

2014

The role of monocyte chemoattractant protein-1 in innate immunity against methicillin resistant *Staphylococcus aureus* pneumonia

Mary Katherine Leissing

Louisiana State University and Agricultural and Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_theses



Part of the [Veterinary Medicine Commons](#)

Recommended Citation

Leissing, Mary Katherine, "The role of monocyte chemoattractant protein-1 in innate immunity against methicillin resistant *Staphylococcus aureus* pneumonia" (2014). *LSU Master's Theses*. 208.

https://digitalcommons.lsu.edu/gradschool_theses/208

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

THE ROLE OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 IN
INNATE IMMUNITY AGAINST METHICILLIN RESISTANT
STAPHYLOCOCCUS AUREUS PNEUMONIA

A Thesis

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
Master of Science

in

The Interdepartmental Program in Veterinary Medical Sciences
through the Department of Pathobiological Sciences

by

Mary Leissingner

B.Sc., Louisiana State University, 2006
D.V.M., Louisiana State University, 2010

May 2014

For my loving family.

ACKNOWLEDGMENTS

In addition to my wonderful biological family, ever supportive and understanding of my career, I would like to acknowledge the tremendous contributions made by my work family, who admittedly, see much more of me. First and foremost I would like to thank my graduate mentor, Dr. Samithamby Jeyaseelan, for inviting me graciously into his laboratory, replete with raw materials, blue prints, and adept scientists. He provided me with the independence to pursue my own new directions, and the tempered council of experience when I was directionless. The challenges of my dual degree program have only stemmed from a shortage of time, never of patience, or support, for which I will always be grateful. To my clinical pathology mentors and graduate committee members past and present including Drs. Stephen Gaunt, Britton Grasperge, and Angela Royal, I owe a debt that will remain largely unpaid for far longer than my student loans. It has been my privilege to work beside and learn from you all, and to be the beneficiary of your uncanny ability to meld mentorship and friendship so seamlessly.

It has also been my privilege to work with and learn from the members of the lung biology laboratory including Drs Shanshan Cai, Sanjay Batra, Ritwij Kulkarni, Theivanthiran Balamayooran, as well as Jin Liliang and Kanapathipillai Jeyagowri, who are always full of kind words and helpful suggestions. Specifically, I would like to thank Ritwij for his writing, and knowledge of all things EndNote[®]. I am especially grateful for the mentorship of Dr. Cai, whose patience, kind-heart, and work ethic continue to impress me, and without whom the completion of this task would have been near impossible. Special thanks also to my fellow resident-mates in anatomic and clinical pathology, especially Khush Banajee, Viviana LeDonne, and Ara Gupta, and the amazing laboratory technologist/friends Essie Mack,

Catherine Caughman Christensen, Suzanne Mouch, Sarah Magee, and Cindy Berry. Finally no list of thank yous would be complete without inclusion of close friends such as Tracy Geiger and Brianna Schur, who always know if a situation calls for council or cocktails, and understand the two are not mutually exclusive.

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	iii
LIST OF FIGURES.....	vii
ABSTRACT.....	viii
CHAPTER 1. INTRODUCTION.....	1
CHAPTER 2. THE ROLE OF NOD-LIKE-RECEPTORS IN THE PULMONARY SYSTEM.....	6
2.1. Abstract.....	6
2.2. Introduction.....	6
2.3. Bacterial pneumonia.....	7
2.4. Host defense.....	8
2.5. The NLR family.....	9
2.6. NOD1/NOD2 signaling.....	12
2.7. Role of NOD receptors in bacterial pneumonia.....	12
2.8. NLR inflammasomes.....	14
2.9. NLRC4 and NAIPs.....	14
2.10. NLRP1.....	17
2.11. NLRP3.....	18
2.12. NLRP6 and NLRP12.....	21
2.13. Perspective.....	22
CHAPTER 3. LITERATURE REVIEW.....	23
3.1. Innate immunity in bacterial pneumonia.....	23
3.1.1. Cellular effectors.....	23
3.1.1.a. Neutrophils.....	23
3.1.1.b. Alveolar macrophages.....	25
3.1.1.c. Epithelial cells.....	26
3.1.2. Pattern recognition receptors.....	27
3.1.3. Cytokines and chemokines.....	30
3.1.3.a. TNF- α	30
3.1.3.b. IL-6.....	31
3.1.3.c. IL-1 β and IL-18.....	32
3.1.3.d. IL-17.....	32
3.1.3.e. IL-8.....	33
3.2. Monocyte chemoattractant protein-1.....	33
3.2.1. MCP-1/CCR2 signaling.....	34
3.2.2. MCP-1 physiologic and pathophysiologic roles.....	36
3.2.2.a. The lung microenvironment.....	37
3.2.2.b. Bacterial pneumonia.....	38
3.2.2.c. Inflammatory and neoplastic disease.....	40
3.2.3. MCP-1 therapies.....	41

3.3. <i>Staphylococcus aureus</i>	41
3.3.1. Ecology and epidemiology	42
3.3.1.a. Community acquired MRSA	43
3.3.1.b. Hospital/Ventilator acquired MRSA	44
3.3.2. Major virulence factors	45
3.3.2.a. Protein A	45
3.3.2.b. α -Hemolysin	46
3.3.2.c. Panton-Valentine leukocidin	47
3.3.2.d. Super antigens	48
3.3.3. Host immune response	48
3.3.4. Animal models	50
 CHAPTER 4. MATERIALS AND METHODS	 52
4.1. Mice	52
4.2. Infection model	52
4.3. Blocking antibodies	52
4.4. Bronchoalveolar lavage fluid collection	53
4.5. Cytokine and chemokine ELISA	53
4.6. Myeloperoxidase activity assay	53
4.7. Histopathology	53
4.8. Statistics	54
 CHAPTER 5. RESULTS	 56
5.1. MCP-1 ^{-/-} mice have increased neutrophils in the airways and lung parenchyma post <i>S.aureus</i> challenge	56
5.2. MCP-1 ^{-/-} mice have increased levels of inflammatory cytokines at 6 hours post <i>S.aureus</i> infection	59
5.3. Local bacterial burden and dissemination are equivocal in MCP-1 ^{-/-} and C57Bl/6 mice	59
5.4. MCP-1 AB-blocked mice trend towards higher BALF leukocyte concentrations and lung MPO activity	61
 CHAPTER 6. DISCUSSION	 64
 REFERENCES	 68
 VITA	 79

LIST OF FIGURES

1. A schematic comparing molecular structures of different NLR family members	10
2. A schematic representation of NLR signaling pathways.....	11
3. Innate immune signaling	29
4. General schematic of a CC chemokine binding a chemokine receptor	35
5. Quantitative scoring of lung histopathology	55
6. Leukocyte recruitment to airways and lungs of MCP-1 ^{-/-} mice.....	57
7. Lung histology in MCP-1 ^{-/-} mice following <i>S. aureus</i> infection.....	58
8. Cytokine and chemokine profile in MCP-1 ^{-/-} mice	60
9. Bacterial burden in MCP-1 ^{-/-} mice	61
10. Neutrophil recruitment to the lungs and airways and local bacterial burden in MCP-1 AB-blocked mice.....	62
11. Cytokine and chemokine profile in MCP-1 AB-blocked mice	63

ABSTRACT

Monocyte chemoattractant protein-1 is critical for monocyte recruitment to the lungs in response to bacterial infection. MCP-1 is also essential for protective neutrophil recruitment to the lungs during *Escherichia coli* and *Klebsiella pneumoniae* infection. *Staphylococcus aureus* pneumonia, specifically strain USA300, carries a high morbidity and mortality rate and is an important pathogen in hospital/ventilator and community acquired pneumonia. In the current study, we investigated the role of MCP-1 in pulmonary innate immunity to *S. aureus* in C57Bl/6, MCP-1^{-/-} and MCP-1 AB blocked mice. As compared to C57Bl/6, MCP-1^{-/-} mice showed increased concentrations of neutrophils in the airways and lung parenchyma as assessed by nucleated cell concentrations in BALF, myeloperoxidase activity (MPO) in lung tissue, and lung histopathology, and increased concentrations of the pro-inflammatory cytokines TNF- α and IL-6. However, this increase in inflammatory cytokines and augmented neutrophilic response did not correlate with increased bacterial clearance, as determined by CFUs from BALF, lung, liver and spleen. MCP-1 AB blocked mice trended towards higher BALF nucleated cell counts and MPO activity in lung tissue, but were not significantly different from negative controls. In conclusion, MCP-1 appears to be differentially regulated during bacterial pneumonia, and in an *S. aureus* model, MCP-1^{-/-} mice have moderately enhanced neutrophilic inflammation which does not improve bacterial clearance.

CHAPTER 1 INTRODUCTION

Staphylococcus aureus is a leading cause of bacterial infection worldwide, and in the United States is the principle isolate from hospital-associated bacterial infections.¹ Among the spectrum of *S. aureus* disease manifestations, the majority are skin and soft tissue infections, however other sites including the bloodstream and lower respiratory tract are also well-described.¹ Notably, while pneumonia is far less common than soft tissue infection, it is responsible for the majority of fatalities associated with methicillin resistant strains of *S. aureus* (MRSA). In 2005 MRSA strains were responsible for 18,000 deaths in the U.S., more than $\frac{3}{4}$ of which were due to pneumonia.²

On the basis of pulse field gel electrophoresis (PFGE), the CDC categorized the most common isolates of *S. aureus* using the USA naming system, which currently includes strains USA100-USA1200, all of which display methicillin resistance with the exception of strains USA900 and USA1200.³ In addition to PFGE classification, *S. aureus* strains are often grouped according to epidemiologic associations, namely community acquired infections (CA-MRSA), or those associated with hospitalization or ventilator support (HA/VAP-MRSA).

Strain USA300, initially described in the 1990s as a sporadic cause of community acquired infection among healthy individuals, continues to be the leading cause of CA-MRSA infections.² Since that time, however, it has also been described as a principle strain in HA/VAP-MRSA cases. In a 2012 analysis of MRSA isolates from 251 intensive care unit (ICU) patients USA300 was the second most common isolate (23.9%).⁴ HA/VAP-MRSA pneumonia has a high mortality rate (up to 37% in one study) compared to other HA bacterial pneumonias, and is also a large drain on health care

resources/costs, with HA/VAP-MRSA cases averaging \$8,000 more in medical costs than pneumonia caused by methicillin sensitive strains.⁴⁻⁶

Gross and histopathologic pulmonary findings in USA300 pneumonia are distinctive among bacterial pathogens, consisting of severe hemorrhage and necrosis with intralesional bacteria but no appreciable suppurative/neutrophilic response.⁷ The severity of USA300 pneumonia is not reliant solely on the immunocompromised nature of hospitalized individuals, as it causes similar lesions in otherwise healthy individuals acquiring infections as a result of CA-MRSA. Methicillin resistance is another unlikely explanation, as many other common HA/VAP MRSA strains actually display broader antibiotic resistance to multiple drug classes.⁴ Ultimately several virulence factors likely contribute although the exact pathologic basis for the necrotizing hemorrhagic features of USA300 remains unclear.

Panton-Valentine leukocidin (PVL), is a virulence factor which has received much research attention, and is expressed by a large number of USA300 isolates, and by relatively few other MRSA or MSSA strains.^{8,9} PVL is a pore-forming toxin shown to cause apoptosis of neutrophils in low doses and neutrophil necrosis in high doses, which may provide an explanation for the paucity of neutrophils seen histologically in USA300 affected lungs.^{9,10} It is unlikely, however, to be the sole mediator, as similar pathologic lesions are described post-infection with USA300 strains not expressing PVL, and other virulence factors including phenol soluble modulins (PSMs) and the super antigen Selx have been shown experimentally to induce similar lesions.^{11,12}

S. aureus pneumonia has an acute clinical course in naturally occurring human infections and animal models.¹³ As such innate immune defenses generated at early

time points are critical for bacterial clearance and host survival. In addition to soluble mediators such as complement, collectins and ficolins, *S. aureus* interacts with pulmonary epithelial cells and macrophages via pattern recognition receptors (PRRs) such as TLR2, NOD2, and NLRP3, initiating pro-inflammatory signaling cascades which upregulate expression of genes important for host defense and leukocyte recruitment. While undoubtedly some degree of pro-inflammatory signaling is necessary for bacterial clearance and neutrophil recruitment, it remains unclear which signaling cascades are vital, dispensable, or actually counter productive for protective immunity. Interestingly while mice deficient in the TLR2 adaptor protein MYD88 are highly susceptible to systemic infection with *S. aureus*, these mice are able to control pulmonary infection with maintained cytokine/chemokine and neutrophil responses.¹⁴ In contrast NOD2^{-/-} mice challenged intratracheally with *S. aureus* do have diminished cytokine/chemokine responses and neutrophil influx, however, reduced inflammatory signaling in this model lead to improved bacterial clearance.¹⁵

Despite its role in neutrophil chemotaxis and ability to augment neutrophil and macrophage mediated microbe killing, TNF- α levels were inversely correlated with outcome in one study, while in another publication mice deficient in TNFR1, the TNF- α receptor, cleared *S. aureus* more efficiently than wild type mice.^{13,16} Similarly there is an ill-defined tipping point at which a robust neutrophilic response transitions from protective to deleterious. Left unchecked, neutrophils can impart as much harm to the surrounding tissue as to the pathogen via toxic mediators such as elastase, collagenase, and free radicals.¹ Ultimately the factors delineating what constitutes a protective versus harmful immune

response to *S. aureus* continue to be elusive, warranting further studies into the pulmonary innate immune response to this important pathogen.

Monocyte chemoattractant protein one (MCP-1) is a chemokine principally described as a monocyte chemattractant, but has also been shown to recruit neutrophils to the airways at early time points in *E. coli* and *K. pneumoniae* infection models, leading to generation of a robust and protective neutrophilic response.^{17,18} MCP-1 is constitutively produced in the lung, but production in epithelial cells and alveolar macrophages can also be upregulated downstream of pattern recognition receptor or inflammatory cytokine signaling mediated by substance including LPS and TNF- α .¹⁹ In a *Pseudomonas aeruginosa* pneumonia model, immunohistochemistry demonstrated strong labeling of intracytoplasmic MCP-1 in murine alveolar epithelial cells and alveolar macrophages post-infection.²⁰

Secreted MCP-1 binds to its principal receptor CC chemokine receptor 2 (CCR2) present on a variety of cells including monocytes/macrophages, fibroblasts, epithelial and endothelial cells.¹⁹ CCR2 is a G-protein coupled receptor which is also integrated with JAK/STAT signaling cascades.^{21,22} While receptor signaling allows for mobilization of intracellular calcium stores necessary for polymerization of cytoskeletal components and chemotaxis, the cascades initiated on CCR2 binding can be multiple and diverse, with other important functions.^{21,22} For example, pulmonary epithelial cells stimulated with MCP-1 can upregulate mucus production, MCP-1 is up-regulated in atherosclerotic lesions, MCP-1 levels correlate with progressive organ fibrosis in some models, and MCP-1 promotes angiogenesis and macrophage infiltration in gastric carcinoma.²³⁻²⁶

Anti-MCP-1 therapies including MCP-1 blocking antibodies and gene therapy have been developed to moderate chronic or deleterious inflammatory responses, fibrosis,

angiogenesis, and tumor burden. To date parenteral administration of anti-MCP-1 or MCP-1 gene therapy have been utilized in a number of animal trials which collectively have produced many promising results including decreasing intimal proliferation in arteriosclerotic plaques, reducing vessel restenosis post-angioplasty, decreasing organ fibrosis, and reducing overall tumor burden and angiogenesis in cancer models.²⁴⁻²⁷ As these therapies progress towards human clinical trials, understanding the role of MCP-1 in innate immune defenses and delineating important risk factors for patients receiving such treatments becomes a priority; especially within the context of a hospitalized population, where *S. aureus* infection is a common cause of comorbidity.

For these reasons we wanted to investigate the role of MCP-1 in pulmonary innate immune responses in *S. aureus* pneumonia and hypothesized that MCP-1 would impart a protective response. C57Bl/6, MCP-1^{-/-} and MCP-1 AB blocked mice were infected intratracheally with *S. aureus* strain USA300 followed by analysis of local and disseminated bacterial burden, pro-inflammatory cytokine, chemokine and pulmonary leukocyte recruitment profiles, and lung histopathology.

CHAPTER 2 THE ROLE OF NOD-LIKE-RECEPTORS IN THE PULMONARY SYSTEM

2.1. Abstract

Innate immunity is the first line of defense against microbes and as such innate responses to infectious stimuli frequently dictate outcomes including survival. Whereas Toll-like receptors have been extensively studied and their importance in pathogen detection and clearance well documented, the importance of NOD-like receptor (NLR) family members is emerging. The lung contains resident immune cells such as leukocytes and epithelial cells and is physiologically positioned to have constant and close contact with inhaled irritants and invading microbial pathogens. As bacterial lung infections are a significant cause of world-wide mortality and innate immune responses often dictate survival from lung infections, understanding the role of NLRs in the pulmonary system is of particular importance. This review highlights recent advances in our understanding of NLR family members, with specific focus on how these proteins sense and respond to pathogens and host-derived substances during respiratory bacterial infections.

2.2. Introduction

The respiratory tract provides a unique microenvironment in which to explore the complex interplay between host immune responses and exogenous stressors such as microbes and environmental irritants is manifest. To be effective, respiratory immune responses must be sensitive, rapid, and diverse; these requirements are driven by constant contact with both commensal and pathogenic microbes and inhaled irritants such as smoke (e.g cigarette, biomass burning) that may possess immuno-modulatory properties. Globally, bacterial pneumonia represents an enormous burden of illness and is associated with substantial morbidity and mortality and the expenditure of significant economic resources. In the last

decade, the role of specific innate immune proteins in protecting the lungs from devastating infections such as bacterial pneumonia has been widely investigated. The availability of resources including specific gene-deficient mice has been a major driving force in this effort. However, the regulation of immune responses during bacterial infection that eventually contribute to host resistance has not been fully delineated. In this review, we focus on recent advances in understanding the importance of NOD-like receptors (NLRs) in orchestrating the innate immune response to bacterial infection.

2.3. Bacterial pneumonia

Pneumonia is an infection of lung parenchyma, usually with bacteria. Bacterial pneumonia is common; in the United States, the incidence of bacterial pneumonia is 4 million adults per year. Moreover, bacterial pneumonia is responsible for significant morbidity and mortality, accounting for 1.1 million hospitalizations, and 50,000 deaths per year.²⁸ In fact, despite treatment with antibiotics and supportive measures, bacterial pneumonia remains the most common infectious cause of death in the U.S. Bacterial pneumonia is characterized clinically by the acute onset of productive cough, fever, and shortness of breath. Severe cases may progress to sepsis and respiratory failure requiring mechanical ventilation. The diagnosis is confirmed by radiographic imaging.

Although the lungs are constantly exposed to inhaled pathogens, a sophisticated host defense system is usually highly effective in the killing and clearance of microorganisms. In the event of exposure to a high burden or high virulence of pathogens or in the setting of a susceptible host, clinical pneumonia can develop. Certain bacteria have developed sophisticated virulence factors to evade a normal host defense system; host characteristics such as underlying structural lung disease, advanced age, and immunocompromised states

such as HIV, chemotherapy, and chronic steroid use predispose to pneumonia. The most common pathogens responsible for bacterial pneumonia are *Streptococcus pneumoniae*, *Haemophilus influenzae*, group A streptococci, *Moraxella catarrhalis*, *Staphylococcus aureus*, anaerobes, and aerobic Gram-negative enteric bacilli such as *Escherichia coli*, *Legionella* spp., *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae*.

In response to bacterial infection, sentinel cells such as macrophages become activated and secrete chemokines, which induce neutrophil migration into the lungs. Neutrophils clear bacteria by phagocytosis followed by killing via proteases and reactive oxygen species. Both the activation of sentinel cells and the phagocytosis and killing by neutrophils are critically dependent on the recognition of pathogens by the innate immune system.²⁹

2.4. Host defense

The innate immune response, the first line of defense against invading pathogens, is initiated when pattern recognition receptors (PRRs) on the surface or in the cytosol of sentinel immune cells sense pathogen-associated molecular patterns (PAMPs) in their vicinity. Following the interaction of PAMPs with PRRs, downstream signaling cascades are activated leading to increased production of cytokines and chemokines that promote recruitment of professional phagocytes and antigen-presenting cells to the site of infection and/or tissue injury. Four major groups of PRRs have been identified: Toll-like receptors (TLRs), Nucleotide recognition domain (NOD)-like receptors (NLRs), RIG (retinoic acid inducible gene)-1-like receptors (RLRs) and lectin receptors. The PAMP ligands for specific PRRs are highly conserved “non-self” molecular motifs of microbial origin; examples include lipopolysaccharide (LPS), peptidoglycan (PGN), flagellin, and CpG nucleotides. As

these motifs are common to both pathogenic and commensal microbes, PAMPs may be regarded as a misnomer for which a more inclusive acronym MAMP (microbe-associated molecular patterns) has been proposed.³⁰ PRRs can also interact with another set of molecular motifs known as damage (or danger) associated molecular patterns which are endogenous (“self”) molecules emanating from stressed (dying/infected/neoplastic) cells.

2.5. The NLR family

The NLR family consists of 22 members in humans, with protein orthologs in both vertebrates and invertebrates.³¹ NLRs are critical to the innate immune response. Unlike the transmembrane TLRs that detect either extracellular or endosomal ligands, NLRs exclusively sense cytosolic ligands.³² All NLR family members are characterized by a tripartite domain structure with C terminal leucine rich repeat (LRR) domain, a central NACHT (NAIP, CIIA, HET-E, TP1) /NOD domain and a variable N-terminal effector domain.³³ NLRs are classified into 4 sub-families based on the N-terminal effector domain they contain: NLRA members have transactivator domains (AD); NLRBs have BIR (baculoviral inhibitor of apoptosis repeat) domains; NLRCs have CARD (caspase activation/ recruitment domains), and NLRPs have PYD (PYRIN domains)^{31,34} (Figure 1). Each domain of the NLR molecule has a unique function. The C terminal LRR sensing domain recognizes and binds to a variety of cytosolic ligands. This is followed by oligomerization of NACHT domains, a crucial but incompletely understood step. Oligomerization leads to the formation of an N-terminal platform where diverse adaptor molecules and downstream effectors may bind.^{31,34} The variable molecular makeup at the N terminus ascribes a degree of structural heterogeneity that is utilized during activation of diverse signaling pathways depending on the specificity of NLR and/or their ligands (Figure 2).

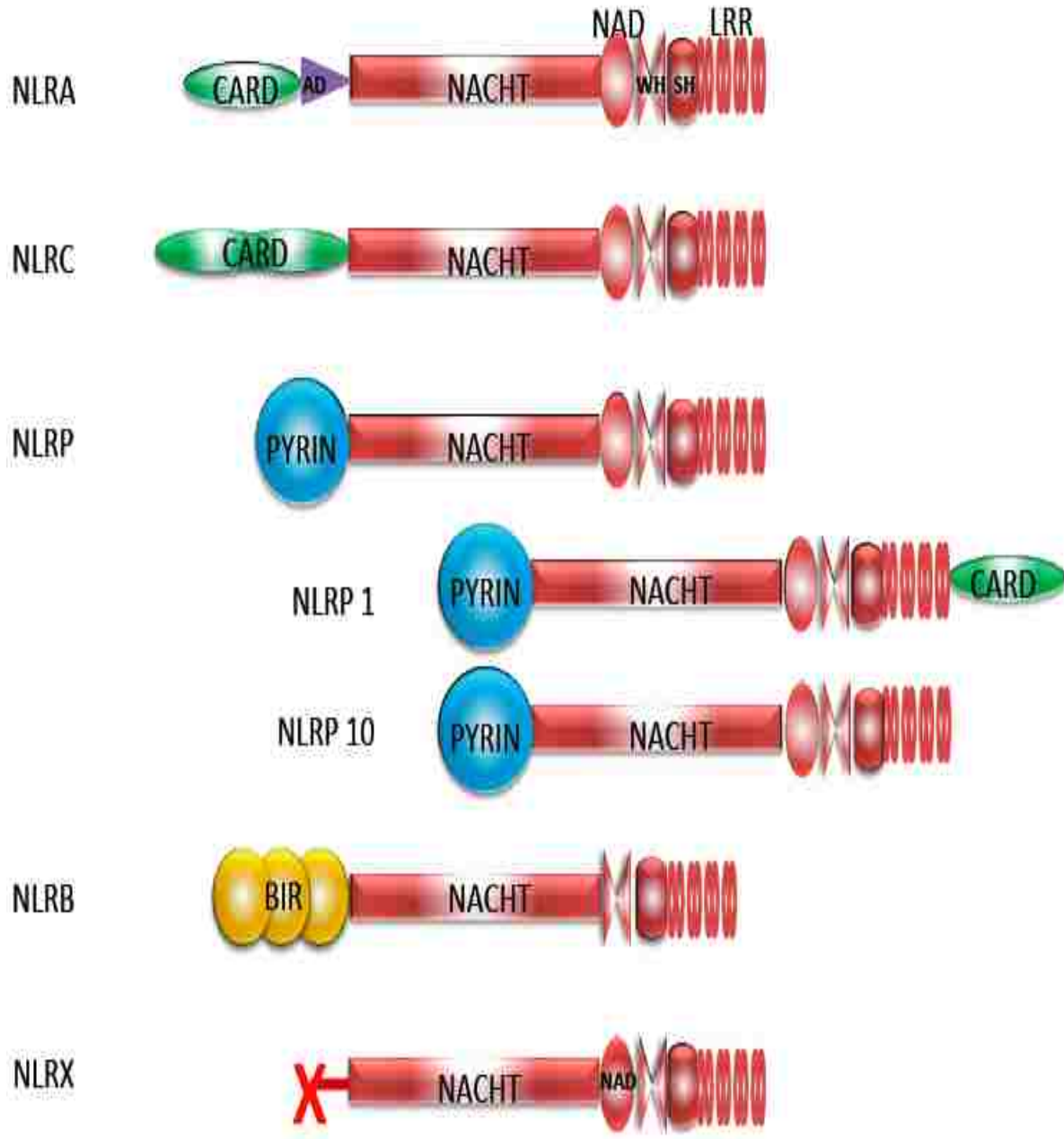


Figure 1. A schematic comparing molecular structures of different NLR family members. All NLR family members have a tri-partite domain organization comprising of C-terminal LRR, middle NACHT and a variable N-terminal domain. The variability of N-terminal domains is the basis for the division of NLRs into distinct subgroups.

Figure 2

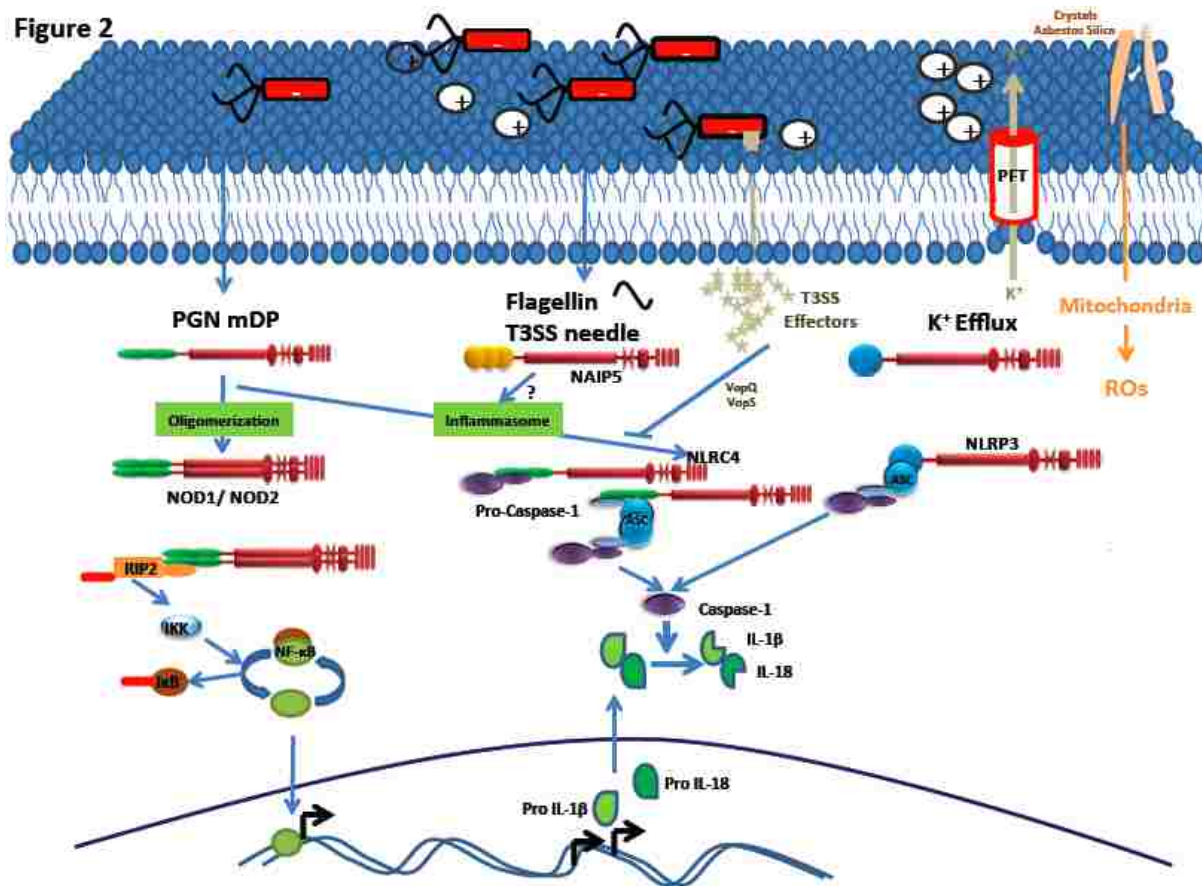


Figure 2. A schematic representation of NLR signaling pathways. Various PAMPs ligands that bind cytosolic NLRs and activate downstream pro-inflammatory signaling pathways in the respiratory tract are indicated.

The NLR ligands range from bacterial and viral components to particulate matter and crystals. For intracellular pathogens and pathogens equipped with trans-membrane secretion systems (e.g., the type III secretion system of *Pseudomonas aeruginosa* and Dot/Icm type IV secretion system of *Legionella pneumophila* (Lp)^{35,36}), the PAMPs are delivered to the cytosol. Recent work demonstrates that extracellular Gram-negative bacteria can shed outer membrane vacuoles or “blebs” containing bacterial products that can be transported by lipid rafts to the cytosol of non-phagocytic cells for interaction with the NOD1 NLRs.³⁷ Apart

from these examples, the exact molecular mechanism(s) for transport of the majority of NLR ligands across the host cell membrane remains elusive.

2.6. NOD1/NOD2 signaling

The cytosolic proteins NOD1 and NOD2 contain CARD domains at their N-termini. While NOD1 is expressed in a wide variety of cells and tissues, NOD2 is expressed in relatively few cell types including macrophages, dendritic cells, keratinocytes, and lung and intestinal epithelium.³¹ Principally described ligands for NOD receptors are components of bacterial peptidoglycan. Specifically, *m*-DAP (L-Ala- γ -D-Glu-*m*-diaminopimelic acid) found in most Gram-negative and some Gram-positive bacteria binds NOD1 while the MDP (muramyl dipeptide) motif present in the peptidoglycans of both Gram-positive and Gram-negative bacteria binds NOD2 LRR.³¹ Peptidoglycan binding is followed by oligomerization of the central NACHT domains and recruitment of the cytosolic adaptor molecule receptor interacting protein 2 (RIP2 at the N-terminus by CARD-CARD interaction. RIP2 is then ubiquitinated, leading to the activation of downstream NF- κ B signaling and upregulation of genes involved in host defense and apoptosis (Figure 2).^{15,31,38,39} In certain infection models, membrane localization of the NOD-RIP2 complex is a prerequisite for activation of NF- κ B signaling⁴⁰ For example, the respiratory syncytial virus (RSV) induces the NOD2-RIP2 complex to bind the adaptor MAVS (mitochondrial viral signaling) on the mitochondrial membrane⁴¹, while the *Shigella flexneri* induced the NOD1-RIP2 complex to bind to the host plasma membrane.⁴⁰

2.7. Role of NOD receptors in bacterial pneumonia

The importance of NOD receptors in pulmonary defense is highlighted by studies using murine models of bacterial pneumonia. In comparison with their WT counterparts,

mice deficient in specific NOD receptors (NOD1^{-/-} or NOD2^{-/-}) or RIP2 (RIP2^{-/-}, which are functionally equivalent to NOD1^{-/-}/NOD2^{-/-} double knockouts) consistently show reduced levels of pulmonary cytokines and chemokines accompanied by reduced inflammation and impaired neutrophil recruitment to the lungs following infection with *Escherichia coli*³⁸, *Staphylococcus aureus*¹⁵ or *Chlamydomphila pneumoniae*³⁹. In contrast, results from pulmonary bacterial burden assays are not as consistent. NOD2^{-/-} mice exhibit reduced neutrophil killing and hence increased bacterial burdens following infection with *E. coli*.³⁸ Similar results (impaired cytokine/chemokine expression, delayed neutrophil recruitment and bacterial clearance) are observed when RIP2^{-/-}, NOD1^{-/-}, and NOD2^{-/-} deficient mice are infected with *C. pneumoniae*.³⁹ Surprisingly, in NOD2^{-/-} mice infected with *S. aureus*, reduced pulmonary neutrophils counts are accompanied by reduced bacterial CFUs.¹⁵ Similarly, in murine models of Legionella pneumonia, NOD1^{-/-}, NOD2^{-/-} and RIP2^{-/-} mice show impaired neutrophil recruitment compared to WT mice, although a small increase in bacterial CFUs was observed only in the lungs of RIP2^{-/-} mice.³⁵ These results suggest a possible co-operation between NOD1 and NOD 2 receptors in mediating *Legionella*-induced activation of RIP2. Taken together, these observations firmly establish NOD receptors at the crossroads of bacterial PAMP identification, pro-inflammatory pathways and neutrophil recruitment. Their importance in resolution of infection by promoting bacterial clearance varies with specific pathogens.

In addition to the well-documented role of NOD receptors in neutrophil recruitment, NOD1/NOD2 signaling also contributes to the production of soluble anti-bacterial and anti-viral molecules. Synthetic PAMPs have been shown to induce human oropharyngeal epithelial cells to produce the antibacterial molecule β -defensin 2 in an NF- κ B dependent

manner *in vitro*.⁴² NOD2 is also involved in induction of type I interferon (IFN) in response to intraphagosomal *Mycobacterium tuberculosis* in murine macrophages⁴³ and also in response to RSV ssRNA, a non-peptidoglycan PAMP.⁴¹

2.8. NLR inflammasomes

Upon ligand binding, NLR proteins NLRC4, NLRP1, and NLRP3 form distinct hetero-oligomeric structures known as inflammasomes, which are platforms for the recruitment of pro-caspase1 zymogen by CARD-CARD interaction followed by activation by proteolytic cleavage. Caspase 1 protease in turn activates pro-IL1 β and pro-IL-18 to IL1 β and IL-18 respectively inducing inflammation and/or cell death, a process termed ‘pyroptosis’. The CARD in an inflammasome may belong to either a constituent NLR such as NLRC4 (NLR family, CARD domain containing 4), or alternatively, to a CARD containing adaptor protein ASC (apoptosis-associated speck like protein containing a CARD 1) recruited by a homotypic PYD interaction between ASC and NLRP1 or NLRP3.^{34,44} The molecular components of different inflammasomes, downstream signaling cascades, and their relevance to bacterial pneumonia will be discussed below.

2.9. NLRC4 and NAIPs

NLRC4 and NAIP (NLR family apoptosis inducing protein) are two structurally dissimilar NLR proteins that form inflammasomes following activation by two bacterial PAMP ligands, flagellin, and the type III secretion system needle apparatus constituent protein PrgJ.^{44,45} Similar to NOD1/NOD2, NLRC4 consists of an N terminal CARD (Figure 1), although oligomerization of the central NACHT/NOD domains by NLRC4 proteins results in the formation of an inflammasome (Figure 2).⁴⁴ The concept of the NAIP-NLRC4 inflammasome originates in observations that the macrophages from A/J mice are highly

permissive to *Legionella* replication in comparison to macrophages from other mouse strains such as C57BL/6, Balb/C and C3H/HeN.⁴⁶ This phenotypic difference was subsequently mapped to the presence of a chromosome 13 locus containing a family of *Naip* (neuronal apoptotic inhibitory protein) genes in non-permissive mouse strains.^{44,46} Today we know that there are four *Naip* paralogs in mice (i. e. *Naip1*, *Naip2*, *Naip5*, and *Naip6*), while only one functional protein, NAIP, has been detected in humans.^{44,47} Polymorphism of the *Naip5* gene in A/J mice has been implicated in their relative susceptibility to *Legionella*, based on the observation that *Naip5*^{-/-} mice in a C57/BL6 background are highly susceptible to Lp.⁴⁸ Similarly, the replication of Lp in human macrophages which precedes the development of Legionnaire's disease in people, is likely explained by the lack of human *Naip* to respond to bacterial flagellin.⁴⁵

Naips exhibit tripartite protein structure with C-terminal LRR, middle NBD and N-terminal BIR domain akin to other NLR family members. Three tandem repeats of BIR at the N-terminus is a feature that Naips share with members of the apoptosis inhibitory protein (AIP) family (Figure 1), although most experimental evidence indicates that the primary role of Naips is in the regulation of innate immunity rather than apoptosis.⁴⁴ *Naip5* and NLRC4 functionally complement one another as inflammasome constituents with *Naip*-LRR acting as a PAMP sensor while NLRC4-CARD recruits and activates pro-caspase1 by CARD-CARD interaction. The role of the BIR domains in the organization of inflammasomes, their downstream signaling and their relevance to immune defense against bacterial pathogens remains to be elucidated, although it is proposed that all three BIR domains are necessary for PAMP-induced oligomerization of NLRC4.⁴⁹

In the case of *Legionella*, murine Naip5 and human NAIP act as cytosolic sensors that oligomerize with NLRC4 in response to flagellin. While the molecular mechanism leading to inflammasome assembly remains nebulous, the NLRC4-Naip interaction has been fairly well-defined.⁴⁴ In contrast, the concept of NLRC4-Naip functional complementarity is well-established. As Naip5 lacks CARD, it is structurally incapable of recruiting and activating caspase-1 by itself. Thus NLRC4^{-/-} mice are defective in mounting an inflammatory response to cytosolic flagellin from *Salmonella typhimurium*.^{44,50} Also, macrophages from Naip5^{-/-} mice fail to activate caspase-1 and IL-1 β and do not undergo pyroptotic death in response to *Legionella*.⁴⁸ Additional biochemical evidence further strengthens this concept: constitutively active NAIP5 Δ LRR induces oligomerization of NLRC4, a step necessary for production of Caspase-1 even in the absence of flagellin ligand, while constitutively active NLRC4 Δ LRR activates caspase-1 even in absence of NAIP5.⁴⁹ The dispensability of Naip5, as observed in certain models of NLRC4 inflammasome activation, spearheaded the investigation of other Naips as potential cytosolic sensors involved in ligand binding. Naip paralogs are proposed to be involved in differential ligand recognition. For example, Naip5 LRR (and to some extent Naip6 LRR) selectively recognizes flagellin, Naip2 LRR recognizes PrgJ (T3SS needle protein) while Naip1 is an orphan member with an unknown PAMP ligand.^{45,49} These observations clearly establish Naips as cytosolic sensors of different PAMPs that act upstream of NLRC4 effectors.

A recent publication has documented that both murine and human macrophages respond to *Klebsiella pneumoniae* via NLRC4 activation, and that NLRC4 activation is critical for development of protective immunity in a murine *in vivo* model.⁵¹ Further studies are needed to determine the importance of NLRC4 in innate immune responses to other

relevant pulmonary pathogens, including both murine models and human cell lines, given the dissimilarities present in NAIP expression between mice and humans.

Since NLRC4 is equipped with its own CARD domain, whether the ASC adaptor is necessary for NLRC4 inflammasome function is not fully established⁴⁴. Legionella activates two independent pathways of caspase1 activation via induction of ASC or NLRC4-NAIP inflammasomes.⁵² Ligands and upstream effectors of ASC-dependent pathway are unknown. Activation of both pathways is required for maximal production of IL1 β and IL18 although NLRC4-NAIP-mediated pyroptosis is independent of ASC activity.⁵² Based on more recent microscopic evidence, ASC appears to be involved in spatial sequestration of NLRC4 - caspase1 complexes.⁵³ This suggests convergence of these two pathways at a centralized ASC platform which may help in maintaining a delicate balance between two caspase 1-induced downstream processes; pyroptosis and proinflammatory cytokine release.

2.10. NLRP1

NLRP1 is structurally distinct from other NLRs in that it has two signal transduction domains; PYD at the N-terminus and CARD at the C-terminus. The LRR domain is positioned between PYD and the central NOD (Figure 1). It is proposed that the C-terminal CARD binds caspase 5 while the N-terminal PYD homotypically binds PYD of ASC. ASC is a bipartite adaptor that in turn contributes CARD for recruitment and induction of pro caspase-1.^{34,54} The NLRP1 inflammasome platform composed of CARD8, ASC, caspase-5, and caspase-1 was first described in a cell-free system.^{34,55} It has been suggested that the activities of both caspase 1 and 5 are necessary for NLRP1 inflammasome mediated IL1 β maturation.^{34,55} Biochemical evidence suggests that NLRP1 inflammasome activation is a two-step process. In the first step, bacterial MDP PAMP binds NLRP1 LRR and primes the

central NACHT domain for NTP binding leading to the second step of NLRP1 oligomerization and procaspase-1 recruitment. ASC may augment the function of NLRP1 but is not required for inflammasome function, as NLRP1 equipped with C-terminal CARD may interact with procaspase-1 bypassing the requirement of ASC.^{34,56}

Unlike humans with a single functional *nlrp1* gene, the murine genome encodes three highly homologous, tandem paralogs; *nrlp1a*, *nrlp1b*, *nrlp1c*. Nlrp1b is a highly polymorphic paralog that is activated by proteolytic cleavage mediated by *Bacillus anthracis* virulence factor, anthrax lethal toxin.⁵⁷ This leads to caspase1 activation which is the molecular event that determines strain-specific susceptibility of murine macrophages to anthrax lethal toxin.^{34,58} Activation of NLRP1 by anthrax lethal toxin has been shown to result in irreversible acute lung injury that is dependent on caspase1 activation and not on IL1 β maturation. In contrast, the ligand for highly conserved NLRP1a is undefined⁵⁹, while the NLRP1c is a truncated protein with unknown relevance to inflammasome assembly/function. Notably, anthrax lethal toxin does not activate human NLRP1. Although the role of NLRP1 in human health and disease remains elusive, the interaction of murine NLRP1 and LT illustrates that NLR activation can be responsible for significant end organ damage.

2.11. NLRP3

The defining feature of NLRP3 (NLR family; PYD containing 3) is the N terminal PYD (Figure 1) that homotypically binds PYD of ASC. The NLRP3 inflammasome is prototypical in its requirement for two distinct signals for activation. The pre-assembly “priming” signal comes from TLR activation that induces NLRP3 expression via NF- κ B activation. Once the cytosolic amount of NLRP3 reaches a threshold, inflammasome assembly is initiated in response to a second signal in the form of one or more of NLRP3

ligands.⁶⁰ The two signal process may act as a cellular safeguard against hyper-activation of the NLRP3 inflammasome. NLRP3 ligands are a curiously heterogeneous group of compounds ranging from exogenous materials such as bacterial PAMPs, ozone, asbestos, silicon and particulate matter to endogenous alarmins such as uric acid from DNA damage, ATP and mitochondrial contents.⁶¹⁻⁶⁶ Hyperactivation of NLRP3 resulting in increased accumulation of pro-inflammatory cytokines such as IL1 β is involved in the pathogenesis of pulmonary fibrosis.^{67,68} The ability of the NLRP3 inflammasome to respond to an array of structurally and chemically diverse signals points to convergence on a common sub-cellular signaling event upstream of inflammasome assembly. Presently, three signaling pathways have been proposed to be involved in these processes: ROS (reactive oxygen species), intracellular electrolyte shift (K⁺ efflux), and lysosomal disruption.⁶⁹⁻⁷¹ Needless to say, each of these models has limitations. ROS production may qualify as the common upstream signaling effector based on the observations that ‘frustrated phagocytosis’ of large crystals (e.g.; asbestos) activates mitochondrial NADPH oxidase (NOX) and that NLRP3 inflammasome assembly is affected by ROS inhibitors.^{65,72} Recently, Bauernfiend *et al* have suggested that ROS activation is an important upstream event required for the priming of NLRP3 inflammasome formation.⁶⁹ Large crystal phagocytosis is also implicated in lysosomal rupture, release of lysosomal protein cathepsin B in the cytosol and subsequent activation of NLRP3 based on the observation that cathepsin B inhibitors abrogate crystal-mediated NLRP3 inflammasome activation while artificial lysosomal disruption (mediated by osmolarity alterations) activates NLRP3 inflammasome.⁷¹ ATP mediated potassium efflux and calcium influx activates ion channel function of purinergic receptor P2X₇ also resulting in the activation of the NLRP3 inflammasome.⁷⁰ Conversely, the presence of high

K⁺ containing buffers blocks NLRP3 inflammasomes.⁶⁶ Pore formation by P2X7-activated Pannexin or by the activity of microbial toxins activates the NLRP3 inflammasome.⁷³ Bacterial pore-forming toxins (PFTs) such as streptolysin O (*Streptococcus pyogenes*), alpha-hemolysin (*S. aureus*), and hemolytic pneumolysin (ply, *Streptococcus pneumoniae*) are particularly relevant virulence factors involved in the pathophysiology of bacterial pneumonia and are established as inducers of NLRP3 inflammasomes.^{61-63,74,75} The variability of PFT expression among bacterial strains in part dictates the immunologic response generated. For instance *S. pneumoniae* strains producing non-hemolytic variants of ply are capable of establishing infection without activating IL-1 β production via NLRP3.⁶² Whether the membrane channels/pores induce K⁺ efflux or function as trans-membrane gateways facilitating entry of ligands into the cytosol allowing for direct interaction with NLRP3 is not clear.

What is apparent, is that NLRP3, through cross-talk with TLRs, and ability to recognize diverse ligands, may play a crucial role in pulmonary immunity. The inflammation resulting from NLRP3 activation may facilitate clearance of pneumococci producing hemolytic pneumolysin, or it may abrogate replication of the atypical mycobacterial pathogen *M. kansasii* in human macrophages.^{61,76} In other instances, such as asbestosis and silicosis, NLRP3 activation is deleterious as it promotes chronic inflammation and fibrosis.⁷² Furthermore, its ability to sense and respond to endogenous alarmins that leak from necrotic cells, indicates it could be activated downstream of any pulmonary condition that induces necrosis.⁶³

2.12. NLRP6 and NLRP12

The newly recognized NLRP6 and NLRP12 are inflammasome-forming NLRs that oligomerize with ASC, although details about the downstream signaling pathways are lacking.⁷⁷⁻⁸⁰ However, in this context the term inflammasome may be a misnomer, as NLRP6 and NLRP12 may induce anti-inflammatory immune responses. Detection of high levels of *nlrp6* transcripts in neutrophils, T cells, macrophages and epithelial cells⁸¹ suggests that NLRP6 may be relevant in the immune defense of respiratory tract and other organs. NLRP6^{-/-} mice display reduced IL-18 levels and colonic dysbiosis (alteration in the structure of normal intestinal microflora) leading to inflammatory colitis.^{77,78} NLRP6 is dispensable for *Listeria monocytogenes* induced neutrophil IL-1 β release.⁸² Moreover, in a mouse model of infection with *Listeria*, NLRP6^{-/-} mice have a survival advantage over their WT counterparts and show increased levels of circulating levels of neutrophils and macrophages accompanied by increased clearance of bacteria from liver and spleen. Whether NLRP6 suppresses innate immune function in response to other bacterial pathogens is unknown.⁸³ NLRP12 is a prototypical member of anti-inflammatory NLRs; it is exclusively produced by eosinophils, granulocytes and monocytes and it is an established negative regulator of the non-canonical pathway of NF- κ B activation.^{80,84} NLRP12 suppresses IL1 β and IL18 production (and IFN γ production via IL18) mediated by *Yersinia pestis*⁷⁹ although no differences were observed in survival profiles, disease progression, and host response mounted by NLRP12^{-/-} and WT mice after airway exposure to *M. tuberculosis* or *K. pneumoniae*.⁸⁵

2.13. Perspective

With the rapidly expanding knowledge of innate immune responses at the molecular level, a more comprehensive approach is needed to link seemingly unrelated molecular effectors and parallel signaling pathways in relation to one another is urgent. This will not only generate comprehensive maps of intricate molecular mechanisms of innate immune responses to invading pathogens, but identify novel targets for future therapeutic interventions. Discovery of inflammasomes, and the description of crosstalk between TLRs and NLRs to generate proinflammatory cytokine responses, represent the initial steps towards a more complete understanding.⁸⁶ It is evident that the specificity between interacting PRRs and PAMP ligands is not exclusive, and that activation of a particular PRR may lead to the simultaneous induction of different signaling pathways.⁸⁷ Despite these major discoveries, many crucial questions remain unanswered, including 1) What are the PAMP ligands activating inflammasomes such as NLRP4 and NLRC4?; 2) How are the molecular patterns of extracellular pathogens transported across the cytoplasmic membrane?; 3) What factors determine whether activation of inflammasome pathways result in an inflammatory response or cell death? A better understanding of interplay between different PRRs in response to a pathogenic insult will pave the way for more sophisticated therapies for a number of infectious, autoimmune, and neoplastic diseases in the lung and other organs.

CHAPTER 3 LITERATURE REVIEW

3.1. Innate immunity in bacterial pneumonia

Innate immunity is the body's first line defense system, and consists of molecular sensors and effectors that are activated in response to invading pathogens or endogenous signals. Cellular components of innate immunity include surface epithelium and leukocytes such as histiocytes (macrophages and dendritic cells), neutrophils, natural killer cells, mast cells, eosinophils and basophils. A wide array of intracellular and extracellular proteins assist in innate immunity including soluble and cell associated pattern recognition receptors, enzymes capable of hydrolyzing cell wall components or creating toxic oxygen/nitrogen compounds, as well as constituents of the complement cascade and acute phase response.²² The ways in which cells sense invading microbes, the signaling cascades which occur downstream of pathogen recognition, and the antimicrobial properties of various innate immune effectors are outlined below, with particular focus on those central to bacterial clearance in pneumonia.

3.1.1. Cellular effectors

3.1.1.a. Neutrophils

Neutrophils are short-lived bone marrow derived granulocytic cells that provide the first line of defense against many invading microbes. Neutrophils are professional phagocytic cells that contain granules replete with bactericidal substances. The primary/azurophilic granules in neutrophils contain myeloperoxidase (MPO), other proteolytic enzymes (cathepsins, elastase), and defensins.¹ Secondary/specific granules, so called as they develop second in neutrophil maturation, contain important membrane proteins including lactoferrin and collagenase as well as receptors for chemotactic molecules,

cytokines, opsonins, and extracellular matrix proteins. In health, murine neutrophils circulate for 10-24hrs before entering tissues where senescent neutrophils become apoptotic and are engulfed by macrophages.¹ During inflammation, microbe and host-derived signals initiate immune cascades resulting in production of neutrophil chemotactic molecules (i.e. IL-8, G-CSF) which can bind to receptors on neutrophils initiating chemotaxis towards sites of inflammation, while altering expression of integrins on endothelial cells to promote neutrophil extravasation into tissues.

Neutrophils are equipped with cell associated pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and Nod-like receptors (NLRs). PRRs recognize highly conserved motifs of pathogens, known as pathogen associated molecular patterns (PAMPs) as well as substances (i.e. mitochondrial DNA, heat shock proteins) liberated from stressed or dying host cells, known as danger associated molecular patterns (DAMPs). Activation of the cell-associated PRRs by PAMPs/DAMPs in neutrophils initiates intracellular signaling cascades resulting in neutrophil phagocytosis, degranulation, reactive oxygen species production, and cytokine/chemokine generation.¹ Additionally neutrophils have receptors for soluble PRRs such as collectins, as well as complement, both of which enhance phagocytosis of bacteria via opsonization.

Once bacteria are inside neutrophil phagosomes, the contents of primary and secondary granules can fuse with the phagosome, exposing organisms to bactericidal products including oxygen dependent (i.e. ROS, superoxide, MPO) and independent (i.e. antimicrobial peptides, elastase, lactoferrin) substances.¹ A more recently identified means of neutrophil-mediated microbe killing is via formation of neutrophil extracellular traps (NETs). NETs are comprised of chromatin, histones, azurophilic granules and cytosolic proteins with

bactericidal activity demonstrated against a variety of pathogens including *S. aureus*. It appears that generation of ROS is a prerequisite in some experimental models for NET formation, however whether NETs constitute a unique form of cell death, or are cast from live cells is still an issue of debate.¹

Neutrophil apoptosis is a form of anti-inflammatory cell death that occurs both in healthy cells as a natural regulator of neutrophil numbers, and in states of inflammation/infection. While the mechanisms governing neutrophil apoptosis differ in health and disease, the net result is caspase-mediated cleavage of nuclear contents, preservation of the cell membrane (preventing liberation of highly inflammatory neutrophil products into the extracellular space) and exposure of phosphatidylserine (PS) onto the extracellular lipid bilayer. PS acts as a signal for macrophages to engulf these senescent cells (efferocytosis). In addition to freeing the extracellular space of the many toxic products present within neutrophils, the action of efferocytosis by macrophages causes these professional phagocytes to produce the anti-inflammatory cytokines IL-10 and TGF- β , further contributing to resolution of inflammation.¹

3.1.1.b. Alveolar macrophages

Alveolar macrophages (AMs) are specialized macrophages that reside in the alveoli. Studies support both monocyte and pulmonary derived sources of AMs. Studies in mice estimate 15% of blood monocytes migrate to the lungs to replace AMs. However maturation of AMs from pulmonary cells of intermediate differentiation has also been demonstrated.¹³ Alveolar macrophages respond to invading pathogens by both phagocytosis and immune signaling via PRRs. Phagocytosis is mediated by binding of opsonized or non-opsonized microbes to a plethora of receptors. Within macrophages nitric oxide is produced via

inducible nitric oxide synthase. Theorized to perhaps protect the lung tissue in their surrounding specialized micro-environment, AMs in humans produce NO to a lesser extent than inflammatory macrophages in other tissues and also have efficient anti-oxidant mechanisms.¹³ While these cells are professional phagocytes, their ability to ingest pathogens is ultimately limited. In addition to phagocytosis, sensing of microbial PAMPs by AMs occurs via PRRs (TLRs and NLRs). These signaling cascades not only enhance microbe killing and phagocytosis by AMs but also recruit neutrophils to the airways.

3.1.1.c. Epithelial cells

A heterogenous population of epithelial cells exists from the trachea to the alveoli including basal cells, secretory and ciliated epithelial cells, neuroendocrine cells and type I and type II alveolar pneumocytes.⁹⁵ The production of mucus and movement of cilia serve to trap inhaled irritants and microbes and propel them back towards the oropharynx. Surfactant proteins A and D produced by type II alveolar pneumocytes are anti-bacterial collectins.¹³ In addition to these constitutive anti-bacterial functions, epithelial cells are able to initiate immune responses via pathogen sensing and subsequent pro-inflammatory signaling cascades. As previously mentioned, NF κ -B is an important transcription factor in pro-inflammatory signaling. In pneumonia models where NF κ -B was selectively blocked in alveolar epithelial cells, generation of cytokines and chemokines and degree of inflammatory cell infiltrate after pathogen challenge were severely blunted.¹³ In separate experiments using mice deficient in the TLR adaptor MYD88, it was shown that the NF- κ B mediated signaling was reestablished after selectively restoring MyD88 function to alveolar epithelial cells, suggesting TLRs are central to NF κ -B cascades generated in epithelial cells.¹³

Another transcription factor important in epithelial cell response in pneumonia is STAT3. Unlike NF κ -B, STAT3 activation does not appear to occur via PAMP/PRR interactions on epithelial cells, but rather via cross talk between epithelial cells and resident leukocytes. The importance of STAT3 in pulmonary immunity is obvious in the naturally occurring human disorder hyperimmunoglobulin E syndrome (HIES) in which patients have severely decreased levels of STAT3. While all cells in the body are deficient in STAT3 expression, the clinical course of this disease is typified by recurrent pulmonary infections, often beginning early in childhood, with pneumonia being the major cause of death in these patients.¹³ Increased susceptibility to pneumonia in these patients may involve epithelial cells directly and indirectly. STAT3 is activated downstream of IL-6 and IL-23 and is vital in differentiation of TH17 T cells. TH17 cytokines (IL-17, IL-22) play important roles in pulmonary immunity, some of which is achieved via signaling with epithelial cells and inducing these cells to produce β -defensins and CXC chemokines.¹³ However, selectively blocking STAT3 in T cells fails to recapitulate the HIES phenotype.¹³ When STAT3 was selectively blocked in the alveolar epithelial cells of mice, animals had more severe lung injury as compared to WT controls, the degree of which did not correlate with neutrophil recruitment between the groups, suggesting a role for STAT3 in maintaining epithelial cell health independent of inflammatory signaling.¹³

3.1.2. Pattern recognition receptors

Pattern recognition receptors (PRRs) are cell associated or secreted molecules that recognize “non-self” signals known as pathogen-associated molecular patterns (PAMPs) including bacterial and viral components or endogenous danger associated molecular patterns (DAMPs) such as mitochondrial DNA and heat shock proteins liberated from dying cells.

Some cell associated PRRs, including CD14 and C-type lectin receptors, are endocytic receptors that initiate internalization and phagocytosis of pathogens upon PAMP binding. Similarly soluble PRRs including collectins and ficolins opsonize pathogens, tagging them for phagocytosis, and also aid in complement associated killing.¹⁴ In contrast the cell associated Toll-like receptors (TLRs) and NOD-like receptors (NLRs) initiate intracellular signaling cascades upon PAMP binding, which culminate in pro-inflammatory chemokine and cytokine production, and in some instances, programmed cell death.

The TLR family includes unique transmembrane receptors expressed by a wide variety of cells such as lymphocytes, histiocytes, epithelial cells, endothelial cells, and fibroblasts, which vary slightly in number across mammalian species (i.e. 12 murine TLRs and 10 human TLRs).¹³ Cell membrane associated TLRs (TLR 1,2,4,5,6) sense an array of peptides and lipopeptides including bacterial cell wall components and flagellin, whereas endosomal TLRs (3,7,9) sense nucleic acids.¹⁴ These receptors are comprised of PRR sensing C terminal leucine rich repeats (LRR), a transmembrane domain, and a N terminal toll and interleukin receptor (TIR) which associates with downstream adaptor proteins (most importantly MyD88) to initiate intracellular signaling. The TLR signaling cascade leads to NF κ -B and MAP kinase activation culminating in increased production of inflammatory cytokines such as TNF- α and IL-6 and neutrophil and monocyte chemotactic molecules such as IL-8 and MCP-1 (Figure 3).¹⁴

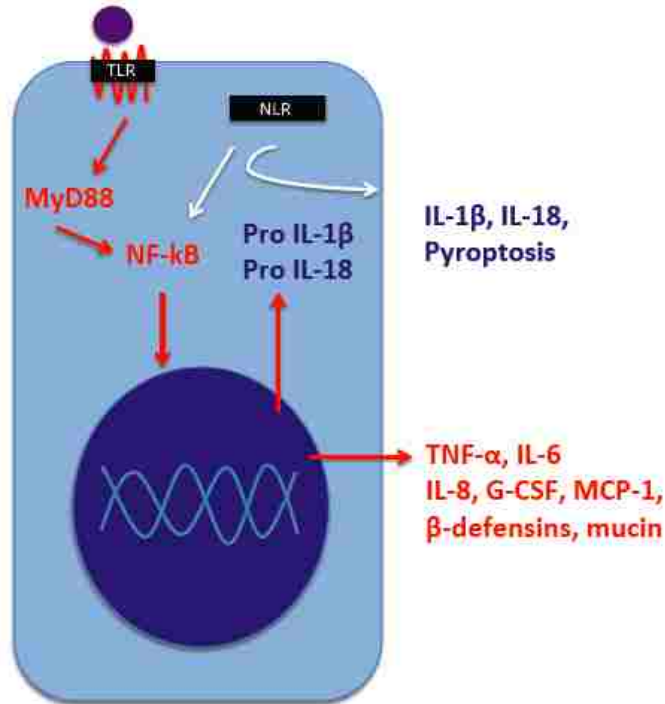


Figure 3. Innate immune signaling. Signaling cascades initiated by *S. aureus* interaction with TLR2 and NLRs (i.e. NOD2, NLRP3) in pulmonary epithelial cells.

NLRs similarly display tripartite structure with a C terminal leucine rich repeat domain, a central NACHT (NAIP, CIIA, HET-E, TP1³³)/NOD domain and a variable N-terminal effector domain.³¹ The NLR ligands range from bacterial and viral components, to particulate matter and crystals. For the well studied NLRs such as NOD1/NOD2, ligands include components of bacterial peptidoglycan. Specifically, *m*-DAP (L-Ala- γ -D-Glu-*m*-diaminopimelic acid) found in most Gram-negative and some Gram positive bacteria, is detected by NOD1 while MDP (muramyl dipeptide) motif, ubiquitously present in the peptidoglycans of both Gram-positive and Gram-negative bacteria, is detected by NOD2 LRR.³¹ PGN binding is followed by oligomerization of the central NACHT domains and recruitment of the cytosolic adaptor molecule RIP2 (receptor interacting protein 2) at the N-terminus by CARD-CARD interaction. RIP2 is then ubiquitinated leading to the activation of

downstream NF- κ B signaling and upregulation of genes involved in host defense and apoptosis (Figure 2).³¹

Upon ligand binding, some NLR proteins (NLRC4, NLRP1, and NLRP3) form distinct hetero-oligomeric structures known as inflammasomes which are platforms for the recruitment of pro-caspase 1 zymogen followed by its activation to caspase 1 by proteolytic cleavage. Caspase 1 protease in turn activates pro-IL1 β and pro-IL-18 to IL-1 β and IL-18 respectively, inducing inflammation and/or inflammatory cell death termed pyroptosis (Figure 3).⁶⁵ Of the inflammasomes NLRP3 arguably has the most relevance to pulmonary pathology, as it is documented to respond to a curiously heterogenous group of compounds ranging from exogenous materials such as bacterial PAMPs, ozone, asbestos, silica and particulate matter to endogenous alarmins such as uric acid from DNA damage, ATP and mitochondrial contents.^{65,75}

3.1.3. Cytokines and chemokines

Cytokines are small soluble proteins secreted by immune (ie monocytes, dendritic cells, granulocytes, lymphocytes) and non-immune cells (fibroblasts, epithelial and endothelial cells). Cytokines can be broadly grouped into five families based on functionality: interleukins, interferons, TNF ligands, growth factors, and chemokines. Major cytokines and chemokines of interest at early time points in pulmonary innate immune responses to bacterial pathogens are described below.

3.1.3.a. TNF- α

Part of the TNF super family, TNF- α is produced predominately by macrophages but is made by a wide array of other cells including neutrophils, mast cells, endothelial cells, fibroblasts, NK cells and CD4+ T cells in response to microbial products and cytokines such

as IL-1, IL-2, and interferon.^{96,97} The cell associated form of TNF- α is released by the action of ADAM 17 (also termed TNF- α converting enzyme) in a homotrimer form, necessary for binding to its receptor.⁹⁷ TNF- α induces endothelial cells to express neutrophil adhesion molecules (ICAM-1 and VCAM-1), augments immune cell function such as macrophage phagocytosis, cytokine secretion, release of reactive oxygen species, and provides positive feed back for NF κ -B activation to increase levels of pro-inflammatory cytokines.⁹⁶ It also has distant effects that include crossing the blood brain barrier to interact with the hypothalamus to induce fever, and causing hepatocytes to upregulate the production of acute phase proteins such as protein C which enhances complement function.⁹⁶

3.1.3.b. IL-6

IL-6 is produced primarily by monocytes and macrophages in response to signaling via other cytokines (i.e. IL-1, TNF- α) or PAMPs (LPS, microbial nucleotides). IL-6 binds its receptor (IL-6R) α chain and the signal transducing component gp130.⁹⁷ It is the most important signaling molecule for induction of the acute phase response. The acute phase response refers to the modulation of protein synthesis, which occurs in the liver upon stimulation with increased concentrations of cytokines such as IL-6, TNF- α and IL-1 β . During times of inflammation the liver down regulates production of albumin (the major negative acute phase protein) presumably to use the amino acid precursors for formation of positive acute phase proteins (APPs). There are many APPs with species specific relative importance but ultimately, regardless of species, the concerted function of these proteins is to enhance multiple facets of innate immunity including complement activation, opsonization, scavenging of free radicals, protease inhibition, and coagulation.⁹⁸ Additionally IL-6

activates cytotoxic T cells and is involved in the differentiation of B cells into immunoglobulin producing plasma cells.⁹⁷

3.1.3.c. IL-1 β and IL-18

IL-1 β and IL-18 are both part of the IL-1 family. These cytokines are unique in that they are synthesized in an inactive pro-enzyme form, requiring the activity of caspase-1 generated by inflammasome assembly to be cleaved into their active forms.⁹⁷ IL-1 β is important in activating T-lymphocytes by enhancing production of IL-2 but also shares biological functions with both TNF- α and IL-6 including upregulation of leukocyte adhesion molecules (ie ICAM-1, VCAM-1, E-selectin) on endothelial cells, and mediating systemic effects such as pyrexia and induction of the acute phase response. The final steps in ICAM-1 expression as mediated by IL-1 and TNF- α requires IL-18. Along with IL-12, IL-18 is also an important inducer of INF- γ .⁹⁷

3.1.3.d. IL-17

The IL-17 family is comprised of 6 members (IL-17A through IL17F) which share virtually no sequence homology to other cytokines. IL-17A is often referred to interchangeably as IL-17 and is primarily produced by a subset of CD4+ T cells (Th17 cells), with other possible cellular sources including neutrophils, eosinophils and CD8+ T cells.⁹⁷ Immune responses of IL-17A include induction of IL-6, IL-8, and granulocyte colony stimulating factor (GM-CSF) in cellular targets (ie fibroblasts, endothelial cells, epithelial cells) bearing the IL-17 receptor.⁹⁷ Via its induction of IL-8 and G-CSF, IL-17 is an important player in neutrophil recruitment to the airways, however its effect on pulmonary immunity extend beyond leukocyte recruitment, as it can also induce goblet cell hyperplasia and increase mucus production by pulmonary epithelium.⁹⁷

3.1.3.e. IL-8

While initially termed as an interleukin, IL-8 is also referred to interchangeably as CXCL-8, a more suitable nomenclature that denotes its major role as a chemokine, as well as its structure. Chemokines are divided into four subclasses (CC, CXC, CX3C, C) where C denotes the arrangement of cysteines within the molecule.⁹⁹ CXC chemokines such as CXCL-8 have one intervening amino acid (ie X) between their cysteine residues. IL-8/CXCL-8 is a potent neutrophil chemoattractant produced by many cell types (i.e. leukocytes, endothelial cells, epithelial cells, fibroblasts) which binds to its receptors (CXCR1 and CXCR2) to stimulate leukocyte transmigration, among other effects.⁹⁹ CXCL-8 is involved in removal of L-selectin and expression of integrins on neutrophils, and facilitates transmigration of neutrophils across fibroblasts, endothelial and epithelial cells. Additionally CXCL-8 induces many neutrophil mediated immune functions including respiratory burst, degranulation, release of leukotriene B4 and synthesis of platelet activating factor.⁹⁹ It should be noted that both mice and rats, do not express CXCL-8, and its orthologs responsible for neutrophil chemotaxis in these species include KC, MIP-2, and LIX.

3.2. Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1) belongs to the chemokine family of cytokines.¹⁰⁰ Chemokines are small (8-10 kD) proteins primarily involved in leukocyte trafficking during inflammation, infection, wound healing and in health. The term chemokine is reserved for proteins with a conserved structure, and many potent chemoattractants (C3a, C5a, leukotriene B₄) are not classified as such. MCP-1 is a CC chemokine, and is referred to interchangeably as CC ligand 2 (CCL2) a pseudonym that reflects its structure rather than functionality.¹⁰⁰ All members of the CC chemokine group

have the first two cysteines adjacent to each other while all members of CXC chemokine group have one amino acid separating the cysteines.¹⁰⁰ The structural similarity of chemokines within subgroups allows for a certain degree of promiscuity in terms of receptor/chemokine interaction. While chemokine receptor 2 (CCR2) is considered the main receptor for MCP-1 (CCL2), human MCP-1 can also bind CCR1, CCR3, and CCR5. Likewise CCR2 has other secondary ligands, including CCL7 and CCL12.^{100,101}

3.2.1. MCP-1/CCR2 signaling

Chemokines such as MCP-1 mediate their effects by binding to seven transmembrane G protein coupled receptors (GPCRs) (Figure 4). The N terminus of the GPCR is present extracellularly and is the site of ligand interaction. The intracellular C terminus interacts with the alpha subunit of a trimeric G protein.^{100,102} The G protein subunits subsequently dissociate and the alpha subunit is free to activate downstream effectors such as phospholipase C (PLC). PLC catalyzes the formation of inositol triphosphate (IP3) and diacyl-glycerol (DAG). IP3 can then open channels to release intracellular calcium into the cytosol while DAG activates protein kinase C (PKC). PKC also mobilizes cellular calcium stores.¹⁰⁰ Increase in intracytosolic calcium is important for the ultimate function of chemokine signaling, namely cell mobility. To move along the chemotactant gradient requires the cell to move its cytoskeletal framework, a process dependent on polymerization of the actin cytoskeleton and calcium/calmodulin interactions.¹⁰⁰

Signaling through the GPCR may have other functions unrelated to mobility (Figure 4).

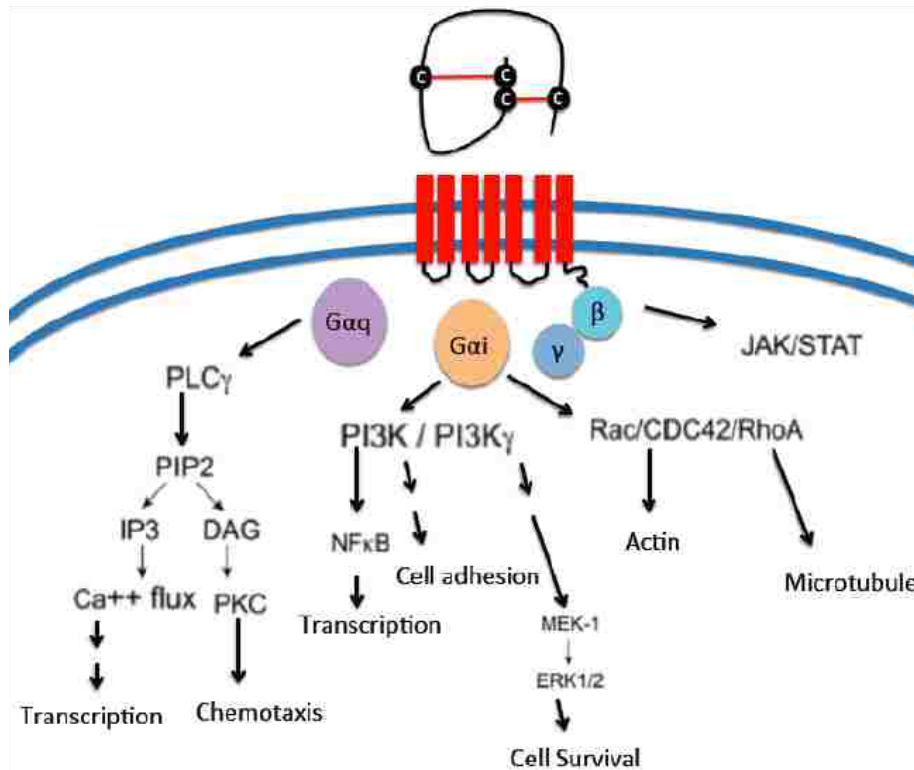


Figure 4. General schematic of a CC chemokine binding a chemokine receptor. Chemokine receptors are transmembrane G protein coupled receptors but are also linked to JAK/STAT signaling pathways. Chemokine binding can lead to activation of multiple downstream signaling cascades resulting in cellular motility and transcriptional activity among other functions.

For instance, PKC also plays important roles in polarization and adhesion, and is also involved in the activation of transcription factor NF- κ B mediated signaling reestablished after selectively restoring MyD88 function to alveolar epithelial cells.¹⁰⁰ G proteins can also activate MAP kinases (p38 and ERK) which in turn activate a number of transcription factors (CREB and c-jun).¹⁰⁰ Additionally, chemokine receptors can directly recruit JAK proteins, thereby allowing the activation of the JAK-STAT pathway, and subsequent STAT dimerization and translocation to the nucleus to upregulate transcription of pro-inflammatory cytokines.¹⁰⁰ This paradigm proves true for murine macrophages that demonstrated tyrosine phosphorylation of JAK2, STAT3 and STAT5 when cultured with MCP-1.²¹ JAK-STAT

and GPCR pathways may in fact work in concert, as in some models where pharmacologic inhibition or mutation of JAK caused defects in both JAK-STAT and GPCR signaling cascades.¹⁰³

The promiscuity of the chemokine ligand/receptor interaction and induction of various intracellular cascades provides a high level of plasticity and intricacy to chemokine signaling, which is further amplified by the finding that CCR2 has two isoforms (CCR2a, CCR2b), and can also form heterodimers.¹⁰⁴⁻¹⁰⁶ Early experiments evaluating isoform mRNA expression, and later work using isoform specific antibodies, support that CCR2b is the predominant form expressed.¹⁰⁵ CCR2b/CCR5 heterodimer formation has also been found to occur in macrophages co-stimulated with the major ligands for these individual receptors, MCP-1 and RANTES (regulated upon activation, normal T cell-expressed and secreted) respectively, and shows preferential induction of leukocyte adhesion rather than chemotaxis.¹⁰⁶

3.2.2. MCP-1 physiologic and pathophysiologic roles

Cellular sources of MCP-1 are diverse and include macrophages and dendritic cells, endothelial cells, smooth muscle, fibroblasts, and some epithelial cells. CCR2 is expressed on many of these same cell types such as macrophages and dendritic cells, endothelial and some epithelial cells and fibroblasts, as well as on other immune cells including T-lymphocytes and neutrophils.^{107-17,108,109} Given the expression of CCR-2 by many MCP-1 producing cell types, studies have demonstrated autocrine feedback.¹⁰⁸ While chemotaxis is an obvious prerequisite for migration of inflammatory cells, it is also important to sessile cell types. Stimulation with MCP-1 resulted in migration of endothelial cells as well as vessel formation and migration of epithelial cells to cover denuded areas.^{107,109} Some of these cells

constitutively produce MCP-1, production can also be upregulated or completely induced in other cell types, with the same being true for CCR2 expression.^{20,108} Stimuli that induce MCP-1 production or CCR2 expression vary with the cell type and model in question often reflecting the underlying pathophysiology. For instance, in a model of vascular disease MCP-1 production was induced by oxidized lipid and shear stress.¹¹⁰ However other known MCP-1 inducers such as LPS, inflammatory cytokines (TNF α , IL-1) and a variety of TLR agonists are more relevant within the context of infection models.^{19,111,112}

3.2.2.a. The lung microenvironment

Pulmonary epithelial cells, alveolar macrophages, and endothelial cells are all possible contributors as well as targets of MCP-1 within the lung. Both constitutive and inducible expression of MCP-1 has been demonstrated in human bronchial epithelial cells and alveolar macrophages (AMs) grown *in vitro*.¹⁹ In a model of bacterial pneumonia with *Pseudomonas aeruginosa*, immunohistochemistry demonstrated strong labeling of intracytoplasmic MCP-1 in murine alveolar epithelial cells 24 hours post infection, and in both epithelial cells and alveolar macrophages by 48 hours post-infection.²⁰ *In vitro* work also supports the expression of the CCR2 receptor on bronchiolar epithelial cells. Human bronchiolar epithelial cell cultures incubated with MCP-1 were shown to upregulate both mucus and MCP-1 production via the CCR2 receptor.²³ In addition to initiating protective responses in the early phases of respiratory insult, interaction between MCP-1 and CCR-2 bearing epithelial cells and AMs may also contribute to healing via epithelial regeneration. When incubated with MCP-1, alveolar epithelial cells from CCR2^{-/-} mice or wild type mice (WT) treated with MCP-1 antibody showed delayed mechanical wound closure compared to WT controls.¹⁰⁹ Additionally, in cell culture, MCP-1 causes murine AMs to increase the

production of hepatocyte growth factor, a mitogen for bronchial and alveolar epithelial cells.²⁰

3.2.2.b. Bacterial pneumonia

To date the role of MCP-1/CCR2 has been investigated in a number of murine pneumonia models using Gram-negative agents such as *Escherichia coli*, *Klebsiella pneumoniae*, *Burkholderia mallei*, and *Pseudomonas aeruginosa* and the Gram-positive agent *Streptococcus pneumoniae*.^{17,18,101,113-115} In *K.pneumoniae* and *E.coli* models, MCP-1^{-/-} mice had increased bacterial burden, decreased neutrophil influx, and attenuated cytokine/chemokine production and NF-κB and MAPK activation following infection as compared to C57Bl/6 mice. Furthermore, through the use of migration experiments and flow cytometry, it was shown that neutrophils present in blood and lung express the CCR2 receptor and migrate in response to MCP-1.¹⁷ In a similar study, *E.coli* endotoxin or MCP-1 were administered as sole agents, or given in concert to immunocompetent Balb/c mice. MCP-1 given alone elicited monocyte recruitment without increasing pro-inflammatory cytokines, whereas *E.coli* endotoxin caused neutrophil influx and increases in pro-inflammatory cytokines (IL-6, TNFα) and the neutrophil chemokine MIP-2 at the 6 hour time point.¹¹³ Interestingly, co-administration of MCP-1 and *E.coli* endotoxin intratracheally resulted in marked increase in pro-inflammatory cytokines/chemokines and a 22-fold higher neutrophil lavage concentration than with endotoxin administration alone, supporting the idea that MCP-1 may be exerting some of its neutrophil chemotactic and pro-inflammatory effects via synergism with endotoxin.¹¹³

The role of MCP-1 in innate immune response to Gram-positive bacterial pneumonia has only been investigated in two studies using various strains of *Streptococcus pneumoniae*,

an important pathogen in community-acquired pneumonia. While these experiments differ in inoculation methods, dosage, strains of *S. pneumoniae*, and timeline, neither demonstrate a strong role for MCP-1 in neutrophil chemotaxis or pro-inflammatory cytokine production at early time points (6-48 hours).^{101,114} In one study *S. pneumoniae* was administered intranasally at varying dosages with no difference in bacterial CFUs, inflammatory cytokine profiles or lethality observed over 0-48 hour time points when comparing C57Bl/6 and MCP-1^{-/-} mice.¹¹⁴ In a second study, mice were inoculated intratracheally with either a highly virulent or less virulent strain of *S. pneumoniae*. While both C57Bl/6 and MCP-1^{-/-} mice were highly susceptible to the more virulent strain, C57Bl/6 mice were protected from mortality when challenged with the less virulent strain, as compared to MCP-1^{-/-} mice.¹⁰¹ In contrast to the *K. pneumoniae* and *E. coli* models, this protective phenotype was most likely conferred by an increased influx of macrophages and dendritic cells observed at later time points, as no appreciable difference in cytokine/chemokine profile were observed at early time points and neutrophil concentrations were actually significantly higher in MCP-1^{-/-} mice at 1 day post-infection.¹⁰¹

From these studies one may speculate that the role of MCP-1 in innate immune response to bacterial pneumonia is dictated by the presence or absence of lipopolysaccharide, however other Gram-negative pneumonia models may not support this conclusion. In a *B. mallei* pneumonia model both MCP-1^{-/-} and CCR2^{-/-} mice had increased mortality, local bacterial burdens and dissemination compared to C57Bl/6 mice at 72 hours, however CCR2^{-/-} mice had increased neutrophils in the lung tissue, and increased TNF α and KC in lung tissue at 48 hours post-infection.¹¹⁵

While the factors dictating how MCP-1 synergizes with pathogens such as *K. pneumoniae* and *E. coli* to produce robust neutrophilic responses remains unknown, a model of *Pseudomonas aeruginosa* pneumonia has provided a plausible explanation for models in which loss of MCP-1/CCR2 has resulted in mild to moderately increased neutrophil influx and cytokine production, and increased mortality.²⁰ Mice administered anti-MCP-1 antibody and *P. aeruginosa* showed increased neutrophilic inflammation, hemorrhage and exudation in lung tissue, increased neutrophil concentrations and MPO activity in BALF, and interestingly, decreased efferocytosis as compared to control Abs-treated mice, despite no differences in BALF macrophage concentrations.²⁰ *In vitro* work by the same group demonstrated macrophages co-cultured with MCP-1 and aged neutrophils exhibited increased phagocytosis of apoptotic cells in a dose dependent manner.²⁰

These findings suggest that in some models of pneumonia increased neutrophil influx, persistence of MPO activity and increases in inflammatory cytokines may perhaps be due to a decreased efficiency of macrophages to phagocytize aged neutrophils in MCP-1^{-/-} or AB-blocked mice, allowing for continued release of inflammatory mediators from these dying cells. Central to all of these studies however, is that among the repertoire of pathogens currently investigated, MCP-1 plays a protective role in innate pulmonary immune responses.

3.2.2.c. Inflammatory and neoplastic disease

MCP-1 and CCR2 are of central importance to many non-infectious disease processes, with the MCP-1/CCR2 axis proving a promising target for pharmacologic blockade and therapeutic intervention. In chronic inflammatory diseases the continued accumulation of macrophages can lead to fibrosis and further end-organ damage. Studies in humans and rodents have demonstrated that MCP-1 levels correlate with increasing degrees

of fibrosis in models of pulmonary and renal fibrosis.²⁵ Additionally MCP-1 is central to the pathogenesis of arteriosclerosis and re-stenosis after vascular injury as it recruits macrophages to the vessel wall.²⁴ MCP-1 may also be important in many neoplastic diseases, owing to expression of MCP-1/CCR2 by endothelial cells and tumor infiltrating macrophages, both key players in neoangiogenesis.²⁶

3.2.3. MCP-1 therapies

Anti-MCP-1 therapies including MCP-1 blocking antibodies and gene therapy have been developed to moderate chronic or deleterious inflammatory responses, fibrosis, angiogenesis, and tumor burden.^{24-26,116} While these therapies contrast in method of delivery, they are functionally similar, as the end result of gene therapy is incorporation and transcription of a DNA sequence encoding for a non-functional MCP-1 competitive antagonist.²⁵ To date parenteral administration of anti-MCP-1 antibodies have been utilized in a number of animal trials as well as a human trial for treatment of rheumatoid arthritis, while gene therapy has only been used in animal models.^{24-26,116} Collectively these trials have produced many promising results including decreasing intimal proliferation in arteriosclerotic plaques, reducing vessel restenosis post-angioplasty, decreasing organ fibrosis, and reducing overall tumor burden and angiogenesis in cancer models²⁴⁻²⁷ As these therapies progress towards human clinical trials, understanding the role of MCP-1 in innate immune defenses and delineating important risk factors for patients receiving such treatments becomes a priority.

3.3. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterial cocci that can be both a commensal or invasive pathogen in humans. Microscopically *S. aureus* appears in clusters,

and when cultured can be differentiated from other types of staphylococci by positive coagulase, mannitol fermentation, and deoxyribonuclease testing.¹¹⁷ While capsular antigens can be used to serotype *S. aureus*, pulse-field gel electrophoresis (PFGE) is considered the method of choice for determining bacterial strain.^{3,117} For the purposes of simplifying nomenclature, the CDC assigned strain names to *S. aureus* based on PFGE results from common isolates within the United States (ie the USA strains).³ Originally 8 strains were identified but this number has since grown ranging from USA100-USA1200.³ Notably the majority of these are methicillin resistant (ie MRSA) with the exception of strains USA900 and USA1200.³

3.3.1. Ecology and epidemiology

The human nares are a well-documented site for *S. aureus* colonization and much of our data concerning prevalence of *S. aureus* colonization come from epidemiologic studies of nasal carriage.^{25,118-120} In one such study conducted in the United States from 2001-2004 approximately 30% of subjects had nasal colonization with *S. aureus*, however a relatively low number of these cases were due to colonization with a MRSA strain (0.8-1.5%).¹²⁰ Colonization with *S. aureus* has long been considered a risk factor for development of invasive disease; although only a low number of those with nasal carriage go on to develop infection.¹¹⁸⁻¹²⁰ The majority of *S. aureus* infections occur in the skin and soft tissues, but other manifestations such as pneumonia and septicemia constitute a substantial cause of MRSA mortality.¹¹⁷ In 2005 MRSA strains were responsible for 18,000 deaths in the United States, greater than 75% of which were caused by pneumonia.² While within a hospitalized setting comorbidity such as underlying disease, immunosuppression, or recent surgery play a role in development of infection, community acquired cases often occur in healthy

individuals with no apparent risk factors.^{3,13,119} Given the dissimilarities between community and hospital acquired *S. aureus* infections, they are addressed individually below with a predominate focus on MRSA.

3.3.1.a. Community acquired MRSA

Community acquired MRSA (CA-MRSA) refers to cases that lack an association with health care. Other traits that typify CA-MRSA often include PFGE type USA300 and genes encoding certain virulence factors that are often expressed by, but not specific to, USA300.^{3,119,120} In addition, while the name itself implies resistance to β -lactam antibiotics, CA-MRSA strains are generally susceptible to a wider range of antibiotics than hospital or ventilator acquired strains (HA/VAP-MRSA).^{119,121} USA300 was recognized as a predominant cause of CA-MRSA in the late 1990s and early 2000s, before which USA400 was the most frequent isolate.^{3,119} While these outbreaks often occurred among seemingly healthy individuals including members of sports teams and military units, these cases did highlight potential risk factors including shared use of personal items (towels/razors), previous antibiotic use, or ineffectual wound care.³ A multi-institutional survey conducted in 2006 indicated MRSA strain USA300 to be the leading cause of skin and soft tissue infections among emergency room patients.¹¹

While USA300 less commonly causes pneumonia, when it occurs, it is often severe, necrotizing and fatal. The necrotizing and hemorrhagic features of USA300 pneumonia are well-documented, however a conserved mechanism to explain development of these lesions has yet to be delineated.⁷ A commonly implicated contributor is Panton-Valentine leukocidin, a virulence factor expressed by many USA300 isolates, but other factors are likely at play, as strains not expressing this virulence factor have been documented to cause

similar lesions.⁸ For individuals developing MRSA as a result of compromised immunity after influenza infection, interferon may play an important role.¹³ In murine models severe *S. aureus* pneumonia can be induced at lower inoculum dosages in animals previously infected with influenza.¹²² Additionally mice deficient in interferon- α/β receptor 1, which are incapable of responding to interferon, were protected from a lethal challenge with *S. aureus* as compared to wild type mice.¹⁶

3.3.1.b. Hospital/Ventilator acquired MRSA

Hospital acquired MRSA (HA-MRSA) and ventilator acquired pneumonia due to MRSA (VAP-MRSA) pose a significant health risk to hospitalized populations, with reported mortality rates as high as 37%.⁴ Prior to the rise of USA300 in CA-MRSA, commonly isolated HA-MRSA strains included USA100, USA200, and USA500.¹¹⁹ While both CA and HA-MRSA strains are resistant to methicillin, the genetic determinants which confer this resistance differ, with HA-MRSA strains often showing resistance to a wider array of antibiotic classes.^{119,121} As USA300 continues to be the most prevalent cause of CA-MRSA cases, these distinctions are becoming less clear and growing epidemiologic evidence supports that USA300 is also now a leading cause of HA-MRSA.⁴ In 2012, MRSA isolates from 251 intensive care unit (ICU) patients were obtained and while USA100 predominated (55%), USA300 was the second most common isolate (23.9%).⁴

Compared to HA/VAP caused by methicillin sensitive strains, HA/VAP MRSA is documented to have increased mortality, and is responsible for increased utilization of hospital resources and cost burden, with treatment of MRSA cases averaging \$8,000 more than MSSA cases in one retrospective.^{5,6} Infection with HA/VAP may occur in patients with nasal colonization, although transient skin colonization of health care workers and patients is

also a proposed mode of transmission. Interestingly a genotypic determinant named arginine catabolix mobile element (acme) present in some isolates of USA300 may improve fitness and increase the ability of the organisms to remain on the skin by encoding an arginine deaminase, which allows for the formation of ammonia. This pathway may allow USA300 to maintain its pH in the relatively acidic environment of human skin.¹²³ If this is indeed the case, much of our current epidemiologic investigations involving MRSA may be far from complete, as they focus largely on screening via nasal carriage.

3.3.2. Major virulence factors

3.3.2.a. Protein A

Protein A (Spa) is a well-studied and abundant *S. aureus* surface protein. Although Spa serotyping was often employed in older epidemiological studies of *S. aureus*, this has largely been supplanted by newer molecular techniques. While PFGE is the gold standard for classification of *S. aureus* strains the Xr region of *spa* is highly genetically diverse, and provides the basis for spa typing.¹³ Immunologically, the Xr region is responsible for promoting a type I interferon response. Spa encoding strains have been shown to induce IFN- β production in pulmonary epithelial cells.¹⁶ Interestingly this type I interferon response increases the pathogenicity of *S. aureus* in the context of pneumonia, as demonstrated by *Ifnar*^{-/-} mice which are protected from lethal *S. aureus* challenge as compared to wild type mice.¹³ These findings provide some mechanistic support for the long held observation that individuals acquiring *S. aureus* pneumonia as a sequel to influenza, a potent inducer of the type I interferon response, suffer dramatically increased morbidity and mortality.¹³

Spa is equipped with 5 IgG binding domains that bind the Fc component of IgG. These structures have proven useful experimentally, as they provide the basis for

immunoprecipitation experiments, however conflicting information exists as to if coating with IgG protects the organism from phagocytosis as originally thought.¹³ Spa is also capable of binding immunoglobulin receptors, notably the B-cell IgM receptor, which has earned it the term B-cell super antigen.¹²⁴ Binding of the B-cell IgM receptor is believed to cause rapid clonal expansion and apoptosis of B-cells, ultimately resulting in the inability to mount an effectual B-cell response to the organism.¹³

The IgG binding domains mentioned above are relatively promiscuous, and have also been demonstrated to bind TNFR1, EGFR, and ADAM17.¹²⁵⁻¹²⁷ Binding of TNFR1 induces TNF production, where as binding to EGFR can lead to cleavage of TNFR-1 and ADAM17 binding produces soluble proteins that neutralize IL-6.^{126,127} Thus, depending on the context, spa can generate both pro and anti-inflammatory cytokine responses.

3.3.2.b. α -Hemolysin

α -Hemolysin (Hla) is a ubiquitously expressed *S. aureus* heptameric pore-forming toxin which penetrates eukaryotic lipid bilayers. It is a well-documented contributor to *S. aureus* induced lung pathology, with a broad range of target cells including histiocytes, epithelial cells and endothelial cells.¹³ The cellular receptor for α -Hemolysin, ADAM-10, is a matrix metalloproteinase which regulates cellular adhesion and mobility.¹²⁸ The Hla-ADAM10 interaction has been shown experimentally to disrupt focal cellular adhesions and integrin signaling in epithelial cells and cause vascular endothelial-cadherin cleavage and loss of endothelial barrier function.^{128,129} This relationship may explain some of the pathology induced by α -Hemolysin including pulmonary hemorrhage and edema formation.¹³ Additionally it provides a putative mechanism for *S.aureus* dissemination throughout the lung, as well as systemically, via interaction with endothelial cells, granting the organism

access to the blood stream.^{128,129} Regardless of the exact mechanisms, experimental evidence supports the important contribution of this interaction to lung pathology, as ADAM-10^{-/-} mice were found to be significantly less susceptible to fatal *S. aureus* pneumonia as compared to wild type mice.¹³ α -Hemolysin also serves as a ligand for the NLRP3 inflammasome, leading to induction of IL-1 β and IL-18 production and pyroptosis.⁷⁵

3.3.2.c. Panton-Valentine leukocidin

Panton-Valentine leukocidin (PVL) is a pore-forming toxin shown to cause apoptosis of neutrophils in low doses and neutrophil necrosis in high doses. These effects appear to vary with species, and have been demonstrated in humans and rabbit models, where as murine neutrophils appear relatively resistant.^{9,10} In addition to inducing inflammation via leukocyte necrosis, PVL has been shown experimentally to induce transcription of genes encoding for pro-inflammatory cytokines in alveolar macrophages. PVL mediated gene transcription occurs in an NF- κ B mediated manner, resulting in upregulation of TNF- α and MIP2.¹³⁰ This function appears to be mediated via binding of PVL to TLR2, and occurs independent of its pore forming capabilities.¹³⁰

While by no means pathognomonic, the severe necrotizing and hemorrhagic pneumonia seen in many cases of CA-MRSA is characteristic of USA300 infection. PVL, which is expressed by a large number of USA300 isolates, and by relatively few other MRSA or MSSA strains, has often been implicated as a major cause of these pathologic changes.^{8,9} It is unlikely, however, to be the sole mediator, as similar pathologic lesions are described post-infection with USA300 strains not expressing PVL.⁸ Furthermore, conflicting information exists as to PVL expression and clinical outcome. Another virulence factor that may contribute are phenol soluble modulins (PSMs). PSMs have been shown experimentally

to have similar leukocytic effects, which may also be relevant in a broader range of host species.^{9,11}

3.3.2.d. Super antigens

Super antigens (SAGs) are molecules that stimulate T-cell hyperactivation resulting in massive cytokine release, and underpin the pathogenesis of toxic shock syndromes associated with certain strains of *Staphylococcus* and *Streptococcus*. SAGs exert this effect by sidestepping traditional antigen presentation, and bind MHC class II molecules of antigen-presenting cells and the variable region of the T-cell receptor β -chain simultaneously.¹² This allows SAGs to activate all T-cells expressing a $V\beta$ -TCR (as much as 50% of cells) rather than the 0.01% of T-cells that would normally respond to an antigen.¹² While most described SAGs are small-secreted molecules encoded on mobile elements with variable strain expression, recently staphylococcal enterotoxin like toxin (Selx) has been discovered, which is a super antigen encoded by the core genome of over 95% of *S. aureus* strains, including MRSA 300. Expression of Selx by USA300 does appear to interact with the $V\beta$ -TCR, and contribute to the necrotizing pneumonia characteristic of USA300 in a rabbit model of pneumonia.¹²

3.3.3. Host immune response

Various cell wall components of *S. aureus* (peptidoglycan, lipoteichoic acid, lipoprotein) are recognized by PRRs including TLR2, NOD2 present on resident epithelial cells and alveolar macrophages, as well as by the soluble PRRs mannose binding lectins and ficolins.¹⁴ While association with lectins and ficolins marks *S. aureus* for complement mediated destruction it may prove ineffectual as the bacteria encodes many complement blocking proteins.¹³ Additionally, while mice deficient in the TLR2 adaptor protein MyD88

are highly susceptible to systemic infection with *S. aureus*, these mice are able to control pulmonary infection with maintained cytokine/chemokine and neutrophil responses.¹⁴ In contrast NOD2^{-/-} mice challenged intratracheally with *S. aureus* do have diminished cytokine/chemokine responses and neutrophil influx, however, reduced inflammatory signaling in this model lead to improved bacterial clearance.¹⁵ These findings illustrate the complex interaction between the pathogen and host, and stress the necessity of *in vivo* models to assess the true relative contributions of putative immune players.

While undoubtedly some degree of pro-inflammatory signaling is necessary for bacterial clearance and neutrophil recruitment, it remains unclear which signaling cascades are vital, dispensable, or actually counter productive for protective immunity. Despite its role in neutrophil chemotaxis and ability to augment neutrophil and macrophage mediated microbe killing, higher TNF- α concentrations are not necessarily protective. In one study TNF- α levels were inversely correlated with outcome in an *S. aureus* pneumonia model.¹³ Likewise mice deficient in TNFR1 clear *S. aureus* more efficiently than wild type mice, however this effect may be mediated by decreased binding of not only TNF- α , but also Protein A, an *S. aureus* virulence factor known to bind and signal through this receptor.¹⁶

Numerous *S. aureus* derived antigens such as capsular antigens, staphylococcal enterotoxin A and B, and lipoteichoic acid, as well as host derived substances such as the complement component C5a contribute to neutrophil chemotaxis and induction of IL-8.¹ In addition to IgG binding domains of protein A and complement inhibitors encoded by *S. aureus* preventing its phagocytosis, the bacteria also encodes proteins that promote its survival post phagocytosis. These include the pore forming HLA or PVL that can liberate the organism from phagocytic cells, and catalase and proteases to combat both oxygen

dependent and independent killing.¹ Additionally phagocytosis of *S. aureus* can accelerate apoptosis of neutrophils, resulting in secondary lysis of neutrophils and release of their inflammatory contents prior to efficient efferocytosis by macrophages.¹

Regardless of these varied immune evading techniques organisms are none the less readily phagocytized and killed by neutrophils, and there is long standing evidence supporting the critical role of neutrophils in clearance of *S. aureus* from the lungs. This point is illustrated in people afflicted with immune-deficiencies involving neutrophil function, such as chronic granulomatous disease and hyper IgE syndromes, in which the incidence of recurrent and unresolving *S. aureus* infections are well-documented.¹ Left unchecked, however, neutrophils can impart as much harm to the surrounding tissue as to the pathogen via toxic mediators such as elastase, collagenase, and free radicals.¹ The factors delineating what constitutes a protective versus harmful immune response to *S. aureus* continue to be elusive, warranting further studies into the pulmonary innate immune response to this important pathogen.

3.3.4. Animal models

The majority of *in vivo* animal studies have been performed in mice.¹³ Given the ready availability of specific gene deficient strains, murine models have offered much insight into immunologic responses during *S. aureus* infection. One major limitation of the murine model is the relative resistance of mice to *S. aureus* infection, developing only mild disease at doses of 1×10^8 CFU/ml, with as much as $3-4 \times 10^8$ CFU/ml constituting a “high dose” causing significant mortality.¹³¹ Additionally while PVL causes neutrophil influx to murine lungs, the toxic pore forming principle does not appear to affect murine leukocytes.¹³⁰ The failure of murine models to recapitulate the severity of human USA300 pneumonic lesions

has lead to the use of a rabbit model. While rabbits also require much higher inoculation dosages of *S. aureus* than would be expected in naturally occurring human pneumonia, they are similarly sensitive to PVL and the T cell superantigen Selx, both of which appear to contribute to severe hemorrhagic pneumonia in this species.^{10,12}

CHAPTER 4 MATERIALS AND METHODS

4.1. Mice

8- to 10-week-old female mice genetically deficient in MCP-1 (Jackson Laboratories) were used, while age- and gender matched C57Bl/6 mice were used as controls.¹³² Animal studies were approved by the Louisiana State University Animal Care and Use Committee. The mice ranged from 19 to 25 g in weight.

4.2. Infection model

Bacteria were prepared for mouse inoculation, as described in previous studies.¹³³ Methicillin resistant *S. aureus* (MRSA) strain USA300 (from F. DeLeo, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT) was grown in Trypticase soy broth (TSB) at 37°C overnight under constant agitation. Bacteria were harvested, washed, and resuspended in sterile 0.9% saline at a concentration of 20×10^8 CFU/ml. Mouse strains were anesthetized with intraperitoneal ketamine-xylazine (250 mg/kg), followed by intratracheal (i.t.) inoculation of 50 μ l of bacteria (10^8 CFU/mouse). The initial mouse inocula were confirmed by plating serial 10-fold dilutions on tryptic soy agar (TSA) plates. For enumerating bacterial CFU in the lung, liver and spleen, whole spleens and whole lung and liver lobes were homogenized in 1 ml sterile deionized water for 30 s, and 20 μ l of the resulting homogenates was plated by serial 10-fold dilutions on TSA plates. Bacterial colonies were counted after incubation overnight at 37°C.

4.3. Blocking antibodies

C57Bl/6 mice were treated i.t. with MCP-1 (R&D systems) or IgG2B antibody (10 μ g/mouse) 30 minutes prior to *S. aureus* infection (10^8 CFUs/mouse).

4.4. Bronchoalveolar lavage fluid collection

Bronchoalveolar lavage fluid (BALF) was collected, and total and differential cell counts and cytokine/chemokine levels were determined. Approximately 3 ml of lavage fluid was retrieved per mouse. Total leukocytes in BALF were determined using a hemocytometer. Cytospin samples were subsequently prepared from BALF cells and stained with Wright-Geimsa. Differential cell counts were determined by direct counting of stained slides. For examination of cytokines/chemokines, the remainder (2 ml) of the undiluted cell-free BALF was used immediately or stored at -80°C.

4.5. Cytokine and chemokine ELISA

We used BALF and lungs that were obtained from animals after *S. aureus* infection or control animals. Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor alpha (TNF-alpha), interleukin-6 (IL-6), Interleukin-1 β (IL-1 β), Interleukin-17 (IL-17), IL-17A, and IL-17F and MCP-1 were obtained from eBiosciences, PA, whereas kits for KC and MIP-2 and LIX were obtained from R&D Systems, MN. The minimum detection limit is 8 pg/ml cytokine protein.

4.6. Myeloperoxidase activity assay

Myeloperoxidase (MPO) release by the neutrophils was measured as previously described.¹³⁴

4.7. Histopathology

The lungs of C57Bl/6 and MCP-1^{-/-} mice were perfused from the right ventricle of the heart with 10 ml isotonic saline at 6 hours and 24 hours post-infection. Lungs were then removed and fixed in 4% phosphate-buffered formalin. Fixed tissue samples were processed in paraffin blocks, and 5 μ m sections were cut with a microtome and stained with

hematoxylin-eosin (H&E). Analysis of histopathology was performed in blinded fashion by a veterinary pathologist using an amended version of a previously published scoring system (Table 1) with scores for each category assigned to individual lung lobes based on microscopic assessment at an objective of x400.¹³⁵

4.8. Statistics

All statistical calculations were performed using GraphPad Prism (version 5.0) software. Data are displayed as median and interquartile ranges. Groups were compared using the Wilcoxon rank-sum test. Differences were considered significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Criterion	0	1	2	3
Intraalveolar inflammatory cell infiltrate	<3 cells	3-10 cells	10-20 cells	>20 cells
Tissue involvement	0%	<10%	10-50%	>50%
Alveolar septae/Perivascular space	All septae are thin and delicate/No perivascular inflammatory cell infiltrate at high power field (HPF)	Thickened alveolar septae in less than one-third of the HPF/Rare (<3) inflammatory cells within the perivascular space in the HPF	Thickened alveolar septae in one-third to two-thirds of the HPF/Low number (3-10) of inflammatory cells within the perivascular space in the HPF	Thickened alveolar septae in greater than two-thirds of the HPF/High number (>10) of inflammatory cells within the perivascular space in the HPF
Alveolar hemorrhage	No hemorrhage	At least 5 erythrocytes per alveolus in 1-5 alveoli	At least 5 erythrocytes per alveolus in 5-10 alveoli	At least 5 erythrocytes per alveolus in >10 alveoli

Figure 5. Quantitative scoring of lung histopathology.

CHAPTER 5 RESULTS

5.1. MCP-1^{-/-} mice have increased neutrophils in the airways and lung parenchyma post *S.aureus* challenge

Recruitment of leukocytes, specifically neutrophils, is an important step in achieving clearance of *S. aureus* as well as other bacterial pathogens. To investigate the extent of leukocyte recruitment to the airways, we determined total and differential leukocyte counts in BALF at 6 and 24 hours post-infection with *S. aureus* (10⁸ CFU/mouse). We observed that both C57Bl/6 and MCP-1^{-/-} mice responded to *S. aureus* challenge with increased leukocyte influx at 6 hours with highest concentrations measured at 24 hours, and that in both groups neutrophils predominated [Figure 6 (6a and 6c)]. Additionally, while total leukocyte and neutrophil concentrations were similar between experimental groups at 24 hours, MCP-1^{-/-} mice had increased numbers of neutrophils at the 6 hour time point [Figure 6 (6a and 6c)]. To assess neutrophil activity and infiltration of lung tissue, a myeloperoxidase activity assay was performed on lung tissue from MCP-1^{-/-} and C57Bl/6 mice. While both experimental groups showed increased MPO activity in lung homogenates, with highest activity at 24 hours post-infection, MCP-1^{-/-} mice had significantly higher MPO activity at both 6 hours and 24 hours [Figure 6 (6a and 6c)]. Intraalveolar inflammation and tissue involvement were assessed in individual lung lobes from C57Bl/6 and MCP-1^{-/-} mice. Higher scores for intraalveolar inflammation and tissue involvement were more frequent in lung lobes from MCP-1^{-/-} mice at 6 hours, at which time 9/10 had a score of 1 for intraalveolar inflammation, and 9/10 a score of 1 or 2 for tissue involvement, compared to a score of 0 in 9/10 lobes from C57Bl/6 mice for both intraalveolar inflammation and tissue involvement (Figure 7). At 24 hours post infection scores of 1 and 2 for both intraalveolar inflammation and tissue

involvement predominated, and occurred in similar frequency between groups. Alveolar hemorrhage and congestion of alveolar septae were also evaluated histologically and appeared similar between groups.

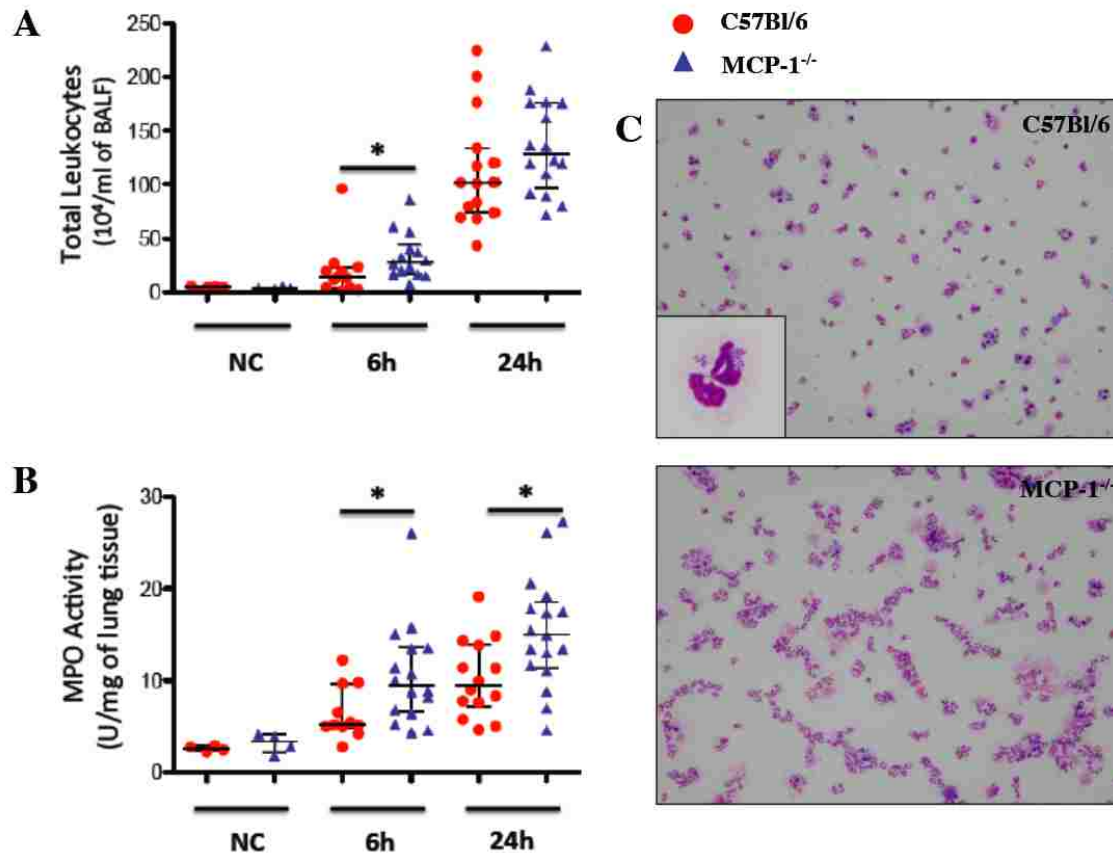
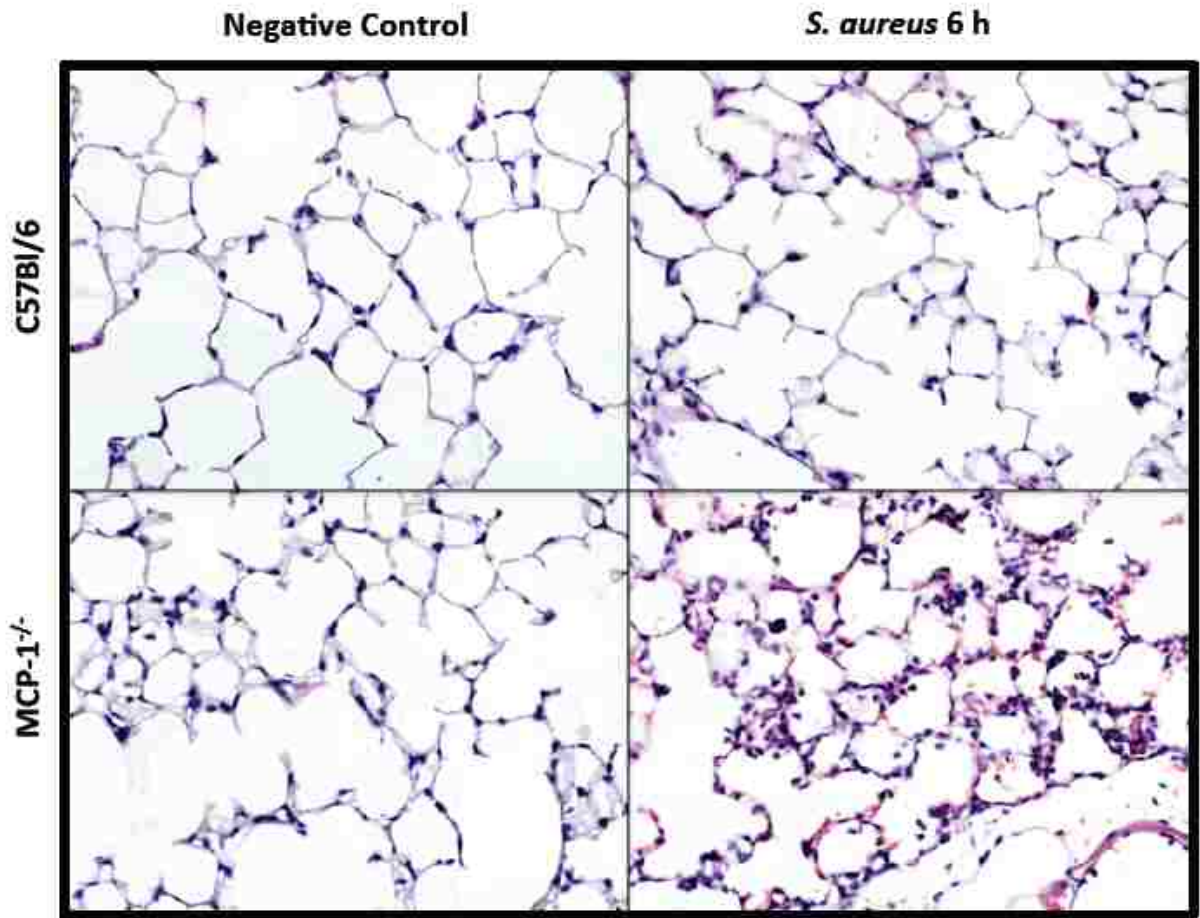


Figure 6. Leukocyte recruitment to airways and lungs of MCP-1^{-/-} mice . A MCP-1^{-/-} mice have increased concentrations of leukocytes in BALF at 6 hours post *S. aureus* infection as compared to C57Bl/6 mice. Both experimental groups have increased concentrations of leukocytes in BALF post- infection as compared to negative controls (NC) (levels of significance not graphically displayed). B MCP-1^{-/-} mice have increased myeloperoxidase (MPO) activity in lung tissue at 6 and 24 hours post *S. aureus* infection as compared to C57Bl/6 mice. Both experimental groups have increased MPO activity as compared to negative controls (NC) (levels of significance not graphically displayed). C Representative cytocentrifuged preparation of BALF from a C57Bl/6 mouse (top) and MCP-1^{-/-} mouse (bottom) 6 hours post *S. aureus* infection. 50x objective, Wright-Geimsa (WG). *Inset*: a neutrophil containing clusters of bacterial cocci. 100x objective WG.



Inflammation Score	
0	0
0	1

Figure 7. Lung histology in MCP-1^{-/-} mice following *S. aureus* infection. Mice were inoculated with *S. aureus* (10⁸ CFU/mouse), lungs were obtained at 6 hours post-infection. This picture is a representative of 2 separate mice with comparable results.

5.2. MCP-1^{-/-} mice have increased levels of inflammatory cytokines at 6 hours post *S. aureus* infection

Numerous cytokines and chemokines produced during inflammation can exert effects on neutrophil dynamics and recruitment. Since neutrophil recruitment appeared enhanced at the 6 hour time point in MCP-1^{-/-} mice, we examined the concentrations of many known inflammatory mediators of neutrophil recruitment in BALF and lung tissue. We found increased concentrations of the inflammatory cytokines TNF- α and IL-6 in MCP-1^{-/-} mice at the 6 hour time point (Figure 8). IL-1 β increased in both experimental groups with infection but was not significantly different between groups. The neutrophil chemoattractant chemokines KC, MIP-2, and LIX increased with infection and LIX concentration were significantly higher in MCP-1^{-/-} mice 24 hours post-infection (Figure 8).

5.3. Local bacterial burden and dissemination are equivocal in MCP-1^{-/-} and C57Bl/6 mice

While adequate neutrophil recruitment is a proven necessity in bacterial clearance, protective immunity does not always correlate directly to neutrophil numbers. In some models excess neutrophil accumulation and/or pro-inflammatory cytokine production are accompanied by poor prognosis and outcome in *S. aureus* infection.¹ For this reason we assessed both local bacterial burden and dissemination as determined by bacterial load in BALF, lung, liver and spleen. Mice were infected with *S. aureus* i.t. and sacrificed at 6 and 24 hours post-infection and BALF, lung, liver, and spleen were collected to quantitate bacterial CFUs. While both experimental groups had highest bacterial burdens in BALF and lung at 6 hours that decreased at the 24 hour time point, MCP-1^{-/-} mice had significantly fewer bacterial CFUs in BALF at 6 hours as compared to C57Bl/6 mice (Figure 9). Dissemination to the liver and spleen was present at 6 hours and persisted at 24 hours in both groups (Figure 9).

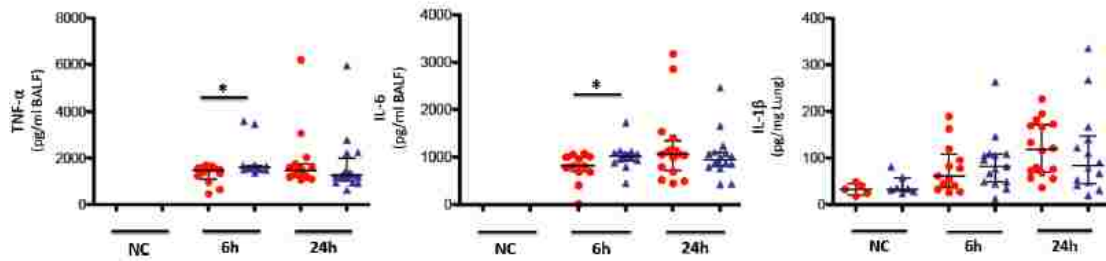
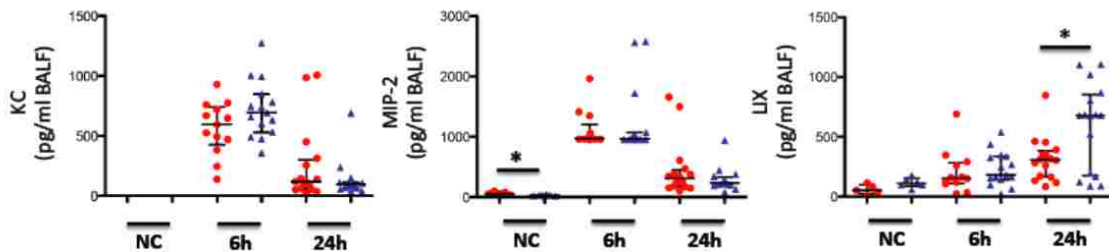
A**B**

Figure 8. Cytokine and chemokine profile in MCP-1^{-/-} mice. A Cytokine Profile. MCP-1^{-/-} mice have increased concentrations of IL-6 and TNF- α in BALF at 6 hours post-infection compared to C57Bl/6 mice. Experimental groups have increased concentrations of IL-6 and TNF- α as compared to negative controls (NC) (levels of significance not graphically displayed, IL-6 and TNF- α NC below detection limit). IL-1 β levels increase with infection but are not different between experimental groups (levels of significance not graphically displayed). B Chemokine Profile. MCP-1^{-/-} mice have higher levels of LIX at 24 hours post-infection compared to C57Bl/6. MIP-2 levels are higher in negative control C57Bl/6 than MCP-1^{-/-} mice. For both groups KC and MIP-2 increase significantly at 6 hours and decrease significantly by 24 hours, whereas LIX continues to increase at 24 hours post-infection (levels of significance not graphically displayed, KC below limit of detection for NC).

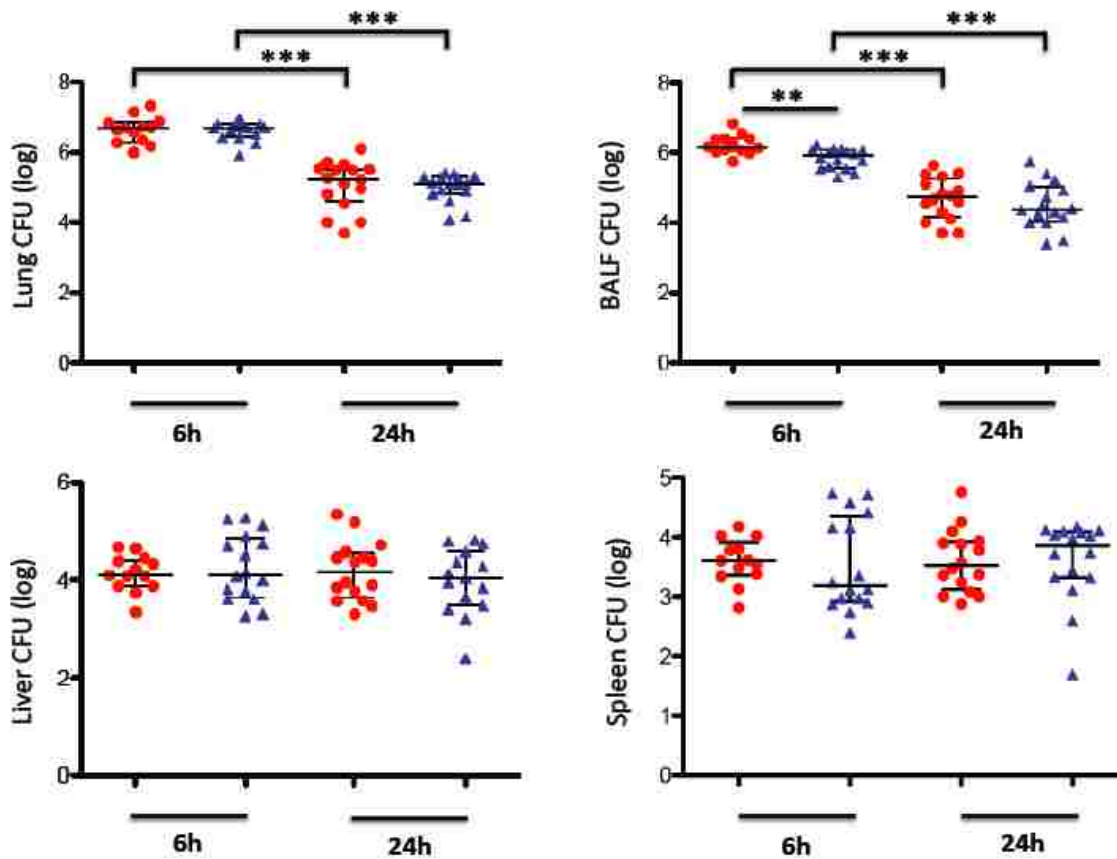


Figure 9. Bacterial burden in MCP-1^{-/-} mice. MCP-1^{-/-} mice have lesser numbers of bacterial CFUs in BALF at 6 hours post *S. aureus* infection as compared to C57Bl/6 mice. BALF and lung CFUs decrease in both experimental groups 24 hours post- infection. Bacterial dissemination is present in liver and spleen at 6 hour and 24 hour time points.

5.4. MCP-1 AB-blocked mice trend towards higher BALF leukocyte concentrations and lung MPO activity

The same infection model was performed using MCP-1 and isotype control AB-blocked mice to ensure that the MCP-1^{-/-} phenotype correlated with that of MCP-1 AB blocked mice. MCP-1 AB blocked mice trended towards higher total leukocyte concentrations in BALF and higher MPO activity in lung tissue, despite similar CFU in BALF and lung homogenates (Figure 10). A cytokine/chemokine profile including IL-6, TNF- α , IL-1 β , KC, MIP2, and LIX revealed no significant differences between groups, with

the exception of LIX, which was higher in MCP-1 AB blocked mice at 6 hours post-infection (Figure 11).

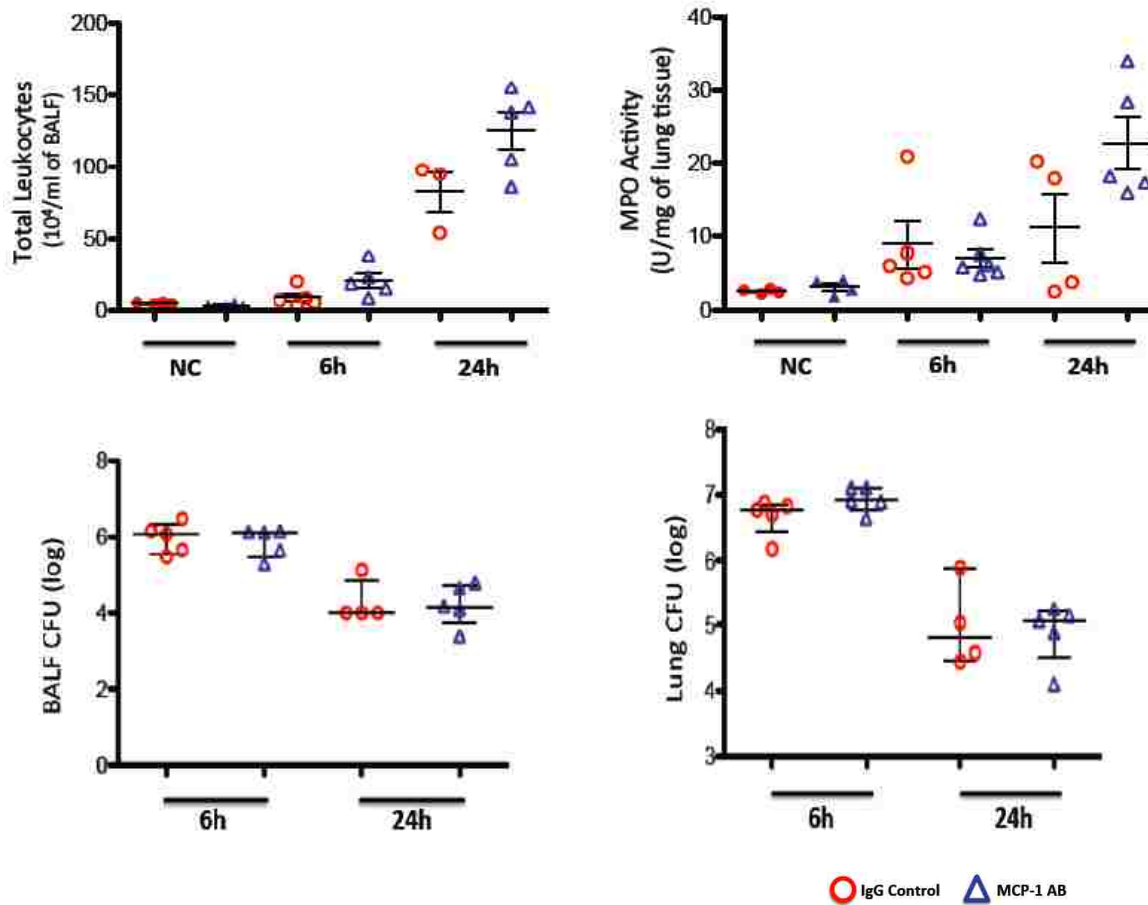


Figure 10. Neutrophil recruitment to the lungs and airways and local bacterial burden in MCP-1 AB-blocked mice. MCP-1 AB blocked mice trend towards higher leukocyte counts in BALF and higher MPO activity in lung tissue as compared to AB-blocked control mice. Bacterial CFUs do not differ between groups, but do decrease significantly by 24 hours post-infection in both experimental groups (level of significance not graphically displayed.)

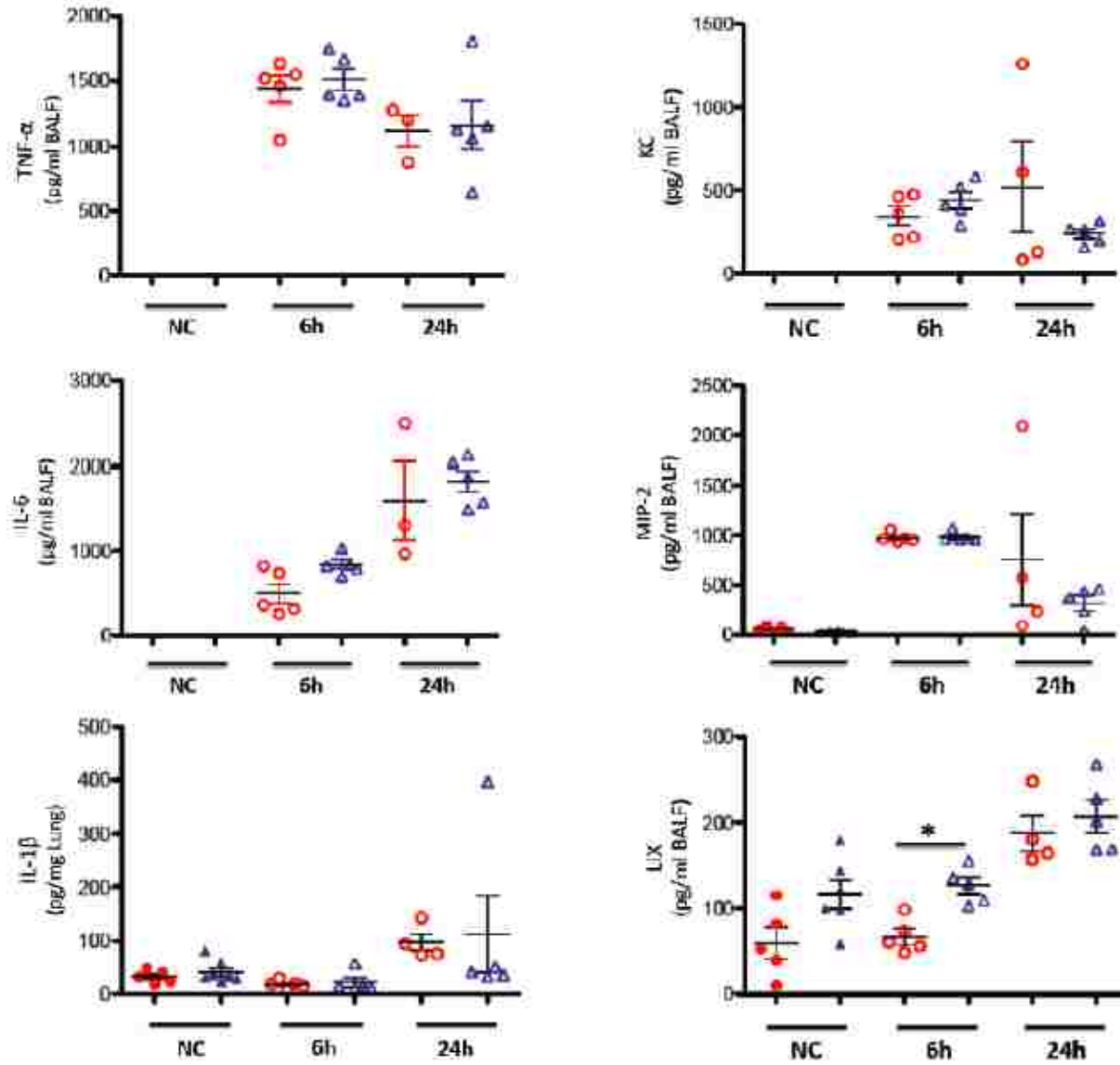


Figure 11. Cytokine and chemokine profile in MCP-1 AB-blocked mice. MCP-1 AB blocked mice have higher levels of LIX at 6 hours post *S. aureus* infection, while levels of IL-6, TNF- α , IL-1 β , KC, and MIP2 do not differ between groups.

CHAPTER 6 DISCUSSION

Adequate neutrophil recruitment to the lungs and airways is vital for protective immunity against a number of pulmonary pathogens including *S. aureus*. In *E. coli* and *K. pneumoniae* models MCP-1 enhances neutrophil recruitment and bacterial clearance, however in our model MCP-1^{-/-} mice had higher neutrophil numbers in BALF, and increased MPO activity in lung tissue, with no appreciable improved bacterial clearance.^{17,18} A number of other pneumonia models support increased neutrophil influx to the lungs and increased pro-inflammatory cytokine profiles in MCP-1^{-/-} or CCR2^{-/-} mice including studies with the gram negative pathogen *Burkholderia mallei* and the gram positive pathogen *S. pneumoniae*. Interestingly, MCP-1 was still protective in these models, with knock out mice demonstrating increased mortality and bacterial burden as compared to wild type controls.^{101,115} Unlike in the *E. coli* and *K. pneumoniae* models, however, the protective role of MCP-1 appeared to be via recruitment of macrophages to airways at later time points, rather than an effect on neutrophil recruitment.

Increased numbers of neutrophils present in the airways and lungs of MCP-1/CCR2^{-/-} mice may be the result of one or more of three general mechanisms. Firstly MCP-1/CCR2^{-/-} mice may produce an altered inflammatory signaling cascade during certain infections leading to the generation of increased neutrophil chemotactic molecules as compared to wild type mice. Second, neutrophil lifespan may be prolonged/enhanced in the absence of MCP-1/CCR2. Thirdly MCP-1/CCR2^{-/-} mice may have impaired clearance of senescent neutrophils.

In our model IL-6 and TNF- α concentrations were higher in MCP-1^{-/-} mice, but neutrophil chemotactic substances (KC, MIP2, LIX) were not significantly different between

groups, with the exception of higher LIX in MCP-1^{-/-} mice at the 24hr time point, and higher MIP2 in C57B/16 mice at baseline. Ultimately, however, sample collection at the 4 hour time point would have been ideal to assess if neutrophil chemotactic molecules were higher in MCP-1^{-/-} mice prior to the increased influx of neutrophils to the lung and airways noted in this group at 6 hours. The increased concentration of LIX at the 24 hour time point may simply reflect the slower kinetics of this chemokine compared to KC and MIP2 as has been previously reported.¹³⁶ A biological reason for the difference in MIP2 between control groups is less evident, and may simply be a statistically relevant but biologically irrelevant difference, reflective of low sample size.

While chemokine receptors generate many down-stream signaling cascades, no experimental evidence currently exists to support the conclusion that neutrophil lifespan is regulated via the MCP-1/CCR2 axis, or would be enhanced in the absence of MCP-1/CCR2 signaling, making this scenario less likely. There is, however, currently experimental support that MCP-1 deficient mice demonstrate decreased efferocytosis of senescent neutrophils, which may contribute to secondary necrosis of these cells, prolonging both pro-inflammatory signaling and neutrophil number and MPO activity in tissue.

Mice administered anti-MCP-1 antibody and *P. aeruginosa* showed increased neutrophilic inflammation, hemorrhage and exudation in lung tissue, increased neutrophil concentrations and MPO activity in BALF, and interestingly, decreased efferocytosis as compared to control Abs-treated mice, despite no differences in BALF macrophage concentrations.²⁰ *In vitro* work by the same group demonstrated macrophages co-cultured with MCP-1 and aged neutrophils exhibited increased phagocytosis of apoptotic cells in a dose dependent manner.²⁰ These findings suggest that in some models of pneumonia

increased neutrophil influx, persistence of MPO activity and increases in inflammatory cytokines may perhaps be due to a decreased efficiency of macrophages to phagocytize aged neutrophils in MCP-1^{-/-} or AB-blocked mice, allowing for continued release of inflammatory mediators from these dying cells.

While our model shares similarities with some of those previously mentioned (*S. pneumoniae*, *B. mallei*, *P. aeruginosa*) in terms of immune response generated, these experiments also demonstrated increased bacterial burden and/or mortality in MCP-1/CCR2^{-/-} mice, findings that were not present in our study. One explanation for this is the relative resistance of mice to *S. aureus* pneumonia. The inoculum dose used in this experiment (1x10⁸ CFU/mouse), which would be exceedingly high for other important pulmonary pathogens such as *K. pneumoniae* or *S. pneumoniae*, is considered a low i.t. dose of *S. aureus* for mice.¹³¹ This is consistent with other pulmonary inoculation models in which *S. aureus* doses of 3-4x10⁸ CFU/mouse are considered a high dose inoculum resulting in significant mortality.¹³¹ If high dose inoculum had been used in the current experiment, it may have allowed for significant differences to emerge between MCP-1^{-/-} and C57Bl/6 mice with respect to bacterial burden and tissue damage as assessed histologically.

The experimental protocol used in MCP-1^{-/-} and C57Bl/6 mice was also performed in antibody-blocked mice for two main reasons. First, administration of anti-MCP-1 antibody has more translational relevance, as it is most similar to the techniques currently employed in experiments assessing efficacy of anti-MCP-1 antibodies and gene therapy. Second, correspondence between MCP-1^{-/-} and antibody blocked phenotypes supports the conclusion that differences present between experimental groups are directly related to MCP-1/CCR2 signaling, rather than any intracellular alterations in gene expression, transcription, or

translation of proteins that may be occurring in MCP-1^{-/-} as a result of gene deletion. While antibody blocked mice did not recapitulate the phenotype of MCP-1^{-/-} mice, BALF leukocyte concentration and MPO activity in lung tissue did trend in similar directions, and LIX was significantly higher in MCP-1 AB blocked mice at the 6 hour time point. We originally attempted antibody blocking at a dose of 1ug/mouse and did not observe this trend (data not shown) supporting the concept that 10 ug/mouse may still be a suboptimal dose of blocking antibody. Ultimately we expect that MCP-1^{-/-} and AB-blocked phenotypes correspond, as this has been demonstrated in other models. However to date these experiments have employed systemic administration of Anti- MCP-1 antibody, and an effective intrapulmonary dose remains to be described.²⁰

Ultimately how MCP-1 is differentially regulated in models of pneumonia, causing robust and protective neutrophil influx in *K. pneumoniae* and *E. coli* models while absence of MCP-1/CCR2 in other models results in ineffectual bacterial clearance despite augmented neutropilic responses, remains unclear. Central to all of these studies however, is that among the repertoire of pathogens currently investigated, MCP-1 plays a protective role in innate pulmonary immune responses. It is likely that MCP-1 plays a protective role in pulmonary *S. aureus* infection as well, however further studies are warranted; both to more clearly delineate the pathology in MCP-1^{-/-} mice using a higher *S. aureus* inoculation dosage, and also to investigate the putative role of MCP-1 in efferocytosis in this model.

REFERENCES

1. Rigby KM, DeLeo FR: Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol* 34:237-259, 2012.
2. Klevens RM, Morrison MA, Nadle J, et al: Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 298:1763-1771, 2007.
3. Tenover FC, Goering RV: Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. *J Antimicrob Chemother* 64:441-446, 2009.
4. Haque NZ, Arshad S, Peyrani P, et al: Analysis of pathogen and host factors related to clinical outcomes in patients with hospital-acquired pneumonia due to methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 50:1640-1644, 2012.
5. Ott E, Bange FC, Reichardt C, et al: Costs of nosocomial pneumonia caused by methicillin-resistant *Staphylococcus aureus*. *J Hosp Infect* 76:300-303, 2010.
6. Shorr AF, Tabak YP, Gupta V, et al: Morbidity and cost burden of methicillin-resistant *Staphylococcus aureus* in early onset ventilator-associated pneumonia. *Crit Care* 10:R97, 2006.
7. Quadri AM, Hock YL: Pulmonary pathology of Panton-Valentine leukocidin gene carrying methicillin-resistant *Staphylococcus aureus* pneumonia. *Histopathology* 51:712-713, 2007.
8. Otto M: A MRSA-terious enemy among us: end of the PVL controversy? *Nat Med* 17:169-170, 2011.
9. Loffler B, Hussain M, Grundmeier M, et al: *Staphylococcus aureus* panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils. *PLoS Pathog* 6:e1000715, 2010.
10. Diep BA, Chan L, Tattavin P, et al: Polymorphonuclear leukocytes mediate *Staphylococcus aureus* Panton-Valentine leukocidin-induced lung inflammation and injury. *Proc Natl Acad Sci U S A* 107:5587-5592, 2010.
11. Wang R, Braughton KR, Kretschmer D, et al: Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* 13:1510-1514, 2007.
12. Wilson GJ, Seo KS, Cartwright RA, et al: A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. *PLoS Pathog* 7:e1002271, 2011.

13. Prince A: Mucosal immunology of acute bacterial pneumonia. New York, Springer, 2012.
14. Sethi S, Chakraborty T: Role of TLR- / NLR-signaling and the associated cytokines involved in recruitment of neutrophils in murine models of Staphylococcus aureus infection. *Virulence* 2:316-328, 2011.
15. Kapetanovic R, Jouvion G, Fitting C, et al: Contribution of NOD2 to lung inflammation during Staphylococcus aureus-induced pneumonia. *Microbes Infect* 12:759-767, 2010.
16. Martin FJ, Gomez MI, Wetzel DM, et al: Staphylococcus aureus activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A. *J Clin Invest* 119:1931-1939, 2009.
17. Balamayooran G, Batra S, Balamayooran T, et al: Monocyte chemoattractant protein 1 regulates pulmonary host defense via neutrophil recruitment during Escherichia coli infection. *Infect Immun* 79:2567-2577, 2011.
18. Balamayooran G, Batra S, Theivanthiran B, et al: Intrapulmonary G-CSF rescues neutrophil recruitment to the lung and neutrophil release to blood in Gram-negative bacterial infection in MCP-1^{-/-} mice. *J Immunol* 189:5849-5859, 2012.
19. Becker S, Quay J, Koren HS, et al: Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. *Am J Physiol* 266:L278-286, 1994.
20. Amano H, Morimoto K, Senba M, et al: Essential contribution of monocyte chemoattractant protein-1/C-C chemokine ligand-2 to resolution and repair processes in acute bacterial pneumonia. *J Immunol* 172:398-409, 2004.
21. Biswas SK, Sodhi A: Tyrosine phosphorylation-mediated signal transduction in MCP-1-induced macrophage activation: role for receptor dimerization, focal adhesion protein complex and JAK/STAT pathway. *Int Immunopharmacol* 2:1095-1107, 2002.
22. Paul WE: Fundamental immunology. New York, Raven Press, 1984.
23. Monzon ME, Forteza RM, Casalino-Matsuda SM: MCP-1/CCR2B-dependent loop upregulates MUC5AC and MUC5B in human airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 300:L204-215, 2011.
24. Kitamoto S, Egashira K: Gene therapy targeting monocyte chemoattractant protein-1 for vascular disease. *J Atheroscler Thromb* 9:261-265, 2002.
25. Wada T, Furuichi K, Sakai N, et al: Gene therapy via blockade of monocyte chemoattractant protein-1 for renal fibrosis. *J Am Soc Nephrol* 15:940-948, 2004.

26. Loberg RD, Ying C, Craig M, et al: Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. *Cancer Res* 67:9417-9424, 2007.
27. Koga M, Kai H, Egami K, et al: Mutant MCP-1 therapy inhibits tumor angiogenesis and growth of malignant melanoma in mice. *Biochem Biophys Res Commun* 365:279-284, 2008.
28. Sligl WI, Marrie TJ: Severe community-acquired pneumonia. *Crit Care Clin* 29:563-601, 2013.
29. Mizgerd JP: Acute lower respiratory tract infection. *N Engl J Med* 358:716-727, 2008.
30. Ausubel FM: Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol* 6:973-979, 2005.
31. Correa RG, Milutinovic S, Reed JC: Roles of NOD1 (NLRC1) and NOD2 (NLRC2) in innate immunity and inflammatory diseases. *Biosci Rep* 32:597-608, 2012.
32. Kawai T, Akira S: Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci* 1143:1-20, 2008.
33. Koonin EV, Aravind L: The NACHT family - a new group of predicted NTPases implicated in apoptosis and MHC transcription activation. *Trends Biochem Sci* 25:223-224, 2000.
34. Franchi L, Munoz-Planillo R, Nunez G: Sensing and reacting to microbes through the inflammasomes. *Nat Immunol* 13:325-332, 2012.
35. Frutuoso MS, Hori JI, Pereira MS, et al: The pattern recognition receptors Nod1 and Nod2 account for neutrophil recruitment to the lungs of mice infected with *Legionella pneumophila*. *Microbes Infect* 12:819-827, 2010.
36. Miao EA, Warren SE: Innate immune detection of bacterial virulence factors via the NLRC4 inflammasome. *J Clin Immunol* 30:502-506, 2010.
37. Kaparakis M, Turnbull L, Carneiro L, et al: Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cell Microbiol* 12:372-385, 2010.
38. Theivanthiran B, Batra S, Balamayooran G, et al: NOD2 signaling contributes to host defense in the lungs against *Escherichia coli* infection. *Infect Immun* 80:2558-2569, 2012.

39. Shimada K, Chen S, Dempsey PW, et al: The NOD/RIP2 pathway is essential for host defenses against *Chlamydomyphila pneumoniae* lung infection. *PLoS Pathog* 5:e1000379, 2009.
40. Kufer TA, Kremmer E, Adam AC, et al: The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction. *Cell Microbiol* 10:477-486, 2008.
41. Sabbah A, Chang TH, Harnack R, et al: Activation of innate immune antiviral responses by Nod2. *Nat Immunol* 10:1073-1080, 2009.
42. Uehara A, Fujimoto Y, Fukase K, et al: Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. *Mol Immunol* 44:3100-3111, 2007.
43. Pandey AK, Yang Y, Jiang Z, et al: NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to *Mycobacterium tuberculosis*. *PLoS Pathog* 5:e1000500, 2009.
44. Kofoed EM, Vance RE: NAIPs: building an innate immune barrier against bacterial pathogens. NAIPs function as sensors that initiate innate immunity by detection of bacterial proteins in the host cell cytosol. *Bioessays* 34:589-598, 2012.
45. Zhao Y, Yang J, Shi J, et al: The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 477:596-600, 2011.
46. Yamamoto Y, Klein TW, Newton CA, et al: Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J mice. *Infect Immun* 56:370-375, 1988.
47. Endrizzi MG, Hadinoto V, Gowney JD, et al: Genomic sequence analysis of the mouse Naip gene array. *Genome Res* 10:1095-1102, 2000.
48. Lightfield KL, Persson J, Brubaker SW, et al: Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. *Nat Immunol* 9:1171-1178, 2008.
49. Kofoed EM, Vance RE: Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477:592-595, 2011.
50. Miao EA, Alpuche-Aranda CM, Dors M, et al: Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol* 7:569-575, 2006.
51. Cai S, Batra S, Wakamatsu N, et al: NLRC4 inflammasome-mediated production of IL-1beta modulates mucosal immunity in the lung against gram-negative bacterial infection. *J Immunol* 188:5623-5635, 2012.

52. Case CL, Shin S, Roy CR: Asc and Ipaf Inflammasomes direct distinct pathways for caspase-1 activation in response to *Legionella pneumophila*. *Infect Immun* 77:1981-1991, 2009.
53. Case CL, Roy CR: Asc modulates the function of NLRC4 in response to infection of macrophages by *Legionella pneumophila*. *MBio* 2, 2011.
54. Mariathasan S, Newton K, Monack DM, et al: Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430:213-218, 2004.
55. Martinon F, Burns K, Tschopp J: The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10:417-426, 2002.
56. Faustin B, Lartigue L, Bruey JM, et al: Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell* 25:713-724, 2007.
57. Kovarova M, Hesker PR, Jania L, et al: NLRP1-dependent pyroptosis leads to acute lung injury and morbidity in mice. *J Immunol* 189:2006-2016, 2012.
58. Boyden ED, Dietrich WF: Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 38:240-244, 2006.
59. Sastalla I, Crown D, Masters SL, et al: Transcriptional analysis of the three Nlrp1 paralogs in mice. *BMC Genomics* 14:188, 2013.
60. Jin C, Flavell RA: Molecular mechanism of NLRP3 inflammasome activation. *J Clin Immunol* 30:628-631, 2010.
61. Harder J, Franchi L, Munoz-Planillo R, et al: Activation of the Nlrp3 inflammasome by *Streptococcus pyogenes* requires streptolysin O and NF-kappa B activation but proceeds independently of TLR signaling and P2X7 receptor. *J Immunol* 183:5823-5829, 2009.
62. Witzentrath M, Pache F, Lorenz D, et al: The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J Immunol* 187:434-440, 2011.
63. Iyer SS, Pulskens WP, Sadler JJ, et al: Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A* 106:20388-20393, 2009.
64. Shimada K, Crother TR, Karlin J, et al: Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36:401-414, 2012.

65. Dostert C, Petrilli V, Van Bruggen R, et al: Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320:674-677, 2008.
66. Petrilli V, Papin S, Dostert C, et al: Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ* 14:1583-1589, 2007.
67. Gasse P, Mary C, Guenon I, et al: IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice. *J Clin Invest* 117:3786-3799, 2007.
68. Artlett CM, Sassi-Gaha S, Rieger JL, et al: The inflammasome activating caspase 1 mediates fibrosis and myofibroblast differentiation in systemic sclerosis. *Arthritis Rheum* 63:3563-3574, 2011.
69. Bauernfeind F, Bartok E, Rieger A, et al: Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *J Immunol* 187:613-617, 2011.
70. Ferrari D, Pizzirani C, Adinolfi E, et al: The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol* 176:3877-3883, 2006.
71. Hornung V, Bauernfeind F, Halle A, et al: Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9:847-856, 2008.
72. Cassel SL, Eisenbarth SC, Iyer SS, et al: The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* 105:9035-9040, 2008.
73. Pelegrin P, Surprenant A: Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J* 25:5071-5082, 2006.
74. Munoz-Planillo R, Franchi L, Miller LS, et al: A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome. *J Immunol* 183:3942-3948, 2009.
75. Craven RR, Gao X, Allen IC, et al: *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* 4:e7446, 2009.
76. Chen CC, Tsai SH, Lu CC, et al: Activation of an NLRP3 inflammasome restricts *Mycobacterium kansasii* infection. *PLoS One* 7:e36292, 2012.
77. Elinav E, Strowig T, Kau AL, et al: NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* 145:745-757, 2011.

78. Chen GY, Liu M, Wang F, et al: A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol* 186:7187-7194, 2011.
79. Vladimer GI, Weng D, Paquette SW, et al: The NLRP12 inflammasome recognizes *Yersinia pestis*. *Immunity* 37:96-107, 2012.
80. Allen IC, Wilson JE, Schneider M, et al: NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity* 36:742-754, 2012.
81. Grenier JM, Wang L, Manji GA, et al: Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-kappaB and caspase-1. *FEBS Lett* 530:73-78, 2002.
82. Meixenberger K, Pache F, Eitel J, et al: *Listeria monocytogenes*-infected human peripheral blood mononuclear cells produce IL-1beta, depending on listeriolysin O and NLRP3. *J Immunol* 184:922-930, 2010.
83. Anand PK, Malireddi RK, Lukens JR, et al: NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. *Nature* 488:389-393, 2012.
84. Lich JD, Williams KL, Moore CB, et al: Monarch-1 suppresses non-canonical NF-kappaB activation and p52-dependent chemokine expression in monocytes. *J Immunol* 178:1256-1260, 2007.
85. Allen IC, McElvania-Tekippe E, Wilson JE, et al: Characterization of NLRP12 during the In Vivo Host Immune Response to *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*. *PLoS One* 8:e60842, 2013.
86. Ferwerda G, Girardin SE, Kullberg BJ, et al: NOD2 and toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. *PLoS Pathog* 1:279-285, 2005.
87. Kawai T, Akira S: Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34:637-650, 2011.
88. Miao EA, Ernst RK, Dors M, et al: *Pseudomonas aeruginosa* activates caspase 1 through Ipaf. *Proc Natl Acad Sci U S A* 105:2562-2567, 2008.
89. Dunne A, Ross PJ, Pospisilova E, et al: Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against *Bordetella pertussis*. *J Immunol* 185:1711-1719, 2010.
90. Levinsohn JL, Newman ZL, Hellmich KA, et al: Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome. *PLoS Pathog* 8:e1002638, 2012.

91. Shimada K, Crother TR, Karlin J, et al: Caspase-1 dependent IL-1beta secretion is critical for host defense in a mouse model of Chlamydia pneumoniae lung infection. PLoS One 6:e21477, 2011.
92. Willingham SB, Allen IC, Bergstralh DT, et al: NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. J Immunol 183:2008-2015, 2009.
93. Mishra BB, Moura-Alves P, Sonawane A, et al: Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol 12:1046-1063, 2010.
94. Gandotra S, Jang S, Murray PJ, et al: Nucleotide-binding oligomerization domain protein 2-deficient mice control infection with Mycobacterium tuberculosis. Infect Immun 75:5127-5134, 2007.
95. Rackley CR, Stripp BR: Building and maintaining the epithelium of the lung. J Clin Invest 122:2724-2730, 2012.
96. Williams A, Hussell T, Lloyd C: Immunology : mucosal and body surface defences. Chichester, West Sussex ; Hoboken, NJ, Wiley-Blackwell, 2012.
97. Commins SP, Borish L, Steinke JW: Immunologic messenger molecules: cytokines, interferons, and chemokines. J Allergy Clin Immunol 125:S53-72, 2010.
98. Cray C, Zaias J, Altman NH: Acute phase response in animals: a review. Comp Med 59:517-526, 2009.
99. Mukaida N: Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. Am J Physiol Lung Cell Mol Physiol 284:L566-577, 2003.
100. Paul WE: Fundamental immunology (ed 6th). Philadelphia, Wolters Kluwer/Lippincott Williams & Wilkins, 2008.
101. Winter C, Herbold W, Maus R, et al: Important role for CC chemokine ligand 2-dependent lung mononuclear phagocyte recruitment to inhibit sepsis in mice infected with Streptococcus pneumoniae. J Immunol 182:4931-4937, 2009.
102. Terashima Y, Onai N, Murai M, et al: Pivotal function for cytoplasmic protein FROUNT in CCR2-mediated monocyte chemotaxis. Nat Immunol 6:827-835, 2005.
103. Soriano SF, Serrano A, Hernanz-Falcon P, et al: Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. Eur J Immunol 33:1328-1333, 2003.

104. Sanders SK, Crean SM, Boxer PA, et al: Functional differences between monocyte chemotactic protein-1 receptor A and monocyte chemotactic protein-1 receptor B expressed in a Jurkat T cell. *J Immunol* 165:4877-4883, 2000.
105. Tanaka S, Green SR, Quehenberger O: Differential expression of the isoforms for the monocyte chemoattractant protein-1 receptor, CCR2, in monocytes. *Biochem Biophys Res Commun* 290:73-80, 2002.
106. Mellado M, Rodriguez-Frade JM, Vila-Coro AJ, et al: Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *EMBO J* 20:2497-2507, 2001.
107. Salcedo R, Ponce ML, Young HA, et al: Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 96:34-40, 2000.
108. Carulli MT, Ong VH, Ponticos M, et al: Chemokine receptor CCR2 expression by systemic sclerosis fibroblasts: evidence for autocrine regulation of myofibroblast differentiation. *Arthritis Rheum* 52:3772-3782, 2005.
109. Christensen PJ, Du M, Moore B, et al: Expression and functional implications of CCR2 expression on murine alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 286:L68-72, 2004.
110. Daly C, Rollins BJ: Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation* 10:247-257, 2003.
111. Yang X, Coriolan D, Murthy V, et al: Proinflammatory phenotype of vascular smooth muscle cells: role of efficient Toll-like receptor 4 signaling. *Am J Physiol Heart Circ Physiol* 289:H1069-1076, 2005.
112. Mitchell D, Olive C: Regulation of Toll-like receptor-induced chemokine production in murine dendritic cells by mitogen-activated protein kinases. *Mol Immunol* 47:2065-2073, 2010.
113. Maus U, Huwe J, Maus R, et al: Alveolar JE/MCP-1 and endotoxin synergize to provoke lung cytokine upregulation, sequential neutrophil and monocyte influx, and vascular leakage in mice. *Am J Respir Crit Care Med* 164:406-411, 2001.
114. Dessing MC, de Vos AF, Florquin S, et al: Monocyte chemoattractant protein 1 does not contribute to protective immunity against pneumococcal pneumonia. *Infect Immun* 74:7021-7023, 2006.
115. Goodyear A, Jones A, Troyer R, et al: Critical protective role for MCP-1 in pneumonic *Burkholderia mallei* infection. *J Immunol* 184:1445-1454, 2010.

116. Haringman JJ, Gerlag DM, Smeets TJ, et al: A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis. *Arthritis Rheum* 54:2387-2392, 2006.
117. Lowy FD: Staphylococcus aureus infections. *N Engl J Med* 339:520-532, 1998.
118. den Heijer CD, van Bijnen EM, Paget WJ, et al: Prevalence and resistance of commensal Staphylococcus aureus, including methicillin-resistant S aureus, in nine European countries: a cross-sectional study. *Lancet Infect Dis* 13:409-415, 2013.
119. Shukla SK, Karow ME, Brady JM, et al: Virulence genes and genotypic associations in nasal carriage, community-associated methicillin-susceptible and methicillin-resistant USA400 Staphylococcus aureus isolates. *J Clin Microbiol* 48:3582-3592, 2010.
120. Gorwitz RJ, Kruszon-Moran D, McAllister SK, et al: Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004. *J Infect Dis* 197:1226-1234, 2008.
121. Fey PD, Said-Salim B, Rupp ME, et al: Comparative molecular analysis of community- or hospital-acquired methicillin-resistant Staphylococcus aureus. *Antimicrob Agents Chemother* 47:196-203, 2003.
122. Lee MH, Arrecubieta C, Martin FJ, et al: A postinfluenza model of Staphylococcus aureus pneumonia. *J Infect Dis* 201:508-515, 2010.
123. Diep BA, Gill SR, Chang RF, et al: Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant Staphylococcus aureus. *Lancet* 367:731-739, 2006.
124. Sasso EH, Silverman GJ, Mannik M: Human IgM molecules that bind staphylococcal protein A contain VHIII H chains. *J Immunol* 142:2778-2783, 1989.
125. Gomez MI, O'Seaghdha M, Magargee M, et al: Staphylococcus aureus protein A activates TNFR1 signaling through conserved IgG binding domains. *J Biol Chem* 281:20190-20196, 2006.
126. Gomez MI, Prince A: Airway epithelial cell signaling in response to bacterial pathogens. *Pediatr Pulmonol* 43:11-19, 2008.
127. Gomez MI, Seaghdha MO, Prince AS: Staphylococcus aureus protein A activates TACE through EGFR-dependent signaling. *EMBO J* 26:701-709, 2007.
128. Wilke GA, Bubeck Wardenburg J: Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. *Proc Natl Acad Sci U S A* 107:13473-13478, 2010.

129. Powers ME, Kim HK, Wang Y, et al: ADAM10 mediates vascular injury induced by *Staphylococcus aureus* alpha-hemolysin. *J Infect Dis* 206:352-356, 2012.
130. Zivkovic A, Sharif O, Stich K, et al: TLR 2 and CD14 mediate innate immunity and lung inflammation to staphylococcal Panton-Valentine leukocidin in vivo. *J Immunol* 186:1608-1617, 2011.
131. Montgomery CP, Boyle-Vavra S, Daum RS: Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. *PLoS One* 5:e15177, 2010.
132. Gu L, Okada Y, Clinton SK, et al: Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2:275-281, 1998.
133. Choi SM, McAleer JP, Zheng M, et al: Innate Stat3-mediated induction of the antimicrobial protein Reg3gamma is required for host defense against MRSA pneumonia. *J Exp Med* 210:551-561, 2013.
134. Hirche TO, Gaut JP, Heinecke JW, et al: Myeloperoxidase plays critical roles in killing *Klebsiella pneumoniae* and inactivating neutrophil elastase: effects on host defense. *J Immunol* 174:1557-1565, 2005.
135. Matute-Bello G, Frevert CW, Kajikawa O, et al: Septic shock and acute lung injury in rabbits with peritonitis: failure of the neutrophil response to localized infection. *Am J Respir Crit Care Med* 163:234-243, 2001.
136. Rovai LE, Herschman HR, Smith JB: The murine neutrophil-chemoattractant chemokines LIX, KC, and MIP-2 have distinct induction kinetics, tissue distributions, and tissue-specific sensitivities to glucocorticoid regulation in endotoxemia. *J Leukoc Biol* 64:494-502, 1998.

VITA

Mary Katherine Leissingner was born in New Orleans, LA in 1984 to Henry and Marilyn Leissingner, the youngest sister to big brother Duane, and big sister Jo Leissingner. She graduated with a Bachelor of Biological Sciences and minor of Latin from Louisiana State University in 2006, followed by her DVM at LSU-SVM in 2010. After completing a rotating small animal internship at the University of Tennessee College of Veterinary Medicine in 2011, Mary returned to LSU to begin her joint residency in veterinary clinical pathology and Master of Science degree. She will complete her residency in May 2014. If she had a life philosophy, it would likely reflect her personal belief of living in the now and her professional love of clinical pathology, as best embodied in the words of Henry David Thoreau “to live deep and suck out all the marrow of life.”