection, 1 µg of murine recombinant CXCL2 or CXCL5 (R&D Systems) i.t. 1 hour post infection, 150 µg of L-selectin sheddase inhibitor (TAPI-O or KD-IX-73-4) (Sigma Aldrich) i.p. at 0 and 24-hours post infection, and 150 µg of anti CD49d mAb (BioXCell) i.p. at 0 and 24-hours post infection. Control mice received PBS or DMSO, or control rat IgG, as appropriate.

2.2.5. BMDM culture and experiment

Tibias and femurs were flushed with PBS to collect bone marrow cells. Cells were RBC-lysed using ACK lysis buffer, washed, and suspended in the DMEM containing 10% FBS and Penicillin/Streptomycin (100U/ml). Undifferentiated bone marrow cells were set up in culture dishes by supplementing MCSF (50 ng/ml) on days 0, 2, 4, 6 for a week until they become macrophages. TLR agonists (Pam2CSK4, Pam3CSK4, HKSA, HKSP, LTA, and LPS) used in BMDM assays were purchased from InvivoGen.
Inhibitors of p38 (SB203580), JNK (SP600125), ERK (PD098059), and NF-κB were purchased from Calbiochem.

2.2.6. Cytokine and chemokine production

Mouse CXCL1, CXCL2, CXCL5 (R&D Systems) and CXCL12 (eBioscience) were quantified according to manufacturers’ protocols.

2.2.7. Flow cytometry

Single cell suspensions were prepared from lungs, bone marrow, and blood. The femur/tibia were flushed thoroughly with PBS (with 1mM EDTA) to completely retrieve BM cells, RBCs were lysed, and then cells were stained. Lung digestion was performed as described previously\textsuperscript{30}. All antibodies were purchased from Biolegend unless otherwise listed. For immunophenotyping, the following antibodies were used: anti-CD45 (clone; 30-F11, eBioscience), CD11b (M1/70), Ly6G (1A8), F-4/80 (BM8), CD115 (AFS98), CD3 (17A2), CD4 (GK1.5), CD8α (53-6.7), γδ-TCR (GL3), and NK1.1 (PK136). For studying granulopoiesis\textsuperscript{8}, the following antibodies were used: anti-Ly6G (1A8), c-Kit (2B8), CD34 (RAM34, eBioscience), and the cocktail of (CD3ε (145-2C11), CD4 (RM4.5), CD19 (6D5), CD8α (53-6.7), B220 (RA3-6B2), and TER119 (Ter119)). For hematopoietic stem cells (HSCs) and myeloid progenitors\textsuperscript{5,8}, BM cells were stained with anti-CD34 (RAM34, eBioscience), FCRγIII/II (93), Sca-1 (D7), c-Kit (2B8), and lineage cocktail (Lin-1). The following antibodies were used to stain adhesion molecules: Ly6G (1A8), CD11b (M1/70), CD11a (121/7), CD29 (HMb1-1), CD18 (GAME-40, BD Biosciences), CD49d (R1-2), and CD62L (MEL-14). Appropriate isotype controls were used for compensation. Cells were acquired either on FACS Calibur or LSRFortessaX20 (BD Biosciences). FlowJo 10 (Treestar) was used to analyze data.
2.2.8. Bone marrow chimeras

Four chimeric groups (WT → WT, WT → Cxcl1/−, Cxcl1/− → WT, and Cxcl1/− → Cxcl1/−) were generated as described previously12,31. In brief, recipient mice were lethally irradiated with a single dose of 1000 rad. RBC-lysed BM cells (4 x10^6/mouse) were injected into each irradiated mouse through tail vein injection. BM-transfused mice were kept on 0.2% neomycin sulfate drinking water for 2 weeks and rested for 2 months before inducing pneumonia. Our routine procedures resulted in least 75 - 85% of the reconstitution of blood leukocytes as observed through GFP-expressing donor cells.

2.2.9. Statistics

Prism 7.0a software (GraphPad Software Inc.) was used for statistical analysis. Unpaired t-test or Mann-Whitney U test (non-parametric) or one-way ANOVA (followed by Bonferroni’s post hoc comparisons) were used to analyze differences between groups, as appropriate. Survival curves (Kaplan-Meier plot) were compared using log-rank tests. Data are expressed as means ±SEM. Data are representative of at least three experiments, but survival and chimeric studies were done twice. A P-value of *<0.05, **p<0.01, and ***p<0.001 was considered significant.

2.3. Results

2.3.1. S. pneumoniae-induced CXCL1 production requires activation of TLR and NF-kB pathways.

To determine whether CXCL1 is produced during pneumococcal pneumonia-induced sepsis, C57BL/6J, A/J, BALB/cJ mice were intratracheally infected with S. pneumoniae 6303 (5x10^4 CFU), a ST3 clinical isolate commonly used in pre-clinical studies. At 48 hours post-infection, we observed increased production of CXCL1 in
bronchoalveolar lavage fluid (BALF) in all mouse strains (Figure 2.1A). In addition to the 6303 strain, infection with other ST3 strains, such as A66.1 and WU2, also resulted in higher levels of CXCL1 in the BALF of C57BL/6J mice (Figure 2.1B).

Figure 2.1. *S. pneumoniae*-induced CXCL1 production requires activation of TLR and NF-κB signaling. (A) C57BL6/J, A/J and BALB/c mice were inoculated intratracheally with 5 X 10^4 CFU of *S. pneumoniae* 6303. (B) C57BL6/J mice were infected intratracheally with *S. pneumoniae* strain 6303 (5 X 10^4 CFU), A66.1 (2 X 10^5 CFU), WU2 (5 X 10^7 CFU), and D39 (5 X 10^4 CFU) strains or PBS. (A-B) Mice were scarified at 48 hours post-infection and CXCL1 was measured in BALF. (C) BMDMs were pre-treated with TLR agonists (Pam2CSK4 (500 ng/ml), Pam3CSK4 (500 ng/ml), HKSA (10^8/ml), HKSP (10^8/ml), LTA (5 µg/ml) and LPS (500 ng/ml) for 4 hours and infected with *S. pneumoniae* 6303 (MOI 10) for 8 hours. CXCL1 level was measured in the supernatant. (D) BMDMs from WT, Nlrp3^-/-, Nlrc4^-/-, and Nlrp6^-/- were infected with *S. pneumoniae* 6303 (MOI 10) for 8 hours. CXCL1 was measured in supernatant. (E) BMDMs from WT, MyD88/Trif^-/- and Asc^-/- were infected with *S. pneumoniae* 6303 (MOI 10) for 8 and 18 hour. CXCL1 was measured in supernatant. (F) WT BMDMs were pre-treated with 10 µM of inhibitors of NF-κB, p38 (SB203580), JNK (SP600125), ERK (PD098059) or DMSO and then infected with *S. pneumoniae* 6303 (MOI 10) for 8 hours. CXCL1 was measured in the supernatant. All experiments were performed three times. (n= 5-6 mice/infection group, n=3 mice/control group). Statistical significance was determined by one-way ANOVA (followed by Bonferroni’s post hoc comparisons). *p<0.05; **p<0.01; ***p<0.001. BALF: bronchoalveolar lavage fluid, MOI: multiplicity of infection, HKSA: heat killed *Staphylococcus aureus*, HKSP: heat killed *Streptococcus pneumoniae*, LTA: lipoteichoic acid.
However, infection with D39 (ST2) did not result in a substantial induction of CXCL1 when compared to the ST3 strain (Figure 2.1B). Since toll-like (TLR) and nod-like receptors (NLRs) are important for *S. pneumoniae*-elicited pro-inflammatory responses\(^{32,33}\), we primed bone marrow-derived macrophages (BMDMs) with known TLR ligands and infected these cells with *S. pneumoniae* 6303. BMDMs stimulated with TLR agonists Pam2CSK4 (TLR2/6), Pam3CSK4 (TLR2/4), HKSA (TLR2/6), HKSP (TLR2), LTA (TLR2), and LPS (TLR4) produced even higher level of CXCL1 (Figure 2.1C).

BMDMs isolated from *Nlrp3*\(^{-/-}\), *Nlrc4*\(^{-/-}\), but not *Nlrp6*\(^{-/-}\) mice produced higher amounts of CXCL1 following infection (Figure 2.1D). BMDMs from *Asc*\(^{-/-}\) mice also produced higher amounts of CXCL1 (Figure 2.1E). Following *S. pneumoniae* infection, BMDMs from *MyD88/Trif*\(^{-/-}\) mice produced markedly less CXCL1 compared to WT-BMDM, further confirming the importance of TLR signaling in CXCL1 induction (Figure 2.1E). Pneumococci are known to activate MAPK/NF-κB signaling, which is implicated in the transcription of cytokines and chemokines\(^{34,35}\). Therefore, we utilized inhibitors of NF-κB and MAPK pathways to examine their contribution to CXCL1 production during *S. pneumoniae* infection. NF-κB inhibition abrogated the *S. pneumoniae*-induced CXCL1 production (Figure 2.1F). Surprisingly, blockade of p38 and JNK, but not ERK, markedly enhanced the CXCL1 production (Figure 2.1F). Taken together, our data indicate *S. pneumoniae*-induced CXCL1 production requires TLR and NF-κB signaling.
2.3.2. CXCL1 controls neutrophil recruitment, bacterial clearance, and survival in pneumococcal pneumonia-derived sepsis.

To elucidate the role of CXCL1 in host defense, we infected Cxcl1 gene-deficient (Cxcl1−/−) and WT mice intratracheally with three commonly used ST3 S. pneumoniae strains (5x10^4 (6303), 2x10^5 (A66.1), and 5x10^7 (WU2) CFU). As anticipated, Cxcl1−/− mice displayed attenuated neutrophil recruitment and impaired bacterial clearance in BALF, lungs, and spleen, which was associated with increased mortality following 6303 infection (Figure 2.2A-C). Similar to the survival observed following 6303 infection, Cxcl1−/− mice infected with A66.1 and WU2 exhibited similar defects in host survival (Figure 2.2D-E). Furthermore, to determine if the protective role of CXCL1 was mouse strain specific, we utilized anti-CXCL1 mAb to neutralize CXCL1 activity in mice of C57BL/6J, A/J, and BALB/cJ backgrounds. As anticipated, anti-CXCL1-treated mice showed reduced neutrophil influx and bacterial clearance in all three backgrounds following S. pneumoniae 6303 infection (Figure 2.2F-G).

Both hematopoietic and non-hematopoietic cells produce CXCL1 during bacterial infection. To study the relative contribution of compartments, we reconstituted lethally irradiated WT or Cxcl1−/− mice with bone marrow from either WT or Cxcl1−/− mice to generate four chimeric mouse groups: 1) WT→WT, 2) WT→Cxcl1−/−, 3) Cxcl1−/−→WT, and 4) Cxcl1−/−→Cxcl1−/−. Compared to the WT→WT group, chimeric groups harboring CXCL1 deficiency in hematopoietic cells (Cxcl1−/−→WT), non-hematopoietic (WT→Cxcl1−/−), and both (Cxcl1−/−→Cxcl1−/−) compartments exhibited defective neutrophil recruitment (Figure 2.2H).
Figure 2.2. CXCL1 controls neutrophil recruitment, bacterial clearance, and survival in pneumococcal lung infection. WT and Cxcl1⁻/⁻ mice were infected intratracheally with *S. pneumoniae* 6303 (5 X 10⁴ CFU) (A-C), A66.1 (2 X 10⁵ CFU) (D), WU2 (5 X 10⁷ CFU) (E) strains or PBS (control). (A) Total number of neutrophils in BALF. (B) The bacterial burden in the BALF and spleen was quantitated at 48 hours post-infection. (C-E) Survival was monitored up to 10 days. A Kaplan Meier plot is used to show survival of mice from each group. (F-G) C57BL/6J, A/J, and BALB/cJ mice were treated with anti-CXCL1/KC mAb or IgG i.p. at 24 and 2 hours prior to infection and infected intratracheally with *S. pneumoniae* 6303 (5 X 10⁴ CFU). (F) Neutrophil counts in BALF were measured through the diff-quick method. (G) The bacterial burden in the lungs was quantitated. (H-J) Bone marrow reconstituted mice were infected intratracheally with *S. pneumoniae* 6303 (5 X 10⁴ CFU). Neutrophil count in BALF (H) and bacterial burden in BALF (I) and lungs (J) were quantitated at 48-hours post-infection. (n= 5-6 mice/infection group, n= 10 mice/survival, n=3 mice/control group). All experiments were performed three times, with exception of the survival and chimera studies, which were performed twice. Statistical significance was determined by unpaired t-test (A and F), Mann-Whitney (B, G), log-rank (C, D, E), and one-way ANOVA (followed by Bonferroni’s post hoc comparisons) (H-J). *p<0.05; **p<0.01. BALF: bronchoalveolar lavage fluid, CFU: colony-forming unit.
Additionally, the expression of CXCL1 in the hematopoietic compartment (WT→Cxcl1<sup>−/−</sup>) was critical for neutrophil accumulation and bacterial clearance as Cxcl1<sup>−/−</sup>→Cxcl1<sup>−/−</sup> group had higher bacterial burden associated with decreased neutrophil influx (Figure 2.2H-I). Similarly, the loss of CXCL1 in the hematopoietic compartment (Cxcl1<sup>−/−</sup>→WT and Cxcl1<sup>−/−</sup>→Cxcl1<sup>−/−</sup>) resulted in higher bacterial burdens in BALF and lungs compared to the WT→Cxcl1<sup>−/−</sup> group (Figure 2.2I-J). However, the chimeric (Cxcl1<sup>−/−</sup>→WT) group had a tendency to recruit less neutrophil and have more bacterial burden in BALF compared to the (WT→Cxcl1<sup>−/−</sup>) group (Figure 2.2H-I). Collectively, these data suggest that hematopoietic compartment-derived CXCL1 might be more important than CXCL1 in the stromal compartment for host defense, such as enhanced neutrophil recruitment and augmented bacterial clearance.

2.3.3. Acute pneumococcal lung infection induces emergency granulopoiesis through CXCL1.

Neutrophils are constantly produced in the bone marrow through granulopoiesis and released to blood or tissue on demand<sup>4</sup>. This process is critical for clearance of <i>S. pneumoniae</i> from the lungs<sup>36</sup>. Thus, the reduced inflammatory response in the lungs of Cxcl1<sup>−/−</sup> mice and resulting increased mortality following pneumococcal infection raised the possibility that emergency granulopoiesis may be altered in these mice. To address this question, we examined the granulopoietic compartments using flow cytometry as described previously<sup>8</sup>. During granulopoiesis, neutrophils lose c-Kit and gradually acquire the Ly6G marker. Similar to Candidemia-induced granulopoiesis<sup>8</sup>, bone marrow cells in pneumococcal pneumonia-derived sepsis can be divided into five subpopulations (#1-5) based on their expression of c-Kit and Ly6G (Figure 2.3A). In granulopoietic compartments, subpopulations #1 (c-Kit<sup>high</sup>Ly6G<sup>neg</sup>), #2 (c-Kit<sup>int</sup>Ly6G<sup>neg</sup>), #3 (c-Kit<sup>int</sup>-
neg$^{\text{Ly6G}^{-}\text{neg-int}}$) represent early granulocytic precursors; whereas subpopulation #4 (c-Kit$^{\text{int}-}$neg$^{\text{Ly6G}^{-}}$) and #5 (c-Kit$^{\text{neg}}$Ly6G$^{\text{high}}$) are immature and mature neutrophils respectively ready to be released (Figure 2.3A).

Figure 2.3. Pneumococcal pneumonia-derived sepsis induces emergency granulopoiesis requires CXCL1. (A-D) WT and Cxcl1$^{-/-}$ mice were infected intratracheally with S. pneumoniae 6303 strain ($5 \times 10^4$ CFU). (A) Flow cytometric analysis of granulopoiesis. First, bone marrow cells that had lost the potential to give rise to granulocytes were removed from the target population. The remaining cells (R5) were then analyzed for expression of c-Kit and Ly-6G. Populations R2 and R4 represent eosinophilic and megakaryocyte-erythroid progenitors respectively. FACS dot plot (B), percentage (C), and number (per femur/tibia) (D) of subpopulations #1–5 within the granulopoietic compartment at 48-hours post infection. (n= 5-6 mice/infection group, n=3 mice/control group). All experiments were performed three times. Statistical significance was determined by unpaired t-test (C, D). *p<0.05; **p<0.01.
At 48 hours after pneumococcal pneumonia-derived sepsis, WT bone marrow exhibited an increase in the cells of subpopulations #1 and #4 and a decrease in subpopulation #5 when compared to steady state control mice (Figure 2.3B-D). In contrast, *S. pneumoniae*-infected *Cxcl1*−/− mice did not exhibit any changes in subpopulations #1 and #5 but had an increase in subpopulation #4 during pneumococcal pneumonia-derived sepsis (Figure 2.3B-D). In fact, the frequency and numbers of cells in subpopulation #5 were greater in *Cxcl1*−/− mice compared to WT mice following infection (Figure 2.3B-D). However, the distributions of cells in subpopulations #1-5 were identical between WT and *Cxcl1*−/− mice at steady state (Figure 2.3B-D). These findings suggest CXCL1 is involved in emergency granulocyte generation, but not steady-state granulopoiesis, during pulmonary pneumococcal infection.

2.3.4. *Cxcl1*−/− mice have reduced amplification of early granulocyte precursors during pneumococcal lung infection-induced emergency granulopoiesis.

Granulocytes are generated from HSCs via common myeloid progenitors (CMPs), granulocyte/macrophage lineage-restricted progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs) in bone marrow through a very sophisticated mechanism, known as hematopoiesis. Compared to WT mice, *Cxcl1*−/− mice had a reduction in subpopulations #1 during pneumonia suggesting a possible defect in the generation of HSCs and early granulocyte precursors (Figure 2.3). We next assessed the composition of the early granulocytic compartments, particularly HSCs and myeloid progenitors, in WT and *Cxcl1*−/− mice after pulmonary pneumococcal infection. Both WT and *Cxcl1*−/− mice showed significant increases in the percentage and total number of HSCs (c-Kit+Sca-1+Lin−) during pneumonia-derived sepsis compared to that at steady state, and the numbers of HSCs were identical in both groups of mice (Figure 2.4A-C).
Figure 2.4. *Cxcl1*<sup>−/−</sup> mice have reduced amplification of early granulocyte precursors in pneumococcal lung infection-induced emergency granulopoiesis. (A-F) WT and *Cxcl1*<sup>−/−</sup> mice were infected intratracheally with *S. pneumoniae* 6303 (5 × 10<sup>4</sup> CFU). FACS analysis plot (A), percentage (B), and number (per femur/tibia) (C) of hematopoietic stem cells ((c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>−</sup>) at 48-hours post infection. FACS dot plot (D), percentage (E), and number (per femur/tibia) (F) of myeloid progenitor cells (c-Kit<sup>+</sup>Sca-1<sup>−</sup>Lin<sup>−</sup>) at 48-hours post infection. (n= 5-6 mice/infection group, n=3 mice/control group). All experiments were performed three times. Statistical significance was determined by unpaired t-test (B, C, E, F). *p<0.05; **p<0.01.
At 48 hours post-pneumococcal infection, the population of GMPs, but not CMPs or MEPs, increased within the c-Kit⁺Sca-1⁻Lin⁻ HSC subpopulation in both WT and Cxcl1⁻/⁻ mice (Figure 2.4D-F). However, the increase in GMPs was significantly higher in WT mice compared to Cxcl1⁻/⁻ mice after infection (Figure 2.4D-F). Moreover, the numbers of these myeloid progenitors in WT and Cxcl1⁻/⁻ mice were identical at steady state (Figure 2.4D-F). Collectively, these data indicate that CXCL1 is required for proliferation of early granulocyte precursors, but not HSCs population, during pneumococcal pneumonia-induced sepsis.

2.3.5. CXCL1 regulates CD62L and CD49d-dependent neutrophil mobilization in pneumococcal pneumonia-derived sepsis.

Next, we investigated the potential role of CXCL1 in release of neutrophils from marrow. To this end, we examined neutrophil retention in the bone marrow of WT and Cxcl1⁻/⁻ mice at 48 hours post-pneumococcal lung infection. Cxcl1⁻/⁻ mice retained a significantly higher percentage of CD11b⁺Ly6G⁺ neutrophils in the bone marrow during pulmonary pneumococcal infection, suggesting a potential defect in the release of mature neutrophils (Figure 2.5A). Accordingly, Cxcl1⁻/⁻ mice also exhibited a decrease in CD11b⁺Ly6G⁺ neutrophils in the blood and lungs (Figure 2.5A) when compared to WT mice. CXCR2 and CXCR4 play dominant but opposing roles in the release of mature neutrophils from bone marrow. Disruption of CXCR4, whose major ligand is CXCL12/SDF1-α, results into spontaneous mobilization of neutrophils into blood. Therefore, we investigated the expression level of CXCR4 on BM neutrophils from Cxcl1⁻/⁻ mice during pneumococcal pneumonia-induced sepsis. Surprisingly, the expression of CXCR4 on marrow neutrophils did not differ between the steady state and during pneumonia-induced sepsis (Figure 2.5B).
Figure 2.5. CXCL1 regulates CD62L and CD49d-dependent neutrophil mobilization in pneumococcal pneumonia-derived sepsis. (A-G) WT and Cxcl1−/− mice were infected intratracheally with *S. pneumoniae* 6303 (5 X 10⁴ CFU). Mice were scarified at 48 hours post-infection. (A) Neutrophil (CD11b⁺Ly6G⁺) counts in bone marrow, blood, and lungs were measured. (B) Mean fluorescence intensity (MFI) of CXCR4 on bone marrow neutrophil was measured. (C) Level of CXCL12 in bone marrow lysates. (D and E) Representative histograms and MFIs of the CD62L in neutrophils. (F and G) Representative histograms and MFIs of the CD49d in neutrophils. (H and I) WT and Cxcl1−/− mice were infected intratracheally with *S. pneumoniae* 6303 (5 X 10⁴ CFU). Mice were treated with L-selectin sheddase inhibitor (TAPI-O or KD-IX-73-4) or monoclonal antibody (mAb) against CD49d or vehicle control (DMSO or IgG) i.p. at 0 and 24 post-infection and then scarified at 48-hour post infection. The total number of BM neutrophil (CD11b⁺Ly6G⁺) per femur/tibia (H) and the percentage of blood neutrophil (CD11b⁺Ly6G⁺) (I) were measured. (n= 5-6 mice/infection group, n=3 mice/control group). All experiments were performed three times. Statistical significance was determined by unpaired t-test (A-G) and one-way ANOVA (followed by Bonferroni’s post hoc comparisons) (H and I). *p<0.05; **p<0.01; ***p<0.001.
Similarly, *Cxcl1<sup>−/−</sup>* mice also produced comparable levels of CXCL12 in the bone marrow following pneumococcal infection (Figure 2.5C). These results indicate that the defects in neutrophil mobilization in *Cxcl1<sup>−/−</sup>* mice are independent of the CXCR4/CXCL12 retention signal in marrow. Neutrophils mobilized in response to infectious stimuli or chemokines shed L-selectin (CD62L) and express elevated level of CD18 or CD49d-integrins<sup>6,40</sup>. Thus, we set out to assess whether there are changes in the expression of adhesion molecules on bone marrow neutrophils during pneumococcal lung infection. At 48-hours post infection, WT neutrophils show marked shedding of CD62L, but this process was impaired in neutrophils from *Cxcl1<sup>−/−</sup>* mice (Figure 2.5D-E). In addition to defective shedding of CD62L, neutrophils from *Cxcl1<sup>−/−</sup>* mice display decreased expression of CD49d compared to WT cells (Figure 2.5F-G).

To further confirm the roles of CD49d and CD62L in our pneumonia-derived sepsis model, we used the L-selectin sheddase inhibitor (TAPI-O) and a specific mAb against CD49d. This inhibition was accompanied by higher retention of neutrophils in bone marrow, which was associated with attenuated release of neutrophils into the blood of WT mice (Figure 2.5H-I). The retention of neutrophils following TAPI-O administration was also observed *Cxcl1<sup>−/−</sup>* mice (Figure 2.5H). Similarly, CD49d blockade resulted in higher neutrophil retention in the bone marrow and reduced mobilization of neutrophils into the blood during pulmonary pneumococcal infection (Figure 2.5H-I). Taken together, our data indicate that CXCL1 regulates neutrophil mobilization in a CD62L and CD49d-dependent manner in pneumococcal infection without disrupting the CXCR4/CXCL12 retention signal.
2.3.6. Administration of recombinant CXCL2 and CXCL5 rescues the impaired host defense and neutrophil release in Cxcl1−/− mice during pneumococcal pneumonia-induced sepsis.

Besides CXCL1, two other ELR+ CXC neutrophil chemokines (CXCL2 and CXCL5) act through CXCR2 receptor\(^4\). Although neutrophil migration from bone marrow to lungs is reduced, there is not a complete abrogation of neutrophil migration in Cxcl1−/− mice, suggesting involvement of either CXCL2 or CXCL5. To this end, we administered recombinant CXCL2 and CXCL5 one hour post-infection with pneumococci. Administration of recombinant CXCL2 resulted in increased neutrophil recruitment, which were associated enhanced bacterial clearance in both WT and Cxcl1−/− mice following pneumococcal pneumonia-derived sepsis (Figure 2.6A-C). However, recombinant CXCL2 induced neutrophil recruitment were very dramatic in WT mice but not in Cxcl1−/− mice following pneumococcal pneumonia-derived sepsis at 48 hour post infection (Figure 2.6A).

Furthermore, exogenous administration of recombinant CXCL5 enhanced neutrophil recruitment, which was associated with enhanced bacterial clearance in Cxcl1−/− mice during pneumococcal infection (Figure 2.6D-F). In addition, recombinant CXCL2 also increased release of mature neutrophils (#P5) from bone marrow (Figure 2.6G-H), suggesting CXCL2 may surrogate the function of CXCL1 in its absence during pneumococcal infection.
Figure 2.6. Administration of recombinant CXCL2 and CXCL5 rescues the impaired host defense seen in Cxcl1−/− mice during pneumococcal lung infection. (A-H) WT and Cxcl1−/− mice were infected intratracheally with S. pneumoniae 6303 (5 X 10⁴ CFU). Mice were treated with murine recombinant CXCL2 and CXCL5 i.t. at one-hour post-infection and then scarified at 48 hours post-infection. BALF neutrophil counts (A) and bacterial burden in lungs (B) and spleen (C) were quantitated. BALF neutrophil counts (D) and bacterial burden in lungs (E) and spleen (F) were quantitated. FACS dot plot (G) and mature neutrophil (subpopulation #5) (Ly6G⁺c-Kit⁻) counts in bone marrow (H) were measured. (n= 5-6 mice/infection group, n=3 mice/control group). All experiments were performed three times. Statistical significance was one-way ANOVA (followed by Bonferroni’s post hoc comparisons) (A-H). *p<0.05; **p<0.01
To validate roles of CXCL2/5, WT mice were treated with either single or both antibodies prior to infection. Mice receiving anti-CXCL2 or anti-CXCL5 or both had enhanced retention of the P5 population in granulocytic compartment and reduced blood neutrophils compared to that of IgG-treated mice (Figure 2.7A-B and 2.7E-F). In addition, the neutralization of both CXCL2 and CXCL5, but not single treatment of either anti-CXCL2 or anti-CXCL5, leads to the decreased GMPs frequency (Figures 2.7C-D). Together, our data suggest CXCL2 and CXCL5 can compensate for the loss of CXCL1.

Figure 2.7. Neutralization of CXCL2 and CXCL5 in pneumococcal pneumonia-induced sepsis (A) WT mice were treated with either single or both i.p. at 24 and 2 hours prior to infection and then infected intratracheally with *S. pneumoniae* 6303. Control groups received IgG. FACS dot plot (A), and number per femur/tibia (B) of subpopulations #5 within the granulopoietic compartment, FACS dot plot (C), and number per femur/tibia (D) of myeloid progenitor cells (c-Kit^Sca-1^-Lin^-), and FACS dot plot (E), and percentage (F) of blood neutrophils at 48-hours post infection are presented. (n= 5-6 infection group, n=3 mice/control group). Statistical significance was determined by one-way ANOVA (followed by Bonferroni’s *post hoc* comparisons) *p<0.05; **p<0.01.
2.4. Discussion

Neutrophil homeostasis is a tightly regulated process that involves granulopoiesis, mobilization, and recruitment to organs/tissues. ELR\(^+\) CXC chemokines display potent neutrophil chemotactic properties, although their role in neutrophilic granulopoiesis and release from the bone marrow in Gram-positive infections is undefined. Thus, we utilized a murine model of pneumococcal pneumonia-derived sepsis to investigate the role of CXCL1 in granulopoiesis and mobilization of neutrophils from the marrow.

A central feature of inflammation is the activation of TLRs, NLRs, transcription factors, and MAP kinases\(^{32-35}\). While our results revealed that CXCL1 production is mediated by the TLR-MyD88/Trif-NF-kB axis, the NLR-ASC and MAP kinase pathways might negatively regulate it during pneumococcal infection. These findings suggest that NLR activation may serve as a molecular switch to dampen TLR-dependent pathways and prevent excessive inflammation. Our observations indicate that CXCL1 plays an essential role in pulmonary host defense against ST3 pneumococci (6303, A66, WU2) regulating neutrophil recruitment and bacterial clearance. Utilizing anti-CXCL1 antibody on A/J and BALB/cJ backgrounds, we confirmed that CXCL1-mediated neutrophil-dependent immunity are not background specific.

The contributions of myeloid cells and stromal cells to neutrophil accumulation in pneumococcal pneumonia-derived sepsis are not known. Myeloid cells produce CXCL1, CXCL2, and type II alveolar cells produce CXCL5\(^{12,26}\). Previously, we have shown that CXCL1-derived from both hematopoietic and stromal cells contributes to host defense during *Klebsiella* infection\(^{12}\). Here, we report that CXCL1-expressing hematopoietic cells primarily contribute to neutrophil recruitment and bacterial clearance, despite the fact that
CXCL1 expression in both compartments is essential for protection against pneumococcal infection.

Although the role of CXCL1 for neutrophil recruitment is well established, its function in granulopoiesis and mobilization from the marrow has not been investigated. We demonstrate that CXCL1 is important during the amplification of granulocytic progenitor cells in bone marrow. Our data show that CXCL1 is not essential at steady state, but is required for emergency granulopoiesis during pneumococcal infection. In addition, CXCL1 is important for proliferation and differentiation of c-Kit+ early granulocytic precursors into Ly6G+ neutrophils. The increased frequency of granulocytic progenitors in WT mice, but not in Cxcl1−/− mice, could be due to either increased proliferation or cell survival or both of these mechanisms following pneumococcal infection. Moreover, STAT3 and C/EBPβ are known to be master regulators of granulopoiesis in mice5,8,9. Future studies will be required to investigate whether the reduced granulopoiesis observed in Cxcl1−/− mice is associated with impaired expression of STAT3 or C/EBPβ. CXCR2, a common receptor for CXC-chemokines, is widely known to participate in neutrophil mobilization in response to infections and cytokines38,41,42. Cxcr2−/− mice have profound defect in neutrophil emigration to sites of inflammation43. Furthermore, CXCR2 negatively regulates CXCR4-mediated neutrophil retention within the bone marrow38. CXCR2 engagement induces a heterologous desensitization to the effects of CXCL12 on CXCR4, thereby inducing neutrophilia44. Since Cxcl1−/− mice have unaltered levels of neutrophil CXCR4 and bone marrow CXCL12, it is possible that the loss of CXCL1-CXCR2 engagement may have compromised CXCR4 desensitization in our model.
Neutrophil mobilization greatly differs between homeostatic and inflammatory settings. Our findings reveal that CXCL1 mediates CD62L and CD49d-dependent release of neutrophils from the marrow during pneumococcal infection. The bone marrow sinusoidal endothelium constitutively expresses cell adhesion molecules\textsuperscript{45}. Although maturation-related loss of both CD49d and CXCR4 have been shown to regulate orderly release of neutrophil in the homeostatic setting\textsuperscript{46}, we believe that massive release of often less mature neutrophils during pneumococcal pneumonia may be much more complex and different under the influence of mediators such as CXCL1. Since we did not see much alteration in marrow CXCL12 and neutrophil CXCR4 expression, we analyzed the panel of adhesion molecules and results of which show marked alteration in expression of CD62L and CD49d only (Figure 2.5). Similar to our study, MIP2 (CXCR2-ligand) has been shown to regulate CD49d-dependent neutrophil mobilization\textsuperscript{6}. In contrast, G-CSF-induced neutrophilia is predominantly associated with disruption of CXCR4/CXCL12 signal\textsuperscript{47}. Thus we speculate that CD49d-dependent neutrophil mobilization may be an important mechanism when inflammatory response is primarily mediated through CXCR2 engagement and the expression of CXCR4/CXCL12 signal remains unaltered. However, further research is needed to confirm our hypothesis.

Our findings indicate: (1) neutrophils shed CD62L during pneumococcal pneumonia-derived sepsis; (2) CXCL1 deficiency impairs this process and leads to the increased retention of mature neutrophils in $\textit{Cxcl1}^{-/-}$ mice. These findings are in support of previous reports showing the relevance of CD62L shedding to neutrophil mobilization\textsuperscript{6,48}. The loss of CD62L surface expression during inflammatory response is primarily associated with proteolytic cleavage of ecto-domain of L-selectin by TNF-\textalpha.
converting enzymes, which may be regulated by p38 MAPK and PKC signaling\textsuperscript{49 50}. Since administration of TAPI-O reduced the mobilization in our model (Figure 2.5), it is likely that CXCL1 may also regulate proteolytic cleavage of CD62L during pneumococcal lung infection. We speculate that the decreased expression of CD49d in \textit{Cxcl1}\textsuperscript{-/-} mice might have also occurred through a similar mechanism, which remains yet to be explored.

In conclusion, the current study identifies a critical role of CXCL1 signaling in mediating not only neutrophil recruitment, but also in the generation of neutrophilic granulocytes and their mobilization from bone marrow in response to Gram-positive lung infection. The results of this study support the concept that the mechanisms regulating neutrophilic granulopoiesis and mobilization in infectious settings are different from those that regulate these events at steady state. Moreover, future studies will be required to further define the molecular and cellular mechanisms by which CXCL1 regulates neutrophil homeostasis upon pulmonary Gram-positive bacterial infection.

2.5. References


3.1. Introduction

Gram-positive pathogens, including Staphylococcus aureus are the major cause of healthcare-associated pneumonia, sepsis, and mortality in post-influenza infection patients \(^1,2\). In particular, the emergence of methicillin-resistant \(S.\) aureus (MRSA) as an endemic, dominant strain poses a menacing therapeutic challenge in the United States \(^3\). Unlike other hospital-acquired \(S.\) aureus strains, MRSA causes a broad spectrum of necrotizing infections of skin and soft tissues, including pneumonia, and these infections are associated with high morbidity and mortality, even in immunocompetent individuals \(^3,4\). \(S.\) aureus pneumonia is characterized by extensive inflammation and localized necrosis leading to loss of alveolar architecture, hemorrhage, and consolidation of lungs \(^5\). Emerging evidence suggests that pathogenesis of \(S.\) aureus pneumonia is largely mediated by over-activation of host inflammatory signaling cascades, although the precise mechanisms underlying this remain elusive.

Despite its antibiotic resistance, the success of \(S.\) aureus as a highly invasive pathogen is largely attributed to its arsenal of virulence factors, including cytolytic and pore-forming toxins \(^6\).

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Reprinted by permission from Macmillian Publishers Ltd on the behalf of Nature Publishing Group: Mucosal Immunology, from NLRC4 suppresses IL-17A-mediated neutrophil-dependent host defense through upregulation of IL-18 and induction of necroptosis during Gram-positive pneumonia. Paudel S, Ghimire L, Jin L, Baral P, Cai S, Jeyaseelan S. 12(1), 2018: permission conveyed through Copyright Clearance Center, Inc
All *S. aureus* strains produce exotoxins or surface proteins, which have diverse targets in multiple cell types. *S. aureus* has been shown to activate NLRP3 and NOD2, and to target CD11b and ADAM10. Unlike Gram-negative pathogen-induced infections, mice deficient in innate immune molecules including ADAM10, NLRP3, NOD2, IFNR-1, and TNFR have improved outcomes in *S. aureus* (Gram-positive) pneumonia. In general, these gene-deficient mice show enhanced bacterial clearance, attenuated pro-inflammatory cytokine production, and decreased lung injury/pathology contributing to increased survival against pulmonary *S. aureus* infection. These observations suggest *S. aureus* may have evolved to utilize host innate immune molecules to dampen immune responses. However, the precise mechanism through which *S. aureus* manipulates the host inflammatory machinery to favor the pathogen is not clear.

Necroptosis is a recently identified, pro-inflammatory mode of cell death regulated by receptor-interacting serine-threonine kinases (RIP1/3) and executed by mixed lineage kinase domain-like protein (MLKL). Recently, toxin-induced rapid loss of macrophages via necroptosis has been suggested as a major mechanism of lung damage in *S. aureus* pneumonia. Similarly, alveolar macrophages exposed to pore-forming toxins from *Serratia marcescens* undergo necroptosis, which contributes to the severity of pneumonia through substantial loss of critical immune cells and extensive bystander tissue injury. Genetic ablation of *Rip3* or treatment with inhibitors of necroptosis protects alveolar macrophages, which is associated with reduced bacterial burden and improved pneumonia outcomes in mice. However, the mechanism
responsible for the necroptosis-mediated depletion of immune cells that impedes bacterial clearance in *S. aureus* pneumonia largely remains unknown.

Regarding inflammasomes, NLRC4 belongs to the NLR family of proteins containing an N-terminal CARD domain, a central NACHT domain and a C-terminal leucine-rich repeat domain and is involved in assembly of the inflammasome complex. It was identified as a cytosolic sensor for bacterial flagellin and type III secretion system (T3SS) \(^{19,20}\). However, *Klebsiella pneumoniae*, a non-flagellated bacterium lacking T3SS, and a flagellin-deficient strain of *Pseudomonas aeruginosa* have been shown to activate the NLRC4, suggesting its activation by other bacterial or endogenous host ligands \(^{21,22}\). Nonetheless, the role of NLRC4 in bacterial pneumonia caused by a Gram-positive organism still remains elusive \(^{21,23}\). Although recent studies linked NLRP3 and ASC to *S. aureus* toxin-induced necroptosis, the precise mechanism for this has not been elucidated either \(^{7,18}\). Furthermore, *Nlrp3*\(^{-/-}\) mice do not show a completely protective phenotype in *S. aureus* pneumonia indicating both NLRP3-dependent and independent mechanisms may be important in eliciting lung damage \(^{13}\). To this end, we investigated whether the activation of the NLRC4 is important in the pathogenesis of *S. aureus* pneumonia in a mouse model. We found that NLRC4-driven necroptosis and IL-18 suppress IL-17A signaling from γδ T cells leading to attenuated neutrophil recruitment, which is necessary to eradicate *S. aureus* from the lung. Thus, these findings provide novel insight into how necroptosis and IL-18 dampens host defense in *S. aureus* pneumonia and suggests the inhibition of NLRC4 as a potential therapeutic approach for control of *S. aureus* infection in the lungs.
3.2. Methods

3.2.1. Animals

*Nlrc4*−/− mice were generated as described24. The generation of *Il-17a*−/−, *Il-17f*−/−, *Il-17ra*−/−, *Casp1*−/−/11−/−, *Il-1β*−/−, *Il-18*−/−, and *Mlkl*−/− were described previously22,25-29. All mouse strains were backcrossed onto the C57BL/6J genetic background at least 10 times. Age and gender-matched WT controls were used. The Institutional Animal Care and Use Committee of the Louisiana State University approved all animal experiments.

3.2.2 Pneumonia model

The *S. aureus* USA300 strain, its isogenic α-hemolysin (*hla*)-deficient, and MSSA Newman strain were used to induce pneumonia, as described previously21,30. Bacterial strains were grown overnight at 37°C in tryptic soy broth, diluted to 1:100 in tryptic soy broth next day, and subculture for 3-4 hours to the mid-logarithmic phase. *S. aureus* strains were harvested by centrifuging the culture at 9000 rpm for 2 minutes, washed twice, and re-suspended in phosphate buffered saline (PBS) to achieve an optical density of 0.5. After anesthesia with xylazine/ketamine mix, mice were intratracheally inoculated with *S. aureus* at a concentration of 5 x10⁷ CFUs/50µl/mouse for pneumonia or 2 x 10⁸ CFUs/50µl/mouse for survival. Neutrophil depletion was achieved with intraperitoneal injection of 500 µg of anti-Ly6G ab (IA8) (Biolegend) 24 hours and 2 hours prior to infection31.

In some experiments, mice were injected intraperitoneally with 300 µg of necrostatin-1 (RIP-1 inhibitor) (Calbiochem), 150 µg of Ac-yvad-cmk (Caspase-1 inhibitor) (Caymen Chemical), 200 µg of murine anti-IL-17A antibody (eBioscience), 100 µg of anti-γδ-TCR antibody (Biolegend), or 200 µg of Rottlerin (PKC-δ inhibitor).
(Calbiochem) 12 hours prior to infection as described with or without slight modifications. In other experiments 100 µl of 10 µM of GW806742X (MLKL-inhibitor) (Synkinase) was administered intraperitoneally 1 hour prior to infection. In additional experiments, mice were treated intratracheally with 1 µg recombinant IL-17A or IL-18 (R&D Systems) 1 hpi. Control mice received PBS or DMSO, or control rat IgG2a, as appropriate.

3.2.3. BALF collection, cell counts, and bacterial burden

BALF and organs were collected as described earlier. In brief, mice were sacrificed, the trachea was exposed and cannulated with 20-gauge catheter, and the lungs were flushed four times with 0.8 mL of PBS containing heparin/dextrose. Total and differential cell counts were performed on BALF using light microscopy utilizing QuikDip Stain. Lungs/spleen were excised, homogenized in PBS, and plated in serial dilutions on TSA plates to count the bacterial burden in organs after overnight incubation at 37 °C.

3.2.4. THP-1 cells and FAM-FLICA caspase-1 assay

THP-1 (#thp-null) and THP-1 cells overexpressing Nlr4 (#thp-nlrc4) were obtained from Invivogen. Culture and maintenance of cells was performed as described in their protocols. The FAM-FLICA caspase-1 assay was performed in THP-1 cells to detect active caspase-1 according to kit’s protocol (ImmunoChemistry Technologies).

3.2.5. Cytokine/Chemokines, LDH, and ROS

Human (IL-1β, IL-18 and IL-17A) and Mouse (IL-1β, IL-1α, IL-18, TNF-α, IL-6, MCP-1, IL-17A, and IL-17F) were measured by ELISA as described in their respective protocols (eBioscience/Invitrogen). CXCL1, CXCL2, CXCL5, G-CSF (R&D systems) and HMGB-1 (Chondrex) were quantified according to the manufacturers’ protocols.
LDH release and % cytotoxicity assays were performed as per manufacturer’s guidelines (Promega). The percentage cytotoxicity was calculated as 100x (Experimental-Spontaneous)/ (Maximum-Spontaneous). Total ROS activity was determined using a commercial kit (AAT Bioquest). In some experiments, cells were infected in the presence or absence of murine recombinant IL-17A (R&D Systems).

3.2.6. BMDM/BMDN, bacterial killing assay, and phagocytosis

Bone marrow-derived cells were flushed from tibia and femurs of mice, RBCs lysed, cells were then washed and resuspended in the DMEM containing 10% FBS and Penicillin/Streptomycin (100 U/ml). Bone marrow cells were then differentiated to macrophages in culture dishes by supplementing MCSF (50 ng/ml) on days 0, 2, 4, 6 for a week. The Mouse Neutrophil Enrichment Kit (Stem Cell) was used to isolate and purify neutrophils through negative selection as described in the manufacturer’s protocol. The intracellular killing abilities of BMDM/Ns were evaluated using a gentamicin protection assay with slight modifications. Briefly, BMDMs were infected with multiplicity of infection (MOI 10) of *S. aureus* for 1 hour, washed, incubated with a medium containing gentamicin (250 µg/ml) for 2 hours, washed, and thereafter incubated with a medium containing gentamicin (50 µg/ml) to kill extracellular bacteria. For BMDNs, a medium containing gentamicin (250 µg/ml) was used for 30 minutes at indicated times. The cells were then washed, lysed, and plated by serial dilution in TSA plates to enumerate the CFU of intracellular bacteria. Net phagocytosis of neutrophils was performed utilizing pHrodo Red *S. aureus* BioParticles (Life Technologies). Net phagocytosis was calculated by deducting the average fluorescence intensity of the no-cell negative-control wells from all positive control and experimental wells.
3.2.7. In vitro stimulation of purified γδ T cells

Naïve γδ T cells were purified from spleens of uninfected WT and Nlrc4−/− mice using pan-T-cell isolation (MAC Miltenyi Biotec) followed by FACS sorting for CD3⁺ γδ-TCR⁺ cells, as described elsewhere 36. Purified γδ T cells (1 × 10⁵ cells/well) were then stimulated with S. aureus (MOI of 10) for 18 hours in presence or absence of IL-23 (40 ng/ml) (R&D systems). Cell supernatants were collected and IL-17A was quantitated by ELISA. The purity of γδ T cells was found to be ≥87% as determined by flow cytometry.

3.2.8. BrdU staining

The BrdU Flow Kit (BD Biosciences, #559619) was used. In brief, WT and Nlrc4−/− mice received a single dose of 1 mg BrdU solution intraperitoneally 1 hour before infection. Lungs were harvested and processed for flow cytometric staining according to the manufacturer’s protocol.

3.2.9. Immunofluorescence microscopy

Immunofluorescence staining for NLRC4 expression on tissue slides was performed as described previously 37. Paraffin-embedded lung sections from healthy or diseased humans (with clinical diagnosis of unspecified bacterial pneumonia) were obtained from Biochain Institute Inc. USA. Following primary antibodies were used: anti-NLRC4 Ab (Cell Signaling), anti-lipocalin-2 Ab for PMNs (R&D systems), anti-CD68 Ab for macrophages (Biolgend) and anti-CD326 Ab for epithelial cells (Biolegend). For mouse slides; we used anti-NLRC4 Ab (EMD Millipore), anti-Ly6G⁺ Ab for PMNs (Biolegend), anti-CD326 Ab for epithelial cells (Biolegend), and the anti-F4/80 Ab for myeloid cells (Biolegend). Similarly, anti-RIP3 Ab (Cell Signaling) and
anti-MLKL (phospho S345) Ab (Abcam) were used to study necroptosis in mouse BMDMs. Alexa conjugated secondary antibodies (Invitrogen) were used.

3.2.10. Flow cytometry

For flow cytometric analysis, lungs were excised, minced, digested for 90 min at 37°C in collagenase (2 mg/ml) (Worthington) and DNase I (20 U/ml) (Roche) to obtain single cell suspensions. The following surface marker antibodies were purchased from eBioscience: anti-CD11b (clone M1/70), Ly6G (1A8,), F-4/80 (BM8), CD3 (17A2), CD4 (GK1.5), CD8α (53-6.7), γδ-TCR (GL3), and NK1.1 (PK136). For intracellular staining, cells were incubated for 4-5 hours with 3 mg/ml of brefeldin A (eBioscience). The BD Cytofix/Cytoperm Kit was used to permeabilize and fix cells before staining with IL-17A (17B7, eBioscience). Isotype controls were used for compensation. Cells were acquired on a FACs caliber (BD Biosciences). FlowJo 10 (Treestar) was used to analyze data.

3.2.11. Western blotting and immunoprecipitation

Western blotting for lung homogenates and cell lysates was performed as described in previous publications. Antibodies to NLRC4 (EMD Millipore), NLRC4 (phospho S533) (ECM Biosciences), Caspase-1 (Adipogen), RIP3 (Cell Signaling), MLKL (phospho S345) (Abcam), and GAPDH (Cell Signaling) were used. The immunoprecipitation Kit, DynabeadsTM Protein G (Invitrogen), was used to immunoprecipitate RIP3 and NLRC4 according to the manufacturer’s protocol.

3.2.12. Bone marrow chimeras

Bone marrow transplantation was performed to create $Nlrc4^{-/-}$ chimera mice wherein the $Nlrc4$ deficiency was confined to either the hematopoietic cells ($Nlrc4^{-/-} \rightarrow$ WT) or non-hematopoietic tissue (WT $\rightarrow$ $Nlrc4^{-/-}$), as described in our previous
publications 21,37. In brief, recipient mice were lethally irradiated with a single dose of 1000 rad. Each recipient mouse received a tail vein injection of marrow cells (4 x10^6/mouse) and was kept on 0.2% neomycin-sulfate drinking water for 2 weeks. Pneumonia was induced at 2 months after the reconstitution. Four groups were generated (WT → WT, WT → Nlrc4^-/-, Nlrc4^-/- → WT, and Nlrc4^-/- → Nlrc4^-/-). Our routine procedure results in at least 75 - 85% reconstitution of blood leukocytes from donor mice, as confirmed by flow cytometry on donor cells expressing GFP.

3.2.13. Statistical analysis

Data are expressed as means ± standard error of mean (s.e.m.) unless otherwise stated. Prism 7.0a software (GraphPad Software Inc.) was used for statistical analyses. The unpaired t-test, Mann-Whitney U test (non-parametric), or one-way ANOVA (followed by Bonferroni’s post hoc comparisons) were used to analyze differences between groups, as appropriate. Survival curves (Kaplan-Meier plot) were compared using log-rank tests. A P-value of *<0.05, **p<0.01, and ***p<0.001 was considered significant.

3.3. Results

3.3.1. NLRC4 expression is enhanced in human and murine pneumonic lungs.

To determine whether NLRC4 expression is upregulated in human pneumonic lungs, we performed immunofluorescence staining of NLRC4 in lung sections from pneumonic patients and healthy controls. As virulence factors from S. aureus are known to target both myeloid and non-myeloid cells 8,10, we assessed NLRC4 expression in myeloid cells (neutrophils, macrophages) and non-myeloid cells (epithelial cells) and found increased expression of NLRC4 in Lipocalin-2^+ neutrophils, CD68^+ macrophages,
and CD326+ epithelial cells (Figure 3.1a). To explore if NLRC4 expression is increased during Gram-positive pneumonia in mouse lungs, we performed immunofluorescence staining in lung sections at 24 hours post- \textit{S. aureus} infection. We observed enhanced expression of NLRC4 in Ly6G+ neutrophils, F4/80+ macrophages, and CD326+ epithelial cells in infected wild-type (WT) mouse lungs compared to that in control lungs (Figure 3.1b). Collectively, our results indicate both myeloid and non-myeloid cells show up-regulation of NLRC4 during bacterial pneumonia.

![NLRC4 Expression in Lungs](image)

**Figure 3.1.** NLRC4 expression is enhanced in human and murine pneumonic lungs. (a) NLRC4 expression was assessed using immunofluorescence in lung sections from healthy controls and from patients with clinical diagnosis of unspecified bacterial pneumonia. Red staining indicates NLRC4 expression. Neutrophils (Lipocalin-2+), macrophages (CD68+), and epithelial cells (CD326+) were stained green. Nuclei were stained with DAPI (blue). Arrowheads (yellow) indicate co-localization of expression of NLRC4 with specific cell markers. Images are representative of five different sections with similar results. (b) NLRC4 expression in lung sections from control and \textit{S. aureus}-infected WT and \textit{Nlrc4}−/− mice at 24 hpi. Red staining indicates NLRC4 expression. Neutrophils (Ly6G+), macrophages (F4/80+), and epithelial cells (CD326+) are stained green. Nuclei were stained with DAPI (blue). Arrowheads (yellow) indicate co-localization of expression of NLRC4 with specific cell markers. Images are representative of five different sections with similar results. Original magnification, 40x. DAPI: 4', 6-diamidino-2-phenylindole, LCN2: lipocalin-2, hpi: hours post infection.
3.3.2. NLRC4 deficiency contributes to host protection against S. aureus pneumonia.

To characterize the importance of NLRC4 in S. aureus pneumonia, WT and Nlrc4−/− mice were infected intratracheally (i.t.) with a lethal inoculum of S. aureus (2x10^8 CFU/mouse) and survival was monitored up to 100 hours. Compared to WT counterparts, Nlrc4−/− mice showed enhanced survival (Figure 3.2a). To determine if the enhanced survival in Nlrc4−/− mice was due to increased bacterial clearance, WT and Nlrc4−/− mice were infected i.t. with a sub-lethal inoculum of S. aureus (5x10^7 CFU/mouse). Compared to WT mice at 12 and 24 hours post infection (hpi), Nlrc4−/− mice showed a diminished bacterial burden in the lung and bronchoalveolar lavage fluid (BALF) along with decreased dissemination in the spleen (Figures 3.2b-d). We next determined whether the enhanced bacterial clearance was due to augmented neutrophil accumulation in the lungs. Our results revealed that Nlrc4−/− mice had increased numbers of total white blood cells, neutrophil, and macrophage recruited to the lungs (Figures 3.2e-g). Furthermore, unlike Nlrc4−/− mice, WT mice had increased protein leakage, an indicator of lung permeability (Figure 3.2h). To assess if the detrimental role of NLRC4 activation is MRSA strain-specific, we infected WT and Nlrc4−/− mice with a methicillin-susceptible S. aureus (MSSA) Newman strain. The protective phenotype seen in Nlrc4−/− mice infected with S. aureus was also seen in pneumonia caused by the MSSA strain (Figures 3.2i, j).

S. aureus targets both myeloid and non-myeloid cells. In Figure 3.1(a-b), we demonstrate increased expression of NLRC4 in both hematopoietic (myeloid cells) and non-hematopoietic (epithelial cells) cells in human and murine pneumonic lungs. Therefore, we next investigated the contribution of hematopoietic versus non-hematopoietic cells to host protection using bone marrow chimeras.
Figure 3.2. NLRC4 deficiency confers host resistance against *S. aureus* pneumonia. (a) WT (control) and *Nlrc4*<sup>-/-</sup> mice were inoculated intratracheally with 2 X 10<sup>8</sup> CFU of *S. aureus* and survival was monitored up to 100 hours. A Kaplan Meier plot is used to show survival of mice from each group. (n= 10 mice/group). (b-h) WT and *Nlrc4*<sup>-/-</sup> mice were inoculated intratracheally with 5 x 10<sup>7</sup> CFU of *S. aureus* or PBS (control). BALF and organs were harvested at the designated time points. Control mice (PBS treated) were sacrificed at 24 hpi. The bacterial burden in the (b) lungs, (c) BALF, and (d) spleen was quantitated at 12 and 24 hpi. (n= 5-6 mice/pneumonia group, n=3 mice/control group). (i and j) WT and *Nlrc4*<sup>-/-</sup> mice were inoculated intratracheally with 5 x 10<sup>7</sup> CFU of *S. aureus* Newman strain (MSSA). (i) Cellular influx in BALF and (j) bacterial burden in lungs and BALF were determined at 24 hpi. (k and l) Bone marrow reconstituted mice were inoculated intratracheally with 5 x 10<sup>7</sup> CFU of *S. aureus*. The bacterial burden in (k) lung and (l) BALF was quantitated at 24 hpi. Each symbol represents a single mouse (b, c, d, j, k, l). Data from a representative experiment are shown. All experiments were performed three times, but survival and chimera study were performed twice. Statistical significance was determined by log-rank (a), Mann-Whitney (b, c, d, j), unpaired t-test (e-i), one-way ANOVA (followed by Bonferroni’s post hoc comparisons) (k, l). *p<0.05; **p<0.01; ***p<0.001. BALF: bronchoalveolar lavage fluid, CFU: colony forming unit, WBC: white blood cell, nd: non-detectable.
In this context, lethally irradiated WT or Nlrc4⁻/⁻ mice were reconstituted with RBC-lysed bone marrow cells (4 X10⁶/mouse) from donor WT or Nlrc4⁻/⁻ mice. Two months after reconstitution, chimeric mice were infected with S. aureus. Interestingly, the Nlrc4⁻/⁻ → Nlrc4⁺/⁺, Nlrc4⁻/⁻ → WT but not WT → Nlrc4⁻/⁻ chimeric groups had decreased bacterial burden in lungs as compared with the WT → WT group (Figures 3.2k). However, the Nlrc4⁺/⁺ → Nlrc4⁻/⁻, Nlrc4⁻/⁻ → WT, and WT → Nlrc4⁻/⁻ groups had reduced bacterial burden in BALF as compared to WT → WT group (Figures 3.2i). Taken together, our results suggest NLRC4 activation, in both hematopoietic and non-hematopoietic compartments, is detrimental, and their deficiency in mice displays robust protective phenotype in S. aureus pneumonia.

3.3.3. S. aureus activates NLRC4 through PKC-δ to induce IL-1β, IL-18, and necroptosis.

S. aureus α-hemolysin has been shown to activate NLPR3 in human and mouse monocytes. Along the same line, to examine the virulence factors associated with NLRC4 activation, we used an isogenic α-hemolysin deficient S. aureus mutant strain, in human and murine cells. To further characterize the role of NLRC4 in response to S. aureus infection, we used THP-1 and THP1-NLRC4 cells (stably overexpressing NLRC4). Compared to THP-1 cells, THP1-NLRC4 produced greater amounts of IL-1β and IL-18 in response to S. aureus (Figure 3.3a, b). In addition, THP1-NLRC4 cells showed increased caspase-1 activity, as evidenced by their marked staining with the fluorescently labeled inhibitor of caspases (FLICA) reagent (Figure 3.3c). We also observed increased expression and phosphorylation of NLRC4 in a dose- and time-dependent manner with S. aureus and purified α-hemolysin in bone marrow-derived macrophages (BMDMs) from WT mice (Figure 3.3d).
Figure 3.3. *S. aureus* activates NLRC4 through PKC-δ to induce IL-1β, IL-18, and necroptosis. Levels of (a) IL-1β and (b) IL-18 in the supernatant of THP-1 and THP1-NLRC4 cells incubated with *S. aureus*, purified α-hemolysin (25 µg/ml), or *Pseudomonas aeruginosa* PAO1 (MOI 10) for 8 hours. (c) Active FLICA-caspase-1 (green) was detected in THP-1 and THP1-NLRC4 cells infected with *S. aureus* (MOI 50) or PBS for 6 hours. 40x. (d) Total NLRC4 and phospho-NLRC4 in cell lysates from WT-BMDMs infected with *S. aureus* (MOI 10 or 50) for 6 or 12 hours or stimulated with commercially purified α-hemolysin (10 or 50 µg/ml) for 4 hours. (e) Immunoblot analysis of total NLRC4 and phospho-NLRC4 in cell lysates from WT-BMDMs infected with *S. aureus* (MOI 10) for 6 or 12 hours with or without one-hour pre-treatment with 10 µM Rottlerin (PKC-δ inhibitor). (f) Immunoblot analysis of pro-caspase-1 and cleaved caspase-1 (p-20) in cell lysates from WT and Nlrc4−/−-BMDMs infected with MOI 10 of *S. aureus* or its isogenic α-hemolysin deficient mutant or stimulated with purified α-hemolysin (50 µg/ml) for 6 hours. (g and h) WT and Nlrc4−/− mice were inoculated with *S. aureus* (5 X 10^7 CFU/mouse). Level of (g) IL-1β and (h) IL-18 measured BALF collected at 12 and 24 hpi. (n= 4-6 mice / pneumonia group, n=3 mice/control group). (i) Cytotoxicity in THP-1 and THP1-NLRC4 cells stimulated with *S. aureus* (MOI 100) for 2 hours with or without 1 hour pre-treatment with 100 uM of necrosulfonamide (NSA). (j) BMDMs from WT mice were left untreated or were incubated with *S. aureus* (MOI 10 or 50) for 6 hours. Cell lysates were immunoprecipitated with ant-NLRC4 Ab or IgG, and then immunoblotted for RIP3 and NLRC4. Whole cell lysates are shown as the input. (k) Immunoblot analysis of phospho-MLKL in the lung homogenates of WT and Nlrc4−/− mice infected with *S. aureus* (5 X 10^7 CFU/mouse). Data from a representative experiment are shown. All experiments were performed three times. *In vitro* experiments have at least four biological replicates. Numeric values in immunoblot indicate ratio of mean grey scale value of protein of interest to its GAPDH control. Statistical significance was determined by unpaired t-test in all experiment except in cell death assay (i), by one-way ANOVA (followed by Bonferroni’s post hoc comparisons). *p<0.05; **p<0.01. Hla: α-hemolysin, ∆Hla: α-hemolysin deficient, MOI: multiplicity of infection, DMSO: dimethyl sulfoxide IP: immunoprecipitation, IB: immunoblotting..
Recently, protein kinase C-δ (PKC-δ) mediated phosphorylation of NLRC4 was shown to be critical for inflammasome activation. As anticipated, S. aureus-induced NLRC4 phosphorylation was dependent on PKC-δ in macrophages, as Rottlerin (PKC-δ inhibitor) reduced expression of phospho-NLRC4 (Figure 3.3e). We also tested whether S. aureus-induced NLRC4 activation leads to caspase-1 processing and IL-1β production, key features of inflammasome activation. To this end, we stimulated BMDMs from WT and Nlrc4−/− mice with S. aureus, its isogenic α- hemolysin deficient mutant, or purified α- hemolysin toxin. As expected, the cleavage of pro-caspase-1 to its active (phospho-20) form was reduced in Nlrc4−/− BMDMs, which was independent of the expression of α-hemolysin by S. aureus (Figure 3.3f). This observation indicates S. aureus may expresses virulence factors other than α-hemolysin that may serve as activators of NLRC4-dependent caspase-1 processing. Furthermore, Nlrc4−/− mice had significant reduction in key inflammasome-dependent cytokines, such as IL-1β and IL-18 (Figures 3.3g, h).

S. aureus has been shown to induce necroptosis in lungs. Therefore, in order to assess the role of NLRC4 in context of necroptosis, we performed cytotoxicity assay in THP-1 and BMDM following S. aureus infection. We detected increased cytotoxicity of THP1-NLRC4 and WT-BMDM compared to THP-1 and Nlrc4−/−BMDMs (Figures 3.3i and 3.4a), providing direct evidence of the involvement of NLRC4 in S. aureus-induced cell death. In addition, pre-treatment with necrostatin-1 (Nec-1: RIP1 inhibitor) or necrosulfonamide (MLKL inhibitor) protected THP1-NLRC4 and WT-BMDM from S. aureus-induced necroptosis respectively (Figures 3.3i and 3.4a). Furthermore, we observed increased co-localization of RIP3 and phospho-MLKL in S. aureus-infected WT-BMDMs compared to Nlrc4−/−BMDMs (Figure 3.4b,c) as well as the co-
immunoprecipitation of RIP3 and NLRC4 indicating their potential interaction in the necrosome complex following *S. aureus* infection (Figure 3.3j). Given that MLKL pore formation is a critical step in necroptosis, we sought to detect the level of phospho-MLKL in *S. aureus*-infected lungs. Corroborating our *in-vitro* results, *Nlrc4*−/− mice show reduced MLKL phosphorylation in their lungs compared to WT controls (Figure 3.3k).

Figure 3.4. *S. aureus* activates NLRC4 through PKC-δ to induce IL-1β, IL-18, and necroptosis. (a) Cytotoxicity in BMDMs, stimulated with *S. aureus* (MOI 100) for 2 hours with or without 1 hour pre-treatment with 100 μM of Necrostatin-1. (b) Representative immunofluorescence images and (c) percentages showing co-expression of RIP3 (green) and phospho-MLKL (red) in BMDMs stimulated with *S. aureus* (MOI 50) or PBS for 12 hours. Nuclei were stained with DAPI (blue). Original magnification, 40x. (d,e) WT and *Nlrc4*−/− mice were inoculated with *S. aureus* (5 × 10⁷ CFU/mouse) and BALF was collected at 12 and 24 hpi. (d) Relative fluorescence units (RFUs) representing LDH release, (e) high mobility group box-1 (HMGB-1) are measured in BALF. (n= 4-6 mice / pneumonia group, n=3 mice/control group). Data from a representative experiment are shown. All experiments were performed three times. *In vitro* experiments have at least four biological replicates. Statistical significance was determined by unpaired t-test in all experiment except in cell death assay (a), by one-way ANOVA (followed by Bonferroni’s *post hoc* comparisons). *p<0.05; **p<0.01. *Hla*: α-hemolysin, MOI: multiplicity of infection, DMSO: dimethyl sulfoxide, LDH: Lactate Dehydrogenase.
In a similar manner, WT mice displayed enhanced LDH release and secretion of necroptosis-related alarmins, such as HMGB-1 in BALF at 12 and 24 hpi (Figure 3.4d,e). Taken together, our results indicate that *S. aureus* phosphorylates NLRC4 in PKC-δ-dependent manner and activation of which leads to production of IL-1β, IL-18, and necroptotic cell death.

3.3.4. NLRC4 deficiency promotes IL-17A-mediated neutrophil recruitment and bacterial clearance.

Neutrophil recruitment is a critical event for bacterial clearance during *S. aureus* infection. *Nlrc4*−/− mice displayed enhanced neutrophil recruitment (Figure 3.2f). Furthermore, neutrophil depletion with anti-Ly6G antibody (IA8) increased the mortality and bacterial burden in *Nlrc4*−/− mice (Figure 3.5a, b). IL-17A has been shown to be critical for neutrophil influx and bacterial clearance at the site of *S. aureus* infection. Thus, we measured the level of IL-17A in BALF and found that IL-17A was increased in *Nlrc4*−/− mice at 12 and 24 hpi (Figure 3.5c). To further implicate IL-17A-mediated neutrophil recruitment in *Nlrc4*−/− mice, administration of recombinant IL-17A in WT mice one-hpi increased neutrophil influx to a level comparable to *Nlrc4*−/− mice (Figure 3.5d). Further, ablation of *Il17a* or blockade with anti-IL-17A antibody in *Nlrc4*−/− mice abrogated neutrophil influx, which was associated with an increased bacterial burden in BALF and lungs (Figures 3.5e-g). To examine if NLRC4 deficiency also alters the phagocytic function of neutrophils, we utilized a phagocytosis assay in bone marrow-derived neutrophils (BMDNs). To our surprise, WT-BMDNs show higher intracellular bacterial survival than *Nlrc4*−/−-BMDNs following *S. aureus* infection (Figure 3.5h).
Figure 3.5. NLRC4 deficiency promotes IL-17A-mediated neutrophil recruitment and bacterial clearance. (a and b) Nlrc4<sup>−/−</sup> mice were injected with anti-Ly6G (IA8) Ab intraperitoneally at 24 and 2 hr prior to intratracheal infection with 2 × 10<sup>8</sup> CFU for survival and 5 × 10<sup>7</sup> CFU for pneumonia. (a) Survival of mice from each group. (n= 10 mice/group). (b) Bacterial burden was determined at 24 hpi. Each symbol represents a single mouse. (c and d) WT and Nlrc4<sup>−/−</sup> mice were inoculated intratracheally with 5 × 10<sup>7</sup> CFU of S. aureus or PBS followed by administration of recombinant murine IL-17A or PBS 1 hour later. (c) Level of IL-17A at 12 and 24 hpi and (d) number of neutrophils at 24 hpi in the BALF were enumerated. (e-g) WT, Il-17a<sup>−/−</sup> and Nlrc4<sup>−/−</sup> mice were inoculated intratracheally with 5 × 10<sup>7</sup> CFU of S. aureus or PBS. Nlrc4<sup>−/−</sup> mice received a single dose of anti-IL17A Ab or IgG at 12 hours prior to infection. (e) Number of neutrophils and (f) bacterial burden in BALF and (g) lungs were determined at 24 hpi. (h-j) BMDNs were isolated from WT, Nlrc4<sup>−/−</sup> and IL-17ra<sup>-/-</sup> mice and stimulated with S. aureus (MOI 1) in the presence or absence of recombinant murine IL-17A (100 or 500 ng/ml) or PBS. (h) Intracellular killing of neutrophils was determined. (i) Net phagocytosis and (j) total ROS production at 1 hpi was determined. (k-n) Wild type, Il-17f<sup>−/−</sup>, and Nlrc4<sup>−/−</sup> mice were inoculated intratracheally with 5 × 10<sup>7</sup> CFU of S. aureus. BALF and lungs were collected at 12 and 24 hpi. (k) Level of IL-17F, (l) number of neutrophils in BALF, and bacterial burden in (m) BALF and (n) lung were determined at 24 hpi. Each symbol represents a single mouse. (n= 5 mice /pneumonia group, n=3 mice/control group). Data from a representative experiment are shown. All experiments were performed three times. Statistical significance was determined by log-rank (a), Man-Whittney (b, h), unpaired t-test (c, k), and one-way ANOVA (followed by Bonferroni’s post hoc comparisons) (d-g, i, j, l-n). *p<0.05; **p<0.01. IgG: immunoglobulin G, ROS: reactive oxygen species.
Further, Nlrc4<sup>−/−</sup>- BMDNs were also effective at particle uptake (Figure 3.5i), suggesting NLRC4 deficiency improved both uptake and clearance in BMDN. Furthermore, the defect in uptake/phagocytosis of WT-BMDN was rescued by pre-treatment with IL-17A, which was associated with increased production of intracellular reactive oxygen species (ROS) (Figures 3.5i, j). Moreover, deficiency of IL-17F, another IL-17 homodimeric cytokine complex that binds to the same receptors (IL-17RA or IL-17RC) <sup>26</sup>, did not alter neutrophil recruitment or bacterial clearance in S. aureus pneumonia (Figures 3.5k-n). Since IL-17A is not directly involved in neutrophil recruitment, we explored whether enhanced recruitment may be due to increased level of CXC chemokines and neutrophil mobilization through IL-17A/G-CSF axis. Accordingly, Nlrc4<sup>−/−</sup> mice also showed increased production of CXCL5, but not CXCL1 and CXCL2, at 12 hpi and higher trend of G-CSF at both 12 and 24 hpi (Figures 3.6a-d). Collectively, our data indicate that the increased level of IL-17A drives the influx and improves the phagocytic abilities of recruited neutrophil in Nlrc4<sup>−/−</sup> mice.

Figure 3.6. Cytokine/Chemokine profiles of Nlrc4<sup>−/−</sup> mice in S. aureus pneumonia. (a-g) WT and Nlrc4<sup>−/−</sup> mice were inoculated intratracheally with 5 x 10<sup>7</sup> CFU of S. aureus or PBS (control). BALF and organs were harvested at the designated time points. Control mice (PBS treated) were sacrificed at 24 hpi. (n= 4-6/group for pneumonia and n=3/group for PBS control). Data are representative of three independent experiments. *p<0.05, ***p<0.001 by unpaired t-test.
3.3.5. NLRC4 deficiency augments IL-17A\(^+\)γδ T cells and neutrophils to regulate neutrophil influx.

Although IL-17A is known as a signature cytokine for CD4\(^+\) T cells (Th17 subsets), emerging evidence has revealed that multiple cell types, such as γδ T cells, NK/NKT cells, neutrophils, innate lymphoid cells, CD8\(^+\) T cells, and colonic epithelial cells, also secrete IL-17A\(^+\)\(^{26}\). To investigate the primary source of IL-17A during the early stage of infection, we utilized intracellular flow cytometry on lung single cell suspensions at 6 and 24 hpi. There was an increase in total and IL-17A producing CD3\(^+\)γδ-TCR\(^+\) cells in the Nlrc4\(^{-/-}\) mice at 6 and 24 hpi (Figures 3.7a-c). However, IL-17A producing neutrophils were higher at 24 hpi in Nlrc4\(^{-/-}\) mice, indicating recruited neutrophils help sustain IL-17A levels and recruit more inflammatory cells (Figure 3.7d and 3.8a). To further implicate γδ T cells in IL-17A-mediated antimicrobial signaling in Nlrc4\(^{-/-}\) mice, we depleted γδ T cells using an anti-γδ-TCR antibody (GL-3). Administration of the GL-3 antibody reduced the level of IL-17A in Nlrc4\(^{-/-}\) mice to a level comparable with WT mice receiving isotype-matched IgG control Ab (Figure 3.7e). Furthermore, depletion of γδ T cells impaired host defense in Nlrc4\(^{-/-}\) mice, as there was decreased neutrophil influx and increased bacterial burden in the BALF of these mice at 24 hpi (Figures 3.7f, g). We next asked if genetic deficiency of NLRC4 alters the production of IL-17A from γδ T cells in addition to their recruitment to the lungs. To this end, we FACS sorted CD3\(^+\) γδ-TCR\(^+\) cells from spleens of naïve mice and stimulated them with S. aureus in the presence or absence of IL-23 \textit{in vitro}. Surprisingly, the γδ T cells from WT and Nlrc4\(^{-/-}\) mice did not show altered production of IL-17A following IL-23 stimulation (Figure 3.7h).
Figure 3.7. NLRC4 deficiency augments IL-17A⁺γδ T cells and neutrophils to regulate neutrophil influx. (a-d) Flow cytometric analysis of the lungs from WT and Nlrc4⁻/⁻ mice either uninfected or intratracheally infected with 5 x 10⁷ CFU of S. aureus. (a) FACS plots of CD3⁺γδ-TCR⁺ cells (larger) and IL-17A⁺CD3⁺γδ-TCR⁺ cells (smaller) at 6 and 24 hpi. Mice receiving PBS were sacrificed at 24-hour time points. (b) The percentage of total γδ T cells, (c) IL-17A producing γδ T cells, and (d) IL-17A producing CD11b⁺Ly6G-1⁺ neutrophils enumerated by flow cytometry in lungs at 6 and 24 hpi. (n= 5 mice / pneumonia group, n= 3 mice / control group). (e-g) WT and Nlrc4⁻/⁻ mice were inoculated intratracheally with 5 x 10⁷ CFU of S. aureus. Nlrc4⁻/⁻ mice received an intraperitoneal injection of anti-γδ-TCR ab (GL-3) or IgG at 12 hours prior to infection. (e) Level of IL-17A, (f) number of neutrophils, and (g) bacterial burden in BALF was determined at 24 hpi. (n= 5 mice / group) (h) The CD3⁺γδ-TCR⁺ cells were sorted from the spleen of the uninfected wild-type and Nlrc4⁻/⁻ mice and incubated with S. aureus (MOI 10) for 18 hours in the presence or absence of IL-23 (40 ng/ml). (h) IL-17A was measured in the supernatant. (i and j) WT and Nlrc4⁻/⁻ mice were inoculated intratracheally with 5 x 10⁷ CFU of S. aureus or PBS. These mice received a single injection of BrdU (1mg/mouse) intraperitoneally 1-hour prior to infection. (i) FACS plot showing BrdU⁺ CD3⁺γδ-TCR⁺ cells and (j) their percentage in the lungs of mice 18 hpi. (n= 5 mice / pneumonia group, n=3 mice / control group). All experiments were performed three times. In vitro experiments have at least four biological replicates. Statistical significance was determined by unpaired t-test (b-d) and one-way ANOVA (followed by Bonferroni’s post hoc comparisons) (e-g) *p<0.05; **p<0.01. BrdU: bromodeoxyuridine.
To examine the possibility of increased $\gamma\delta$ T cell proliferation in lungs of $Nlrc4^{-/-}$ mice, we injected BrdU intraperitoneally before $S. aureus$ infection and stained for BrdU positive $\gamma\delta$ T cells. We found that NLRC4 deficiency did not regulate the proliferation of this cell type in the lungs (Figures 3.7i, j). While neutrophils (CD11b$^+$ Ly6G$^+$), CD4$^+$ T cells, CD8$^+$ T cells, and NK1.1$^+$ cells also produce IL-17A during $S. aureus$ infection, but NLRC4 deficiency did not alter their number in our model (Figures 3.8a-d).

Figure 3.8. Multiple immune cell types produce IL-17A in the lungs during $S. aureus$ pneumonia. (a-d) Flow cytometric analysis of lungs from WT and $Nlrc4^{-/-}$ mice either untreated or intratracheally infected with $5 \times 10^7$ CFU of $S. aureus$. At 24 hpi, lungs were harvested and processed for flow cytometric analysis. (a) Dot plots representation of neutrophils (Ly6G$^+$ CD11b$^+$ cells) and IL-17A$^+$ neutrophils, (b) CD4$^+$ CD3$^+$ cells (CD4 T cells) and IL-17A$^+$ CD4 T cells, (c) CD8$^+$ CD3$^+$ cells (CD8 T cells) and IL-17A$^+$ CD8 T cells, and (d) NK1.1$^+$ CD3$^+$ cells (NK cells) and IL-17A$^+$ NK cells. (e) Purified human neutrophils were stimulated with $S. aureus$ (MOI 10 or 20) or PBS for 12 hours and IL-17A level in supernatant was measured. (n= 5-6 mice/ $S. aureus$ group, 3 mice/PBS group). Data are representative of three independent experiments.
Thus, these data indicate increased frequency of γδ T cells from early stage and neutrophil at late stage in Nlrc4−/− mice produce IL-17A that is critical of neutrophil recruitment to the lungs in S. aureus pneumonia.

3.3.6. NLRC4-driven necroptosis and IL-18 suppress IL-17A-dependent neutrophil recruitment by limiting γδ T cell expansion.

We showed that S. aureus-induced NLRC4 activation results in the production of IL-1β, IL-18, and necroptosis in macrophages and lungs (Figure 3.3). Necroptosis-mediated loss of immune cells in WT mice, but not in the Rip3−/− mice, has been shown to worsen the outcome of S. aureus pneumonia 17. Here, we sought to elucidate whether NLRC4-coupled necroptosis suppress IL-17A-mediated antibacterial defense in pneumonia. To confirm necroptosis in vivo, we administered Nec-1 intraperitoneally to block RIP1 and found that it inhibited LDH release in BALF of WT mice at 24 hpi (Figure 3.9a). To identify if necroptosis alters γδ T cell-derived IL-17A signaling axis, we enumerated percentage of total and IL-17A producing γδ T cells in the lungs of S. aureus infected Mlkl−/− mice. As anticipated, Mlkl−/− mice had a higher percentage of IL-17A+ γδ T cells, but not total γδ T cells, at 24 hpi (Figures 3.9b, c). Furthermore, the genetic ablation or blockade of MLKL with GW806742X impaired IL-17A production, neutrophil recruitment and bacterial clearance from BALF and lungs of Nlrc4−/− mice (Figures 3.9d-g). Previous reports have shown that NLRC4 processed IL-18 is deleterious in P. aeruginosa-induced pneumonia 23,40. In this regard, mice lacking expression of Ili8 had increased total and IL-17A producing γδ T cells compared to WT controls, with numbers comparable to those of Nlrc4−/− mice (Figures 3.9h, i). Similarly, administration of recombinant IL-18 in Nlrc4−/− mice decreased both total and IL-17A producing γδ T cells in the lungs at 24 hpi (Figures 3.9h, i).
Figure 3.9. NLRC4-driven necroptosis and IL-18 suppress 17A-dependent neutrophil recruitment by limiting γδ T cell expansion. (a) WT and Nlrc4<sup>−/−</sup> mice were treated with Necrostatin-1 or DMSO intraperitoneally 12 hours prior to infection with <i>S. aureus</i> (5 x10<sup>7</sup> CFU/mouse). RFU of LDH release in BALF was measured at 24 hpi. (n= 5 mice/group). (b-g) WT, Mlkl<sup>−/−</sup>, and Nlrc4<sup>−/−</sup> mice were infected with <i>S. aureus</i> (5 x10<sup>7</sup> CFU/mouse) intratracheally with administration of GW806742X (MLKL inhibitor) or DMSO intraperitoneally 1 hour prior. BALF and lungs were harvested at 24 hpi. (b) The percentage of total γδ T cells and (c) IL-17A producing γδ T cells in the lungs were determined by flow cytometry. (d) IL-17A level, (e) number of neutrophils, and (f) bacterial burden in BALF, and (g) lungs were quantitated. Each symbol represents a single mouse. (n= 4-6 mice/group). (h-m) WT, Il-18<sup>−/−</sup>, Il-1β<sup>−/−</sup>, and Nlrc4<sup>−/−</sup> mice were infected with <i>S. aureus</i> (5 x10<sup>7</sup> CFU/mouse) intratracheally. At 24 hpi, the lungs were harvested and processed for flow cytometric analysis. (h) The percentage of total γδ T cells and (i) IL-17A producing γδ T cells in the lungs of WT, Il-18<sup>−/−</sup>, and Nlrc4<sup>−/−</sup> mice receiving recombinant murine IL-18 (1 µg/mouse) or PBS 1 hpi were enumerated. (j) The percentage of total γδ T cells and (k) IL-17A producing γδ T cells in the lungs of WT, Il-1β<sup>−/−</sup>, and Nlrc4<sup>−/−</sup> mice. (l) The number of neutrophils in BALF and (m) bacterial burden in the lungs at 24 hpi. Each symbol represents a single mouse. (n= 4-6/group). Data from a representative experiment are shown. All experiments were performed three times. Statistical significance was determined by one-way ANOVA (followed by Bonferroni’s post hoc comparisons) in all experiments. *p<0.05; **p<0.01.
However, deficiency of IL-1β, a key NLRC4-dependent cytokine, did not alter the frequency of γδ T cells in *S. aureus* pneumonia (Figures 3.9j, k). Intriguingly, IL-1β−/− mice had enhanced neutrophil recruitment, similar to *Nlrc4−/−* and *IL-18−/−* (Figures 3.9l). Taken together, our results indicate that NLRC4-mediated necroptosis and IL-18 act synergistically, independent of IL-1β, to suppress the recruitment of γδ T cells, which dampens IL-17A-mediated antibacterial defense in *S. aureus* pneumonia.

3.3.7. Blockade of NLRC4 signaling or necroptosis improves host defense in *S. aureus* pneumonia.

Like other inflammasomes, activation of NLRC4 results in cleavage of caspase-1 leading to production of IL-1β and IL-18 during *S. aureus* infection (Figure 3.3). To determine if deficiency of caspase-1 (downstream of NLRC4) results in a similar outcome in *S. aureus* pneumonia, mice lacking expression of *Casp1/11* or pre-treated with Ac-yvd-cmk (caspase-1 inhibitor) were infected with *S. aureus*. As compared to WT controls, mice lacking *Casp1/11* or WT mice pre-treated with Ac-yvd-cmk demonstrate reduced bacterial burden in the BALF and lungs as well as improved survival (Figures 3.10a-c). To determine if blockade of necroosome components also had a similar effect in *S. aureus* pneumonia, we infected mice pre-treated with Nec-1 with *S. aureus*. As anticipated, blockade of RIP1 with Nec-1 enhanced neutrophil recruitment and subsequent bacterial clearance in the BALF, which was associated with improved host survival rates (Figures 3.10d-f). As *S. aureus*-induced NLRC4 activation was dependent on PKC-δ (Figures 3.3), we investigated the role of PKC-δ inhibition in host defense. To this end, mice were pretreated with Rottlerin or DMSO at 12 hours prior to infection. As expected, Rottlerin pre-treated mice show reduced IL-1β production, increased neutrophil influx and bacterial clearance at 24 hpi (Figures 3.10g-i). Together,
our results demonstrate the blockade of NLRC4 and necroptosis signaling improve pneumonia outcome in *S. aureus* pneumonia.

Figure 3.10. Blockade of caspase-1 and RIP1 improve the outcome of *S. aureus* pneumonia. (a-c) WT, Casp1/11^-/-^ and Nlrc4^-/-^ mice were treated with Ac-yvad-cmk (150 µg/mouse) or DMSO i.t. 12 hours prior the infection with *S. aureus* (5 x 10^7 CFU or 2 x 10^8 CFU/mouse) i.t. (a) Bacterial burden in the lungs, (b) BALF at 24 hpi, and (c) survival over 100 hours were determined. (d-f) WT and Nlrc4^-/-^ mice were treated with Necrostatin-1 (300 µg/mouse) or DMSO i.p. 12 hours prior to infection with *S. aureus* (5 x10^7 CFU/mouse for pneumonia or 2 x 10^8 CFU/mouse) intratracheally. (d) Number of neutrophils, (e) bacterial burden in BALF at 24 hpi, and (f) survival over 100 hours were determined. (g-i) WT mice were treated with Rottlerin (200 µg/mouse) or DMSO i.p. 12 hours prior to infection with *S. aureus* (5 x 10^7 CFU/mouse) i.t. (g) Level of IL-1β, (h) number of neutrophils, (i) bacterial burden in lungs and BALF at 24 hpi were determined. Data are representative of three independent experiments. Statistical significance was determined by one-way ANOVA (followed by Bonferroni’s *post hoc* comparisons (a, b, d, e), log-rank test (c,f), unpaired t-test (g, h), and Man-Whittney (i). *p<0.05; **p<0.01; ***p<0.001. (n= 4-6/group for pneumonia and n= 10/group for survival).
3.4. Discussion

The pathogenesis of Gram-positive (*S. aureus*) pneumonia is mediated by its several virulence factors, such as exotoxins and surface proteins. These virulence factors target different innate signaling molecules in infiltrating leukocytes and resident epithelial cells. Moreover, growing evidence indicates that *S. aureus* is able to exploit innate signaling pathways, as mice deficient in *Adam10, Nlrp3, Nod2, Ifnr-1*, and *Tnfr* expression showed augmented host defense against this pathogen \(^{10-14}\). Although toxin-induced necroptotic lung damage has been implicated in the pathogenesis of *S. aureus* pneumonia \(^{17}\), the precise mechanism underlying the subversion of host immunity is largely unknown. We report that NLRC4 activation leads to necroptotic cell death and IL-18 production, which in turn impedes host defense in *S. aureus* pneumonia. Furthermore, we demonstrate a novel mechanism by which NLRC4-driven necroptosis and IL-18 production suppress γδ T cell-derived IL-17A-dependent neutrophil recruitment during *S. aureus* infection. Finally, we show that blockade or genetic deficiency in NLRC4 signaling cascade and necroptosis pathway improve host defense in the context of *S. aureus* pneumonia.

Pulmonary host defense regulated by the NLRC4 is protective against Gram-negative pathogens, such as *K. pneumoniae* \(^{21}\), *L. pneumophila* \(^{41,42}\) and *B. pseudomallei* \(^{43}\), but deleterious against *P. aeruginosa* \(^{23,44}\). In contrast, our study reports NLRC4 activation is deleterious in the context of Gram-positive pathogens, as *Nlrc4* \(^{-/-}\) mice show improved host defense and survival. In accordance with this, activation of other NOD-like receptors has also been shown to be detrimental during *S. aureus* infection. For example, *Nod2* \(^{-/-}\) and *Nlrp3* \(^{-/-}\) mice are less susceptible to *S. aureus* pneumonia \(^{12,13}\).
Because NLRP3 recognizes a broad range of pathogens, including *S. aureus* \(^{45}\), NLRP3 activation was thought to be critical for the pathogenesis of *S. aureus* pneumonia. However, *Nlrp3* \(^{-/-}\) mice do not have a remarkable phenotype in *S. aureus* pneumonia, suggesting involvement of an NLRP3-independent host defense mechanism in the response to this pathogen \(^{13}\). We showed both myeloid and resident cells express NLRC4 during *S. aureus* pneumonia. Our bone marrow chimeras reveal that NLRC4 expression in both compartments is detrimental with regards to bacterial control. It is important to note that WT $\rightarrow Nlrc4^{-/-}$ group (hematopoietic) had defective bacterial control in the lungs compared to other chimeric groups. Since neutrophil are a major player of *S. aureus* clearance and *Nlrc4^{-/-}* neutrophils are more efficient in controlling bacterial burden, it is possible that NLRC4 expression in myeloid cells of WT $\rightarrow Nlrc4^{-/-}$ (only hematopoietic expression) group may have dampened phagocytic function of these cells.

Based on data from experiments in THP-1 cells and BMDMs, the NLRC4 was important for caspase-1 activity and IL-1$\beta$ production in response to *S. aureus* infection. Our findings also indicate that $\alpha$-hemolysin induces phosphorylation of the NLRC4, which is a PKC-$\delta$-dependent process like in *S. typhimurium*-induced NLRC4 activation in macrophages \(^{38}\). However, $\alpha$-hemolysin expression in *S. aureus* was dispensable for caspase-1 processing. In addition, pulmonary infection with *K. pneumoniae* or *P. aeruginosa* (non-flagellated strains) has been shown to activate the NLRC4 in vivo \(^{21,22}\), indicating that virulence factors other than flagellin can activate NLRC4. In this regard, recent reports related to the involvement of NLRC4 activation in obesity-associated tumors, in progression of melanoma tumors, and in diabetic nephropathy suggest endogenous host proteins may serve as signals for NLRC4 activation \(^{46-48}\). Therefore, it is
possible that unidentified bacterial components of S. aureus or host-derived proteins may act as homologues to known NLRC4-activating bacterial signals.

A number of S. aureus toxins are known to activate necroptosis, a highly pro-inflammatory mode of cell death. S. aureus-derived pore-forming toxins like Hla, Leukocidin AB, and PSMs leading to cytotoxicity has been well characterized. Further, necroptosis results in irreparable loss of alveolar macrophages and anti-inflammatory signals, impeding clearance of S. aureus. In addition to reduced phosphorylation of MLKL in the lungs of Nlrc4−/− mice, we also found that NLRC4 interacts with RIP3 suggesting a potential role of NLRC4 in necrosome formation. However, it remains unknown if NLRC4 activation also alters the K+ efflux or contributes to membrane destabilization leading to cell lysis. Unlike Nlrp3−/−, Nod2−/−, or Rip3−/− mice infected with S. aureus, the central feature of protection in Nlrc4−/− mice is their ability to recruit sufficient numbers of neutrophils to the lungs, which is impaired in WT mice. In Nlrc4−/− mice, this occurs through the increased frequency of γδ T cells, which provide the IL-17A signal to drive neutrophil influx. Previous studies have shown that IL-17A stimulates granulopoesis in bone marrow and regulates expression of CXC chemokines in lungs to recruit neutrophils. In our model, it appears that IL-17A may have contributed to neutrophil recruitment via increased CXCL5 and G-CSF production in Nlrc4−/− mice. While numerous immune cells can produce IL-17A at different stages of infection, many studies have highlighted the importance of early IL-17A production by γδ T cells during infection with S. aureus and other extracellular bacteria. In addition to γδ T cells, the increased frequency of IL-17A+ neutrophils at 24 hours post-infection may explain that neutrophils are also involved in
the recruitment through an autocrine loop. Interestingly, MLKL deficiency or blockade of necroptosis with Nec-1 or MLKL inhibitors improves bacterial clearance, which is also associated with an increased frequency of IL-17A+ γδ T cells and neutrophil recruitment. In addition to necroptosis, NLRC4-induced IL-18 production was found to suppress the γδ T cell-IL-17A-axis, as IL-18 deficiency resulted in improved neutrophil recruitment and clearance of *S. aureus*. Similar to our results, IL-18 has also been shown to impede host defense in pneumonia and other disease models. However, IL-1β, another NLRC4-dependent cytokine, was surprisingly found to be dispensable for this pathway. Thus, these two seemingly disparate NLRC4-dependent mechanisms, the necroptosis and IL-18, work synergistically to impede neutrophil recruitment in *S. aureus* pneumonia. However, future studies will be required to determine how deficiency of necroptosis and IL-18 leads to the increased frequency of IL-17A producing γδ T cells as early as 6 hpi.

The precise mechanism through which PKC-δ-dependent NLRC4 phosphorylation occurs following Gram-positive (*S. aureus*) infection remains unclear. It should be noted that a wide range of pathogens or host-derived factors, along with environmental pollutants, can activate NLRP3. Along the same lines, it is possible that NLRC4 may recognize host-derived ligands during bacteria-induced inflammation in addition to recognizing pathogens /or their components. For example, since *S. aureus* -derived pore forming toxins destabilize the membrane of cells, it is possible that endogenous inflammatory ligands are released and subsequently activates NLRC4. However, elucidation of the specific ligands and mechanisms responsible for NLRC4 activation during Gram-positive *S. aureus* infection of the lung will require future studies.
3.5. References


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4.1. Introduction

Sepsis, a dysregulated systemic host inflammatory response to infection with organ failure, is leading cause of death in non-coronary intensive care unit worldwide\textsuperscript{1-3}. Despite the improvement in antibiotic therapies and critical care management, severe sepsis and sepsis induced organ failure still accounts for 30-50\% mortality in critically ill patients\textsuperscript{1, 4}. Uncontrolled activation of host response accompanied with loss of immune homeostasis and subsequent immune suppression are critical pathogenesis of sepsis\textsuperscript{5}. Furthermore, septic survivor patients have been known to develop long-term cognitive impairment and functional disabilities with significant social and economic implications\textsuperscript{6}.

Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins are involved in pathogen recognition and promotion of host inflammatory responses. Of note, NLRP3 is critical player of innate immunity, and surprisingly \textit{Nlrp3}\textsuperscript{-/-} mice are resistant to polymicrobial sepsis\textsuperscript{7, 8}. NLRP3 intensifies acute inflammation, impairs synthesis of lipid resolvers, and phagocytosis during sepsis\textsuperscript{7, 8}. Additionally, the neutralization of inflammasome-dependent cytokine (IL-1\beta and IL-18) has additive effect on preventing LPS-induced mortality\textsuperscript{9}. As lymphocyte depletion/dysfunction being a major observation in sepsis, the role of NLR in lymphocyte depletion is largely remains unknown. NLRC4 is widely expressed NLR in hematopoietic compartment and senses bacterial pathogens\textsuperscript{10-12}. Given reports of NLRC4 modulating T cells responses in different disease setting, the role of NLRC4 in sepsis-induced T cells dysfunction and overall host defense is elusive.
In this study, we utilized cecal-ligation and puncture method in $Nlrc4^{-/-}$ mice to induce polymicrobial sepsis. Our result shows NLRC4 deficiency improves host survival and bacterial clearance during sepsis. $Nlrc4^{-/-}$ mice are protected from sepsis induced cytokine storm and macrophage dysfunction. $Nlrc4^{-/-}$ mice are protected from sepsis induced cytokine storm, macrophage dysfunction, and T cell exhaustion. Collectively, our data indicates NLRC4 contributes to sepsis-induced hyper-inflammation and T cell exhaustion, and the pharmacological inhibition of NLRC4 may be an attractive therapeutic target in treating septic patients.

4.2. Methods

4.2.1. Animals

$Nlrc4^{-/-}$ mice, generated as described previously\(^\text{13}\), were obtained from Millennium Pharmaceuticals and backcrossed onto the C57BL/6J genetic background at least 10 times. Age- and sex-matched C57BL6/J mice were purchased from Jackson Laboratory and used as WT controls. The Institutional Animal Care and Use Committee of the Louisiana State University approved the sepsis related animal protocol (16-028).

4.2.2. Cecal ligation and puncture method

Cecal ligation and puncture method, a murine model of polymicrobial sepsis, was utilized to induce a moderate sepsis (50% mortality) in mice as described previously\(^7\). In brief, mice were anesthetized with the Xylazine/Ketamine mix, abdomen was shaved, and midline incision was made. Cecum was located, exteriorized, ligated away from the blunt end, punctured with a 21-guage needle, a small amount of fecal content was extruded, and the cecum was repositioned in peritoneal cavity. Sham-operated mice underwent the
exteriorization of cecum only, no ligation and puncture. All animals received 1 ml of warm saline injection subcutaneously to compensate fluid loss in procedure.

4.2.3. E.coli-induced Sepsis

WT (control) and Nlrc4−/− mice were injected with E.coli (5 x10⁸ CFU/kg BW) intraperitoneally and survivals were monitored for 10 days.

4.2.4. Peritoneal lavage, cell count and bacterial burden

Peritoneal cavity lavage was collected with 7 ml of warm PBS containing heparin/dextrose as described previously⁷. Number of total cells, neutrophils, and macrophages were counted with use of haemocytometer and Diff-quick staining under a light microscope⁷, 11, 14. Cell free supernatants from peritoneal lavage fluid were stored for ELISA. At indicated time points, spleen, lungs, and liver (right lobe) were collected, homogenized in 1 ml of PBS, and 20 µl of serial dilutions of homogenates were plated on tryptic soy agar plates. The number of colonies was counted following incubation at 37 °C overnight.

4.2.5. In vivo depletion method

Macrophages were depleted with an intraperitoneal injection of 200 µl of Clodronate liposomes (www.clodronateliposomes.org) as described previously¹⁵. In some experiments, mice were injected with 100 µg of anti-IFN-γ mAb (Biolegend, clone XMG1.2) or 150 µg of anti-CD8α mAb (Bioxcell, 2.43), or 150 µg of anti-NK1.1 mAb (Bioxcell, PK136) intraperitoneally 18-hr prior and at the time of CLP induction as described previously¹⁴, 16. Control mice were injected with PBS liposomes or control rat IgG2a, as appropriate.
4.2.6. ELISA and BUN

IL-1β, TNF-α, MCP-1 (eBioscience/Invitrogen) and CXCL1, CXCL2 (R&D systems) were measured by ELISA as described in their respective protocols. Blood urea nitrogen (Invitrogen) was measured in serum as described in its commercial kit.

4.2.7. Phagocytosis

Intracellular bacterial burden of peritoneal macrophages were measured as described previously\textsuperscript{11}. Briefly, thioglycolate-elicited peritoneal macrophages were incubated with multiplicity of infection (MOI 10) of \textit{S. aureus} for 1 hour. Medium containing gentamicin (250 µg/ml) was replaced for 2 hours, washed, and thereafter incubated with a medium containing gentamicin (50 µg/ml) to kill extracellular bacteria.

4.2.8. Flow cytometry

For flow cytometric analysis, peritoneal fluid and spleen were harvested. Spleen was excised, minced, RBC-lysed, and washed to obtain a single cell suspension. The following surface marker antibodies were purchased from Biolegend otherwise noted: anti-CD45 (clone; 30-F11, eBioscience), CD11b (M1/70), F-4/80 (BM8), MHCII (M5/114.15.2), CD86 (24F), CD80 (16-10A1), PD1 (29F.1A12), CD3 (17A2), CD4 (GK1.5), CD8α (53-6.7), γδ-TCR (GL3), and NK1.1 (PK136). For intracellular staining, cells were incubated with cell activation cocktail (Biolegend) for 4-5 hours in presence of 3 mg/ml of brefeldin A (eBioscience). The BD Cytofix/Cytoperm Kit was used to permeabilize and fix cells before staining with IFN-γ (XMG1.2, eBioscience). Isotype and auto fluorescence controls were used for compensation. Cells were acquired on a Fortessa X20 (BD Biosciences). FlowJo 10 (Treestar) was used to analyze data.
4.2.9. Co-housing

Co-housing experiments were performed as described previously\textsuperscript{14, 17}. In brief, age- and sex-matched WT and \textit{Nlrc4}\textsuperscript{-/-} mice were mixed in 1:1 ratio along with their bedding and co-housed for 4 weeks prior to infection.

4.2.10. 16S rDNA-based phylogenetics.

Stool samples were collected for the sequencing of 16S rDNA and phylogenetic analysis. The V4 hypervariable region was amplified using 515F: GTGCCAGCMGCCGCGGTAA and 805R: GGACTACHVGGGTWTCTAA 16S primers along with standard Illumina sequencing adapters. Amplicons were indexed, pooled, and sequenced on an Illumina MiSeq using a V2 2x250 paired end (500 Cycle) sequencing kit. Raw sequencing reads were processed through the DADA2 pipeline\textsuperscript{18} using trimLeft = 20 to remove 16S primers and truncLen = 210 for forward reads and 170 for reverse reads to discard low sequencing quality tails. Inferred sequence variants were merged and placed into a sequence variant table. Diversity metrics and taxonomic classification were performed using the QIIME\textsuperscript{2} pipeline\textsuperscript{19} and Ribosomal Database Project Classifier\textsuperscript{20}.

4.2.11. Statistical analysis

Data are expressed as means ± standard error of mean (s.e.m.) unless otherwise stated. Prism 7.0a software (GraphPad Software Inc.) was used for statistical analyses. The unpaired t-test and one-way ANOVA (followed by Bonferroni’s \textit{post hoc} comparisons) were used to analyze differences between groups, as appropriate. Survival curves (Kaplan-Meier plot) were compared using log-rank tests. A \textit{P}-value of *\textless{}0.05, **\textless{}0.01, and ***\textless{}0.001 was considered significant.
4.3. Results

4.3.1. NLRC4 deficiency improves host survival and bacterial clearance in sepsis.

To understand the role of NLRC4 in sepsis, WT and Nlrc4−/− mice were subjected to CLP-induced poly microbial sepsis and survivals were monitored for 10 days. Nlrc4−/− mice displayed reduced mortality compared to WT controls after CLP (Figure 4.1A).

Figure 4.1. NLRC4 deficiency improves survival and bacterial clearance in sepsis. (A) WT (control) and Nlrc4−/− mice were subjected to CLP-induced polymicrobial sepsis and survivals were monitored for 10 days. A Kaplan Meier plot is used to show survival of mice from each group. (n= 12-18 mice/group). (B) WT (control) and Nlrc4−/− mice were injected with E.coli (5 x10^8 CFU/kg BW) survivals were monitored for 10 days. A Kaplan Meier plot is used to show survival of mice from each group. (n= 8-9 mice/group). (C-F) The bacterial burden in the (C) peritoneal fluid, (D) liver, (E) spleen, and (F) lung was quantitated at 12 and 24 hpi. (5-9 mice/group). Each symbol represents a single mouse (D-F). Statistical significance was determined by log-rank (A,B), unpaired t-test (C-F), *p<0.05; **p<0.01; ***p<0.001. CFU: colony forming unit.
Similarly, reduced mortality of \( Nlrc4^{+/−} \) mice was also verified in \( E.\) \( coli \)-induced peritonitis (Figure 4.1B). Further, \( Nlrc4^{−/−} \) mice had significantly less bacterial burden in peritoneal lavage fluid and spleen along with reduced dissemination to lungs after 12 and 24 hours after CLP (Figure 4.1C-F). Collectively these data suggests that NLRC4 activation impairs bacterial clearance and survival in sepsis.

4.3.2. NLRC4 deficiency protects from hyper-inflammation and cytokine storm during CLP-sepsis

NLRC4 regulates pro-inflammatory response to infections. To evaluate if NLRC4 mediates cellular recruitment during CLP-induced sepsis, we performed lavage of peritoneal fluid of WT and \( Nlrc4^{−/−} \) mice following CLP sepsis. \( Nlrc4^{−/−} \) mice displayed reduced numbers of total cells and neutrophils compared to WT mice at 12 and 24-hour post CLP (Figure 4.2A,B). However, \( Nlrc4^{−/−} \) mice recruited higher numbers of macrophages at 12-hour post CLP (Figure 4.2C). Given pro-inflammatory cytokine storm are detrimental in sepsis\(^{21} \), we evaluated their levels in peritoneal lavage fluid after 12 and 24 hours after CLP. As expected, \( Nlrc4^{−/−} \) mice show reduced inflammasome-dependent (IL-1β) and other cytokines/chemokines (TNF-α, MCP-1, CXCL1, CXCL2) (Figure 4.2D-H). Since sepsis-induced lethality is accompanied with severe organ damage, we subsequently investigated blood urea nitrogen (BUN). WT mice had increased level of BUN at 12 and 24 hours after CLP (Fig 4.2I). Collectively these data suggests NLRC4 activation contributes to hyper inflammatory response and organ damage in sepsis.
Figure 4.2. NLRC4 protects from hyper inflammation and cytokine storm sepsis. (A-I) WT (control) and Nlrc4<sup>-/-</sup> mice were subjected to CLP-induced polymicrobial sepsis. (A) Total numbers of cells, (B) neutrophils, (C) macrophages were enumerated in peritoneal lavage fluid at 12 and 24-hour post CLP. Level of (D) IL-1β, (E) TNF-α, (F) MCP-1, (G) CXCL1, (H) CXCL2 were measured in peritoneal fluid at 12 and 24-hour post CLP. (I) BUN level was measured in serum. (5-9 mice/group). Statistical significance by Unpaired t-test (A-I), *p<0.05; **p<0.01; ***p<0.001. CFU: colony forming unit.

4.3.3. Nlrc4<sup>-/-</sup> mice have limited dysbiotic microbiome, which are neither sepsis promoting nor transferable to co-housed WT mice.

Microbiota dysbiosis are known to increase host susceptibility in multiple human diseases, including sepsis<sup>22,23</sup>. Although debatable, recent studies have shown that loss of NLRP6 and ASC inflammasomes in mice results in microbiome dysbiosis, which are disease promoting and transferable to co-housed mice in the experimental model of
colitis and colitis-associated cancer, hepatic steatosis, and obesity\textsuperscript{24-26}. The role of NLRC4 in the gut health as shown in various colitis models is discrepant \textsuperscript{27-29}. We speculate these discrepancies can be reconciled by considering microbiome differences in $Nlrc4^{-/-}$ mice, thereby urging the routine assessments of microbiome in the NLRC4 biology. Additionally, the polymicrobial septic-peritonitis in CLP is gut-propagated illness model, which lends itself well to examine the effect of gut microbiome in disease phenotype. To this end, we collected stool samples from WT and $Nlrc4^{-/-}$ mice for the sequencing of 16S rDNA and phylogenetic analysis. Compared with naïve WT mice, Naïve $Nlrc4^{-/-}$ mice had higher bacterial community richness as their alpha diversity metrics i.e, observed species, Chao1, and Shannon indices are significantly higher (Figure 4.3A-C). However, the induction of CLP-induced sepsis in $Nlrc4^{-/-}$ mice before or after co-housing resulted similar bacterial community richness as in their respective WT mice (Figure 4.3A-C). However, a PCoA analysis of microbial OTUs using weighted and un-weighted UniFrac distance revealed a limited difference in the microbial community composition between WT and $Nlrc4^{-/-}$ mice (Figure 4.3 D-E). Consistent with previous reports\textsuperscript{24}, taxonomic analysis showed $Nlrc4^{-/-}$ mice had increase in the abundance of species $Prevotella$ and $Paraprevotellacea$ following the CLP, which are absent in WT controls (Figure 4.3F). Unlike in $Nlrc4^{-/-}$ mice, septic WT mice had increased abundance of species $Akkermansia$ (Figure 4.3F).
Figure 4.3. *Nlrc4*−/− mice have limited dysbiotic microbiome, which are neither sepsis promoting nor transferable to co-housed WT mice. (A-F) WT and *Nlrc4*−/− mice were either naïve, or CLP-operated, or co-housed and CLP-induced. At 24-hour post CLP or sham, fecal samples were collected and the microbiota was analyzed using 16S rDNA-based phylogenetics method. Observed species (A), Chao1 (B), and Shannon index (C) are shown. (D,E) PCoA plots based on the weighted and unweighted Unifrac distance matrix are presented. (D) Heat maps showing taxonomic composition of microbial communities at the genus level. (n=10 mice/group). (G-I) Total number of white blood cells (G) and bacterial burden in PF (H) and spleen (I) were enumerated at 24-hour post-CLP of separately or co-housed mice (n=5–6 mice/group). Statistical significance was determined by unpaired t-test (G-J). *, p<0.05; **, p<0.001; ****, p<0.0001. Error bars represent SEM.

To examine whether differences in microbiome observed between WT and *Nlrc4*−/− mice are disease promoting and transmissible, we co-housed WT and *Nlrc4*−/− mice for eight weeks as described previously. Similar to phenotype observed with the separately housed mice, co-housed *Nlrc4*−/− mice also had reduced inflammation and fewer bacteria in the peritoneal cavity and spleen compared to the co-housed WT mice (Figure 4.3G-I). Additionally, co-housing influences neither host resistance in *Nlrc4*−/−
mice nor susceptibility in WT mice following CLP, suggesting microbiome are not disease promoting and transmissible (Figure 4.3G-I). To further confirm dispensable role of microbiome in host resistance, we depleted microbiome in Nlrc4−/− mice with broad-spectrum antibiotics and induced CLP. These results suggest that NLRC4 regulates host defense in CLP-induced sepsis independently of its microbiome composition.

4.4. NLRC4 mediates sepsis-induced immune dysfunction in macrophages.

As macrophage/monocyte deactivation is a significant observation of sepsis-induced immune dysfunction30, we depleted macrophages to investigate their contributions in survival of Nlrc4−/− mice. Surprisingly, macrophages depleted Nlrc4−/− mice lost their survival advantage, suggesting critical role of Nlrc4−/− macrophages in host survival (Figure 4.4A). However, macrophage depletion in WT control did have impact in survival (Figure 4.4A). To further confirm importance of Nlrc4−/− macrophages, we adoptively transferred Nlrc4−/− macrophages to WT mice and vice versa 24 hours before CLP. WT and Nlrc4−/− mice complemented with Nlrc4−/− macrophages displayed reduced bacterial burden in the peritoneal fluid compared to mice complemented with WT macrophages (Figure 4.4B). As defective bacterial clearance is associated sepsis-induced macrophage dysfunction, we investigated whether NLRC4 deficiency improved the phagocytic function of peritoneal macrophages isolated mice. Nlrc4−/− peritoneal macrophages displayed enhanced clearance of S. aureus under gentamicin protected phagocytosis assay (Figure 4.4C).
4.4. NLRC4 mediates sepsis-induced immune dysfunction in macrophages. (A) WT and Nlrc4−/− mice were subjected to CLP-induced polymicrobial sepsis with or without 48 hour prior clodronate-mediated macrophage depletion and survivals were monitored for 10 days. A Kaplan Meier plot is used to show survival of mice from each group. (n= 9-17 mice/group). (B) WT and Nlrc4−/− mice were adoptively transferred with WT or Nlrc4−/− peritoneal macrophages 24 hours before CLP. Bacterial burden in PF is shown. (n=5-6 mice/group) (C) Peritoneal macrophages were isolated and phagocytosis was performed with S.aureus (MOI: 10). Intracellular bacterial burden is shown at indicated time points. (D,E) WT and Nlrc4−/− mice were subjected to CLP-induced polymicrobial sepsis. Representative flow cytometry histograms (D) and mean frequency interval (MFI) (E) of surface markers on the peritoneal macrophages (CD11b+F4/80+) are presented (n=5-6 mice/group). Statistical significance was determined by log rank test (A), ANOVA followed Bonferroni’s multiple comparison tests (B,E), unpaired t-test (C). *, p<0.05; **, p<0.001; ****, p<0.0001. Error bars represent SEM.

To identify molecular signatures of macrophage dysfunction in our model, we investigated changes in MHCII, co-inhibitory molecules/receptors (PD-1, PD-L1/2), costimulatory molecules (CD86, CD80) in peritoneal macrophages31,32. MHCII (HLA-DR in human) down-regulation is a major observation in sepsis-induced immunoparalysis31. Peritoneal macrophage (CD11b+F4/80+) from WT mice had decreased MHCII expression compared to Nlrc4−/− macrophages following CLP (Figure
4.4D,E). Additionally, Peritoneal macrophages from septic \textit{Nlrc4}\textsuperscript{−/−} mice were protected from sepsis-induced loss of CD86, but no changes in CD80 and PD1 were seen (Figure 4.4D,E). Taken together, these data suggest that NLRC4 deficiency protect macrophages from sepsis-induced dysfunction.

4.5. NLRC4 mediates the loss and dysfunction of lymphocytes in polymicrobial sepsis.

Lymphocytes, which produce IFN-\(\gamma\) for macrophage activation, are profoundly lost and dysfunctional in sepsis\textsuperscript{33}. To investigate whether NLRC4 modulates sepsis-induced lymphoid depletion, we investigated the dynamic changes of lymphoid cells in the spleen of \textit{Nlrc4}\textsuperscript{−/−} mice. Compared to WT mice, \textit{Nlrc4}\textsuperscript{−/−} mice were protected from sepsis-induced depletion of CD4 and CD8 T cells at 24-h post CLP (Figure 4.5A-C). Furthermore, septic \textit{Nlrc4}\textsuperscript{−/−} mice had increased level of CD4 and CD8 T cells compared to septic WT mice at 24-h post CLP (Fig 4A and B). NK cells are also important player in sepsis as they produce IFN-\(\gamma\), which has a double-edged function in sepsis\textsuperscript{34}. Compared to WT mice, \textit{Nlrc4}\textsuperscript{−/−} mice had increased frequency of NK1.1 cells at 24-h post CLP (Figure 4.5D). Furthermore, CD8 T cells, but not CD4 T cells, depleted \textit{Nlrc4}\textsuperscript{−/−} mice had increased bacterial burden in peritoneal fluid and spleen compared to control \textit{Nlrc4}\textsuperscript{−/−} mice (Figure 4.5E,F). In addition to the depletion, sepsis impairs the effector function of lymphocytes. IFN-\(\gamma\) plays a critical role in modulating and restoring macrophage function in sepsis\textsuperscript{31,35,36}. Flow cytometric method revealed that INF-\(\gamma\)+CD8 T cells were significantly higher in the \textit{Nlrc4}\textsuperscript{−/−} mice at 24 hours after CLP (Figure 4.5G). Taken together, our data suggests NLRC4 contributes to loss and dysfunction of CD8 T cells in polymicrobial sepsis.
4.5. NLRC4 mediates the loss and dysfunction of lymphocytes in polymicrobial sepsis. (A-D, G) WT and Nlrc4−/− mice were subjected to CLP-induced polymicrobial sepsis (n=5 mice/group). Representative FACS plots of CD4, CD8 T cells (A), percentage of CD4 T cells (B), percentage of CD8 T cells (C), and percentage of NK cells (D) at 24-hour post-CLP-induced sepsis. (E-F) Nlrc4−/− mice were injected with anti-NK1.1, anti-CD4, anti-CD8 mAb or IgG prior CLP. Bacterial burden at spleen (E) and peritoneal fluid (F) were enumerated at 24-hour post-CLP-induced sepsis. (n=5-6 mice/group). Statistical significance was determined by unpaired t-test (B-D, G), ANOVA followed Bonferroni’s multiple comparison tests (E, F). *, p<0.05; **, p<0.001; Error bars represent SEM.

4.4. Discussion

Septicemia and septic shock is the leading cause of deaths in non-coronary intensive care units². Sepsis, a dysregulated host inflammatory response to infection, is a devastating disorder with organ dysfunction³. Molecular and cellular pathogenesis of sepsis involves hyper inflammation, cytokine storm, and cellular dysfunction³⁷,³⁸. Numerous attempts of human clinical trials with various specific anti-cytokine therapies have failed because the progression of sepsis is accompanied by activation of several redundant pro- and anti-inflammatory pathways leading to organ damage³⁹. A better understanding of dysregulated host defense mechanisms may reveal unique molecular pathways that may be exploited as a novel biomarker for diagnosis and treatment of sepsis.
NLRC4 is a cytoplasmic pathogen sensor\textsuperscript{10} and shown to mediates host inflammatory response during various diseases\textsuperscript{11, 12}. In this study, we report that activation of NLRC4 has detrimental role in CLP-induced polymicrobial sepsis. \textit{Nlrc4}\textsuperscript{-/-} mice have survival advantage, reduced cytokine storm, and enhanced bacterial clearance compared to WT mice during sepsis. NLRC4-dependent IL-1\(\beta\) and IL-18 cytokines are pro-inflammatory and involves in hyper inflammation and activation of leukocyte \textsuperscript{10, 11, 40, 41}. \textit{Nlrc4}\textsuperscript{-/-} mice displayed reduced level of IL-1\(\beta\) and other pro-inflammatory cytokines/chemokines. It is possible that the activation of NLRC4/IL-1\(\beta\)/IL-18 axis in sepsis is detrimental. Given NLRs are redundant in activating inflammasome cascades, further studies on relative contribution of NLRC4 using double or triple knock out mice in sepsis are desirable.

Host microbiome are involved in shaping host immune responses in different disease models\textsuperscript{23, 24}. Although debatable, the genetic deletion of NLRP6 and ASC results in microbiome dysbiosis and mediates susceptibility of these knock out mice to colitis and colorectal cancer\textsuperscript{24, 26}. Several studies reported the divergent role for NLRC4 in the gut health \textsuperscript{27-29}. We report that \textit{Nlrc4}\textsuperscript{-/-} mice have limited dysbiotic microbiome, which are neither sepsis promoting nor transferable to co-housed WT mice. Co-housed \textit{Nlrc4}\textsuperscript{-/-} mice displayed similar bacterial clearance as co-housed WT mice following sepsis. Consistent with previous reports\textsuperscript{24}, the microbiome analysis revealed that \textit{Nlrc4}\textsuperscript{-/-} mice displayed expansion of species \textit{Prevotella} and \textit{Paraprevotellacea} following the CLP, which are absent in WT. Studies performed after antibiotic mediated microbiome depletion or usage of germ free mice may reveal the precise role of microbiota in sepsis pathobiology.
The loss and dysfunction of cellular responses, especially macrophages and T cells, contributes to immunosuppressive or hypo responsive state of leukocytes during sepsis\textsuperscript{33, 39}. Selective depletion of macrophages increased bacterial burden and mortality in septic mice\textsuperscript{42}. We demonstrate that the deletion of NLRC4 protects from sepsis–induced monocyte/macrophage deactivation. \textit{Nlrc4}\textsuperscript{-/-} macrophages have better bacterial clearance and their adoptive transfer to septic-WT mice lowered bacterial burden. IFN-\(\gamma\) is key cytokine that is essential for sustained macrophage activation\textsuperscript{31}. \textit{Nlrc4}\textsuperscript{-/-} mice were protected from sepsis-induced loss of T cells. Indeed, the increased frequencies of IFN-\(\gamma\)+ CD8 T cells were observed in \textit{Nlrc4}\textsuperscript{-/-} mice, and depletion of CD8 T cells increased bacterial burden in \textit{Nlrc4}\textsuperscript{-/-} mice. We speculate that deficiency of NLRC4 contributes to IFN-\(\gamma\) production by T cells, which may be independent of IL-18\textsuperscript{43}, since its level is unchanged in \textit{Nlrc4}\textsuperscript{-/-} mice. Studies are needed to understand if NLRC4 modulates the expression of IFN-\(\gamma\)R in macrophages, thereby regulating their activation and phagocytosis.

Our study has several caveats and these conjectures need to be confirmed. It is possible that deficiency of NLRC4 contributes to expansion of regulatory T cells, which may lead further immune suppression. Sepsis-derived PAMPs/DAMPs that may activate NLRC4 was not investigated in this study. Since CLP is polymicrobial model, several Gram-negative bacteria and even endogenous ligands may be the direct or indirect activator.

In conclusion, our works demonstrate that NLRC4 contributes to sepsis-induced disorder and mortality through mediating hyper inflammation and impairing cellular immunity. These finding are of great importance, as they greatly enhances our
understanding of pathophysiology of sepsis and may present that NLRC4 inhibition as a potential mechanism to treat systemic diseases, including sepsis.

4.5. References


5.1. Introduction

Despite the significant advancements in health care system, bacterial pneumonia remains as major global health problems\textsuperscript{1-3}. Acute respiratory infections are a major cause mortality and morbidity worldwide\textsuperscript{1}. In addition, the emergence of multidrug resistant strains of bacteria (superbugs) and their community spread have made it very difficult to treat these diseases\textsuperscript{4}. In addition to peritonitis, bacterial lung infection, can also lead to septicemia and sepsis. Sepsis is a stage of uncontrolled inflammatory response in response to an infection leading to organ dysfunction\textsuperscript{5}. With the failure of common antibiotics, the understanding of the unfolding of host responses may reveal the molecular and cellular players of these diseases, which may inspire novel therapeutic targets.

Presented in this dissertation, we have utilized clinically relevant experimental mouse models to understand innate host defense mechanisms of bacterial pneumonia and polymicrobial sepsis. Chapter 1 provides a detail description on how host defense mechanisms are generated during an infection, specifically from the perspective of neutrophil granulopoiesis and immunity (review to be submitted). Chapter 2 elucidates the role of chemokine CXCL1 in emergency granulopoiesis and neutrophil- dependent immunity in a murine model of pneumococcal pneumonia (published in Blood). Chapter 3 describes the unsuspected role of NLRC4 in suppressing neutrophil-dependent mechanisms in MRSA induced pneumonia (published in Mucosal Immunology). Chapter 4 explores how NLRC4 contributes to sepsis-induced impairment of host defense in experimental model of CLP-induced polymicrobial sepsis (manuscript to be submitted).
Each chapter has been summarized in following section to highlight seminal findings and how it contributes to our knowledge of the evolving field of bacterial pneumonia and sepsis.

5.2. Summary of Results

Chapter 2 determines the unique role of chemokine CXCL1 in context of emergency granulopoiesis and neutrophil homeostasis in a murine model of pneumonic sepsis caused by *Streptococcus pneumoniae* serotype 3. CXCL1 has been long known to mediate the recruitment of neutrophil and other inflammatory cells to a site of infection and injury\(^6,7\). Using multiparametric flow cytometry in *Cxcl1*\(^{-/-}\) mice we demonstrate that CXCL1 regulates neutrophil generation in the bone marrow and their subsequent release to blood, which is essential for protective immunity against pneumococcal pneumonia. *Cxcl1*\(^{-/-}\) mice displayed abrogated neutrophil recruitment, which were associated with increased bacterial burdens in lungs and host mortality. We precisely demonstrate CXCL1-mediated neutrophil release were dependent upon the shedding of CD62L and upregulation of CD49d following pneumococcal infection. Furthermore, two other CXCR2 ligands such as CXCL2 and CXCL5 restored CXCL1-dependent neutrophil immunity and granulopoietic response when exogenously administered to *Cxcl1*\(^{-/-}\) mice at the time of pneumococcal pneumonia.

Chapter 3 explores the unsuspected role of NLRC4 in MRSA-induced pneumonia. We demonstrate MRSA infection induces NLRC4 activation though PKC-\(\delta\) signaling and NLRC4 mediates IL-18 secretion and induction of necroptosis. NLRC4 activation in MRSA pneumonia is deleterious as it contributes to reduced the neutrophil recruitment and phagocytosis, the bacterial clearance from lungs and extra-pulmonary
organs, subsequent leading to increased mortality. Enhanced neutrophil recruitment in Nlrc4⁻/⁻ mice was associated with increased frequency of IL-17A-producing γδ T cells and neutrophils. Mechanistically, NLRC4-driven IL-18 and necroptosis contributed to reduced number IL-17A-producing γδ T cells in lungs and subsequent neutrophil-dependent bacterial clearance. Furthermore, the blockade of caspase-1 signaling with caspase-1 inhibitor (AC-yvad-cmk) and necroptosis with Nec-1 (necroptosis inhibitor) improved the survival in WT controls following MRSA infection. Collectively, our data suggests the detrimental role for NLRC4 in MRSA pneumonia.

Chapter 4 characterizes the role of NLRC4 in a murine model of experimental sepsis. Nlrc4⁻/⁻ mice, when induced with polymicrobial sepsis with cecal ligation and puncture method, exhibited improved survival and bacterial clearance. Nlrc4⁻/⁻ mice were protected from sepsis-induced cytokine storm, hyper inflammation, and organ damage. Peritoneal macrophages isolated from Nlrc4⁻/⁻ mice were protected from sepsis-induced macrophage dysfunction and performed efficient bacterial killings under phagocytosis assay. Clodronate-mediated macrophage depletion removed the survival advantage of Nlrc4⁻/⁻ mice during sepsis. Nlrc4⁻/⁻ mice retained CD8 T cells, which were lost in WT controls during sepsis. Depletion of CD8 T cells in Nlrc4⁻/⁻ mice also enhanced bacterial burden. Nlrc4⁻/⁻ mice also had an increased frequency of IFN-γ-producing CD8 T cells, which may have contributed to the sustained activation of macrophages. Co-housing experiments revealed that the protective phenotype of Nlrc4⁻/⁻ mice were not microbiome dependent. Taken together, our data indicates that NLRC4 contributes to sepsis-induced mortality and cellular dysfunction. Thus the inhibition of NLRC4 activity may represent attractive therapeutic target to curtail sepsis-induced hyper inflammation and dysfunction.
Identification of NLRC4 inflammasome and elucidation of the role of chemokines has broadened our understanding on the role of these innate immune molecules in the pathogenesis of bacterial pneumonia and sepsis and to assess the need to develop improved therapeutic strategies to reduce pathogen-induced organ damage in combating these diseases.

5.3. Conclusion and Future Directions

Results from this dissertation work have significantly advanced our understanding of the role of innate mediator such as CXCL1 and NLRC4 during bacterial pneumonia and sepsis. Based on our data, CXCL1 is not only a chemokine but also a molecular messenger that converts pathogen signals into molecular cues of emergency granulopoiesis. In contrast, NLRC4 appears to be a pathogen sensor, which could be exploited by Gram-positive bacteria to dampen host defense mechanisms, specifically neutrophil recruitment. The detrimental role of NLRC4 can be extended to sepsis as our study reveals that NLRC4 contributes to hyper inflammation and lymphoid depletion. Therefore, the understanding these innate immune mechanisms may provide attractive targets for the development of therapeutics in order to mitigate severe organ damage. However, understanding of the big picture of bacterial pneumonia and septic peritonitis is far from complete, and these additions to the field of bacterial pathogenesis will provide a platform for future investigations into the pathogenesis of pulmonary lung infection and septic peritonitis.

Continued research should be focused on identifying the S. aureus-derived products that NLRC4 recognizes and mediates inflammatory response during pneumonia. Since NLRs have redundant roles in inflammatory cascades, relative contribution of
NLRC4 should be explored using double or triple knockout mouse models. Our data generated using a preclinical model should be verified in humanized mice to translate these observations in humans.

5.4. References


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8.5 The licensing transaction described in the Order Confirmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court. If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-750-8400 or send an e-mail to info@copyright.com.

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VITA

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