NLRP6 in Gram-positive Pneumonia and Sepsis

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NLRP6 IN GRAM-POSITIVE 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
Laxman Ghimire
B.V.Sc & A.H. Tribhuvan University, 2013
December 2019
Dedicated to my parents, brothers, sisters, and my wife
Their love, support, and motivations are responsible for every accomplishment of my life
ACKNOWLEDGMENTS

I would like to extend my sincere gratitude to my mentor, Dr. Samithamby Jeyaseelan (Jey), for his guidance, financial support, patience, motivation, and enthusiasm in each and every step of my research. I could not imagine a better mentor than Dr. Jey for pursuing a PhD degree. Beside my mentor, I am eternally indebted to Dr. Ingeborg Langohr, Dr. Rhett Stout, Dr. Michael Behnke, and Dr. Guoshan Wang (LSUHSC, New Orleans) for their guidance and constructive criticism throughout my research.

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ABSTRACT

Acute lower respiratory infections (pneumonia) and pneumonia-derived sepsis are among the leading causes of death in the world causing 7.8 million deaths annually. In this regard, Methicillin-resistant *Staphylococcus aureus* (MRSA) is endemic in the US and implicated for causing high mortality-associated necrotizing pneumonia and aggravating viral pneumonia with superinfection. Additionally, sepsis is the 7th leading cause of death among newborns in the US and is responsible for more than 750,000 hospitalization cases every year. Although there is a plethora of research in both pneumonia and sepsis, the detailed pathophysiology still remains elusive. Understanding the host defense mechanism will help designing better therapeutic interventions to treat such infections.

In this context, NLRP6 is a member of Nod-like receptor (NLR) family that has been proposed to regulate microbiota, gut-epithelial integrity, and host defense against *Listeria* infections. While NLRP6 is expressed in lungs, liver, spleen, and kidneys, its role in sepsis and MRSA-induced pneumonia remains unexplored. Neutrophils play critical role to limit the Staphylococcal infection; however how neutrophils homeostasis is orchestrated during pulmonary MRSA infection remains elusive. Using NLRP6 gene-deficient mice, we demonstrate that NLRP6 negatively regulates Gram-positive bacteria-induced pneumonia via regulating phagocytic oxidase activity and cell death mechanism of neutrophils. NLRP6 triggers necroptosis and pyroptosis that reduces phagocytic cells in the lungs. In addition, in a murine model of CLP-induced polymicrobial sepsis, we found that NLRP6 is detrimental for lymphocyte survival and function in the spleen. Furthermore, our data illustrate that MRSA upregulates CXCL5 in order to suppress granulopoiesis and neutrophil trafficking from bone marrow to the lungs.
In conclusion, MRSA exploits CXCL5 to suppress the neutrophil accumulation in the lungs as an immune evasion strategy. NLRP6 inflammasome is detrimental during MRSA-induced pneumonia and polymicrobial sepsis. Blocking of NLRP6 could be an effective therapeutic strategy to treat both septic and MRSA-infected patients.
CHAPTER 1. NLRP6 INFLAMMASOME IN HEALTH AND DISEASES

1.1 Introduction

The mammalian immune system is endowed with the germline-encoded pattern recognition receptors (PRR) that sense both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to initiate proinflammatory responses as a first line of defense. PRRs can be classified into two groups based on their cellular localization. The first group includes Toll-like receptors (TLRs) and C-type lectin (CTL) receptors that surveil the extracellular and endosomal compartments. The second class of PRRs includes Retinoic acid-inducible gene (RIG)-like receptors (RLRs) and nucleotide-oligomerization domain (NOD) like receptors (NLRs), which survey intracellular compartments for the presence of PAMPs and DAMPs [1]. Thus far, 23 NLRs have been identified in humans and more than 30 have been identified in mice [2]; however, only a few of these have been extensively studied. Predominantly expressed in first responder cells like neutrophils, macrophages and dendritic cells, NLRs are multi-domain protein complexes consisting of a central nucleotide oligomerization domain (NOD or NACHT) flanked by C-terminal leucine-rich repeats (LRRs) for sensing PAMPs and DAMPs, and a variable N-terminal region consisting of either a caspase activation and recruitment domain (CARD), pyrin domain (PYD), or baculovirus inhibitor repeats (BIR) [2-7]. Unlike other classes of receptors, NLRs are unique in the sense that many of them can form a supramolecular complex, known as an inflammasome, by recruiting apoptosis-associated speck-like protein (ASC) and caspase-1 or 11 after sensing PAMPs or DAMPs. The activated caspase converts the inactive forms of IL-1β and IL-18 into their respective active forms to initiate inflammatory signaling [8, 9]. Based on the phylogenetic structure of the central NACHT domain, NLRs can be classified into three broad categories: NODs, NLRPs or NALPs
(NLRP1-14), and the IPAF family of NLRs [NLRC4 and NLR family apoptosis inhibitory proteins (NAIPs)] [3] (Fig. 1.1).

Figure 1.1 Different inflammasomes and their domain organization. Based on the structure of central NACHT domain, NLRs can be classified into three broad categories: the NODs (NOD1, NOD2, NOD3, NOD4, NOD5, and CIITA), the NLRPs or NALPs (NLRP1-14), and the IPAF family of NLRs [NLRC4 and NLR family apoptosis inhibitory proteins (NAIPs)]. NOD1 and NOD2 contains CARD, NBD, and LRR domain whereas NOD3-5 and CIITA lacks card domain. In addition to NB and LRR, NAIPs contain BIR domain whereas IPAF contains CARD instead of BIR. NLRP10 only contain PYD and NBD whereas NLRP2-9 and NLRP11-14 contain PYD, NBD, and LRR. Interestingly, NLRP1 has FIIND and CARD domains in addition to PYD, NBD, and LRR [3, 10]. ASC consists of PYD and CARD domains and caspase 1/11 have CARD, p10, p20 fragments. CIITA: class II transactivator; FIIND: function to find domain; NBD: nucleotide-binding domain, PYD: pyrin domain, LRR: leucine-rich repeats, CARD: caspase activation and recruitment domain, and BIR: baculovirus inhibitor of apoptosis protein repeat.
Because NLRP6 is a relatively new member of the NLR family its detailed signaling mechanism is yet to be established. During its initial identification, NLRP6 was shown to activate caspase-1, like other NLRs, and NF-kB, like TLRs, suggesting that it is unique in the NLR family as it can perform functions of both NLRs and TLRs [11]. This notion is supported by other studies demonstrating a wide scope of physiological functions of NLRP6 ranging from modulation of the host-microbial interface [12] and host defense against pathogens [13, 14] to inhibition of carcinogenesis [15] and neuroinflammation [16]. However, NLRP6 has also been shown to negatively regulate NF-kB signaling in mouse model [14]. In this review, we discuss the recent advancements in understanding of NLRP6 inflammasome signaling, summarize the diversified roles of this receptor in host defense and inflammation, and cover recent updates regarding its structure, inflammasome assembly, and regulation of microbiota.

1.2 NLRP6 assembles an inflammasome

The initial report of NLRP6 by Grainer et al. in 2002 using human cell lines demonstrated that co-expression of NLRP6 (PYPAF5) and ASC leads to caspase-1 and NF-kB activation in COS-7L and 293T cells, respectively [11]. Based on this observation, it was assumed that NLRP6 could form an inflammasome like other members of the NLR family, although in vivo evidence was lacking. The in vivo evidence of this was later provided by two different laboratories at the same time in 2011 when they demonstrated that NLRP6−/− mice have reduced serum IL-18 levels under steady state conditions [17] and after DSS-induced colitis [15, 17] compared to that of WT counterparts. However, IL-1β, which is regarded as one of the signature cytokines of inflammasome activation, was found to be upregulated in the colon of NLRP6−/− mice [15]. This may be due to redundant functions of other inflammasome complexes. Nonetheless, Levy et al. demonstrated that NLRP6 co-localizes with ASC in intestinal cells
under steady-state conditions to form an inflammasome thus, confirming that NLRP6 assembles an inflammasome in vivo [18]. However, this finding did not hold true during microbial infection. Anand et al. reported that they did not observe any obvious difference in the levels of IL-1β or caspase-1 processing between NLRP6<sup>−/−</sup> and WT mice during different bacterial infections including Salmonella, Listeria, and E. coli [14]. Therefore, at this time, it remained unclear whether NLRP6 forms an inflammasome during systemic bacterial infections.

Recently, we [13] and others [19] reported that the NLRP6 inflammasome is activated during bacterial infections. In our study, NLRP6<sup>−/−</sup> bone marrow-derived macrophages (BMDMs) showed reduced caspase-1 processing and IL-1β secretion compared to that of WT BMDMs when infected with methicillin-resistant Staphylococcus aureus (MRSA). Consistently, MRSA-infected NLRP6<sup>−/−</sup> mice had reduced IL-1β secretion in bronchoalveolar lavage fluid (BALF) compared to that of WT mice. This is strong evidence that NLRP6 assembles an inflammasome during microbial infection. We observed that the NLRP3 inflammasome was intact in NLRP6<sup>−/−</sup> BMDMs as treatment with NLRP3 agonist enhanced IL-1β production in these macrophages. As reported by Greiner et al. and Levy et al. [11, 18], we found that NLRP6 colocalizes with ASC in bone marrow-derived macrophages (BMDMs) after infection [13]. Similarly, an excellent study from Hara et al. reported that Gram-positive bacteria, such as Listeria monocytogenes, activate the NLRP6 inflammasome. More importantly, the authors demonstrated that lipoteichoic acid (LTA) from L. monocytogenes upregulates the expression of NLRP6 and caspase 11 via type 1 IFN signaling, which could be regarded as the first signal. As a second signal, LTA serves as a ligand to bind NLRP6 in order to activate the inflammasome via the ASC-caspase 11-caspase 1 signaling cascade [19] (Fig. 1.2). However, further investigations are warranted to determine how S. aureus upregulate NLRP6 expression in macrophages. Being a gram-positive bacterium,
it is possible that toxins from *S. aureus* could also activate type 1 IFN signaling, as in the case of *Listeria* [19], or regulate TLR signaling, as in the case of NLRP3 [2], to upregulate expression of NLRP6.

![Diagram](image)

**Figure 1.2. Activation of NLRP6 inflammasome during *Listeria* and *S. aureus* infection.** Activation of NLRP6 inflammasome during *Listeria* and *S. aureus* infection. Cytosolic delivery of *Listeria* or its toxin (LTA) upregulates the expression of caspase-11 and NLRP6 via triggering Type 1 IFN signaling in a macrophage. NLRP6 will recruit ASC (via PYD-PYD interaction), and caspase 1 and caspase 11 (via CARD-CARD interaction) to form NLRP6 inflammasome complex. The activated inflammasome cleaves pro-IL-1β and pro-IL-18 into active form of IL-1β and IL-18 which are then secreted from the cell for further immune response. During *S. aureus* infection, NLRP6 is upregulated and recruits ASC and caspase-1 as mentioned above to form NLRP6 inflammasome complex. Inactive form of IL-1β and IL-18 will be converted into their respective active form for further immunological response. PYD: pyrin domain, LRR: leucine-rich repeats; CARD: caspase activation and recruitment domain; LTA: lipoteichoic acid.
1.3 Mechanism of NLRP6 inflammasome activation

In general, an inflammasome consists of a sensor component, an adapter molecule, and an effector component (Fig. 1.1). The sensor molecule consists of either a PYD or CARD at its N-terminus, the adapter molecule, usually ASC, consists of PYD and CARD domains, and the effector component has a CARD at its end. Based on the sensor component, inflammasomes can be broadly classified into three groups: NLR-associated inflammasomes, absent in melanoma-2 (AIM-2)-like receptor (ALR)-associated inflammasomes, and the Pyrin inflammasome [20, 21]. Based on the requirement of adapter molecules, inflammasomes can be either ASC-dependent or ASC-independent (NAIP/NLRC4 inflammasome, where NAIP acts as a sensor and NLRC4 acts as the adaptor molecule) [22]. Similarly, based on the effector component, inflammasomes can be canonical (activate caspase 1) or non-canonical (activate caspase 11). All NLR-related inflammasomes are canonical inflammasomes; however, all canonical inflammasomes are not NLR-associated inflammasomes (for example, AIM2).

Inflammasome auto activation is thought to be prevented by the closed conformation of the LRR and NACHT domains of NLR (autoinhibition) in the absence of ligand [23]. Upon stimulation by ligands, the homotypic interaction between component proteins leads to the assembly of an inflammasome. During assembly of the NLRP6 inflammasome, NLRP6 protein recruits the bipartite ASC adapter molecule via a homotypic PYD-PYD interaction. ASC then recruits caspase-1 via a CARD-CARD interaction to form the NLRP6-ASC-caspase-1 supramolecular complex known as the NLRP6 inflammasome, which facilitates the autocleavage of caspase-1 [24]. Biochemical studies have reported prion-like properties of PYD and CARD filaments that facilitate inflammasome assembly. Recently, Cai et al. and Lu et al. demonstrated that prion-like polymerization of ASC into filamentous structures is an important
step in the activation of ASC-dependent inflammasomes [20, 25]. Based on the cryoelectron microscopy structure and structure-based mutagenesis, ASC-dependent inflammasomes were found to share a unified assembly mechanism that involves two nucleation-induced polymerization steps [20, 26]. The first step being nucleation of ASC\(^\text{PYD}\) filaments by NLR\(^\text{PYD}\) through a PYD-PYD interaction that leads to polymerization of ASC. In the second step, the polymerized ASC\(^\text{CARD}\) nucleates caspase-1\(^\text{CARD}\) filaments via a CARD-CARD interaction for caspase-1 activation. Activated caspase-1 is responsible for maturation of pro-IL-1β and pro-IL18 as well as gasdermin-D mediated pyroptosis [27]. Recently, the detailed filamentous and crystal structure of the NLRP6 inflammasome was resolved by Shen et al. using biochemical and biophysical methods. The authors purified full-length NLRP6 (NLRP6\(^\text{FL}\)), NLRP6\(^\text{PYD}\), and NLRP6\(^\text{PYD+NBD}\) through affinity and size exclusion chromatography and tested their ability to induce ASC\(^\text{PYD}\) polymerization. All NLRP6 constructs were able to promote ASC\(^\text{PYD}\) polymerization. However, compared to NLRP6\(^\text{PYD}\), NLRP3\(^\text{PYD}\) alone was not sufficient to induce ASC\(^\text{PYD}\) polymerization [24]. NLRP6\(^\text{PYD}\) filaments possess a hollow cylindrical structure that assembles through a right-handed helix, and a single NLRP6\(^\text{PYD}\) contains six anti-parallel α helices. During activation, most of the conformational changes in NLRP6 occur between the α2 and α3 loop to facilitate interactions [24].

Compared to AIM2\(^\text{PYD}\), the α2-α3 loop of NLRP6\(^\text{PYD}\) is longer suggesting that NLRP6\(^\text{PYD}\) permits more degrees of conformational change during filament formation than does AIM2\(^\text{PYD}\) [24, 28]. Both NLRP6 and NLRP3 share similar molecular structures with slight differences in the PYD component. However, while NLRP6\(^\text{PYD}\) is capable of nucleating ASC\(^\text{PYD}\) by itself, NLRP3\(^\text{PYD}\) is not able to provide a sufficient platform for ASC assembly [20, 24]. Like NLRC4, upon overexpression the full-length NLRP6 assembles into an open and active filamentous
conformation with the PYD filament in the center and LRR+NBD outside. However, the LRR+NBD ring was more ordered in NLRC4 than in NLRP6 [24, 29]. Since, NLRP6 has been shown to recruit both caspase 1 and caspase 11 during inflammasome assembly [19], future biochemical studies are needed to provide additional mechanistic insight into NLRP6 inflammasome assembly.

1.4 NLRP6 in host defense

Inflammasomes have been shown to regulate host defense mechanisms under different disease conditions [13, 30-32]. The role of the NLRP3 inflammasome in response to bacterial, viral, and fungal infections has been well established [33-37], and there is mounting evidence that NLRP6 also regulates host defense during different microbial infections (Table 1.1). An interesting study by Anand et al. reported that NLRP6 negatively regulates innate immunity during bacterial infections [14]. In this study, deletion of NLRP6 was found to enhance NF-κB and MAPK activity resulting in increased neutrophil recruitment to the lungs. This augmented neutrophil accumulation was associated with enhanced bacterial clearance and improved survival in NLRP6−/− mice infected with Listeria, Salmonella, or E.coli. However, the authors neither depleted neutrophils nor blocked NF-κB or MAPK pathways to confirm this claim. Interestingly, the authors did not find differences in the level of IL-1β or caspase-1 processing indicating that the host protection conferred by NLRP6 deficiency was inflammasome independent. In addition, NLRP6 regulated bacterial infection independently of microbiota composition as the co-housed NLRP6−/− mice demonstrated a reduced bacterial burden compared to that of singly housed mice. However, this study did not elucidate how NLRP6 recognizes these bacteria to initiate immune signaling cascades.
Table 1.1 NLRP6 in host defense following microbial infection

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Model used</th>
<th>Phenotype observed</th>
<th>Proposed mechanism of action</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>Pneumonia (IT)</td>
<td>NLRP6−/− mice protected</td>
<td>NLRP6 augments pyroptosis and necroptosis of neutrophils and macrophages thereby reducing the number of phagocytes to clear bacteria. In addition, NLRP6 deficiency enhances IFN-γ secretion in the lungs.</td>
<td>[13]</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>Systemic infection (IV)</td>
<td>NLRP6−/− mice protected</td>
<td>NLRP6 inflammasome enhances IL-18 secretion, which is detrimental during <em>Listeria</em> infection.</td>
<td>[19]</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>Systemic infection (IV)</td>
<td>No change in phenotype</td>
<td></td>
<td>[19]</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>Systemic infection (IP)</td>
<td>NLRP6−/− mice protected</td>
<td>NLRP6 deficiency augments NF-κB mediated neutrophil recruitment, which is important to limit <em>Salmonella</em>.</td>
<td>[14]</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Systemic infection (IP)</td>
<td>NLRP6−/− mice protected</td>
<td>NLRP6 negatively regulates the NF-κB signaling pathway to enhance neutrophil recruitment</td>
<td>[14]</td>
</tr>
<tr>
<td><em>C. rodentium</em></td>
<td>Enteritis model</td>
<td>NLRP6−/− mice susceptible</td>
<td>NLRP6−/− mice have a thin mucus layer as a result of defective goblet cell function. This facilitates prolonged adherence of <em>C. rodentium</em> in the intestinal epithelium.</td>
<td>[12]</td>
</tr>
<tr>
<td>Encephalomyocarditis virus (EMCV), Norovirus</td>
<td>Systemic infection (EMCV: IP &amp; oral; Norovirus: oral)</td>
<td>NLRP6−/− mice susceptible</td>
<td>NLRP6 recognizes cytosolic long dsRNA and activated mitochondrial antiviral signaling proteins (MAVS) to enhance the antiviral response. NLRP6-Dhx15-MAVs axis augments the expression of interferon-stimulated genes to limit virus in the intestine.</td>
<td>[38]</td>
</tr>
</tbody>
</table>

We recently demonstrated that NLRP6 serves as a negative regulator of pulmonary host defense during Gram-positive bacterial infection of the lungs [13]. MRSA has been shown to induce necroptosis in the lungs resulting in pathology [39]; however, how the necroptosis
machinery is regulated in the lungs has remained unclear. We reported that activated NLRP6 triggers necroptosis and pyroptosis of macrophages and neutrophils in the lungs, which ultimately reduces neutrophil numbers thereby compromising bacterial clearance (Fig. 1.3). Through co-housing of NLRP6−/− mice with WT mice we further demonstrated that NLRP6 aggravates MRSA infection independent of microbiota composition. It is interesting that pyroptosis, which was reported to be essential for protection against intracellular pathogens [40], was found to be detrimental during pulmonary MRSA infection. These observations indicate that role of pyroptosis may depend on the lifestyle of a pathogen. For intracellular pathogens, pyroptotic cell death may kill the pathogen along with the cell itself. However, extracellular pathogens might hijack NLRs, such as NLRP6, NLRC4 [30] or NLRP3 [34], to induce pyroptotic cell death that exacerbates inflammation-induced pathology resulting in detriment to the host. In addition to S. aureus-induced pneumonia, it would be revealing to study whether the NLRP6 inflammasome exerts deleterious or protective effects during Gram-negative bacteria-induced pneumonia, since Gram-negative pathogens have their own lifestyles, virulence factors, and immune evasion strategies.

Recently, an elegant study from Hara and colleagues demonstrated that LTA from Listeria binds with NLRP6 and activates the NLRP6 inflammasome via ASC to regulate host defense during Gram-positive bacterial infection [19]. It is interesting that NLRP6 activates both caspase 11 and caspase 1 upon binding of LTA or Listeria for processing of IL-1β and IL-18. In contrast, NLRP3, AIM2, and pyrin inflammasomes do not activate caspase 11. Upon infection with Listeria, NLRP6−/− and caspase 11−/− mice had reduced bacterial burdens in several organs and better survival compared to that of WT mice, thus confirming that NLRP6 is detrimental during systemic Gram-positive pathogen infection. This protection was abolished when these
mice received recombinant IL-18, but not IL-1β, suggesting that the NLRP6 inflammasome exacerbates *Listeria* infection via IL-18 production (Fig. 1.3). In addition, the authors co-housed NLRP6−/− mice with WT mice for 4 weeks to determine if the protection observed in NLRP6−/− mice is microbiota dependent. Interestingly, NLRP6/caspase 11 signaling regulates *Listeria* infection independently of microbiota. However, future studies are essential to understand how IL-18 exacerbates *Listeria* infection as IL-18 has been shown to play protective as well as deleterious roles during such infections. In the same study, the authors also infected WT and NLRP6−/− mice with *Salmonella* and observed bacterial growth in the liver and spleen. In contrast to what was observed by Anand et al, no differences were observed between WT and NLRP6−/− mice [14]. It is difficult to explain this discrepancy in results as both studies used similar bacteria, and doses, and almost similar routes of infection (IV vs IP). One explanation could be due to difference in route used; however, further investigations are warranted to resolve this controversial finding.

Although most studies of microbial infections have presented NLRP6 as a negative regulator of host defense, some studies have reported essential roles for this molecule. Wlodarska and colleagues utilized a murine model of *Citrobacter*-induced enteritis and found that NLRP6 deficiency results in impaired host defense [12]. Intestines from NLRP6−/− mice were extensively colonized with *C. rodentium* and the NLRP6−/− mice displayed extensive mucosal ulceration, edema, and hyperplasia compared to WT mice. Interestingly, the authors found that NLRP6 inflammasome signaling was essential for mucus secretion from goblet cells, which is required as a physical barrier between microbiota and the underlying epithelium. Thus, the thin mucus layer observed in NLRP6−/− mice appears to facilitate persistent adherence of *C. rodentium* (Fig. 1.3). However, it is important to note that the authors did not investigate the functions of
innate immune cells such as macrophages and neutrophils in the clearance of *C. rodentium*. Therefore, whether NLRP6 regulates the recruitment or functions of these cells during such infections remain unclear.

Figure 1.3 NLRP6 inflammasome in host defense during microbial infection. A) Pulmonary infection with *S. aureus* activates NLRP6 inflammasome that triggers inflammatory modes of cell death particularly necroptosis and pyroptosis in the lungs. NLRP6 increases the expression of MLKL and RIP3 to induce necroptosis and caspase 1 and gasdermin-d to induce pyroptosis. As a result of increased cell death, the number of phagocytic cells will be reduced in the lungs that impairs Staphylococcal clearance. B) Systemic infection with *Listeria monocytogens* activates NLRP6-ASC-caspase1/11 inflammasome complex in the macrophage. The activated caspase 1 cleaves inactive IL-18 into active IL-18 which is then secreted from the cell. Thus produced IL-18 enhances mortality during Listeria infection. C) NLRP6 recognizes cytosolic dsRNA of encephalomyelitis virus via NLRP6-Dhx15 viral sensing complex in the enterocyte. This complex will further activate Mavs to induce the transcription of Type I/II interferons and IFN-stimulated genes for antiviral response. GSMD: Gasdermin d; MAVs: mitochondrial antiviral signaling protein; Dhx15: DEAH-box helicase 15.

In addition to bacterial infections, a role for NLRP6 in viral infections has also been reported. Wang and colleagues were the first to demonstrate that NLRP6 plays a crucial role in
limiting viral infections [38]. While WT and NLRP6−/− mice exhibited no difference in survival when infected intraperitoneally with encephalomyocarditis virus (EMCV), a +ssRNA virus, the NLRP6−/− mice had higher viral loads in the intestine, but not in blood, brain, or heart. This suggests that NLRP6 may have an important role in viral clearance from the intestine.

Interestingly, NLRP6−/− mice display increased susceptibility to EMCV when it is administered orally, and similar results were obtained for oral infection with murine norovirus. In addition, the authors performed co-housing experiments to determine if this phenotype is dependent on the composition of the gut microbiome in mice and found that this was not the case. Interestingly, NLRP6 interacts with the Dhx15 helicase to form a viral sensing complex that recognizes cytosolic long dsRNA and activates mitochondrial antiviral signaling proteins (MAVS) to initiate the antiviral response. Activation of the NLRP6-Dhx15-MAVs axis enhances the expression of interferon-stimulated genes essential for limiting virus replication in the intestine (Fig. 1.3). Further, expression of NLRP6 in the intestine following viral infection is regulated by type I/III IFNs via the IRF3/7 transcription factors [38]. Given that NLRP6 has detrimental roles in Gram-positive bacterial pneumonia [13], it would be of interest to investigate whether NLRP6 exhibits a protective or detrimental role in pulmonary host defense during infection with influenza or rhinoviruses.

While it is clear that NLRP6 executes essential roles to protect the host against bacterial and viral infections in the intestine, where it is highly expressed, in systemic and pulmonary infections, NLRP6 expression appears to have detrimental effects. Thus, NLRP6-mediated host defense regulation is likely context-dependent. In bacterial infections, where myeloid cells are most important, NLRP6 seems to trigger destructive inflammation; however, during enteritis, where non-hematopoietic cells (epithelial cells) play crucial roles, the NLRP6-mediated immune
response is protective. Nonetheless, more studies will be necessary to further define the role of NLRP6 in these systems. Moreover, whether NLRP6 exerts similar effects in viral, fungal, or Gram-negative bacterial pneumonia and sepsis remains obscure. Defining the roles of NLRP6 in these infections will allow for better generalization of these conclusions.

1.5 NLRP6 in intestinal microbiota

NLRP6 is highly expressed in the small and large intestine, especially in enterocytes, colonic goblet cells, and myofibroblasts [15, 17, 41] and hence, most studies on NLRP6 have concentrated on defining its role in these organs. A pioneer study by Elinav et al. involving 16S rRNA analysis of fecal samples from non-littermate NLRP6−/−, ASC−/−, and IL-18−/− mice revealed distinct microbiota composition in the guts of these mice compared to that of wild-type (WT) mice. For example, there was a higher prevalence of anerobic taxa Prevotellaceae and TM7 [17]. These mice are also more susceptible to DSS-induced colitis than are WT mice. This alteration in the microflorae is considered to be colitogenic since transfer of microbiota from NLRP6−/−, ASC−/−, or IL-18−/− mice to WT mice via cohousing for 4 weeks results in enhanced susceptibility of WT mice to colitis. Intriguingly, depletion of microbiota using broad-spectrum antibiotics reversed this phenomenon. Moreover, reduced IL-18 and increased CCL5 secretion in NLRP6−/− mice led to altered microbiota and spontaneous intestinal inflammation [17] (Fig. 1.4). This finding was crucial to document the prominent role of NLRP6 in maintaining intestinal microbiota. In line with this finding, several studies using mouse models confirmed that the NLRP6 inflammasome regulates gut microbiota [14, 18, 42]. Furthermore, Levy et al. demonstrated that ex-germ-free NLRP6−/− mice develop dysbiosis as early as 3 weeks after spontaneous recolonization compared to their ex-germ-free WT counterparts [18]. Since microbiota can influence various physiological conditions [43] and diseases, this finding opened
a new research platform for investigation of the possible roles of the NLRP6 inflammasome in several physiological conditions such as colitis, tumorigenesis, and metabolic diseases including non-alcoholic fatty liver disease and obesity [42, 44-46].

Figure 1.4 NLRP6 inflammasome orchestrating microbiota-epithelium interface. NLRP6 inflammasome maintains healthy microbiota in the gut via IL-18 secretion. Microbial metabolite such as taurine activates NLRP6 inflammasome in the enterocyte to secrete IL-18. Genetic ablation of NLRP6 will result into dysbiosis formation and increased secretion of CCL5 in the intestine that lead to spontaneous inflammation and susceptibility to chemical-induced colitis. Additionally, NLRP6 inflammasome maintains physical and chemical barrier between microbiota and underlying epithelium to maintain healthy microbiota-epithelium interface. Microbiota-derived LPS, Lipid A, and flagellin activates Myd88-ROS pathway to activate NLRP6 inflammasome in the goblet cells. Activated NLRP6 inflammasome facilitates the exocytosis of muc2 from goblet cells that forms physical barrier above the epithelium. On the other hand, IL-18 derived from NLRP6 inflammasome in the enterocyte triggers the production of antimicrobial peptides (AMP) that forms chemical barrier between microbiota and underlying epithelium. ROS: reactive oxygen species, CCL5: CC-motif chemokine ligand-5; LPS: lipopolysaccharides.
Non-genetic factors such as differences in animal housing [47], familial transmission [48], and diet [49] can modulate microbiota composition, suggesting that littermates are the best control animals to use when investigating the impact of host genetic factors on microbiota regulation. Recently, a study by Robertson and colleagues compared co-housing and F2-generation littermate methods for standardization of microbiota between two different sources of mice. Interestingly, the littermate method yielded uniform normalization of microbiota in feces, colon, and ileum. However, extended co-housing was only able to normalize fecal microbiota and the microbial community in the colon and ileum remained stable [50]. Using separately housed, non-littermate WT and NLRP6−/− mice, Mamantopoulos et al. did not observe any overall difference in microbiota composition, which is in contrast with the findings from Elinav et al. [17, 51]. However, Porphyromonadaceae and Bacteroidaceae, but not the Prevotellaceae, bacteria were differentially represented in these mice. Using distance-based redundancy analysis, they found that these differences are due to mother and cage covariates rather than host genetic factors (NLRP6). Furthermore, they did not observe obvious differences in microbiome composition when using littermates (NLRP6+/−) even after lifetime separate housing [51]. In support of this finding, Lemire et al. also demonstrated that NLRP6 does not impact gut microbiota composition by using littermate NLRP6−/− and NLRP6+/− mice [52]. Similarly, Galvex et al. also reported similar microbial compositions of WT, NLRP6−/−, and Rag2−/− mice under pathobiont-free conditions. Recently, results contradicting these were reported both by Levy et al. and by Seregin et al. [45, 46]. These two studies also used littermate NLRP6−/− and NLRP6+/+ mice and analyzed their microbiota but found different microbial populations in NLRP6−/− compared to NLRP6+/+ littermates. It is difficult to explain these contradictory results as both studies used littermate NLRP6−/− and NLRP6+/+ mice and 16s RNA sequencing to analyze
microbiota composition. Nonetheless, one possible explanation could be differences in microbiota among different facilities due to unique husbandry practices, including chow formulations and housing conditions, which could influence phylogenetic diversity and richness. Another explanation could be the differences in algorithms or analysis software used. These factors need to be considered when investigating microbiota-driven phenotypes in mouse models. Moreover, use of littermate control animals should be utilized over co-housing to normalize microbiota between different mice.

1.6 NLRP6 in epithelial integrity, and microbiota-epithelial crosstalk

The intestine contains trillions of commensal microbes that are beneficial to the host under normal conditions [53]. Two sheets of mucosal layers and underlying epithelium prevent direct contact of these commensal microbes with the lamina propria of the intestine. The outer mucosal layer is blanketed by muc2 mucin secreted by goblet cells and serves as a habitat for commensal microbiota [54-56]. Breaching of the mucin layer leads to aberrant crypt morphology and can result in subsequent invasive adenocarcinoma and colorectal cancer [57]. Interestingly, the NLRP6 inflammasome has been linked to epithelial integrity through regulation of goblet cell function [12, 58] and secretion of antimicrobial peptides by enterocytes [18]. Wlodarska et al. reported that NLRP6 inflammasome signaling is essential for optimal mucin secretion by goblet cells. NLRP6−/− mice demonstrate reduced autophagy and hyperplasia of goblet cells with a failure to exocytose mucin granules. As a result, these mice have only a thin mucus layer over the epithelium and increased susceptibility to enteric infections [12] (Fig. 1.4). However, how the NLRP6 inflammasome is activated in the intestine in response to microbiota was not clearly defined in this study. To this end, Birchenough et al. demonstrated that metabolites from bacteria activate the NLRP6 inflammasome for regulation of goblet cell function. Microbial metabolites
such as LPS, lipid-A, P3CSK4 and flagellin, but not LTA or bacterial DNA, are endocytosed by sentinel goblet cells via the TLR-MyD88 pathway and then induce intracellular reactive oxygen species (ROS). These ROS further activate the NLRP6 inflammasome to regulate compound exocytosis of goblet cells for muc2 secretion [58] (Fig. 1.4). Since ROS plays an upstream role in NLRP3 inflammasome activation [59], it is possible that they serve as a common upstream mediator of inflammasome activation. In the study by Birchenough and colleagues, the nature of the cell death involving the extrusion of goblet cells was not delineated. Thus, it may be non-inflammatory in nature, occurring through a mechanism such as autophagy, as documented by Wlodarska et al. [12]. Together, these studies propose NLRP6 as a pivotal regulator of the goblet cell-derived protective mucin layer, which serves as a physical barrier to protect underlying epithelial integrity (Fig. 1.4).

NLRP6 has also been shown to regulate antimicrobial peptide secretion to form a protective chemical barrier between microbiota and epithelium [18]. For example, microbiota-derived metabolites such as Taurin acetate induce IL-18-mediated AMP secretion via activation of the NLRP6 inflammasome (Fig. 1.4). However, how these AMPs restore normal microbiota in the dysbiotic environment remains elusive. Thus, further investigation is warranted to determine whether AMPs selectively kill harmful bacteria or act as a probiotic to nourish useful bacteria in the gut. Similarly, studies are needed to further confirm the role of IL-18 in intestinal homeostasis and colitis as activation of IL-18 has also been shown to enhance loss of goblet cells resulting in decreased epithelial integrity [60]. It is possible that NLRP6-dependent IL-18 plays a protective role in maintaining epithelial integrity, while inflammasome-independent IL-18 has a deleterious effect. Thus, future studies are essential to resolve the current controversy regarding the function of IL-18 in the intestine.
1.7 NLRP6 in metabolic diseases and carcinogenesis

Studies using mouse models have demonstrated that NLRP6 augments progression of non-alcoholic fatty liver disease (NAFLD) and obesity through regulation of gut microbiota [42]. After feeding mice a methionine-choline-deficient diet (MCDD) for 4 weeks, Henao-Mejia and colleagues found increased severity of NAFLD and non-alcoholic steatohepatitis (NASH) in inflammasome-deficient mice (Asc\(^{-/}\) and Casp1\(^{-/}\)) compared to WT mice. Further, this was mediated by IL-18 since IL-18\(^{-/}\), but not IL-1b\(^{-/}\), mice displayed a phenotype similar to Asc\(^{-/}\) and caspase1\(^{-/}\) mice. Moreover, co-housing WT mice with Asc\(^{-/}\), Casp1\(^{-/}\), IL18\(^{-/}\), or NLRP6\(^{-/}\) mice enhanced the severity of NAFLD and NASH compared to singly housed WT mice indicating that the augmented susceptibility is mediated by differences in microbiota composition regulated through the NLRP6-IL-18 signaling axis. Translocation of metabolites from the altered microflora in the gut of these mice via the portal vein activated TLR4 and TLR9 in the liver to exacerbate NAFLD and NASH via TNF-α secretion [42] (Fig. 51.). This study provides strong evidence that microbiota composition can dictate metabolic conditions, including diet induced NAFLD and obesity. Consistent with this finding, a study by Lee and colleagues showed increased levels of IL-1β upon LPS treatment of mice fed a high-fat diet suggesting involvement of inflammasomes in NAFLD. Moreover, treatment of hepatic cell lines (Hep G2) with palmitic acid and LPS upregulated the NLRP6 inflammasome, an effect which was reversed by pretreatment with peroxisome-proliferator activated receptor-δ agonist (PPAR-δ), a nuclear receptor protein [61]. However, this study did not reveal how PPAR-δ communicates with NLRP6 to induce inflammasome activation.
Figure 1.5 NLRP6 inflammasome regulating gut-liver axis. In a normal WT mouse, NLRP6 inflammasome maintains healthy epithelial cell-microbiota axis via mucin and AMP production by goblet cells and enterocyte respectively. When these mice are co-housed with NLRP6<sup>−/−</sup> mice, colitogenic bacteria will outnumber normal healthy microbiota. TLR2 and TLR9 agonists secreted from dysbiotic microbiota will reach liver through portal circulation where they activate TLR2 and TLR9 respectively to induce TNF-α secretion. TNF-α will further enhance the progression of NAFLD to NASH. AMP: antimicrobial peptide; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis

Another elegant study using a murine water avoidance stress (WAS) model reported that NLRP6 inflammasome signaling is inhibited in WAS-induced enteritis via corticotrophin
releasing hormone (CRH) secretion from the brain as a result of stress [62]. Further, this inhibition of NLRP6 signaling led to dysbiosis-induced enteritis in stressed mice, which was transferrable to non-stressed mice upon co-housing. Interestingly, WAS-induced NLRP6 inflammasome inhibition and associated intestinal pathology was reversed upon treatment with PPAR-γ agonists [62]. However, how CRH inhibits NLRP6 inflammasome activation in the intestine remains unknown. It is possible that dysbiotic microflora observed in co-housed, non-stressed mice could affect the expression of the NLRP6 inflammasome. Future studies are needed to shed light on these issues.

NLRP6 deficiency has been implicated in the aggravation of chemical-induced colitis and subsequent tumorigenesis [15]. Chen and colleagues induced chemical colitis in WT and NLRP6−/− mice using dextran sulfate sodium (DSS) and found that NLRP6−/− mice had increased colitis-induced mortality compared to WT mice. Further, NLRP6−/− mice displayed increased susceptibility to colitis-mediated tumorigenesis and NLRP6 in hematopoietic cells was found to be important for the prevention of inflammation-induced tumorigenesis [15]. However, the authors did not investigate the molecular mechanism underlying the increased susceptibility to tumorigenesis in NLRP6−/− mice. Soon after this report was published, Normand et al. also reported that NLRP6 performs essential functions in the regulation of tissue repair necessary for protection against chemical-induced colorectal carcinogenesis [41]. This study showed that NLRP6 deficiency leads to dysregulated colonocyte proliferation and migration, thereby facilitating tumor formation. RT-PCR analysis revealed increased expression of genes involved in cell proliferation such as casein kinase ε (csnk1ε) and SMARRC1 along with the proto-oncogene Myc11 in the DSS-treated colon of NLRP6−/− mice [41] (Fig. 1.6). One year later, another well-designed study by Hu et al. reported that the enhanced susceptibility to
tumorigenesis in NLRP6−/− mice is microbiota-dependent. The aberrant microflora in these mice caused increased transcription of CCL5 which, in turn, augmented epithelial proliferation via increased IL-6 secretion [44] (Figure 6). However, future studies will be necessary to understand the components of the altered microbiota that contribute to tumorigenesis. In another study, overexpression of NLRP6 in gastric cancer cells enhanced the expression of P14ARF, P53, and P21 while reducing the expression of Mdm2 and Cyclin D1, indicating that NLRP6 negatively regulates carcinogenesis via the P14ARF-Mdm2-P53 tumor suppressor axis [63] (Figure 6). Together, these observations establish NLRP6 as a negative regulator of colorectal cancer and suggest that modulation of NLRP6 may be a promising therapeutic alternative for its treatment.

1.8 NLRP6 in neuroinflammation

In addition to its role in host defense against bacterial and viral infection-induced inflammation, NLRP6 has been shown to modulate neuroinflammation [16, 64]. In a murine model of acute peripheral nerve injury, Ydens and colleagues reported that deletion of NLRP6 strongly impairs nerve function [16]. Further, the expression of both NLRP6 and NLRP3 was found to be increased 4 hours after surgery-induced acute neurodegeneration. Intriguingly, NLRP6−/−, but not NLRP3−/−, mice had a dramatic drop in the sciatic functional index (SFI) and needed a longer recovery time, compared with WT mice, after crushing of the right sciatic nerve. Furthermore, it was demonstrated that NLRP6 enhanced this recovery independent of inflammasome activation. Thus, the authors proposed that enhanced MAPK activity observed in NLRP6−/− mice could exacerbate inflammation resulting in delayed recovery from nerve injury. Future studies will be necessary to shed more light on how NLRP6 enhances recovery from peripheral nerve injury and how this could be manipulated for therapeutic intervention.
Figure 1.6: NLRP6 regulating different mechanisms to prevent carcinogenesis. A and B) NLRP6 deficiency increased the expression of genes involved in cell proliferation such as casein kinase ε (csnk1ε), ccl20, and SMARRC1 along with proto-oncogene Myc11 in the DSS-treated colon. C) NLRP6 prevents dysbiosis-induced IL-6 secretion thereby preventing abnormal cell proliferation in the intestine. D and E) NLRP6 augments the expression of P14ARF, P21, and P53 and suppresses Mdm2 and Cyclin-D in gastric cancer cells to prevent tumor progression. Ccl20: CC-chemokine ligand 20; SMARRC1: a member of switch/sucrose nonfermentable chromatin remodeling complex; Ccl5: CC-chemokine ligand 5; Mdm2: murine double minute 2.

In addition to the peripheral nervous system, a role for NLRP6 in the central nervous system (CNS) has also been reported. In a murine model of intracerebral hemorrhage (ICH)-induced brain injury, Wang et al. analyzed the expression profiles and biological functions of NLRP6 [64]. NLRP6 expression was increased mainly in glial fibrillary acidic protein (GFAP)-
positive astrocytes in the perihematomal brain after ICH downstream of TLR4 activation. Interestingly, NLRP6−/− mice displayed severe brain injury as evidenced by increased edema, NF-κB-mediated pro-inflammatory cytokine production, and aggravated neurological-deficit scores compared to their WT counterparts. Since microbiota has been shown to regulate neurodevelopmental disorders and promote the autism phenotype [65], it will be interesting to explore whether microbiota in the gut has any influence on ICH-induced brain injury or in acute peripheral nerve injury.

1.9 NLRP6 in human diseases

Most of the studies intended to define the expression and function of NLRP6 have been performed in mouse models and very little research to date has involved human patients. Consistent with the mouse model, genome-wide analysis based on gastrointestinal transcriptomics revealed high expression of NLRP6 in the small intestine of healthy humans [66]. Thus, as in mice, NLRP6 appears to have a specific role in the intestines of humans. Recently, we reported higher expression of NLRP6 in neutrophils, macrophages, and epithelial cells in the lungs obtained from pneumonia patients [13]. Consistent with this, MRSA-infected mice also displayed enhanced expression of NLRP6 in these cells. While expression of NLRP6 has been shown to prevent colorectal cancer in murine models, gene expression analysis performed in colorectal cancer patients showed no change in the expression of NLRP6 [67]. In contrast, a genome-wide study linked a single nucleotide polymorphism in NLRP6 with platelet volume [68] suggesting that NLRP6 could have an important role in thrombopoiesis or platelet function. Interestingly, the expression of NLRP6 and IL-1β is increased in adipose tissue obtained from portal fibrosis patients compared to that from control patients [69]. This is
consistent with the important role of NLRP6 in NAFLD and obesity previously identified in mice [42].

In another study of patients undergoing endodontic microsurgery, analysis of tissues associated with apical periodontitis revealed higher expression of NLRP6 [70]. In addition, when NRP6 was silenced in periodontal ligament cells and then stimulated with E coli-derived LPS, both NF-κB and ERK signaling were found to be augmented as were secretion of IL-6 and TNF-α. This suggests that NLRP6 suppresses the inflammatory response during periodontitis, which is in line with mouse studies where NLRP6 has been reported to negatively regulate NF-κB and ERK pathways [14]. A similar anti-inflammatory role of NLRP6 has been reported in rheumatoid arthritis patients. In this study it was demonstrated that NLRP6 is downregulated in synovial tissues and fibroblast-like synoviocytes (FLS) at both the transcription and translational levels compared to that of osteoarthritis patients [71]. Silencing of NRP6 in FLS from rheumatoid arthritis patients enhanced proinflammatory cytokine production via increased activity of NF-κB in response to TNF-α. Furthermore, the authors reported that NLRP6 acts as a docking site to facilitate the interaction between transforming growth factor-b-activated kinase-1 binding protein 2/3 and tripartite motif 38 (TRIM38) to promote lysosome-dependent degradation [71]. It is interesting that NLRP6 was found to regulate lysosome-mediated degradation, although future studies are needed to investigate if these findings have clinical relevance in other diseases.

1.10 Concluding remarks

NLRP6 has multifaceted functions ranging from regulation of host defense during microbial infections, composition of the epithelium-microbiota interface, and modulation of inflammatory modes of cell death such as pyroptosis and necroptosis, to regulation of metabolic
diseases, neuroinflammation, and cancer. Thus, the multitasking ability of NLRP6 makes this inflammasome unique among NLR-family members. Although NLRP6 was first reported to form an inflammasome in vitro in 2002, the long-standing conundrum regarding whether it can assemble an inflammasome in vivo or during microbial infection was only recently resolved. It is now known that NLRP6 recognizes Listeria (LTA) and S. aureus and forms an inflammasome complex along with ASC to regulate host defense. Of interest is the finding that NLRP6 recruits both caspase 1 and caspase 11 to form an inflammasome, whereas other NLR family members recruit only one or the other. Thus, future biophysical and biochemical studies are essential to understand how caspase 11 is recruited along with caspase 1 during NLRP6 inflammasome assembly. Additionally, there likely remain many unknowns regarding this pathway as new inflammasomes, sensors, and downstream signaling pathways are constantly being elucidated.

Currently there are large discrepancies regarding the role of NLRP6 in the composition of gut microbiota. Future studies are essential to address this controversy. Moreover, a unanimous, comprehensive protocol should be established through a consortium in order to bring uniformity in microbiota research.

While most of the research studies regarding NLRP6 have concentrated on the gut, the role of this molecule in host defense has also gained considerable attention. For example, a detrimental role of NLRP6 in innate immunity of the lungs has recently been described [13]. However, the function of this inflammasome in host defense during pathogen infections in other organs, including heart, brain, kidneys and neurons, remains unexplored. In addition, it will be of interest to investigate how NLRP6 regulates immunity during fungal infections and sepsis. Future studies in these areas will enhance our current understanding of the role of NLRP6 in host defense mechanism.
Several studies have suggested that the function of NLRP6 is context dependent. While this molecule is important for limiting infections in the intestines, it appears to be detrimental to host defense in the lungs. This may be explained by the cell types present in these tissues. During infections, expression of NLRP6 in myeloid cells such as macrophages and neutrophils appears to be detrimental; however, epithelial cells expressing NLRP6 might play protective roles. Further investigation will be required to understand the cell and organ-specific functions of NLRP6. Studies involving deletion of NLRP6 in specific cell compartments, such as myeloid cells, epithelial cells or lymphocytes, could generate more conclusive findings. The future challenge will be to apply our current knowledge of inflammasome biology to attenuate the tissue injury induced by uncontrolled inflammation through the use of specific treatment strategies in the context of the acute and chronic stages of both infectious and non-infectious diseases.

1.1 Notes


CHAPTER 2. NLRP6 NEGATIVELY REGULATES PULMONARY HOST DEFENSE DURING GRAM-POSITIVE BACTERIAL INFECTION THROUGH MODULATING NEUTROPHIL RECRUITMENT AND FUNCTION

2.1 Introduction

Acute pneumonia is a leading cause of childhood mortality (<5 years of age) accounting for the death of 920,136 children annually [1], and methicillin-resistant Staphylococcus aureus (MRSA) has been implicated in severe life-threatening infections, including necrotizing pneumonia and sepsis [2]. Further, S. aureus infection is also one of the major causes of secondary pneumonia following influenza infection [3]. In addition, S. aureus has developed resistance to multiple antibiotics and effective treatment strategies against this bacterium are limited [2, 4]. Therefore, S. aureus is a serious threat to human health and novel therapeutic strategies are needed.

The lung pathology induced by S. aureus is attributed to virulence factors, the intense inflammatory response, and evasion of host defense mechanisms, including neutrophil-mediated ROS-dependent bacterial killing [5, 6]. Regarding innate immune responses, nucleotide-binding oligomerization domain-like receptor (NLR) pyrin domain-6 (NLRP6) is a recently identified NLR present in the cytosol of innate immune cells [7]. Co-transfection of plasmids containing NLRP6 and apoptosis-associated speck-like protein containing card (ASC) in 293T cells led to activation of NF-κB and in COS-7L cells to a synergistic increase in caspase-1 activation and IL-1β secretion [8]. Under homeostatic conditions Levy et al have demonstrated that NLRP6 co-localizes with ASC and caspase-1 to form a complex resulting in IL-18 secretion from intestinal

epithelial cells, which is essential to prevent development of dysbiosis [9]. Together these results suggest that NLRP6 can co-localize with ASC for inflammasome formation and activation. However, the mechanisms of NLRP6 inflammasome activation and its role in host defense specifically during pulmonary microbial infection has not been explored. Furthermore, it remains unknown whether the NLRP6 inflammasome is activated by microbial infections and whether NLRP6 co-localizes with ASC and caspase-1 during such infections to induce pyroptosis.

In a mouse model of systemic infection, Anand et al [7] found that the NLRP6 negatively regulates host defense against *Listeria* and *Salmonella* infections as NLRP6 KO mice showed higher survival, decreased bacterial burden, and attenuated pathology compared to WT mice. In contrast, a study reported by Wlodarska et al [10] revealed a positive regulatory role of the NLRP6 inflammasome on immune function during enteric infection with *Citrobacter rodentium*. In this investigation, NLRP6 KO mice were shown to have an increased *C. rodentium* burden in the intestine, which correlated with extensive mucosal damage in the KO mice compared with WT controls. However, these results cannot be extrapolated to other bacterial infections in different organs, such as the lung, because Gram-positive bacteria have unique virulence factors and immune evasion strategies and the route of administration of bacteria also dictates host responses. Thus, the role of the NLRP6 inflammasome in pulmonary immunity against Gram-positive infections remains unclear. To this end, we have used NLRP6 KO mice to demonstrate how *S. aureus* exploits the NLRP6 inflammasome to dampen neutrophil function and enhance pyroptosis and necroptosis to increase mortality during acute bacterial pneumonia. Our results show NLRP6 as a potential therapeutic target for treatment of *S. aureus*-infected pneumonic patients.
2.2 Methods

2.2.1 Ethics Statement. Mouse experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University (protocol number 16-072). All animal experiments were performed in a manner to ensure minimal pain and distress.

2.2.2 Immunofluorescence microscopy. Immunofluorescence microscopy of lung sections was performed as described previously [11]. Human lung sections from lungs without evidence of infection or injury (control) or from patients who died due to bacterial infection with ALI/ARDS (pneumonic) were used. Mouse lung sections were from saline-challenged or S. aureus-infected mice. Lung sections were incubated with anti-NLRP6 (Abgent, CA) and antibodies for surface markers including anti-lipocalin Ab for neutrophils (R&D Systems, MN), or anti-CD68 Ab for macrophages (BioLegend, CA), and anti-CD326 Ab for alveolar epithelial cells (BioLegend, CA). For mouse lung sections, we used antibodies for surface markers including anti-Ly6G for PMNs (BioLegend, CA), anti-F4/80 for macrophages (BioLegend, CA), and anti-CD326 for alveolar epithelial cells along with anti-NLRP6 Ab (Sigma, MO). Appropriate Alexa-conjugated secondary antibodies (Invitrogen, CA) were used. For detection of necroptosis and pyroptosis through an immunofluorescence assay, antibodies against mouse NLRP6 and ASC (Sigma, MO), p-MLKL, (Abcam, MA), RIP3 (Cell signaling, MA), caspase-1 (Adipogen, CA), and gasdermin-D (Santa cruz, CA) were used. Excess antibodies were washed off, and the cells were labeled with secondary antibodies, such as mouse IgG/IgM (H+L) Alexa fluor 488, 568 (Invitrogen, CA). Images were obtained using an Axiocam digital camera (Zeiss, NY) connected to a Zeiss Axioskop 2 Plus microscope.
2.2.3 Immunoblotting and cytokine measurement. BMDMs or lungs were harvested at designated time points and homogenized in PBS containing 0.1% Triton X-100 (phosphatase and protease inhibitor cocktail added). After centrifugation the supernatants were used for immunoblotting. Total protein content in the supernatant was measured using a BCA protein assay kit (Thermofisher, NY) to ensure that equal amounts of proteins were loaded onto 10% SDS-PAGE gels. Proteins were transferred to polyvinylidene fluoride membrane according to the protocol provided by Bio-Rad. Appropriate primary antibodies against mouse NLRP6 (Sigma, MO), phospho-MLKL (Abcam, MA), RIP3, RIP1, P47\textsuperscript{phox}, P67\textsuperscript{phox}, gp91\textsuperscript{phox}, phospho-p38 MAPK, phospho-JNK, phospho-Stat3, ICAM-1, VCAM-1, caspase-8, GAPDH (Cell Signaling, MA), caspase-1 (Adipogen), and gasdermin-D (Santa Cruz, CA) were added to the membrane and incubated overnight at 4\textdegree C. Appropriate secondary antibodies were used, and the films were developed using ECL plus western blot detection system (ThermoFisher, NY). IL-1\textbeta, TNF-\textalpha, IFN-\gamma, IL-1\alpha, and IL-6 were measured in BALF supernatants by ELISA according to the manufacturer’s protocol (eBioscience, CA).

2.2.4 Animals. Eight to twelve-week-old WT mice (C57BL/6 background) were used. Equal age- and gender-matched NLRP6 KO, ASC KO, and Caspase-1/11 DKO mice on the C57BL/6 background were used throughout the experiments. Mice were kept on a 12:12 hour light/dark cycle under specific pathogen-free condition with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University.

2.2.5 Pneumonia model. To induce pneumonia, mice were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg) prior to intratracheal inoculation of S. aureus (USA 300 strain). A small midline incision was made on the ventral aspect of the neck and excess fat was separated
to expose the trachea. Fifty microliters of bacterial suspension containing $5 \times 10^7$ CFU of log phase *S. aureus* in isotonic saline was injected into the lungs by piercing trachea using a 28.5-gauge needle. At 12- and 24-hours post-infection, mice were euthanized to collect BALF, lungs, and liver for quantification of bacterial burden and leukocyte recruitment. BALF and homogenized organs were serially diluted and plated onto Tryptic soy agar (TSA) plates for bacterial enumeration. For survival experiments, we used $2 \times 10^8$ CFUs/mouse of *S. aureus* and observed survival for 12 days post-infection.

2.2.6 BALF collection. BALF was collected as described in our previous publication [11]. Briefly, after specific time points, mice were humanly euthanized, and the trachea was exposed. Using a 20-gauge catheter, 0.8 ml of PBS (heparin and dextrose added) was instilled inside the lungs and collected in a clean tube. The process was repeated a total of four times so that a minimum of 2.8-3 ml of BALF was collected from each mouse. Total leukocyte count was performed in a hemocytometer using 10 µl of BALF and the differential count was done under light microscopy using cytospin slides stained with DiffQuik reagent. The remaining cell-free BALF was preserved at -80°C for cytokine analysis.

2.2.7 Co-housing experiments. Co-housing experiments were performed as described by Anand et al [7]. In brief, age and sex matched WT and NLRP6 KO mice were co-housed together in 1:1 ratio for 4 weeks. After co-housing, WT and the KO mice were infected with *S. aureus* (i.t.) and euthanized 24 hours post-infection to measure the bacterial burden.

2.2.8 Bone marrow chimeras. Bone marrow chimeras were generated as described previously [11, 12]. In brief, the recipient mice were lethally irradiated with a 1000-rad inoculum from a cesium source. Bone marrow cells collected from healthy donor mice were injected into recipient mice via tail vein (8 million cells per mouse). The chimeric mice were kept under 0.2%
neomycin sulfate treatment for 15 days after transplantation. After 8 weeks of transplantation, the chimeric mice were infected with 5X 10^7 CFU of *S. aureus*. The mice were euthanized at 24-hour post-infection to estimate cellular recruitment and bacterial burden in the lungs.

2.2.9 Neutrophil depletion. For neutrophil depletion, mice were treated with 500 µg of anti-Ly6G antibody (clone 1A8, BioLegend, CA) [13] intra-peritoneally 24 and 2 hours prior to infection with lethal inoculum of *S. aureus* (2 X 10^8 CFUs/mouse). For IFN-γ inhibition, mice were treated with 100µg of IFN-γ antibody (BioXCell, NH) 12 hours prior to infection with *S. aureus*. For caspase-1 inhibition, mice were treated with 100 µg of caspase-1 inhibitor (Ac-YVAD-CMK, Cayman chemical, MI) 12 hours prior to infection with *S. aureus*. For MLKL inhibition, 100 µl of 100 µM MLKL inhibitor (GW806742X, Adipogen, CA) [14] was injected into each mouse i.p. 12 hours prior to infection with *S. aureus*. For RIP1 inhibition, mice were treated with 300 µg of necroptosis inhibitor (Nec-1/necrostatin-1s, Calbiochem, MA) [15] 18 hours before and at the time of bacterial infection, as published elsewhere [15]. Mice were euthanized to collect BALF and organs 24 hours post infection.

2.2.10 Neutrophil killing assay. The intracellular killing assay was performed as described [16] with slight modification. Briefly, bone marrow neutrophils from NLRP6 KO and WT mice were isolated, infected with *S. aureus* (MOI: 10), and treated with gentamicin for 30 minutes at designated time points post-infection (30 min, 60 min, 90 min, and 120 min) to kill extracellular bacteria. Cells were washed several times with sterile PBS to remove excess gentamicin and were then lysed with 0.1% triton X to release intracellular bacteria. The lysate was serially diluted with PBS, plated onto the TSA, and incubated at 37°C overnight for bacterial load estimation.
2.2.11 ROS detection. Total neutrophils, isolated from bone marrow of WT and NLRP6 KO mice using a magnetic negative selection cell isolation kit (STEMCELL Technologies, Vancouver, Canada), were infected with *S. aureus* (MOI 10). Total intracellular ROS production was quantified using a Fluorometric kit (AA Bioquest, CA). The effect of IFN-γ on ROS production was determined by pretreating neutrophils with either 20 ng/ml of recombinant mouse IFN-γ (R&D Systems, MN) or an equal volume of PBS for 30 minutes before infection with *S. aureus*. The total ROS production was quantified after 30 minutes of infection using a spectrophotometer.

2.2.12 Measurement of cell death. BMDNs were isolated from WT and NLRP6 KO mice and pretreated with Nec-1s (300 µM) or Ac-YVAD-CMK (100 µg/ml) or DMSO for 30 minutes before infection with *S. aureus* (MOI: 20). The percentage of cytotoxicity in BMDNs and LDH release into the alveolar space after *S. aureus* infection were measured using the Cytotox-ONE™ homogenous membrane integrity assay kit (Promega, WI). HMGB-1 was measured using a commercially available kit from Chondrex Inc, WA.

2.2.13 Statistics. Data are represented as mean ± SEM. The Mann-Whitney test was used to compare the bacterial burden between two groups. Student’s t-test was used whenever the data were parametric in nature. We used one-way ANOVA followed by Bonferroni’s multiple comparison test wherever more than two groups were involved. All statistical analyses were performed using GraphPad Prism 7 software. The survival curve was analyzed using Log-rank (Mantel Cox) test. All experiments were performed thrice. Significant differences are indicated by * (p<0.05), ** (p<0.01), *** (p<0.001), and **** (p<0.0001).
2.3 Results

2.3.1 NLRP6 expression is increased in the lungs of pneumonic patients and *S. aureus*-infected mice

To determine whether NLRP6 is upregulated in the lungs of pneumonic patients, we stained bacterial pneumonic and normal lung tissue sections with anti-NLRP6 antibody and found that NLRP6 was upregulated in key innate immune cells in the lungs, such as neutrophils (lipocalin-2⁺), macrophages (CD68⁺), and epithelial cells (CD326⁺) (Fig. 2.1A). Next, we determined if NLRP6 expression is upregulated in *S. aureus*-induced pneumonia in mice. In line with the results seen in human pneumonic lung sections, NLRP6 was upregulated in neutrophils (Ly6G⁺), macrophages (F4/80⁺), and epithelial cells (CD 326⁺) of mouse lungs following *S. aureus* infection (Fig. 2.1B). Consistent with the immunofluorescence results, the expression of NLRP6 was also increased in lung lysates of human pneumonic patients, *S. aureus*-infected human cell lines (THP-1 and HL-60), and mouse bone marrow-derived macrophages (BMDMs) (Figs. 2.1C-E).

To investigate whether NLRP6 triggers inflammasome activation during *S. aureus* infection, we infected BMDMs from WT and NLRP6 KO (KO) mice with *S. aureus* (MOI: 10) and measured the extent of caspase-1 activation at 8 hours post-infection. Both cleaved caspase-1 (p-20) in macrophage lysates and IL-1β levels in culture supernatants following infection were attenuated in the NLRP6 KO samples compared to the WT control (Figs. 2.1F and G) indicating the activation of the NLRP6 inflammasome by *S. aureus*. *S. aureus* has several virulence components, such as α-hemolysin (hla), clumping factor B, β toxin, phenol-soluble modulins, and panton-valentine leukocidins [5] that could potentially activate the NLRP6 inflammasome. Because hla has been reported to activate the NLRP3 inflammasome in human and mouse
monocytic cells under similar conditions [17, 18], we investigated whether hla can also activate the NLRP6 inflammasome in BMDMs and found this to be the case (Figs. 2.1E and F).

Figure 2.1. Upregulation and activation of NLRP6. (A) Lungs tissue sections from healthy and pneumonic patients were stained with antibodies against neutrophils (lipocalin-2+), macrophages (CD68+), and epithelial (CD326+) cells. NLRP6-positive cells are indicated by white arrowheads. (B) Immunofluorescence microscopy was performed on lung sections from healthy and S. aureus-infected mice to assess expression of NLRP6 as in A. NLRP6-positive cells are indicated by white arrowheads. (C) NLRP6 expression was analyzed in lysates of human healthy control tissue, pneumonic lung tissue, and HL-60 (human neutrophil-like) cells infected with S. aureus (MOI10) for 4 hours. (D) THP-1 (human monocytic) cells were infected with S. aureus (MOI 20) or purified α-hemolysin (hla) for 8 hours and expression of NLRP6 was assessed by western blotting. (E, F) Bone marrow-derived macrophages (BMDMs) were infected with S. aureus (MOI: 20) or hla from S. aureus (50 µg/ml) for 8 hours (E). Expression of NLRP6 protein was analyzed by immunoblotting. (F) Caspase-1 processing by the NLRP6 inflammasome in response to S. aureus or hla in BMDMs. (G) IL-1β secretion by WT and NLRP6 KO BMDMs following S. aureus infection. (H) IL-1β secretion by WT and NLRP6 KO mice after S. aureus (5 X 10^7 CFU/mouse) infection (N=6-8/group). (I) BMDMs from WT and NLRP6 KO mice were infected with S. aureus (MOI: 50) and stained for NLRP6 (red) and ASC (green). The white arrowheads demonstrate co-localization of NRP6 and ASC. (J) BMDMs from WT and NLRP6 KO mice were infected with S. aureus (MOI: 50) and stained for NLRP6 (red) and caspase-1 (green). The white arrowheads show co-localization of NRP6 and caspase 1. The graphs show the mean ± SEM of three independent experiments. The images shown are the representative of five different fields from three independent experiments. Magnification: 40X. SA: S. aureus, hla: α-hemolysin. *, p<0.05, **, p<0.01, and *** p<0.001.
Consistent with the *in vitro* results, NLRP6 KO mice showed lower levels of IL-1β in bronchoalveolar lavage fluid (BALF) after infection with *S. aureus*, providing evidence of NLRP6 inflammasome activation *in vivo* (Fig. 2.1H). ASC is known to be an integral part of NLRP3 inflammasome signaling although it is dispensable for NLRC4 inflammasome activation [19]. We infected BMDMs with *S. aureus* and performed immunofluorescence assay to observe whether NLRP6 co-localizes with ASC and caspase 1. We found that NLRP6 co-localized with ASC and caspase-1 during *S. aureus* infection (Figs. 2.1I and J).

### 2.3.2 NLRP6 deficiency confers host protection during *S. aureus*-induced pneumonia

To assess the role of the NLRP6 inflammasome in pulmonary host defense against *S. aureus*, we infected WT and NLRP6 KO mice intratracheally with a lethal dose of *S. aureus* (USA 300) (2x10⁸ CFUs per mouse) and observed the survival patterns for 10 days. Although all WT mice died within 3 days, 70% of KO mice survived longer than 10-days post-infection (Fig 2A). Furthermore, ASC KO mice displayed a survival pattern similar to that of the NLRP6 KO mice (Fig 2A). To determine whether the difference in survival is due to differences in the bacterial burden of various organs, we measured the bacterial burden in the lung, BALF, and extra-pulmonary organs after infecting mice with a sub-lethal inoculum (5x10⁷ CFU) of *S. aureus*. As compared to NLRP6 KO mice, WT mice had higher bacterial burdens in the lungs, BALF, and liver at both 12 and 24-hours post-infection (Fig 2B-D). Accordingly, the total protein in the BALF, which is a measure of pulmonary leakage, was higher in WT mice compared to their NLRP6 KO counterparts (Fig 2E).
Figure 2.2. NLRP6−/− mice are resistant to pulmonary *S. aureus* infection. (A) WT, NLRP6 KO, and ASC KO mice (N = 13-15/group) were infected intratracheally with a lethal inoculum of *S. aureus* (USA 300) (2X10^8 CFU/mouse) and survival was then observed for 10 days. WT and NLRP6 KO mice (N = 5-8/group) were infected intratracheally with a sublethal inoculum of *S. aureus* (5 X 10^7 CFU/mouse) and then were euthanized 12 or 24 h post-infection to quantitate the bacterial burden in (B) lungs, (C) BALF, and (D) liver. Total protein in BALF was measured (E). WT and KO mice (N=6-8/group) were infected intratracheally with 5X10^7 CFU/mouse of *S. aureus* (MSSA strain). Twenty-four-hours post-infection, the bacterial burden was measured in lungs (F) and BALF (G). WT and KO mice (N=5/group) were co-housed for 4 weeks and infected with a sublethal dose of *S. aureus*. Mice were euthanized at 24 hours post-infection to estimate bacterial burden in the lungs (H) and BALF (I). Bone marrow chimeras were generated as described in the methods (N=6-8/group) and then were infected with a sublethal dose of *S. aureus*. At 24-hours post-infection, mice were euthanized to estimate bacterial burden in the lungs (J) and BALF (K). *, p<0.05, **, p<0.01, and *** p<0.001.

To determine whether the detrimental effects of NLRP6 are bacterial strain specific, we infected WT and KO mice with a methicillin-susceptible *Staphylococcus aureus* strain (MSSA, Newman strain) and measured the bacterial burden in lungs and BALF at 24-hours post-infection. Consistent with the results seen with the USA 300 strain, the bacterial burden of the methicillin-sensitive strain was also higher in the lungs and BALF of WT mice compared to that
of KO mice (Fig 2.2F and G). NLRP6 inflammasome has been implicated in regulating intestinal microbiota [9, 20]. To determine if observed difference in the phenotype between WT and NLRP6 KO mice is due to difference in gut microbiota, we co-housed WT and NLRP6 KO mice for 4 weeks and infected them with S. aureus. As observed with non-co-housed mice, co-housed NLRP6 KO mice had significantly less bacterial burden in the lungs and BALF as compared to co-housed WT mice (Fig 2.2H and I). These data suggest that NLRP6 regulates pulmonary S. aureus infection independent of microbiota composition.

2.3.3 Both hematopoietic and non-hematopoietic cells contribute to host susceptibility.

Since we found upregulation of NLRP6 in both hematopoietic (neutrophils and macrophages) and non-hematopoietic cells (epithelial cells) (Fig 1.1B), we sought to determine if NLRP6 from each of these compartments is detrimental to bacterial clearance of S. aureus-induced pneumonia. Using bone marrow chimeric mice, we found that WT mice that received KO bone marrow (KO→WT) had lower bacterial burdens in the lungs and BALF than WT mice that received WT bone marrow (WT→WT) (Fig 2.2J and K). However, KO mice that received WT bone marrow (WT→KO) showed no increase in bacterial burden in the lungs and BALF compared to KO mice that received KO bone marrow (KO→KO) (Fig 2.2J and K). Together, these results suggest that NLRP6 derived from both hematopoietic and non-hematopoietic compartments is detrimental for bacterial clearance during S. aureus pneumonia.

2.3.4 Enhanced host protection in NLRP6 KO mice is dependent on neutrophils.

Neutrophils have been shown to be essential for containing pulmonary Staphylococcal infections [21, 22]. To determine if neutrophils confer augmented host protection in NLRP6 KO mice, we depleted neutrophils in the KO mice prior to infection with a lethal dose of S. aureus
and observed survival. We found that depletion of neutrophils reversed the survival benefit observed in the NLRP6 KO mice suggesting that protection is neutrophil-dependent (Fig 2.3A).

Figure 2.3. Neutrophils confer host protection in NLRP6 KO mice. (A) NLRP6 KO mice (N=10-12/group) were treated either with anti-Ly6G antibody or IgG2a, 24 and 2 hours prior to infection with a lethal dose of *S. aureus* (USA300). Survival was monitored for 10 days. NLRP6 KO mice (N = 5-8/group) were infected intratracheally with a sublethal inoculum of *S. aureus* (5 X 10^7 CFU/mouse). Mice were euthanized 12- or 24-hours post-infection to estimate total leukocytes (B), neutrophils (C), and macrophages (D) in the BALF. (E) Myeloperoxidase assay was performed in the lungs. *, p<0.05, **, p<0.01, and *** p<0.001.

Since neutrophils are essential for survival, we investigated if disruption of NLRP6 affects recruitment of neutrophils into alveolar spaces during *S. aureus* pneumonia. Compared to WT mice, KO mice had more leukocytes, including neutrophils, and macrophages recruited into alveolar spaces (Figs 2.3B-D). Furthermore, to measure neutrophil accumulation in the lung parenchyma, we performed a myeloperoxidase (MPO) assay and that found KO mice had more MPO activity than WT mice (Fig 2.3E).
2.3.5 Deletion of NLRP6 enhances bacterial killing of neutrophils through increased IFN-γ and ROS production.

Since NLRP6 was found to be upregulated in neutrophils and macrophages in the lungs, we wanted to know whether deletion of NLRP6 affects the function of these cells. To this end, we compared the intracellular killing ability of bone marrow-derived neutrophils (BMDNs) and BMDMs isolated from both WT and NLRP6 KO mice following infection with *S. aureus* (MOI:10). Our results indicate that neutrophils from NLRP6 KO mice had improved killing ability compared to WT cells as depicted by the reduction in intracellular CFUs in KO neutrophils (Fig. 2.4A). It is known that neutrophils use NADPH oxidase-dependent reactive oxygen species (ROS) to kill *S. aureus* intracellularly [23-25]. To determine if differences in killing ability of WT and NLRP6 KO neutrophils is due to an alteration in ROS production, we compared ROS production by neutrophils from WT and KO mice after infection with *S. aureus* and found that KO neutrophils produced more ROS than WT neutrophils (Fig 2.4B). To confirm these *in vitro* findings, we assessed the expression of NADPH oxidase components in lung homogenates from infected mice by western blotting. The expression of p47\(^{phox}\), p67\(^{phox}\), and gp91\(^{phox}\) was increased in the lungs from NLRP6 KO mice compared to those from WT mice, supporting the finding of higher ROS production by KO neutrophils (Fig 2.4C). IFN-γ has been shown to activate phagocytic cells during intra-pulmonary *S. aureus* infection [26]. Therefore, we measured the levels of IFN-γ secreted in the BALF of WT and NLRP6 KO mice after infection with *S. aureus*. Interestingly, we found that IFN-γ production was higher in KO mice compared to that of WT mice (Fig 2.4D). The production of IFN-γ requires activation of MAPK pathways [27]. Accordingly, we found higher MAPK activity in NLRP6 KO lungs than in WT lungs (Fig 2.4E).
Figure 2.4. NLRP6<sup>-/-</sup> neutrophils exhibit enhanced intracellular killing through increased IFN-γ and NADPH oxidase-dependent ROS production. (A) BMDNs from WT and KO mice were infected with <i>S. aureus</i> (MOI 10) and the intracellular killing ability of neutrophils was measured at the indicated time points. (B) ROS production by BMDNs from WT and KO mice were compared after infection with <i>S. aureus</i> (MOI 1). (C) WT and KO mice (N=6-8/group) were infected with a sublethal dose of <i>S. aureus</i> (5 X 10<sup>7</sup> CFU/mouse). At designated time points, lungs were collected and processed for immunoblotting to compare the expression of NADPH oxidase enzyme components between WT and KO mice. (D) IFN-γ in BALF obtained from mice in 3C was measured. (E) Lungs obtained from <i>S. aureus</i>-infected WT and KO mice were homogenized and expressions of phospho-38, phospho-JNK, and phospho-STAT3 were measured through immunoblotting. (F) BMDNs from WT and KO mice were isolated and pre-treated with either IFN-γ or PBS for 30 minutes before infection with <i>S. aureus</i> (MOI 10). Two-hours post-infection, WT neutrophils were lysed and immunoblotted to measure the expression of NADPH oxidase enzyme components. (G) ROS production by neutrophils in 3F were measured as described in the methods. All figures are representative of three independent experiments. ROS: reactive oxygen species, RFU: relative fluorescence unit. WT: Wild-type, KO: NLRP6 KO. *, p<0.05, **, p<0.01, and ### p<0.001

IFN-γ has been shown to induce ROS production in human mast cells during <i>Staphylococcal</i> infection [28]. Based on this observation, we assessed if IFN-γ contributes to the enhanced bacterial killing by neutrophils through induction of ROS. To this end, BMDNs from WT and KO mice were pre-treated with IFN-γ and subsequently infected with <i>S. aureus</i> (MOI of 10) followed by assessment of ROS production. Treatment of neutrophils with IFN-γ increased NADPH oxidase activity and ROS production (Fig 2.4F and G). The activation of phagocytes by IFN-γ involves activation of signal transducer and activator of transcription (STAT) proteins.
Supporting this observation, higher expression of phospho-STAT3 was found in the lungs of KO mice compared to WT (Fig. 2.4E). We also compared intracellular killing ability of BMDMs, but no difference was observed between WT and KO BMDMs after infection with S. aureus (Fig 2.4H). Collectively, these results suggest that genetic ablation of NLRP6 increases bacterial killing by neutrophils through increased IFN-γ and ROS production, which are associated with higher NADPH oxidase activity.

### 2.3.6 IFN-γ mediates bacterial clearance in NLRP6 KO mice

We examined if blocking IFN-γ hinders bacterial clearance in the KO mice following infection with S. aureus. In this context, we administered anti-mouse IFN-γ antibody (100 µg/mouse i.p.) to one group of the KO mice and a similar volume of isotype antibody to another group of mice 12 hours before infection with S. aureus. Blocking of IFN-γ in the KO mice increased the bacterial burden in the lungs and BALF suggesting that IFN-γ mediates bacterial clearance in the KO mice (Fig 2.5A and B).

![Graph](image)

Figure 2.5. IFN-γ mediates bacterial clearance in NLRP6 KO mice during S. aureus-induced pneumonia. KO mice (n=6-8/group) were treated with either anti-IFN-γ antibody (100 µg/mouse, i.p.) or isotype control antibody 12 hours prior to infection with S. aureus (5 X 10^7 CFU/mouse). WT mice were treated with equal volume of isotype control antibody 12 hours prior to infection with S. aureus (5 X 10^7 CFU/mouse). Twelve-hours post-infection, mice were euthanized to collect lungs and BALF. Bacterial burden in the (A) lungs, and (B) BALF were compared. All figures are representative of three independent experiments. *, p<0.05, **, p<0.01, *** p<0.001.
2.3.7 Natural killer and CD4⁺T cells are the primary sources of IFN-γ

We next sought to identify the cellular sources of IFN-γ during *S. aureus* infection. To this end, we performed flow cytometric analysis of lung cells from WT and KO mice following infection with *S. aureus* and found that NLRP6 KO mice had more IFN-γ-positive NK and CD4⁺T cells (Fig 2.6A-D).

Figure 2.6. NK cells and CD4⁺ T cells are the major sources of IFN-γ production during pulmonary *S. aureus* infection. WT and KO mice (N=6-8/group) were infected with *S. aureus* (5 X 10⁷ CFU/mouse). Twelve-hours post-infection, mice were euthanized to harvest lungs. Single cell suspensions obtained through digestion of lungs were treated with PMA/Ionomycin and GolgiStop for 5 hours and then stained for flow cytometry. (A) Representative zebra plot showing CD3⁻ IFN-γ⁺ NK 1.1⁺ cells. (B) Quantification of A. (C) Representative zebra plot showing CD3⁺ IFN-γ⁺ CD4⁺ T cells. (D) Quantification of C. (E) WT and KO mice (N=6-8/group) were infected with *S. aureus* (5 X 10⁷ CFU/mouse). Twenty-four hours post-infection, mice were euthanized to collect lungs tissues. Single cell suspensions obtained after lung digestion were stained to estimate NK and CD4 T cells using flowcytometry. The figures shown above are representatives of three independent experiments. *, p<0.05, **, p<0.01.
In this regard, Nguyen et al reported NK cells as the major source of IFN-γ during *S. aureus* infection [26]. Furthermore, we measured the number of NK and CD4 T cells in the lungs after infection through flow cytometry and found that the KO mice had more NK and CD4 T cells accumulated in the lungs compared to that of WT mice (Fig. 2.6E). These results together suggest that increased IFN-γ observed in the KO mice is due to enhanced numbers of NK and CD4 T cells recruited during infection. We also found CD8⁺T cells and γδT cells produce IFN-γ, although there was no difference between WT and KO mice in the total number of IFN-γ-positive CD8 or γδT cells in the lungs (Fig 2.7A-D).

Figure 2.7. Cellular source of IFN-γ in pulmonary MRSA infection. Single cell suspensions obtained from lungs of *S. aureus* infected WT and KO mice (N=9-11/group) were stimulated with PMA/ionomycin along with brefeldin A for 4 hours and then stained intracellularly for IFN-γ. (A) Gating strategy to obtain cell positive for both γδT cells and IFN-γ. (B) IFN-γ positive CD8⁺T cells. (C) Quantification of A. (D) Quantification of B. (E) NK cells and CD4 T cells (F) were isolated from WT and KO mice and pre-treated them with MAPK inhibitor (10µM) prior to infection with *S. aureus*. Percentage of IFN-γ positive cells are shown. Each figure is a representative of 3 independent experiments.
To determine whether increased IFN-γ production in the KO mice is due to higher MAPK activity in NK and CD4 T cells, we isolated these cells from the lungs of WT and KO mice and treated them with MAPK inhibitor prior to infection with *S. aureus*. However, blocking MAPK did not reduce IFN-γ secretion by NK and CD4 T cells (Figs 2.7E and F).

### 2.3.8 Deletion of NLRP6 decreases *S. aureus*-induced pyroptosis and necroptosis

Since we found decreased neutrophil recruitment and increased protein leakage in the lungs of WT mice compared to KO mice, we hypothesized that cell death in the lungs may be responsible for these results during *S. aureus* infection. In this regard, LDH, IL-1α, and HMGB-1 are intracellular molecules released exclusively after cell death and are thus termed *alarmins* [30, 31]. Therefore, we measured the levels of these alarmins in an *in vivo* setting and found their expression to be increased in WT mice compared to NLRP6 KO mice. This suggests that NLRP6 enhances inflammatory modes of cell death during *S. aureus*-induced pneumonia (Fig 2.8A-C).

We also measured the extent of cell death in BMDNs following infection with *S. aureus*. Neutrophils from WT mice exhibited increased cell death, as seen by increased cytotoxicity (LDH release), compared to that of KO neutrophils (Fig 2.8D). Next, to determine the nature of cell death, BMDNs from WT and KO mice were pre-treated with either Ac-YVAD-CMK (Caspase-1 inhibitor) or Necrostatin-1s (Nec-1s, necroptosis inhibitor) and infected with *S. aureus*. Pre-treatment of neutrophils with Ac-YVAD-CMK or Nec-1s reduced the cell death in WT neutrophils suggesting that the nature of cell death is both pyroptosis and necroptosis (Fig. 2.8D). In this regard, it is reported that caspase-1 and gasdermin-D mediate pyroptosis during bacterial infection [32-34].
Figure 2.8. NLRP6−/− mice display reduced pyroptosis and necroptosis during *S. aureus* induced pneumonia. WT and KO (N=6-8/group/time point) mice were infected with a sublethal dose of *S. aureus* (5 X 10⁷ CFU/mouse). (A) LDH released in BALF, (B) IL-1α in BALF, and (C) HMGB1 in serum were measured. (D) BMDNs from WT and KO mice were isolated and pre-treated with either Ac-YVAD-CMK (100µg/ml), Nec-1s (300 µM), or an equivalent amount of DMSO for 30 minutes prior to infection with *S. aureus* (MOI 50). Cytotoxicity was measured 2 hours post-infection. (E) Expression of cleaved gasdermin-D in BMDMs obtained from WT and KO mice after infection with *S. aureus* for 8 hours. (F) BMDMs from WT and NLRP6 KO mice were infected with *S. aureus* (MOI 50) for 8 hours. Caspase-1 activation was observed through fluorescence microscopy. Caspase-1 positive cells are shown by white arrowheads. (G) BMDMs from WT and NLRP6 KO mice were infected with *S. aureus* as in F. Gasdermin-D positive cells were observed through fluorescence microscopy. (H) Western blots to show expression of phospho-MLKL, RIP3, RIP1, and cleaved caspase-8 in lungs homogenates obtained from *S. aureus*-infected mice. WT and KO mice (N=6-8/group/time-point) were infected with *S. aureus* for 2 and 24 h post-infection, mice were euthanized to collect lungs and processed for western blotting. (I) Fluorescent microscopy showing expression of molecules involved in necroptosis in macrophages after infection with *S. aureus*. BMDMs obtained from WT and KO mice were infected with *S. aureus* (MOI 50) for 8 hours and then stained for phospho-MLKL (red) and RIP3 (green) antibody. Necroptotic cells are represented as orange (indicated by white arrowheads). (J) Human pneumonic lungs showing necroptosis. Lungs tissue sections from healthy and pneumonic patients were deparaffinized and stained with phospho-MLKL, RIP3, and DAPI. Tissue undergoing necroptosis is shown by white arrowheads. Immunofluorescence pictures were taken at 40X. All figures are representative of three independent experiments. Ac-YVAD-CMK: caspase-1 inhibitor, Nec-1s: Necrostatin-1s, CL-GSMD: Cleaved Gasdermin-D, FL-GSMD: Full-length Gasdermin-D, WT: *, p<0.05, **, p<0.01, and *** p<0.001.
To validate that NLRP6 induces pyroptosis, we assessed the expression of caspase-1 and gasdermin-D in BMDMs from WT and NLRP6 KO mice after infection with *S. aureus* (MOI of 10) for 8 hours. Both cleaved caspase-1 (Fig 2.1F) and gasdermin-D (Fig 2.8E) expression were higher in WT mice compared to KO mice. Also, we performed immunofluorescence microscopy on BMDMs to quantify caspase-1 and gasdermin-D expression and found increased caspase-1 and gasdermin-D-positive cells in BMDMs from WT mice compared to NLRP6 KO mice (Figs 2.8F and G).

It has been reported that *S. aureus* induces pathology in the lungs by a distinct cell death mechanism known as necroptosis [15]. Receptor-interacting-serine-threonine kinase-1 (RIP1), RIP3, and mixed lineage kinase-domain like protein (MLKL) are the core protein kinases that initiate and execute necroptosis [14, 15, 35]. Recently, caspase-8 has been shown to negatively regulate necroptosis during *Salmonella* infection in an enteritis model [36]. Thus, to explore whether NLRP6 can enhance necroptosis in the lungs during *S. aureus* infection, we assessed the expression of phospho-MLKL, RIP1, RIP-3, and caspase-8 in lung homogenates obtained from *S. aureus*-infected WT and NLRP6 KO mice through western blotting. Interestingly, both phospho-MLKL and RIP-3 were higher in the lungs of WT mice compared to NLRP6 KO mice (Fig. 2.8H). To further confirm this finding at the cellular level, we infected both WT and NLRP6 KO BMDMs with *S. aureus* for 8 hours and quantified necroptosis using immunofluorescence microscopy. The immunofluorescence assay also revealed more phospho-MLKL and RIP-3-positive cells in WT macrophages compared to NLRP6 KO macrophages after *S. aureus* infection, confirming that NLRP6 increases necroptosis (Fig 2.8I).

Since *S. aureus* has been shown to induce necroptosis in human cells, such as THP-1 cell lines [15], we examined if activation of the necroptosis pathway occurs in lungs of human
patients during pneumonia. Immunofluorescence microscopy conducted on lung sections obtained from bacterial pneumonic patients displayed more necroptosis, as evidenced by increased phospho-MLKL and RIP-3 expression, compared to that of healthy control lungs (Fig. 2.8J).

2.3.9 Blocking pyroptosis and necroptosis in WT mice augments host defense

We found that NLRP6 enhances both pyroptosis and necroptosis pathways during pulmonary *S. aureus* infection (Fig. 2.8A-J). Because pyroptosis has been found to be beneficial for bacterial clearance during intracellular bacterial infections [34], we examined whether pyroptosis is advantageous during *S. aureus* infection. Blockade of pyroptosis in mice by intraperitoneal administration of Ac-YVAD-CMK (caspase-1 inhibitor) 12 hours prior to infection with *S. aureus* resulted in reduced pulmonary leakage, LDH release, and bacterial burden in lungs and BALF (Figs 2.9A-D). In addition, blockade of pyroptosis suppressed cytokine secretion and enhanced survival of WT mice (Figs. 2.9E-H), confirming that pyroptosis is detrimental during *S. aureus* infection. It should be noted that the bacterial burden in WT mice receiving Ac-YVAD-CMK was still high when compared with NLRP6 KO mice (Fig. 2.9C), suggesting that an NLRP6-dependent, but caspase-1-independent, mechanism also exists to increase susceptibility to *S. aureus* infection.

Next, to confirm that NLRP6-mediated necroptosis is detrimental for host defense, we blocked necroptosis using an MLKL inhibitor (GW806742X) 12 hours before infection with *S. aureus*. There was a decrease in total protein and LDH release in the BALF of WT mice treated with GW806742X, which was comparable to that seen in NLRP6 KO mice (Fig. 2.10A and B).
Figure 2.9. NLRP6-mediated pyroptosis is detrimental during pulmonary \textit{S. aureus} infection. WT and KO mice (N=6-8/group) were administered either Ac-YVAD-CMK (100 µg/mouse) or an equal amount of DMSO 12 hours prior to infection with a sublethal dose of \textit{S. aureus}. Caspase-1/11 double knock out mice (N=6-8/group) received an equal amount of DMSO 12 hours prior to infection with a sublethal dose of \textit{S. aureus}. Mice were euthanized 24 hours post-infection to collect BALF and lungs. (A) Total protein and (B) LDH release in the lungs, bacterial burden in (C) lungs and (D) BALF were measured. (E) IL-1β, (F) TNF-α, and (G) MCP-1 in the BALF were measured through standard ELISA procedure. (H) WT mice (N=15/group) were treated with either Ac-YVAD-CMK (100 µg/mouse 12 hours prior to infection with \textit{S. aureus}), or an equivalent amount of DMSO prior to infection with a lethal dose of \textit{S. aureus} (2 X 10^8 CFU/mouse, i.t.). Survival was monitored up to 80 hours post infection. Each figure is a representative of at least three independent experiments. Ac-YVAD-CMK: caspase-1 inhibitor. TNF-α: Tumor necrosis factor-α, MCP-1: Monocyte chemoattractant protein-1, *, p<0.05, **, p<0.01, *** p<0.001, and **** p<0.0001.

Moreover, blocking necroptosis reduced the bacterial burden in lungs and BALF and ameliorated the inflammatory cytokine levels in WT mice (Figs. 2.10C-G). Furthermore,
leukocyte recruitment (neutrophils and macrophages) and survival were also increased in WT mice treated with necroptosis inhibitor (Figs. 2.10H-K). Collectively, these results reveal that NLRP6-mediated pyroptosis and necroptosis are detrimental to bacterial clearance and host survival during pulmonary *S. aureus* infection.

Figure 2.10. Blocking NLRP6-mediated necroptosis in WT mice improves host defense during *S. aureus* infection. WT and KO mice (N=6-8/group) were treated with either GW806742X (100 µl of 100 µM solution, i.e., 12 hours prior to infection) or an equivalent amount of DMSO prior to infection with a sublethal dose of *S. aureus*. Mice were euthanized 24 hours post infection to collect lungs and BALF. (A) Total protein and (B) LDH release in the BALF, bacterial burden in (C) lungs and (D) BALF were measured. (E) IL-1β, (F) TNF-α, and (G) MCP-1 in the cell-free BALF were measured. (H) Total leukocytes, (I) neutrophils, and (J) macrophages in the BALF was determined. (K) WT mice (N=15/group) were treated with either Nec-1s (300 µg/mouse) or an equivalent amount of DMSO, 18 hours before and at the time of infection with *S. aureus* (2X10⁸ CFU/mouse). Survival was monitored for up to 80 hours. Each figure is representative of three independent experiments. GW806742X: MLKL inhibitor, Nec-1s: Necrostatin-1s. *, p<0.05, **, p<0.01, and *** p<0.001.
2.4 Discussion

Lung diseases induced by Gram-positive pathogens are an important cause of morbidity and mortality in both immunocompetent and immunocompromised individuals [19, 37]. Although antibiotics decrease the morality rates of bacterial pneumonia, the efficacy is somewhat limited due to the substantial number of immunocompromised individuals, growing number of elderly patients, and the rise of multi-antibiotic resistant bacterial strains. Thus, alternative therapeutic approaches, including the manipulation of host signaling events, are needed. However, detailed understanding of the host innate immune response is critical for the design of potential therapeutic interventions. Because the lung is continuously exposed to pathogens and their virulence factors, this organ possesses a multifaceted host defense system. Moreover, a successful immune response in the lung is critical for efficient clearance of microbial pathogens and therefore, the innate immune system possess germline-encoded pattern-recognition receptors.

The NOD-like receptors (NLRs), including inflammasomes, are specialized cytosolic pattern-recognition receptors/sensors necessary for clearance of invading cytosolic pathogens. Under normal homeostatic conditions, the NLRP6 inflammasome is highly expressed in intestinal epithelial cells [9, 20, 38, 39] where it co-localizes with ASC and caspase-1 [9]. It is also expressed in immune cells including neutrophils, T-cells, macrophages, and dendritic cells [7]. Despite high expression of NLRP6 in the lower respiratory tract, the role of NLRP6 in lung inflammation has not previously been explored. In the current study, we demonstrate that NLRP6 is upregulated in neutrophils, macrophages, and epithelial cells in the lungs of bacterial pneumonic patients. Further, NLRP6 is upregulated in myeloid and non-myeloid cells in the
lungs and co-localizes with ASC in a mouse model of pulmonary *S. aureus* infection. We also found that the important virulence factor, α-hemolysin, can activate the NLRP6 inflammasome.

The immune response to *S. aureus* is manifested by vascular leakage, neutrophil recruitment into the alveolar space, and upregulation of cytokines and chemokines [5, 6]. The current study demonstrates that the NLRP6 inflammasome increases susceptibility to *S. aureus*-induced lung infection. Delving into the mechanisms underlying this, we found that NLRP6 dampens NK cell-mediated IFN-γ secretion thereby hindering ROS-dependent bacterial clearance by neutrophils. Moreover, our study identifies NK cells and CD4 T cells as the primary source of IFN-γ during acute pulmonary *S. aureus* infection. In agreement with these findings, studies of *Listeria* and *Salmonella* infections [7] have also shown that NLRP6 signaling is detrimental to host defense. Nonetheless, in a non-infectious model, the NLRP6 inflammasome was found to be important for epithelial self-renewal, proliferation, and mucus secretion, which were essential for protection against chemical-induced colitis [9, 38].

Studies from different groups have shown that NLRP6 inflammasome regulates gut microbiota composition [7, 9, 20]. NLRP6 KO mice were found to have different microbiota configuration which make them more susceptible to chemical-induced colitis compared to the WT mice [20]. In contrast, recent studies have demonstrated that NLRP6 and ASC-related inflammasome do not regulate gut microbiota composition [40, 41]. Although it remains debatable whether the NLRP6 inflammasome influence gut microbiome, the reported difference in microflora composition in the KO mice can be nullified by co-housing the mice together with WT for 4 weeks [7]. In addition to colitis, microbiota have been shown to influence various disease conditions such as rheumatoid arthritis [42], diabetes [43], inflammatory bowel disease [44], and colorectal cancer [45]. In our study, however, co-housing of WT with NLRP6 KO mice did not
change the resistant phenotype of the KO mice against \textit{S. aureus}. In this context, similar report has been demonstrated by Anand \textit{et al}, showing that co-housing does not alter NLRP6 mediated immune mechanism during \textit{Salmonella} and \textit{Listeria} infection [7].

It is widely accepted that hematopoietic and non-hematopoietic (stromal) cells in the lung produce numerous proinflammatory mediators, including cytokines and chemokines. Although hematopoietic cells secrete chemokines or neutrophil chemo-attractants, including CXCL1/KC and CXCL2/MIP-2, the stromal cells (alveolar epithelial cells) secrete other neutrophil chemo-attractants, such as CXCL5/LIX and CXCL15/lungkine [46]. Our observations indicate that in both cell types, NLRP6 contributes to the enhanced susceptibility to \textit{S. aureus}-induced pneumonia. These conclusions are consistent with previous studies of the role of hematopoietic and non-hematopoietic cells in the context of bacterial infections in the lungs. In this context, NLRP6 in both hematopoietic and non-hematopoietic cells increases susceptibility to \textit{Listeria} and \textit{Salmonella} infections [7]. Similarly, CXCL1/KC secreted by both hematopoietic and stromal cells was found to be crucial for bacterial elimination and neutrophil accumulation in the lungs following \textit{Klebsiella pneumoniae} infection [47]. Nevertheless, it is clear from this investigation that neutrophil accumulation and function are critical for host protection against \textit{S. aureus}.

Pyroptosis and necroptosis are two distinct inflammatory modes of cell death. While pyroptosis is mediated by caspase-1 [32-34] and executed by gasdermin-D [32, 33], necroptosis is regulated by RIP1, RIP3, and MLKL (caspase-1 independent) [15, 35]. Pyroptosis has been shown to play an essential role in limiting several intracellular bacterial infections such \textit{Salmonella typhimurium}, \textit{Legionella pneumophila}, and \textit{Burkholderia thailandensis} [34]. However, extensive caspase-1 activation and subsequent pyroptosis have also been associated with the severity of several diseases such as myocardial infarction [48], inflammatory bowel
disease [49], and endotoxic shock [50]. Pertaining to these observations, we report that during S. aureus infection, NLRP6-mediated pyroptosis is detrimental for host survival. Furthermore, blocking pyroptosis reduced the hyper-inflammatory milieu and subsequently increased survival suggesting that pyroptosis triggers exaggerated inflammation during S. aureus infection. This difference in the role of pyroptosis can be attributed to differences in pathogenic properties and lifestyles of bacterial pathogens. While S. aureus is predominantly an extracellular pathogen, studies have shown that it can also survive intracellularly [51] and can resist anaerobic conditions [52]. Necroptosis induced by S. aureus is responsible for pathology in the lung [15]; however, its relationship with inflammasomes was previously unknown. Although ASC and NLRP3 have been linked with pore-forming toxin-induced necroptosis [14], the precise role of inflammasomes in the induction of necroptosis is not clear. In this study, we used both in vivo and in vitro experiments to show that NLRP6 mediates necroptosis of immune cells during acute pulmonary S. aureus infection. Moreover, S. aureus exploits NLRP6 to drive necroptosis, which is accompanied by an intense inflammatory response and loss of macrophages and neutrophils. It is possible that reduced cell death in NLPR6 KO mice attributed to higher leukocyte accumulation in the lungs of these mice. Since TNF-α has been shown to induce necroptosis [53, 54], the reduction of TNF-α found in the NLRP6 KO mice suggests that NLRP6 can trigger necroptosis via the TNF-α pathway. However, more comprehensive future studies will be needed to identify the detailed molecular mechanisms underlying NLRP6-mediated necroptosis. Future studies are also needed to determine whether other toxins or virulence factors produced by S. aureus can also activate the NLRP6 inflammasome.

In conclusion, the present study reveals the detrimental role of NLRP6 during S. aureus pneumonia (S6 Fig). Further, NLRP6 in both hematopoietic and resident lung compartments
contribute to *S. aureus*-induced lung inflammation. Not only does NLRP6 subdue neutrophil function by dampening IFN-γ and ROS production, it also triggers pyroptosis and necroptosis in the lungs that may lead to hyper-inflammation, loss of neutrophils, and mortality. However, future studies are essential to determine whether NLRP6 interacts with other inflammasomes such as NLRP3 and/or NLRC4 to induce pyroptosis and necroptosis during *S. aureus* infection. Comprehensive studies using specific double- or triple-KO mice would be useful to delineate these interactions in a conclusive manner. Further, extending upon our findings, we propose that functional single nucleotide polymorphisms in human NLRP6 may have effects on host defense mechanisms against gram-positive microbes.

2.5 Notes


CHAPTER 3. NLRP6-DERIVED IL-18 ENHANCES MORTALITY DURING POLYMICROBIAL SEPSIS

3.1 Introduction

Sepsis is a leading cause of death in intensive care units (ICU) [1] and the most expensive disease as it accounted for more than $20 billion hospital expenses in 2011 [2]. The third consensus conference has defined sepsis as “a life-threatening organ dysfunction caused by a dysregulated host response to infection” [1]. The estimated annual incidence of sepsis is around 19 million worldwide [3] and the incidence is increasing due to increased number of immunocompromised patients and improvement in identification of septic patients [1]. Numerous clinical trials have been performed in past 20 years; however, none of these trials succeed to provide an effective drug that could be used to treat the sepsis patients. In this situation, research to understand the detailed pathophysiology of sepsis is warranted in order to identify potential new drug targets of treatment strategies.

Pattern recognition receptors like Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are germline encoded innate immune components which play important roles in initiating the innate immune response to infections. Unlike TLRs, certain NLRs have ability to bind with an adaptor molecule like ASC (Apoptosis-associated speck-like protein containing CARD) and recruit an enzyme called caspase-1 to form a multiprotein complex known as inflammasome [4]. The active caspase-1 cleaves inactive form of IL-1β and IL-18 to their active form in order to initiate immune responses [4, 5]. While the roles of TLRs (especially TLR2, TLR3, TLR4, and TLR9) [6-9] in the outcome of sepsis are well defined, very few studies [10, 11] have focused to determine the roles of different NLRs in the pathophysiology of sepsis.

NLRP6 is a relatively new member of NLR family which is shown to form inflammasome during both infectious [12, 13] and non-infectious conditions [14]. In a recent
study using mouse model of pulmonary *S. aureus* infection, NLRP6 inflammasome was found to negatively regulate neutrophil-dependent host immunity [12]. Similar negative roles of NLRP6 were also reported during other bacterial infections such as *Salmonella* and *Listeria* [13, 15]. However, a study using an enteric pathogen, *Citrobacter rodentium*, NLRP6 inflammasome was found to be important for host defense [16]. Similarly, during viral infection, NLRP6 KO mice showed increased viral burden in the intestine compared to their WT counterparts [17]. These observations suggest that the role of NLRP6 inflammasome could be model or pathogen specific. Although NLRP6 is well expressed in the intestines, kidneys, liver, and lungs [18], its potential roles in sepsis remain elusive.

Using mouse model of CLP-induced polymicrobial sepsis, we demonstrate for the first time that NLRP6 inflammasome is detrimental during sepsis. NLRP6-driven IL-18 elicits hyperinflammation, lymphocytic cell death, and mortality. In addition, NLRP6 suppresses IFN-γ production by both CD4 and CD8 T cells. These results suggest that NLRP6 inflammasome serves as a negative regulator of host protection during polymicrobial sepsis.

### 3.2 Methods

#### 3.2.1 Mice. C57BL/6 (wild type) mice were purchased from Jackson Laboratories (Bar Harbor, ME). NLRP6−/− and IL-18 KO−/− mice were purchased from the Millennium Pharmaceuticals (Cambridge, MA) and then backcrossed 10 times with C57BL/6 mice. Animals were kept in specific pathogens free environment with access to food and water all the time. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The animal protocol (16-028) was approved by Institutional Animal Care and Use Committee (IACUC) at Louisiana State University.
3.2.2 Human tissue samples. Human splenic tissue blocks (septic and non-septic) were kindly granted by Dr. Richard S Hotchkiss, Washington University School of Medicine at St. Louis, MO.

3.2.3 Induction of sepsis. Sepsis was induced in mice using CLP technique as described in our previous publication [11]. It is further discussed in detail in supplemental section. For single bacterium induced sepsis, we injected *E. coli* at the dose rate of 5 X 10⁸ CFU/kg of mouse intraperitoneally and observed the survival for next 12 days. Recombinant IL-18 was administered immediately after CLP (5 ug/mouse, IP) [48]. For IL-18 blocking, mice received 200 µg of anti-IL-18 antibody (BioXcell, NH) 12 and 2 hours prior to induction of CLP. CD4 or CD8 T cells were depleted by treating each mouse with 200 µg of anti-CD4 or anti-CD8 antibody (BioXcell, NH) 12 and 2 hours prior to CLP surgery.

3.2.4 Collection of peritoneal lavage fluid. After specific time points, mice were euthanized. The peritoneal cavity was washed by injecting 7 ml of PBS containing heparin and dextrose. The lavage fluid was collected in a sterile conical tube. A total of 6ml of lavage fluid was collected from each mouse. The total and differential leukocyte count was performed in the peritoneal fluid as described in our previous publication [12].

3.2.5 Flow cytometry. Flowcytometric staining was performed as recommended by manufacturer’s protocol (BioLegend, San Diego, CA). The single cell suspensions obtained from septic WT and NLRP6 KO mice were stained with antibody against CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), T-bet (4B10), IFN-γ (XMG1.2) (BioLegend, San Diego, CA), and PI (Thermo Fisher Scientific, Waltham, MA). The stained cells were fixed with 2% paraformaldehyde and analyzed using either a FACs Caliber or LSRFortessaX20 (BD).
Appropriate isotype antibodies were used each color. The data obtained were analyzed using FlowJo_V10 (Treestar).

**3.2.6 Western blotting.** Western blotting was performed as described previously. Briefly, spleens from septic mice were homogenized with beads in the presence of protease/phosphatase inhibitor cocktail. Equal amount of protein from each spleen sample was loaded on to 10% SDS-PAGE gel. The proteins were then transferred to polyvinylidene fluoride membrane. Primary antibodies against mouse cleaved caspase-3, caspase-7, RIP-3, MLKL, gasdermin-D, and BCL2 were added to the membrane and incubated for overnight at 4°C. Next day, appropriated secondary antibodies were added, and films were developed using ECL plus western blot detection system.

**3.2.7 Immunofluorescence assay.** Parafilm-embedded splenic tissue blocks from septic and non-septic patients were provided by Dr. Richard S Hotchkiss, Washington University School of Medicine at St. Louis, MO. Immunofluorescence assay was performed in spleen tissue section from human patients and mice as described in our previous publication [12]. The following primary antibodies were used: human NLRP6, CD3, CD4, CD8 and mice NLRP6, CD3, CD4, and CD8. After overnight incubation with these primary antibodies, the excess antibodies were washed off with cold PBS. The slides were then stained with appropriate secondary antibodies against human and mouse antigens for 30 minutes at room temperature. Image were taken under florescent microscope (Zeiss Axioskop 2 Plus microscope).

**3.2.8 In vivo BrdU incorporation.** BrdU incorporation experiment was performed in accordance with the manufacturer’s recommendations (BD Biosciences). Briefly, WT and KO mice were treated with BrdU (1mg/mouse, i.p.) immediately after CLP. At designated time point, mice were
euthanized to collect splenic cells and stained with fluorescently labelled antibodies including BrdU. The data were acquired using an LSRFortessaX20 (BD).

3.2.9 Cytokine measurement. Cytokines were measured using standard ELISA procedure as described in manufacturer’s protocol (ebioscience/Thermofisher scientific, MA).

3.2.10 Cell death assay. Measurement of cell death was performed using Cytox-One Homogenous Membrane Integrity Assay Kit (Promega, WI) following the manufacturer’s protocol. LDH release was expressed in relative fluorescence units (RFU).

3.2.11 Statistics. Data are presented as Mean ± SEM. Unpaired two-tailed students t test was used to compare the data between two groups. One-way ANOVA followed by Tukey’s multiple comparisons test was used whenever there were more than two groups. Survival analysis was performed using Log-rank test. P value less than 0.05 was considered significant.

3.3 Results

3.3.1 NLRP6 is upregulated in the spleens of septic patients and CLP-induced septic mice

To determine whether NLRP6 is upregulated in the spleen of human patients, we obtained spleen tissue sections from septic and non-septic patients and performed immunofluorescence assay to determine the upregulation of NLRP6 in these tissue sections. Interestingly, NLRP6 (Red) was upregulated in CD3, CD4, and CD8 T cells (Green) of septic patients compared to that of non-septic patients (Figure 3.1 A). Consistent with this finding, NLRP6 (Red) was also upregulated in splenic CD3, CD4, and CD8 T cells (Green) of CLP-induced septic mice compared to sham-operated mice (Figure 3.1 B).
Figure 3.1: Expression of NLRP6 in lymphocytes of septic human patients and septic mice. A) Tissue section from septic and non-septic patients were processed for immunofluorescence assay. Tissue was stained against anti-human CD3, CD4, CD8 T cells (Green), anti-human NLRP6 (Red), and DAPI (Blue). The white arrowheads represent NLRP6+ cells. B) C57BL/6 wild type (WT) were either subjected to CLP or sham surgery. Twenty-four hours post-CLP, mice were euthanized to collect spleen for histopathological processing. Paraffin embedded spleen sections were processed for immunofluorescence microscopy. Antibodies used were anti-mouse CD3, CD4, CD8 T cells (Green), anti-mouse NLRP6 (Red), and DAPI (Blue). The white arrowheads represent NLRP6+ cells. Each image is a representative image from 4 different fields from 3 independent experiments. Magnification: 40X.

3.3.2 NLRP6 deficiency confers host protection during murine model of sepsis

To investigate the role of NLRP6 in polymicrobial sepsis, we induced moderate sepsis in NLRP6 KO and WT mice through CLP technique and observed the survival for 10 days. Interestingly, the KO mice had better survival (80% vs 40%) compared to that of WT mice (Figure 3.2A). To determine whether the resistant phenotype in the KO mice is model specific, we infected KO and WT mice with sub-lethal dose of E. coli intraperitoneally and observed the survival for 10 days. Consistent with the CLP model, the KO mice had improved survival compared to that of WT mice during E.coli-induced sepsis (Figure 3.2B). Furthermore, we measured bacterial burden in major organs and peritoneal lavage fluid (PF) after inducing sepsis
through CLP. The KO mice had less bacteria in the PF, spleen, and kidney compared to that of WT mice at both 12- and 24-hours post-CLP (Figures 3.2C-E). NLRP6 inflammasome has been implicated in regulating gut microbiota composition [15, 16]. To determine whether differences in gut microbiota can influence outcome of sepsis, we co-housed WT and KO mice for 4 weeks and induced sepsis through CLP technique. The co-housed KO mice still had less bacterial burden in the PF, spleen, and Kidneys compared to that of WT mice suggesting that the phenotype observed in the KO mice is not microbiota dependent (Figures 3.2F-H).

### 3.3.3 NLRP6 mediates hyperinflammation during polymicrobial sepsis

Sepsis is characterized by initial hyper inflammatory phase followed by prolonged hypo-inflammmatory or immunosuppressive stage [19, 20]. Both these phases are implicated for death of septic patients. Therefore, we determined whether NLRP6 initiates hyperinflammation to enhance mortality during sepsis. To this end, we induced sepsis through CLP and measured inflammatory cell recruitment in the PF. Interestingly, WT mice had more total WBCs and neutrophils recruited in the peritoneal cavity compared to that of KO mice after sepsis (Figure 3.3A). In addition, we measured major proinflammatory cytokines in the cell free PF obtained from septic mice. The WT mice had massive cytokine storm as seen by increased levels of IL-1β, TNF-α, MCP-1, and CXCL1 compared to that of KO mice (Figures 3.3B-E). Reduction in IL-1β in the PF of KO mice compared to WT mice indicates that NLRP6 inflammasome is activated during sepsis. Moreover, IL-10, which is considered as a predictive of fatal outcome in sepsis [21, 22], was also more abundant in WT compared to that of KO at 24 hours post-CLP (Figure 3.3F).
Figure 3.2. The impact of NLRP6 in host protection in a murine model of sepsis. A) C57BL/6 wild-type (WT) and NLRP6 KO mice (N=14 per group) were subjected to CLP-induced polymicrobial sepsis and survival was monitored for 12 consecutive days. The curve shown is a representative figure of 3 independent experiment (N=14-16 per group each time). Data analyzed using Log-rank (Mantel-Cox) test (*P<0.05). B) WT and NLRP6 KO mice (N=10 per group) were injected with $10^7$ CFUs of *E. coli* per mouse intraperitoneally (IP) and monitored for 12 days post-infection. The figure shown is a representative of 3 independent experiments (N=10 per group each time). Log-rank (Mantel-Cox) test was used to analyze the data (*P<0.05). C-E) WT and KO mice (N=6-10 per group) were subjected to CLP-induced polymicrobial sepsis. Sham operated animals were used as control. Twelve- and 24-hours post infection, mice were sacrificed to collect peritoneal lavage fluid (PF), spleen, and kidneys for estimating bacterial burden. Each figure is a representative figure of 3 independent experiments. Data were analyzed using unpaired two-tailed student’s *t* test for each time points (*P<0.05, **P<0.01, and ***P<0.001). F-H) WT and NLRP6 KO mice (N=5 per group) were co-housed for 4 weeks and induced polymicrobial sepsis via CLP. Twenty-four hours post-CLP, mice were euthanized to enumerate bacterial burden in PF, spleen, and kidneys. Data represented as Mean ± SEM. Each scatter plot is a representative figure of 3 independent experiments. Data were analyzed as described for C-E.
Figure 3.3. Role of NLRP6 in inflammation during polymicrobial sepsis. A) CLP- and sham operated WT and NLRP6 KO mice (N=6-10 per group) were euthanized at designated time points to collect PF. Total and differential cell counting were performed in PF using Diff-Quik staining and light microscopy. The figure displayed is a representative figure of 3 independent experiments. Data analyzed using Multiple t test: one per row (*P<0.01). B) IL-1β, C) TNF-α, D) MCP-1, E) CXCL-1, and F) IL-10 were measured in PF using standard ELISA procedure. All data represented as Mean ± SEM. Each bar-diagram is a representative figure of 3 independent experiments. Data analyzed using unpaired two-tailed student’s t test for each time points (*P<0.05 and **P<0.01). CLP=Cecal ligation and puncture, PF=Peritoneal lavage fluid, IL-1β=Interleukin-beta, TNF-α=Tumor necrosis factor-alpha, MCP-1=Monocyte chemoattractant protein-1, CXCL-1=C-X-C motif chemokine ligand 1, and IL-10=Interleukin-10.

3.3.4 NLRP6 augments sepsis-induced lymphocytic cell death in the spleen

Sepsis causes extensive loss of T-cells in the spleen that leads to immunosuppression and mortality [19, 23, 24]. Based on this fact, we determined whether NLRP6 inflammasome enhances lymphocyte death during sepsis. For this, we first measured the number of T cells in the spleen after inducing sepsis and found that the WT mice had less CD3 positive lymphocytes compared to that of KO counterparts (Figures 3. 4A and B). Interestingly, the normal CD4 to CD8 T cells ratio (2:1) observed in sham animals was reduced to 1:1 in both WT and KO mice after sepsis suggesting that sepsis alters the proportion of CD4 and CD8 T cells in the spleen.
Figure 3.4: NLRP6 augments sepsis-induced lymphocytic cell death in the spleen. WT and NLRP6 KO mice (N=6-8 per group) were subjected to CLP-induced polymicrobial sepsis. At designated time points, mice were sacrificed to collect spleen. The single cell suspensions obtained from septic and sham spleen were stained with appropriated fluorochrome-tagged antibodies and subjected to flowcytometric analysis. A) A representative pseudo-color plot showing total CD3+ T cells. B) Quantification of A. C) Representative figure showing the proportion of CD4 and CD8 T cells within CD3 cells. D) Quantification of C. E) A flowcytometric figure showing CD3+CD4+ T cells. F) Quantification of E. G) A representative flowcytometric figure displaying CD3+CD8+ T cells. H) Quantification of G. I) A representative zebra plot showing PI+CD3+ T cells. J) Quantification of I. All bar diagrams are expressed as Mean ± SEM. Each figure is a representative figure of 3 independent experiments. Figure B, F, H, and J were analyzed using unpaired two-tailed student’s t test (*P<0.05, and **P<.001). K) LDH release in the PF obtained from septic WT and KO mice (N=6-8 per group) was measured using LDH assay kit. Data expressed as Mean ± SEM. The graph is a representative graph of 3 independent experiments. Unpaired two-tailed student’s t test was used to analyze the data (*P<0.05). LDH=Lactate dehydrogenase.

(Figures 3.4C and D). Similar observation was reported from the blood of septic patients [25].

The number of CD4 T cells significantly reduced after sepsis in both WT and KO mice;
however, this reduction was less pronounced in the KO mice (Figure 3.4E and F). In contrast, the number of CD8 T cells increased in both WT and KO mice after sepsis; however, the increase was more pronounced in KO compared to that of WT counterparts (Figure 3.4G and H). The increased numbers of CD4 and CD8 T cells observed in the spleens of KO mice could be due to reduced cell death and/or increased proliferation. To this end, we induced sepsis in WT and KO mice and measured the extent of cell death in the spleen through flowcytometry. Interestingly, WT mice had significantly more PI+ T cells compared to that of KO mice (Figs. 3.4I and J). In addition, the PF obtained from septic KO mice displayed less LDH compared to that of WT counterparts confirming that NLRP6 enhances cell death during sepsis (Fig. 3.4K). Next, to determine whether NLRP6 affect the proliferation of T cells in the spleen during sepsis, we measured BrdU+ T cells after inducing CLP and found that KO mice had more BrdU+ cells in the spleen (Fig. 3.5).

### 3.3.5 NLRP6-mediated increased susceptibility to polymicrobial sepsis is IL-18 dependent.

IL-18 and IL-1β have been implicated for aggravating septic conditions [26-31]. Based on this fact, we measured levels of IL-18 and IL-1β in PF of WT and KO mice and found that these cytokines were less abundant in KO mice compared to that of WT mice (Figs 3.6A and B). Moreover, we hypothesized that reduced IL-18 and/or IL-1β in the KO mice could account for higher survival in these mice. To this end, we administered recombinant IL-1β or IL-18 to the KO mice (IP) immediately after CLP and observed the survival. Treatment with IL-18 reversed the survival advantage observed in the KO mice following sepsis. However, no difference in the survival was observed in WT mice upon IL-1β treatment suggesting that NLRP6 increases susceptibility via IL-18 (Fig. 3.6B).
Figure 3.5: Role of NLRP6 deficiency in the proliferation of CD4 and CD8 T cells in the spleen during sepsis. WT and NLRP6 KO mice (N=6-8 per group) were subjected to CLP surgery to induce polymicrobial sepsis. BrdU was injected right after CLP procedure. Single cell suspensions obtained from septic mice were stained to obtain BrdU positive cells. A) Percentage of BrdU+ CD3 T cells. B) Quantification of A. C) Percentage of BrdU+ CD4 T cells. D) Quantification of C. E) Percentage of BrdU+ CD8 T cells. F) Quantification of E. The experiment was repeated thrice. Data in bar diagram are expressed as Mean ± SEM. Data analyzed using unpaired two-tailed student t test (*P<0.05).

3.3.6 NLRP6-driven IL-18 enhances destructive inflammation during polymicrobial sepsis.

Next, we sought to determine the mechanism how IL-18 enhances mortality in NLRP6 KO mice following sepsis. Since NLRP6 KO mice displayed attenuated inflammation and less T cell death, we hypothesized that IL-18 could contribute to hyperinflammation and high lymphocyte loss in the WT mice ultimately leading to mortality. To this end, we administered recombinant IL-18 in the KO mice immediately after sepsis and determined the extent of inflammation. Interestingly, mice that received IL-18 had higher levels of inflammatory
cytokines such as IL-6, TNF-α, MCP-1, and CXCL1 compared to the groups that didn’t receive IL-18 (Figs. 3.7A-D). In addition, we measured the extent of cell death by determining the levels of cell death alarmins (LDH and IL-1α) [12] in the PF after administration of IL-18.

Fig. 3.6: NLRP6-mediated increased susceptibility to polymicrobial sepsis is IL-18 dependent. A) Polymicrobial sepsis was induced in WT and NLRP6 KO mice (N=6-8 per group) using CLP technique. At designated time points, mice were humanely euthanized to collect PF. IL-18 in cell free PF supernatants was measured using ELISA. The figure is a representative of 3 independent experiments. Data expressed as Mean ± SEM and analyzed using unpaired two-tailed student’s t test (*P<0.05). B) WT and NLRP6 KO mice (N=10 per group) were subjected to CLP-induced polymicrobial sepsis. WT mice received PBS right after CLP whereas, NLRP6 KO mice either received PBS, IL-18, or IL-1β immediately after CLP surgery. Survival was monitored for next 10 days. The experiment was repeated 3 times (N=10 per group each). The survival analysis was performed using Log-rank (Mantel-Cox) test (*P<0.05).

Interestingly, the KO group that received IL-18 had more cell death compared to that of WT mice (Figs 3.7E and F). Since NLRP6 KO mice had less cell death, we measured the T cells population in the spleen after induction of sepsis. Intriguingly, the KO mice that received IL-18 had less CD3+ T cells in the spleen compared to the group that received PBS only (Figure 3.7G and H). To confirm this finding, we used IL-18 KO mice and observed more T cells in the spleen compared to that of WT after sepsis (Fig. 3.8A). We further examined CD4 and CD8 T cells within the T cells population and found that IL-18 specifically led to depletion of CD8 T cells in the spleen of NLRP6 KO mice during polymicrobial sepsis (Figs. 3.7I and J). However, no change in CD4 T cells population was observed after IL-18 treatment (Figs 3.8B and C)
suggesting that IL-18 does not affect CD4 T cell death during murine model of polymicrobial sepsis.

Figure 3.7: NLRP6 inflammasome driven IL-18 enhances inflammation and CD8 T cell depletion during polymicrobial sepsis. We induced sepsis in WT and NLRP6 KO mice (N=6-8 per group) through CLP procedure. WT mice received PBS whereas the KO mice either received PBS or IL-18. At 36 hours post-CLP, mice were sacrificed to collect PF. A) IL-6, B) TNF-α, C) CXCL1, and D) MCP-1 were measured in the PF using ELISA procedure. E) LDH released in the PF was measured using Fluorimetric kit. F) IL-1α was measured in PF using ELISA procedure. Each figure is a representative figure of 3 independent experiments. Data are expressed as Mean ± SEM in each bar diagram. Data from A-F were analyzed using one-way ANOVA followed by Tukey’s multiple comparisons test (*P<0.05, and **P<0.01). G-J) Spleens obtained from mice in A were processed to generate single cell suspensions. These cells were then stained with antibodies to estimate the numbers of T cells subpopulations using flowcytometry. G) Representative plot showing percentage of CD3+ T cells. H) Absolute numbers of T cells per 300,000 events. I) Representative plot displaying percentage of CD3+ CD8+ T cells. J) Absolute numbers of CD3+ CD8+ T cells per 300,000 events. Data are represented as Mean ± SEM. Each figure is a representative of 3 independent experiments. Figures H and I are analyzed using one-way ANOVA followed by Tukey’s multiple comparisons test (*P<0.05 and **P<0.01).
Figure 3.8: Effect of IL-18 on lymphocyte population during sepsis. A) We induced sepsis in WT and IL-18 KO mice (N=5 per group) through CLP technique. At 36 hours post-CLP, spleens were collected to obtain cell suspensions. These cells were then stained to analyze total CD3+ T cells through flowcytometry. The figure is representative of 3 independent experiments. Unpaired two-tailed student’s t test was used to analyze the data (*P<0.05). B-C) We used WT and NLRP6 KO mice (N=6-8 per group) and induce sepsis through CLP procedure. WT mice received PBS whereas the KO mice either received PBS or IL-18. At 36 hours post-CLP, mice were sacrificed to estimate CD4 T cells population within spleen through flowcytometry. Experiment was repeated thrice. Data in the bar graphs are represented as Mean ± SEM. One-way ANOVA followed by Tukey’s multiple comparison tests was used to analyze the data (*P<0.05).

3.3.7 CD8 T cells but not CD4 mediates bacterial clearance during sepsis

Since CD4 and CD8 T cells were higher in NLRP6 KO mice compared to that of WT counterparts, we sought to determine their roles in bacterial clearance during polymicrobial sepsis. In this regard, we depleted CD4 and CD8 cells by using specific antibodies. Although depletion of CD4 T cells did not affect bacterial clearance during polymicrobial sepsis (Figs. 3.9A and B), depletion of CD8 T cells increased bacterial burden in the PF and spleen in the KO mice (Figs. 3.9C and D).
3.3.8 NLRP6 suppresses T cells mediated IFN-γ production via regulating T-bet expression during sepsis

Since NLRP6 was found to affect T cells survival and proliferation, we were interested to investigate whether it can modulate T cell function during sepsis. Several studies have reported critical role of IFN-γ in sepsis [32-34] and reduced ability of septic lymphocytes to produce this cytokine is an indication of immunosuppression [34]. To determine whether NLRP6 affects IFN-γ production, we determined the level of this Th-1 specific cytokine after inducing sepsis and found that KO mice had more IFN-γ in the peritoneal fluid compared to that of WT mice (Fig. 3.10A). Since T cells are an important source of IFN-γ production, we compared IFN-γ produced by these cells in WT and KO mice after induction of sepsis. Consistent with the result in PF, the KO mice had more IFN-γ positive CD4- and CD8 T cells compared to WT mice suggesting that NLRP6 not only affects T cells survival, it also impairs T cell function (Figures 3.10B–E).
Figure 3.10: NLRP6 suppresses T cells mediated IFN-γ production via regulating T-bet expression during sepsis. A) IFN-γ was measured in PF obtained from CLP-induced septic WT and NLRP6 KO mice (N=6-10 per group). B-E) Spleens obtained from septic WT and NLRP6 KO mice (N=6-8 per group) were processed to obtain single cell suspensions. The cells were then stimulated with leukocyte activation cocktail for 4 hours and intracellularly stained to enumerate IFN-γ producing T cell populations. A total of 250,000 events were collected. B) Zebra plot showing percentage of IFN-γ+CD4 T cells within CD3+CD4+ T cells. C) Quantification of B. D) A representative zebra plot showing IFN-γ+CD8 T cells within CD3+CD8+ T cells. E) Quantification of D. F-I) Single cells suspensions obtained from spleens of CLP-induced septic WT and NLRP6 KO mice (N=6-8 per group) were stained with appropriate antibodies to analyze the expression of T-bet within CD4 and CD8 T cells. F) A representative flowcytometric plot showing percentage of T-bet+CD4 T cells within CD3+CD4+ T cells. G) Quantification of F. H) A representative zebra plot showing T-bet+CD8 T cells within CD3+CD8+ T cells. I) Quantification of H. Each figure is a representative of 3 independent experiments. Data represented as Mean ± SEM. Bar diagrams A, C, E, G, and I were analyzed using unpaired two-tailed student’s t test (*P<0.05, and **P<0.01). PF=Peritoneal fluid.
Next, we determined how NLRP6 suppresses IFN-γ secretion by T cells. Studies have shown that IFN-γ secretion by T cells is regulated by T-bet expression [35-37]. T-bet directly activates Ifng that encodes for IFN-γ [35]. Based on these observations, we determined whether NLRP6 regulates T-bet expression to modulate IFN-γ secretion. For this, we compared the expression of T-bet on splenic CD4 and CD8 T cells from WT and KO mice after sepsis and found that the KO mice had more T-bet expressed on CD4 and CD8 compared with their WT counterparts (Figs. 3.10F-I).

3.3.9 Blocking IL-18 in WT mice reduces cytokine storm, bacterial burden, and improves survival during polymicrobial sepsis

Since NLRP6-mediated host susceptibility to polymicrobial was IL-18 dependent, we determined whether blockage of IL-18 in the WT mice could improve the outcome of sepsis in the WT mice. For this, we neutralized IL-18 in the WT mice by using anti-IL-18 antibody, induced sepsis through CLP method, measured inflammatory cytokines, and enumerated bacterial burden in the PF, liver, and spleen. As observed in NLRP6 KO mice, WT mice that received blocking antibody showed attenuated cytokines production and cell death (Figs. 3.11A-E). Furthermore, bacterial burden in PF and spleen were significantly reduced after neutralizing IL-18 (Figs. 3.11F and G). Finally, blocking IL-18 enhanced the survival in the WT mice suggesting that IL-18 is indeed, detrimental during polymicrobial sepsis (Figure3.11H). Consistent with our findings, IL-18 was found to enhance lung inflammation and bacterial burden in murine model of Pseudomonas aeruginosa infection [38].
Figure 3.11: Blocking IL-18 in WT mice reduces cytokine storm, bacterial burden, and improves survival during polymicrobial sepsis. WT and NLRP6 KO mice (N=5 per group) were subjected to CLP-induced polymicrobial sepsis. WT mice received either IgG1 or anti-IL-18 antibody at 12 and 2 hours prior to CLP whereas KO mice received IgG1 at those time points. A) TNF-α, B) IL-6, C) MCP-1, D) CXCL1, and E) LDH release in the PF were determined. Bacterial burden in F) Peritoneal fluid and G) Spleen was enumerated. All data are represented as Mean ± SEM. Each figure is a representative figure of 3 independent experiments. Data in figure A-G were analyzed using one-way ANOVA followed by Tukey’s multiple comparisons test (*P<0.05 and **P<0.01). H) WT mice (N=10 per group) received either IgG1 or IL-18 antibody and NLRP6 KO mice (N=10 per group) received isotype antibody 12 and 2 hours prior to CLP surgery. Survival was monitored for next 12 days. The experiment was repeated thrice (N=10 per group each time). Data analyzed using Log-rank (Mantel-Cox) test (*P<0.05). PF=Peritoneal fluid.

3.4 Discussion

Sepsis is a complex disorder characterized by initial hyperinflammatory response followed by immunosuppressive stage both of which contribute to mortality. However, the
mechanism behind the initiation of exaggerated inflammation and subsequent immunosuppression remains elusive. Through this investigation, we have demonstrated that NLRP6 is upregulated in different lymphocytic cells of septic patients and septic mice. NLRP6 inflammasome triggers destructive inflammatory response and enhances sepsis-induced immunosuppression through increased loss of T cells and reduced IFN-γ production. In addition, we have shown that NLRP6-driven IL-18 enhances mortality in sepsis through mediating both inflammation and cell death.

Using CLP-induced polymicrobial sepsis and *E. coli*-induced septic shock, we have confirmed that NLRP6 KO mice are protected from sepsis. In line with our findings, negative roles of NLRP6 inflammasome have been reported from other infection models including pulmonary [12] and systemic bacterial infections [13, 15]. Similarly, we and others have demonstrated detrimental roles of NLRP3 inflammasome during sepsis [10, 11]. Although these models have similar phenotypes, the mechanisms associated with these findings are model specific. In systemic bacterial infection model, NLRP6 was found to regulate MAPK and canonical NF-kB pathway to enhance neutrophil recruitment and bacterial clearance [15]. In MRSA-induced pneumonia model, NLRP6 KO mice had higher neutrophil accumulation in the lungs due to reduced necroptosis and pyroptosis [12]. In both models, higher neutrophil recruitment was found to be important to clear bacteria. However, in our polymicrobial sepsis model, NLRP6 KO mice had reduced neutrophil recruitment, attenuated cytokine storm, and higher survival. In support of our findings, higher neutrophil recruitment along with excessive cytokine storm have been shown to worsen the outcome of sepsis [21, 39, 40]. In contrast to our finding, NLRP6 was found important to clear *Citrobacter rodentium* [16] and encephalomyocarditis viral infection from the intestine [17] suggesting that NLRP6 is important
to clear enteric pathogens. These observations suggest that the role of NLRP6 could be site or organ specific. In localized infection model such as intestinal inflammation, NLRP6 could play crucial role; however, in case of septic model, NLRP6-induced inflammation is detrimental. This spatiotemporal response of NLRP6 has made this NLR unique among its family members.

Different studies have reported that NLPR6 inflammasome regulates gut microbiota composition and such differences in microbiota composition render the mice susceptible to colitis and tumorigenesis [14-16, 18]. However, recent studies have challenged these observation stating that NLRP6 inflammasome do not shape the microbiota composition [41, 42]. Although NLRP6 inflammasome regulating the microbiota composition is debatable, we confirmed that the resistant phenotype observed in the NLRP6 KO mice is not dependent on their microbiota composition. The co-housed KO mice displayed similar phenotype as that of singly housed KO mice confirming that NLRP6 regulate sepsis independent of microbiota composition. Consistent with our findings, NLRP6 has been shown to regulate host defense against microbial infections independent of microbiota composition [12, 15].

Loss of lymphocytes, particularly through apoptosis and inflammatory modes of cell death is a hallmark of sepsis-induced immunosuppression [23, 43, 44]. Here we have shown that NLRP6 inflammasome leads to loss of lymphocytes via reducing their proliferation and enhancing both inflammatory and non-inflammatory modes of cell death. Additionally, we found that NLRP6-driven IL-18 enhances sepsis-induced cell death ultimately leading to loss of lymphocytes, particularly CD8 T cells which were found to be crucial for host defense. However, IL-18 did not affect CD4 T cells population suggesting that reduced CD4 T cells observed in WT mice could be due to reduced proliferation and non-IL-18-mediated cell death.
Consistent with our findings, IL-18 has been shown to play detrimental roles in septic conditions [26-28, 45] including pneumonia [46].

IFN-γ has been shown to enhance antigen presenting capacity of septic monocytes through enhancing HLA-DR expression [32]. In addition, IFN-γ also enhances intracellular killing ability of phagocytic cells [12]. These observations suggest that IFN-γ plays essential role during sepsis. One of the hallmarks of immunosuppression in sepsis is reduced capacity of lymphocytes to produce IFN-γ [34]. However, the exact mechanism behind this phenomenon is not understood. In our study, we found that NLRP6 suppressed IFN-γ production by CD4 and CD8 T cells. Consistent with these findings, increased IFN-γ production in NLRP6 KO mice was reported during Gram-positive pneumonia [12]. However, the mechanisms underlying this observation was not clear. Here we have demonstrated that NLRP6 reduces IFN-γ production by suppressing T-bet expression in these cells. However, further research is needed to understand in detail how NLRP6 interacts and suppresses T-bet expression in these cells. IL-18 has been shown to enhance IFN-γ production by T cells [47]. However, in our model, NLRP6 KO mice had higher IFN-γ production despite less IL-18. This result suggests that IFN-γ production in NLRP6 KO mice is IL-18 independent. Since IFN-γ was higher in NLRP6 KO compared to the WT mice, it is possible that this increased IFN-γ could further enhance the functions of neutrophils and monocytes in these mice which ultimately leads to better bacterial clearance observed in these mice.

Taken together, the current study discloses an unrecognized role of NLRP6 inflammasome during septic condition. Using appropriate human and mouse samples, we have demonstrated that NLRP6 is upregulated in different cell types during sepsis and negatively regulates host protection mechanism through modulating lymphocyte survival and function.
Based on these results we propose that blocking NLRP6 or its downstream cytokine, IL-18 could be an effective therapy to reduce sepsis-related mortality.

### 3.5 Notes


CHAPTER 4. NEUTROPHIL HOMEOSTASIS DURING GRAM-POSITIVE BACTERIAL PNEUMONIA

4.1 Introduction

Multiple studies using mouse models have demonstrated that neutrophils are essential for containing Gram-positive bacterial pneumonia-induced sepsis including *Streptococcus pneumoniae* (1) and *S. aureus* (2, 3). However, excessive neutrophil recruitment could be hazardous to the host as these cells secrete enzymes and inflammatory mediators that can damage tissues (4-6). Therefore, tight control of neutrophil homeostasis is important to contain infections while causing minimum damage to the host. Since neutrophils have a short lifespan (7), they are constantly generated in the bone marrow by granulopoiesis and released into the circulation through a highly regulated process. In humans, $1 \times 10^{11}$ neutrophils are mobilized from the bone marrow every day as a part of *steady-state granulopoiesis* (8). During infection a dramatic increase in bone marrow hematopoiesis is observed, called *emergency granulopoiesis*, together with increased release of neutrophils from the bone marrow into the circulation to contain the infection (9). However, the mechanism how granulopoiesis and neutrophil trafficking is regulated during *S. aureus* infection remains elusive.

*Staphylococcus aureus* remains one of the major causes of acute pneumonia, skin infection, and post-influenza superinfection (10-13). To date, antibiotics are the only therapeutic option to treat *S. aureus*-induced pneumonia; however, their efficacy has been jeopardized by the emergence of multiple antibiotic-resistant and hypervirulent strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). In this regard, optimal neutrophil accumulation into the lung is a critical event for host defense and is a multi-step process that includes granulopoiesis, their mobilization from bone marrow, and recruitment to the lungs. These processes rely on the functions of chemokine ligands, their receptors, and different transcription factors (14-18).
Specifically, CXC-chemokines such as CXCL1/Keratinocyte-derived chemoattractant (KC), CXCL2/Monocyte inhibitory protein-2 (MIP-2), and CXCL5/Lipopolysaccharides-induced CXC-chemokine (LIX) have been reported to mediate neutrophil recruitment during acute lung injury via their common receptor, CXCR2 (19-22). CXCL5 is different from other CXC-chemokines because it is chiefly secreted by type-II alveolar epithelial cells whereas other chemokines, such as CXCL1 and CXCL2, are predominantly secreted by myeloid cells. We and others have reported important roles of CXCL1, CXCL2, and MCP-1 in murine models of pneumonia (21, 23-27). Human CXCL5 (also called epithelial cell-derived neutrophil-activating peptide-78, ENA-78) has been implicated in various disease conditions such as COPD (28), asthma (29), and acute coronary syndromes (30). CXCL5 has been reported to drive neutrophil recruitment during murine models of LPS-induced lung inflammation (22), *M. tuberculosis* (31), and crescentic glomerulonephritis (32). However, using an *E coli*-induced pneumonia model, CXCL5 was found to suppress neutrophil recruitment and subsequent host defense (33) suggesting that the function of CXCL5 could be model or pathogen specific. Nonetheless, the role of CXCL5 in host defense against Gram-positive pneumonia-induced sepsis, including *S. aureus*, remains unknown.

Under steady state conditions, CXCL5 has been shown to regulate neutrophil trafficking along with GCSF (34). However, its role in a clinically relevant pathogen-induced emergency granulopoiesis and neutrophil mobilization remains elusive. In this study we reveal previously unrecognized roles of CXCL5 in a murine model of Gram-positive pneumonia-induced sepsis, including in host defense, emergency granulopoiesis, and neutrophil mobilization. Using CXCL5 knockout (KO) mice, we found that CXCL5 attenuates neutrophil-mediated host defense by suppressing IL-17A secretion by γδ T cells and neutrophils. In addition, our findings demonstrate
that CXCL5 suppresses *S. aureus*-induced emergency granulopoiesis via IL-17A and neutrophil trafficking from the bone marrow through the IL-17A/CXCR2/CD62L signaling axis.

4.2 Methods

4.2.1 Mice. Eight-twelve-week-old female C57Bl/6 (Wild-type) and CXCL5 KO mice were used throughout the experiments. WT mice were purchased from Jackson Laboratories (Bar Harbor, ME) and CXCL5 KO mice were generated as described (33). Mice were kept in specific pathogen-free housing with free access to food and water. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge.

4.2.2 Pneumonia-induced sepsis model. Age and sex-matched WT and CXCL5 KO mice were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg) and then were infected with a sublethal dose (5X10^7 CFU/mouse) of *S. aureus* intratracheally to induce pneumonia-induced sepsis. For survival experiments, age-matched WT and CXCL5 KO mice were infected with 1-2X10^8 CFUs of *S. aureus* and survival observed for 150 hours. The mouse lung epithelial cell lines (MLE12) were purchased from ATCC.

4.2.3 BALF collection and cell counting. BALF was collected as described in our previous publication (3). Total and differential cell counting was performed in BALF using light microscopy after Diff-quik staining.

4.2.4 Bone marrow chimera. Bone marrow chimeric mice were generated as reported in our previous publication (3, 24). Briefly, recipient mice were subjected to a lethal dose of irradiation (1000 rad) from a cesium source. RBC-free bone marrow cells from donor animals were injected into the recipient animals through tail vein (8 million/mice) after irradiation. Four groups of
chimeric mice were generated (WT→WT, KO→KO, WT→KO, KO→WT). These chimeric mice were kept on 0.2% neomycin sulphate for 2 weeks. Eight weeks after transplantation, chimeric mice were infected with \textit{S. aureus} (5X10^7 CFU/mouse, i.t.). Mice were euthanized 24 hours post-infection to collect BALF for cytokine analysis.

\textbf{4.2.5 Granulopoiesis.} The granulopoiesis experiments were performed in accordance with published guidelines (16, 17). Briefly, femurs and tibias obtained from control and infected WT and CXCL5 KO mice were flushed with PBS (EDTA included) to obtain a single cell suspension. After lysing RBCs, these cells were stained with fluorescently labelled antibodies including live/dead aqua, Ly6G (IA8), c-Kit (2B8) (BioLegend), CD34 (RAM34, eBioscience), and the cocktail of CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), and TER119 (Ter119) (BioLegend). For analyzing HSCs and myeloid progenitors, bone marrow cells were stained with anti-FCRγIII/II (93), Sca-1 (D7), c-Kit (2B8), lineage cocktail (Lin-1) (BioLegend), and CD34 (RAM34, eBioscience).

\textbf{4.2.6 Antibodies and reagents.} For blocking experiments, mice received 500ug of anti-Ly6G antibody (BioXcell; clone IA8), or anti-IL-17A (BioXcell; clone 17F3) intraperitoneally (i.p.) at 12 and 2 hours prior to infection (3, 35). Mice received 150 µg of anti-CXCR2 or L-selectin sheddase inhibitor (TAPI-O; Peptide internationals) i.p. at 0 and 12 hours post infection (27). Control mice received PBS or dimethyl sulfoxide (DMSO) or isotype antibody as appropriate.

\textbf{4.2.7 Flow cytometry and lung digestion.} For flow cytometric analysis, RBC-free single cell suspensions were made from bone marrow, blood, and lungs. These cells were treated with Fc block prior to surface staining with the following antibodies for immunophenotyping: anti-CD11b (M1/70), Ly6G (1A8), CD3 (145-2C11), CD4 (GK1.5), CD8α (53-6.7), and γδ-TCR (UC7-13D5) (BioLegend). Appropriate isotype controls were used for each. The cells were fixed
with 2% paraformaldehyde after staining and analyzed using either a FACs Caliber or LSRFortessaX20 (BD). For intracellular staining, lungs obtained from S. aureus-infected WT and KO were digested using collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) and DNase I Recombinant (Sigma Aldrich, CA) by incubating in 37 °C 5% CO2 incubation for 90 minutes. The single cell suspension obtained after digestion was stimulated with leukocyte activation cocktail containing Brefeldin A (BD Biosciences, San Jose, CA) for 4 hours at 37 °C 5% CO2 prior to intracellular staining.

4.2.8 In vivo BrdU incorporation. BrdU incorporation experiment was performed in accordance with the manufacturer’s recommendations (BD Biosciences). Briefly, WT and KO mice were treated with BrdU (1mg/mouse, i.p.) immediately after infection with S. aureus. At designated time points, mice were euthanized to collect bone marrow cells and stained with fluorescently labelled antibodies including BrdU. The data were acquired using an LSRFortessaX20 (BD).

4.2.9 Intracellular killing. The intracellular killing assay was performed as discussed in our previous publication (3). Briefly, BMDNs obtained from WT and KO mice were pretreated with either recombinant IL-17A (100 or 500 ng/ml) or vehicle control for 30 minutes prior to infection with S. aureus (MOI of 10). At the indicated time points, extracellular bacteria were killed using gentamicin. The cells were washed several times to remove excess gentamicin, lysed with 0.1% triton-X to expose bacteria, serially diluted with PBS, and plated onto tryptic soy agar to estimate intracellular bacterial loads.

4.2.10 ROS measurement. Total intracellular ROS produced from neutrophils was measured as described previously (3, 36). Briefly, bone marrow-derived neutrophils (BMDNs) were isolated from WT and KO mice through negative magnetic selection (STEMCELL technologies).
BMDNs were then pretreated with either recombinant IL-17A (R & D System) (100 ng/ml and 500 ng/ml) or vehicle control for 30 minutes prior to infection with S. aureus (MOI:10). One-hour post-infection, ROS production was measured using a fluorometric kit (AA Bioquest).

4.2.11 Statistics. All statistical analyses were performed using GraphPad Prism 7.0 software. All data are presented as mean ± SEM. Student t-test was used to compare means between two groups. Whenever more than two groups were involved, ANOVA followed by Tukey’s multiple comparison test was used. Survival data were analyzed using Log-rank (Mantel-cox) test. Flowcytometric data were analyzed using FlowJo_V10 (Treestar). Significant differences are indicated by * whenever p<0.05, ** p<0.01, *** p<0.001, and **** p<0.0001.

4.3 Results

4.3.1 CXCL5 deficiency enhances host protection during pulmonary S. aureus-induced sepsis.

To investigate whether CXCL5 is upregulated in the blood and lungs following S. aureus-induced pneumonia-derived sepsis, we first infected WT mice with S. aureus and measured CXCL5 protein levels in these organs. CXCL5 was upregulated in blood and lungs following infection with S. aureus (Fig. 4.1 A and B). Next, we sought to investigate the signaling pathways essential for secretion of CXCL5 during MRSA infection. Since, TLRs have been shown to enhance inflammatory cytokines production (37), we stimulated MLE12 cells with different TLR agonists before infection with MRSA. Pretreatment with lipoteichoic acid (TLR2), Pam3CSK (TLR1/2/4), and Pam2CSK (TLR2/6) significantly enhanced CXCL5 secretion suggesting that that TLRs signaling is important for CXCL5 secretion (Fig. 4.1C). In line with the in vitro result, MyD88/Triff⁻/⁻ also displayed reduced CXCL5 production (Fig. 4.1D). Since inflammasomes also regulate cytokine production (37), we used caspase 1/11⁻/⁻ and NLRP6⁻/⁻ mice to determine if the secretion is inflammasome dependent. Interestingly, we found...
that CXCL5 production is reduced in these mice suggesting that inflammasomes regulates MRSA-induced CXCL5 production (Fig. 4.1D). Further, to determine the signaling pathways involved in secretion of CXCL5, we treated MLE12 cells with inhibitors of NF-κB, MAPK, JNK, and p38 prior to infection with S. aureus. Inhibition of these pathways blocked the production of CXCL5 indicating that these signaling pathways regulate CXCL5 production during S. aureus infection (Fig. 4.1E).

Next, we sought to determine the role of CXCL5 in pulmonary S. aureus infection. To this end, we infected WT and CXCL5 KO mice with a lethal dose of S. aureus (USA 300) and observed survival for 150 hours. Compared to WT mice, CXCL5 KO mice had higher survival rates (Fig. 4.1F). To determine whether the difference in survival is due to better clearance of bacteria, we estimated bacterial burden in the lungs, BALF, and blood obtained from infected WT and KO mice. Consistent with the survival data, KO mice demonstrated reduced bacterial burden in these organs after infection (Fig. 4.1 G-I). To explore whether the phenotype displayed by the KO mice is MRSA-specific, we infected WT and KO mice with similar dose of methicillin-susceptible strain of S. aureus (MSSA, Newman strain). Consistent with MRSA strain, KO mice displayed reduced bacteria in the lungs and BALF compared to that of WT mice after infection with MSSA (Fig. 4.1 J and K). Taken together, these data suggest that CXCL5 is detrimental to host survival during S. aureus-induced pneumonia.

To determine the cellular sources of CXCL5 during S. aureus infection, we generated bone marrow chimeras between CXCL5 KO mice (KO) and WT mice (WT). Only WT recipient mouse groups (WT→WT and KO→WT) showed measurable levels of CXCL5 in BALF after infection with S. aureus. This indicates that primarily non-hematopoietic cells contribute to CXCL5 secretion in the lung following pneumonia (Fig. 4.1 L).
Figure 4.1. CXCL5 deficiency enhances host protection during *S. aureus*-induced pneumonia-derived sepsis. (A and B) CXCL5 levels in blood and BALF from *S. aureus*-infected (5X10^7 CFUs) WT mice (N=6-8/group) were measured using ELISA. (C) MLE12 cell lines were pre-treated with TLR agonist such as Pam3CSK (500 ng/ml), Pam2CSK (500 ng/ml), lipoteichoic acid (5 µg/ml), or PBS before infection with MRSA (MOI: 20). Ten hours post-infection, CXCL5 was measured. (D) WT, MyD88/Triff\(^{-/-}\), Caspase 1/11\(^{-/-}\) and NLRP6\(^{-/-}\) mice (N=5-6/group) were infected with *S. aureus*. 24 hours post-infection, CXCL5 was measured in the BALF fluid. (E) MLE12 cells were pre-treated with inhibitors of NF-κB, MAPK, JNK, and p38 (10µM each) for 4 hours and infected with *S. aureus* (MOI: 20). 10 hours post-infection, CXCL5 was measured. (F) WT and CXCL5 KO mice (N=12/group) were infected with 1-2X10^8 *S. aureus* and survival was monitored for 150 hours. (G-I) Bacterial burden was measured in lungs (G), BALF (H), and blood (I) of WT and CXCL5 KO mice (N=6-8/group) 24 hours post-infection with 5X10^7 CFUs of *S. aureus* (i.t.). (J and K) WT and CXCL5 KO mice (N=6-8/group) were infected with the Methicillin-susceptible strain of *S. aureus* (MSSA, Newmann Strain) to induce pneumonia. Twenty-four hours post-infection, mice were euthanized to collect BALF and lungs to measure bacterial burden. (L) Chimeric mice were generated as described in Experimental Procedures. Eight weeks post-transplantation, the chimeric mice were infected with *S. aureus* (5X10^7 CFUs/mouse). Twenty-four hours post-infection, mice were euthanized to collect BALF. Levels of CXCL5 in cell-free BALF supernatants were measured using ELISA. Data are presented as mean ± SEM. Each figure is a representative figure of 3 independent experiments. * p<0.05; ** p<0.01; *** p<0.001; and **** p<0.0001.
4.3.2 Neutrophils confer host protection in CXCL5 KO mice.

It is well established that neutrophils are critical for limiting *Staphylococcal* infections (2, 3, 38). Therefore, we measured the extent of leukocyte accumulation in the alveolar space after infection with *S. aureus* and found that CXCL5 KO mice had more accumulated neutrophils in the BALF (Fig. 4.2 A and B). Similarly, the flow cytometric analysis of lungs tissue after infection with *S. aureus* showed higher neutrophil recruitment in the lungs of CXCL5 KO mice compared to that of WT mice (Fig. 4.2 C and D). To assess the role of neutrophils in host protection, we depleted neutrophils and found that this reversed the survival advantage of CXCL5 KO mice, indicating that the enhanced host protection observed in these mice is neutrophil-dependent (Fig. 4.2 E).

4.3.3 γδ T cell- and neutrophil-derived IL-17A mediates neutrophil recruitment and bacterial clearance in CXCL5 KO mice.

We next sought to determine the mechanism underlying the enhanced neutrophil influx in CXCL5 KO mice. To this end, we analyzed cytokine and chemokine profiles in the BALF following infection. To our surprise, KO mice had reduced CXCL1, MIP-2, TNF-α, IL-17F, and IL-6 levels in BALF compared to the WT mice (Fig. 4.3A-F). In contrast, IL-17A was higher in CXCL5 KO mice than in their WT counterparts (Fig. 4.4A). Because IL-17A has been shown to mediate neutrophil recruitment to the lungs during bacterial infections (35, 39, 40), COPD (41), and ischemia-reperfusion injury (42), we blocked IL-17A with an antibody in CXCL5 KO mice and found attenuated neutrophil recruitment in the BALF (Fig. 4.4 B and C). Moreover, blocking IL-17A also increased the bacterial burden in the lungs, BALF, and blood of CXCL5 KO mice (Fig. 4.4 D-F), indicating that IL-17A is necessary for optimal bacterial clearance.
Figure 4.2. Neutrophils confer host defense in CXCL5 KO mice. (A and B) WT and CXCL5 KO mice (N=6-8/group) were infected with *S. aureus* (5X10^7 CFUs per mouse, i.t.) to induce pneumonia. Twenty-four hours post-infection, mice were euthanized to collect BALF. Total and differential counting were performed using light microscopy. (C) Lungs from *S. aureus* infected WT and CXCL5 KO mice (N=5-7/group) were collected 24 hours post-infection to measure the number of neutrophils recruited through flow cytometry. (D) Quantification of C. (E) Survival following neutrophil depletion in CXCL5 KO mice. WT and KO mice (N=10/group) were either treated with anti-Ly6G (IA8) or isotype control antibody at 24 and 2 hours prior to infection with *S. aureus* (2X10^8 CFUs/mouse, i.t.). Survival was monitored for 150 hours. Data in the bar diagrams are represented as Mean ± SEM and analyzed using two tailed unpaired student t-test. Each figure is a representative of 3 independent experiments. The survival analysis was performed using Log-rank (Mantel-cox) test. * p<0.05; ** p<0.01
Figure 4.3: CXCL5 regulates cytokines and chemokines during pulmonary *S. aureus* infection. (A-F) WT and KO mice (N=6-8/group) were infected with *S. aureus* to induce pneumonia. Twenty-four hours post infection, mice were euthanized to collect BALF. CXCL1 (A), MIP-2 (B), TNF-α (C), IL-6 (D), MCP-1 (E), and IL-17F (F) were measured in cell-free BALF supernatants by ELISA. Each figure is representative of three independent experiments. Data are presented as mean ± SEM. * p<0.05; ** p<0.01; *** p<0.001; and **** p<0.001.

In addition to neutrophil recruitment, we designed experiments to investigate whether IL-17A modulates neutrophil function during *S. aureus* infection. Our findings show that pretreatment of neutrophils with recombinant IL-17A significantly increases ROS production (Fig. 4.4 G) and their intracellular killing ability during *S. aureus* infection (Fig. 4.4 H). Collectively, these results suggest that IL-17A not only enhances neutrophil accumulation, but also increases neutrophil function in CXCL5 KO mice during pulmonary *S. aureus* infection. Since IL-17A production was higher in CXCL5 KO mice, we sought to determine the cellular origin of this cytokine. To accomplish this, lungs from infected WT and KO mice were digested to obtain single cell suspensions and then stained intracellularly with fluorescently labelled antibodies to determine the source of IL-17A. Compared to WT mice, CXCL5 KO mice had more IL-17A⁺ γδ T cells and neutrophils (Fig. 4.5 A-D), suggesting that these cells are the major
sources of IL-17A production in KO mice during *S. aureus* infection. Furthermore, compared to WT mice, CXCL5-deficient mice had increased numbers of γδ T cells (Fig. 4.5 E) in the lungs after infection.

Figure 4.4. CXCL5 suppresses IL-17A production by γδ T cells and neutrophils during *S. aureus* infection. (A-E) WT and KO mice (N=5-6/group) were infected with *S. aureus* (5X10^7 CFUs per mouse, i.t.) or treated with PBS. Twenty-four hours post-infection, lungs were collected and digested to obtain a single cell suspension. Cell were then re-stimulated with leukocyte activation cocktail along with Brefeldin-A for 4 hours and processed for intracellular staining. IL-17A-positive γδ T cells (A and C) and neutrophils (B and D) were quantified. (E) Total γδ T cells per 250,000 events were calculated. Data presented as mean ± SEM. * p<0.05; ** p<0.01; *** p<0.001.

4.3.4 CXCL5 deficiency increases emergency granulopoiesis during *S. aureus*-induced acute pulmonary infection.

Since CXCL5 KO mice had augmented neutrophil accumulation in the lungs, we explored whether CXCL5 deficiency could enhance emergency granulopoiesis or neutrophil mobilization from the bone marrow during *S. aureus* pneumonia. Therefore, we assessed the
total Ly6G-positive cells in the bone marrow after infection and found more neutrophils in the bone marrow of CXCL5 KO mice than in WT mice (Fig. 4.6 A and B).

Figure 4.5: γδ T cell- and neutrophil-derived IL-17A mediates neutrophil-dependent host immunity in CXCL5 KO mice. (A) IL-17A was measured in BALF obtained from *S. aureus*-infected WT and CXCL5 KO mice (24 hours post-infection). (B-F) WT and CXCL5 KO mice (N=5-6/group) were treated with either anti-IL-17A or isotype control antibody 12 hours prior to infection with *S. aureus* (5x10^7 CFUs per mouse, i.t.). Twenty-four hours post-infection, mice were euthanized to collect blood, BALF, and lungs to measure leukocyte recruitment (B and C) and bacterial burden (D-F). (G) BMDNs obtained from WT and CXCL5 KO mice were pretreated with either recombinant mouse IL-17A (rIL-17A, 100 or 500 ng/ml) or vehicle control for 30 minutes and then infected with *S. aureus* (MOI: 10). ROS production was measured using a spectrophotometer. (H) BMDNs obtained from WT mice were treated with either rIL-17A (100 or 500 ng/ml) or vehicle control for 30 minutes prior to infection with *S. aureus* (MOI of 10). At designated time points, intracellular killing ability was measured by estimating bacterial CFUs. Each experiment was repeated thrice. Data represented as Mean ± SEM. * p<0.05; ** p<0.01; *** p<0.001; and **** p<0.0001.
Next, we used a novel flow cytometric method described by Satake et al (17) to analyze different developmental stages of neutrophils within the bone marrow of WT and KO mice. During development, granulocytes gradually lose C-kit expression and acquire Ly6G on their surface and thus, early neutrophil precursors are C-kit$^{\text{high}}$Ly6G$^{-}$ (subpopulation #1) and mature neutrophils are Ly6G$^{\text{high}}$C-kit$^{\text{low}}$ (subpopulation #5) (Fig. 4.6 C). Compared to the controls, both WT and CXCL5 KO mice had significant reductions in matured bone marrow neutrophils after infection suggesting the release of neutrophils from bone marrow to the blood. However, CXCL5 KO mice had significantly more mature neutrophils (subpopulation #5) in the bone marrow compared to that of WT mice even after infection (Fig. 4.6 D and E). This increase in bone marrow neutrophils in KO mice could either be due to increased granulopoiesis and/or defective release to the blood. Therefore, we assessed Ly6G$^{+}$ neutrophils in the blood after infection and found that KO mice had significantly more neutrophils than WT counterparts (Fig. 4.6 F and G). This suggests more neutrophils were mobilized from the bone marrow in CXCL5 KO mice than in WT mice. Furthermore, we enumerated progenitor cells number in subpopulation#1(C-kit$^{\text{high}}$Ly6G$^{-}$) and found that the KO mice had more progenitor cells compared to that of WT mice (Fig. 4.6 H). These results together indicate that the increased number of mature neutrophils in the bone marrow of CXCL5 KO mice is due to increased granulopoiesis and is not caused by defective release of neutrophils to the blood.

4.3.5 Deletion of CXCL5 augments S. aureus-induced amplification of early granulocyte precursors.

It has been reported in previous studies that neutrophils are derived from hematopoietic stem cells (HSCs) through common myeloid progenitors (CMPs) and granulocyte and macrophage lineage restricted progenitor cells (GMPs) (16).
Figure 4.6. CXCL5 deficiency increases emergency granulopoiesis following infection. WT and CXCL5 KO mice (N=6-8/group) were infected with *S. aureus* (5X10^7 CFUs per mouse, i.t.) or with PBS. Twenty-four hours post-infection, mice were euthanized to collect blood, bone marrow, and lungs. (A and B) Bone marrow (BM) cells obtained from control and *S. aureus*-infected animals were stained with Ly6G antibody to assess the total neutrophil population through flow cytometry. (C) Gating strategy to analyze different stages of neutrophils during *S. aureus*-induced emergency granulopoiesis. Cells that lost the potential to differentiate into neutrophils (cells expressing lineage markers for T and B cells, eosinophils, and erythroid cells) were removed to identify the region 5 population (R5). Cells in R5 were gated for C-kit and Ly6G markers and divided into five subpopulations based on C-kit and Ly6G expression. Subpopulation #1 (C-kit^high^Ly6G^-^), #2 (C-kit^int^Ly6G^-^), and #5 (C-kit^low^Ly6G^high^). (D and E) Flow cytometric analysis of *S. aureus*-induced emergency granulopoiesis. Mature neutrophils (subpopulation #5) from BM of WT and CXCL5 KO mice (control and infected) were quantified. (F and G) Blood from control and *S. aureus* infected WT and CXCL5 KO mice were collected and processed for flow cytometry to assess the total number of neutrophils. (H) Numbers of cells in subpopulation #1 from Figure 4D were quantified. Data in the graphs are represented as mean ± SEM. Each figure is a representative of three independent experiments. * p<0.05; ** p<0.01; *** p<0.001.
Figure 4.7. CXCL5 deficiency enhances *S. aureus*-induced amplification of early granulocyte precursors. (A) BM cells obtained from WT and CXCL5 KO mice (control and *S. aureus* infected) were subjected to flow cytometric analysis to investigate early progenitor cells. Lineage-negative BM cells were analyzed. Numbers indicated on the flow cytometry plots are the percentage among Lin cells. (B and C) C-kit+Sca-1-lin BM cells (hematopoietic stem cells, HSCs) were quantified from (A). (D) C-kit+Sca-1-lin BM cells were further analyzed to enumerate different granulocyte precursor populations. Number within the gates shows the percentage among C-kit+Sca-1-lin BM cells. (E) Quantification of (D). (F) *In vivo* BrdU incorporation. Numbers of BrdU-positive cells within each population of progenitor cells are shown. Data are presented as mean ± SEM. Each figure is a representative of three independent experiments. BM: bone marrow, HSC: hematopoietic stem cells, CMP: common myeloid progenitors, GMP: granulocyte-macrophage progenitors, MEP: megakaryocyte-erythroid progenitors, Con: control, SA: *S. aureus* *p*<0.05; ** *p*<0.01.
To determine the specific precursor population regulated by CXCL5, we analyzed C-kit^Sca-1^lin^ HSCs and C-kit^Sca-1^lin^ myeloid progenitor cells obtained from the bone marrow of WT and CXCL5 KO mice after infection. Interestingly, KO mice had more HSCs than WT mice (Fig. 4.7 A-C). Among myeloid progenitor cells, CXCL5 deficiency selectively enhanced the population of GMP cells but not CMP (Fig. 4.7 D and E). However, the megakaryocyte-erythroid progenitor (MEP) cells that give rise to thrombocytes and erythrocytes were significantly reduced in the KO mice compared to that of WT counterparts (Fig. 4.7 D and E). BrdU staining also demonstrated more BrdU^+GMP cells in KO bone marrow than in WT bone marrow after infection (Fig. 4.7 F). Together, these results suggest that genetic ablation of CXCL5 enhances amplification of early granulocyte precursor cells during *S. aureus*-induced emergency granulopoiesis.

4.3.6 IL-17A mediates both emergency granulopoiesis and neutrophil mobilization from bone marrow in CXCL5 KO mice during infection.

Next, we sought to investigate the underlying mechanisms that led to increased granulopoiesis and mobilization from the bone marrow in CXCL5 KO mice. Upregulation of IL-17A via adenovirus-mediated delivery of murine IL-17A cDNA has previously been shown to result in increased granulopoiesis under homeostatic conditions (43). Based on this observation and the fact that IL-17A is higher in CXCL5 KO mice, we hypothesized that IL-17A could mediate granulopoiesis and subsequent mobilization from the bone marrow during *S. aureus* infection. To this end, we blocked IL-17A in CXCL5 KO mice using an antibody and observed granulopoiesis after *S. aureus* infection. KO mice that received the blocking antibody showed increased neutrophil numbers in the bone marrow compared to the group that received isotype control antibody (Fig. 4.8 A and B).
Figure 4.8. IL-17A regulates granulopoiesis and neutrophil mobilization from bone marrow in CXCL5 KO mice following infection. WT and CXCK5 KO mice were treated with either anti-IL17A or isotype control antibody prior to infection with *S. aureus*. Twenty-four hours post-infection, mice were euthanized to collect bone marrow and blood. Single cell suspensions obtained from RBC-free bone marrow and blood were subjected to flowcytometric analysis. (A and B) Bone marrow cells were stained to quantify total Ly6G positive cells. (C-E) BM cells were stained to identify different stages of neutrophil maturation (C). Numbers of cells in subpopulation #5 (D) and #1 (E) were quantified. (F and G) Neutrophils in the blood of WT and CXCL5 KO mice were quantified. (H and I) C-kit^+^Sca-1^−^lin^−^ BM cells were analyzed to compare different progenitor cell populations. Cells within the GMP population were quantified. Data in the graph is presented as the mean ± SEM. Each figure is representative of three independent experiments. CMP: common myeloid progenitors, GMP: granulocyte-macrophage progenitors, MEP: megakaryocyte-erythroid progenitors. * p<0.05; ** p<0.01; *** p<0.001.

Moreover, the number of cells in subpopulation #5 (mature neutrophils) increased in CXCL5 KO mice treated with anti-IL-17A antibody compared to those receiving the isotype control (Fig. 4.8 C and D). In contrast, cells in subpopulation #1 were significantly reduced after blocking IL-17A in CXCL5 KO mice, but not in WT mice (Fig. 4.8 E). These results suggest
that blocking IL-17A increases neutrophil retention and reduces granulopoiesis in the bone marrow of KO mice. However, inhibition of IL-17A in WT mice led to increased retention of neutrophils but did not affect the early progenitor cell population (Fig. 4.8D and E). To further confirm this result, we measured neutrophils in the blood after blocking IL-17A and found significantly fewer neutrophils in the blood of antibody receiving group (Fig. 4.8F and G) compared to those given the isotype control. This neutrophil hyperplasia in bone marrow and neutropenia in blood following antibody treatment confirms that IL-17A is required for neutrophil mobilization from bone marrow during *S. aureus* infection. Similarly, to confirm that IL-17A enhances granulopoiesis in CXCL5 KO mice, we further analyzed early granulocyte precursor populations in these mice and found that IL-17A blockade caused a reduction in GMP cells after infection (Fig. 4.8H and I). Taken together, these data indicate that IL-17A mediates granulopoiesis as well as neutrophil trafficking from the bone marrow in CXCL5 KO mice during pulmonary *S. aureus*-induced pneumonia.

### 4.3.7 CXCL5 regulates neutrophil mobilization via IL-17A/CXCR2/CD62L signaling axis.

The chemokine receptors CXCR2 and CXCR4 act antagonistically to regulate the release of neutrophils from the bone marrow into circulation (44-47). Therefore, we investigated whether CXCL5 regulates the expression of CXCR2 or CXCR4 during pulmonary *S. aureus* infection. No difference between WT and KO mice was observed in case of CXCR4 expressing neutrophils in the bone marrow (Fig.4.9A). However, higher number of CXCR2-expressing neutrophils in bone marrow (Fig. 4.9B) and blood (Fig. 4.9C) of KO mice was observed compared to that of WT mice indicating that the enhanced mobilization of neutrophils involves CXCR2 signaling. To confirm this result, we blocked CXCR2 in the KO mice after infection and observed increased retention of neutrophils in the bone marrow (Fig. 4.9D). Accordingly,
neutrophil number in blood of antibody receiving group was reduced compared to isotype antibody receiving mice (Fig. 4.9E). To further understand the involvement of the CXCR2/CXCR4 axis, we assessed CXCR2+ and CXCR4+ neutrophils in the bone marrow and blood of CXCL5 KO mice treated with IL-17A blocking antibody. The CXCR2+ neutrophil population was significantly reduced in the blood of mice treated with blocking antibody (Fig. 4.9C); however, no significant changes were observed in the expression of CXCR4 in the bone marrow following antibody treatment (Fig.4.9A).

Figure 4.9. IL-17A and CXCR2 signaling is critical for neutrophil mobilization in CXCL5 KO mice. (A-C) WT and CXCL5 KO mice (N=6-8/group) were treated with either anti-IL-17A or isotype control antibody prior to infection with S. aureus. Twenty-four hours post-infection, mice were euthanized to collect bone marrow and blood for flow cytometric analysis of CXCR4 and CXCR2 on neutrophils. CXCR4 (A) and CXCR2 (B) positive neutrophils in the bone marrow are shown. (C) CXCR2-positive neutrophils in the blood. (D and E) WT and CXCL5 KO mice (N=5-7 per group) were either treated with isotype antibody or anti-CXCR2 at 0- and 12-hours post-infection with S. aureus. Twenty-four hours post-infection, mice were euthanized to collect bone marrow and blood. Total neutrophils in bone marrow (D) and blood (E) were quantified using flowcytometry. Each figure is a representative figure of 3 independent experiments. Data in the graph are represented as mean ± SEM. * p<0.05; ** p<0.01; *** p<0.001.
In addition to conventional CXCR4/CXCR2 axis, neutrophil trafficking from bone marrow is also regulated by adhesion molecules such as selectins and integrins. During mobilization from bone marrow in response to chemokine or infection, neutrophils shed selectins (CD62L) and increase the expression of integrins (CD49d or CD18) on their surface (48, 49). Therefore, we assessed whether neutrophil mobilization in response to \textit{S. aureus} infection involves changes in these adhesion molecules. No changes in the levels of \(\beta2\) integrins (CD11a/CD18) were found on the surface of neutrophils after MRSA infection (Figs. 4.10A-D). Compared to the controls, neutrophils from infected groups displayed reduced CD62L and elevated CD29d on their surface (Figs. 4.10E-H) suggesting that expression of these molecules are altered during \textit{S. aureus} infection. In addition, shedding of CD62L was higher in the KO mice compared to their WT controls indicating that CXCL5 deficiency enhances CD62L sheddase activity to augment neutrophil mobilization from bone marrow (Figs. 4.10E and F). To confirm this result, we administered L-selectin sheddase inhibitor (TAPI-O) in both WT and KO mice after infection and found that the inhibition was associated with enhanced retention of neutrophils in bone marrow along with reduced neutrophil recruitment in the blood and lungs (Figs. 4.10I-K). Taken together, these results suggest that neutrophil mobilization from bone marrow into the blood and lungs in \textit{S. aureus}-infected CXCL5 KO mice requires IL-17A/CXCR2/CD62-L signaling axis. G-CSF has been shown to play an important role in both steady-state and emergency granulopoiesis (50-53). Thus, we were interested to determine whether CXCL5 regulates GCSF during pulmonary \textit{S. aureus} infection. Compared to the controls, both WT and CXCL5 KO mice had significantly more GCSF secreted in the bone marrow, blood, and the BALF after infection, suggesting that GCSF is involved during \textit{S. aureus}-induced emergency granulopoiesis (Fig. 4.11 A-C).
Figure 4.10: CXCL5 deficiency augments shedding of CD62L to enhance neutrophil trafficking from bone marrow. WT and CXCL5 KO mice (N=5-7 per group) were infected with *S. aureus* to induce pneumonia. Twenty-four hours post-infection, mice were euthanized to collect bone marrow. The single cell suspension obtained from bone marrow were stained with fluorescent antibodies to measure the expression levels of CD11a (A and B), CD18 (C and D), CD62L (E and F) and CD49d (G and H). (I-K) WT and CXCL5 KO mice (N=5-6 per group) were either treated with DMSO or L-selectin sheddase inhibitor (TAPI-O) at 0- and 12-hours post-infection with *S. aureus*. Twenty-four hours post-infection, mice were euthanized to collect bone marrow, blood, and the lungs. Total neutrophils in bone marrow (I), blood (J), and lungs (K) were quantified using flow cytometry. Each figure is a representative figure of 3 independent experiments. Data in the graph are represented as mean ± SEM. BM: bone marrow, DMSO: dimethyl sulfoxide, TAPI-O: TNF-α protease inhibitor, MFI: mean florescence intensity. * p<0.05; ** p<0.01; *** p<0.001.

Although, CXCL5 KO mice had increased granulopoiesis and neutrophil mobilization, we did not find differences in the levels of GCSF in the bone marrow, blood, or BALF between WT and CXCL5 KO mice after infection (Fig. 4.11 A-C). However, blocking of IL-17A significantly reduced GCSF levels in bone marrow, serum, and BALF of KO mice, suggesting
that GCSF works downstream of IL-17A for neutrophil mobilization from bone marrow (Fig. 4.11 A-C).

Figure 4.11: Levels of GCSF before and after infection with S. aureus. (A-C) WT and KO mice (N=6-8/group) were treated with either isotype antibody or anti-IL-17A before infection with S. aureus. Twenty-four hours post-infection, mice were euthanized to collect BALF, bone marrow, and blood. GCSF in cell-free BALF supernatants, bone marrow, and serum were measured using standard ELISA procedure. Each figure is a representative of three independent experiments. Data are presented as mean ± SEM. * p<0.05 and *** p<0.001.

4.4 Discussion

Community-acquired MRSA (CA-MRSA) is endemic in US and remains a significant cause of mortality and morbidity worldwide (11). Currently, the efficacy of antibiotics used to treat this superbug-induced disease has been reduced due to increased resistance among bacterial species along with a substantial increase in the number of immunocompromised patients. In this context, understanding the host defense mechanism against Gram-positive bacteria could help inform the design of effective host-targeted therapeutics to treat such infections.

Neutrophils are a crucial component of the innate immune defense mechanism that respond quickly during bacterial pneumonia by migrating into the lungs. This migration is directed by chemokines of which, CXCL5 (or ENA-78) has been strongly correlated with neutrophil recruitment to the lungs of patients with acute respiratory distress syndrome (54). In
mice, *Cxcl5* was initially identified as a glucocorticoid-attenuated response gene (55), and has since been shown to mediate neutrophil recruitment in a rat model of ischemia-reperfusion injury (56) and during LPS-induced lung inflammation (22, 57). In this study, we illustrated the signaling mechanism regulating CXCL5 secretion during *S. aureus* infection. We found that TLRs and NF-κB/MAKP pathways are essential for CXCL5 section. Furthermore, we unveiled an unexpected role of CXCL5 where CXCL5 suppressed neutrophil influx into the lungs, thereby hindering staphylococcal clearance. A similar negative function of CXCL5 in neutrophil recruitment was reported in Gram-negative *E. coli*-induced pneumonia model (33). In contrast, CXCL5 was found to be important for neutrophil recruitment in models of *M. tuberculosis* infection (31) and crescentic glomerulonephritis (32). This recruitment, however, was shown to increase pulmonary inflammation and renal tissue injury, respectively. These disparities in neutrophil functions and the role of CXCL5 in neutrophil recruitment could be explained by the differences in models and pathogens used in these studies.

Using a well-established flow cytometric technique (16, 17), we report that *S. aureus* exploits CXCL5 to suppress emergency granulopoiesis and neutrophil mobilization from bone marrow. This is evident from the fact that CXCL5 deficiency selectively increased the proliferation of HSCs and C-kit⁺Sca-1⁻lin⁻ GMP cells within the granulocyte progenitor compartments and enhanced the trafficking of mature neutrophils from bone marrow. The increased neutrophil recruitment in the lungs of CXCL5 KO mice is a net result of both increased granulopoiesis and augmented release into the circulation. Our data further revealed that IL-17A mediates granulopoiesis and neutrophil trafficking into the blood and lungs of CXCL5 KO mice ultimately leading to increased neutrophil recruitment and enhanced bacterial clearance. Various cell types, including γδ T cells, Th-17 cells, NK cells, and neutrophils, can secrete IL-17A (34).
In the model employed in the current study, $\gamma\delta$ T cells and neutrophils are major sources of IL-17A secretion, which is in agreement with earlier findings of Staphylococcal infection (58-60). As reported in different studies (35, 59, 60), we suggest that $\gamma\delta$ T cells are the early source of IL-17A production that drives initial neutrophil recruitment to the lungs. At later time points, these recruited neutrophils contribute for more IL-17A production. We further suggest that increased amount of IL-17A in CXCL5 KO mice could be due to increased numbers of $\gamma\delta$ T cells and neutrophils recruited in the lungs during infection. Further research is needed to understand in detail how CXCL5 could suppress IL-17A production during S. aureus-induced pneumonia.

The CXC-chemokines CXCL1, CXCL2, and CXCL5 bind to their common receptor, CXCR2, and are thought to have redundant roles in neutrophil homeostasis. Multiple studies have demonstrated that CXCR2 and CXCR4 reciprocally regulate neutrophil mobilization from bone marrow into the circulation (44, 45). Studies have further shown that disruption of CXCR2 leads to retention of neutrophils in the bone marrow while disruption of CXCR4 causes increased mobilization into the circulation (45, 61). In accordance with these observations, we found increased CXCR2-expressing neutrophils in the bone marrow of CXCL5 KO mice, which correlated with higher neutrophil mobilization in these mice. Blocking of IL-17A led to reduction in numbers of CXCR2-expressing neutrophils in the blood but no change was observed in neutrophil that expressed CXCR4 in the bone marrow indicating that CXCR2 plays critical role for trafficking of neutrophils in our model. Essential role of CXCR2 in neutrophil trafficking has been shown in other model of studies too (45, 46). Several cell adhesion molecules are involved in neutrophil mobilization from bone marrow during homeostatic and infectious conditions (48, 49). However, we only observed alteration in the expression levels of CD62L and CD49d during S. aureus-induced pneumonia-derived sepsis suggesting that S. aureus specifically
modulates these molecules during infection. Furthermore, CXCL5 KO mice had altered expression of CD62L compared to that of WT mice. In addition, blocking of L-selectin sheddase activity impeded the trafficking of neutrophils from bone marrow to the blood and lungs. These observations together suggest that CXCL5 deficiency enhanced neutrophil trafficking via IL-17A, CXCR2, and CD62L signaling axis. In normal condition, CXCL5 mediated neutrophil homeostasis requires GCSF signaling (34). However, in our model of S. aureus-induced pneumonia, CXCL5 did not affect GCSF levels in the bone marrow, blood, or the lungs suggesting that CXCL5 could regulate emergency granulopoiesis independent of GCSF signaling.

Several studies have reported the involvement of IL-17A in mediating neutrophil recruitment to the lungs during infections (24, 39, 41, 42). However, since IL-17A itself is not an established chemokine, the mechanism underlying IL-17A mediated neutrophil recruitment during infection remained elusive. Although the involvement of IL-17A in steady-state granulopoiesis has been reported from several studies (34, 43, 62), the importance of this cytokine in pathogen-induced emergency granulopoiesis has yet remained unexplored. By using S. aureus induced-pneumonia model, we demonstrate for the first time that IL-17A enhances emergency granulopoiesis and augments neutrophil mobilization via CXCR2 and CD62L to enhance neutrophil accumulation in the lungs during infection.

In conclusion, this is a pioneer study to report the detail mechanism of neutrophil homeostasis during S. aureus-induced pneumonia-derived sepsis. Our findings illustrate that CXCL5 orchestrates MRSA-induced granulopoiesis via IL-17A and subsequent mobilization of neutrophils into the lungs via IL-17A/CXCR2/CD62L axis. Particularly, previous studies on other members of CXC-chemokines, especially CXCL1 and CXCL2, have reported essential
roles of these chemokines for host defense as well as neutrophil homeostasis (25, 27, 48, 63). In contrast, our study has pointed out opposite roles of CXCL5 in both neutrophil homeostasis and host defense in response to S. aureus infection. Based on this observation, we propose that within the CXC-chemokine family, CXCL5 could act as a negative regulator of neutrophil-mediated host defense to prevent overt pathology during microbial infection. Nonetheless, we have unveiled a previously unrecognized role of CXCL5 in S. aureus-induced pneumonia and subsequent emergency granulopoiesis and blocking of CXCL5 could be a promising therapeutic strategy to enhance bacterial clearance in patients with Gram-positive pneumonia-derived sepsis. Moreover, upregulation of CXCL5 could be a novel immune modulation strategy of S. aureus to evade neutrophil-mediated host defense in the lungs.

4.5 Notes


CHAPTER 5. CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Introduction

Nod-like receptors (NLRs) are germline encoded intracytoplasmic pattern recognition receptors that sense danger signals including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [1, 2]. Although 23 NLRs have been identified in humans and more than 30 in the mice, only a few of them have been studied extensively. NLRP6 was identified to activate caspase 1 in human cell lines [3] but the signaling mechanism remained elusive. While the roles of NLRP6 in regulation of the intestinal microbiota were widely studied, its role in the host defense in the context of acute lung injury and sepsis largely remained unknown. In this dissertation, we have used the mouse model of MRSA-induced pneumonia and cecal ligation and puncture (CLP)-induced polymicrobial sepsis to delineate the roles of NLRP6 in host defense mechanism during acute lung injury and sepsis respectively. In chapter 1, we introduced NLRP6 and discussed the recent advancements in its signaling mechanism, the host defense during pathogenic insults, and regulation of microbiota by this receptor (review manuscript currently under revision in Mucosal Immunology). In chapter 2, we investigated the role of NLRP6 in pulmonary host defense during Gram-positive bacterial infection (published in PLoS Pathogens). Chapter 3 describes the role of NLRP6 in polymicrobial sepsis (manuscript to be submitted), and chapter 4 demonstrates how MRSA regulate neutrophil homeostasis to evade host immunity (under revision in Proceedings of National Academy of Sciences). We used MRSA as a model bacterium to investigate the host defense mechanism in pneumonia because MRSA is the leading cause of pneumonia in health-care settings. The summary of each chapter along with implication of results have been discussed below.
5.2 Discussion of results

In chapter 2, we elucidated the roles of NLRP6 inflammasome in pulmonary host defense during Gram-positive pneumonia. By using the mouse model of MRSA-induced acute lung injury model, we demonstrated for the first time that the NLRP6 is activated during Gram-positive bacterial infection in the lungs [4]. NLRP6 expression was increased in neutrophils, macrophages, and epithelial cells of pneumonic patients as well as in MRSA-infected mice. We found that NLRP6 is detrimental to host survival as NLRP6−/− mice displayed enhanced survival and reduced bacterial burden in lungs and extra-pulmonary organs. Further, we have shown that NLRP6−/− mice accumulate more neutrophils in the lungs that are essential to limit MRSA infection. The augmented neutrophil accumulation in the lungs was not chemokine-dependent as NLRP6−/− mice displayed less chemokines compared to that of WT mice. Using in vivo and in vitro techniques, we found that NLRP6 triggers necroptosis and pyroptosis in the lungs that deplete macrophages and neutrophils. Blocking necroptosis and pyroptosis using specific inhibitors enhanced the survival in WT mice suggesting that NLRP6 induces detrimental effects via triggering inflammatory modes of cell death. In addition, NLRP6 also enhanced killing ability of neutrophils via augmenting NADPH-oxidase-mediated ROS production. NLRP6 was found to negatively regulate host immunity during systemic bacterial infection [5]; however, the mechanism was not clear. Through this study we showed that NLRP6 is detrimental during MRSA infection and delineated the mechanism by which NLRP6 drives detrimental effects. We believe that the results of chapter 2 will help to fill the knowledge gap and provide significant advancement regarding the role of NLRP6 in host defense during microbial infection.

The chapter 3 delineates the role of NLRP6 in sepsis by using the murine model of polymicrobial sepsis. Sepsis is a life-threatening medical condition caused by bacteria leading to
multiple vital organ’s dysfunction and death. Despite continuous research, the detailed pathophysiology of sepsis is still elusive. Sepsis is characterized by an early hyperinflammatory condition followed by a delayed immunosuppressive stage [6-8]. However, how these two phases are developed in septic patients and how they contribute to mortality is not clear. Using the mouse model of CLP-induced polymicrobial sepsis, we demonstrated that NLRP6 elicits hyperinflammation and lymphocyte loss during the septic condition. We have shown that NLRP6 is upregulated in splenic T cells in human septic patients and CLP-induced septic mice. Based on this, we hypothesized that NLRP6 regulates immunity during sepsis. NLRP6−/− mice were resistant to polymicrobial sepsis as evident by increased survival and reduced bacterial burden in these mice. Further investigating the mechanism, we found that NLRP6 elicits destructive inflammation via IL-18 as the addition of recombinant IL-18 to these mice reduced inflammation and enhanced the survival. Moreover, T cells from NLRP6−/− mice produced more IFN-γ as a result of higher expression of transcription factor T-bet. Finally, blocking IL-18 in WT mice reduced cell death, inflammation, and mortality during polymicrobial sepsis. IL-18 was found to be detrimental in different murine septic models [9, 10]; however, how IL-18 exerts detrimental effects was not known. The results of the chapter 3 demonstrate that NLRP6-derived IL-18 increases susceptibility to polymicrobial sepsis via exaggerating inflammation and cell death. These results have clinical implications as excessive lymphocyte loss is one of the causes of mortality in septic patients.

In chapter 4, we demonstrate how neutrophil homeostasis is regulated during MRSA-induced pneumonia-derived septic condition. Neutrophil accumulation in the lungs is a critical step that involves neutrophil production in bone marrow (granulopoiesis), their mobilization into the blood, and recruitment to the lungs. Since neutrophils are essential to limit MRSA infection,
it is important to study how neutrophil homeostasis is regulated during MRSA infection. Using well-designed flow cytometric technique, we demonstrate that MRSA regulates CXCL5 to suppress emergency granulopoiesis. CXCL5 was found to dampen granulopoiesis via suppressing the numbers of hematopoietic progenitor cell population in the bone marrow. As a result, neutrophil numbers were inadequate in the lungs to combat the infection. In addition, a compensatory increase in IL-17A in the absence of CXCL5 was found to enhance granulopoiesis and neutrophil mobilization from bone marrow. Our data further indicated that CXCL5 regulates CXCR2, CD62L, and CD49d to suppress the neutrophil release from bone marrow. Together, these data indicate that MRSA exploits CXCL5 as a part of its immune evasion strategy to dampen neutrophil number in the lungs.

5.3 Conclusions and future directions

While chapters 2 and 3 demonstrate how NLRP6 enhances susceptibility to sepsis derived from pulmonary MRSA infection and CLP-induced peritonitis respectively, chapter 4 delineates the mechanisms by which neutrophil homeostasis is regulated during pulmonary MRSA infection. The research findings described in this body of work has advanced the field of NLRP6 inflammasome considerably by establishing its role in host defense against bacterial infections and septic peritonitis. However, understanding of the big picture of MRSA pneumonia and septic peritonitis is far from complete, and these additions to the field of host-pathogen interaction will provide a platform for future research into the pathogenesis of pulmonary MRSA infection and CLP-induced peritonitis. Continued research should focus on understanding (1) the molecular mechanism of how NLRP6 triggers necroptosis, and (2) whether NLRP6 itself interact with MLKL and RIP3 or induces necrosome assembly via indirect mechanisms. Another avenue is to characterize if NLRP6 exerts detrimental or beneficial effect in fungal and Gram-negative
bacterial pneumonia as each pathogen has its own virulence factors and immune evading or stimulating strategies. Since NLRP6 regulates CXCL5 secretion, the other avenue is to determine if NLRP6 has any direct effect on granulopoiesis and neutrophil release from bone marrow.

5.4 Notes


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LIST OF REFERENCES


Birchenough GM, Nystrom EE, Johansson ME, Hansson GC. A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion. Science.


Laxman Ghimire was born in Bardaghat-10, Nepal and received his Bachelor’s in Veterinary Sciences and Animal Husbandry (BVSc & AH) degree from the Tribhuvan University, Nepal in 2013. He joined the Center for Lung Biology and Diseases, Department of Pathobiological Sciences at Louisiana State University in 2014 to pursue a PhD degree in Pathobiological Sciences. Ghimire has a keen interest in innate immunity in lungs specifically during bacterial infections. Under the mentorship of Dr. Samithamby Jeyaseelan, Ghimire was able to delineate how multi-drug resistant bacterial species such as Methicillin-resistant *Staphylococcus aureus* (MRSA) modulate host defense mechanisms in order to evade innate immune responses. Ghimire plans to graduate in December 2019 and pursue his future career in the field of immunology at Harvard Medical School.