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Christopher Jondle

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MECHANISTIC INSIGHTS INTO NEUTROPHIL TURNOVER DURING GRAM-NEGATIVE PNEUMONIC SEPSIS

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A dissertation
Submitted to the Graduate Faculty

Of the

University of North Dakota

In partial fulfillment of the requirements

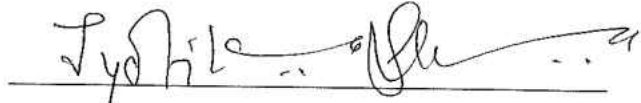
For the degree of

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This dissertation, submitted by Christopher Jondle in partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Microbiology and Immunology from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.



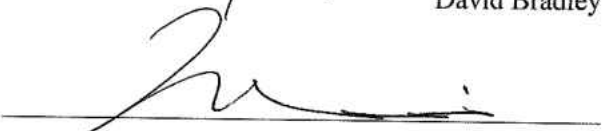
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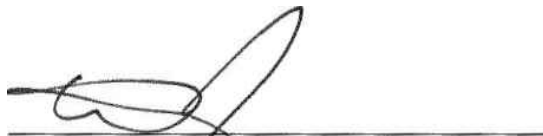


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ABSTRACT

Sepsis is one of the oldest and most elusive syndromes in medicine. It is characterized by a systemic inflammatory response leading to acute organ dysfunction. In the United States alone there are over 750,000 cases of sepsis each year, with a mortality rate between 20-50%. Sepsis is the second leading cause of death in the ICU and the tenth leading cause of death overall in the United States. Over half of all ICU resources are consumed in the treatment of sepsis. There are currently no effective therapies available against sepsis, all attempts to develop an effective therapy have failed. Respiratory infections are the leading cause of sepsis, leading to what is called pneumonic sepsis. The Gram-negative bacteria *Klebsiella pneumoniae* (KPn) is associated with upwards of 20% of all pneumonic sepsis cases. Utilizing a mouse model of pneumonic sepsis induced by intranasal infection of KPn, I studied the role that mammalian C-type lectin receptors (CLRs) play in the development and clearance of pneumonic sepsis. CLRs are primarily expressed on phagocytic immune cells of myeloid origin and are considered pattern recognition receptors that have the ability to shape the immune response by recognizing both pathogen associated molecular patterns and host damage associated molecular patterns in many different pathological conditions. From the pathogen perspective, I studied the effect KPn has on the immune cells and how that may impact the ability of KPn establish the infection.

In my first project, I examined the role of two CLR, Macrophage Galactose Type Lectin 1 (MGL1) and Clec4e otherwise known as Mincle, in the development and clearance of pneumonic sepsis caused by KPn. This project was built on previous observations made by my advisor Dr. Jyotika Sharma which showed these two CLR upregulated in the lung during KPn induced pneumonic sepsis. In regards to MGL1, I am the first author on the paper that we recently published in the Journal of Immunology “Macrophage Galactose-Type Lectin-1 Deficiency Is Associated with Increased Neutrophilia and Hyperinflammation in Gram-Negative Pneumonia” (J Immunol. 2016 Apr 1;196(7):3088-96. doi: 10.4049/jimmunol.1501790.). In this paper we showed that MGL1^{-/-} mice were more susceptible to KPn infection, a phenotype that did not correlate with the systemic and local bacterial burden; the ability of macrophages and neutrophils to phagocytose and kill bacteria or neutrophil NETs. We demonstrated that the mechanism underlying the increased mortality of MGL1^{-/-} mice was increased ability of neutrophils to infiltrate the lungs causing their overwhelming accumulation and severe neutrophil-mediated pathology in the lungs in the absence of MGL1. Mechanistic insights into the potential negative regulatory function of MGL1 in neutrophil infiltration, their clearance by efferocytosis as well as the process of granulopoiesis are some of the future directions of this work that are being perused in the laboratory. In the paper I co-authored on Mincle, “Protective Role of Mincle in Bacterial Pneumonia by Regulation of Neutrophil Mediated Phagocytosis and Extracellular Trap Formation” (Infect Dis. (2014) 209 (11): 1837-1846.doi: 10.1093/infdis/jit820), we showed that Mincle mediates two important bacterial clearance mechanisms i.e.

bacterial phagocytosis and extracellular trap (NET) formation by neutrophils. As a result, Mincle^{-/-} mice are more susceptible to KPn infection as compared to the Mincle sufficient wild-type mice. This project is being led by Dr. Atul Sharma, a postdoctoral fellow in the lab.

My second project examined the effect of KPn infection on the clearance of neutrophils by a process called efferocytosis. While performing experiments for the role of MGL1 in efferocytosis, I found that in comparison with the uninfected neutrophils, KPn infected cells were engulfed less efficiently by macrophages. As efferocytosis is a receptor mediated process, I discovered that while KPn infection increases the expression of repressive molecules called “Don’t Eat Me” signals on neutrophils, it is the modulation of distribution of a key “Eat Me” signal, phosphatidylserine (PtdSer) and the subsequent delay of apoptosis in neutrophils that is partially involved in KPn mediated inhibition of efferocytosis. Accordingly, KPn infected neutrophils also induce an alternative route of programmed cell death called necroptosis. It is the combination of the “eat me” signal downregulation and the induction of necroptosis in KPn infected neutrophils that inhibit their efferocytic uptake by macrophages. A part of this data has been communicated for publication. Current work that I have led in the lab on this project involves determining what signaling mechanisms KPn is activating to decrease PtdSer as well as activation of necroptosis to inhibit the efferocytosis of infected PMNs.

The overarching goal of this work was to increase the overall knowledge on the role CLR plays in pneumonic sepsis in terms of neutrophil turnover and how KPn

subverts these processes to its own advantage. I believe that this knowledge can be used to identify novel targets for effective therapy of sepsis as well as other inflammatory conditions.

CHAPTER 1

INTRODUCTION

Sepsis and Pneumonia: Healthcare burden and treatment

Sepsis has been described for thousands of years and despite massive amounts of effort to better understand and treat sepsis, it is still a major challenge in medicine (1, 2). A recent study determined that there were over 31.5 million cases of sepsis worldwide, of which 19.4 were severe sepsis, resulting in 5.3 million deaths annually (3). In the United States sepsis is the 11th leading cause of death and is considered a major healthcare burden by the Centers for Disease Control and Prevention, causing over an estimated 750,000 cases annually with a mortality rate of 20-50% depending on certain risk factors. Sepsis is the leading cause of death in non-coronary ICU patients in the US and affects 1 out of 3 ICU patients. An estimated \$24.3 billion dollars is spent each year treating sepsis (2, 4-10). Patients who are immune-compromised, have chronic diseases such as cancer, diabetes, liver disease, or chronic obstructive pulmonary disease, as well as elderly individuals have the highest risk of developing sepsis or severe sepsis (11).

Respiratory infections and pneumonia in the lungs are the most common site of infection for sepsis, accounting for 40%-50% of all sepsis cases (2, 12, 13). Both Gram-positive and Gram-negative bacteria can cause sepsis, but in the lung Gram negative bacteria are the primary cause of infection. Gram negative infections also have the highest rate of mortality (14-16). Gram negative bacteria that belong to the Enterobacteriaceae family, such as *E. coli* and *Klebsiella* species are the leading cause of sepsis (12, 17, 18).

The incidence of sepsis is on the rise with a 13% increase annually in the number of sepsis cases seen (11, 19, 20). While there has been an increase in the occurrence of sepsis over the past two decades, there has also been a decrease in the mortality rate of sepsis going down from 46.9% from 1991-1995 to 29% from 2006-2009 (10). The decrease in mortality rates has partially been due to advances in supportive care as well as better recognition of early sepsis due the improved definition of sepsis by the American College of Chest Physicians and Society for Critical Care Medicine Consensus Conference in 1991 and 2001. The 1991 conference defined systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and septic shock as a disease complex with varying degrees of severity based on a number of different criteria such as inflammation and organ failure. The 2001 conference revised the criteria for sepsis making it more specific by using clinical and laboratory criteria to identify sepsis (11, 21, 22). Despite this decrease in mortality rate, the morbidity associated with sepsis and the development of antibiotic resistance by bacterial pathogens continues to pose a healthcare threat and highlight the importance of understanding immune responses in order to identify new avenues for treatment strategies. The clinical signs of sepsis today include but are not limited to, increase or decrease in body temperature, heart rate, or white blood cell count, and elevated levels of plasma C-

reactive protein due to an infection and systemic inflammation. Sepsis progresses to severe sepsis when organ dysfunction is seen. Septic shock occurs when there is a dangerous drop in blood pressure, due to complications of severe sepsis (11, 21, 22). Hallmarks used in the lab to identify sepsis include hypercytokinemia, with an initial increase in pro-inflammatory cytokines and later increase in anti-inflammatory cytokines in conjunction with high pro-inflammatory cytokine levels, indicating a dysregulation of the immune response. The cytokine storm is followed closely by a dysregulation of the complement and coagulation systems, the dysregulation of the coagulation system can lead to vascular injury caused by disseminated intravascular coagulation (DIC). Vascular injury along with other organ dysfunctions leads to bacteremia or the presence of bacteria in the blood, as well as cell death. Bacteremia can spread the infection to other organs further driving the development of sepsis. Cell death causes the release of damage associated molecular patterns (DAMPs) and alarmins, endogenous molecules that are released upon tissue damage, which further drives inflammation and recruits cells neutrophils and other innate immune cells causing further sepsis development (Figure 1). All of these outcomes if not identified and treated quickly, ultimately leads to organ failure culminating in death. The development of sepsis occurs in a feed forward loop. As the disease progresses, the result of the initial inflammation recruits more innate immune cells, which lead to exaggeration of inflammation, cell death, and tissue injury. That in turn drives further inflammation and dysregulation of the immune system. This cycle will continue to feed on itself, until death or the cycle is broken in some way via treatment (1, 23).

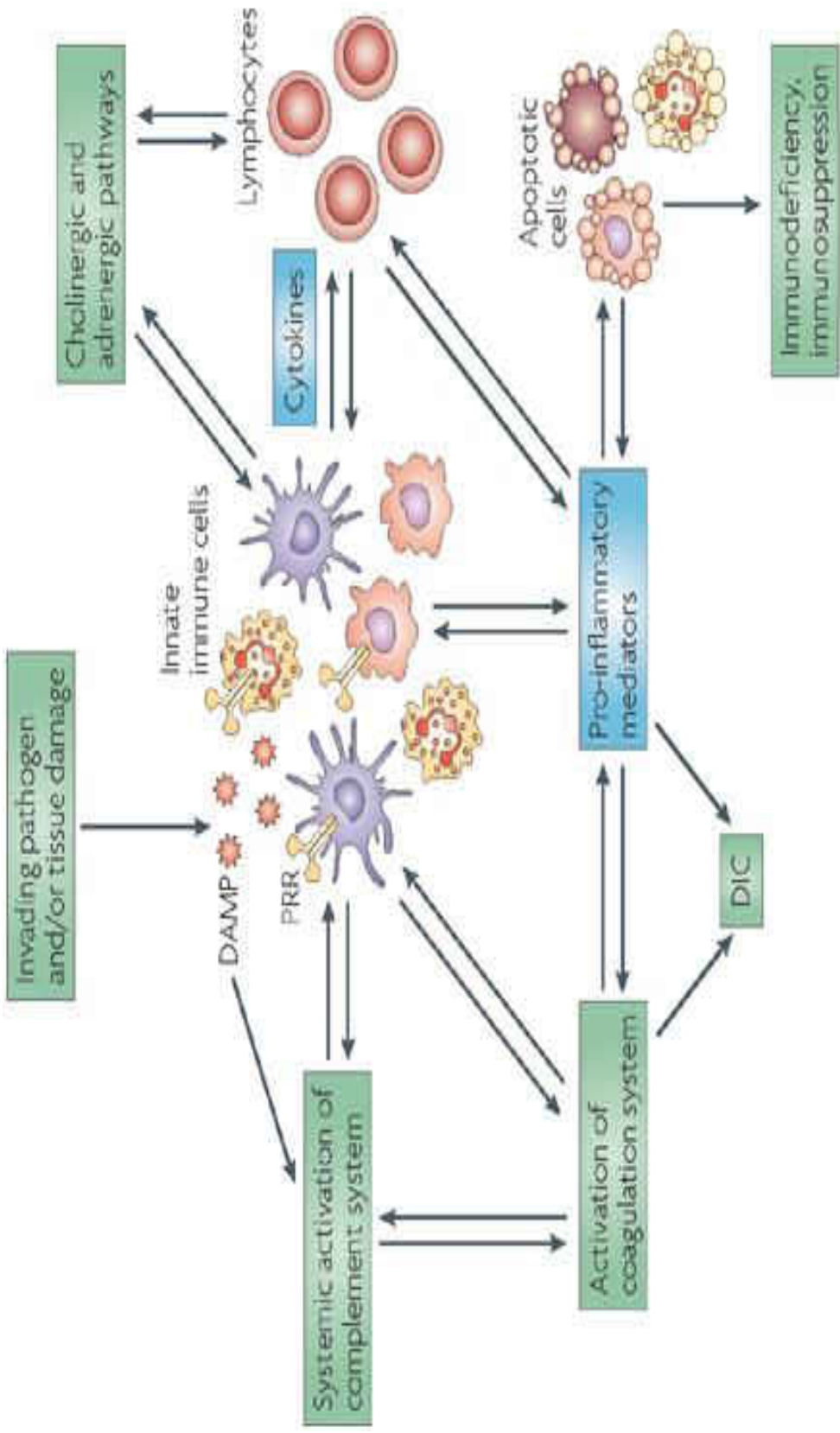


Figure 1. The inflammatory network in sepsis

During sepsis, homeostasis between the various biological systems of the inflammatory network is highly imbalanced. In the initiation of sepsis, the release of a large amount of damage-associated molecular patterns (DAMPs) from invading microorganisms and/or damaged host tissue results in the overstimulation of pattern-recognition receptors (PRRs) on immune cells. Activated immune cells release excessive amounts of pro-inflammatory mediators (resulting in a 'cytokine storm'), free radicals and enzymes, which converts the normally beneficial effects of inflammation into an excessive response that damages the host. Activation of the adrenergic branch of the autonomic nervous system (ANS) and/or decreased activity of the cholinergic anti-inflammatory pathway (of the parasympathetic branch of the ANS) further amplifies the pro-inflammatory responses of neutrophils, macrophages and dendritic cells in sepsis. The presence of invading microorganisms or their products in the blood can cause systemic activation of the complement system, which results in the excessive generation of complement anaphylatoxins, which, at high concentrations, induce numerous harmful effects. Simultaneous activation of the coagulation system and the inhibition of fibrinolysis as a result of the pro-inflammatory environment and/or damaged endothelium can result in disseminated intravascular coagulation (DIC), which is a major complication of sepsis, and in the amplification of the inflammatory response. The complement, coagulation and fibrinolysis systems are tightly connected through direct interactions of serine proteases, and imbalances in each cascade are intensified in a positive-feedback loop.

Figure 1 cont. The inflammatory network in sepsis

Finally, the sustained pro-inflammatory environment affects the functional state of immune effector cells, eventually causing the dysfunction of neutrophils and immunoparalysis. Alterations in leukocyte apoptosis in the later stages of sepsis further account for immunosuppression, which increases the susceptibility to secondary infections. Reproduced with permission from Nature Reviews Immunology. Permission # 4126831477050 (23).

Current treatment guidelines for sepsis dictate that antibiotic therapy be started within one hour of diagnosis and mortality increases by 6% every hour after that. Beyond, early recognition, broad-spectrum antibiotic therapy, and treatment of any symptoms, there are no other treatment options. There have been over 100 Phase II and Phase III clinical trials in the past 40 years and yet no FDA-approved drug for the use in treatment of sepsis has passed clinical trials (24-27). Despite significant efforts, the field is still a long way off from finding a treatment for this complex and deadly disorder. Sepsis, a complex immune disorder, is associated with dysregulation of many immune regulatory processes, which are not completely understood (23, 26-29). The majority of clinical trials have focused on one aspect of what causes sepsis, either the hyperimmune response, dysregulation of the coagulation system, or attempting to stimulate a specific immune function, all have failed. There have been 15 clinical trials utilizing anti-tumor necrosis- α (TNF- α) antibodies, due to the central role played by TNF- α in mediating inflammation. These studies were designed to target and prevent the “cytokine storm or hypercytokinemia” observed in sepsis patients, which is characterized by dysregulation and massive upregulation of both pro-

and anti-inflammatory cytokines (23, 27, 30). All these studies failed because they did not recognize the complexity of the disease, especially given the heterogenous patient population(28, 29). Xigris, an FDA approved drug to treat sepsis, was a recombinant activated protein C (APC) that targeted the disseminated intravascular coagulation (DIC), that caused vascular injury, a contributing factor to sepsis development. Unfortunately, in October 2011, the results of the PROWESS-SHOCK clinical trial were revealed, which failed to show overall efficacy of this drug for APC, and Xigris was pulled from the market (18, 27, 31). All this goes to show that sepsis as an immune disorder is too complex to be treated by targeting only one aspect of the dysfunction. There, there is a critical need to identify additional targets for drug development, which can be used as combination therapies to treat this complex immune disorder, as single molecule therapies have failed.

Inflammatory Mechanisms in Sepsis

Sepsis is characterized as an initial hyperimmune response; therefore, it is essential to understand the innate immune components that play a role in inflammation. The inflammatory response against an invading pathogen is initiated when pathogen associated molecular patterns (PAMPs) on the bacteria are recognized by pattern recognition receptors (PRRs) on tissue resident cells. The recognition of PAMPs by PRRs causes PRR-induced signal transduction pathways to be activated with the end result being the release of chemokines and cytokines, which recruit and stimulate the innate immune cells needed to fight of the infection. The innate immune cells recruited to the site of infection by the released chemokines are phagocytes and antigen-presenting cells (APCs) such as neutrophils, macrophages, and dendritic cells (DCs). Those cells work in conjunction with one another to kill the pathogen intracellularly by phagocytosis or extracellularly by

releasing DNA traps and other noxious cargo, present antigen to activate the adaptive immune response, and clear dead/dying cells via efferocytosis to prevent further inflammation (32-34).

Neutrophils are the first responders for any infection and while circulating they are constantly probing the endothelial cells of the vasculature searching for stimulated endothelial cells, which indicate sites of inflammation (35-38). The neutrophils then cross the endothelial barrier and becomes activated while making its way to the site of infection by following a chemoattractant gradient (39, 40). During the activation process, a number of pattern recognition receptors (PRRs) are activated, which also aids in the activation and induction of oxidative burst, degranulation is also initiated during PRR activation (41-45). This activation process during migration along the chemoattractant gradient allows for the neutrophil to be primed and ready to kill when it reaches the site of infection. Once at the site of infection the neutrophil has a few different antimicrobial weapons at its disposal. Phagocytosis, a receptor mediated process, that leads to the engulfment and internalization of bacteria and cellular debris into a phagosome is main mechanism utilized by neutrophils to clear pathogens (38, 46, 47). After entrapment of the pathogen inside the phagosome, the phagosome must undergo a maturation process in order to kill the pathogen. During that maturation process, granules filled with antimicrobial peptides, that aid in the killing of the pathogen, fuse to the phagosome (48). These granules also contain components of the NADPH oxidase machinery in its membrane, which is required for oxidative burst and the production of reactive oxygen species (ROS) (49). The granules also fuse with the plasma membrane, releasing antimicrobial peptides into the milieu at the site of inflammation as well as bringing necessary components of the NADPH oxidase complex

to produce ROS. This creates an inhospitable environment for the invading pathogen, while also affecting other cells causing the production of neutrophil and monocyte chemoattractant and other immune mediators that can enhance the antimicrobial effect of other immune cells (38, 50-58). Besides killing the pathogen through phagocytosis and the release of granular content and production of ROS inside the phagosome as well as outside the plasma membrane, neutrophils are also able to release extracellular traps or NETs, that are composed of DNA, decondensed chromatin, histones, and granule proteins. These NETs ensnare the pathogen, preventing it from disseminating, and giving time for other immune cells to come along and remove it (59, 60). The exact mechanism of NET formation and whether it is an active form of cell death or something viable neutrophils can do is not completely understood and is currently an active area of research in our lab (38, 46, 61-66). ROS and the NADPH oxidase complex are thought to be essential components of NETosis, though some reports, including ours indicate that it may depend on the stimulus (61, 65-71). Other processes such as the Raf-MEK-ERK pathway and autophagy have been implicated in NET formation (65, 66, 72, 73). The studies on neutrophil functions, including NET formation, have been hampered by the short life span of these cells and a lack of proper cell lines that faithfully reproduce all neutrophil functions. Due to these reasons, even though neutrophils were first described in 1846, the NETs were only discovered in the past two decades, indicating that there is still more to learn about neutrophil function (59, 74).

Another key aspect of any innate immune response leading to a pro-inflammatory response is the role of pattern recognition receptors (PRRs) on host immune cells. These receptors are essential in recognizing pathogen associated molecular patterns (PAMPs) as well as

damage associated molecular patterns (DAMPs) and initiating an inflammatory response. There are thought to be five families of PRRs. Those include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and AIM2-like receptors (ALRs). ALRs, NLRs, and RLRs are classified as unbound intracellular receptors and are found inside the cytoplasm, while TLRs and CLRs are classified as membrane bound receptors (75-78) (Figure 2). As relevant to my work, I will only describe TLR and CLR functions and signaling in the sections below.

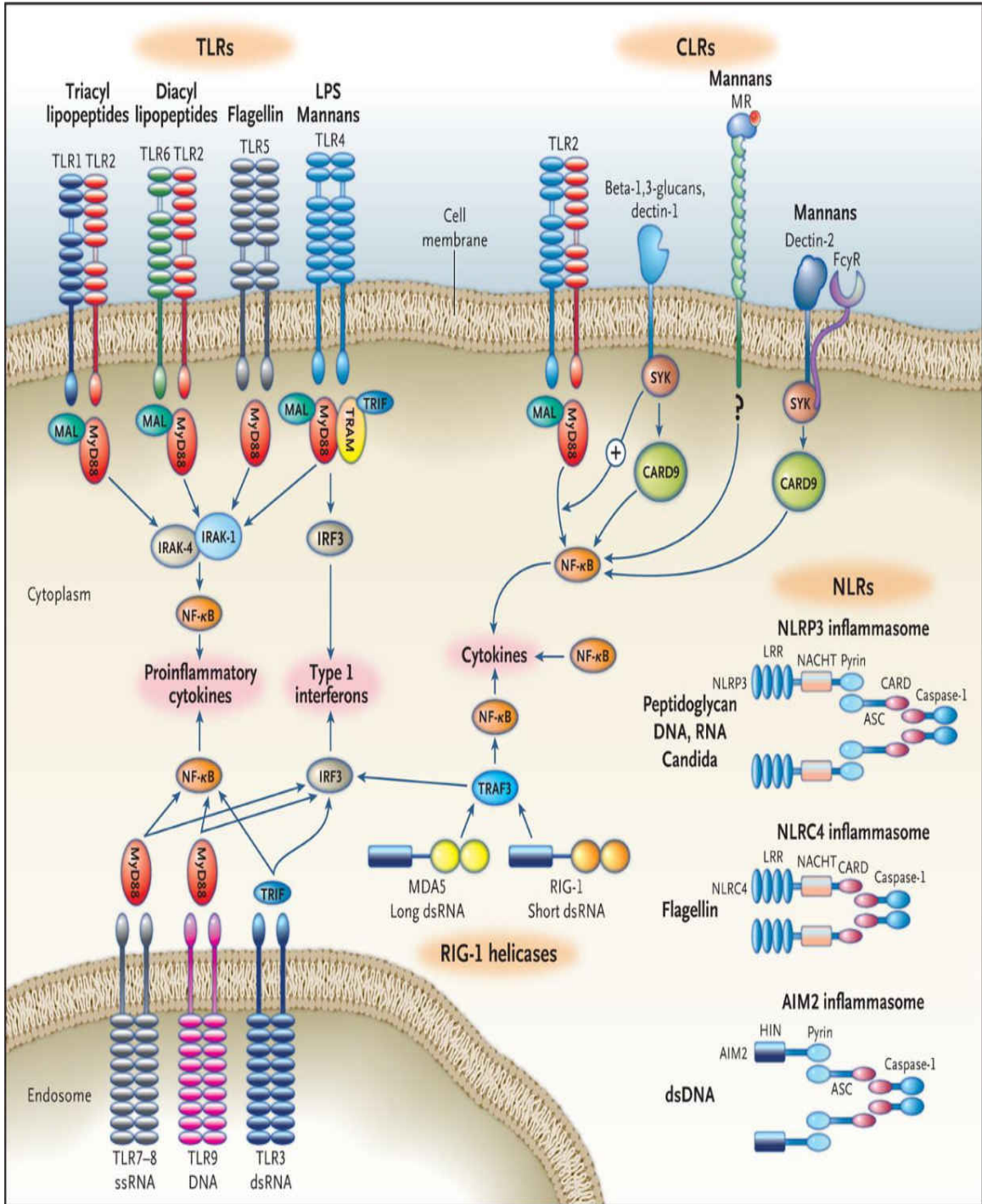


Figure 2. The Four Major Classes of Pattern-Recognition Receptor and Their Most Important Ligands.

The four classes are toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD) leucine-rich-repeat (LRR)-containing receptors (NLRs), and retinoic acid-inducible gene I protein (RIG-I) helicase receptors. NLRs, the central components of the inflammasomes, are complex protein platforms that lead to the activation of caspase 1 and interleukin-1 β processing. The most extensively studied inflammasomes are as follows: the NOD leucine-rich-repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome, activated by bacterial and fungal pathogen-associated molecular patterns; the NLR family caspase recruitment domain-containing protein (CARD) 4 (NLRC4) inflammasome, activated during intracellular bacterial infections by flagellin; and the absent in melanoma 2 (AIM2) inflammasome, activated by double-stranded (ds) DNA. ASC denotes apoptosis-associated speck-like protein containing a CARD, HIN hematopoietic interferon-inducible nuclear protein, IRF3 interferon regulatory factor 3, LPS lipopolysaccharide, MAL myelin and lymphocyte protein, MDA5 melanoma differentiation-associated protein 5, MR mineralocorticoid receptor, MyD88 myeloid differentiation factor 88, NF- κ B nuclear factor- κ B, NLRC4 NLR family CARD-domain-containing protein 4 (also known as IPAF), ss single-stranded, SYK spleen tyrosine kinase, TRAM TRIF-related adaptor molecule, and TRIF toll-like-receptor adaptor molecule. Reproduced with permission from the New England Journal of Medicine, Copyright Massachusetts Medical Society (78).

TLRs are the most well characterized PRR. There are 10 TLRs expressed in humans and 12 in mice. The localization and signal transduction pathway for each has been elucidated, while the major ligand for everyone, except TLR 10, is known (33, 76). TLRs signal through two main signal transduction pathways, the MyD88 dependent pathway and the TRIF dependent pathway. Only TLRs 3 and 4 signal through the TRIF pathway, but TLR4 is unique in that it can also signal through the MyD88 pathway. Signaling through the MyD88 dependent pathway begins with MyD88 forming a complex with IRAK4 and IRAK1. The IRAK complex acts on TRAF6, which in turn polyubiquitinates TAK1 to activate it. TAK1 activation in turn leads to the activation of two pathways, the IKK complex NF- κ B pathway and the MAPK pathway. Those pathways lead to induction of pro-inflammatory gene expression and release of pro-inflammatory cytokines. Activation of the TRIF dependent pathway begins when TRIF interacts with TRAF6 and TRAF3. TRAF6 then recruits RIP-1 kinase in order to activate TAK1 and the NF- κ B and MAPK pathways. TRAF3 on the other hand interacts with TBK1, IKKi, and NEMO to phosphorylate IRF3, thereby activating it. Activated IRF3 forms a dimer and induces the expression of type I INF genes in the nucleus. Activation of either MyD88 or TRIF pathways leads to the activation of transcription factors AP-1, IRF3, and NF- κ B, which causes the release of pro-inflammatory cytokines such as IL-1 β , IL-6, IP-10, INF- γ , and TNF- α (34, 77-80) (Figure 3).

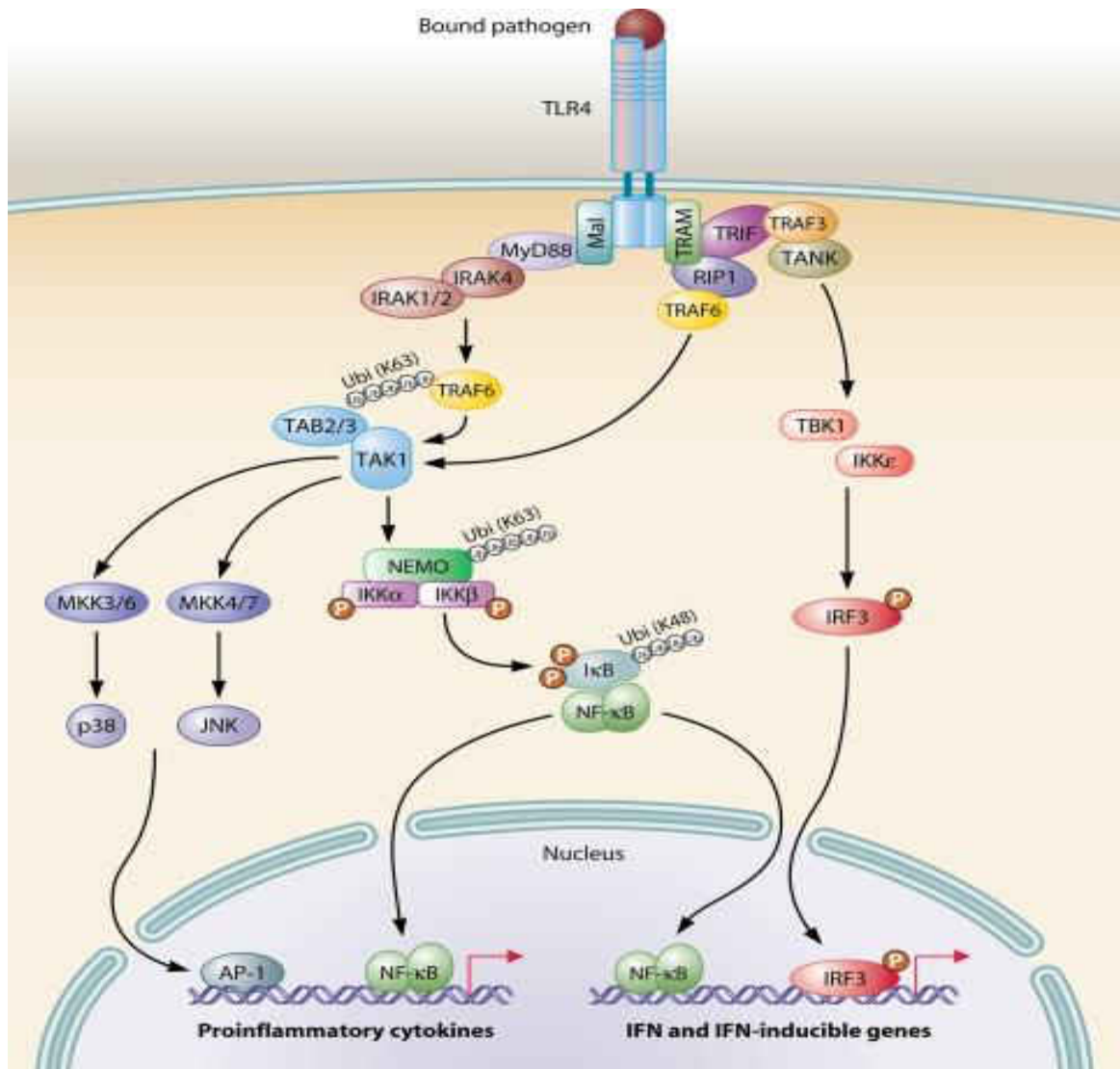


Figure 3. Principles in TLR signaling.

TLR4 activates both the MyD88-dependent and MyD88-independent, TRIF-dependent pathways. The MyD88-dependent pathway is responsible for early-phase NF-κB and MAPK activation, which control the induction of proinflammatory cytokines.

Figure 3 cont. Principles in TLR signaling.

The MyD88-independent, TRIF-dependent pathway activates IRF3, which is required for the induction of IFN- β - and IFN-inducible genes. In addition, this pathway mediates late-phase NF- κ B as well as MAPK activation, also contributing to inflammatory responses. Reproduced with permission from the American Society of Microbiology (34).

The other membrane bound PRR, CLRs belong to a broader family of receptors and have not been as well characterized as TLRs have. There are 17 different groups of CLRs based on the structure of the carbohydrate recognizing domain (CRD) (81). Given the number of different groups of CLRs, it is no surprise that the family includes both secreted and transmembrane proteins. CLRs are most often expressed on myeloid cells and have a wider range of functions than TLRs. CLRs have been shown to play a role in PAMP recognition, phagocytosis, cytokine and chemokine production, antigen presentation, and development of the adaptive immune response (82-85). In addition to pathogen recognition, CLRs have been shown to be receptors for self-ligands and to play a role in maintaining homeostasis. For example, Mannose receptor (MR) has been shown to play a role in the clearance of apoptotic cells via efferocytosis (86, 87). Likewise, soluble CLR, Galectin-3 (Gal3) has been shown to play a role in the clearance of apoptotic neutrophils via efferocytosis (88). Work from our lab showed that galectin-3 and -9 can act as DAMPs to increase inflammatory response during bacterial pneumonia (89, 90). Other CLRs, such as LOX-1, MGL1, and DEC-205, recognize apoptotic cells, but have not been further implicated in homeostatic process that is efferocytosis (91-93). Further, soluble lectins such as Collagenous lectins (Collectins) act as opsonins along with complement proteins in order

to opsonize and take up apoptotic cells (94-97). Beyond just apoptotic cell recognition, CLEC-2, DC-SIGN, and MGL have been implicated in tumor immune surveillance by binding tumor antigens (98-101). The CLR Mincle (Clec4e), is a sensor for SAP-130, an alarmin released by necrotic cell bodies (102, 103). We and others have shown its role in various disease conditions (65, 66, 104-109). Soluble CLRs, Gal3 and Gal9, have both been characterized as alarmins (89, 90).

Given the numerous ways in which CLRs play a role in inflammation and homeostasis, it is important to understand what role CLRs play in sepsis development and neutrophil function. These topics have been researched to some extent with 195 and 430 search results for sepsis and C-type lectins or neutrophils and C-type lectins respectively in PubMed as of June 2017. While those search results indicate that research is being done on this topic, given the sheer number of CLRs it is important to continue to characterize their function in sepsis development. Doing so will not just increase our understanding on CLRs and their function, but highlight new therapeutic targets in the treatment of sepsis.

Resolution of Inflammation: Efferocytosis

A vital part of any infection is the clearance of the infiltrating immune cells that fought the infection in order to achieve a state of homeostasis. Failure to efficiently clear the infiltrating neutrophils at the site of infection can lead to further inflammation. Apoptotic neutrophils can undergo secondary necrosis, causing the release of damage associated molecular patterns (DAMPS) and pathogen associated molecular patterns (PAMPS). The release of those pro-inflammatory markers will help drive further inflammation, contributing to the development of sepsis (110-114). The process of efferocytosis involves

clearing of apoptotic cells by professional phagocytes such as macrophages and immature dendritic cells. Efferocytosis plays a vital role in controlling inflammation, by clearing pathogens not destroyed through phagocytosis (115) in addition to preventing the release of pro-inflammatory mediators from necrotic cells (110). Upon engulfment of dead cells, the efferocytic phagocytes undergo phenotypic changes that leads to the production of TGF-beta and IL-10, anti-inflammatory mediators, which are able to shut down the inflammatory immune response and prevent further cellular influx (112, 116-120). Not surprisingly, defects in the efferocytic process has been associated with development of a variety of inflammatory diseases including atherosclerosis, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) (121). Interestingly, products released by necrotic neutrophils during infection and inflammation inactivate anticoagulants such as activated protein C, which can further drive sepsis development by deregulating the coagulation system, causing vascular injury, and contributing to organ failure and death (122). Given that sepsis is characterized by massive cell death culminating in organ failure, one might speculate that a deregulation of efferocytosis causing inefficient neutrophil turnover might lead to the development of sepsis. A PubMed search for “sepsis and efferocytosis” turned up only 10 articles as of June 2017. Of those 10 articles, 3 are reviews that only speculate on the effect efferocytosis has on the development of sepsis. 3 of the other 7 articles deal directly with the efferocytosis of neutrophils, while the 4 identify different ways to modulate efferocytosis. This clearly highlighted that this field is highly understudied.

Efferocytosis and the resolution of inflammation is a complex and well-orchestrated process. The first step in efferocytosis is the recruitment of the phagocytic cells by the release of "find me" signals by the apoptotic cell, which creates a gradient that leads the

phagocytic cell to migrate to the apoptotic cell. Once the phagocytic cells are in the vicinity of the apoptotic cells, they are able to recognize and bind a number of different "eat me" signals. "Eat me" signal binding to the "eat me" signal receptor activates a signaling pathway leading to Rac activation, which in turn causes to actin polymerization and eventual cytoskeletal rearrangement that is required for internalization and clearance (110, 123-125) (Figure 4).

Find-me signaling	Eat-me signaling	Post-engulfment
<p>Find-me signaling</p>	<p>EAT-ME SIGNALING</p>	<p>Post-engulfment</p>
<p>↑ cAMP, PPARγ ↓ NFκB</p> <p>↑ Ni4a1, Thbs1 ↓ CXCL1, CXCL2, IL1 ? TNF, IL10, TGFF</p> <p>↑ Efferocytosis ↓ Neutrophil influx ? "M2" polarization</p>	<p>↑ Rac ↑ PI3K ↓ NFκB</p> <p>TNF, IL1, IL6, IL33 CCL5</p> <p>↑ PI3K ↑ TLR4, IL4</p> <p>TNF, IL6, CCL5</p> <p>↑ Rac, RhoA</p> <p>↑ STAT1-SOCS TWIST TLR-NFκB, IFNα/β</p> <p>↑ Efferocytosis ↓ Organ-specific inflammation ↓ Cellitis</p>	<p>↑ ABCA1, MFGE8, MERTK, C1q, CD36, GASS, IL10, TGFF ↓ TNF, IL6, NOS2, IL12, IL1, CCR2</p> <p>↑ Efferocytosis ↓ Autoimmunity</p>
<p>↑ Efferocytosis ↓ Neutrophil influx ? "M2" polarization Fibrosis</p>	<p>↑ Rac, RhoA</p> <p>↑ PI3K ↑ TLR4, IL4</p> <p>TNF, IL6, CCL5</p> <p>↑ STAT1-SOCS TWIST TLR-NFκB, IFNα/β</p> <p>↑ Efferocytosis ↓ Auto-immunity Collitis</p>	<p>↑ ABCA1, MFGE8, MERTK, C1q, CD36, GASS, IL10, TGFF ↓ TNF, IL6, NOS2, IL12, IL1, CCR2</p> <p>↑ Efferocytosis ↓ Autoimmunity</p>

Effects on phagocyte signalling pathways

Effects on immune mediators

Immune regulation

Figure 4. Immunomodulatory roles of efferocytosis signals.

Depiction of the tree key stages of efferocytosis (dashed boxes) with detailed illustrations showing some of the known signaling molecules/pathways relevant to immune modulation for each of the three stages. The lower table indicates some of the key effects of these efferocytosis signaling mechanisms on the following: immune signaling in phagocytes, production of immune mediators, and some of the prominent immune outcomes of the indicated molecules/pathways. Arrows in the table indicate whether signaling via these pathways generally increases or decreases the effects listed. Please note this diagram represents only a portion of the efferocytosis signals and their immunoregulatory effects that have been described. MMP, matrix metalloprotease; PS, phosphatidylserine (PtdSer); RTK, receptor tyrosine kinase. Reproduced with permission of The American Association of Immunologist. Copyright 2017. The American Association of Immunologist, Inc. (126).

While the general process of efferocytosis is straight forward, there are numerous factors that play a role, many of those factors are not fully understood or not even identified yet (110, 123). There are a few different "find me" signals, lipids such as lysophosphatidylcholine (LPC), which binds to G-protein-coupled receptor G2A (127) or S1P that too binds G-protein-coupled receptors (123, 128). Other "find me" signals include a classical chemokine Fractalkine (CX3CL1) (129) and nucleotides such as ATP and UTP that bind the P2Y2 receptor on the phagocytic cell (130). Several "eat me" signals, including ICAM3 (110, 123, 131), thrombospondin, compliment C1q, Calreticulin (110, 123), changes in glycosylation (132), and the most well characterized one is phosphatidylserine (PtdSer) (110, 123, 133, 134). Most of the known and characterized

"eat me" signal receptors, such as TIM1&4, BAI-1, Sabilin-2 bind PdtSer (110, 123, 135-140). CD14 binds to the "eat me" signal ICAM3, while CD36 with the help of integrins binds to thrombospondin (123). The signaling mechanisms activated by "eat me" signal binding to receptor leading to apoptotic cell internalization are not well known. There are two semi characterized pathways, both of which lead to the activation of Rac, which is required for actin polymerization and cytoskeletal rearrangement needed for apoptotic cell internalization and clearance (110, 123, 124). The CrkII–Dock180–ELMO pathway is the better characterized pathway of the two. Dock180 and ELMO complex together in order to form an unconventional guanine nucleotide exchange factor complex, needed to activate Rac (Figure 5).

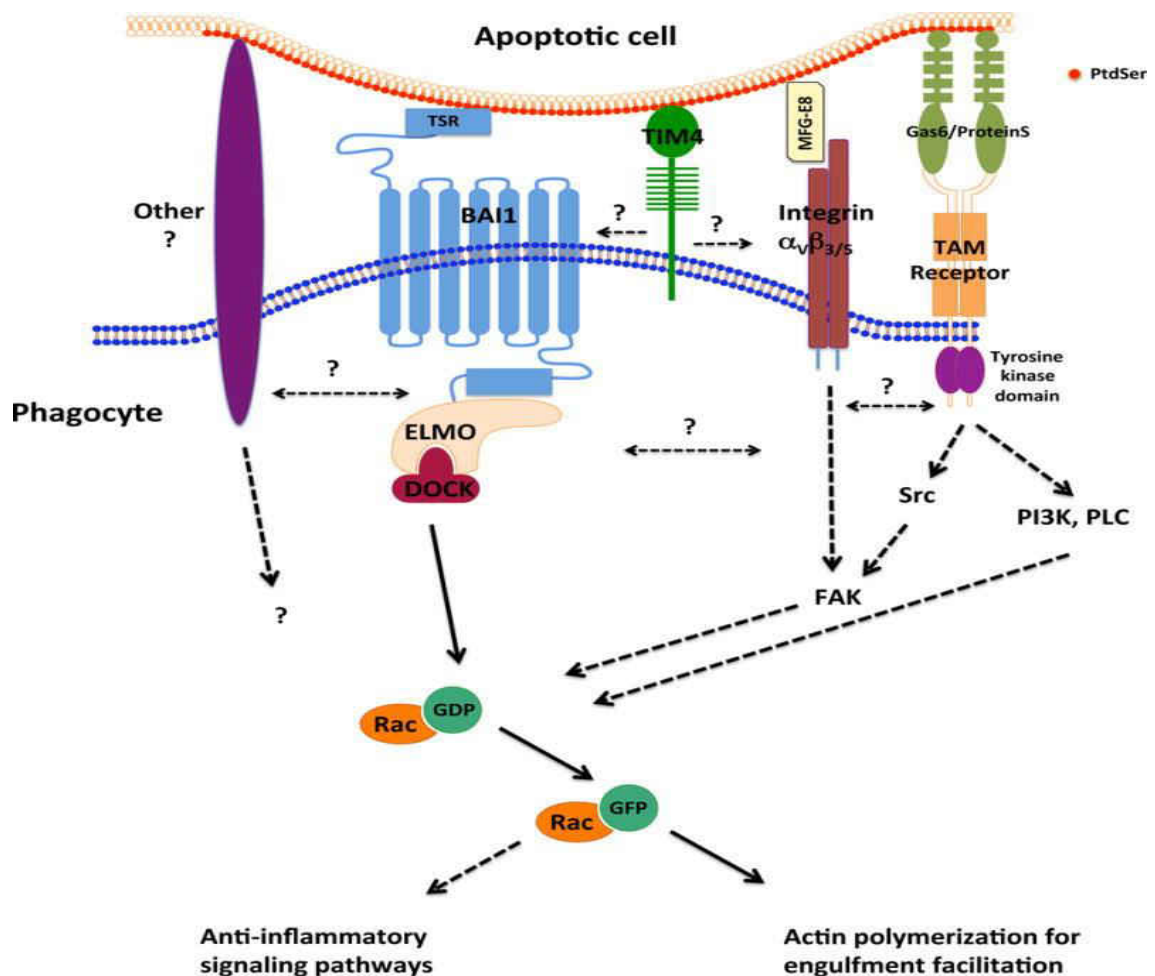


Figure 5. Signaling pathways elicited by three PtdSer recognition receptors.

Binding of the apoptotic cell to the phagocyte triggers signaling pathways. BAI1 is a 7-transmembrane receptor that directly binds the PtdSer on the surface of an apoptotic cell, resulting in the recruitment of the Engulfment and cell motility (ELMO)/Downstream of Crk (DOCK) complex, which functions as a guanine exchange factor for the small GTPase Rac. Rac activation promotes actin cytoskeleton remodeling required for the engulfment of the apoptotic corpse. Integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$ and the Tyro Axl Mer (TAM) family receptors bind apoptotic cells indirectly, via PtdSer-bound bridging molecules MFG-E8, Gas6 or ProteinS, resulting in the activation of the focal adhesion kinase (FAK) and contributing to the activation of Rac. TAM receptors are tyrosine kinases that also activate cell signaling pathways involving the kinases Src and phosphatidylinositol-3-kinase (PI3K) and phospholipase C (PLC). TIM4 functions as a tethering receptor bringing the apoptotic cell in contact with signaling engulfment receptors, and signal through co-receptors. The extent of the connection between the signals elicited by different engulfment receptors awaits further characterization. Reproduced with permission from Nature Reviews Immunology. Permission # 4126831301692 (141).

In order to activate Rac, Dock180 and ELMO, sometimes CrkII, are recruited to the cell membrane upon "eat me" signal ligation to the carboxyterminal of the receptor (110, 123, 140, 142). The second pathway recruits the adapter protein GULP, the molecular components in that pathway are not fully understood (110, 123). Efferocytosis is a complex process with numerous components that could easily become deregulated and disrupt the resolution of inflammation and return to homeostasis, which would drive the development

of chronic inflammation and eventually lead to sepsis. Therefore, it is a promising area of research in regards to understanding the development of sepsis and how a beneficial host response aimed at restoring the body to homeostasis could lead to massive inflammation and tissue destruction if deregulated.

Bacteria Fighting Back

In order to survive inside the host, bacteria have developed numerous ways by which to evade the host immune response. The host immune response is initiated when the pathogen is recognized by various pattern recognition receptors (PRRs) on the innate immune cell as described above. This activates the innate immune response, leading to an inflammatory response in order to kill the invading pathogen. The pathogen can be killed directly via phagocytosis and phagolysosomal fusion or indirectly by being trapped via NETosis or cleared after escaping the phagosome via efferocytosis. Below are just a few cases to highlight the many ways bacteria are able to evade host recognition, engulfment, and killing. One of the first steps in fighting of the infection is recognition of the pathogen and subsequent release of chemokines and cytokines meant to recruit phagocytic cells to the site of infection. Streptococcal species of bacteria produce streptolysin, which is able to suppress neutrophil chemotaxis and even kill phagocytes (143). Other bacteria such as *Treponema pallidum*, the causative agent of syphilis, cloak themselves in host protein in order to evade phagocytosis (144, 145). Once the pathogen is recognized and phagocytic cells recruited to the site of infection the next step in clearing the pathogen is to phagocytosis. Some bacteria such as *Klebsiella pneumoniae* produce capsules that prevent phagocytic engulfment (146-148). While others such as *Pseudomonas aeruginosa* and *Yersinia enterocolitica* utilize type III secretion systems to inject effector molecules into

the host to prevent phagocytosis (149, 150). If the host is unable to phagocytose and kill the bacteria, other host defense weapons are deployed. For example, neutrophils have the ability to produce extracellular traps (NETs) by spewing out their DNA and histones as a way to trap extracellular pathogens, prevent their dissemination, and allow for them to be cleared by other means (151). Several pathogens have devised mechanisms to escape entrapment or killing by NETs. *Staphylococcus aureus* produces a nuclease which is able to degrade NETs and allow *S. aureus* to escape (152). In addition to there, several bacterial pathogens are able to survive inside the phagocyte upon phagocytosis, whether that be in the phagosome, the phagolysosome, or in the cytoplasm. Rickettsiae for example, escapes from the phagosome to survive in the cytoplasm, through the use of bacterial enzyme phospholipase A (153). *Listeria monocytogenes* also uses its own hemolysin enzyme listeriolysin O to escape the phagosome (154). *Mycobacterium tuberculosis* on the other hand produces sulfatides that arrest the phagosome and prevent phagosome-lysosome fusion (155). The bacterium *Salmonella typhimurium* has the ability to adapt to extremely low pH values and survive within phagolysosome (156). Despite the pathogens best efforts to survive inside the phagocyte, the host is still able to clear the pathogen through efferocytosis. As mentioned above *Mycobacterium tuberculosis* is able to arrest the phagosome, evade the host response, replicate inside the cell, and disseminate when the cell become necrotic over time. The host can clear the bacteria via the uptake of infected apoptotic phagocytes by the process of efferocytosis. The *M. tuberculosis* is already compartmentalized inside an arrested phagosome and is unable to prevent lysosomal fusion to the new phagosome, thus killing the bacteria (157). *Pseudomonas aeruginosa* is also cleared through efferocytosis. In this case the bacterium binds to apoptotic cells or

apoptotic bodies and then internalized and killed via efferocytosis (158). Just as with phagocytosis, bacteria have found a way to hijack efferocytosis. *Listeria monocytogenes* can escape the phagosome with its listeriolysin O toxin and then hijack host actin regulation via its ActA protein. From there it is able to escape the host cell in a membrane-derived vesicle covered with exofacial phosphatidylserine (PS). That vesicle is recognized by the PS-binding domain TIM-4 and is then efferocytosed into a new cell, where it can continue to disseminate from cell to cell (159). *S. aureus* on the other hand takes a different approach to exploit efferocytosis. It acts on neutrophils to upregulate the expression of “don’t eat me” signal CD47 on the cell surface in order to prevent the efferocytosis of the infected cell as well as driving the cell away from apoptosis and toward necroptosis (160). This account of host defense strategies and the bacterial evasion mechanisms briefly highlights the tug of war between the host and the pathogen and describes some of the mechanisms by which bacteria are able to survive and cause disease. While several advances have been made on different bacterial strategies used to evade phagocytosis or survive inside the phagocyte (161-164), the same cannot be said about how bacteria manipulate efferocytosis. There are only 83 published articles on efferocytosis and bacteria as of June 2017. Of those, only one study describes the effect bacteria has on neutrophils to inhibit their efferocytosis (160). Bacterial manipulation of efferocytosis is a promising area of research that is only just beginning to be explored. This research could identify new targets for antimicrobial therapies as well as identify novel virulence factors in bacteria. Our work utilizing *Klebsiella pneumoniae* (KPn) to induce pneumonic sepsis in mice allows us to not only look at the role C-type lectin receptors (CLRs) play in pneumonic sepsis; it allows us to

study the pathogenicity of KPn and the virulence mechanisms that it utilizes to subvert host defenses in order to become one of the leading causes of sepsis (12, 17, 18).

Animal Model of Sepsis

Three murine models of sepsis have been described to recapitulate the disease development in humans. The endotoxemia model, uses administration of exogenous toxins such as bacterial lipopolysaccharide (LPS) to induce sepsis like symptoms in mice. It can only mimic some of the pathophysiological alterations seen in sepsis patients, but is utilized owing to its ease and reproducibility. This model shows a rapid increase in systemic cytokine levels over a period of 8 hours, peaking between 1.5 to 4.5 hours post injection (165). Unfortunately, this differs from clinical manifestations of sepsis where there is prolonged systemic cytokine levels that is orders of magnitude lower than what is seen in the endotoxemia model. Also, the therapeutic targets identified using this model have proven ineffective in human clinical trials, strongly suggesting that this model while convenient and reproducible may not be the most relevant model to use in regards to human disease (165-172).

The second model is a breach in the endogenous protective barriers, such as cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP) models. These models were once considered the “gold standard” of animal sepsis models because they were able to reproduce a number of key hallmarks of sepsis in mice, such as hyperinflammation, bacteremia, systemic organ damage, and mortality. However, these models induce polymicrobial sepsis, while relevant to some forms of sepsis is not relevant to hospital-acquired pneumonias, which are usually caused by a single bacterial species. While these

models replicate some aspects of sepsis, they do not provide insight into the host pathogen response mechanisms underlying sepsis (165-173).

The third model is the live bacterial infection model, which includes the pneumosepsis model. This model focuses on one of the leading causes of sepsis, pneumonia, accounting for almost 50% of all cases (2, 12). This model is able to reproduce the hallmarks of sepsis seen in humans such as hyperinflammation, bacteremia, systemic organ damage, and mortality. Importantly, this model uses only a single pathogen, which is more frequently the case in human sepsis, unlike the CLP and CASP models that induce polymicrobial sepsis (12). That allows examination of the host pathogen interaction mechanisms. In our lab, we utilize the pneumoseptic model of *Klebsiella pneumoniae* (KPn) infection. This model focuses on pulmonary bacterial infection, which is a leading cause of sepsis (2, 12, 14, 172). Intranasal delivery of this pathogen in WT (C57/BL6) mice produces major characteristics of sepsis i.e., hyperinflammation, bacteremia, systemic organ damage, and mortality.

Klebsiella pneumoniae (KPn), is a Gram-negative, nonmotile, encapsulated, bacterium that belongs to the Enterobacteriaceae family of bacteria (174-176). Members of that family, including *Klebsiella* species and *E. coli* are the leading cause of sepsis (12, 17, 18). KPn resides all over the environment and readily colonizes human mucosal surfaces (174, 175). Pneumonias and Urinary Tract Infections are the primary infections caused by KPn. This opportunistic pathogen causes both community-acquired pneumonias (CAP) and hospital-acquired pneumonias (HAP), which are the leading cause of mortality among nosocomial pneumonias (176-179). Approximately 12% of all HAPs are due to KPn, while approximately 4% of CAPs are caused by KPn (180-182). Interest in KPn has increased in

recent years due to the emergence of multidrug resistant and hypervirulent strains. This is worrisome given the number of HAPs caused by KPn and how it is more likely that the KPn will be multidrug resistant due to many of those patients having already been treated with antibiotics (177, 183, 184).

KPn has four well characterized virulence factors, which are capsule, lipopolysaccharide (LPS), fimbriae, and siderophores. It uses these virulence factors to evade early host immune responses (176, 185). The capsule is a protective polysaccharide coating that covers the bacterium and is thought to be necessary for virulence. There are 78 serotypes of the K antigen that makes up the capsule, of those 25 make up 70% of all isolated strains, with K1 and K2 being associated with the most virulent strains. K1 and K2 strains are more virulent due to a lack of mannose in their capsules. The lack of mannose means no mannose receptor binding, which inhibits efficient phagocytosis and downstream signaling (146-148, 186-190). The capsule of KPn prevents opsonization and phagocytosis of the bacteria by neutrophils and other phagocytic cells (191, 192). The capsule also protects the bacterium from complement mediated killing as well as other antimicrobial peptides. Early inflammatory response is also suppressed due to the capsule and its ability to inhibit TLR2 and TLR4 signaling. Inhibition of those PRR signaling pathways, removes key aspects of the innate immune response, thereby reducing the early inflammatory response to the pathogen when compared to non-capsulated bacteria (146, 192, 193). It is clear from these examples that the capsule is an essential tool for the bacteria to subvert and suppress the early host immune response.

KPn is a Gram-negative bacterium and as such the endotoxin LPS is a part of its cell membrane. LPS is both detrimental and beneficial to KPn virulence. KPn LPS has 9 O

antigens, with O1 being the most common. Full-length O antigen on LPS prevents complement mediated killing by hiding complement activators on the bacterial cell surface (192, 194-196). The lipid A portion of LPS is recognized by the pattern recognition receptor (PRR) TLR4, which leads to the production of cytokines and chemokines as well as cellular recruitment (197). Some KPn strains with particular K antigens are able to mask their LPS inside the capsules, which dampens TLR4 signaling (196). KPn also modulates its LPS, like other bacteria, such as *Yersinia pestis*, so that it is no longer recognized by TLR4 (198, 199). Interestingly, Lipid A along with the core polysaccharide of LPS can resist phagocytosis in the lungs by alveolar macrophages (200). LPS is an endotoxin and activates the innate immune system through TLR4 to recruit cells via the release of chemokines and cytokines. It also plays a protective role on the bacterial surface by preventing phagocytosis and complement mediated killing.

KPn has both type 1 and type 3 fimbriae as virulence factors. These fimbriae are important for adhesion and biofilm formation. Type 1 fimbriae are thin, thread like protrusions on the cell surface and are expressed biased on environment. They are expressed while the bacteria is in the urinary tract, but not the GI tract or lungs (201, 202). Type 3 fimbriae on the other hand are helix like filaments, but similar to type 1 fimbriae are not needed for GI track infection or virulence in the lungs. Type 3 fimbriae can bind tracheal cells and therefore assist KPn in the colonization of the lungs (203-205).

Siderophores are used to acquire iron from the host and are essential for bacterial survival and virulence. They are secreted and have a higher affinity for iron than host transport proteins. KPn has a number of different siderophores, such as enterobactin, yersiniabactin, salmochelin, and aerobactin. More common KPn strains usually have only enterobactin as

its siderophore, while more hypervirulent strains also utilize the siderophores yersiniabactin, salmochelin, and aerobactin (206-210). Other possible virulence factors in KPn have been identified, but have yet to be fully characterized (176).

KPn virulence factors described above help the bacteria subvert and suppress the early inflammatory host response, allowing the bacteria to establish an infection and survive. These virulence factors have been well characterized and the ways in which they aid in KPn pathogenesis are known. With the emergence of multidrug resistant and hypervirulent strains of KPn over the past couple of decades, it is essential to identify new virulence factors and pathogenic mechanisms that can be targeted for new therapies against KPn infection.

Outline of dissertation

Sepsis is a complex immune disorder, the roles in which CLR and bacterial pathogenesis play in the development of sepsis is understudied. The aim of this dissertation is to study the role two particular CLR, Macrophage Galactose Type Lectin-1 (MGL1) and Mincle (Clec4e), play in pneumonic sepsis as well as identify novel pathogenic strategies used by KPn, a causative agent of pneumonic sepsis, to establish an infection in the host. In the following chapters, we show that CLR play an important protective role in KPn induced pneumonic sepsis by regulating neutrophil turnover and that the pathogen employs a strategy to help overcome host defense mechanisms to successfully establish infection.

In Chapter 2 the role of MGL1 in pneumonic sepsis will be described. MGL1 has not been characterized in bacterial infections and disease pathogenesis to date. It is known to be expressed on macrophages and immature dendritic cells and play a role in recognition of

tumor antigens as well as apoptotic cells (100, 211, 212). Here we show that MGL1 is protective in KPn induced pneumonic sepsis and that protection is due to its role in neutrophil migration and turnover. Chapter 3 will focus on the protective role of Mincle during KPn induced pneumonic sepsis. Mincle is an activating receptor for damage-associated and pathogen-associated molecular patterns, called DAMPs and PAMPs and can be expressed in neutrophils, macrophages, and dendritic cells. Most of the functional analysis of the receptor has been done in macrophages. Its role in chronic bacterial infections has been studied, but how it functions in acute pneumonic infections is not known (102, 109, 213-215). We show that in pneumonic infections leading to sepsis, Mincle plays a protective role via bacterial clearance mechanisms phagocytosis and NETosis in neutrophils.

Chapter 4 transitions from the host response in pneumonic infection to bacterial pathogenic mechanisms used to establish infection. KPn, a causative agent of sepsis, has virulence factors that help it evade and suppress early host immune response (146, 191-196). Here we show a novel mechanism by which KPn modulates the route of cell death in infected neutrophils to inhibit efferocytosis of those infected cells, indicating another pathogenic mechanism KPn utilizes to evade the host response. Chapter 5 contains a summary along with a discussion of these new findings and conclusions in this dissertation.

CHAPTER 2

MACROPHAGE GALACTOSE-TYPE LECTIN-1 (MGL1) DEFICIENT IS ASSOCIATED WITH INCREASED NEUTROPHILIA AND HYPERINFLAMMATION IN GRAM- NEGATIVE PNEUMONIA

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Abstract

C-type lectin receptors (CLRs), the carbohydrate recognizing molecules, orchestrate host immune response in homeostasis and in inflammation. In the present study we examined the function of macrophage galactose-type lectin-1 (MGL1), a mammalian CLR, in pneumonic sepsis, a deadly immune disorder frequently associated with a non-resolving hyperinflammation. In a murine model of pneumonic sepsis using pulmonary infection with *Klebsiella pneumoniae* (KPn), the expression of MGL1 was upregulated in the lungs of KPn infected mice and the deficiency of this CLR in MGL1^{-/-} mice resulted in significantly increased mortality to infection than the MGL1-sufficient wild-type mice, despite a similar bacterial burden. The phagocytic cells from MGL1^{-/-} mice did not exhibit any defects in bacterial uptake and intracellular killing and were fully competent in neutrophil extracellular trap formation, a recently identified extracellular killing modality of neutrophils. Instead, the increased susceptibility of MGL1^{-/-} mice seemed to correlate with severe lung pathology, indicating that MGL1 is required for resolution of pulmonary inflammation. Indeed, the MGL1^{-/-} mice exhibited a hyperinflammatory response, massive pulmonary neutrophilia and increase in neutrophil-associated immune mediators. Concomitantly, MGL1 deficient neutrophils exhibited an increased influx in pneumonic lungs of KPn infected mice. Together these results show a previously undetermined role of MGL1 in controlling neutrophilia during pneumonic infection thus playing an important role in resolution of inflammation. This is the first report depicting a protective function of MGL1 in an acute pneumonic bacterial infection.

Introduction

Pattern recognition receptors (PRRs) activate innate immune responses upon detection of conserved pathogen motifs as well as self-molecules released during a pathological insult. Among the PRRs, the Toll-like receptor (TLR) family is the most characterized group of receptors known to be involved in activation and maturation of innate immune cells such as dendritic cells and macrophages, among others (216). Another family of PRRs consists of transmembrane and soluble C-type lectin receptors (CLRs), which have selective affinity for self and non-self glycan motifs in a Ca^{2+} -dependent manner (217). Due to their abundant expression on sentinel cells of innate immune system that contribute to inflammation and maintenance of immune homeostasis, CLRs act as key sensors of tissue integrity and pathological insult and play a central role in orchestrating immune responses (218).

Macrophage galactose-type C lectin 1 (MGL1) is a type 2 transmembrane CLR expressed on macrophages and immature dendritic cells with a binding specificity for galactose and/or its monosaccharide derivative, that are abundantly expressed on tumor antigen MUC1 as well as self-antigens such as gangliosides (211). Not surprisingly, MGL1 is included in the family of scavenger receptors along with macrophage mannose receptor (212) that plays a role in recognition of tumor antigens and apoptotic cells (93, 100). Although this CLR can bind antigens from *Neisseria gonorrhoeae* (219), *Campylobacter jejuni* (220) and *Bordetella pertussis* (221), its role in overall disease pathogenesis is unknown. An anti-inflammatory function of this CLR in colitis via its interaction with commensal bacteria has been reported (222). However, the current knowledge of MGL1

function in infectious diseases, particularly the pathogenesis of pneumonia and sepsis is virtually non-existent.

Sepsis is a deadly disorder characterized by a systemic hyperinflammation resulting usually from an infectious insult. An estimated 20-50% of the 750,000 sepsis and severe sepsis patients die in the US owing to the lack of effective treatment strategies (2). Infections of the lung and the respiratory tract are the main causes of severe sepsis, and 41% of these infections are due to Gram-negative bacteria (223-225). Nosocomial infections with *Klebsiella pneumoniae* (KPn) account for 5-20% of Gram-negative sepsis cases (223, 225). KPn-induced lung infection is a clinically relevant animal model of sepsis and a better understanding of this model may help to increase the knowledge about sepsis pathophysiology (169). Additionally, emergence of multidrug resistant isolates of KPn in clinical settings is a serious health concern. In this scenario an understanding of the functioning of host innate immune components that influence the outcome of KPn pneumonia might provide targets for modulation of host immune system in a beneficial manner.

In this study we examined if MGL1 plays a role in orchestrating host defense against KPn pneumosepsis. Indeed, our results suggest that MGL1-mediated responses are required for the resolution of pneumonia and an increased susceptibility of MGL1^{-/-} mice correlates with the increased inflammatory response and with massive accumulation of neutrophils in the lungs of infected mice, despite similar bacterial burden. These results identify for the first time, a protective role of MGL1 in regulation of neutrophil influx and inflammatory response against pneumonic KPn sepsis.

Materials and Methods

Bacterial strains and Mice

The KPn (ATCC strain 43816) were grown to log phase in LB medium at 37°C. All in-vivo experiments were performed using 6-8 weeks old female wild-type C57BL/6 (WT) or MGL1^{-/-} mice on same background obtained from JAX mice (Jackson Laboratory), and bred in the animal facility of the University of North Dakota. The animals were used according to institutional and federal guidelines.

Infection of Mice, survival and bacterial burden

Mice were anaesthetized with a mixture of 30mg/ml ketamine and 4 mg/ml xylazine in PBS and were infected intranasally with 3.0×10^4 CFUs in 20ul of saline, of KPn or with 20 µl of saline alone. Survival of the mice was recorded for up to 2 weeks post-infection (p.i.). Death was recorded as infection induced mortality. Mice displaying severe signs of distress (labored breathing, non-responsiveness to cage tapping, failure of grooming and feeding) were humanely sacrificed and also recorded as infection induced mortality. In some experiments, the mice were euthanized at indicated times p.i. and blood, lungs and liver were aseptically homogenized in cold PBS with Complete™ protease inhibitor cocktail (Roche Diagnostics, Germany). For the bacterial burden analyses, serially diluted homogenates and blood were plated on LB agar and incubated at 37°C overnight.

Quantitative real-time PCR

Total RNA from lungs of infected and mock control mice harvested at various times p.i. was extracted using Trizol reagent (Invitrogen) according to the manufacturers' instructions. Real-time PCR analysis was performed using SYBR green (Applied Biosystems, CA, USA) to measure the MGL1-specific mRNA by using specific primers

(sense) 5'- 5'-TCTCTGAAAGTGGATGTGGAGG-3' and (anti-sense) 5'-CACTACCCAGCTCAAACACAATCC-3' as described by us (226). The target gene expression levels were normalized to levels of the house keeping 18S gene in the same sample. Expression of MGL1 in infected samples was determined as fold change over that in control samples as calculated by using the formula $2^{-(\Delta\Delta Ct)}$.

Multi-analyte profile analysis

The lung homogenates were prepared as described for the bacterial burden analysis above and were centrifuged at 2000 x g for 15 min to clear cellular debris. The supernatants were immediately frozen at -80° C. The biomarker levels in lung homogenates were determined commercially by Myriad Rules-based Medicine (Austin, TX, USA) utilizing a multiplexed analysis.

Histological and Immunofluorescence analysis

For histological analysis, frozen lung tissues were processed as previously described (227, 228). Serial horizontal sections (10 µm thick) of frozen lung tissues thus obtained were stained with hematoxylin and eosin for pathological analysis as previously described (229, 230) . For Immunofluorescence staining, the frozen lung tissue sections were stained for the detection of MGL1, using an affinity purified anti-mMGL1/CD301 goat IgG (R&D Systems, Minneapolis, MN) and visualized with Alexa Fluor® 546 donkey anti-goat IgG (Life Technologies, Grand Island, NY). The images were acquired using a Nikon eclipse 80i upright microscope (Nikon Corporation, Tokyo, Japan) with an attached cooled RTke Spot 7.3 three spot color camera (Diagnostic Instruments Inc., Sterling Heights, MI). The images were processed and analyzed using Adobe Photoshop 7.0 software (Adobe, Mountain View, CA).

Flow Cytometry

Lungs cells were harvested from mice at 3 days p.i. and processed as previously described by us (227, 228, 231). Cells types in the lungs were quantified by staining with Pacific Blue™ anti-mouse CD11b (Clone M1/70), APC-Cy7 anti-mouse CD11c (Clone N418), FITC anti-mouse CD19 (Clone 6D5), Brilliant Violet 570 anti-mouse CD3 (Clone 17A2), APC anti-mouse Ly6G (Clone 1A8), PerCP/Cyc5.5 anti-mouse Ly6C (Clone HK1.4), PE-Cy7 anti-mouse F4-80 (Clone BM8) antibodies (Biolegend, San Diego, CA), and PE anti-mouse TCR β (Clone H57-597) antibody (Becton Dickinson Pharmingen, San Jose, CA). Enumeration of neutrophils by flow cytometry (using a BD LSR II, Becton Dickinson, San Jose, CA) was done by quantitating Ly6G+CD11b+ cells stained with Pacific Blue™ anti-mouse CD11b (Clone M1/70) and APC anti-mouse Ly6G (Clone 1A8) antibodies (Biolegend, San Diego, CA). FlowJo (Tree Star) software was used to analyze all data.

Bacterial phagocytosis and killing by neutrophils and macrophages

Bacterial phagocytosis of WT and MGL1^{-/-} neutrophils and macrophages was assessed by plating on LB media, incubating at 37°C overnight, and counting colonies. For this, peritoneal neutrophils from C57BL/6 and MGL1^{-/-} mice were isolated using an established method of thioglycollate-induced peritonitis. Sterile 4% thioglycollate was injected in peritoneal cavity of mice and neutrophils enriched 16-18h following the injection were isolated. Five x10⁵ Neutrophils were incubated with KPn (MOI 50) for 1 hour to determine bacteria uptake and 3 hours to determine bacteria killing. After 1h the neutrophils were washed two times with warm PBS and one time with RPMI before adding 2μg/mL gentamicin in RPMI (Gibco) with 10% FBS for the remaining 2h (for the 3h samples) and for 15 minute for the 1h samples. The cells were washed extensively with warm PBS before

lysing with 0.1% TritonX100. Serial dilutions of the lysates were plated on LB media overnight at 37°C before colonies were counted.

Bone marrow was isolated from wild-type and MGL1^{-/-} mice and the cells were differentiated to macrophages as previously described (232). On day 6 of culture, 3x10⁵ macrophages were plated in 24-well flat bottom plates and were infected with KPn (MOI 50) for 1 hour to determine bacteria uptake and 3 hours to determine bacteria killing as described above.

Neutrophil Extracellular Traps

For detection of neutrophil NETs in-vivo, the bronchoalveolar lavage (BAL) was performed in WT and MGL1^{-/-} mice at 3dp.i. (66). The lavage cells were cytocentrifuged on glass slides and were stained with Sytox Green (Molecular Probe, Eugene, OR) to visualize NETs. The percent NET formation was quantitated by dividing the number of NET-forming neutrophils by total number of cells in 8-10 random microscopic fields and multiplying the values by 100. The experiment was repeated 3 times.

Neutrophil Adoptive Transfer

Adoptive transfer of bone marrow neutrophils was performed by methods previously described with modifications (233-235). Briefly, bone marrow neutrophils were isolated from WT and MGL1^{-/-} mice by gradient separation. Purified neutrophils (~80% purity determined by flow cytometry) were labeled with CellTracker Orange CMTMR (Life technologies) or CellTracker Green CMFDA (Life technologies) at 37°C for 10 minutes. Labeled cells were mixed in 1:1 ratio using 2x10⁶ each group and then injected intravenously via tail vein into WT and MGL1^{-/-} mice that had been infected with 3.0 x 10⁴ CFUs of KPn intranasally 24h prior. Lungs were harvested 2h after the tail vein injections

and processed for flow cytometry as described above. Adoptively transferred neutrophils recruited to the lungs were enumerated using LSR II flow cytometer and analyzed using FlowJo software (BD Biosciences, San Jose, CA). Relative recruitment of WT and MGL1^{-/-} neutrophils was calculated as the ratio of indicated populations.

In-vivo Efferocytosis

Peritoneal neutrophils from WT and MGL1^{-/-} mice were isolated and labelled with Carboxyfluorescein succinimidyl ester (CFSE; Cell Trace™ CFSE Cell Proliferation Kit from Invitrogen) per manufacturer's instructions. CFSE-labelled WT and MGL1^{-/-} neutrophils were suspended in complete RPMI and 35uL/3.5x10⁶ cells were administered intranasally into mice anaesthetized using a mixture of 30mg/ml ketamine and 4 mg/ml xylazine in PBS. Lungs were lavaged as described by us (65, 236) 2 hrs after the instillation and alveolar macrophages analyzed by flow cytometry using PE-Cy7 conjugated anti-F4/80 and APC conjugated anti-Ly6G antibodies (BioLegend, San Diego, CA). Efferocytic uptake by macrophages was quantitated by flow cytometry to enumerate Ly6G-F4/80+ CFSE+ cells. All other statistical analyses were performed using the Student t test (SIGMA PLOT 8.0, Systat Software, San Jose, CA).

Statistical Analysis

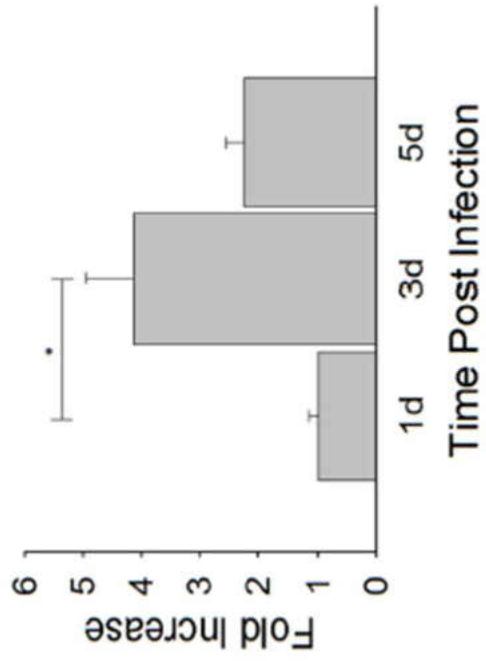
Statistical analysis of survival studies was performed by Kaplan Meir log-rank test; bacterial burdens by non-parametric Mann-Whitney Test. All other statistical analyses were performed using the Student t test (SIGMA PLOT 8.0, Systat Software, San Jose, CA).

Results

MGL1 expression in mouse lungs during pneumonic KPn infection

To examine the role of MGL1 in KPn induced pneumonic sepsis, we first determined its transcript-level expression by real-time quantitative PCR using RNA from lungs of KPn-infected wild-type (WT) mice. The results showed that MGL1 mRNA was maximally transcribed by 3dp.i. (Fig. 6A), a time when infiltration of immune cells is peaked during infection (66, 230). To examine the expression of MGL1 at the protein level, in-situ IF microscopy was performed on lung cryosections of mock control and KPn infected WT mice at indicated post-infection times. In uninfected mock controls, the expression of MGL1 was at low basal level (Fig. 6B). Upon infection, this CLR was found to be abundantly expressed at 3dp.i., consistent with the high transcript level in lungs at that time (Fig. 6B). Interestingly, in addition to some infiltrating immune cells, MGL1 appeared to be expressed prominently by endothelial and alveolar epithelial cells in the lungs of these mice. This infection induced upregulation in the expression of MGL1 in pneumonic lungs during KPn infection indicated that this CLR likely plays a role in the pathogenesis of KPn pneumonia.

A



B

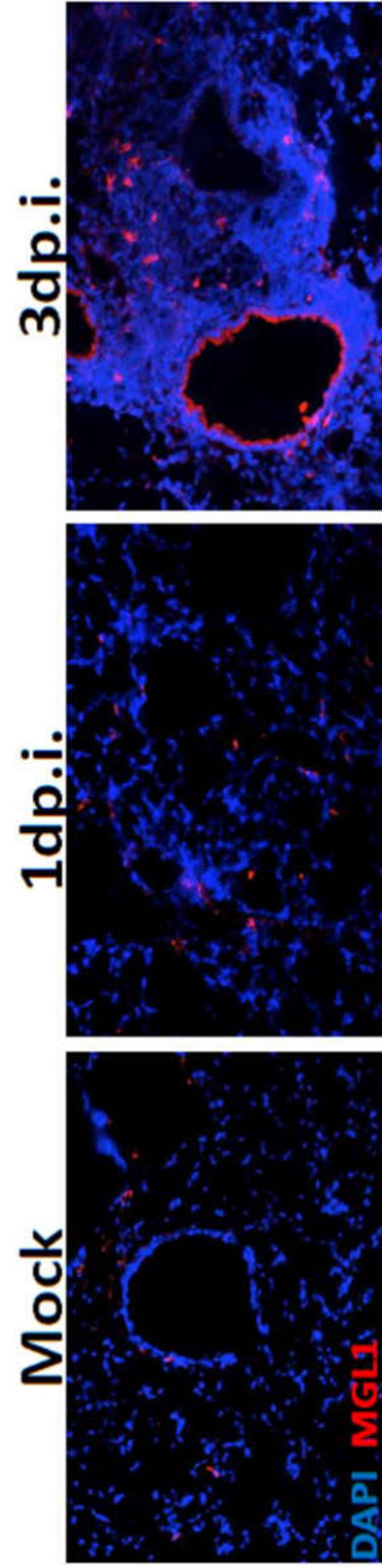


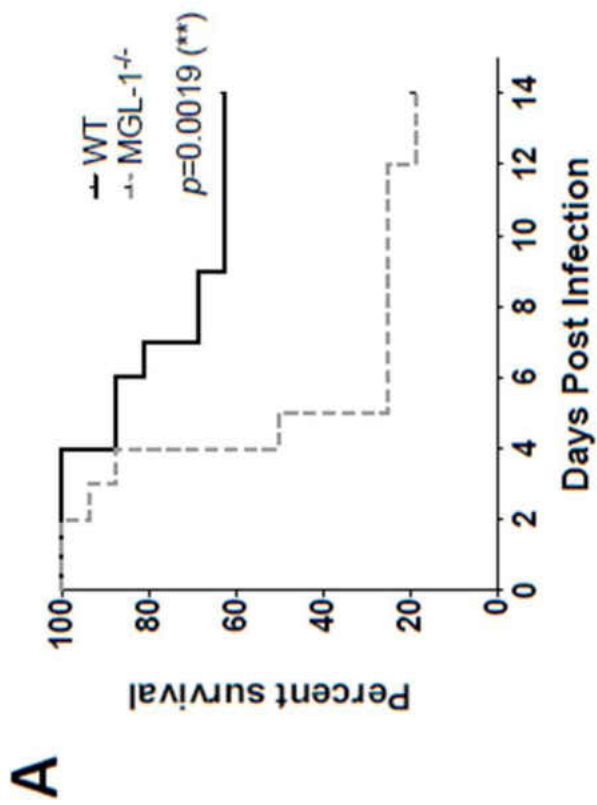
Figure 6. MGL1 is upregulated in the lungs of *Klebsiella pneumoniae* (KPn) infected pneumonic mice

(A) Total RNA was extracted by Trizol method from the lungs of KPn infected wild-type (WT) C57/BL6 mice, harvested at indicated times post-infection. The mRNA levels of MGL1 were analyzed by real-time PCR as described in Methods and are expressed as fold changes over the levels in mock control mice calculated by using the formula $2^{-(\Delta\Delta Ct)}$. Data shown are the mean \pm SEM of 3-4 mice per time point in two independent experiments. Significant differences were measured by Student's *t* test ($p < 0.05$) (B) MGL1 expression was examined by immunofluorescence staining on lung cryosections of mock control and KPn infected wild-type mice harvested at indicated times post-infection using an affinity purified anti-mouse MGL1 goat IgG followed by Alexa Fluor® 546 labelled (red) donkey anti-goat IgG. Nuclei (blue) were stained with 4',6'-diamidino-2-phenylindole dilactate. Images shown are representative of 3 independent experiments with 3-4 mice each.

Increased susceptibility of MGL1^{-/-} mice to KPn pneumonia

To examine the role of MGL1 in disease development, overall disease severity and survival was compared in wild-type (WT) and MGL1^{-/-} mice infected with KPn. As the WT mice are also susceptible to KPn infection, in order to dissect the role of MGL1, we experimentally standardized a sublethal dose of KPn at which 60-70% of the WT mice resolve the infection and display only transient signs of disease (ruffled fur, lethargy) early during infection. We found that MGL1^{-/-} mice were significantly more susceptible to this dose and majority of mice succumbed to infection by day 5p.i. (Fig. 7A). These mice exhibited progressive development of disease and overt signs of infection (weight loss,

piloerection, hunched gait, lethargy, increased respiratory rate). The increased susceptibility of MGL1^{-/-} mice clearly indicated a protective role played by this CLR during pneumonic KPn infection. To examine the impact of MGL1 deficiency on the host antibacterial defense mechanism, comparison of local and systemic bacterial burden in the organs of infected WT and MGL1^{-/-} mice was performed. Intriguingly, there were no statistically significant differences in the bacterial loads in lungs, liver or in blood of WT and MGL1^{-/-} mice, although the MGL1^{-/-} mice tended to exhibit higher burdens at later times post-infection (Fig. 7B). Based on this data, the protective function of MGL1 did not appear to correlate with reduced bacterial burden or systemic dissemination.



B

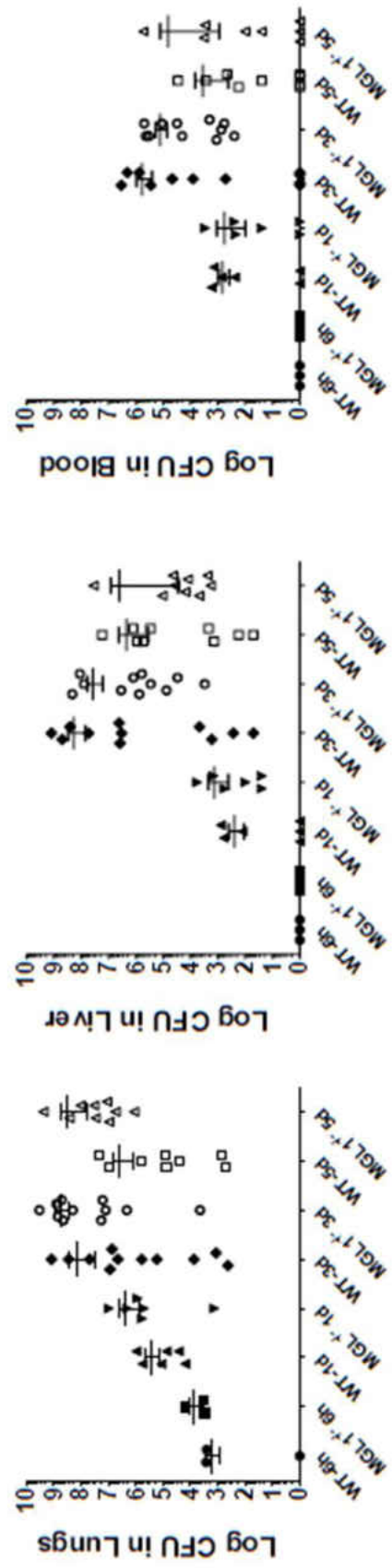


Figure 7. MGL1^{-/-} mice exhibit reduced survival despite similar bacterial burden as the WT mice

(A) WT and MGL1^{-/-} mice were intranasally infected with 3.0x10⁴ CFUs of KPn in 20μl of sterile PBS and were assessed daily for disease severity. The survival was monitored for two weeks. Statistical comparison of susceptibility was done by Kaplan-Meier survival curve statistical analysis (p=0.0019 **). The data shown is from 3 independent experiments (n=16). (B) WT and MGL1^{-/-} were intranasally infected with KPn. At indicated times post infection the mice were sacrificed, systemic organs were isolated, homogenized and plated as described in Materials and Methods. Bacterial burden was enumerated after incubating the plates overnight at 37°C. No significant differences in bacterial burden (using non-parametric Mann-Whitney test) in WT and MGL1^{-/-} were found. Each symbol in the scatter plots represents individual mouse and the data is from 3-4 independent experiments.

Role of MGL1 in phagocyte function

We next examined the direct effect of MGL1 deficiency on the bacterial uptake and killing capability of phagocytic cells i.e. neutrophils and macrophages. As shown in Fig. 8A and B, WT and MGL1^{-/-} neutrophils and macrophages were equally competent in phagocytosis and clearance of KPn. Studies from our and other laboratories have shown that KPn infection induces Neutrophil Extracellular Trap (NET) formation in-vivo in lungs of mice and that NETs can effectively inhibit the growth of KPn (66, 237). We thus compared the extent of NET formation in-vivo in the lungs of KPn infected WT and MGL1^{-/-} mice. As shown in Fig. 8C, neutrophils isolated from BAL of infected MGL1^{-/-} mice did not exhibit

any defect in extracellular trap formation. Together with our observation of similar bacterial burden in WT and *MGL1*^{-/-} mice in-vivo, these data indicated that protective function of MGL1 was likely independent of the bacterial clearance mechanisms during pneumonic KPn infection.

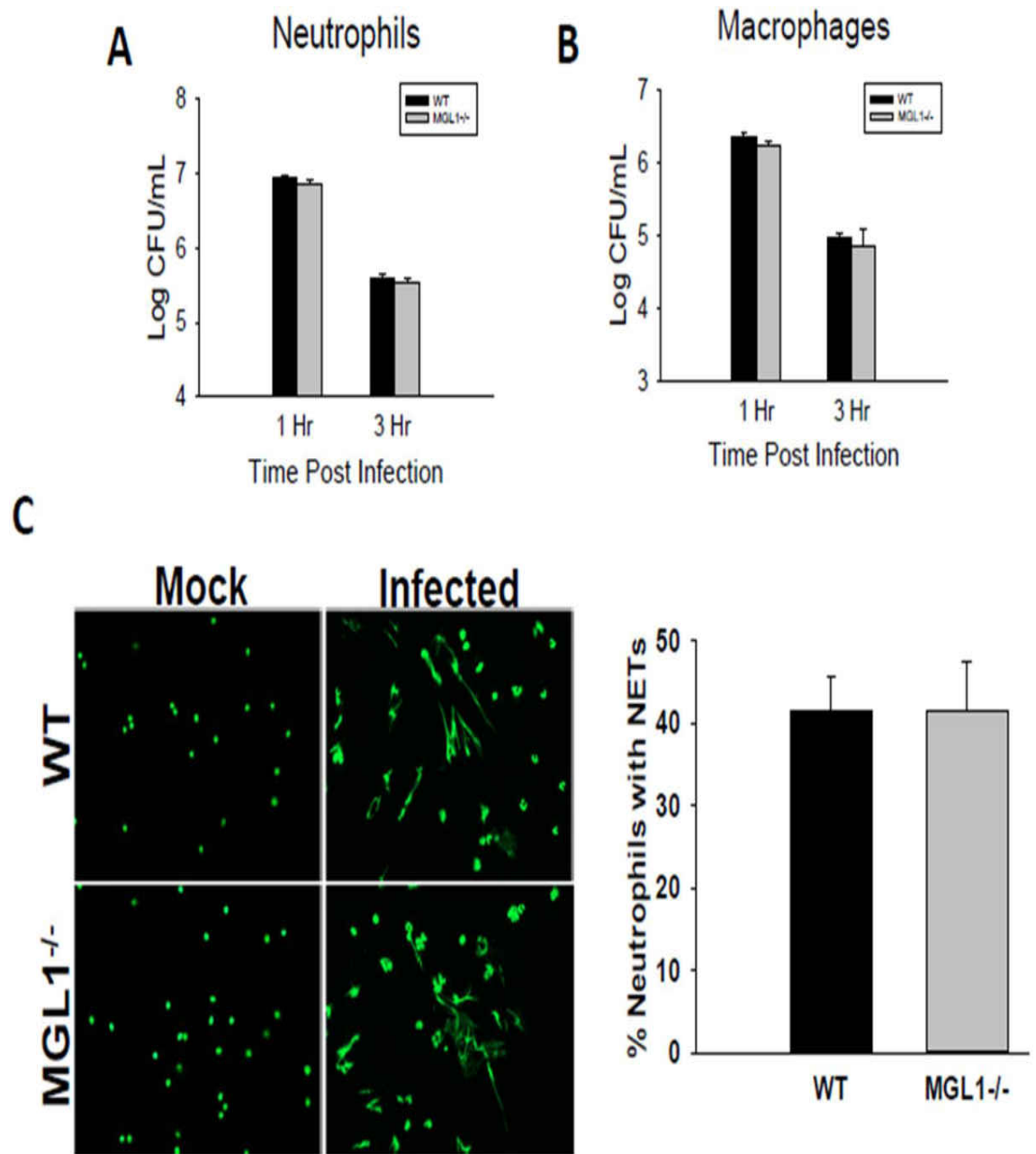


Figure 8. MGL1 deficiency does not impair bacterial clearance by phagocytes

Bacterial uptake and killing capacity of MGL1^{-/-} and WT neutrophils (A) or bone-marrow derived macrophages (B) was determined at 1h and 3h by assessing intracellular CFUs in these cells as described in Methods. Data from a representative of 3 independent experiments is shown. (C) Neutrophils isolated from bronchoalveolar lavage fluid (BAL) of WT (upper panel) and MGL1^{-/-} (lower panel) mice infected with KPn, were cytocentrifuged and stained with Sytox Green to visualize extracellular traps (NETs). Magnification 200X. Bar graph shows quantitation of NET forming neutrophils in BAL from KPn infected WT and MGL1^{-/-} mice. Data are mean ± SEM from 8 mice per group in 3 independent experiments.

MGL1^{-/-} mice exhibit severe lung pathology and hyperinflammatory response

We next compared the gross immunopathological changes and immune cell infiltration in KPn infected WT and MGL1^{-/-} mice. The mock control mice of both strains displayed similar normal lung tissue morphology in H&E stained cryosections (Fig. 9a, 9e). A moderate transient infiltration of immune cells was observed in infected WT mice by day 3p.i. which was reduced substantially by 5dp.i. (Fig. 9b-d). The overall architecture of the lungs was largely preserved in the WT animals throughout the course of infection. In MGL1^{-/-} mice on the other hand, an increased immune cell infiltration was evident even at 1dp.i. which progressively increased and resulted in pulmonary inflammation characterized by highly confluent immune cell infiltrates (Fig. 9f-h). To further examine the impact of MGL1 deficiency on inflammatory response, levels of multiple inflammatory

cytokines in lung homogenates were compared between KPn infected WT and MGL1^{-/-} mice. While the mock controls displayed low basal levels of these cytokines, KPn infected WT mice exhibited increased amounts at 3dp.i., a time of peak bacterial burden and cellular infiltration (Fig. 9B). Infected MGL1^{-/-} mice on the other hand, exhibited significantly higher levels of inflammatory cytokines at that time compared to the WT mice (Fig. 9B). The levels of IL-10, an anti-inflammatory cytokine were also significantly higher in MGL1^{-/-} mice, suggesting a condition of “cytokine storm” typical of sepsis, where anti-inflammatory host mediators are upregulated in an attempt to counter-balance the systemic inflammatory response (238-240). Together, our results show that MGL1 deficiency did not render the mice defective in their ability to mount an anti-inflammatory response to counter the on-going inflammation; instead these mice displayed a hyperinflammatory phenotype typically associated with sepsis. Our observations thus raised the possibility that despite a similar bacterial burden, increased lung pathology and hyperinflammatory response is causing increased susceptibility of MGL1^{-/-} mice to KPn infection.

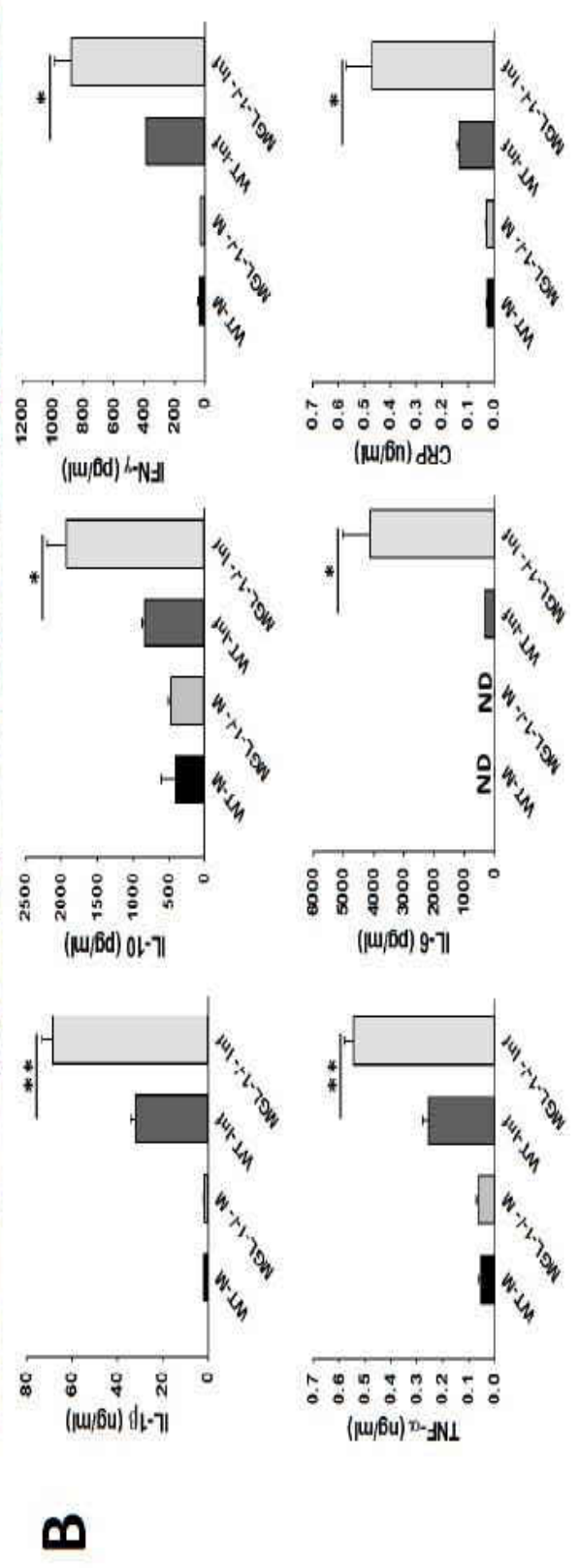
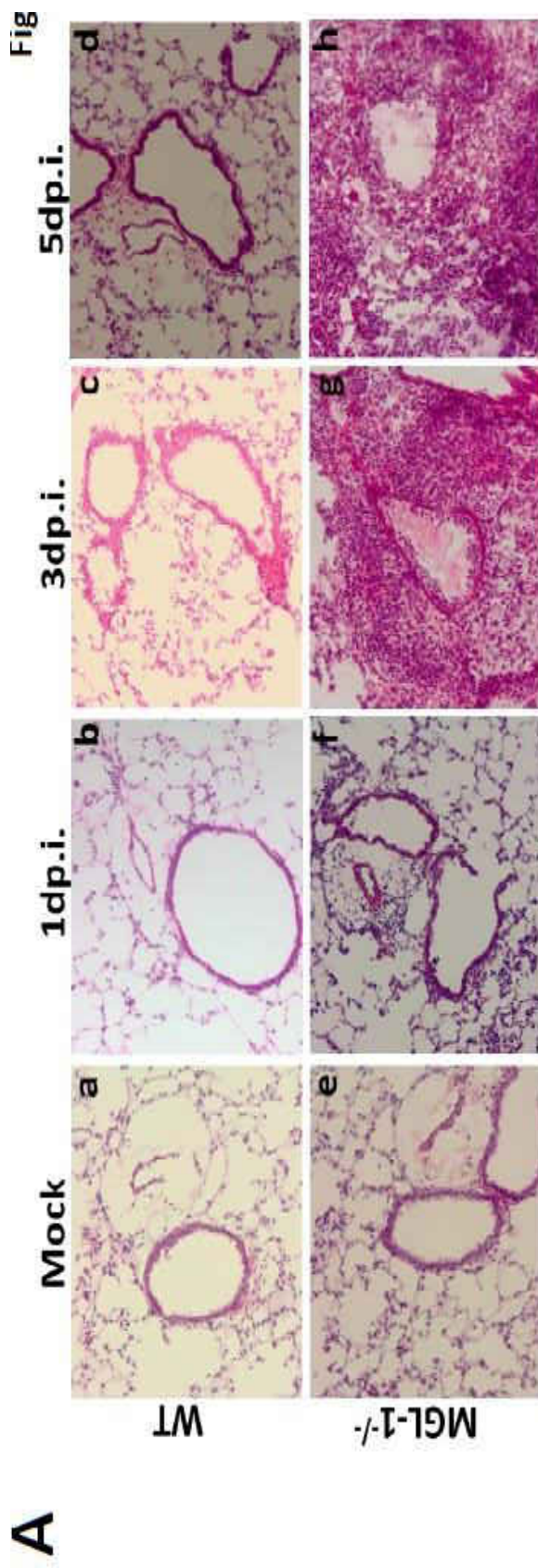


Figure 9. Pneumonic MGL1^{-/-} mice exhibit severe lung pathology and hyperinflammatory response

(A) Hematoxylin & Eosin staining of lung cryosections from mock control (a and e) and KPn infected WT (b,c,d) and MGL1^{-/-} (f,g,h) mice isolated at indicated times post-infection. Magnification 200X. (B) The lungs from mock control (WT-M and MGL1^{-/-} M) and KPn infected WT and MGL1^{-/-} (WT-Inf and MGL1^{-/-} Inf) mice isolated at 3d p.i. were homogenized in PBS with protease inhibitors and analyzed commercially for host immune mediators by rodent multi-analyte profile (MyriadTM Rules-Based Medicine, Austin, TX). Results shown are mean ± SEM of 3-4 each infected and mock control mice from 2-3 independent experiments. Statistical significance are denoted by asterisks (*, p<0.05; **, p<0.005). CRP; C-reactive protein.

MGL1^{-/-} mice display altered neutrophil accumulation and neutrophilic immune mediators

Pneumonic infection with KPn typically results in immune cell infiltration in lungs. To examine if there were differences in the immune cell types infiltrating in the lungs of WT versus MGL1^{-/-} mice, flow cytometry analysis of total lung cells was performed. The results revealed that the percent as well as absolute numbers of Ly6G⁺; Ly6C⁺ and CD11b⁺ myeloid cells were significantly higher in the infected MGL1^{-/-} lungs than those in the WT mice (Fig. 10A and B). We found no significant differences in the numbers of macrophages (F4/80⁺), dendritic cells (CD11c⁺), αβ T cells (TCRβ⁺) or B cells (CD19⁺). Further analysis of the data revealed that the percent as well as total numbers of Ly6G⁺CD11b⁺ neutrophils were increased 2-3 fold in the lungs of MGL1^{-/-} mice upon

infection, as compared to the WT mice (Fig. 10C). This indicated that MGL1 likely plays a role in regulating neutrophil infiltration and that increased susceptibility of these mice may be due to excessive neutrophil accumulation contributing to exacerbated lung pathology. This was further corroborated with the analysis of neutrophil associated immune mediators which revealed significantly higher levels of neutrophil chemoattractants (CXCL1, CXCL6), neutrophil survival mediator (GM-CSF) and neutrophil activation markers (MMP9, MPO) in lung homogenates of MGL1^{-/-} mice, as compared to their WT counterparts (Fig. 11). Together our data suggested a neutrophilia-promoting effect of MGL1 deficiency in KPn pneumosepsis, which results in severe pathology and hyperinflammatory response contributing to the increased susceptibility of MGL1^{-/-} mice to KPn pneumonia.

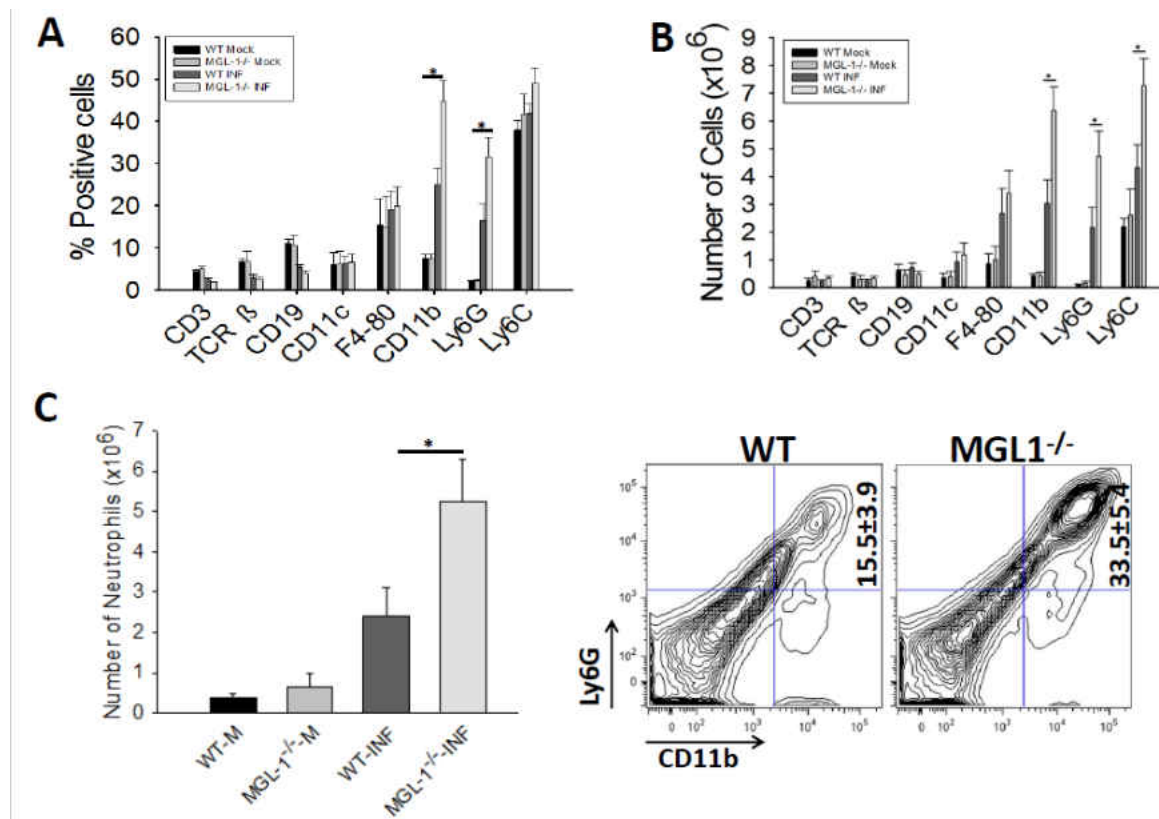


Figure 10. Pneumonic MGL1^{-/-} mice exhibit increased neutrophil accumulation in their lungs

(A) Flow cytometry analysis of total lung cells from mock and KPn infected WT and MGL1^{-/-} at 3dp.i. Total lungs cells were isolated from mice by collagenase treatment followed by staining with antibodies against indicated cell markers as described in methods. Each bar represents percent cells positive for individual markers in lungs of indicated experimental mice. (B) Shows total number of cells positive for indicated cell markers. Data shown are the mean \pm SEM of 5-6 mice from 3-4 independent experiments. Statistical significance are denoted by asterisks (*, $p < 0.05$). (C) Ly6G+CD11b+ neutrophils in lungs of KPn infected WT and MGL1^{-/-} mice at 3dp.i. The cells were double-stained with anti-Ly6G-APC and anti-CD11b-Pacific Blue antibodies as markers for neutrophils. The bar graph shows mean \pm SEM of total number of neutrophils in lungs of 3-4 mock control and 4-5 KPn infected WT and MGL1^{-/-} mice each from 3 independent experiments. Contour plots shown on the right are from one representative experiment. Statistical significance are denoted by asterisks (*, $p < 0.05$).

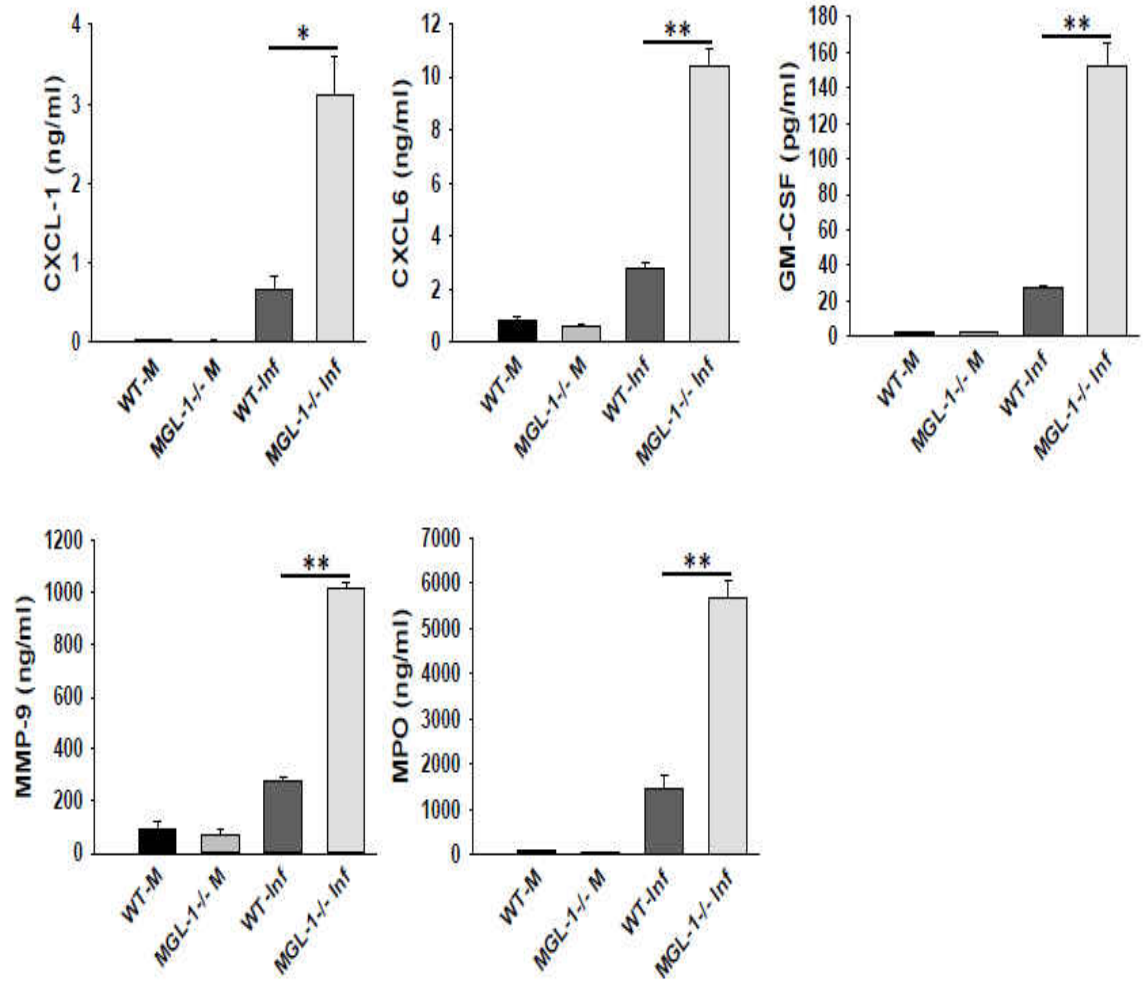


Figure 11. Pneumonic MGL1^{-/-} mice exhibit increased levels of neutrophil associated immune mediators

The lungs from mock control and KPn infected WT and MGL1^{-/-} mice were harvested at 3dp.i. and analyzed commercially for host immune mediators by rodent multi-analyte profile (MyriadTM Rules-Based Medicine, Austin, TX). Levels of neutrophil chemoattractants and growth factor (CXCL1, CXCL6, GM-CSF) and activation markers (matrix metalloproteinase-9, MMP-9 and myeloperoxidase, MPO) are shown. Results shown are mean ± SEM of 3-4 each infected and mock control mice from 2-3 independent experiments. Statistical significance are denoted by asterisks (*, p<0.05; **, p<0.005).

MGL-1 deficiency causes increased neutrophil influx into the lungs during KPn pneumonia

In order to gain mechanistic insights into the role of MGL1 in neutrophil accumulation, we performed neutrophil adoptive transfer using WT and MGL1^{-/-} neutrophils labelled with two different intracellular dyes. Injecting a mix of the two types of neutrophils and calculating the ratio of MGL1^{-/-} neutrophils to WT neutrophils and vice versa, allowed us to assess the relative migration efficiency of WT and MGL1^{-/-} neutrophils in the exact same microenvironment (Fig. 12A). Consistent with our pathological and flow cytometry findings, we observed that MGL1^{-/-} neutrophils were recruited at a higher rate than their WT counterparts in pneumonic lungs of recipient WT as well as MGL1^{-/-} mice (Fig.12B). Deficiency of MGL1 on endothelial and possibly other resident cells did not appear to affect neutrophil recruitment, as evidenced by similar migration of WT neutrophils in MGL1^{-/-} and WT mice (Fig. 12B black bar). These results further solidified the fact that MGL1^{-/-} neutrophils exhibit an increased capacity to infiltrate the lungs during KPn pneumonia indicating a previously undetermined role of MGL1 in controlling neutrophilia during pneumonic infection.

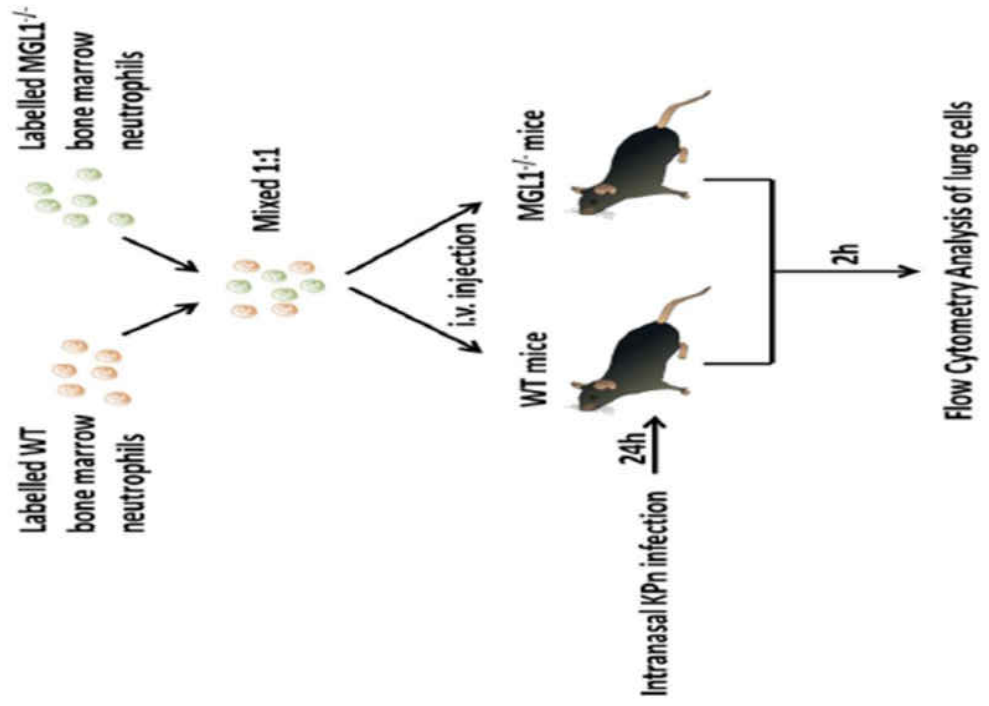
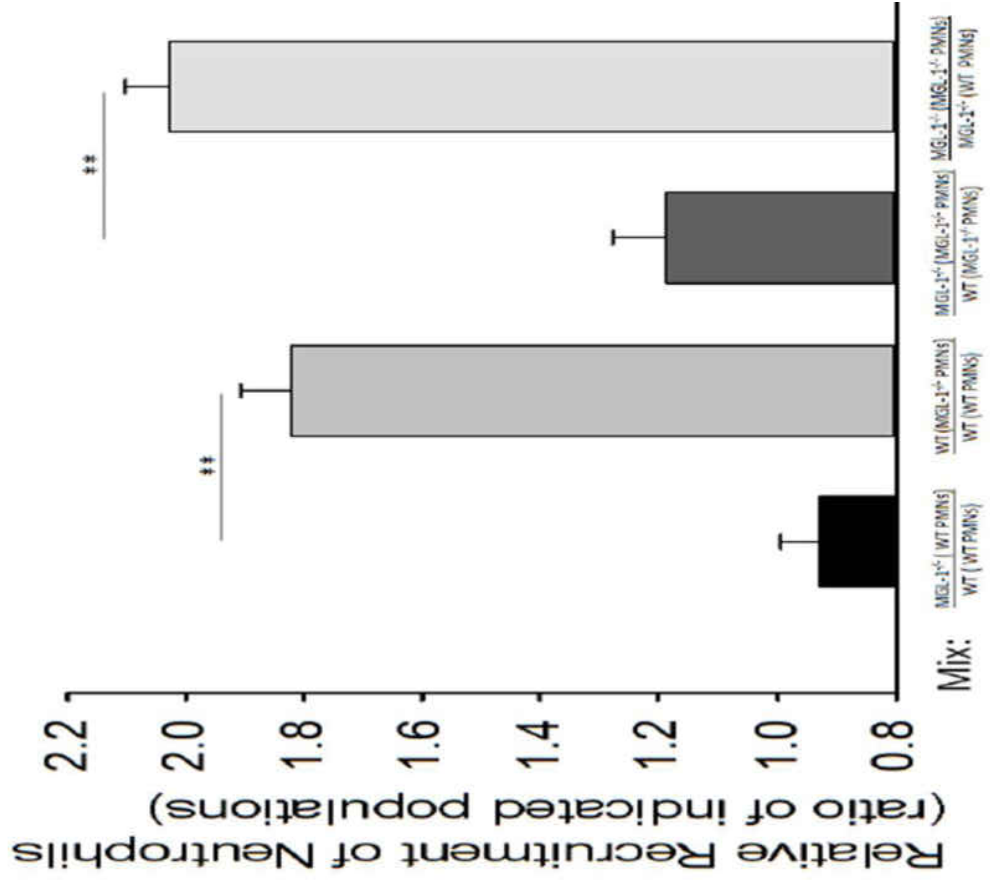
A**B**

Figure 12. MGL1 deficiency causes increased neutrophil influx in the pneumonic lungs

(A) Schematic representation of the adoptive transfer experiment as described in the Methods. Purified bone marrow neutrophils from WT mice were labelled with CellTracker Orange CMTMR and those from MGL1^{-/-} mice labeled with CellTracker Green CMFDA, mixed in 1:1 ratio and injected intravenously (i.v.) into WT or MGL1^{-/-} recipient mice infected intranasally with KPn 24h prior to injection. Lungs were harvested 2 hrs after the adoptive transfer and processed for flow cytometry. (B) Relative recruitment of MGL1^{-/-} and WT neutrophils in each mouse strain is depicted as the ratio of indicated populations in the KPn infected lungs. Data shown are mean \pm SEM from 9 mice per group in 3 independent experiments. Statistical significance are denoted by asterisks (**, $p < 0.005$).

Discussion

Gram-negative pneumonic sepsis remains a serious healthcare challenge despite effective antibiotic treatments. This underscores that erratic host response to the infectious insult is an important element in determining the outcome of the disease. Here we report that MGL1, a host CLR, impacts the outcome of *Klebsiella* induced pneumosepsis as evidenced by a reduced survival, severe neutrophilic lung pathology and hyperinflammation of MGL1^{-/-} mice. While the antimicrobial functions of neutrophils and macrophages remain unaffected by MGL1 deficiency, the mechanistic basis for the observed differences between the WT and MGL1^{-/-} mice appeared to be de-regulated neutrophil influx and excessive, non-resolving inflammation. Our study shows, for the first time, a protective role of MGL1 in Gram-negative pneumonic sepsis by controlling neutrophil influx into the lungs.

CLRs, by way of their abundant expression on phagocytic cells, can mediate pathogen uptake and antigen presentation (241-243). MGL1 has been shown to mediate the attachment and entry of influenza A virus (244) and recombinant MGL1 can bind intestinal commensal bacteria (222). We examined if this CLR plays any role in KPn uptake and clearance by phagocytic cells which may explain the increased susceptibility of MGL1^{-/-} mice to this infection. Strikingly, we observed no difference in the phagocytic and bacterial killing capacity of neutrophils and macrophages, two cells types involved in KPn phagocytosis and clearance. Neutrophils can also kill pathogens by the release of NETs composed of chromatin decorated with neutrophil derived proteins (245). The NETs have been demonstrated to play a role in controlling KPn growth (59, 66, 237). However, similar to the phagocytosis and intracellular killing, we did not find any defect in NETs formation

in MGL1^{-/-} neutrophils in-vivo. Together, these observations correlated with similar local and systemic bacterial burden in WT and MGL1^{-/-} mice and led to the possibility that the poor disease outcome in the absence of MGL1 is due to its impact on host immune responses other than those directly affecting antibacterial mechanisms.

Unlike humans, who express only one form of MGL, mice have two orthologues of this CLR: MGL1 and MGL2 (211). MGL1 and MGL2 have been suggested to perform non-redundant functions with MGL1 being most widely studied for its role in inflammatory and infectious disease conditions (211). To the best of our knowledge, there are no reports to suggest that MGL2 expression is modulated due to a deficiency of MGL1 in MGL1^{-/-} mice. While this will require detailed structural and functional studies utilizing MGL2^{-/-} mice, our data presented in this manuscript shows a clear phenotype of increased susceptibility and poor disease outcome in the absence of MGL1 in MGL1^{-/-} mice. Moreover, unlike MGL1, KPn infection did not cause any increase in expression of MGL2 at the transcript level in infected mouse lungs as compared to the uninfected control mice (data not shown). Whether or not MGL2 is involved synergistically, directly or indirectly; could be a topic of future investigation.

Despite a similar bacterial burden, MGL1 deficiency resulted in more severe lung pathology and heightened inflammatory response. These mice displayed hyperinflammation and a lack of resolution in their lungs, suggesting a protective, pro-resolving role of MGL1 in pneumosepsis. Both anti- as well as pro-inflammatory functions of MGL1 have been described in distinct pathological conditions. Increased susceptibility of MGL1^{-/-} mice to helminth parasitic infection with *Taenia crassiceps* was shown to correlate with reduction in the levels of proinflammatory cytokines (246). An MGL-

dependent induction of pro-inflammatory cytokines, TNF- α , IL-6 and IFN- γ in *Bordetella pertussis* stimulated mast cells was reported (221), however, the relevance of these findings to overall disease outcome was not examined. In contrast to these studies, an anti-inflammatory role of MGL1 was described in experimental colitis, where the protective function of MGL1 was attributed to induction of IL-10 by colonic lamina propria macrophages in response to commensal bacteria (222). We observed an excessive upregulation of both pro- as well as anti-inflammatory cytokines in MGL1^{-/-} mice undergoing KPn pneumoseptic infection. It is likely that the presence of multiple PAMPs and alarmins released due to bacterial growth and/or inflammation contributes to the effect of MGL1 deficiency and causes stimulation of multiple PRRs on immune cells resulting in a mixed pro-and anti-inflammatory hyper response reminiscent of characteristic cytokine storm. Indeed, a cross-talk between CLR and Toll-like receptors (TLR)-induced immune responses has been reported where CLR mediated uptake of *Schistosoma* antigens suppresses TLR-mediated dendritic cell maturation (247). Whether an inverse relationship between MGL1 and TLR signaling exists or if MGL1 negatively regulates TLR stimulation in KPn pneumonia remains to be determined. Nonetheless, the fact that a hyperinflammation and more severe pathological changes were observed in MGL1^{-/-} mice despite a similar bacterial burden indicate that MGL1 negatively regulates the inflammation and a perpetuation of inflammation rather than the bacterial growth contributes to lethality of MGL1^{-/-} mice.

We found that KPn infected MGL1^{-/-} mice exhibited significantly increased neutrophil accumulation in their lungs. Neutrophil mediated responses are essential for combating pneumonic bacterial infection and their protective role in sepsis and KPn infection in

particular has been described elegantly (248, 249). However, persistent accumulation of neutrophils can lead to bystander tissue destruction, owing to their tissue destructive cargo. Accumulation of large numbers of neutrophils in the lungs of MGL1^{-/-} mice and higher amounts of their associated inflammatory mediators suggested that there is a defect in neutrophil turnover in these mice. This could either be due to increased influx of neutrophils into the lungs; due to a defect in their clearance by efferocytosis; or a combination of both. While we are currently investigating the role of MGL1 in efferocytosis, our adoptive transfer experiment showed that MGL1 deficiency on neutrophils significantly increases their capacity to infiltrate the lungs of pneumonic mice. This suggested a novel role of MGL1 as a negative regulator of neutrophil influx, although previous studies have implicated this CLR in trafficking of other myeloid cell types. Relevant to our study, mouse MGL has been reported to impede migration of immature dendritic cells (DCs) via its interaction with its glycan ligands on endothelium, and blockage of MGL results in an enhanced random mobility of these cells (250). Mature DCs lose MGL expression (251), thus enabling these cells to overcome the negative effect of MGL and egress. Similar mechanisms could be at play in our model where MGL1 negatively regulates epithelium-neutrophil interactions thereby impeding neutrophil infiltration into the intrapulmonary space and retaining them into circulation, and only after MGL1 expression is lost, are these neutrophils able to infiltrate into the lungs. This could also explain why we could not detect MGL1 expression on neutrophils infiltrating the lungs of KPn infected WT mice (data not shown). Curiously, while MGL1 was expressed on bronchial epithelial and endothelial cells in lungs, we did not see a significant effect of endothelial cell expressed MGL1 on neutrophil influx (evidenced by similar infiltration of

labelled WT neutrophils in MGL1^{-/-} and WT mice). While the role of MGL1 expressed on other resident cells cannot be ruled out at this time, the significance of this endothelial expression pattern remains to be experimentally determined.

In the absence of MGL1 we also observed significantly higher induction of neutrophil chemoattractants (CXCL1 and CXCL6), neutrophil survival mediator (GM-CSF) and neutrophil activation markers (MMP9, MPO) in MGL1^{-/-} mice, as compared to their WT counterparts. These results further support the notion that MGL1 is likely involved in neutrophil turnover and the absence of MGL1 results in greater and prolonged accumulation of neutrophils in lungs of mice. This leads to a greater neutrophil-mediated inflammation and lung pathology observed in MGL1^{-/-} mice as compared to their WT counterparts where the neutrophils infiltrate transiently and are cleared off as the infection resolves. These observations clearly suggest that while deficiency of MGL1 does not impair bacterial phagocytosis and activation of neutrophils, this CLR is required for regulating neutrophil trafficking.

In summary, this study shows that MGL1 plays an important role in mitigating the inflammation during a pneumonic infection, by negatively regulating the neutrophil influx. This study opens up new avenues of research on the role of MGL1 in neutrophil trafficking. This can have major implications in the therapeutic measurements of inflammation associated disorders.

Acknowledgements

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Current Work

I am currently working to understand the mechanistic underpinnings of massive neutrophil accumulation in the lungs of MGL1^{-/-} mice. We hypothesized that the accumulation of neutrophils in the lungs of MGL1^{-/-} mice could be due to either an increase in influx into the lungs or a defect in their ability to be cleared via efferocytosis; or a combination of both. I tested both possibilities and found that:

MGL1 deficiency leads to an increase in neutrophil mobilizing factors during KPn induced pneumonia

The adoptive transfer experiment described above indicated that MGL1 plays a role in negatively regulating neutrophil influx. In order to gain mechanistic insight into this, we analyzed the levels of neutrophil mobilizing factors Granulocyte Chemotactic Protein-2 (GCP-2) and Granulocyte-Colony Stimulating Factor (G-CSF) levels in the lung homogenates of uninfected mock control and KPn infected WT and MGL1^{-/-} mice at 3dp.i. The basal levels of these mediators in the lung homogenate of uninfected WT and MGL1^{-/-} uninfected mice was similar. However, by 3dp.i. MGL1^{-/-} mice had significantly elevated levels of both neutrophil mobilizing factors in their lungs compared to WT mice (Fig 13). Elevated G-CSF levels are known to cause emergency granulopoiesis (252-261) which is, a form of hematopoiesis for granulocytes and occurs constantly to maintain a steady state

of mature granulocytes in the blood. In the case of severe systemic infection a demand-adapted granulopoietic response (emergency granulopoiesis) kicks in to drastically increase the number of neutrophils to meet the current situation (252). We next analyzed granulopoiesis in WT and MGL1^{-/-} mice upon KPn infection.

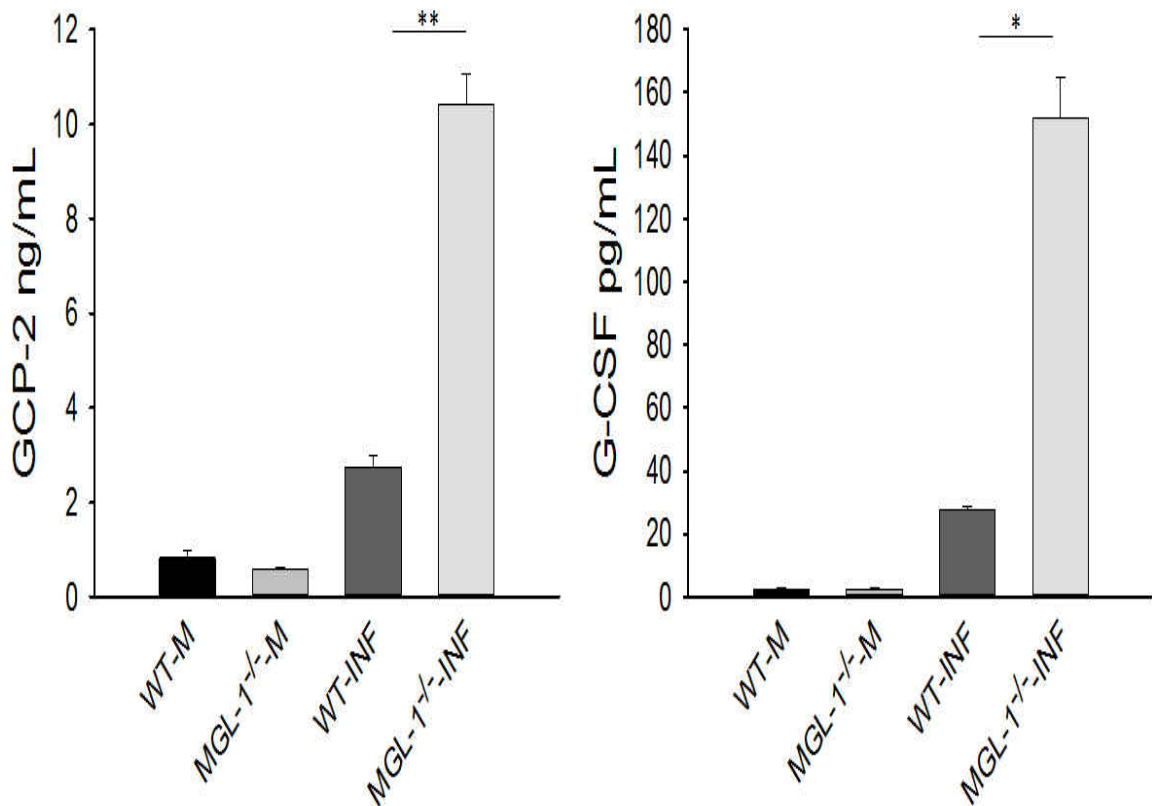


Figure 13. Pneumonic MGL1^{-/-} mice exhibit increased neutrophil mobilizing factors in their lungs

Lung homogenate from *K. pneumoniae*-infected WT and MGL1^{-/-} mice at 3 d p.i. were analyzed for a number of chemokines, cytokines, and growth factors. Data represented above shows Granulocyte Chemotactic Protein-2 (GCP-2) and Granulocyte-Colony Stimulating Factor (G-CSF). ($n = 3$) * $p < 0.05$, ** $p < 0.005$.

Pneumonic MGL1^{-/-} mice display an increase in mature neutrophil mobilization from reserves in the bone marrow

We analyzed the process of granulopoiesis in WT and MGL1^{-/-} mice over a 3-day time course beginning at 6 hours post KPn infection via flow cytometry. Using the hematopoietic stem cell marker c-Kit, granulocyte marker Ly6G, and gating on different subpopulations; we quantitated the number of progenitor cells (c-Kit⁺⁺ Ly6G⁻), early granulocytes (c-Kit^{Low} Ly6G^{Low}), and mature neutrophils (c-Kit⁻ Ly6G⁺) in the bone marrow and blood of WT and MGL1^{-/-} mice. As shown in Figure 14, there was no difference in the numbers of progenitor cells, early granulocytes, or mature neutrophils in bone marrow or blood of uninfected mock as well as at 6hr p.i. and 1dp.i. in WT and MGL1^{-/-} mice. However, by 3dp.i. MGL1^{-/-} mice had increase numbers of progenitor cells, early granulocytes, and mature neutrophils in the blood compared to the infected WT mice. These results suggested that in the absence of MGL1, increase in neutrophil mobilizing factors in the lungs, there is increased mobilization of mature neutrophils from the bone marrow.

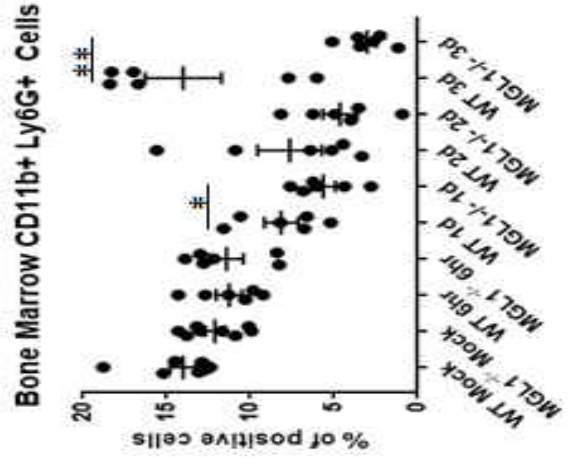
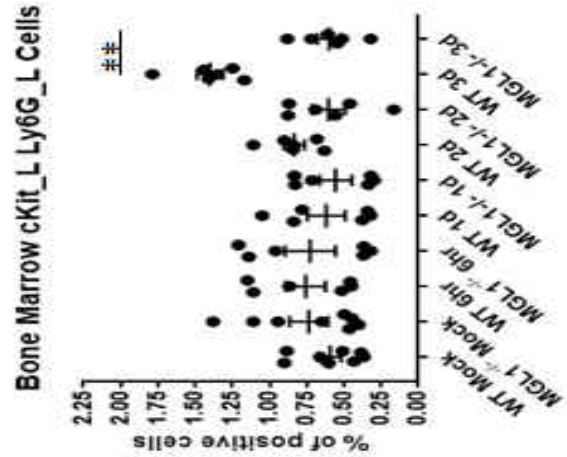
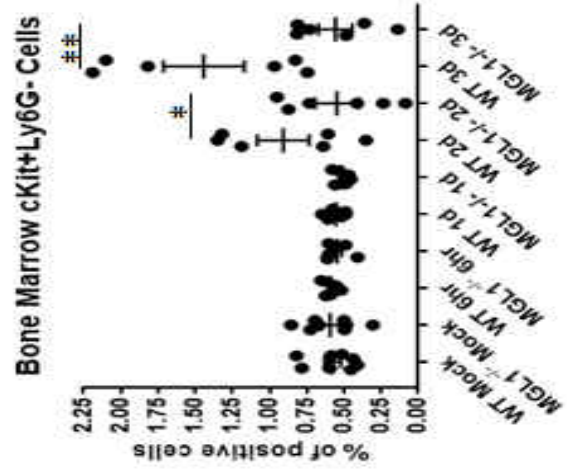
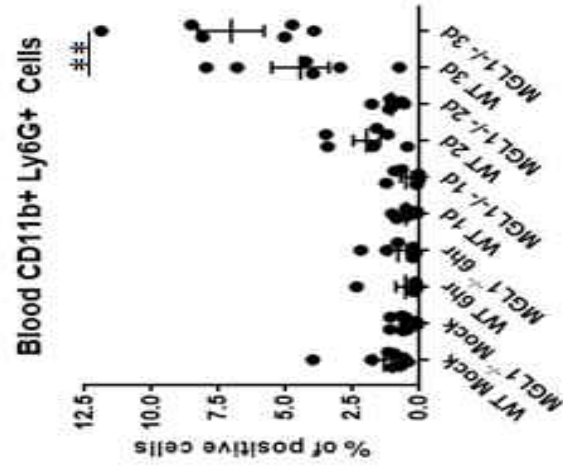
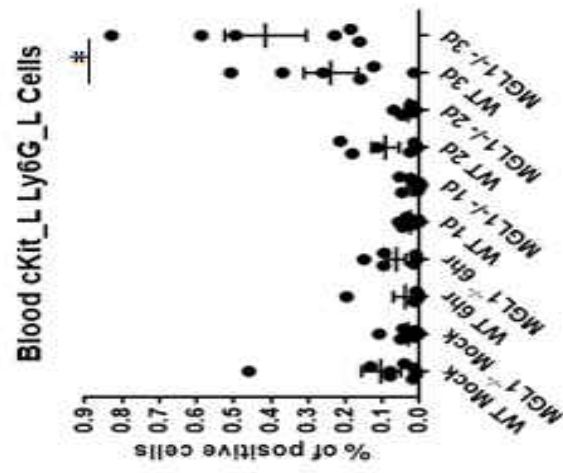
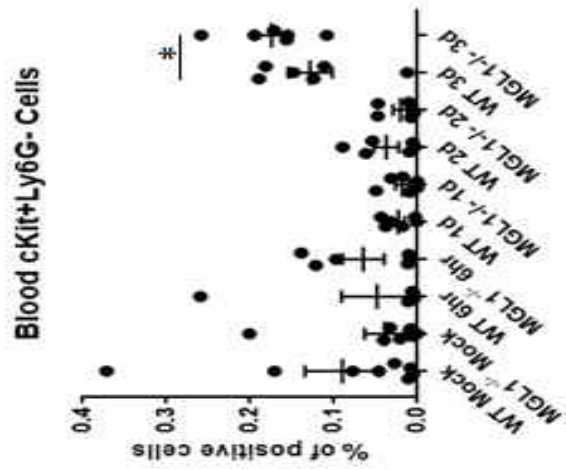


Figure 14. Pneumonic MGL1^{-/-} mice showed an increased mobilization of neutrophil reserves from bone marrow

WT and MGL1^{-/-} mice were infected with *K. pneumoniae* and the generation and mobilization of hematopoietic progenitor cells to mature neutrophils via the process of granulopoiesis was checked at the indicated time points in the blood and bone marrow. ($n = 6$). Data was analyzed as shown above, using cKit to identify hematopoietic stem cells and Ly6G to identify neutrophils. cKit⁺ Ly6G⁻ cells are progenitors, cKit^{Low} Ly6G^{Low} cells are early granulocytes, and CD11b⁺ Ly6G⁺ cells are mature neutrophils. * $p < 0.05$, ** $p < 0.005$.

Absence of MGL1 on neutrophils negatively impacts the uptake of neutrophils by alveolar macrophages

Previous reports have shown that the efferocytosis of apoptotic neutrophils is able to regulate granulopoiesis by reducing the production of granulopoietic stimulating factors such as G-CSF (262). To examine this we performed an in-vivo efferocytosis experiment by administering CFSE labelled WT or MGL1^{-/-} neutrophils intranasally into WT mice followed by isolation of alveolar macrophages by lung lavage. The macrophages were then analyzed by flow cytometry to quantitate Ly6G-F4/80+CFSE+ efferocytic cells. As shown in Figure 15, MGL1^{-/-} neutrophils were efferocytosed less than WT neutrophils by WT alveolar macrophages, indicating a defect in the clearance of neutrophils in the absence of MGL1 expression on their surface.

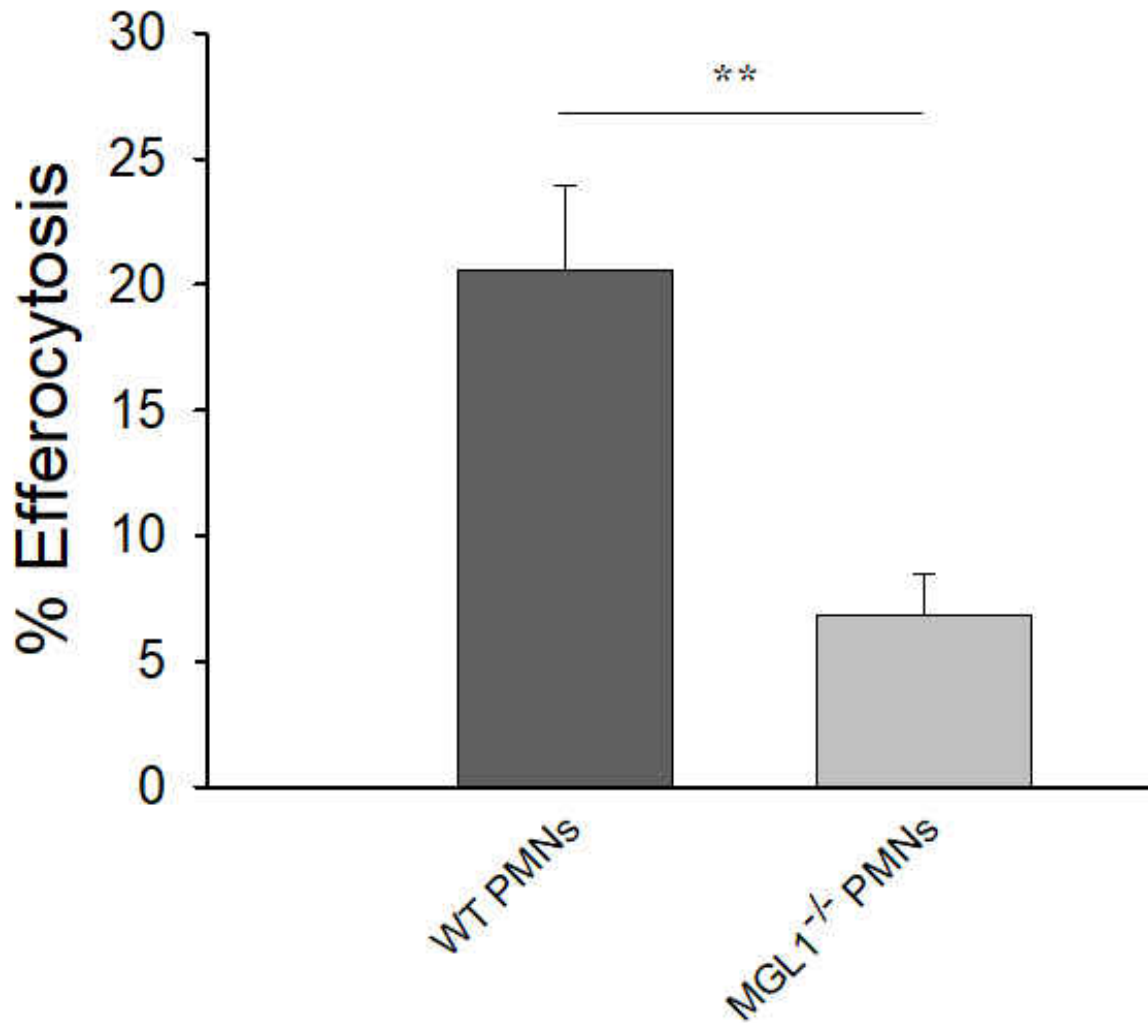


Figure 15. MGL1^{-/-} neutrophils are efferocytosed less by alveolar macrophages *in vivo* compared to WT neutrophils

CFSE labelled WT or MGL1^{-/-} neutrophils were instilled intranasally in anaesthetized mice (3.5 million cells in 35 μ l PBS). Two hours later lungs were lavaged as described in methods and cells were stained with PE-Cy7 conjugated anti-F4/80 and APC conjugated anti-Ly6G antibodies for flow cytometry.

Figure 15 cont. MGL1^{-/-} neutrophils are efferocytosed less by alveolar macrophages in-vivo compared to WT neutrophils

Percentage of efferocytic uptake by macrophages was quantitated by enumeration of Ly6G⁻ F4/80⁺ CFSE⁺ cells. Data are from 4 mice per group in 2 independent experiments (**, p<0.005).

CHAPTER 3

PROTECTIVE ROLE OF MINCLE IN BACTERIAL PNEUMONIA BY REGULATION OF NEUTROPHIL MEDIATED PHAGOCYTOSIS AND EXTRACELLULAR TRAP FORMATION

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Conflict of Interest

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Abstract

Background: Nosocomial infections with *Klebsiella pneumoniae* (KPn) are a frequent cause of Gram-negative sepsis. To understand the functioning of host innate immune components in this disorder, we examined a previously uninvestigated role of C-type lectin receptor Mincle, in pneumonic sepsis caused by KPn.

Methods: Disease progression in wild-type and Mincle^{-/-} mice undergoing pulmonary infection with KPn was compared.

Results: While the wild-type mice infected with a sublethal dose of bacteria could resolve the infection with bacterial clearance and regulated host response, the Mincle^{-/-} mice were highly susceptible with a progressive increase in bacterial burden despite their ability to mount an inflammatory response which turned to an exaggerated hyperinflammation with the onset of severe pneumonia. This correlated with severe lung pathology with a massive accumulation of neutrophils in their lungs. Importantly, Mincle^{-/-} neutrophils displayed a defective ability to phagocytize non-opsonic bacteria and an impaired ability to form extracellular traps (NETs), an important neutrophil function against invading pathogens, including KPn.

Conclusion: Our results demonstrate protective role of Mincle in host defense against KPn pneumonia by coordinating bacterial clearance mechanisms of neutrophils. A novel role for Mincle in regulation of NETosis may have implications in chronic disease conditions characterized by deregulated NET formation.

Key words: Mincle, Clec4e, *Klebsiella*, pneumonia, sepsis, neutrophils, NETs, phagocytosis

Introduction

Lower respiratory tract infection with bacteria can lead to sepsis development, which is a complex immune disorder characterized by a systemic hyperinflammation. There are currently no effective therapies for sepsis that results in 750,000 hospitalizations annually in the United States with a mortality rate of 20-50% (2, 224). Nosocomial infections with opportunistic pathogen *Klebsiella pneumoniae* (KPn) account for 5-20% of Gram-negative sepsis cases (223, 225, 263). Additionally, emergence of multidrug resistant isolates of KPn in clinical settings is a serious health concern. As innate mucosal immunity plays a direct role in bacterial killing and immunomodulation in this acute infection (237, 248, 264-266), an understanding of functioning of host innate immune components might provide targets for modulation of host immune system in a beneficial manner.

Mincle is a C-type lectin receptor (CLR) belonging to the Dectin-2 subfamily of innate immune receptors that can function as an activating receptor for host- as well as pathogen-associated molecular patterns, termed alarmins and PAMPs respectively (reviewed in (213, 214). It is an inducible receptor, expressed mainly by myeloid cells such as macrophages, neutrophils, myeloid dendritic cells as well as some B-cell subsets (213, 215, 267). Functional analysis of this receptor in macrophages has received the most attention, where its association with Fc γ activates downstream signaling cascades involving Syk kinases resulting in induction of protective inflammatory response (84, 109, 268). While the function of Mincle in chronic bacterial infections such as tuberculosis and fungal infections was examined in these studies, its role in acute pneumonic infections leading to sepsis development has not been explored. Furthermore, its functions other than as an inflammatory PRR (**P**attern **R**ecognition **R**eceptor) have received little, if any, attention.

Neutrophil mediated responses are essential for combating pneumonic bacterial infection and their protective role in sepsis and KPn infection in particular has been described (249, 269). The professional antimicrobial program of neutrophils mainly constitutes phagocytosis of infectious agents followed by production of noxious agents such as reactive oxygen species which kill the internalized microbes. Another recently established mechanism of microbial killing by neutrophils is by formation of extracellular traps (termed neutrophil extracellular traps or NETs) which are DNA fibrils expelled by these cells that are decorated with granular contents such as various proteases and can ensnare and kill the microbes without phagocytosis (59, 270, 271). Mincle has been shown to be expressed by neutrophils and while shown to play a role in neutrophil mediated protective responses against *Candida* and mycobacteria (105, 107), its direct role in bacterial phagocytosis and NET formation is not known. Since neutrophils are a key cell type in controlling KPn infection, Mincle signaling in neutrophils may be a key event in control of KPn infection and sepsis.

In this study, we examined the role of Mincle in acute KPn infection causing pneumonic sepsis. Our results suggest novel protective function of Mincle as non-opsonic phagocytic receptor for the bacteria and in regulation of neutrophil NET formation indicating the importance of this CLR in neutrophil-specific bacterial clearance mechanisms in pneumonic infections.

Materials and Methods

Bacterial strains and Mice

The KPn (ATCC strain 43826) were grown to log phase in LB medium at 37°C. All in-vivo experiments were performed using 6-8 weeks old female wild-type C57BL/6 or Mincle^{-/-} mice on same background obtained from the Consortium of Functional Genomics, Scripps, La Jolla and bred in the animal facility of the University of North Dakota. The animals were used according to institutional and federal guidelines.

Infection of Mice, survival and bacterial burden

Mice were anaesthetized with a mixture of 30mg/ml ketamine and 4 mg/ml xylazine in PBS and were infected intranasally with sublethal dose (2.5 x 10⁴ bacteria in 20ul of saline, determined experimentally) of KPn or with 20 µl of saline alone. Survival of the mice was recorded for up to 2 weeks post-infection (p.i.). In some experiments, the mice were euthanized at indicated times p.i. and blood, lungs and liver were aseptically homogenized in cold PBS with CompleteTM protease inhibitor cocktail (Roche Diagnostics, Germany). For the bacterial burden analyses, serially diluted homogenates and blood were plated on LB agar and incubated at 37°C overnight.

Quantitative real-time PCR

Total RNA from lungs of infected and mock control mice harvested at various times p.i. was extracted using Trizol reagent (Invitrogen) according to the manufacturers' instructions. Real-time PCR analysis was performed using SYBR green (Applied Biosystems, CA, USA) to measure the expression levels of Mincle-specific mRNA by using specific primers (sense) 5'- ACC AAA TCG CCT GCA TCC -3' and (anti-sense) 5'- CAC TTG GGA GTT TTT GAA GCA TC -3' (as described by us in (89)). The target gene

expression levels were normalized to levels of the house keeping 18S gene in the same sample. Expression of Mincle in infected samples was determined as fold change over that in control samples as calculated by using the formula $2^{-(\Delta\Delta Ct)}$.

Multi-analyte profile analysis

The lung homogenates were prepared as described for the bacterial burden analysis above and were centrifuged at 2000 x g for 15 min to clear cellular debris. The supernatants were immediately frozen at -80° C. The biomarker levels in lung homogenates were determined commercially by Myriad Rules-based Medicine (Austin, TX, USA) utilizing a multiplexed analysis.

Histological analysis

For histological analysis, frozen lung tissues were processed as previously described (227, 228). Serial horizontal sections (10 µm thick) of frozen lung tissues thus obtained were stained with hematoxylin and eosin for pathological analysis as previously described (229, 230).

Flow Cytometry

Lungs or BAL cells were harvested from mice at 3 days p.i. and processed as previously described by us (227, 228, 231). Enumeration of neutrophils by flow cytometry (using a BD LSR II, Becton Dickinson, San Jose, CA) was done by quantitating Ly6G+CD11b+ cells stained with Pacific Blue™ anti-mouse CD11b and APC anti-mouse Ly6G (Clone 1A8) antibodies (Biolegend, San Diego, CA). Mincle expression was examined by using a rat anti-mouse Mincle monoclonal antibody (Clone 6G5, InvivoGen, CA) followed by goat anti-rat Alexa-488 secondary antibody (InvitroGen, OR). FlowJo (Tree Star) software was used to analyze all data.

Bacterial phagocytosis by neutrophils

Bacterial phagocytosis of WT and Mincle^{-/-} neutrophils was assessed by flow cytometry. For this, peritoneal neutrophils were isolated using an established model of thioglycollate-induced peritonitis. Sterile 4% thioglycollate was injected in peritoneal cavity of mice and neutrophils enriched 8-12h following the injection were isolated (95-99% pure as assessed by flow cytometry using GR1 and CD11b antibodies). Neutrophils were incubated with GFP-labeled KPn (kindly provided by Dr. Steven Clegg, University of Iowa) for 1 hour and washed three times with ice-cold FACS-buffer (PBS+10% fetal bovine serum). Fluorescence of the attached but non-internalized bacteria was quenched by treating the cells with 0.04% Trypan Blue. The % positive cells containing fluorescent bacteria were determined by flow-cytometry using uninfected neutrophils as control.

Neutrophil NETs

For detection of neutrophil NETs in-vivo, the bronchoalveolar lavage (BAL) was performed in WT and Mincle^{-/-} mice at 3dp.i.. The lavage cells were cytocentrifuged on glass slides and were co-stained with Sytox Green (Molecular Probe, Eugene, OR) and rabbit anti-neutrophil elastase (NE) polyclonal antibody (Abcam) followed by goat anti-rabbit Alexa546 antibody. The percent NET formation was quantitated by dividing the number of NET-forming neutrophils by total number of cells in 8-10 random microscopic fields and multiplying the values by 100.

Statistical Analysis

Statistical analysis of survival studies was performed by Kaplan Meir log-rank test; bacterial burdens by non-parametric Mann-Whitney Test. All other statistical analyses were performed using the Student t test (SIGMA PLOT 8.0, Systat Software, San Jose, CA).

RESULTS

Mincle is highly expressed in lungs during pneumonic KPn infection.

To examine the role of innate immune receptors in pathogenesis of KPn induced pneumonic sepsis, we initially screened a panel of 52 membrane-bound and soluble CLR by Taqman Low-density arrays which showed an upregulated expression of Dectin-2 family CLR, Clec4e (also called Mincle), among others in the lungs of mice undergoing respiratory KPn infection. To further confirm the Mincle expression, real-time quantitative PCR was performed using RNA from lungs of KPn infected wild-type mice. The results showed a progressive increase in the transcript level of Mincle mRNA which was maximally transcribed by 3dp.i. and remained at high level throughout the course of infection (Figure 16A). Flow cytometry analysis further confirmed the increased numbers of Mincle-positive cells, majority of which were CD11b+Ly6G+ neutrophils, in the lungs of KPN-infected mice (Figure 16B). This indicated that Mincle was highly expressed on neutrophils and played a role in pathogenesis of KPn pneumonia.

Mincle deficient mice are highly susceptible to KPn pneumonia.

To examine the role of Mincle in disease development, overall disease severity and survival was compared in wild-type and Mincle^{-/-} mice infected with a sub-lethal dose of KPn. This dose was experimentally determined at which the WT mice displayed minimal morbidity and mortality (230). As shown in Figure 16C, 76% of WT mice infected with 2.5×10^4 CFUs of KPn survived the infection with transient signs of disease (ruffled fur, lethargy) early during infection and appeared healthy later. The Mincle^{-/-} mice, in contrast, were extremely susceptible to this dose and all mice succumbed to infection by day 6p.i. While majority of the infected WT mice cleared the infection by day 5 p.i., Mincle^{-/-} mice exhibited

progressive development of disease and overt signs of infection (weight loss, piloerection, hunched gait, lethargy, increased respiratory rate). The increased susceptibility of *Mincle*^{-/-} mice clearly indicated a protective role played by this CLR during pneumonic KPn infection.

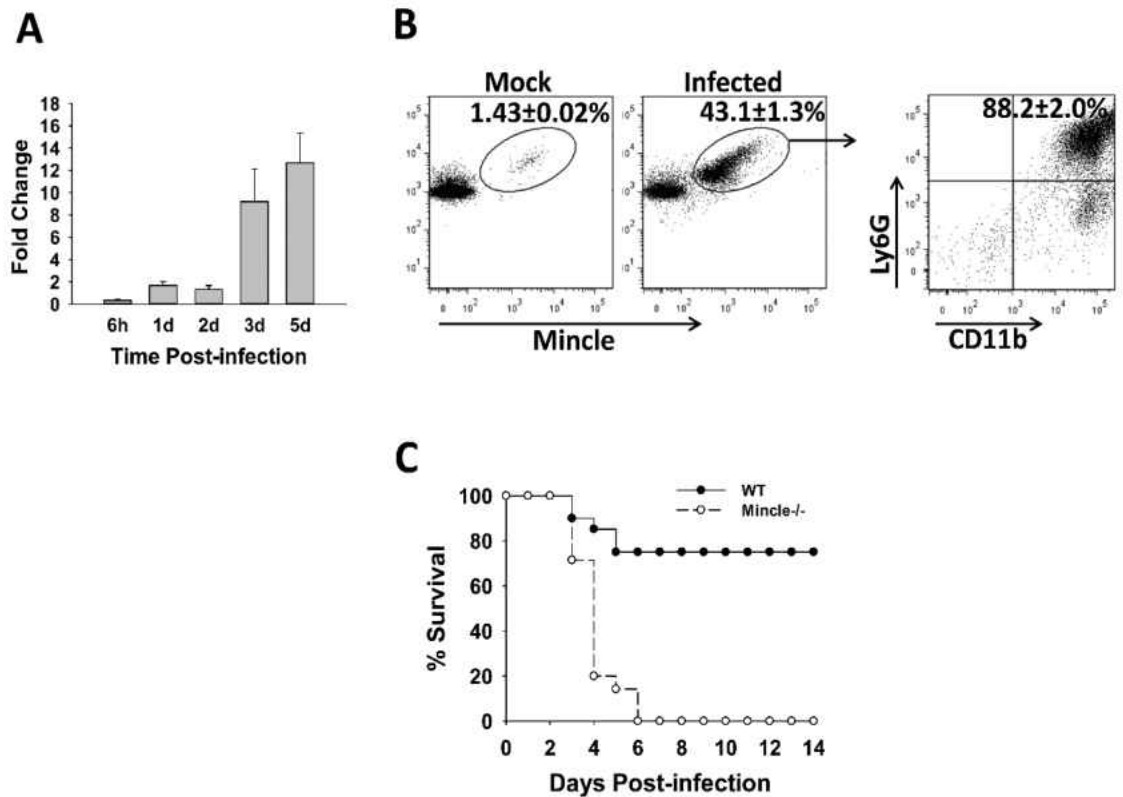


Figure 16. MinCLE is highly induced in the lungs of KPn infected pneumonic mice and *Mincle* deficiency increases susceptibility to the infection

A. Total RNA was extracted by Trizol method from the lungs of KPn infected wild-type C57/BL6 mice, harvested at indicated time post-infection. The mRNA levels of MinCLE were analyzed by real-time PCR as described in Methods and are expressed as fold changes over the levels in mock control mice calculated by using the formula $2^{-(\Delta\Delta C_t)}$. Data shown are the averages of 6-8 mice per group in two independent experiments.

Figure 16 cont. Mincle is highly induced in the lungs of KPn infected pneumonic mice and Mincle deficiency increases susceptibility to the infection

B. Mincle expression was examined on infiltrating lung cells from KPn infected wild-type C57/BL6 mice harvested at 3dp.i. by flow cytometry using a rat anti-mouse Mincle antibody followed by goat anti-rat secondary antibody labeled with Alexa-488. Mincle positive cells were gated and further analyzed for expression of CD11b and Ly6G as mentioned in the Methods section. The dot plots shown are representative of 3 independent experiments with 3 mice each. **C.** Fifteen each WT and Mincle^{-/-} mice were intranasally infected with 2.5X10⁴ CFUs of *Klebsiella pneumoniae* (KPn) in 20µl of sterile PBS and were assessed daily for disease severity. The survival was monitored for two week. Statistical comparison of susceptibility was done by Kaplan-Meier survival curve statistical analysis (p<0.001).

Mincle deficiency results in increased bacterial burden and systemic dissemination

In order to examine if increased susceptibility of Mincle^{-/-} mice to KPn infection correlated with inefficiency to clear bacteria, homogenized lungs, liver and blood from infected Mincle^{-/-} and WT mice collected at various times post infection were plated on LB agar. Up to 2dp.i., Mincle^{-/-} and WT animals displayed similar bacterial burdens in their lungs (Figure 17A). By 3dp.i., however, lungs of Mincle^{-/-} mice exhibited significantly higher bacterial counts as compared to their WT counterparts. The bacterial burden in these mice remained high at 5dp.i., the time when majority of mice had become moribund. In contrast, the WT mice displayed 3-5 logs lower bacterial burden at 3dp.i. and the counts continued to drop through 5dp.i., indicating clearance of bacteria and resolution of the infection in

these mice. The *Mincle*^{-/-} mice also displayed a higher systemic dissemination of bacteria as depicted by significantly higher bacterial load in liver (Figure 17B) and a more severe bacteremia (Figure 17C). In contrast, no viable bacteria were detected in the blood of WT mice by 5dp.i. These data indicated that *Mincle* mediated responses directly or indirectly influenced bacterial clearance in pneumonic infection with KPn.

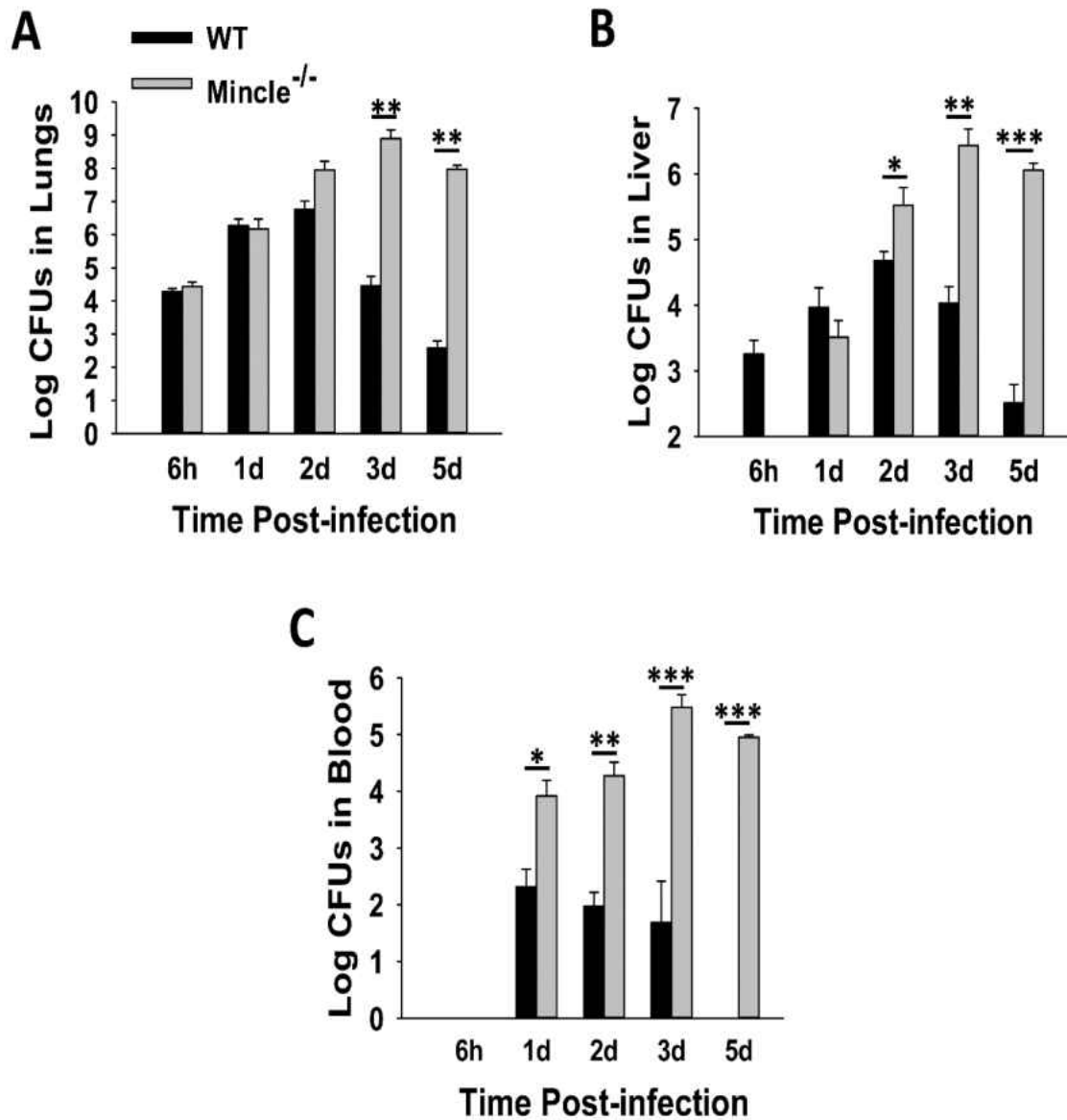


Figure 17. Mincle^{-/-} mice display increased bacterial burden and systemic dissemination during pneumonic KPn infection

WT and Mincle^{-/-} were intranasally infected with 2.5X10⁴ CFUs of KPn. At indicated times post infection the mice were sacrificed, systemic organs were isolated, homogenized and plated as described in Materials and Methods. Bacterial burden was enumerated after incubating the plates overnight at 37°C. The data shown is from three independent experiments with 3-5 mice at each time point per experiment. Significant differences in bacterial burden (using non-parametric Mann-Whitney test) in WT and Mincle^{-/-} are denoted by asterisks (*, $p < 0.05$; **, $p < 0.005$, *** $p < 0.001$).

Mincle^{-/-} mice exhibit hyperinflammatory response

We next examined if the inability of Mincle^{-/-} mice to clear the bacteria was due to a defect in mounting inflammatory response. In both WT and Mincle^{-/-} strains, mock infected mouse lungs displayed similar low basal levels of inflammatory cytokines tested (Figure 18). Upon KPn infection, WT mice exhibited increased levels of these cytokines at 1dp.i., which started to drop by 3dp.i. and were reduced to minimal levels by 5dp.i. (Figure 18). This was consistent with the reduced bacterial burden in these mice at these times p.i.. In contrast, infection of Mincle^{-/-} mice resulted in a progressive increase in levels of these cytokines through the course of infection, which remained high till the mice became moribund. These mice in fact exhibited an overwhelming inflammatory response at 3d and 5dp.i. (Figure 18). The levels of inflammatory cytokines and chemokines tested in lungs of these mice were significantly higher than WT mice at these time points. The levels of IL-

10, an anti-inflammatory cytokine were also significantly higher in *Mincle*^{-/-} mice, suggesting a condition of “cytokine storm” typical of sepsis, where anti-inflammatory host mediators are upregulated in an attempt to counter-balance the systemic inflammatory response (238-240). These results show that *Mincle* deficiency did not render the mice defective in their ability to mount an inflammatory response but these mice rather displayed a hyperinflammatory phenotype typically associated with sepsis. Our observations thus raised the possibility that *Mincle* likely plays a direct role in bacterial clearance and the hyperinflammation resulted due to activation of other PRRs and inflammatory receptors in response to persistent overwhelming bacterial burden in *Mincle*^{-/-} mice undergoing pneumonic KPn infection.

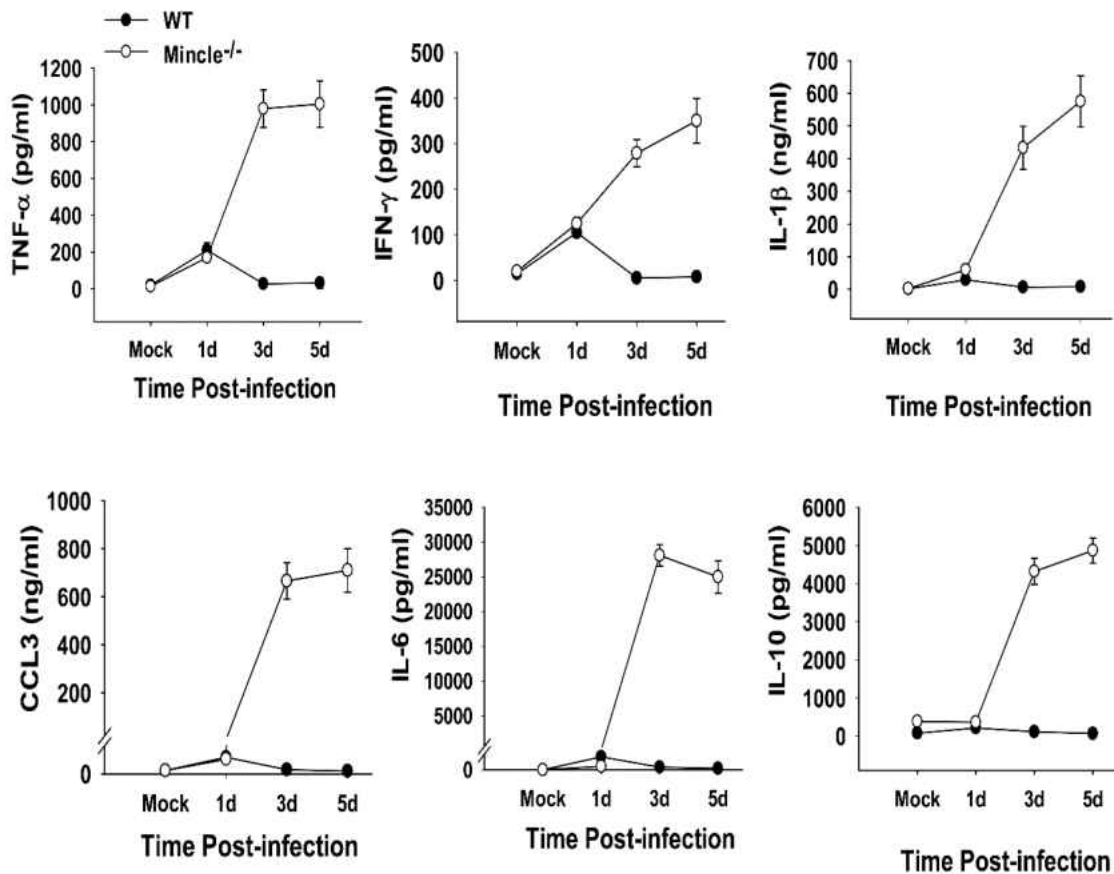


Figure 18. Pneumonic Mincle^{-/-} mice exhibit hyperinflammatory response

The lungs from mock control and KPn infected WT and Mincle^{-/-} mice were harvested at indicated time points post-infection, homogenized in PBS with protease inhibitors and analyzed commercially for host immune mediators by rodent multi-analyte profile (MyriadTM Rules-Based Medicine, Austin, TX). Results shown are average of 3-4 each infected and mock control mice from 3 independent experiments. Amounts of mediators shown were significantly higher (**p<0.001) in KPn infected Mincle^{-/-} mice at 3dp.i. and 5dp.i. in comparison with their levels in the infected WT mice at those time points tested.

Effect of Mincle deficiency on neutrophil infiltration and overall lung pathology

Since neutrophils are a key cell type involved in bacterial clearance and initiation of protective immune response during KPn pneumonia, we next compared neutrophil infiltration and gross immunopathological changes in KPn infected WT and Mincle^{-/-} mice. The mock control mice of both strains displayed similar normal lung tissue morphology in H&E stained sections (Figure 19). A moderate transient infiltration of immune cells was observed in infected WT mice by day 3p.i. which was reduced substantially by 5dp.i. The overall architecture of the lungs was largely preserved in the WT animals throughout the infection. The Mincle^{-/-} mice, on the other hand, displayed a progressive increase in immune cell infiltration, which were mainly neutrophils, based on characteristic multi-lobed nuclei (Figure 19 inset). By day 3 p.i. substantially increased influx of cells was

observed in large lesions and by 5 p.i., extensive foci of consolidation were visible with massive accumulation of neutrophils around alveolar spaces (Figure 19).

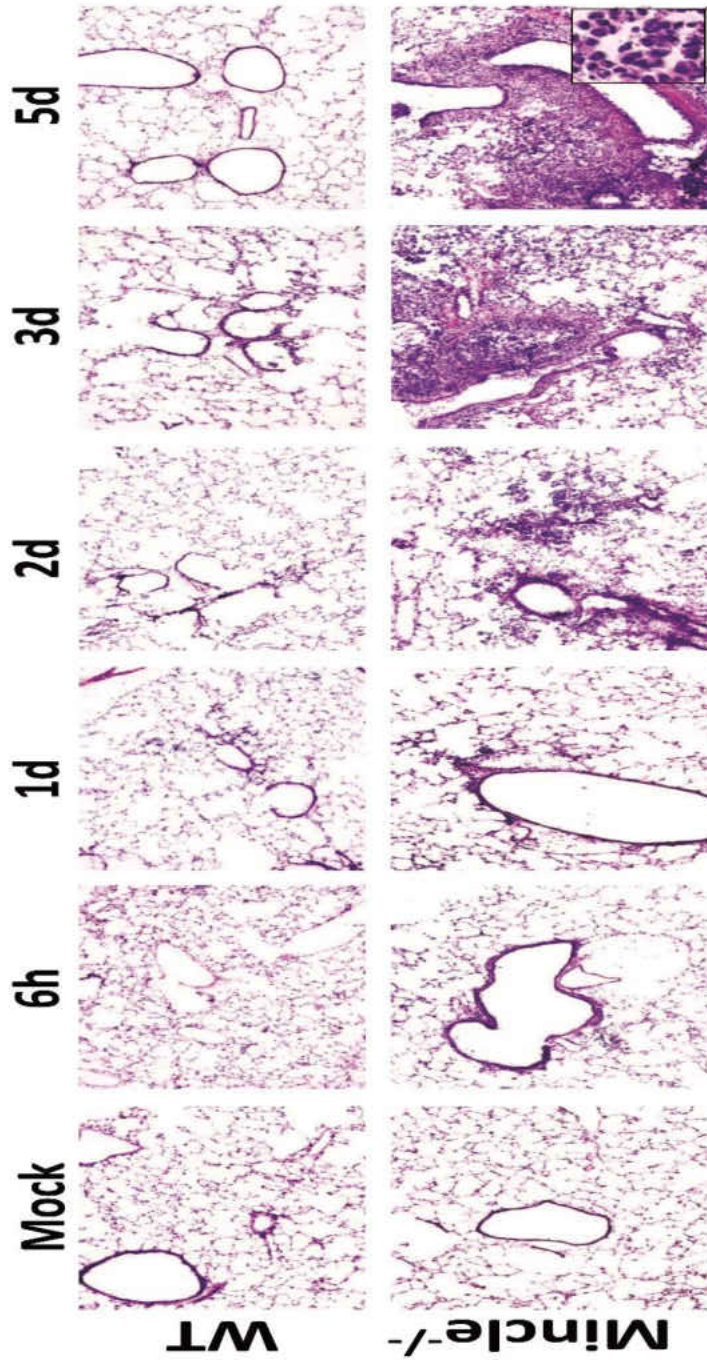


Figure 19. Pneumonic Mincle^{-/-} mice exhibit severe lung pathology characterized by massive neutrophil accumulation

Hematoxylin & Eosin staining of lung cryosections from mock control and KPn infected WT and Mincle^{-/-} mice isolated at indicated times post-infection. Magnification 100X. Inset shows a highly magnified area (1000X) of a lesion in infected Mincle^{-/-} lung depicting neutrophils as indicated by characteristic multilobed nuclear morphology.

Flow cytometry analysis of infiltrating cells in lungs confirmed that the majority of these cells were Ly6G+CD11b+ neutrophils (Figure 20A). The numbers of these cells were significantly higher in the infected Mincle^{-/-} lungs than those in the WT mice (Fig. 20A, bar graph). This correlated with significantly higher levels of neutrophil chemoattractants (CXCL1, CXCL6), neutrophil survival mediator (GM-CSF) and neutrophil activation markers (MMP9, MPO) in these mice, as compared to their WT counterparts (Figure 20B).

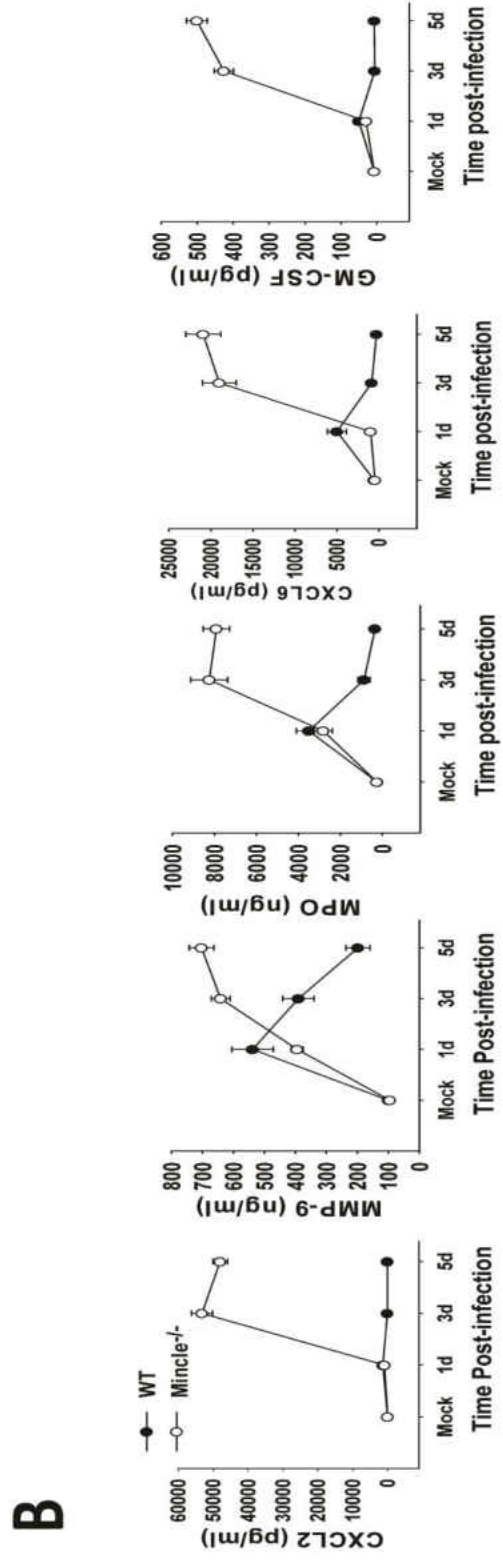
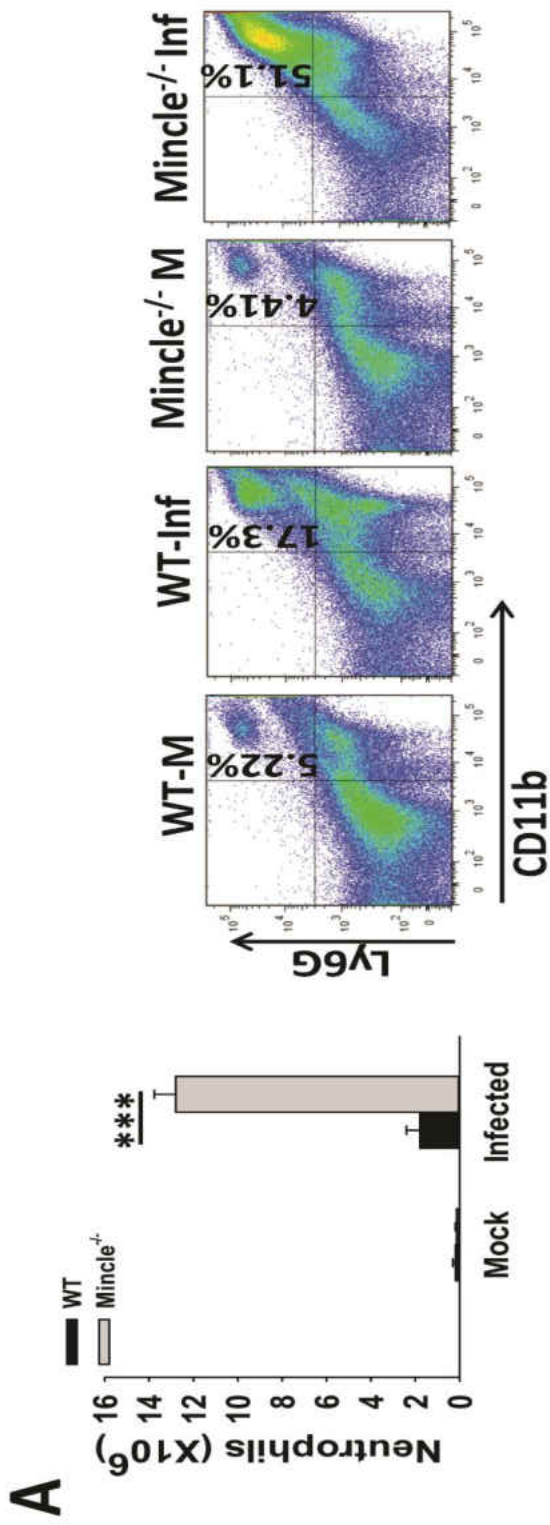


Figure 20. Increased neutrophil accumulation coincides with elevated expression of neutrophil chemoattractant and activation markers in lungs of KPn infected Mincle^{-/-} mice **A.** Flow cytometry analysis of Ly6G+CD11b+ neutrophils in mock control (WT-M and Mincle^{-/-}-M) and KPn infected (WT-Inf and Mincle^{-/-}-Inf) WT and Mincle^{-/-} mice. Total lungs cells were isolated from mice by collagenase treatment at 3dp.i.. The cells were stained with anti-Ly6G-APC and anti-CD11b-Pacific Blue antibodies as markers for neutrophils. The bar graph shows average of total number of neutrophils in lungs of 2-3 mock control and 3-4 KPn infected WT and Mincle^{-/-} mice each from 3 independent experiments. Dot plots shown on the right are from one representative experiment. Statistical significance are denoted by asterisks (***, p<0.001). **B.** The lungs from mock control and KPn infected WT and Mincle^{-/-} mice were harvested at indicated time points post-infection and analyzed commercially for host immune mediators by rodent multi-analyte profile (MyriadTM Rules-Based Medicine, Austin, TX). Levels of neutrophil chemoattractants (CXCL2, CXCL6, GM-CSF) and activation markers (matrix metalloproteinase 9, MMP-9 and myeloperoxidase, MPO) shown are average of 3-4 each infected and mock control mice from 3 independent experiments. Amounts of mediators shown were significantly higher (***p<0.001) in Mincle^{-/-} mice at 3dp.i. and 5dp.i. in comparison with their levels in the WT mice at those time points tested.

Mincle^{-/-} neutrophils are defective in KPn phagocytosis

We next examined the bacterial uptake by Mincle^{-/-} neutrophils, in light of an increased bacterial burden in Mincle^{-/-} mice. For this, phagocytosis of GFP-labeled KPn was

compared between WT and Mincle^{-/-} neutrophils by flow cytometry. As shown in Figure 21, Mincle deficiency resulted in significantly reduced phagocytosis of non-opsonized bacteria by neutrophils. The uptake of opsonized bacteria was also reduced in Mincle^{-/-} neutrophils as compared to the Mincle-sufficient WT cells, however the differences were not statistically significant. These results indicate that Mincle is likely a novel non-opsonic phagocytic receptor for KPn and plays an important role in bacterial uptake by neutrophils.

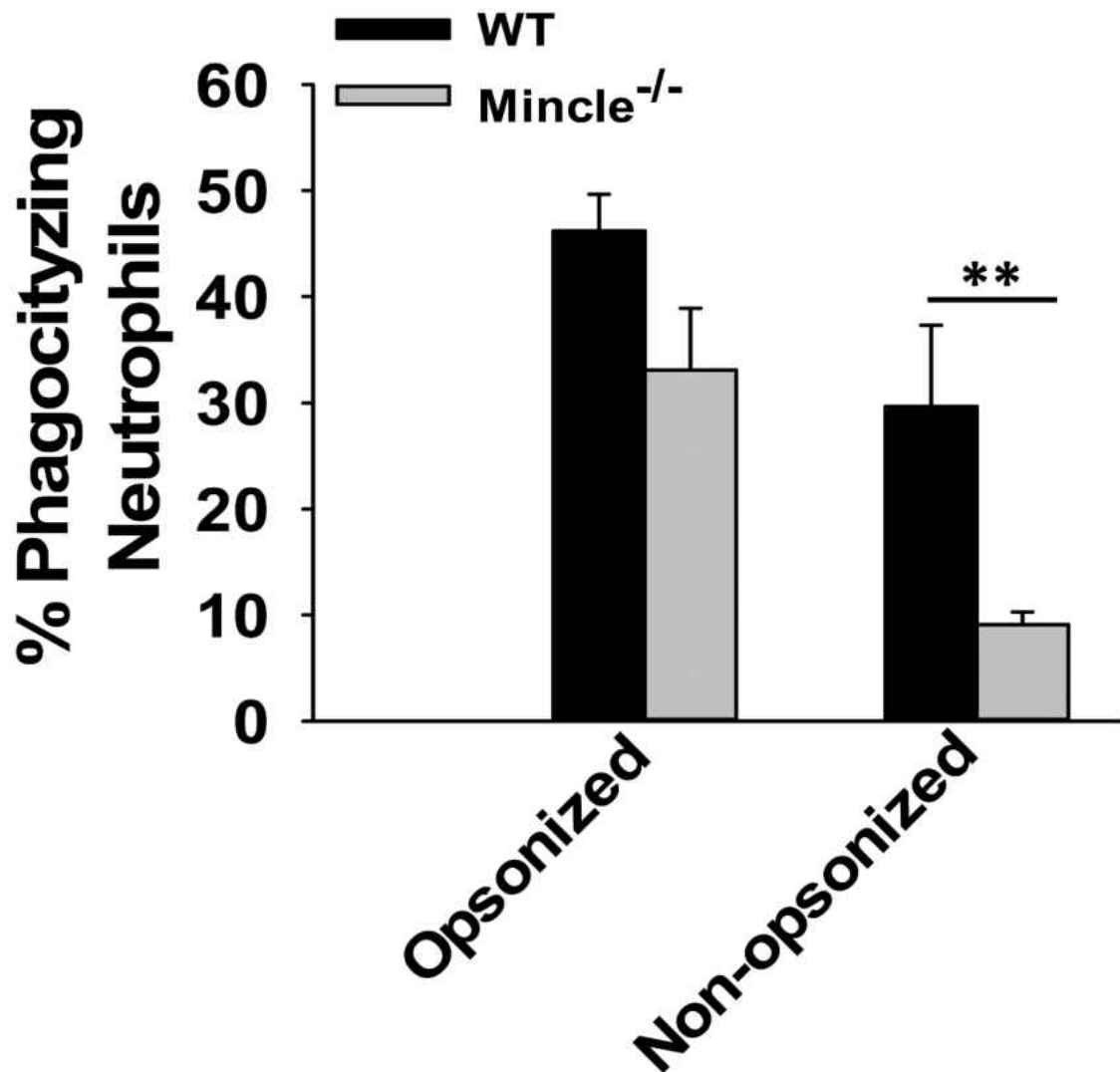
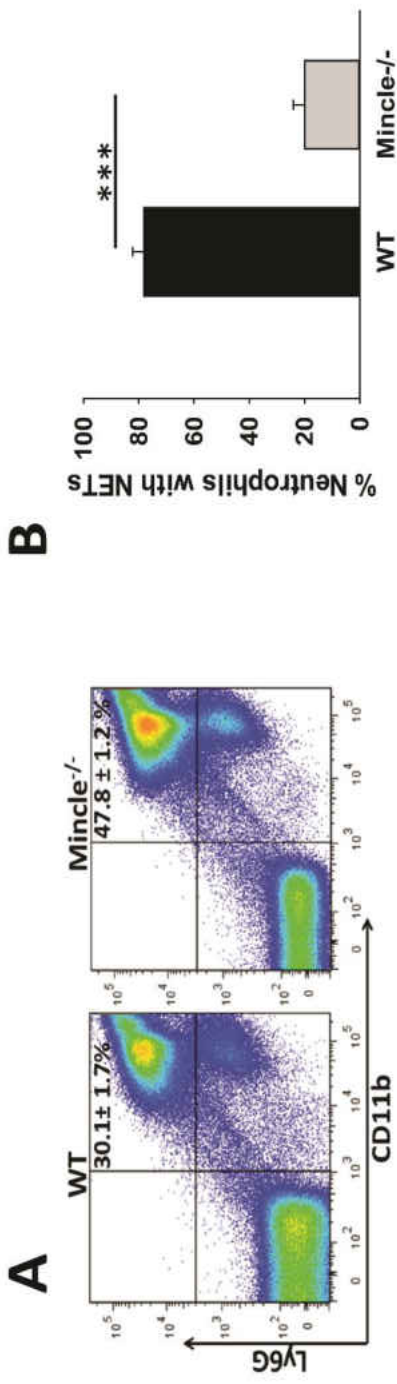


Figure 21. Mincle deficiency impairs neutrophil phagocytosis of non-opsonized bacteria Peritoneal neutrophils from WT and Mincle^{-/-} mice were incubated with GFP (Green Fluorescent Protein)-labeled KPn with (opsonized) or without (non-opsonized) 10% normal mouse serum for 1 hour followed by quantitation of phagocytosis by flow cytometry. The results are expressed as % cells positive for fluorescent bacteria. Significant differences are denoted by asterisks (**, p<0.005).

Mincle^{-/-} neutrophils are defective in NET formation

Extracellular trap formation is an important mechanism by which neutrophils clear extracellular bacteria. Since Neutrophil NET mediated killing has been shown to play a role in KPn clearance (237, 272), we sought to determine if Mincle deficiency resulted in a defect in NET formation. In order to minimize the tissue processing to avoid degradation of NETs, neutrophils isolated from BAL were used. Flow cytometry analysis showed that neutrophils were a predominant cell-type in the BAL of infected WT and Mincle^{-/-} mice (Figure 22A). A quantitative comparison of BAL neutrophils showed that significantly higher numbers Mincle sufficient WT neutrophils produced NETs (Figure 22B) which stained positive for neutrophil-specific enzyme neutrophil elastase (Figure 22C), showing that these fibrillar structures originated mainly from neutrophils. Furthermore, the NETs observed in Mincle^{-/-} neutrophils appeared dwarfed and lacked the web-like appearance as observed in the WT mice. This observation, together with reduced phagocytic ability of Mincle^{-/-} neutrophils shows that Mincle deficiency severely impairs neutrophil mediated bacterial uptake and clearance mechanisms in lungs during pneumonic KPn infection.



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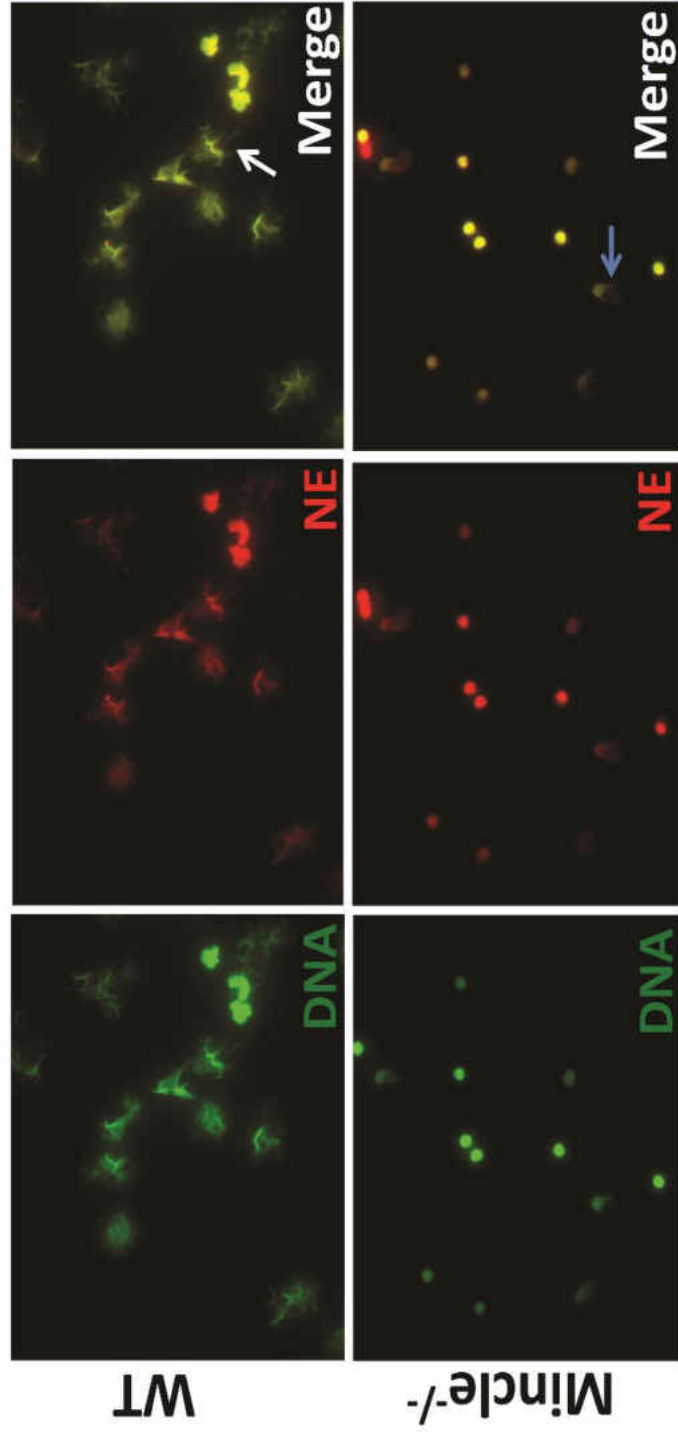


Figure 22. Mincle deficiency causes a defect in formation of neutrophil extracellular traps (NETs) in the lungs of mice upon pneumonic KPn infection

A. Enumeration of CD11b+Ly6G+ neutrophils by flow cytometry in BAL isolated from KPn infected WT and Mincle^{-/-} mice. **B.** Quantitation of neutrophils showing NETs in BAL isolated from WT and Mincle^{-/-} mice infected with KPn. (***)p<0.001). **C.** Representative fluorescence images of the neutrophils isolated from BAL fluid of WT (upper panel) and Mincle^{-/-} (lower panel) mice infected with KPn, and stained with Sytox Green to label DNA (green) and a rabbit anti-neutrophil elastase (NE) polyclonal antibody followed by goat anti-rabbit Alexa546 (red). The neutrophils from WT mice showed web-like structures that stained positive for NE and Sytox green (white arrow), while the Mincle^{-/-} neutrophils appeared inactive and displayed occasional small DNA fibers that lacked the typical web-like appearance of NETs (blue arrow). The experiment was repeated 3 times with 3-4 mice in each group. Magnification 400X.

DISCUSSION

Pneumonic sepsis is a major health care burden worldwide and *K. pneumoniae* (KPn) is the most frequent Gram-negative sepsis-associated opportunistic pathogen (224). An imbalance of innate immune responses resulting in deleterious and prolonged inflammation and impairment of protective functions of first responder cells such as neutrophils have been directly correlated with sepsis-associated mortality (23, 273, 274). This warrants an improved understanding of functioning of innate immune components in this deadly disease. In this study, we sought to determine the role of Mincle, an innate immune C-type lectin receptor in KPn pneumonia. Here we report several novel findings: 1) We show, for the first time, a clear phenotype in terms of a severely reduced survival rate of Mincle^{-/-} mice upon pneumonic KPn infection; 2) the reduced survival is not due to a defect in ability to mount an inflammatory response in absence of Mincle; 3) Mincle acts as a non-opsonic phagocytic receptor mediating uptake of KPn by neutrophils; and 4) Mincle deficiency results in a defect in neutrophil NET formation upon KPn infection. Our results thus show that Mincle is required for defense against KPn induced pneumonic sepsis and that lack of Mincle causes a defect in neutrophil mediated bacterial clearance mechanisms such as phagocytosis and NET formation.

Mincle has been previously shown to play a role in eliciting inflammatory responses against *Mycobacterium*, *Candida albicans* and skin fungal pathogens, *Malassezia* and *Fonsecaea* (104, 108, 109, 275). In these infections, Mincle expressed on macrophages, upon recognition of its ligands triggers FcR γ -Syk-Card9 pathway to induce production of protective Th1/Th17 responses as well as chemokines required for recruitment of

inflammatory cell types (85, 268, 275). The increased susceptibility to these infections in the absence of Mincle was measured in terms of increased bacterial and fungal burden which was attributed to reduced inflammatory response and defective pathogen clearance in these studies. However, overall survival of the experimental animals was not affected by Mincle deficiency. Our study for the first time, reports a clear outcome where Mincle seems to play a non-redundant role in survival of KPn infected pneumonic mice. Moreover, the reduced survival of Mincle^{-/-} mice is not due to their inability to mount an inflammatory response. These mice, instead exhibit hyperinflammation in their lungs suggesting that the protective ability of Mincle was independent of its role in eliciting inflammatory response. It is likely that the redundant function of other PRRs, upon recognition of bacterial PAMPs and endogenous alarmins generated from increased bacterial growth and accumulation of dead cells over time, is sufficient to induce inflammation in the absence of Mincle. Indeed, Mincle^{-/-} mice in our studies, exhibited overwhelming local as well as systemic bacterial burdens.

Concomitant to increased bacterial burden, Mincle^{-/-} mice exhibited extensive neutrophil accumulation, the primary cell type shown to play an important role in mediating protective immune response against Kpn infection (248, 263, 269, 276). We thus examined if Mincle^{-/-} neutrophils were defective in performing cellular functions such as internalization of bacteria via phagocytosis which would explain increased bacterial burden in Mincle^{-/-} mice despite a heightened inflammation. Indeed, Mincle^{-/-} neutrophils showed a mitigated phagocytosis of non-opsonized, but not opsonized KPn, suggesting a non-redundant and direct role of Mincle for internalization of non-opsonized bacteria. To the best of our knowledge, this is the first study reporting Mincle as a non-opsonic phagocytic receptor.

Ongoing studies in our lab are currently investigating Mincle specific ligand of KPn, nature of this interaction and production of specific antibodies that can inhibit this interaction. Lectinophagocytosis or lectin-mediated uptake by macrophages has been reported previously for several pathogens (reviewed in (148)). However, the receptors or mechanisms of non-opsonized phagocytosis of bacteria by neutrophils are poorly understood. Non-opsonic phagocytosis by receptors like Mincle may be important during early stages of infection before the onset of humoral immunity to generate opsonins, and in complement deficient or immunosuppressed patients. This mode of phagocytosis is particularly significant for inhaled bacteria as serum and complement components are as such limited in the alveolar space (277). Although appearance of serum components in alveolar space is common during severe KPn pneumonia, owing to high binding capacity of Mincle to mannose and N-acetylgucosamine (278), uptake by Mincle of KPn with mannan-rich capsule in lungs could be a major mechanism of bacterial clearance in lungs. Absence of Mincle and a resulting defect in initial phagocytic uptake of KPn, as observed in our studies likely contributes to the increased bacterial burden and subsequent inflammation via activation of other PRRs such as TLRs.

One of the more recently defined mechanisms of antimicrobial activity of neutrophils is extrusion of fibrous mesh of chromatin that entraps extracellular pathogens (59). These structures, termed NETs, are decorated with antimicrobial factors normally contained within neutrophil granules and represent an important strategy of neutrophils to immobilize and kill pathogens. Our observation reported in this study that Mincle^{-/-} neutrophils are defective in NET formation in-vivo during KPn infection coincides with overwhelming bacterial burdens in these mice. These results are in line with previous reports indicating

that NET mediated killing is an important mechanism of bacterial clearance and protection against KPn induced pneumonia (237, 272). How Mincle regulates NET formation is currently under investigation in our laboratory and is expected to provide novel insights into mechanism of NETosis. This will have important implications in chronic disease conditions where deregulated NET formation is associated with the pathophysiology.

Taken together, our results show that Mincle plays a protective role in KPn induced pneumonic sepsis by regulating neutrophil phagocytosis and NET formation, two important mechanisms of antimicrobial activity of neutrophils. Particularly, the novel observation of Mincle as a potential new component of NETosis pathway implicates this CLR in a much wider range of biological functions than initially surmised.

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CHAPTER 4

KLEBSIELLA PNEUMONIAE INFECTION OF NEUTROPHILS INHIBITS THEIR EFFEROCYTIC CLEARANCE BY MODULATING PHOSPHATIDYLSERINE EXPOSURE AND NECROPTOSIS ACTIVATION

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Conflict of Interest

The authors have no financial conflict of interest.

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Abstract

A profound neutrophilia and neutrophilic inflammation is characteristic of pneumoseptic infections with bacterial pathogens including *Klebsiella pneumoniae* (KPn). In homeostatic conditions, macrophages clear neutrophils by efferocytosis, via recognition of “eat me” signal phosphatidylserine (PS) exposed exofacially on these cells. We demonstrate here that infection with live, but not heat-killed KPn inhibits the efferocytic uptake of neutrophils in-vitro and in-vivo by macrophages. This infection-induced inhibition of efferocytosis correlated with a drastic decrease in surface exposed PS as well as induction of nonapoptotic cell death by necroptosis in KPn infected neutrophils. Concomitantly, treatment with sulfhydryl agent N-ethylmaleimide (NEM), which enhances PS externalization on plasma membrane, as well as necrostatin-1, a necroptosis inhibitor rescued the infected neutrophils from KPn mediated inhibition of efferocytic clearance. To our knowledge, this is the first report of inhibition of efferocytosis by KPn by PS redistribution and necroptosis, which likely promotes neutrophilia and persistent neutrophilic inflammation in this infection.

Keywords: Efferocytosis; *Klebsiella pneumoniae*; neutrophil turnover; phosphatidylserine; necroptosis, pneumonia, sepsis, neutrophilia

Introduction

Sepsis is one of the oldest and most elusive syndromes and despite extensive efforts to better understand and treat sepsis, it is still a major challenge in medicine (279). With no effective therapies there are over 750,000 cases of sepsis each year in the United States alone, which accounts for 10% of all ICU patients, leading to a mortality rate between 20-50% depending on certain risk factors (2). Pneumonia is the most frequent cause of sepsis (223-225). In particular *Klebsiella pneumoniae* (KPn), an opportunistic pathogen, accounts for 5-20% of all Gram-negative sepsis cases (223, 225). A notable emergence of antibiotic resistant strains of KPn in clinical settings has caused concerns over an already dwindling armamentarium of antibiotics. In this scenario, elucidation of protective host immune responses and pathogen-mediated manipulation thereof will likely provide novel therapeutic targets.

Neutrophils are the first cell types to infiltrate the site of infection and contribute to initial protective response. Indeed, in murine models of KPn infection, neutrophil-mediated responses are shown to be essential for initial control of the infection (248, 249). However, persistent accumulation of neutrophils and their over activation later causes neutrophil-dependent inflammatory diseases in the lungs, including bacterial pneumonia and sepsis (114, 280). A profound neutrophilia and the role of released alarmins in perpetuation of inflammation in septic bacterial infections has been shown by us recently (65, 66, 89, 90, 236). In *Klebsiella* infection, neutrophils have been reported to constitute a reservoir for this pathogen and aide in systemic dissemination of this infection (281). This underscores the importance of neutrophil turnover and resolution of neutrophil associated responses in

KPn pneumonia and sepsis. In this regard, clearance of neutrophils by phagocytic cells, mainly macrophages, occurs via efferocytosis which is a highly regulated receptor-dependent process (282, 283). Recognition of surface exposed “eat-me” signal phosphatidylserine (PS), which replaces the native “don’t eat me” signals on apoptotic cells, by macrophage cell surface receptors initiates engulfment and engulfment-associated downstream signaling events that induce a pro-resolving macrophage phenotype (284-286). Concomitantly, efferocytosis has been documented to play a vital role in controlling inflammation, by preventing the release of pro-inflammatory mediators from dead cells as well as by clearing pathogens not destroyed through phagocytosis (114, 157). While there is considerable research done on the efferocytosis pathways and its effect on disease development, studies on pathogen subversion of this important protective host response and the underlying mechanisms are extremely limited. Whether KPn can directly manipulate efferocytosis to promote disease is completely unknown.

In this study we determined if KPn infection of neutrophils modulates their clearance via efferocytosis by macrophages. Our results presented in this manuscript show that KPn inhibits efferocytic clearance of neutrophils by causing a live infection- and time-dependent decrease in the surface exposure of “eat-me” signal PS on the infected neutrophils as well as by driving the cell death away from immunologically silent apoptosis to a more inflammatory necroptosis pathway. Concomitantly, restoration of PS on KPn infected neutrophils as well as inhibition of necroptosis rescued the efferocytic uptake of these cells. Our studies present evidence for novel virulence mechanisms of KPn causing neutrophil turnover deficit which likely contributes to the genesis of KPn dissemination

and neutrophilic inflammation. PS and necroptosis targeted therapies may be useful to treat KPn infections in the face of constant emergence of antibiotic resistant strains of this bacterium.

Materials and Methods

Bacterial strains and Mice

The KPn (ATCC strain 43816) were grown to log phase in LB medium at 37°C. For isolation of cells and in-vivo experiments 6-8 weeks old wild-type C57BL/6 bred in the animal facility of the University of North Dakota were used. The animals were handled according to the institutional and federal guidelines.

Flow cytometry analysis of “Eat-me and “Don’t eat me” signals

Peritoneal neutrophils isolated from mice 12-16 hrs after intraperitoneal injection with sterile 4% thioglycollate (BD Biosciences, San Jose, CA) as described by us previously (65, 236). Purity was ensured by quantitating Ly6G+CD11b+ cells by flow cytometry (85-90 % neutrophils). Isolated neutrophils were left uninfected or infected with 10 MOI of KPn. Cells were stained with PerCP-Cy5.5 labelled anti-CD31 (Biolegend, San Diego, CA) or FITC abelled anti-CD47 (Biolegend, San Diego, CA) antibodies to detect “Don’t eat-me” signals by flow cytometry as described by us (65, 236). A FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA) was used according to manufacturer’s instructions followed by flow cytometry analysis at indicated times to enumerate percentage of PI- Annexin V+ cells with surface exposed “Eat-me” signal PS using BD LSR II flow cytometer (Becton Dickinson, San Jose, CA). FlowJo (Tree Star) software was used to analyze the data.

In-vivo Efferocytosis

Peritoneal neutrophils were infected with KPn (MOI 10) for 2 hours before killing of extracellular, non-internalized bacteria with antibiotics, then labelled with Carboxyfluorescein succinimidyl ester (CFSE; Cell Trace™ CFSE Cell Proliferation Kit

from Invitrogen) per manufacturer's instructions. CFSE-labelled infected or uninfected neutrophils were suspended in complete RPMI and $35\mu\text{L}/3.5 \times 10^6$ cells were administered intranasally into mice anaesthetized using a mixture of 30mg/ml ketamine and 4 mg/ml xylazine in PBS. Lungs were lavaged as described by us (65, 236) 2 hrs after the instillation and alveolar macrophages analyzed by flow cytometry using PE-Cy7 conjugated anti-F4/80 and APC conjugated anti-Ly6G antibodies (BioLegend, San Diego, CA). Efferocytic uptake by macrophages was quantitated by flow cytometry to enumerate Ly6G-F4/80+ CFSE+ cells. Efferocytic index was calculated as the percent difference between uninfected and KPn infected neutrophils. All other statistical analyses were performed using the Student t test (SIGMA PLOT 8.0, Systat Software, San Jose, CA).

In-vitro Efferocytosis

Peritoneal neutrophils and macrophages were isolated 12-16hr and 5 days respectively after intraperitoneal injection of sterile 4% thioglycollate. Macrophages were seeded on 6 well plates at a density of 0.5×10^6 cell/ml for 1-2 hr before adding CFSE-labelled uninfected or KPn infected neutrophils (prepared as described above) at a ratio of 5:1. After 2 hrs cells were thoroughly washed, scraped and stained with PE-Cy7 conjugated anti-F4/80 and APC conjugated anti-Ly6G antibodies for flow cytometry. For some experiments, uninfected and infected neutrophils were treated with 5 mM of N-ethylmaleimide (NEM) or neutralizing antibodies against CD31 and CD47 (BioLegend, San Diego, CA), or with necroptosis inhibitors necrostatin-1 (100 μM) or GSK'872 (3 μM) (Millipore Sigma, St. Louis, MO) before incubating with macrophages. Efferocytic index was calculated as described above.

Western blot Analysis

For detection of necroptosis markers, peritoneal neutrophils uninfected or infected with 10 MOI KPn for 3 hours were lysed with RIPA buffer and probed with anti-mouse Caspase 8, RIPK1 antibodies (Cell Signaling Technology, Danvers, MA) and pRIP3 S232 & T231, pMLKL S345 antibodies (Abcam, Cambridge, MA) by immunoblot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Peritoneal neutrophils treated with 1 μ M Smac mimetic AT-406 and 20 μ M Q-VD for 30 min prior to stimulating with 100 ng/mL mouse TNF- α were used as positive control for necroptosis induction (287).

Statistical analyses

Statistical analyses were performed using the Student t test (SIGMA PLOT 8.0, Systat Software, San Jose, CA). A p value of ≤ 0.05 was considered statistically significant.

Results

KPn infection inhibits efferocytosis of neutrophils

In order to determine the effect of KPn infection on efferocytosis of neutrophils, we compared the uptake of CFSE labelled uninfected or KPn infected neutrophils by macrophages using flow cytometry. As shown in Figure 23A, after 3 hrs of incubation with CFSE labelled neutrophils, $32.7\% \pm 5.4\%$ macrophages stained positive for uptake of uninfected neutrophils. On the other hand, only $13.9\% \pm 1.6\%$ macrophages had efferocytosed KPn infected neutrophils which was significantly lower than their uninfected counterparts. This indicated that infection of neutrophils with KPn caused a significant reduction in their efferocytic uptake by macrophages. Importantly, this infection-induced inhibition was largely dependent on live bacteria and/or active bacterial replication because heat-killed KPn were significantly less potent in inhibiting efferocytosis of neutrophils causing a reduction only by $29.1\% \pm 2.3\%$ of the uninfected neutrophils, compared to live bacteria which inhibited the efferocytosis by $57.2\% \pm 1.4\%$ (Figure 23B).

We next investigated if KPn infection can inhibit efferocytic clearance of neutrophils in-vivo in lungs by alveolar macrophages. For this, CFSE labelled uninfected or KPn infected neutrophils were inoculated intranasally into anesthetized mice followed by isolation and flow cytometry analysis of alveolar macrophages as described in methods. Consistent with our in-vitro results, KPn infection of neutrophils reduced their efferocytic uptake by $45.1\% \pm 1.6\%$ compared to the uninfected cells (Figure 23C). Together, these data strongly suggested that KPn infection inhibits the efferocytic clearance of neutrophils by macrophages in-vitro and in-vivo.

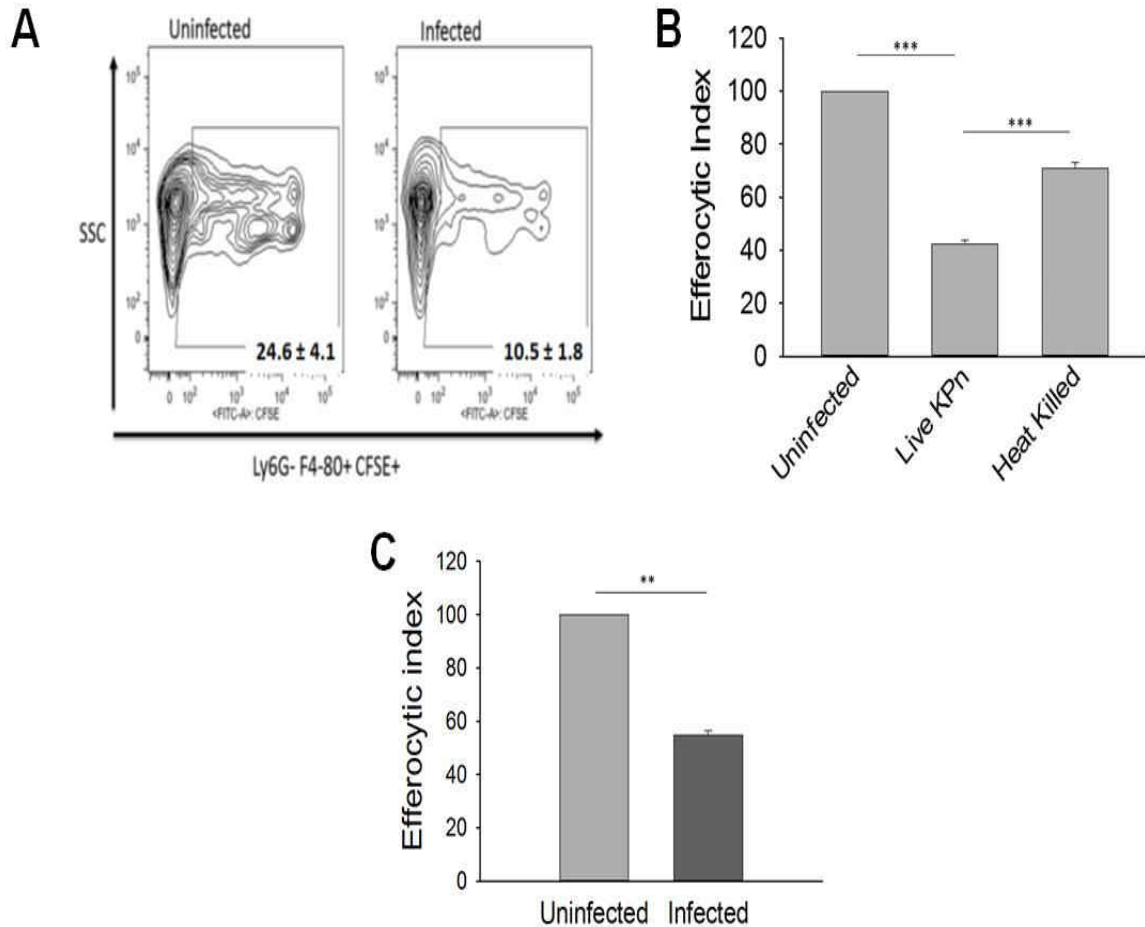


Figure 23. Live KPn infection inhibits efferocytosis of neutrophils

Neutrophils left uninfected or infected with live (A) or heat-killed (B) KPn at MOI 10 for 2 hours were labelled with CFSE and incubated with macrophages at 5:1 ratio for 2 hours. The cells were washed thoroughly and the macrophages were processed for flow-cytometry by staining with anti-F4/80 and anti- Ly6G antibodies. The percent of Ly6G-F4/80+CFSE+ cells indicating macrophages that have internalized CFSE labelled neutrophils calculated from 5 independent experiments is shown in the representative density plots. The efferocytic index was calculated as the percent difference between uninfected and KPn infected neutrophils, (**, $p < 0.005$; ***, $p < 0.001$).

Figure 23 cont. Live KPn infection inhibits efferocytosis of neutrophils

(C). CFSE labelled uninfected or KPn infected neutrophils were instilled intranasally in anaesthetized mice (3.5 million cells in 35 μ l PBS). Two hours later lungs were lavaged as described in methods and cells were stained with PE-Cy7 conjugated anti-F4/80 and APC conjugated anti-Ly6G antibodies for flow cytometry. Efferocytic uptake by macrophages was quantitated by enumeration of Ly6G- F4/80+ CFSE+ cells. Efferocytosis of uninfected neutrophils was taken as 100% and efferocytic index was calculated as the percent difference between uninfected and KPn infected neutrophils. Data are from 3 mice per group in 3 independent experiments (**, $p < 0.005$).

Effect of KPn infection on “don’t eat me” signals expressed by neutrophils

Expression of specific cell surface molecules called the “don’t eat me” signals on healthy cells prevents their efferocytosis by phagocytes (288). We determined if impaired efferocytosis of KPn infected neutrophils could be attributed to these negative regulatory signals. Flow cytometry analysis showed that KPn infected neutrophils exhibited significantly increased surface expression of CD47 and CD31, two well-defined “don’t eat me” signals, as compared to the uninfected neutrophils (Figure 24A). However, upregulation of these signals on KPn infected neutrophils did not appear to contribute to the inhibition of their efferocytic uptake since the inhibitory effect of KPn infection remained intact even after blocking neutralization of CD47 and CD31 individually or in combination (Figure 24B). This indicated to us that “don’t eat me” signals do not play any significant role in KPn mediated inhibition of neutrophil efferocytosis.

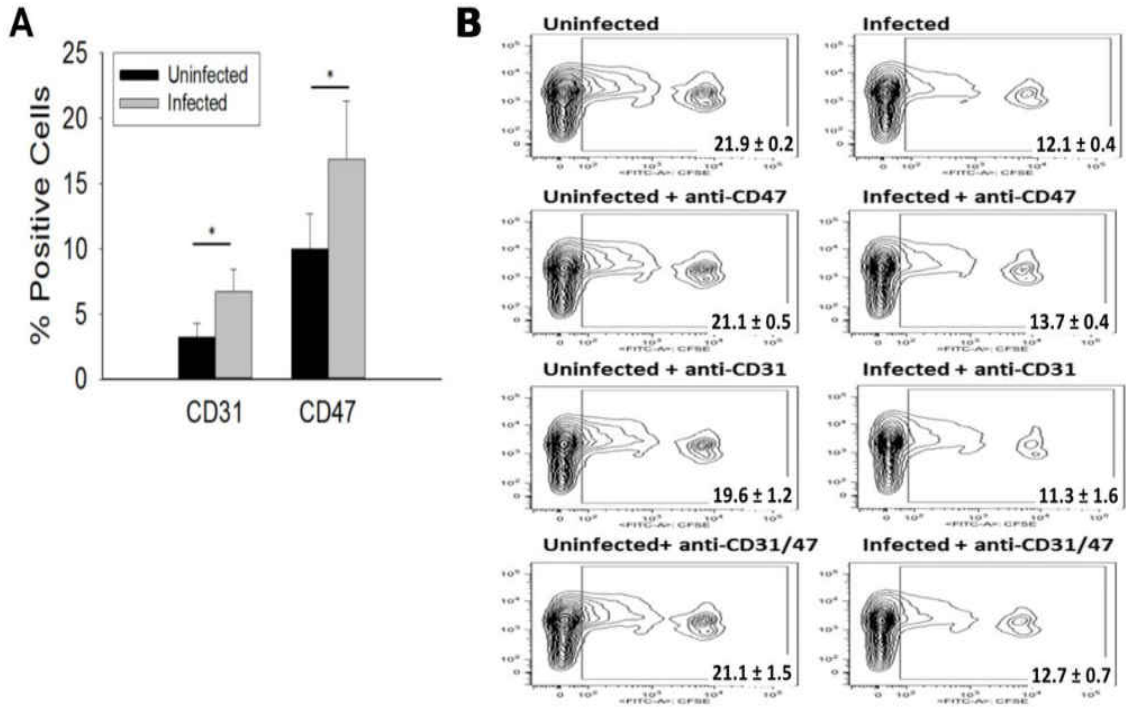


Figure 24. “Don’t eat me” signals do not play any role in efferocytic inhibition of KPn infected neutrophils

(A). Neutrophils were left uninfected or infected with KPn (MOI 10) for 3 hrs. Cells were then co-stained with antibodies against neutrophil marker Ly6G and the “don’t eat me” signals CD31 and anti-CD47. Each bar represents percent of Ly6G positive neutrophils expressing CD31 or CD47. Data are mean ± SEM from 4 independent experiments (*, $p < 0.05$). (B). CFSE labelled neutrophils uninfected or infected with KPn were incubated with 5 μ g/mL anti-CD47 or anti-CD31 blocking antibodies individually or in combination 30 min. prior to incubating with macrophages for 2 hrs. Efferocytic uptake by macrophages was quantitated by flow cytometry using neutrophil specific anti-Ly6G and macrophage specific anti-F4/80 antibodies to enumerate percent of efferocytic cells. Numbers on the representative density plots show percent of Ly6G- F4-80+ CFSE+ cells from 3 independent experiments.

KPn infection of neutrophils causes reduced exposure of “eat me” signal phosphatidylserine

We next examined if modulation of “eat-me” signal on neutrophils played any role in KPn mediated inhibition of efferocytosis. Phosphatidylserine (PS) is externalized to the plasma membrane of apoptotic cells, and has been classically characterized as an “eat me” signal essential for the recognition and efferocytosis by phagocytes (288). We thus compared the kinetics of PS externalization in uninfected and KPn infected neutrophils by flow cytometry using Annexin V which binds to PS. Propidium iodide was used to exclude necrotic cells from analysis. As shown in Figure 25A, uninfected neutrophils exhibited surface exposure of PS after 30 min. of culture, which gradually increased over a period of 5 hrs. The KPn infected neutrophils, on the other hand, showed similar levels of PS exposure as their uninfected counterparts for the initial two hours ($19.7\% \pm 3.3\%$ in KPn infected and $19.3\% \pm 4.6\%$ in uninfected neutrophils). By 3 hrs post-infection, however, a drastic reduction in PS exposure was observed in KPn infected neutrophils ($9.7\% \pm 1.9\%$ in KPn infected compared to $21.8\% \pm 6.5\%$ in uninfected neutrophils) which remained significantly lower than that in the uninfected cells up to 5 hrs ($2.5\% \pm 0.3\%$ in KPn infected compared to $33\% \pm 7.1\%$ in uninfected neutrophils), the time at which the experiment was terminated. Importantly, this sharp decrease in the PS exposure in KPn infected neutrophils at 3 hrs coincided with the time by which these neutrophils were incubated with macrophages for efferocytic uptake, indicating that this phenomenon is likely responsible for the reduced uptake of KPn infected neutrophils by macrophages. This reduced PS exposure was not due to necrosis as the number of PI positive cells did not increase over time (data not shown).

KPn infection activates necroptosis in neutrophils

As PS exposure is regarded a measure of apoptotic cell death, given the reduced PS exposure we examined if KPn infection modulates the cell death mechanism of neutrophils. In this regard, Caspase 8 is centrally positioned to control the extrinsic pathway of apoptosis as well as necroptosis by activating caspase 3 and 7 and by degradation of RIPK1, which is required for recruitment of RIPK3, eventuating in phosphorylated mixed lineage kinase domain-like protein (MLKL)-dependent necroptosis. Western blot analysis of Caspase 8 and RIPK1 in uninfected and KPn infected neutrophils showed reduced processing of procaspase 8 into active Caspase 8 (Figure 25B). This coincided with the reduced degradation of RIPK1 in these cells, indicating inhibition of apoptosis and activation of necroptosis. Indeed, phosphorylation of RIPK3 and MLKL was increased in KPn infected neutrophils confirming the activation of necroptosis in these cells upon infection (Figure 25B). As a positive control neutrophils treated with TNF-alpha in combination with caspase inhibitor Q-VAD were analyzed for necroptosis activation in the absence of caspase-8 activity, which showed a similar inhibition of caspase 8 processing and RIPK1 cleavage and increased phosphorylation of RIPK3 and MLKL (Figure 25B first lane). Taken together these data strongly suggested that KPn infection inhibits apoptosis, a cell death favorable for efferocytic clearance and instead activates pro-inflammatory necroptosis of neutrophils.

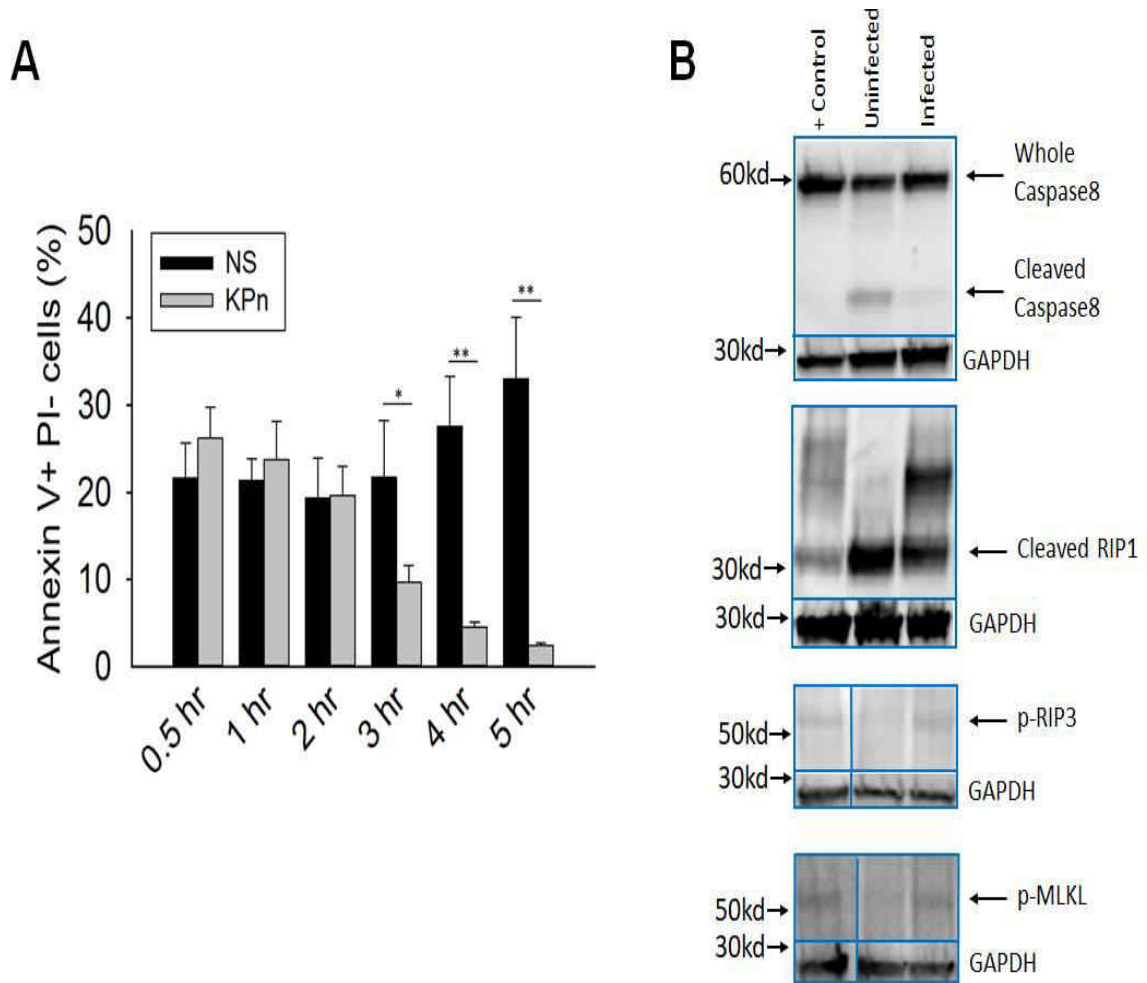


Figure 25 KPN infection decreases the level of surface exposed “eat me” signal phosphatidylserine (PS) on infected neutrophils and induces necroptosis

(A). Neutrophils were left uninfected or were infected with KPn (MOI 10). The level of “eat me” signal PS was monitored by over time by Annexin V staining using a Annexin V Apoptosis Detection followed by flow cytometry analysis. Propidium iodide (PI) was used to exclude necrotic cells. Percent of Annexin V+ PI- cells at indicated times is shown (mean \pm SEM from 5 independent experiments; *, $p < 0.05$; $p^{**} < 0.005$).

Figure 25 cont. KPn infection decreases the level of surface exposed “eat me” signal phosphatidylserine (PS) on infected neutrophils and induces necroptosis

(B). Immunoblots for caspase-8, RIPK1, pRIPK3 and pMLKL in whole cell lysates from uninfected and KPn infected neutrophils are shown. Lysate from primary neutrophils treated with TNF- α with pan-caspase inhibitor Q-VD and Smac mimetic was used as positive control for necroptosis activation. GAPDH was used as loading control.

Restoration of PS exposure and inhibition of necroptosis rescues the efferocytic uptake of KPn infected neutrophils

Given our data showing KPn-infection induced necroptosis and reduced externalization of PS, we determined if restoration of PS and necroptosis inhibition will rescue the efferocytic uptake of KPn infected neutrophils. In this regard, treatment with a sulphydryl modifying agent N-ethylmaleimide (NEM) triggers flipping of PS to outer plasma membrane of cells (289-291). As shown in Figure 26A, NEM treatment of KPn infected neutrophils reversed the PS exposure on their surface as $52.1\% \pm 7.7\%$ cells stained positive for Annexin V staining by flow cytometry at 3 hrs as compared to the untreated KPn infected neutrophils on which the PS exposure remained low ($9.9\% \pm 2.1\%$). Having established that NEM treatment could reverse KPn infection-induced reduction of PS surface exposure, we next tested if efferocytic uptake of KPn infected neutrophils was improved following NEM treatment. Indeed, reversal of PS externalization upon NEM treatment restored the efferocytic uptake of KPn infected neutrophils to the levels of uninfected neutrophils which was significantly higher than the untreated KPn infected cells ($26.5\% \pm 1.7\%$ NEM treated KPn infected Vs $10.1\% \pm 1.8\%$ of untreated KPn infected) (Figure 26B).

To establish the role of KPn induced necroptosis in efferocytosis inhibition, we similarly treated KPn infected neutrophils with necrostatin-1, a RIPK1 inhibitor which suppresses necroptosis (292). As shown in Figures 26 C and D, treatment with necrostatin-1 reversed the inhibitory effect of KPn infection on efferocytic uptake of neutrophils by restoring it to the levels similar to uninfected cells. Necrostatin-1 treatment itself did not affect the efferocytosis of uninfected cells. Because necroptosis-independent functions of RIPK1 have been reported (293), we also tested the effect of RIPK3 inhibitor GSK'872 to further confirm the role of necroptosis in inhibition of efferocytosis of KPn infected neutrophils. Indeed, GSK'872 treatment of KPn infected neutrophils increased their efferocytic uptake by macrophages to 80% (Figure 26 E and F). This strongly implicated the activation of necroptosis as an important mechanism underlying impaired efferocytosis of KPn infected neutrophils. Interestingly, RIPK1 or RIPK3 inhibition did not affect the level of externalized PS in the infected neutrophils (data not shown), indicating that necroptosis induction and PS exposure are, likely two independent mechanisms which KPn employs to inhibit the efferocytosis of neutrophils. Taken together, these data showed that KPn infection inhibits the efferocytic clearance of neutrophils by reducing the surface exposure of PS, a classical “eat-me” signal as well as by driving the cells to necroptosis. Consequently, restoration of exofacial PS as well as suppression of necroptosis pathway reverses this inhibitory effect of KPn infection.

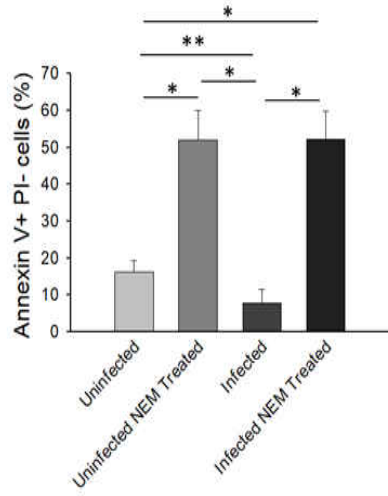
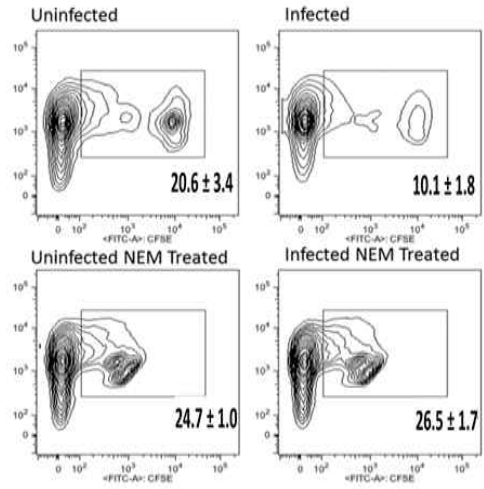
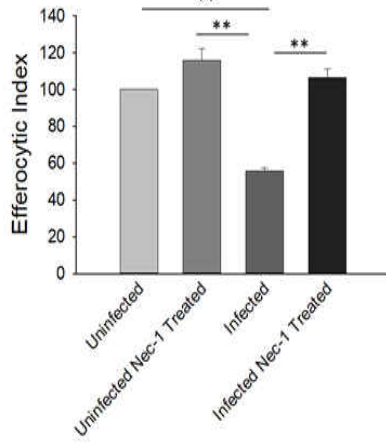
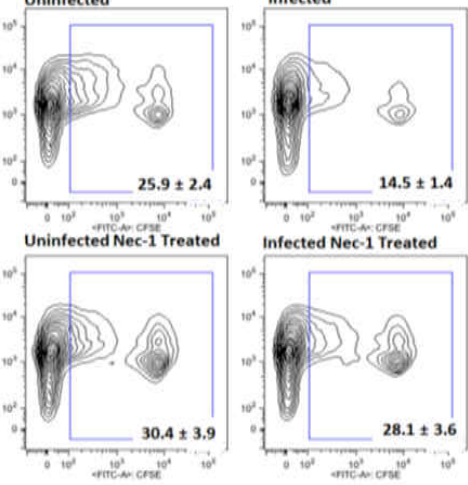
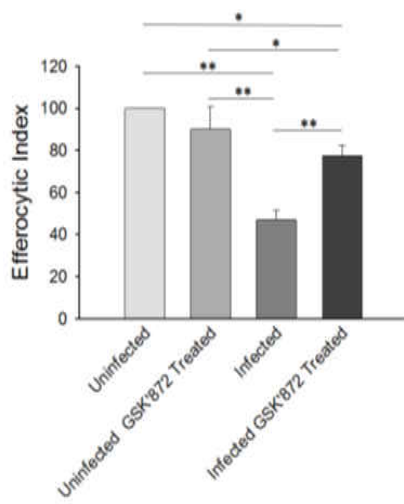
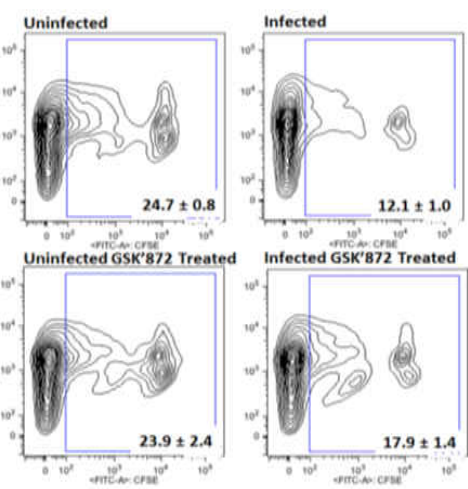
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Figure 26. Restoration of PS exposure and inhibition of necroptosis rescues the efferocytic uptake of KPn infected neutrophils

(A). Treatment with N-ethylmaleimide (NEM) rescues surface exposed “eat me” signal PS in KPn infected cells and restores efferocytosis. The level of surface exposed PS in uninfected or KPn infected neutrophils with or without NEM treatment (5mM for 30 min) was measured by Annexin V staining followed by flow cytometry. Percent of Annexin V+ PI- cells at 3hrs post-infection is shown (mean \pm SEM from 3 independent experiments, ** $p < 0.005$; ***, $p < 0.001$). (B) Uninfected and KPn infected neutrophils were treated or left untreated with NEM. The efferocytosis of these cells by macrophages was performed followed by flow cytometry as described above. Numbers on the representative density plots show percent of Ly6G- F4/80+ CFSE+ efferocytic cells (mean \pm SEM) from 3 independent experiments. (C&E) Efferocytic index and (D&F) percent of Ly6G- F4/80+ CFSE+ cells efferocytosing uninfected and KPn infected neutrophils with and without treatment with RIPK-1 inhibitor Necrostatin-1 (C&D) or RIPK3 inhibitor GSK’872 (E&F). Data is presented as (mean \pm SEM) from 3 independent experiments.

Discussion

During an acute injury neutrophils are promptly recruited to the site which, upon the resolution of insult, are cleared by efferocytosis to maintain homeostasis (114). Given its host protective consequences, subversion of efferocytosis may be advantageous for pathogens to establish infection. Here we report that *Klebsiella pneumoniae*, an opportunistic pathogen causing pneumonic sepsis characterized by neutrophilia and hyperinflammation, prevents efferocytic clearance of neutrophils. We show that a down modulation of cell surface exposure of PS, an “eat-me” signal recognized by the phagocytes for efferocytic internalization of neutrophils, and modulation of cell death mode toward necroptosis are the underlying mechanisms by which KPn subverts efferocytosis. Our study provides important insights into pathogenic mechanisms that can be targeted for future antimicrobial therapies for this infection.

KPn has recently gained attention as a “successful” pathogen owing to an emergence of hypervirulent strains as well as antibiotic resistance (294). The wide range of infections caused by this pathogen in immunocompromised and immune-competent individuals have become increasingly difficult to treat owing partly to the arsenal of virulence factors exhibited by this pathogen that it utilizes to protect itself from host immune response (295). Based on these virulence factors KPn has been categorized as an “evader” rather than an “offender” pathogen. For example, a polysaccharide capsule, lipopolysaccharide, Type 1 and 3 fimbriae and outer membrane proteins have been identified and characterized as factors required for survival and growth of the pathogen within the host (176). Our results presented in this manuscript, show for the first time, strategies employed by this pathogen

to actively suppress an important host defense i.e. efferocytic clearance of neutrophils by modulation of cell death pathway and PS externalization. Our data showing downregulation of PS exposure, reduced activation of apoptosis executioner Caspase 8 and activation of necroptosis in neutrophils infected with KPn highlight strategies employed by this pathogen to impair efferocytic clearance of these cells by macrophages. In this regard it is important to note that a recent report showed neutrophils as vehicles for KPn for its dissemination to establish liver abscess, a severe clinical complication of KPn infection (281). Efferocytic clearance of KPn-infected neutrophils thus may aid in circumventing these distant metastatic complications, as has been shown in case of mycobacterial infection (157). These findings open new avenues to treat and prevent the systemic spread of this infection.

Efferocytic clearance of apoptotic cells is a highly orchestrated event involving specific receptors on phagocytic cells that discriminate live cells from dead/dying cells by recognizing “don’t eat-me” and “eat me” signals respectively (288). Phosphatidyleserine (PS) is considered the most well-characterized “eat-me” signal recognized by the phagocytes via receptors such as Bai1 and Tim4 for removal of apoptotic cells (296). Owing to an efficient and immunologically silent nature of this event, many viruses and parasites have been reported to use PS mimicry by concentrating PS on their surface and hijack efferocytic machinery of host cells to promote their internalization and cell-to-cell spread (159, 297, 298). In contrast to this, pathogen -mediated skewing of efferocytic clearance of immune cells via PS recognition is much less studied. *Staphylococcus aureus* is shown to inhibit efferocytic clearance of neutrophils via upregulation of CD47, a “don’t

eat me” signal and necroptosis (160). In our study “don’t eat me” signals, although upregulated on neutrophils upon infection, did not appear to play any significant role in inhibition of efferocytosis. Instead, KPn infected neutrophils showed a drastic down modulation in the externalization of “eat-me” signal PS. PS distribution on the lipid bilayer in biological membranes is controlled by the activity of ATP-dependent flippases and scramblases (299). Flippases contribute to PS maintenance in the inner leaflet via ATP dependent transport of phospholipid from the outer to inner membrane. Scramblases on the other hand, transport PS to the outer leaflet. Whether the decreased PS exposure on the outer membrane of KPn infected neutrophils is due to KPn mediated modulation of flippases and scramblases, is an exciting area of mechanistic research which we are currently pursuing. Notwithstanding the molecular mechanism, the relevance of this event was confirmed by reversal of PS exposure on KPn infected neutrophils by NEM treatment which rescued their efferocytic clearance. To the best of our knowledge, this is the first report of pathogen mediated inhibition of PS exposure on neutrophils to avoid their efferocytic clearance. PS-dependent clearance of immune cells results in the release of anti-inflammatory and pro-resolving factors which downregulate the inflammation (300). In addition, recent studies have shown that efferocytic uptake of apoptotic neutrophils regulates granulopoiesis and peripheral neutrophilia via an IL-23/IL-17/G-CSF axis (262). KPn mediated inhibition of neutrophil clearance may result in the loss of this negative regulatory axis thus promoting the neutrophilia and inflammation characteristic of this infection. In addition, maintenance of neutrophilic niche may help disseminate the infection to systemic sites.

Necroptosis is defined as a caspase-independent non-apoptotic form of regulated cell death which requires RIPK1 and RIPK3-mediated activation of pseudokinase, mixed lineage kinase-like protein (MLKL) (301). Extrinsic pathway of apoptosis activated by death ligands such as FasL or TLR ligands promotes processing of caspase-8 which degrades RIPK1 and drives apoptotic cell death (302). Conversely, inhibition of caspase 8 spares RIPK1 to promote RIPK3 autophosphorylation and recruitment of MLKL to form a necroptosome complex (303). Phosphorylation of the C-terminal pseudokinase domain of MLKL by RIPK3 promotes MLKL translocation to plasma membrane which then disrupts the membrane integrity (304). Our results showed reduced processing of caspase 8 in KPn infected neutrophils indicating the inhibition of apoptosis in these cells. This coincided with increased RIPK1 levels, RIPK3 phosphorylation and MLKL phosphorylation strongly suggesting the activation of necroptosis pathway in KPn infected neutrophils. This supports the notion that KPn drives the cell death away from immunologically silent apoptosis which favors efferocytosis, toward necroptosis which lacks “eat-me” signals and promotes inflammation (305). While inhibition of apoptosis by KPn has recently been reported (306), our studies present physiological consequence of this phenomenon eventuating in reduced PS externalization and modulation of cell death pathway relevant to KPn infection induced inhibition of efferocytosis. This is supported by increased efferocytic uptake of infected neutrophils upon treatment with NEM to increase PS exposure as well as with necrostatin-1 and GSK’872, which are inhibitors of necroptosis. It is noteworthy here that necroptotic fibroblasts and monocytic cell lines were recently reported to expose PS on their surface in pMLKL-dependent fashion (307, 308). While these findings indicate a novel function of necroptosis pathway in PS exposure in these

cells, the inhibition of PS exposure and induction of necroptosis in KPn infected primary neutrophils in our studies appears to be via two independent pathways since inhibition of necroptosis did not affect the levels of PS on KPn infected neutrophils (data not shown). In light of our results showing the rescue of efferocytosis upon increased PS exposure by NEM treatment as well as upon inhibition of necroptosis it is highly likely that KPn utilizes two independent strategies to inhibit the clearance of neutrophils and to drive the neutrophilia and inflammation characteristic of pneumonic infection. As the antibiotic resistance is a serious problem associated with Klebsiella infections, elucidation of mechanism by which this pathogen manipulates efferocytosis, as reported here, might provide novel therapeutic targets.

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CHAPTER 5

DISCUSSION

General Discussion

As described in the Introduction section, sepsis continues to be a major healthcare burden. Despite decades of dedicated research efforts, occurrence of sepsis and septic shock is rising and the mortality rate has not changed over the past three decades (309, 310). A likely reason as to why it has been so difficult to find effective treatment for sepsis is that immune regulatory processes that respond to sepsis are not completely understood (273, 311). An overwhelming inflammatory response, termed “cytokine storm” or hypercytokinemia resulting from stimulation with pathogen-derived (PAMPs) or host-derived factors (alarmins) is thought to be responsible for shock, multiple-organ failure and eventual death in septic patients (23). However, anti-inflammatory interventions have not been a successful treatment option for sepsis. This highlights the need to take into account, not only the initiation of inflammation, but also the resolution thereof, which favors tissue repair. In this regard, neutrophil mediated responses are essential for combating pneumonic bacterial infection and their protective role in sepsis has been described (248, 249). However, persistent accumulation of neutrophils can lead to bystander tissue destruction, owing to their tissue destructive cargo. Neutrophil turnover and resolution of neutrophil

associated responses is thus, an integral part of a resolving inflammatory response to an infectious insult such as pneumonic infection. My dissertation revolves around the hypothesis that a failure of this process likely contributes to the acceleration of inflammation resulting in the development of sepsis. This is a highly understudied area of research. The studies described here provide an understanding of the host factors regulating neutrophil mediated responses and their perturbations by pathogen in a setting of pneumonic sepsis, which will likely provide new targets for therapeutic intervention of this deadly immune disorder.

In chapter 1 I presented an overview of the disease burden, inflammatory immune responses, clearance of dead cells via efferocytosis, mechanisms by which bacteria subvert host immune response to establish infection, and animal models used to study sepsis. Chapters 2 and 3 elucidated the protective roles of the CLR, MGL1 and Mincle, in pneumonic sepsis. In chapter 4, a novel mechanism by which the pathogen KPn, modulates the host response in order to prevent its clearance via efferocytosis was presented. The remainder of chapter 5 will summarize and discuss the new findings from this work.

Protective role of CLR during inflammation and sepsis

C-type lectin receptors (CLR) are considered pattern recognition receptors (PRR) and have been shown to play a role in numerous inflammatory and homeostatic host functions (82). Surprisingly, little is known about the role of CLR in the development of pneumonic sepsis. We set out to characterize the function of CLR in pneumonic sepsis using our KPn infection model. Initial transcript level screening of more than 60 CLR showed several CLR upregulated at in the lungs of these pneumonic septic mice. I focused on two of those

CLRs namely Macrophage Galactose Type Lectin 1 (MGL1) and Mincle (Clec4e). We found that in the absence of either of these CLRs, mice were highly susceptible to KPn infection compared to WT mice and most mice succumbed to infection by 5 days post infection. This data suggested that both MGL1 and Mincle play a protective role in pneumonic sepsis. In both MGL1^{-/-} and Mincle^{-/-} mice infected with KPn we observed severe lung pathology along with a hyperinflammatory response with massive increases in pro- and anti-inflammatory cytokines when compared to infected WT mice 3 days post infection. There was also a massive accumulation of neutrophils in the lungs of both infected CLR knockout mice compared to infected WT mice. However, a kinetic analysis of bacterial burden in these knockout mice showed disparate phenotypes. Whereas MGL1^{-/-} mice showed no difference in bacterial burden when compared to WT mice, Mincle^{-/-} mice displayed increased systemic and local bacterial burden compared to WT mice. This indicated distinct roles of these two CLRs in KPn infection induced pneumonic sepsis. Considering a massive neutrophil influx in the absence of both MGL1 and Mincle, and the fact that both CLRs are expressed mainly by myeloid cells, we further analyzed the function of neutrophils in MGL1^{-/-} and Mincle^{-/-} mice. We found that while MGL1^{-/-} neutrophils were fully competent in bacterial clearance mechanism (bacterial uptake by phagocytosis as well as NET formation), Mincle^{-/-} neutrophils were defective in these functions. No functional defect seen in MGL1^{-/-} neutrophils, but a massive influx of neutrophils into the lungs of infected MGL1^{-/-} mice, indicated to us two possible scenarios. One possibility is that MGL1 plays a role in neutrophil influx and in the absence of MGL1 the neutrophils infiltrate the lungs in increased numbers. The other possibility is a defect in the ability of MGL1^{-/-} mice to clear apoptotic cells via efferocytosis thus causing their

increased accumulation in their lungs. To this end, we began by looking at the role of MGL1 in neutrophil influx by adoptive transfer experiments of WT and MGL1^{-/-} neutrophils. Those experiments showed that the absence of MGL1 on neutrophils and not endothelial cells, led to the increased neutrophil influx as seen in MGL1^{-/-} mice. Additional experiments looking into neutrophil mobilization markers, showed that by 3 days post infection MGL1^{-/-} had significantly higher levels of GCP-2 and G-CSF, when compared to WT mice. The massive increase of G-CSF in the lungs of KPn infected MGL1^{-/-} mice, suggested that emergency granulopoiesis could be occurring (252-261). We therefore examined the process of granulopoiesis in WT and MGL1^{-/-} mice over a 3-day time course. The results of the experiments showed that by 3 days post infection, MGL1^{-/-} mice exhibit an increase in mature neutrophil mobilization from the bone marrow to the blood when compared with WT mice. This could possibly be due to the increased levels of G-CSF observed in the lungs of KPn infected MGL1^{-/-} mice. It has been shown that defects in efficient clearance of apoptotic neutrophils via efferocytosis leads to an increase in G-CSF production (262). It is possible then that a defect in MGL1^{-/-} mice ability to clear apoptotic neutrophils drives the increased levels of G-CSF in MGL1^{-/-} mice. In-vivo efferocytosis experiments to determine if MGL1^{-/-} neutrophils are cleared as efficiently as WT neutrophils showed that indeed in the absence of MGL1, neutrophils were cleared less efficiently than the WT neutrophils. This result indicated to us that a defect in the clearance of apoptotic neutrophils in MGL1^{-/-} mice during KPn infection, lead to increased production of G-CSF, and ultimately increased neutrophil influx into the lungs. Combined with the results of the adoptive transfer experiment it is possible that MGL1 negatively regulates neutrophil influx as well as aids in efficient clearance of apoptotic neutrophils via

efferocytosis. Future studies in the lab will determine the mechanisms by which MGL1 plays a role in these processes. This study identified a novel role of MGL1 in pneumonic sepsis by regulating neutrophil influx through either the negative regulation of neutrophil influx, clearance of apoptotic neutrophils, or a combination of both.

Our findings show that both MGL1 and Mincle play a protective role during KPn induced pneumonic sepsis and inflammation. MGL1 by regulating neutrophil influx into the lungs and Mincle by regulating bacterial clearance mechanisms through neutrophil phagocytosis and NET formation. These discoveries help identify novel mechanisms to be used in the development of treatments and therapies for sepsis.

KPn's modulatory effect on neutrophils and impact on efferocytosis

Klebsiella pneumoniae (KPn) is an opportunistic pathogen known for causing hospital acquired pneumonic sepsis. It has become a pathogen of interest recently due to the emergence of multidrug resistant strains, which are even resistance to colistin, an antibiotic considered a last line of defense (177, 183, 184). Given that, it is critical that we understand the virulence factors, which aid in the ability of this pathogen to infect and survive in the host tissue. KPn has four well characterized virulence factors; capsule, lipopolysaccharide (LPS), fimbriae, and siderophores. KPn utilizes these virulence factors to evade rather than subvert an early host immune response. With our studies described in Chapter 4, we have identified a novel virulence mechanism by which KPn subverts a protective host response i.e. efferocytosis of neutrophils thereby maintaining a replicative niche (281). Our results

showed that KPn infected neutrophils were cleared less efficiently via efferocytosis than uninfected neutrophils. The mechanism underlying this inhibition appears to be different from what has been reported in case of *Staphylococcus aureus* where the pathogen inhibits the efferocytic uptake of infected cells by upregulating the surface expression of the “don’t eat me” signal CD47, while also inducing necroptosis (160). Our results instead showed that while KPn does increase the expression of two “don’t eat me” signals in CD31 and CD47, neutralization of these signals has no effect on KPn induced inhibition of efferocytosis of infected neutrophils. Instead, KPn infection drastically reduced the surface exposure of the most well characterized “eat me” signal, phosphatidyleserine (PS). This decrease in cell surface PS exposure in KPn infected neutrophils coincided with the timing of our efferocytosis assay and suggested to us that KPn modulation of cell surface PS exposure was leads to the KPn mediated inhibition of efferocytosis of infected neutrophils. Indeed, reversal of PS exposure by treatment with NEM rescued the efferocytic uptake of infected cells. In this regard a recent report showed that KPn infection delays apoptosis in human neutrophils (306). AS PS exposure is a characteristic measure of apoptotic cell death, our studies present the first evidence of physiological relevance of this phenomenon in KPn infection.

We next determined if KPn could induce alternative routes of cell death Western blot analysis of caspase 8 and RIP1 via Western blot analysis in uninfected and KPn infected neutrophils showed that KPn infection inhibited caspase 8 activation, which in turn prevented RIP1 inactivation through cleavage. That indicated an activation of necroptosis in KPn infected neutrophils. An increased phosphorylation of downstream kinases RIPK3 and MLKL confirmed this notion. Concomitantly, inhibition of necroptosis with

necrostatin-1 (RIPK1 inhibitor) as well as GSK'872 (RIPK3 inhibitor), rescued the defect in the efferocytosis of KPn infected neutrophils. The results of this study indicate that KPn infection inhibits apoptosis resulting in reduced PS exposure, while inducing an alternative cell death by necroptosis in neutrophils in order to inhibit their efferocytic clearance. The inhibition of efferocytosis through these mechanisms likely contributes to the development of a pro-inflammatory environment, which allows the pathogen to establish an infection, thus driving the development of sepsis. To our knowledge this is the first instance of a pathogen modulating PS exposure mechanisms in conjunction with induction of necroptosis to avoid efferocytic clearance. Understanding the mechanisms behind KPn modulation of PS exposure and induction of necroptosis could lead to novel therapeutic targets against KPn, which given the rise of antibiotic resistant strains is essential. Work is underway in the lab to determine the mechanisms leading to the molecular pathway underlying these aspects of KPn mediated inhibition of efferocytosis.

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