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# Role Of Lectins Receptors In Development Of Sepsis During Pulmonary Bacterial Infection

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# ROLE OF LECTINS RECEPTORS IN DEVELOPMENT OF SEPSIS DURING PULMONARY BACTERIAL INFECTION

By  
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Bachelor of Science, North Dakota State University, 2010

A dissertation  
Submitted to the Graduate Faculty

Of the  
University of North Dakota

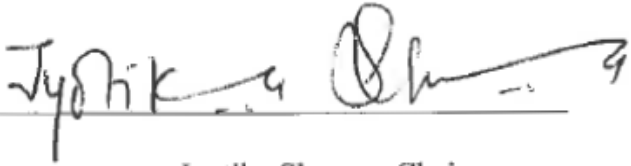
In partial fulfillment of the requirements

For the degree of

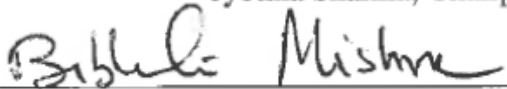
Doctor of Philosophy

Grand Forks, North Dakota  
December  
2015


This dissertation, submitted by Anthony Steichen 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.



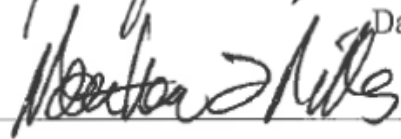
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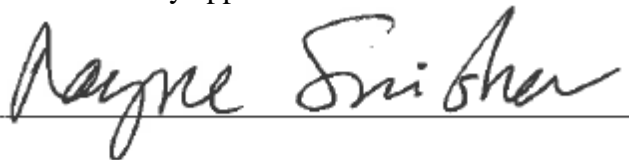


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This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.



Wayne Swisher

Dean of the School of Graduate Studies

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## PERMISSION

Title                      Role of Lectins Receptors in Development of Sepsis During  
Pulmonary Bacterial Infection

Department              Microbiology and Immunology

Degree                      Doctor of Philosophy

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Anthony Steichen

10-8-15

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# Abstract

A major focus of my work has been to understand the mechanism/s of sepsis development during respiratory bacterial infections with the goal of identifying novel alarmins/alarmin receptors. Specifically, I have analyzed the role of C-type lectins as novel alarmin/alarmin receptors likely involved in the development of sepsis resulting from acute pulmonary infection with Gram negative bacteria *Francisella novicida* and *Klebsiella pneumonia* (Kpn). In that regard, the **first project** was built upon a previous observation, made by Dr. Sharma, that two soluble lectins galectin-3 and -9 were upregulated and released in extracellular milieu in lungs of mice upon pulmonary infection with *Francisella novicida* (Fn). As alarmins are endogenous molecules released from dead/dying cells under pathological conditions, we wanted to examine if galectin-3 and -9 act as alarmins to exacerbate the inflammatory response during Fn infection. I was brought in as a co-author on the studies with galectin-3 where I examined the effect of this lectin on myeloid cell activation. My work showed that pre-treatment of primary neutrophils and macrophages with recombinant galectin-3 augmented Fn-induced activation of these cells in terms of increased reactive oxygen species and inflammatory cytokine production. Together with improved lung architecture, reduced cell death and improved survival of galectin-3<sup>-/-</sup> mice in comparison with the wild-type mice, these findings suggested that galectin-3 functions as an alarmin by augmenting the

inflammatory response in sepsis development during pulmonary Fn [1]. Similar studies with galectin-9 showed that galectin-9 also acts as an alarmin during Fn infection-induced sepsis [2]. In the **second project**, we specifically examined the function of Clec4d, a mammalian C-type lectin receptors (CLRs) as possible alarmin receptor in the development of Gram-negative pneumonic sepsis caused by KPn. In our initial analysis, we found that this CLR was upregulated in the lungs of mice infected with KPn. In our follow up studies we observed a protective role of this receptor in pneumonic sepsis. In my paper as the first author we showed that Clec4d<sup>-/-</sup> mice have an increased susceptibility to KPn infection, increased bacterial burden in systemic organs as well as blood, increased lung pathology as evident by increased accumulation of Ly6G<sup>+</sup>/CD11b<sup>+</sup> neutrophils and hyperinflammatory response [3]. While we observed no defect in bacterial phagocytosis in the absence of Clec4d, we found a possible role of Clec4d in turnover of neutrophils by efferocytosis. In another paper that I co-authored as second author, we showed a protective role of another CLR Mincle. In this study I was primarily involved in demonstrating the increased susceptibility of Mincle<sup>-/-</sup> mice to KPn pneumonia by reduced survival, increased bacterial burden and hyperinflammatory response [4]. On the basis of these findings, further studies are currently ongoing in Sharma lab to characterize the specific functions of Clec4d and Clec4e as part of separate projects.

# INTRODUCTION

## **Sepsis and Pneumonia: Healthcare burden and treatment**

A precise estimation of the number of cases in the US has been difficult partly due to the heterogeneity of the disease and the methodology used. Regardless of the range of estimated cases (256,000-750,000 annually in the US) and the associated mortality (28.6%-37.7%) reported [5-8], one thing is clear: despite decades of dedicated research efforts, occurrence of sepsis and septic shock has increased significantly over the past two decades. An accurate estimation of the disease incidence and mortality is critical for proper distribution of health care resources and a consistent definition of this complex disorder would help identify sepsis cases uniformly. Hence the American College of Chest Physicians and Society for Critical Care Medicine defined sepsis, severe sepsis, and septic shock as a disease complex with varying degrees of organs dysfunction resulting from a host response to systemic infection [9-11]. As a result of this uniform definition, sepsis has been recognized as a major US healthcare burden and according to Centers for Disease Control and Prevention, it is the 11<sup>th</sup> leading cause of deaths in the US, and the leading cause of non-coronary deaths in ICU patients [12,13]. Moreover, severe sepsis, defined as sepsis associated with organ dysfunction and hypoperfusion/hypotension with extremely high ICU admission rate has been estimated to cost the U.S. healthcare system \$24.3 billion in 2007 [14]. A mortality rate of approximately 50% is

associated with septic shock, which is highly unacceptable [5]. Individuals at the highest risk of developing sepsis or severe sepsis include older adults of over 65 years of age, patients with chronic disease conditions such as cancer, diabetes, chronic obstructive pulmonary disease, chronic liver or kidney diseases and individuals with abnormal immune response to infections [15,16].

Respiratory tract infections and pneumonias are the most common cause of sepsis (accounting for almost 50% of all sources of infections) and are associated with highest mortality rates [15,17] . Despite a recent increase in the numbers of Gram positive bacterial infection cases, Gram negative infections remain the cause of the highest mortality [18,19]. Interestingly, a recent study found that a lung infection was more commonly the primary cause of sepsis in patients with a negative culture than in patients with a positive culture [20]. This may indicate the critical need for biomarkers and improved methodologies for detection and prognosis of pneumonia. Gram negative bacteria belonging to Enterobacteriaceae family (e.g. E.coli and Klebsiella species) are the most common causes of sepsis [21,22].

Despite continuous and dedicated research on sepsis (a total of 139,200 Pubmed entries with the keyword “sepsis”; first article published in 1886), and hundreds of clinical trials, we are still ways from finding a treatment for this deadly disorder. This may partly be due to the complexity of this disease and the fact that immune regulatory processes controlling sepsis are not completely understood. Due to the overactivation of pro-inflammatory cascades occurring during a microbial infection, the initial attempts to treat sepsis centered around dampening or blockage of inflammatory cytokines (reviewed in [23]). Due to its central position in auto-and paracrine loops of numerous inflammatory

mediators, tumor necrosis- $\alpha$  (TNF- $\alpha$ ) has been the target of most anti-cytokine therapies which have failed in clinical trials [24,25]. We have come a long way since the recognition of “cytokine storm or hypercytokinemia”; a condition of co-existence of both pro- and anti-inflammatory cytokines. Moreover, with strong evidence of the complexity of sepsis, characterized by a cross-talk between multiple biological systems (reviewed in [26]), it is now clear that targeting a single biomolecule is highly unlikely to provide cure for heterogeneous sepsis patient population. For a number of years, recombinant activated protein C was the only FDA approved drug for sepsis treatment that targeted disseminated intravascular coagulation (DIC)”, a major complication of sepsis. However, in October 2011, this drug (Xigris) was withdrawn from market following the much anticipated results of the clinical trial (PROWESS-SHOCK) where it failed to show any survival benefit for severe sepsis and septic shock patients [27]. This underscores the need for a better understanding of immune dysfunction that will likely identify novel targets for successful treatment of this disorder.

### **Inflammatory Mechanisms in sepsis: Role of alarmins**

Sepsis is mainly characterized by an imbalanced, hyper-inflammatory response to an infection. As for any pathogenic insult, innate immune components play pivotal role in initiating the pro-inflammatory response required to combat infection. The key players in this process are host Pattern Recognition Receptors (PRRs) and pathogen-associated molecular patterns (PAMPs). Three families of host PRRs involved in the recognition of PAMPs are Toll-Like Receptors (TLRs), NOD-Like receptors (NLRs) and RIG-I like receptors (RLRs). Regardless of the origin of PAMPs or the localization of PRRs, their interaction results in an inflammatory response that serves as a first line of defense

against a wide variety of pathogens (Reviewed in [28]). On the flip side, the non-specificity of innate immune response and the antimicrobial mediators produced by the first responder cells such as neutrophils can result in severe damage to by-stander cells at the site of acute inflammation. This causes the release of endogenous host factors in the extracellular milieu. These endogenous molecules are termed alarmins, and typically perform homeostatic functions when contained within cellular compartments. However under pathological conditions, these molecules can be released either passively from dead cells or actively via non-classical secretion pathways. Once in the extracellular milieu, they exhibit immune modulatory properties such as induction of pro-inflammatory cytokines, immune cell chemotaxis and regulation of cell death [29,30]. The interaction of Toll-Like receptors (TLRs) as well as NOD-Like receptors (NLRs) with pathogen derived PAMPs during sepsis has been studied extensively (reviewed in [28,31]). However, the recognition of self-molecules (alarmins) by signaling receptors and the concomitant inflammatory response is an area of research which is still in its infancy. The role of several alarmins such as High Mobility Group Box 1 (HMGB1) and S100 family of calcium binding proteins has been demonstrated in various inflammatory conditions [32,33]. However, in a complex immune disorder like sepsis which is an interplay of several host immune pathways such as the coagulation system, complement cascade and even the autonomic nervous system, it is likely that several alarmins are involved at the intersections of these pathways. Thus, identification of novel alarmins and their receptors may aid in understanding this complex disorder and may present additional targets for effective therapeutics. Moreover, depending upon the type of infectious insults detected

in septic patients, the identification of these alarmins and alarmin receptors may present opportunities to customize the treatment options in septic patients.

## **Resolution of inflammation**

Once the pathogenic insult has been taken care of, it is imperative that the immune cells are cleared off the milieu, in order to restore the quiescent state of homeostasis. This is achieved by the process of efferocytosis, where the innate immune cells such as neutrophils, once they have completed their task of combating pathogens, start to undergo apoptosis and are eventually phagocytosed by professional phagocytes in the vicinity [34]. Deregulation of this process can result in secondary necrosis of these apoptotic cells causing the release of host alarmins in the extracellular milieu which, we now know, play an important role in exaggeration of an ongoing inflammation [35,36]. As sepsis is often characterized by massive cell death in systemic organs, it is tempting to speculate that a deregulation of efferocytosis process and turnover of neutrophils contributes to sepsis development. However, studies correlating these two processes with sepsis are surprisingly few. In addition to clearing dead cell carcasses, the process of efferocytosis also modifies the phenotype of phagocytic cells from inflammatory to anti-inflammatory nature resulting in production of host mediators such as TGF-beta and IL-10 that not only shut the inflammatory response but also inhibit further influx of immune cells [37]. All of these events constitute the complex process of resolution. In contrast to an initial belief of resolution of inflammation being a passive process resulting from mere dilution of chemokine gradients over time causing a cessation of cellular recruitment to the site of injury, recent studies have revealed that the resolution of inflammation is a well-orchestrated active process [38]. Deficiency in any of its components may lead to over-

active, uncontrolled chronic inflammation. It is thus tempting to assume that, in the context of an inflammatory disorder such as sepsis, this regulated process of resolution is disrupted and progresses towards an accelerated and sustained condition of chronic inflammation. This is an area of investigation which may hold some key answers to queries regarding the mechanisms of sepsis development and how an otherwise beneficial host response turns to a harmful process of excessive inflammation and overt tissue destruction.

### **C-Type Lectins: Role in inflammation and resolution**

Innate C-type lectins are emerging as immune determinants that can shape the balance between inflammation and homeostasis. These are  $\text{Ca}^{++}$  dependent glycan-binding proteins that share a homologous carbohydrate-recognition domain. With over 1000 members, the C-type lectin superfamily includes secreted as well as transmembrane proteins. Most commonly expressed on the cells of myeloid origin, C-type lectin receptors (CLRs) detect the PAMPs on microbes and, unlike TLRs, phagocytose the microbes, leading to activation of tyrosine kinases which in-turn coordinate multiple downstream signaling pathways leading to myeloid cell activation [39-41]. This has implications not only for clearance of the internalized microbes via phagolysosomal pathway and generation of innate immune mediators, but also for processing and presentation of antigens to activate the adaptive immune system [42-44]. While in most instances, this pathogen-binding capacity, internalization and signaling by CLRs mediates host defense against infection, there is evidence that myeloid CLRs can be exploited by pathogens to evade or suppress the immune response [45,46]. Beyond pathogen binding, accumulating evidence now suggests that many CLRs can bind to



endogenous or self-molecules from damaged/altered cells, thus acting as sensors of injury or cellular stress. For example, Mincle (Clec4e), a dectin-2 family CLR recognizes SAP130, an alarmin released from damaged cells causing activation of inflammatory response [47,48]. While Mincle has been shown to act in concert with another Dectin-2 family CLR Clec4d in recognition of mycobacterial PAMP and activation of inflammatory response [49], the role of Clec4d in Mincle-mediated recognition of alarmin SAP130 is unknown. MGL-1 and Lox-1 have been shown to bind to and internalize aged and apoptotic cells [50,51]. These CLRs thus have been speculated to play a role maintaining homeostasis. Furthermore, DC-SIGN and MGL have been shown to bind tumor antigens and possibly contribute to tumor surveillance [52,53]. Mannose receptor plays a role in clearance of mannosylated proteins from serum [54]. Apart from membrane-bound CLRs, many soluble C-type lectins such as Collectins (Collagenous Lectins) have also been shown to engage in recognition and clearance of apoptotic cells [55-57]. The outcome of this interaction however, varies depending upon the tissue type and the interacting proteins. For example, surfactant proteins in the immune-privileged tissues such as lungs can opsonize and facilitate clearance of cellular material such as DNA without evoking an immune reaction [58]. Involvement of complement proteins in this soluble lectin mediated opsonization and phagocytosis, however, results in an inflammatory response [59,60]. Despite these pleotropic functions in balancing the inflammation and homeostasis, the role of CLRs in sepsis, a dynamic disease marked with widespread cell death, inflammation and loss of homeostasis, is surprisingly understudied.

Another family of soluble lectins that has emerged as pleotropic regulators of immune responses are Galectins ( $\beta$ -galactoside binding lectins). These are one of the most ubiquitously expressed lectins which regulate a variety of inflammatory and homeostatic processes such as cell growth, proliferation, cell-cell interaction, acute and chronic inflammation and immune tolerance [61-63]. Interestingly, consistent with the property of alarmins, galectins can be released by “leaderless”, non-classical secretion pathway and play homeostatic roles in regulation of cell cycle and apoptosis, phagosome formation and stabilization of intracellular signalling when contained in intracellular compartments, but display inflammatory and T cell apoptotic activities upon extracellular release [63,64]. Moreover, the extracellular release of galectins seems to correlate with the virulence of invading pathogen [65], as well as influences immune responses through chemotaxis and activation of innate immune cells [66]. Despite the progress in our understanding of inflammatory properties of galectins, the role of these lectins as alarmins in the development of sepsis disorder has not been studied.

### **Central Hypothesis**

In light of these pleotropic immune regulatory functions of CLR, the central hypothesis of my dissertation proposal is that C-type lectin receptors regulate inflammatory response in pneumonic sepsis by functioning as alarmins (soluble lectins) and alarmin receptors (cell-surface CLR). Before joining my lab, a large scale screening of over 200 soluble and membrane-bound CLR in the lungs of mice undergoing pneumonic sepsis revealed upregulation of several of these molecules at transcript level. My project involved examination of the function of two soluble lectins Galectin-3 and -9 as putative alarmins

and two membrane-bound lectins Mincle and Clec4d as putative alarmin receptors in pneumonic sepsis.

## **Animal Models of Sepsis**

Various animal models of sepsis have contributed significantly to our understanding of host response and inflammatory cascade in the pathogenesis of sepsis. Endotoxemia model developed by bolus injection of LPS mimics pathophysiological alterations reported in sepsis patients to some extent, but the rapid and transient increase in inflammatory cytokines in this model differs from a prolonged systemic response observed in sepsis patients [67,68]. In fact it has been difficult to extrapolate the findings of many endotoxin mouse models (cecal ligation puncture, colon ascendens stent peritonitis) due to a relative resistance of mice to endotoxin shock in comparison with humans [69-71]. We chose to utilize pneumosepsis model of pulmonary bacterial infection because lung infections are a major source of sepsis in humans [72]. This model produces detectable bacteremia, systemic organ damage and mortality with severe lung pathology, observed in human patients. Two bacterial pathogens that we have used in our studies are *Klebsiella pneumoniae* (KPn) and *Francisella novicida* (Fn). Pulmonary infection of C57/BL6 mice with these pathogens causes bacteremia, extensive cell death leading to severe lung pathology, hypercytokinemia and systemic organ dysfunction. The excessive cell death is a hallmark of these pulmonary bacterial sepsis model infection, regardless of the bacterial strain used [73] additionally, Dr. Sharma's previous studies showed *Francisella* infected phagocytes defective in efferocytosis, resulting in accumulation of dead cells and their contents in the milieu [74]. *We thus hypothesize that during pneumosepsis host cells undergo death due to bacterial replication and*

*inflammatory response. Membrane-bound CLRs such as Clec4d and Mincle play a protective role by mediating clearance of dead cells by phagocytes. On the other hand, galectins released from dead cells play a pathogenic role and function as alarmins to activate myeloid cells causing them to secrete proinflammatory mediators and exacerbation of inflammatory response culminating in sepsis.*

# **Galectin-3 functions as an alarmin: Pathogenic role for sepsis development in murine respiratory tularemia**

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## Abstract

Sepsis is a complex immune disorder with a mortality rate of 20-50% and currently has no therapeutic interventions. It is thus critical to identify and characterize molecules/factors responsible for its development. We have recently shown that pulmonary infection with *Francisella* results in sepsis development. As extensive cell death is a prominent feature of sepsis, we hypothesized that host endogenous molecules called alarmins released from dead or dying host cells cause a hyper inflammatory response culminating in sepsis development. In the current study we investigated the role of galectin-3, a mammalian  $\beta$ -galactoside binding lectin, as an alarmin in sepsis development during *F. novicida* infection. We observed an upregulated expression and extracellular release of galectin-3, in the lungs of mice undergoing lethal pulmonary infection with virulent strain of *F. novicida* but not in those infected with a non-lethal, attenuated strain of the bacteria. In comparison with their wild-type C57Bl/6 counterparts, *F. novicida* infected galectin-3 deficient (galectin-3<sup>-/-</sup>) mice demonstrated significantly reduced leukocyte infiltration, particularly neutrophils in their lungs. They also exhibited a marked decrease in inflammatory cytokines, vascular injury markers and neutrophil associated inflammatory mediators. Concomitantly, in-vitro pre-treatment of primary neutrophils and macrophages with recombinant galectin-3 augmented *F. novicida*-induced activation of these cells. Correlating with the reduced inflammatory response, *F. novicida* infected galectin-3<sup>-/-</sup> mice exhibited improved lung architecture with reduced cell death and improved survival over wild-type mice, despite similar bacterial burden. Collectively, these findings suggest that galectin-3 functions as an

alarmin by augmenting the inflammatory response in sepsis development during pulmonary *F. novicida* infection.

## Introduction

Sepsis results in 750,000 hospitalizations every year in the US and is the second leading cause of mortality in patients admitted to intensive care units [75]. Pulmonary infections, in turn, are a major cause of sepsis [76]. However, the mechanisms responsible are not well understood. This is underscored by a lack of effective therapeutics against this immune disorder despite more than two decades of active research. Our recent studies have shown that pulmonary infection of mice with fully virulent *Francisella tularensis* as well as the murine model organism *F. novicida* (F.n.) a Gram negative bacterial pathogen, leads to development of severe sepsis characterized by hyperinflammation, T cell depletion, and extensive cell death in systemic organs [73,77,78]. We are thus using a murine inhalation model of F.n. infection to understand the mechanism/s responsible for pulmonary infection-induced sepsis development.. Intriguingly, this pathogen is not known to produce any exotoxin, which can account for the lethality of this infection. Moreover, the lipid A of Francisella LPS does not stimulate TLR4 and is thus hypo-inflammatory [79]. Studies from our and other laboratories have shown that extensive tissue damage and wide-spread cell death is a hallmark of Francisella infection, regardless of the bacterial strain [78,80-83]. Additionally, our studies show that Francisella infected macrophages are defective in clearance of dead cell debris, a process termed efferocytosis, leading to accumulation of these dead cells and their contents [74]. We thus hypothesized that in the absence of any bacterial toxins, host endogenous molecules released from these dead or dying cells contribute to the inflammatory response culminating in sepsis development during respiratory infection with Francisella.



Alarmins are host endogenous factors which perform homeostatic functions when contained within cellular compartments [29]. However, under pathological conditions, these molecules can be released either passively from dead cells or actively via non-classical secretion pathways [84]. Once in the extracellular milieu, they exhibit immune modulatory properties such as induction of pro-inflammatory cytokines, immune cell chemotaxis, and regulation of cell death [29,30,84]. The overt inflammation during sepsis is primarily a result of the interaction between innate immune receptors with pathogen derived molecules (Pathogen Associated molecular patterns (PAMPs) and alarmins. PAMPs and alarmins together constitute Danger-Associated Molecular Patterns (DAMPs). The interaction of Toll-Like receptors (TLRs) as well as NOD-Like receptors (NLRs) with pathogen derived PAMPs during sepsis has been studied extensively (reviewed in [26,31]. However, the recognition of self-molecules (alarmins) by signaling receptors and the concomitant inflammatory response is an area of research which is still in its infancy. Moreover, in a complex immune disorder like sepsis which is an interplay of several host immune pathways such as the coagulation system, complement cascade and even the autonomic nervous system [26], it is likely that several alarmins are involved at the intersections of these pathways. Thus, identification of novel alarmins may aide in understanding this complex disorder and may present additional targets for effective therapeutics. As sepsis developed during pulmonary infection with Francisella is associated with extensive cell death in lungs and other systemic organs, we sought to identify novel alarmins that might be released during this infection and may contribute to disease development.

Galectins constitute a soluble mammalian  $\beta$ -galactoside binding lectin family which play homeostatic roles in regulation of cell cycle and apoptosis, as well as display inflammatory and immune modulatory activities in various pathological conditions [63,64,66,85]. Previous studies have implicated galectin-3 in regulation of various inflammatory conditions including endotoxemia and airway inflammation [86-88]. In this study we show that galectin-3, a mammalian galactoside binding soluble lectin is upregulated and released in lungs of mice undergoing lethal respiratory infection with F.n. but not in mice vaccinated with an attenuated mutant strain of the bacteria that protects these mice from an otherwise lethal challenge. We thus hypothesized that galectin-3 exacerbates the inflammatory response during lethal infection. The outcome of this study, with use of galectin-3 deficient mice, shows that galectin-3 plays the role of an alarmin in Francisella infection induced sepsis development.

## **Materials and Methods**

### Ethics Statement

The animal usage protocols were approved by the Institutional Animal Care and Usage Committee at the University of North Dakota (protocol no. 1108-3) and the University of Texas at San Antonio (protocol no. MU066). All the procedures strictly followed the institutional and federal guidelines and all efforts were made to minimize animal suffering.

### Bacterial strains and Mice

The F.n. strain U112 and an attenuated transposon mutant lacking a 58kDa protein of hypothetical function (kindly provided by Dr. Larry Gallagher, University of Washington) were grown on Trypticase Soy Agar (TSA) medium supplemented with L-cysteine at 37°C. After overnight growth, the bacteria were harvested and suspended in a freezing medium (250 mM sucrose, 10 mM sodium phosphate pH 7.2 and 5 mM glutamic acid). Stocks were aliquoted and frozen at -80°C for further use.

All *in-vivo* experiments were performed using 6-8 wk old female C57Bl/6 wild-type and galectin-3<sup>-/-</sup> mice. Galectin-3<sup>-/-</sup> mice were purchased from Jackson Laboratories (Bar Harbor, ME). Sex- and age-matched galectin-3<sup>+/+</sup> mice with the same genetic background were used as control.

### Antibodies and Reagents

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. For detection of galectin-3 by immunofluorescence (IF) staining, a purified rat anti-mouse galectin-3 antibody (eBioscience, San Diego, CA) followed by Alexa-546 conjugated chicken anti-rat antibody (Molecular Probes, OR) was used. A rat anti-mouse CD11b antibody conjugated to PE (BD Pharmingen) and a purified rat anti-mouse Gr1 monoclonal antibody, clone Ly-6G (Clone Accurate Chemical, Westbury, NY, USA), followed by the secondary antibody RRX-conjugated Affipure goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used for double staining of activated neutrophils. The terminal deoxyribonucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining kit was purchased from Chemicon International, CA. Purified recombinant galectin-3 was purchased from R&D

Systems, MN. The endotoxin level was <1.0 EU per µg of protein. For detection of reactive oxygen species, Fc OxyBURST assay reagent was purchased from Molecular Probes, Eugene, OR. Mouse IL-6 and TNF-α ELISA kits (BD OptEIA) were from BD Biosciences, San Diego, CA.

#### Infection of Mice, survival and bacterial burden

Mice were anaesthetized with a mixture of ketamine HCL and xylazine (30mg/ml ketamine, 4 mg/ml xylazine in PBS) and were infected intranasally with 50-70 CFUs of the wild-type F.n.

strain U112 in 20 µl of PBS or with 20 µl of PBS alone. Mice were monitored daily for signs of disease, which typically included piloerection, hunched gait, lethargy and eye discharge. The survival of infected mice was recorded for up to 2 weeks post-infection (p.i.). Mice displaying severe signs of distress (labored breathing, non-responsiveness to cage tapping, failure of grooming and severe eye discharge) were humanely sacrificed by injecting a mixture of ketamine (90-120mg/kg) and xylazine (10mg/kg) followed by cervical dislocation. The death was recorded as tularemia induced mortality. For non-lethal infection, the mice were similarly inoculated with the mutant bacteria followed 3 weeks later by challenge with similar dose of the wild-type organisms. In some experiments, the mice were euthanized at 3 days p.i. and blood, lungs and liver were aseptically harvested. The organs were homogenized aseptically in cold PBS with Complete™ protease inhibitor cocktail (Roche Diagnostics, Germany). For the bacterial burden analyses, the homogenates and blood were serially diluted in PBS and plated on TSA. CFU counts per mouse were calculated after incubating the plates at 37°C overnight.

### Quantitative real-time PCR

Lungs from infected and mock control mice at various times post-infection were immediately removed after perfusion and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis of the samples was performed using SYBR green (Applied Biosystems, CA, USA) as the detection dye to measure the expression levels of Galectin-3-specific mRNAs. Briefly, one microgram of total RNA from either infected or mock infected mice was reverse transcribed into cDNA by using a high capacity cDNA reverse transcription kit according to the manufacturer's instructions (Applied Biosystems, CA, USA). Transcript levels of the housekeeping ribosomal 18S and galectin-3 were PCR amplified in each sample by using specific primers (Advanced Nucleic Acids Core Facility, UTHSCSA, TX): 18S (sense) 5'-CATGTGGTGTGAGGAAAGCA-3' and (anti sense) 5'-GTCGTGGGTTCTGCATGATG-3'; Gal-3 (sense) 5'-CAGTGCAGAGGCGTCGGGAAA-3' and (anti-sense) 5'-CTGCCCCAGCAGGCTGGTTT-3'. The target gene expression levels were normalized to levels of the house keeping 18S gene in the same sample. Expression of galectin-3 in infected samples was determined as fold change over that in control samples as calculated by using the formula  $2^{-(\Delta\Delta Ct)}$ .

### Histological and Immunofluorescence staining

For histological and immunofluorescence staining, frozen lung tissues were processed as previously described [77,82]. Frozen lung sections thus obtained were stained with hematoxylin and eosin for pathological analysis, or for detection of galectin-3 and

activated neutrophils (CD11b<sup>+</sup>Gr1<sup>+</sup>) by immunofluorescence staining, as previously described [89] . For detection of cell death, TUNEL method was used according to manufacturer's instructions (Chemicon International, CA). The images were acquired using a Leica DMR epifluorescent microscope (Leica Microsystems, Wetzlar, Germany) with an attached cooled CCD SPOT RT camera (Diagnostic Instruments Inc., Sterling Heights, MI). The images were processed and analyzed using Adobe Photoshop 7.0 software (Adobe, Mountain view, CA).

#### Enumeration of cellular infiltration in lungs

Lungs were harvested from infected and mock control mice at 3 days p.i. after perfusion with PBS and were treated with collagenase to obtain single cell suspensions as previously described [77,78,90]. Total numbers of viable immune cells in lungs of infected and mock control galectin-3<sup>-/-</sup> or WT mice were counted by trypan blue exclusion staining.

#### 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 flow-based system: Mouse MAP<sup>TM</sup> (Multi-Analyte Profiles) analysis technology.

#### Neutrophil and macrophage activation

Cells were isolated from the peritoneal cavities of naïve C57BL/6 mice 12-14h after intraperitoneal injection with sterile 4% thioglycollate. Neutrophil percentage was

determined by flow cytometry using neutrophil specific anti-mouse Gr-1 (anti-Ly-6G and Ly6C). Additionally, the lavage cells were cytocentrifuged on glass slides and were stained with H&E as described above. The cells were plated at the density of  $1 \times 10^6$  cells and were infected with wild-type F.n. strain U112 at MOI 50 with or without pretreatment of the cells with  $10 \mu\text{g/ml}$  of purified recombinant galectin-3. Cells stimulated with galectin-3 alone or with  $10 \text{ng/ml}$  of phorbol myristate acetate (PMA) served as controls. One hour after stimulation, production of reactive oxygen species (ROS) was measured in the cells by flow cytometry using Fc OxyBURST reagent according to the manufacturer's instructions. A minimum of 10,000 events was read for each sample and all the cells fluorescing positive in FITC channel (excitation and emission maxima of  $\sim 490 \text{ nm}$  and  $\sim 520 \text{ nm}$ , respectively) were gated to get the percentage of ROS producing cells.

Bone marrow was isolated from wild-type and galectin-3<sup>-/-</sup> mice and the cells were differentiated to macrophages as previously described [91]. On day 6 of culture 90-95% cells were macrophages as determined by flow cytometry using macrophage specific markers CD11b and F4/80. The cells were plated at  $8 \times 10^4$  cells per well in 96-well flat-bottom plates and were stimulated as described above for the neutrophils. Culture supernatants were collected 24h after stimulation and measurement of IL-6 and TNF- $\alpha$  was performed by ELISA according to the manufacturer's instructions (BD OptEIA, BD Biosciences).

### Statistical Analysis

Survival of the infected wild-type and galectin-3<sup>-/-</sup> mice was compared using Kaplan Meier log Rank test. Statistical comparison between levels of host mediators in different experimental groups was performed by Student's *t* test using Sigma Plot 8.0

### **Results**

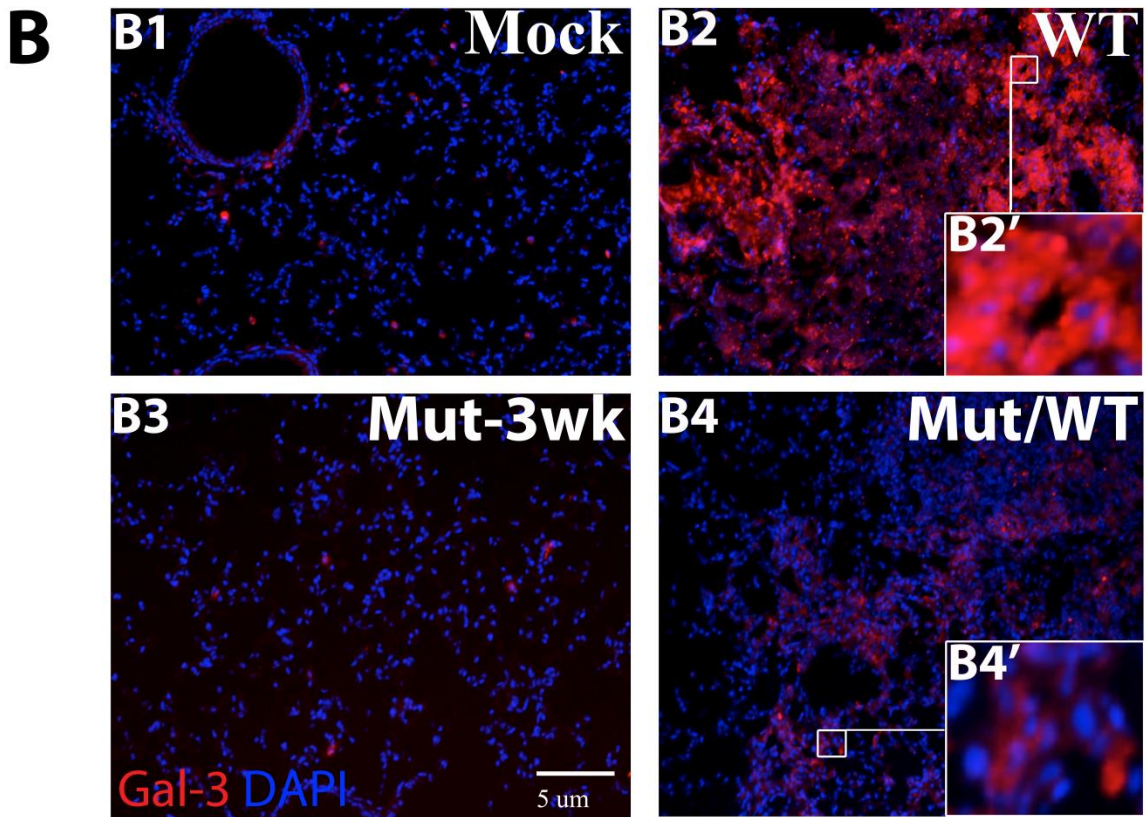
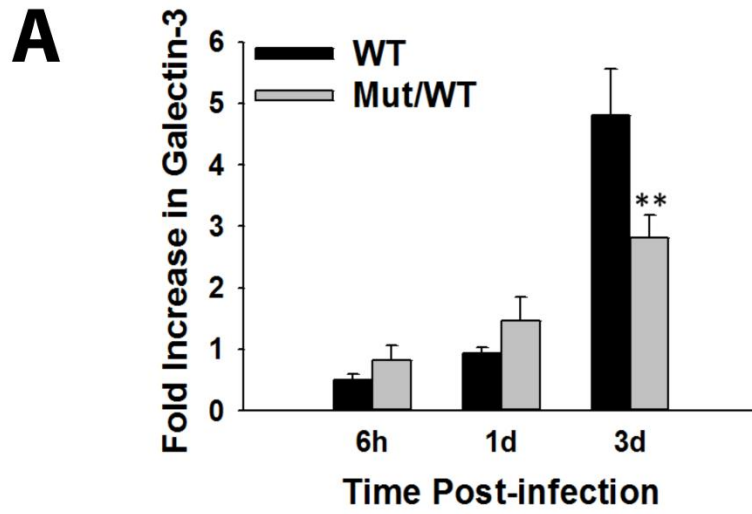
Galectin-3 is highly expressed and is localized extracellularly in lungs during the septic phase of F.n. infection.

Alarmins are characterized as intracellular host factors which display extracellular release under pathological conditions. To examine if galectin-3 exhibits this alarmin property in pulmonary tularemia, the expression and distribution of this lectin was analyzed. We compared the transcript and protein level expression of galectin-3 at various times post-infection (p.i.) in lungs of mice undergoing lethal pulmonary infection with the wild-type strain of F.n. versus the mice vaccinated with an attenuated mutant of F.n. (Mut/WT mice), which protects the mice from sepsis. This mutant has been characterized extensively in our previous studies [77]. As shown in Figure 1A, galectin-3 transcript levels showed maximal increase at 3 days p.i. (dp.i.) in the lungs of mice infected with the wild-type bacteria as well as in the Mut/WT mice. However, this increase was significantly higher in mice undergoing lethal infection as compared to the protected

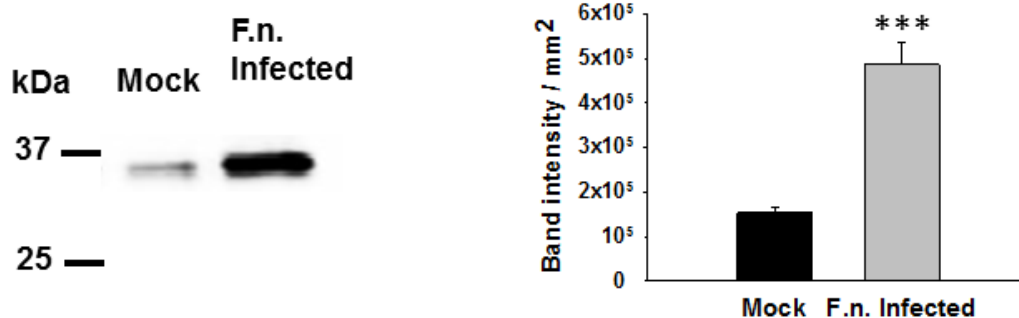


**Figure 1. Upregulated expression and extracellular release of Galectin-3 in lungs during respiratory *F. novicida* infection**

. (A) Total RNA was extracted by Trizol method from lungs harvested at the indicated times after infection with the Wild-type bacteria (WT) or from mice vaccinated with an attenuated mutant strain followed by challenge with WT bacteria (Mut/WT mice). The mRNA levels of Galectin-3 were analyzed by real-time PCR as described in Materials and Methods and are expressed as fold changes over the levels in mock control mice. Data shown are the averages of 3- 4 mice per group. Statistically significant differences are denoted by asterisks (\*\*,  $p < 0.005$ ). (B) In-situ IF staining of frozen lung sections from mock infected and WT U112 infected or Mut/WT mice harvested at 3 d. p.i Lung harvested 3 weeks after vaccination with the mutant alone (Mut-3wk) served as controls for Mut/WT mice. The sections were stained for galectin-3 (red) using a purified rat anti-mouse galectin-3 antibody followed by Alexa-546 conjugated chicken anti-rat antibody. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindol-dilactate (DAPI). Magnification X 200. Insets depict extracellular galectin-3 in WT *F. novicida* infected mouse lungs (B2') and cytosolic galectin-3 in Mut/WT (B4') mouse lungs.



Mut/WT mice. This increase in galectin-3 expression at 3 dp.i. is consistent with the appearance of other sepsis features (extensive cell death, hyperinflammatory response, increased vascular injury) at this time, as shown in our previous studies with F.n. as well as the fully virulent *F. tularensis* [77,78]. We thus termed this as septic phase of Francisella infection and carried out the rest of our analysis at this time point. Immunofluorescence (IF) analysis of galectin-3 protein expression in frozen sections of lungs harvested at 3dp.i. showed a low basal level expression in mock infected mice inoculated with PBS alone (Fig. 1.B1). Consistent with the transcript data, lungs of mice undergoing septic infection with the wild-type F.n. exhibited upregulated expression of this lectin at 3d p.i. (Fig. 1. B2). This increase in expression was substantially higher than that in Mut/WT mice (Fig.1.B4). The mice infected with mutant alone for 3 weeks and without challenge with WT bacteria (Mut-3wk) served as control for the Mut/WT mice. In these mice, galectin-3 was observed to be expressed at low basal level similar to mock control animals (Fig. 1B3). Importantly, most of galectin-3 expressed in septic mice was localized extracellularly in large granuloma-like areas of cellular infiltration, undergoing extensive cell death (Fig. 1.B2'). The non-septic Mut/WT mice, on the other hand, showed intracellular galectin-3 associated with live cells (Fig. 1.B4'). Western blot analysis of bronchoalveolar lavage (BAL) from mice infected with the WT *F. novicida* also showed a significantly high extracellular release of galectin-3 (Fig. 2). Taken together, these data clearly showed that galectin-3 exhibits a characteristic alarmin property of extracellular release during septic phase of pulmonary F.n. infection.



**Figure 2. Upregulated expression and extracellular release of Galectin-3 in lungs during respiratory *F. novicida* infection.**

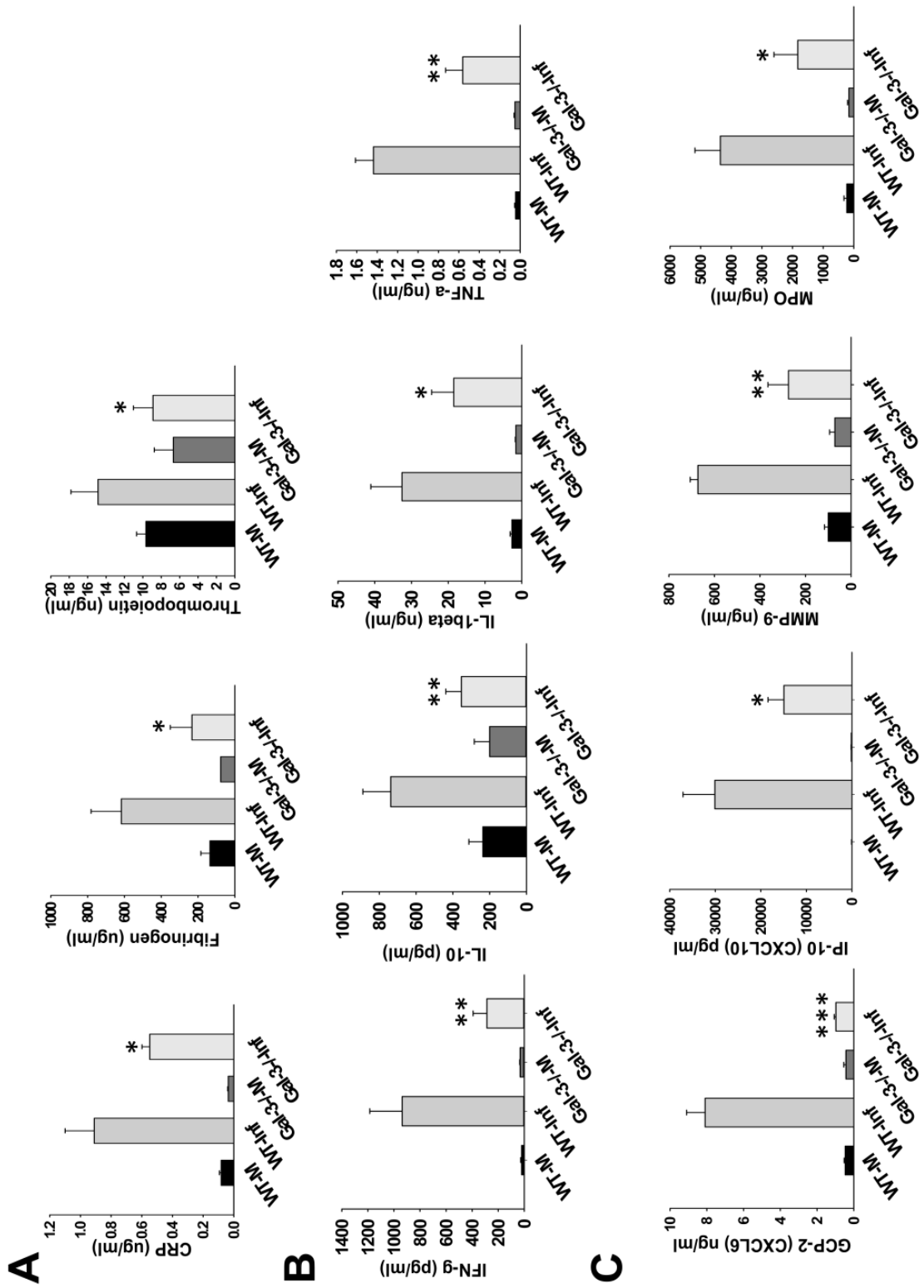
Bronchoalveolar lavage (BAL) was obtained from lungs of mice infected with the wild-type *F. novicida* strain U112 or PBS alone as previously described (9). Galectin-3 was immunoprecipitated from BAL using a purified rat anti-mouse galectin-3 antibody (eBioscience, San Diego, CA) by previously described method [92] with modifications. Briefly 1 mg of total BAL proteins were incubated with 10 µg anti-galectin-3 antibody at 4°C overnight. Immune complexes were pulled down with using 30 µl of 30% Protein A Plus agarose beads (Pierce) for 2h at 4°C. The beads were washed, solubilized in 1× SDS gel loading buffer and resolved on 12% acrylamide gels (BioRad). The gels were processed for western blotting as described previously [77] for detection of galectin-3 using anti-mouse galectin-3 antibody. Densitometric analysis of bands was performed using the Lumi-Imager software (Roche Applied Science). Bar graph depicts densitometry analysis of galectin-3 bands represented in arbitrary units. Statistically significant differences are denoted by asterisks (\*\*\*, p<0.001).

### **F.n. infected Galectin-3<sup>-/-</sup> mice display reduced inflammatory response and neutrophil accumulation**

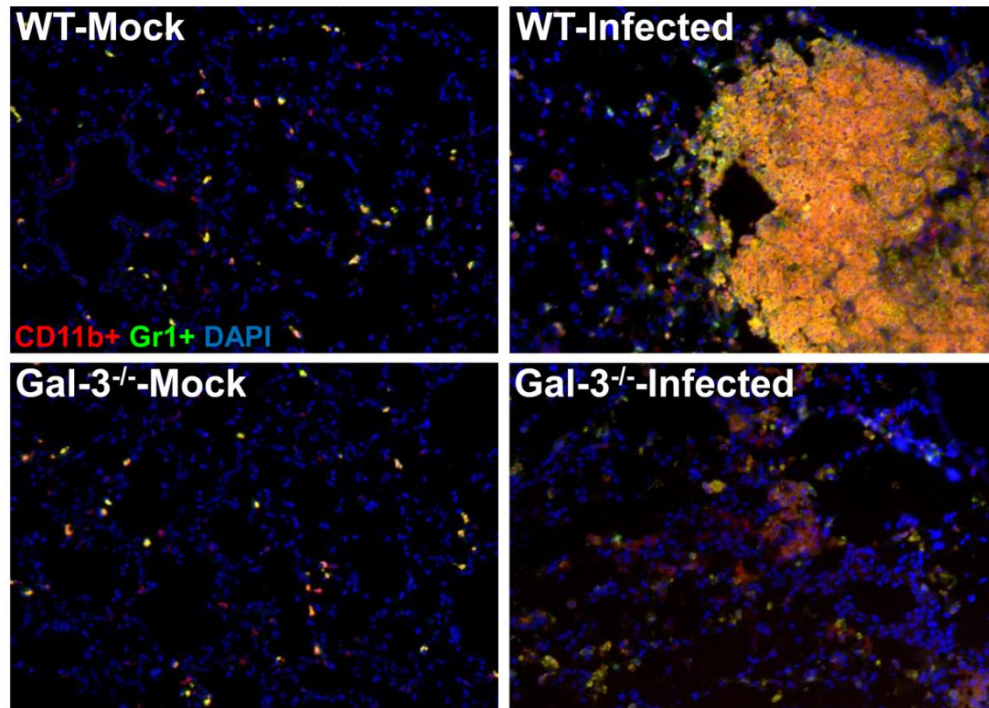
We hypothesized that, similar to the function of alarmins, increased expression and extracellular localization of galectin-3 may be contributing to the hyperinflammatory response culminating in sepsis during lethal *Francisella* infection. In order to analyze this, lungs were harvested at 3d.p.i. from F.n. infected galectin-3<sup>-/-</sup> and wild-type mice and the levels of multiple cytokines, chemokines as well as vascular injury markers were measured using a multiplex assay. Galectin 3<sup>-/-</sup> mice displayed significant reduction in levels of several vascular injury markers in comparison with their wild-type counterparts (Fig. 3A). In addition, levels of several inflammatory cytokines (TNF- $\alpha$ , IL-10, IL-1 $\beta$ ), described as markers of sepsis, were reduced in galectin-3<sup>-/-</sup> mice (Fig. 3B). These observations strongly suggested an immune-stimulatory role of galectin-3 during pulmonary *Francisella* infection. Interestingly, in comparison with wild-type mice, infected galectin-3<sup>-/-</sup> mice displayed a reduction in several chemokines involved in neutrophil recruitment (Fig. 3C). Furthermore, levels of myeloperoxidase (MPO), a neutrophil associated protease and marker of neutrophil activation, was also reduced in infected galectin-3<sup>-/-</sup> mice. In order to correlate these observations with cellular infiltration in-vivo, IF staining for co-expression of CD11b and Gr1, markers for activated neutrophils [93], was performed on lung sections from galectin-3<sup>-/-</sup> and wild-type mice. Consistent with the chemokine data, cells infiltrating the lungs of infected wild-type mice showed high co-expression of CD11b and Gr1, suggesting an activated neutrophil phenotype. These cells were mostly accumulated in large lesion like areas in the lungs of these mice. The cells in infected galectin-3<sup>-/-</sup> mice, on the other hand,

**Figure 3. Galectin-3<sup>-/-</sup> mice display reduced levels of inflammatory mediators in lungs after pulmonary infection with F.n.**

The lungs from WT mock infected (WT-M), galectin-3<sup>-/-</sup> mock infected (Gal3<sup>-/-</sup>M), WT *F. novicida* infected (WT-Inf) or galectin-3<sup>-/-</sup> *F. novicida* infected mice (Gal3<sup>-/-</sup>Inf) were harvested at 3 d.p.i., homogenized with protease inhibitors in PBS and analyzed commercially for rodent multi-analyte profiles (Rules-Based Medicine, Austin, TX). (A), levels of vascular injury markers; (B), levels of inflammatory cytokines; and (C), levels of neutrophil attractant chemokines and activation markers in lung homogenates. Results shown are from 3-4 mice per group from 3 different experiments. CRP; C-reactive protein, MMP-9; matrix metalloproteinase-9, MPO; myeloperoxidase. \*  $p < 0.05$ ; \*\*  $p < 0.00$ .







**Figure 4. Galectin-3<sup>-/-</sup> mice display reduced accumulation of neutrophils in lungs during F.n. infection.**

Frozen sections of lungs harvested at 3 d. p.i. from mock infected and *F. novicida* infected WT or galectin-3<sup>-/-</sup> mice were co-stained with antibodies against myeloid cell markers CD11b (red) and Gr1 (green). A high co-expression of both markers is depicted by yellow color in infected WT lungs while cells infiltrating lungs of galectin-3<sup>-/-</sup> mice exhibited expression of only CD11b. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindol-dilactate (DAPI). Magnification X 200. Asterisks depict lesions in the lungs.



expressed CD11b, but low or no Gr1 (Fig. 4). These results suggested a role of galectin-3 in regulation of myeloid cell accumulation, particularly neutrophils, in the lungs of mice during pulmonary F.n. infection.

#### Galectin-3 regulates F.n. infection induced inflammatory response in-vitro

In order to further investigate the immune stimulatory properties of galectin-3, we examined the role of this lectin in in-vitro activation of myeloid cells, particularly neutrophils and macrophages. These are the major cell types that infiltrate the lungs of Francisella infected mice [77,94]. In-vitro infection of WT bone marrow derived macrophages (BMDMs) with wild-type F.n. U112 resulted in an inflammatory response in terms of increased TNF- $\alpha$  and IL-6 production (Fig. 5A). Galectin-3<sup>-/-</sup> macrophages on the other hand, produced significantly lower amounts of these cytokines in response to infection (Fig. 5A). As the extracellularly released galectin-3 may be playing a role in activation of myeloid cells in-vivo, we examined if pretreatment of these cells with galectin-3 has any effect on Francisella infection induced cytokine production. Stimulation of macrophages with purified galectin-3 induced minimal amount of TNF- $\alpha$  and IL-6 production (Fig. 5B). The optimal concentration of galectin-3 was experimentally determined by using 1-20 $\mu$ g/ml of the recombinant protein (data not shown). Infection with wild-type F.n. strain U112 infection, on the other hand, induced substantial amounts of these cytokines in macrophages. Interestingly, pre-treatment of macrophages with purified galectin-3 exacerbated this Francisella-induced inflammatory cytokine production (Fig. 5B). Immune stimulatory effect of galectin-3 was also examined on peritoneal neutrophils. Cells collected by peritoneal lavage following

**Figure 5.** Galectin-3 regulates F.n. infection induced inflammatory response *in-vitro*.

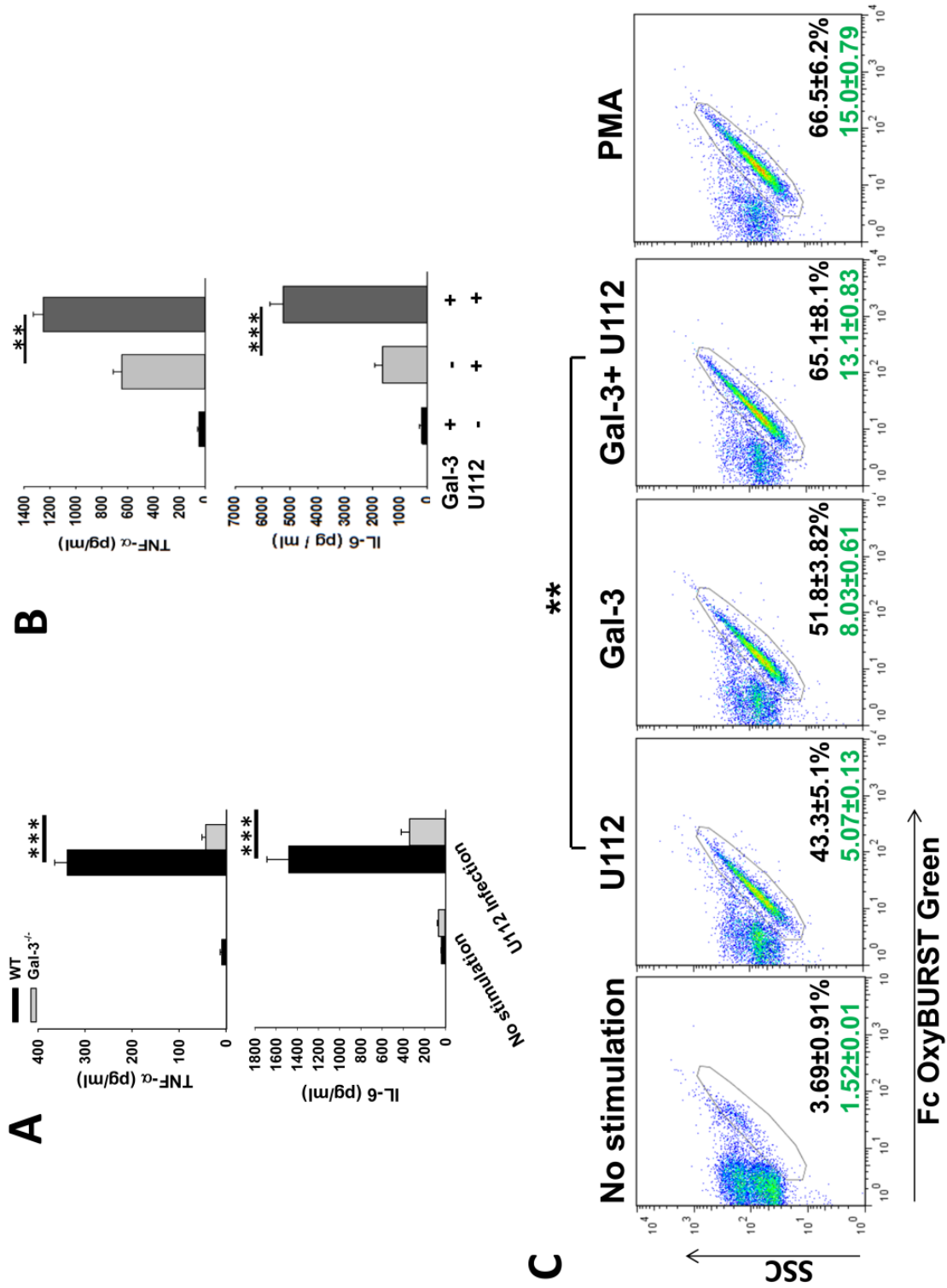
(A). Bone marrow derived macrophages (BMDMs) were isolated from wild-type and galectin-3<sup>-/-</sup> mice as described in Methods. The cells were infected with wild-type F.n. Strain U112 at MOI of 50 and the culture supernatants were collected 24h after infection.

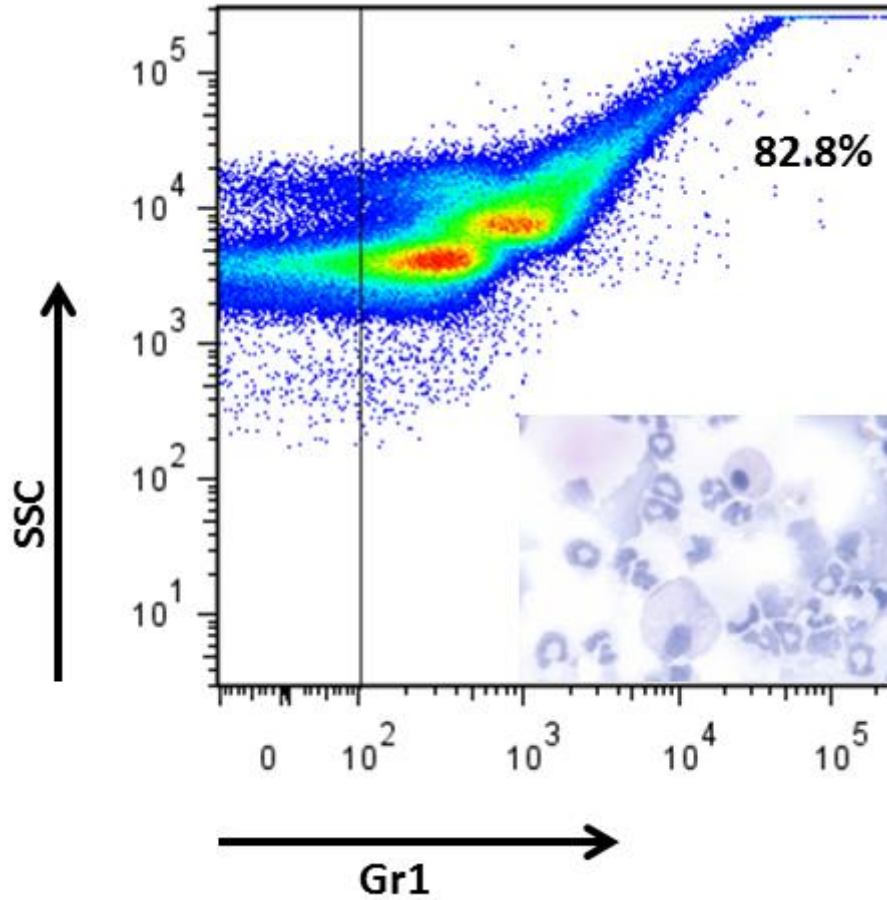
The amount of TNF- $\alpha$  and IL-6 were measured in the supernatants by Sandwich ELISA.

(B). BMDMs from C57Bl/6 wild-type mice were infected with wild-type F.n. Strain U112 at an MOI of 50 with or without pretreatment with 10 $\mu$ g/ml of purified recombinant galectin-3. Culture supernatants were collected 24h after infection and the amount of TNF- $\alpha$  and IL-6 were measured by ELISA. The experiment was repeated three times with similar results.

(C). Peritoneal neutrophils were isolated from mice 12-14h after injection with 4% thioglycollate and were stimulated with *F. novicida* at an MOI 50 with or without pre-treatment with purified recombinant galectin-3 (10 $\mu$ g/ml). Stimulation with galectin-3 alone or phorbol myristate acetate (PMA, 10ng/ml) was used as a control. Production of reactive oxygen species was measured one hour post-stimulation by flow-cytometry using Fc-OxyBURST dye following the manufacturer's instructions. Numbers in black on the plots depict percent of ROS positive cells and the numbers in green represent median fluorescence intensity (MFI) of individual cells. Dot plots from a representative of 3 independent experiments are shown.

Statistical analysis between the data sets was performed by Student's t test where \*\* $p < 0.005$ ; \*\*\* $p < 0.001$ .





**Figure 6. Flow cytometry analysis of peritoneal neutrophils.**

Mice were injected intraperitoneally with sterile 4% thioglycollate. Peritoneum was lavaged 12-14hrs later and cells were analyzed by flow cytometry using neutrophil specific anti-mouse Gr1 (Ly6G+Ly6C) antibody. In addition, cells were cytocentrifuged and stained with H&E for morphological analysis.

intraperitoneal injection of thioglycollate were 80-85% neutrophils as determined by flow cytometry and morphological analysis with characteristic multilobed nuclei (Fig. 6). Unlike macrophages, treatment of neutrophils with purified galectin-3 alone activated these cells to produce substantial levels of ROS as determined by oxidation of Fc OxyBURST dye (Fig. 5C). Importantly, pre-treatment of neutrophils with this lectin primed these cells to produce further increased amounts of ROS in response to F.n. infection, which was significantly higher than that elicited by F.n. infection alone (Fig. 5C). This cell-type specific response of galectin-3 indicates involvement of distinct receptors and/or signaling pathways, which is currently being investigated in our laboratory. Nonetheless, this augmentation of Francisella infection-induced myeloid cell activation by galectin-3 likely has implications in exacerbation of inflammation culminating in sepsis development during this infection.

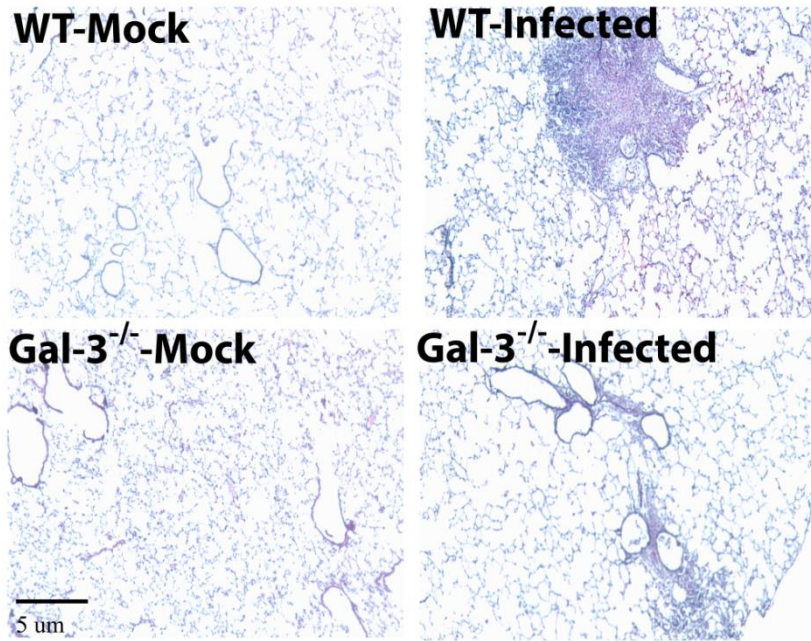
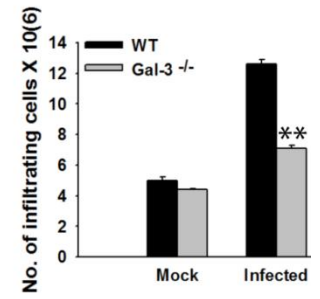
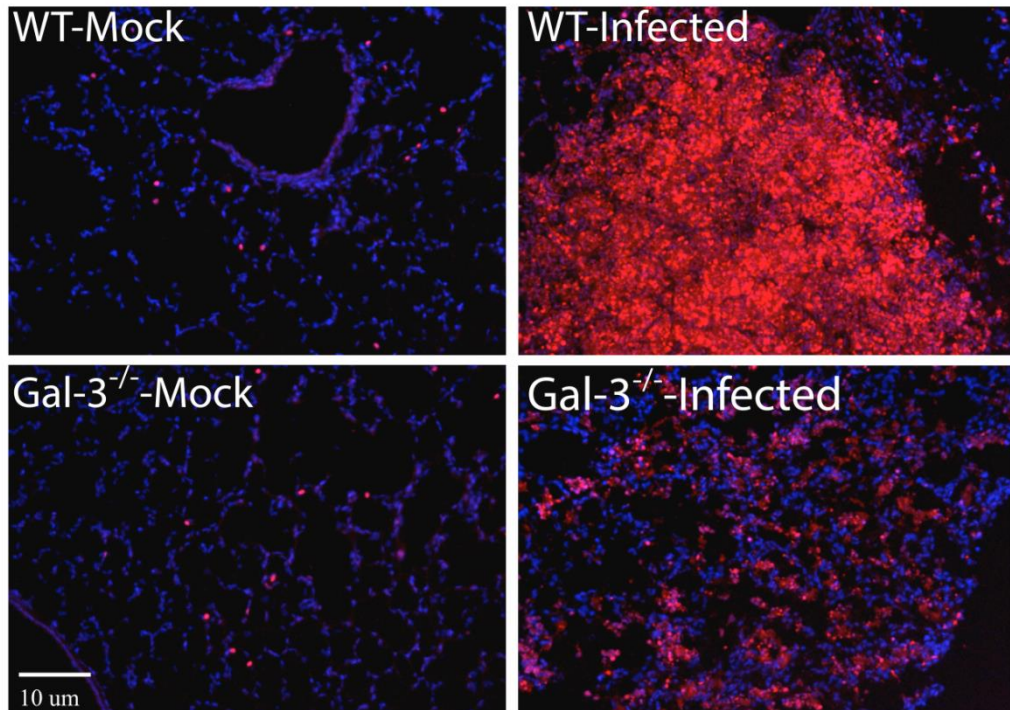
#### Galectin-3<sup>-/-</sup> mice exhibit reduced lung pathology after F.n. infection.

Lung cryosections from wild-type and galectin-3<sup>-/-</sup> mice infected with a lethal dose of F.n. were stained with H&E and processed for histopathological analyses as described in Materials and Methods. Mock infected wild-type and galectin-3<sup>-/-</sup> mice exhibited similar normal lung architecture with minimal cellular infiltration and clear air spaces (Fig. 7A). As expected, a massive increase in cellular infiltration and extensive pathology, along with severe bronchopneumonia and massive cell death occurring in the center of large granuloma-like areas of infiltration, was evident in the lungs of wild-type mice at 3 dp.i. (Fig 7A). The lungs of galectin-3<sup>-/-</sup> mice, on the other hand, showed moderate peribronchial and perivascular infiltration (Fig. 7A). The infiltrating cells in these areas

**Figure 7. Galectin-3 deficiency leads to improved lung pathology, reduced leukocyte accumulation and reduced cell death upon pulmonary F.n. infection.**

(A) Lungs from mock infected and F.n. infected wild-type (WT) or galectin-3<sup>-/-</sup> mice were harvested at the septic phase (3 d. p.i.), embedded in optimal-cutting-temperature (OCT) compound, and sectioned as described in Materials and Methods. The frozen sections were stained with Hematoxylin and Eosin. The images obtained are representatives of three experiments performed, and in each experiment each group contained three mice. Magnification, ×200. (B). Lungs from mock infected and F.n. infected WT or galectin-3<sup>-/-</sup> mice were harvested 3 days after intranasal infection. Total immune cells infiltrating the lungs were isolated by collagenase treatment of lungs as described in Materials and Methods. Total numbers of viable immune cells were counted by trypan blue exclusion staining (n = 5-6). Statistical analysis between the data sets was performed by Student's t test where \*\**p* < 0.005. (C). Frozen lung sections from mock infected and Francisella infected WT or galectin-3<sup>-/-</sup> mice were processed for in-situ TUNEL staining for detection of DNA fragmentation (red) in nuclei. Nuclei (blue) were stained with 4',6'-diamidino-2-phenylindole dilactate. Magnification, ×100.



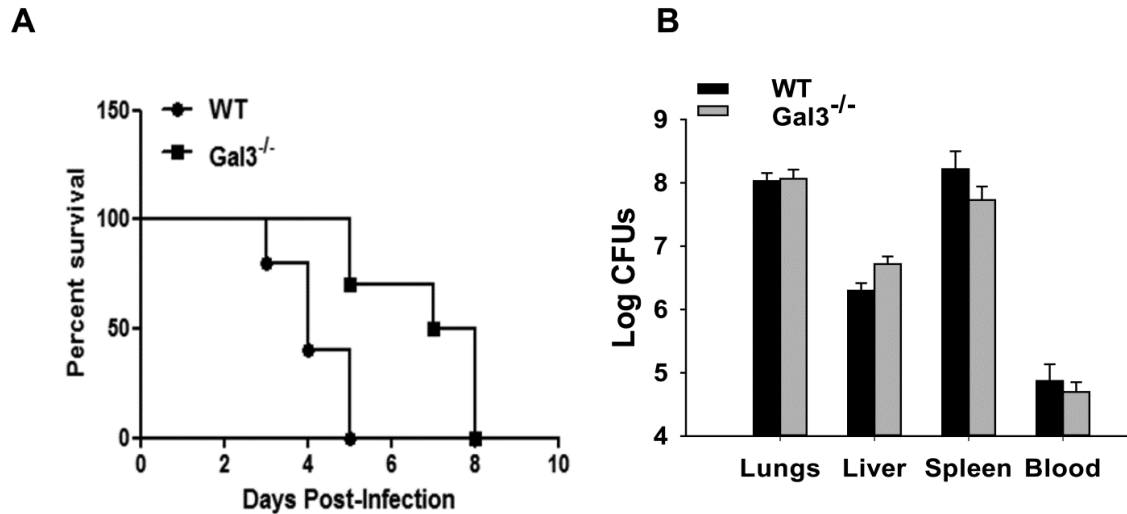
**A****B****C**

appeared to be viable and the areas of infiltration lacked cellular debris that is typical of extensive apoptosis and necrosis in the wild-type mice. This was consistent with reduced numbers of leukocytes enumerated after collagenase treatment of the lungs harvested from galectin-3<sup>-/-</sup> mice (Fig. 7B). Galectin-3 deficiency did not affect the basal number of cells as mock infected wild-type and galectin-3<sup>-/-</sup> animals showed similar low number of cells in the lungs. To further analyze the extent of cell death in the lungs of infected wild-type and galectin-3<sup>-/-</sup> mice, TUNEL assay was performed on frozen sections of lungs harvested at 3 dp.i. As shown in Fig. 7C, mock infected wild-type and galectin-3<sup>-/-</sup> mice showed minimal numbers of TUNEL positive cells in their lungs. On the other hand, septic lungs of F.n. infected wild-type mice showed extensive cell death within perivascular and peribronchial lesions which are the main sites of immune cell infiltration during infection (Fig. 7C). In contrast, the numbers of apoptotic TUNEL positive cells in infected galectin-3<sup>-/-</sup> mice were much less as compared to their wild-type counterparts following infection with F.n. The improved lung architecture and reduced cell death in the absence of galectin-3 indicates a pathological role of this lectin during pulmonary Francisella infection.

#### Galectin-3<sup>-/-</sup> mice show improved survival following F.n. infection.

In order to see the effect of improved lung pathology and reduced inflammatory responses in the absence of galectin-3, overall disease severity and survival was compared in C57BL/6 wild-type and galectin-3<sup>-/-</sup> mice infected with a lethal dose of F.n. In the infected wild-type mice, visible signs of disease started to appear by day 3 p.i. which typically included piloerection, hunched gait, lethargy, and eye discharge. All of these mice succumbed to infection by day 5 p.i. (Fig. 8A). By contrast galectin-3<sup>-/-</sup> mice





**Figure 8. Galectin-3<sup>-/-</sup> mice show improved survival during pulmonary F.n. infection.**

(A). Fifteen C57Bl/6 WT and 17 galectin-3<sup>-/-</sup> mice in 3 separate experiments were inoculated intranasally with *F. novicida* and were monitored for survival daily for 2 weeks. The improved survival of galectin-3<sup>-/-</sup> mice compared to WT mice was statistically significant, as determined by Kaplan-Meier log-rank analysis ( $P$  value\*\*\* = 0.0003). (B) Bacterial burdens in lungs harvested from *F. novicida* infected WT and galectin-3<sup>-/-</sup> mice at 3 d.p.i. Lungs and liver were harvested from infected mice, homogenized as described in Materials and Methods. Tissue homogenates and blood harvested at the same time, were serially diluted and plated on TSA plates to enumerate bacterial burdens. In this representative of three independent experiments, each group contained three to five mice.

exhibited delayed appearance of disease symptoms and showed significantly improved survival as compared to the infected wild-type mice (Fig. 8A). Intriguingly, enumeration of bacterial burden in the organs of these mice at the peak of infection, i.e., 3 d.p.i. showed that both galectin-3<sup>-/-</sup> and the wild-type animals exhibited similar bacterial burdens in their systemic organs as well as in blood (Fig. 8B).

## Discussion

Sepsis is the 2<sup>nd</sup> leading cause of death in ICU patients and pulmonary infections in turn are a major source of sepsis [5]. It is a complex immune disorder resulting from deregulation of multiple host defense pathways. Accumulating evidence suggests that host endogenous molecules termed alarmins, likely play an important role in pathophysiology of sepsis [95]. In this study we show that galectin-3, a mammalian  $\beta$ -galactoside binding lectin acts as a novel alarmin in development of sepsis during pulmonary infection with *F. novicida*. Consistent with characteristic properties of alarmins, galectin-3 was upregulated and extracellularly released during the septic phase of infection and could amplify the Francisella infection-induced inflammatory response of neutrophils and macrophages. Furthermore, galectin-3<sup>-/-</sup> mice showed improved pathology, reduced inflammation and improved survival during pulmonary Francisella infection. These results suggest that galectin-3 functions as an alarmin and plays a pathogenic role in development of sepsis in pulmonary bacterial infection.

Alarmins are structurally diverse multifunctional host proteins with some common properties (12-15). These are endogenous proteins performing homeostatic functions that lack any signal sequence for active secretion and have chemoattractant and immune activating properties, once released in extracellular milieu. With advances in our

understanding of host responses to pathogenic events, the list of alarmins has continued to grow over the past decade. Several well characterized alarmins such as High Mobility Group Box1 (HMGB-1), S100 family of proteins, and heat shock proteins have been shown to perform dual functions as factors controlling homeostatic processes like transcriptional regulation when localized to intracellular compartments and as pro-inflammatory factors upon their release from necrotic cells during a pathogenic insult [32,33,96]. Similarly, galectin-3 when localized in the nucleus, has been shown to act as an RNA splicing factor [97] and performs homeostatic functions such as embryogenesis and cell cycle regulation [63]. The results of current study showed that galectin-3 can be released extracellularly in lungs under pathogenic conditions such as an infection. Curiously, galectin-3 does not contain any signal sequence for golgi mediated classical secretion. Thus active secretion of this lectin is likely via a yet unclear non-classical secretion pathway, a property shared by most alarmins characterized to date [66]. However, in F.n. infection, this lectin is likely released passively from dead/dying cells since the extracellular galectin-3 is detected only during lethal infection with F.n., which typically results in extensive cell death [77,78,80]. On the other hand, the mice vaccinated with an attenuated F.n. mutant causing little or no cell death showed this lectin to be intracellular and largely associated with live cells, with minimal levels in BAL. Notwithstanding the mechanism involved, release of galectin-3 in the septic phase of F.n. infection indicates a role for this molecule in pathogenesis of this infection. Consistent with alarmin properties, galectin-3 exhibited immune activating properties such as stimulation of oxidative burst in neutrophils and inflammatory cytokine production in macrophages. Importantly, this lectin was able to augment F.n. infection

induced inflammatory response from neutrophils as well as macrophages, which can have important implications under in-vivo conditions. Previous studies from our laboratory have shown that pulmonary infection with F.n., as well as *F. tularensis*, results in extensive cell death and that F.n. infected phagocytes are defective in efferocytosis, the process of clearing dead cell debris [74]. Coinciding with this, we have further shown that pulmonary Francisella infection is characterized by hyperinflammatory response with an unbridled increase in levels of several inflammatory cytokines as well as vascular injury markers [77,80]. It is likely that during pulmonary Francisella infection, galectin-3 released from these dead cells primes the bystander myeloid cells to produce heightened levels of inflammatory mediators in response to the bacteria, resulting in further tissue damage and ultimately organ failure, characteristic of sepsis. Curiously, while the macrophages did not respond to galectin-3 alone, neutrophils displayed ROS production upon stimulation with galectin-3 or F.n. infection alone, which was amplified upon combination of the two. This cell-specific nature of galectin-3 activity in the context of Francisella infection is interesting and is in line with a previous study showing the role of prototype alarmin HMGB1 in promoting the inflammatory response of monocytes elicited by external stimuli [98]. Intriguingly, in that study as well, the monocytes did not respond to HMGB1 alone. In light of several biologically distinct functions performed by alarmins ranging from inflammation to tissue repair and wound healing, association with other stimuli possibly adds another layer to the regulation of their mechanisms of action. Although speculative at this stage, it is possible that the activating receptor for galectin-3 on neutrophils is constitutively expressed while

that on macrophages likely gets expressed in an infection-specific manner. Further studies to test this hypothesis are currently underway in our laboratory.

As mentioned earlier, one of the characteristic properties of alarmins is to mediate immune cell influx. The reduced number of leukocytes in infected galectin-3<sup>-/-</sup> animals in this study indicates that this lectin likely plays a role in recruitment of these cells in sepsis. Reduced levels of neutrophil chemoattractants and activation markers in galectin-3<sup>-/-</sup> mice support this notion. This observation is in line with the role of prototypic alarmin HMGB1 in neutrophil migration by regulating the levels of chemoattractants such as IL-8 [99]. Additionally galectin-3 may also play a direct role in extravasation of neutrophils from blood vessels into the lungs, as shown in a previous study with pulmonary *S. pneumonia* infection [65]. Other proinflammatory alarmins such as S100 proteins have also been shown to directly promote migration of myeloid cells by binding to their cell surface receptors [100,101]. Another possibility is that galectin-3 may have a role in inhibiting neutrophil turnover. Under the conditions of a resolving inflammatory response to an infectious insult, once the neutrophils have performed their antimicrobial function, they undergo programmed cell death. The failure of this process is the root cause of several inflammatory disorders, as prolonged exposure to neutrophilic factors can result in non-specific tissue damage [102]. In this regard a recent study showed a defect in neutrophil turnover and thus the lack of resolution of inflammation during *Francisella* infection [103]. Furthermore, a previous study has shown the involvement of galectin-3 in decreasing neutrophil death as well as reducing macrophage cell death in response to apoptotic stimuli [104,105]. This suggests that expression of galectin-3 in inflammatory cells may lead to their enhanced survival, resulting in exacerbated

inflammation. Thus it is tempting to speculate that galectin-3, in addition to activating neutrophils, may be playing a role in inhibition of neutrophil turnover during *Francisella* infection by prolonging the lifespan of these cells. Consistent with this hypothesis, F.n. infected galectin-3<sup>-/-</sup> mice show reduced numbers of neutrophils, lower levels of neutrophil associated immune mediators and consequently reduced tissue pathology. These observations further support proinflammatory and pathogenic role of galectin-3 in pulmonary F.n. induced sepsis.

Extensive tissue pathology is a major complication of acute respiratory infections which are associated with severe sepsis [106,107]. This is caused by hyper activation of the inflammatory immune response that results in capillary leakage, tissue injury, and ultimately lethal organ failure [108,109]. Results from the current study showed that F.n. infected galectin-3<sup>-/-</sup> animals exhibited a reduction in the levels of sepsis mediators such as vascular injury markers thrombopoietin, fibrinogen, as well as acute phase protein CRP and inflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-1. This observation further suggested that galectin-3 mediates upregulation of these sepsis markers during *Francisella* infection and thus plays a role in development of sepsis. As a result of this mitigated inflammatory response and tissue pathology, galectin-3<sup>-/-</sup> mice are able to survive the infection for a significantly longer duration as compared to infected wild-type mice. Galectin-3<sup>-/-</sup> mice have been shown to be highly resistant to infection with another Gram negative bacterium *Salmonella* [86] with KO mice exhibiting lower bacterial burdens. Although the mechanism of this protection was not shown, it was speculated that galectin-3 binds to and masks bacterial PAMPs resulting in immune suppression and unchecked bacterial growth. Previous studies have shown the ability of galectin-3 to bind

to a variety of pathogens [110-113] owing to its specificity for  $\beta$ -galactosides which are a common constituent on pathogen membranes. This interaction can serve to activate immune response as well as result in direct killing of the pathogen as shown in case of *Candida* [112]. Since we observed similar bacterial burdens in *F. novicida* infected galectin-3<sup>-/-</sup> and wild-type mice, it seems unlikely that galectin-3 is involved in direct killing of bacteria. It is, however, possible that galectin-3 could be binding to a *Francisella* factor thus potentiating its interaction with immune activating receptor/s, as has been shown in case of HMGB1 [98]. Studies regarding the identity of *Francisella* factors possibly engaged by galectin-3 and immunological consequences of these interactions are currently on-going in our laboratory. The survival advantage of *F. novicida* infected galectin-3<sup>-/-</sup> was observed to be only transient as these mice ultimately succumbed to infection, possibly due to overwhelming bacterial burdens. Interestingly, this survival advantage of galectin-3<sup>-/-</sup> mice was dependent on the infection dose of bacteria. The galectin-3<sup>-/-</sup> mice succumbed to the infection at a similar rate as WT mice when infected with 300-500 CFUs of bacteria (data not shown). It is possible that at that dose, a higher bacterial burden leads to a further increase in cell death leading to an increased accumulation of other alarmins which mask the advantageous effect of the absence of galectin-3. This observation highlights the complex nature of sepsis syndrome where multiple host and pathogen derived factors cross talk and regulate various immune pathways. It is also consistent with previous studies showing partial or no protection upon blocking single alarmin such as HMGB1 [114-116]. Nonetheless, as the bacteria can be cleared by successful antibiotic therapy, the complications often arise from tissue damage during sepsis. Thus, a combinatorial approach using blockage of galectin-3 along with

antibiotics could prove to be a successful therapy for treating Francisella infection induced sepsis.

In Toto, our findings indicate that galectin-3 plays a pathogenic role as an alarmin to exacerbate the inflammatory response during pulmonary infection with Francisella and contributes to sepsis development. Galectin-3 thus may represent a potential target for treatment of sepsis during this infection.

### **Acknowledgements**

We thank Mr. Steve Adkins at the University of North Dakota and Dr. UmaMahesh Gundra at New York University for technical help with flow cytometry. We also thank Dr. Arun Rajput for his assistance with immunoprecipitation and western blot analysis.



# **C-type lectin receptor Clec4d plays a protective role in resolution of Gram negative pneumonia**

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Summary sentence: In this brief conclusive report the authors present data to implicate an orphan C-type lectin receptor in resolution of inflammation by facilitating neutrophil turnover

Abbreviated Title: Protective role of Clec4d in pneumonia

## Abstract

Pneumonia is frequently associated with sepsis characterized by a non-resolving hyperinflammation. However, specific host components of the pulmonary milieu that regulate the perpetuation of inflammation and tissue destruction observed in this immune disorder are not clearly understood. We examined the function of Clec4d, an orphan mammalian C-type lectin receptor, in Gram pneumonic sepsis caused by *Klebsiella pneumoniae*. While the wild-type mice infected with a sublethal dose of bacteria could resolve the infection, the Clec4d<sup>-/-</sup> mice were highly susceptible with a progressive increase in bacterial burden, hyperinflammatory response typical of sepsis and severe lung pathology. This correlated with a massive accumulation of neutrophils in lungs of infected Clec4d<sup>-/-</sup> mice which was in contrast with their wild-type counterparts where neutrophils transiently infiltrated the lungs. Interestingly, the Clec4d<sup>-/-</sup> neutrophils did not exhibit any defect in bacterial clearance. These results suggest that Clec4d plays an important role in resolution of inflammation, possibly by facilitating neutrophil turnover in lungs. This is the first report depicting physiological function of Clec4d in a pathological condition. The results can have implications not only in sepsis but also other inflammatory diseases where non-resolving inflammation is the root cause of disease development.

## Introduction

Sepsis poses a major health care burden with 750,000 cases annually in the United States with a mortality rate of 20-50% [5]. Currently there are no effective therapies to treat this deadly immune disorder. Pneumonia is the most frequent source of sepsis [117]. In that regard, nosocomial infections caused by the opportunistic pathogen *Klebsiella pneumoniae* (KPn) account for 5-20% of Gram-negative sepsis cases. In light of constant occurrence of antibiotic resistant strains of this pathogen, an understanding of functioning of host innate immune components might provide targets for modulation of host immune system in a beneficial manner [118,119].

Sepsis is now perceived as interplay of pathogen derived (pathogen associated molecular patterns, PAMPs) as well as endogenous host factors (termed alarmins [120]). C-type lectin receptors (CLRs) are emerging as pattern recognition receptors which can shape immune responses by recognizing a variety of PAMPs as well as alarmins [121]. However, their function in development of sepsis is largely unexplored. In that regard, Clec4e (Mincle) of Dectin-2 subfamily has recently been shown to function as an activating receptor for host endogenous factors released from dead cells and PAMPs of bacterial and fungal origin [49]. Clec4d, another Dectin-2 family member is localized close to Mincle in NK gene complex region of chromosome 12p13 and its function is yet to be defined [122,123]. Based on the close vicinity of this receptor with Mincle, we hypothesized that it likely plays a role in regulating immune responses during pathogenic conditions.

In this study we tested the role of Clec4d in Gram negative sepsis induced by pulmonary infection with KPn. Our results show that Clec4d-mediated responses are required for the resolution of pneumonia and to mitigate mortality in pulmonary KPn induced sepsis. This may have implications for other immune disorders associated with non-resolving, persistent inflammation.

## **Materials and Methods**

### Infection of mice and survival

Six to 8 weeks old female wild-type C57BL/6 and Clec4d<sup>-/-</sup> mice (obtained from the Consortium of Functional Genomics) bred in the animal facility of the University of North Dakota were used according to institutional and federal guidelines. Mice were infected intranasally with sublethal dose ( $5 \times 10^4$  bacteria in 20ul of saline, determined experimentally) of KPn (ATCC strain 43826). The bacteria were grown to log phase in LB medium. Mock infected mice received saline only. Mice were monitored daily for signs of disease, which typically included piloerection, hunched gait, lethargy, weight loss and increased respiratory rate. The survival of infected mice was recorded for up to 2 weeks post-infection (p.i.).

### Bacterial Burden and Multi-analyte profile analysis

Lungs and livers from infected and mock mice at various times post-infection (p.i.) were immediately removed after perfusion were homogenized aseptically in cold PBS with Complete<sup>TM</sup> protease inhibitor cocktail (Roche Diagnostics, Germany), all described by us previously [77,124]. For the bacterial burden analyses, the organ homogenates and blood were serially diluted in PBS and plated on LB agar. The immune mediators in lung homogenates were determined commercially by Myriad Rules-based Medicine (Austin,

TX, USA) utilizing a multiplexed flow-based system: Rodent MAP<sup>TM</sup> (Multi-Analyte Profiles) analysis technology.

#### qRT-PCR

Lungs from infected and mock mice at various times post-infection (p.i.) were immediately removed after perfusion and were used to extract total RNA by Trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis of the samples was performed, as described previously [124] using SYBR green (Applied Biosystems, CA, USA) to measure the expression levels of Clec4d-specific mRNAs by using specific primers Clec4d (sense) 5'- GAA CAA ATT CTT GCC GTC CTG ACC-3' and (anti-sense) 5'-TCC ATC ACA AGG ACC ACT TTC TGA G-3' (Integrated DNA Technologies, Inc., Iowa). The target gene expression levels were normalized to levels of the house keeping 18S gene (sense) 5'-CATGTGGTGTGAGGAAAGCA-3' and (anti sense) 5'-GTCGTGGGTTCTGCATGATG-3' in the same sample. Expression of Clec4d in infected samples was determined as fold change over that in control samples as calculated by using the formula  $2^{-(\Delta\Delta Ct)}$ .

#### Histological Analysis

Lungs from infected and mock mice at various times post-infection (p.i.) were isolated after perfusion were snap-frozen in OCT resin. For pathological analysis 10 µm thick serial horizontal sections of frozen lungs were stained with hematoxylin and eosin (H&E) as described previously [77].

#### Flow Cytometry

For enumeration of neutrophils by flow cytometry, lungs from mock control and infected animals isolated 3 days p.i. (dp.i.) were processed to get total cellular infiltrates as

previously described by us [77]. Ly6G+CD11b+ neutrophils were quantified using Pacific Blue™ anti-mouse CD11b and APC anti-mouse Ly6G antibodies (Biolegend, San Diego, CA).

#### Bacterial phagocytosis and killing activity of neutrophils

For assessing KPn phagocytosis, peritoneal neutrophils were isolated by previously described method [125] and were incubated with GFP-labeled KPn (kindly provided by Dr. Steven Clegg, University of Iowa) at MOI of 50 with or without opsonization in 10% normal mouse serum. After 1 hour, the cells were washed twice with ice-cold PBS followed by two washes with FACS-buffer (PBS+10% fetal bovine serum). The % positive cells containing fluorescent bacteria were determined by flow-cytometry using BD LSR II (Becton Dickinson Immunocytometry, San Jose, CA.). For bacterial killing activity, intracellular CFUs in *Clec4d<sup>-/-</sup>* and WT neutrophils were enumerated at 1h and 3hp.i. by gentamycin protection assay as described previously [126].

#### Statistics

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 AND DISCUSSION

In order to investigate the role of Clec4d in KPn pneumonia, we first examined if the Clec4d<sup>-/-</sup> and WT mice respond to pulmonary KPn infection differentially. For this we initially infected the mice intranasally with a lethal dose of  $1 \times 10^5$  CFUs (determined experimentally). One hundred percent of the WT mice died within 5 dp.i. at this dose while all the Clec4d<sup>-/-</sup> mice reproducibly succumbed to infection 1-2 days earlier than the WT mice (data not shown). In order to finalize a dose at which the WT mice would display minimal morbidity and mortality while the Clec4d<sup>-/-</sup> mice would all succumb to infection, mice were infected with sublethal dose of  $5 \times 10^4$  CFUs. As shown in Fig. 9A, 70-80% of WT mice infected with  $5 \times 10^4$  CFUs of KPn survived the infection with transient signs of disease (ruffled fur, lethargy) early during infection and appeared healthy later. The Clec4d<sup>-/-</sup> mice, in contrast, were extremely susceptible to this dose. Majority of these mice died within 5dp.i. and 100% of mice succumbed to infection by day 7 with progressive development of disease and overt signs of infection (piloerection, hunched gait, lethargy, increased respiratory rate). Importantly while the disease signs appeared at similar times (2dp.i.) in WT and Clec4d<sup>-/-</sup> mice, they became divergent by 3dp.i. While the WT mice appeared to enter recovery phase at that time, the Clec4d<sup>-/-</sup> mice continued to display disease progression. The recovery phase of WT mice strongly correlated with the expression level of Clec4d in their lungs where Clec4d mRNA was maximally transcribed by 3dp.i. (Fig. 9B). This was followed by a downregulation in Clec4d transcript that was reduced to only marginal levels by 5dp.i. These results indicated a protective role of this receptor in the resolution of KPn pneumonia.

Next, serial dilutions of homogenized lungs, liver and blood from infected Clec4d<sup>-/-</sup> and WT mice were plated on LB. For initial 2 days, the Clec4d<sup>-/-</sup> and WT animals displayed similar bacterial burdens in their lungs (Fig. 9C). Overwhelming loads were detected in lungs of Clec4d<sup>-/-</sup> mice at 3dp.i. which 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 a resolution of the infection in these mice. The Clec4d<sup>-/-</sup> mice also displayed higher and earlier systemic dissemination of bacteria as depicted by significantly higher bacterial load in liver even at 2dp.i. and a more severe bacteremia (Fig. 9C). In contrast, no viable bacteria were detected in the blood of WT mice by 5dp.i. These data indicated that Clec4d mediated responses directly or indirectly influenced bacterial clearance in pneumonic infection with KPn.

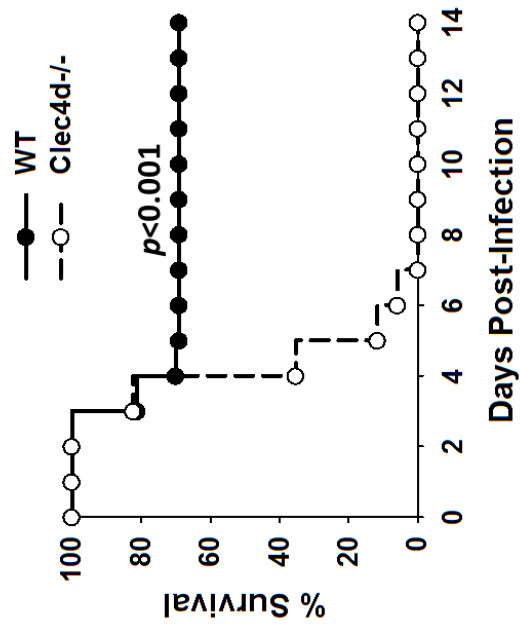
To examine if the inability of Clec4d<sup>-/-</sup> mice to clear bacteria was due to a defect in mounting inflammatory response, pro-inflammatory cytokines in infected mice lungs were analyzed. In both strains, mock infected mouse lungs displayed similar low basal levels of inflammatory cytokines tested (TNF- $\alpha$ , IFN- $\gamma$ , IL-6, IL-17) (Fig. 10A). Upon KPn infection, WT mice exhibited increased levels of these cytokines at 1dp.i., which



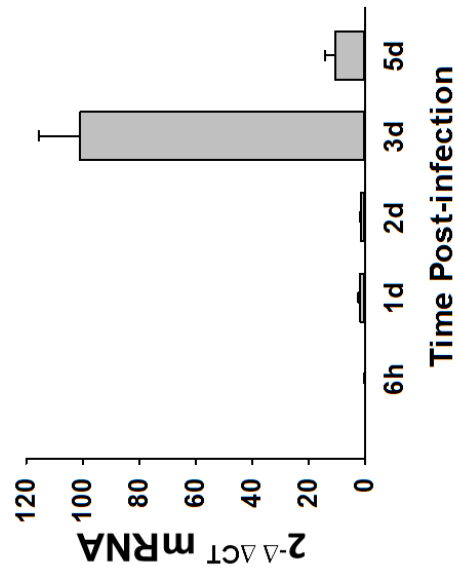
**Figure 9. Clec4d<sup>-/-</sup> mice display increased mortality accompanied with overwhelming bacterial burden in systemic organs during Gram negative pneumonia.**

(A). Sixteen WT (C57BL/6) and seventeen Clec4d<sup>-/-</sup> mice were intranasally infected with 5X10<sup>4</sup> CFUs of *Klebsiella pneumoniae* (KPn) in 20µl of sterile PBS and were assessed daily for disease severity. The increased susceptibility of Clec4d<sup>-/-</sup> mice as compared to WT mice is statistically significant as determined by Kaplan-Meier survival curve statistical analysis (p<0.001). (B) Total RNA was extracted by Trizol method from lungs harvested at the indicated times after infection with 5X10<sup>4</sup> CFUs of KPn. The mRNA levels of Clec4d were analyzed by real-time PCR as described in Materials and 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 averages of 6-8 mice per group in two independent experiments. (C). WT and Clec4d<sup>-/-</sup> were intranasally infected with 5X10<sup>4</sup> 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 one representative experiment (5 animals at each time point) out of three performed with similar results. Significant differences in bacterial burden (using non-parametric Mann-Whitney test) in WT and Clec4d<sup>-/-</sup> are denoted by asterisks (\*, p<0.05; \*\*, p<0.005, \*\*\*p<0.001).

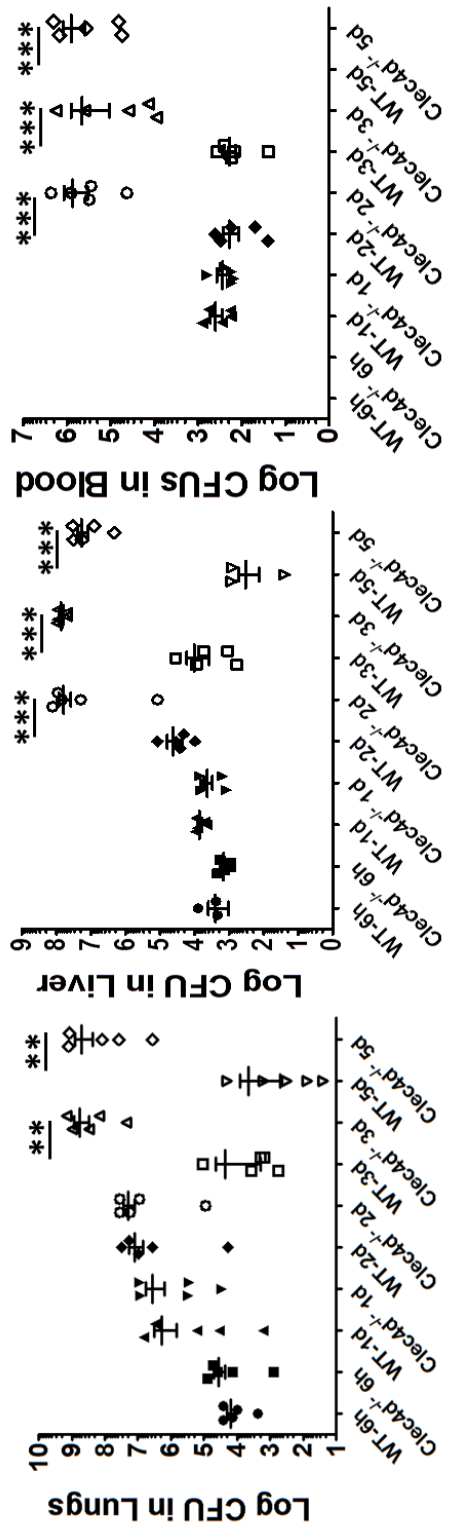
**A**



**B**



**C**

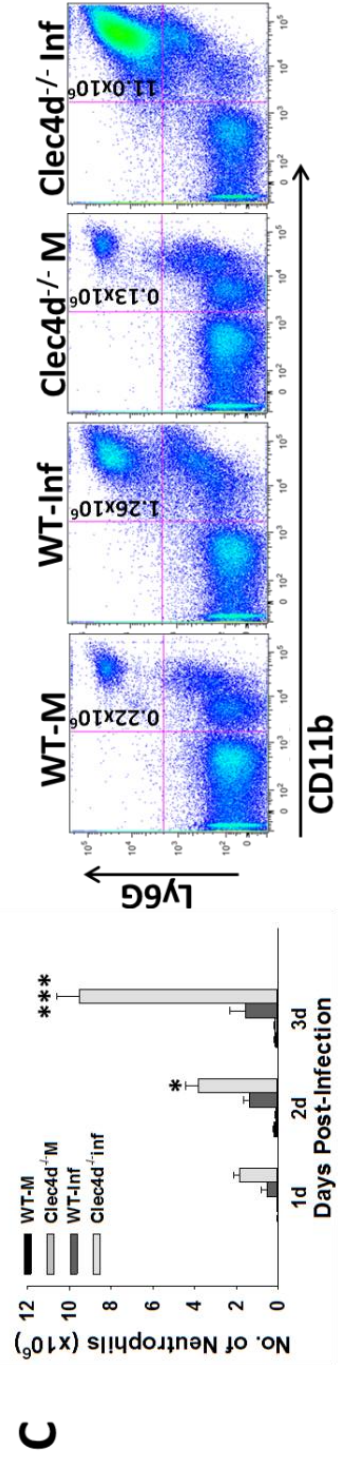
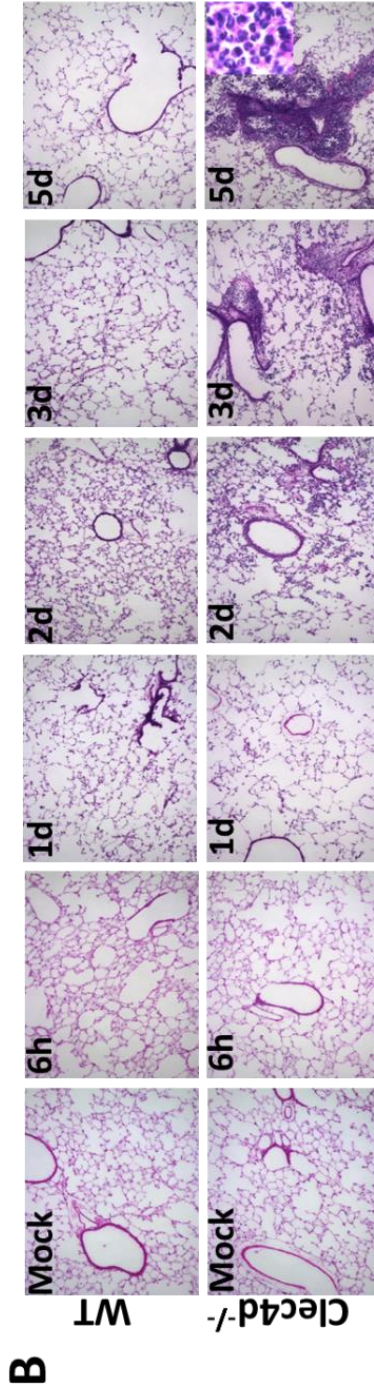
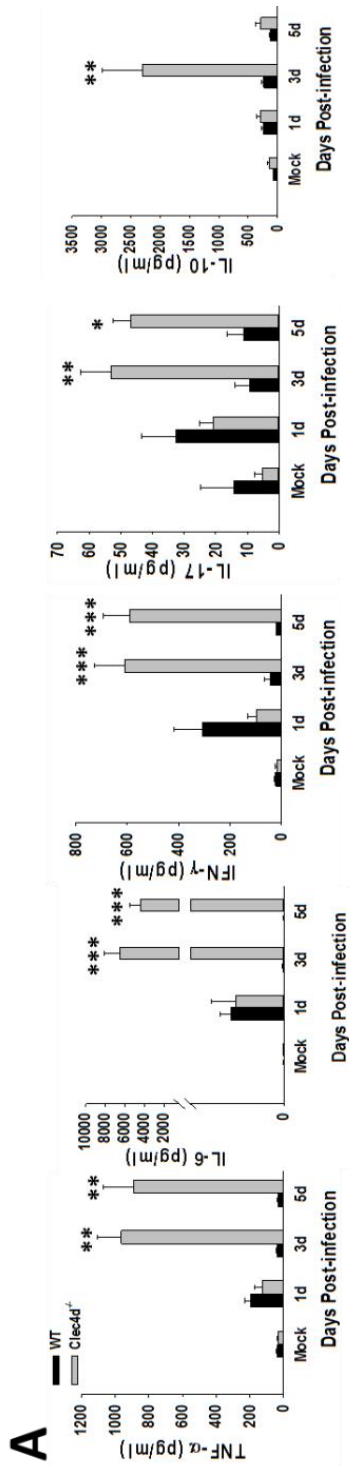


started to drop by 3dp.i. and were reduced to minimum by 5dp.i. (Fig. 10A). This was consistent with the reduced bacterial burden in these mice at these times post-infection. In contrast, infection of Clec4d<sup>-/-</sup> mice resulted in a progressive increase in levels of these cytokines through the course of infection, showing a massive upregulation at 3d and 5dp.i. Interestingly, at 1dp.i., the levels of these cytokines were slightly lower than those in the WT mice at that time p.i., however the differences were not statistically significant. Of note, unlike the pro-inflammatory cytokines tested, the amount of IL-10, a regulatory cytokine was significantly reduced at 5dp.i. in comparison to its level at 3dp.i. in Clec4d<sup>-/-</sup> mice. This was in contrast to other cytokines and chemokines whose levels continued to increase or stayed at similar high levels through day 5p.i. in these mice. In light of a recent study implicating STAT-1 regulated IL-10 production in resolution of KPn pneumonia [127], reduced level of this cytokine, either due to depletion of IL-10 producing cells in Clec4d<sup>-/-</sup> mice or due to the role of this receptor in continuous production of this cytokine in a positive feedback loop manner, possibly plays a role in lack of resolution in these mice. Notwithstanding the mechanism, our results show that Clec4d<sup>-/-</sup> mice do not display a defect in their ability to mount an inflammatory response but rather display a hyperinflammatory phenotype akin of cytokine storm typically associated with sepsis. Moreover, levels of sepsis marker mediators such as Factor VII, C-reactive protein and fibrinogen were also massively increased in these mice (data not shown). Importantly, in the absence of Clec4d, these mice are unable to resolve the infection and the resulting inflammation. This further indicates a protective role of this receptor in resolution of KPn pneumonia.

Next, H&E staining was performed to determine immunopathological changes in WT and Clec4d<sup>-/-</sup> mice. As shown in Fig. 10B, mock control WT and Clec4d<sup>-/-</sup> mice display similar normal lung tissue morphology. A moderate peribronchial and perivascular infiltration of immune cells was observed in WT mice, which was reduced substantially by 5dp.i. The overall architecture of the lungs was largely preserved in the WT animals throughout the infection. The Clec4d<sup>-/-</sup> mice, on the other hand, displayed a moderate immune cell infiltration for initial 2 days after infection. However, at later time points these mice exhibited much more severe pathological changes in their lungs with progressive increase in leukocytes (mainly neutrophils, as depicted by multilobed nuclear morphology in high magnification inset) infiltration through 3dp.i. By day 5 p.i., a time when the mice had become moribund, extensive foci of consolidation were visible with massive accumulation of neutrophils around alveolar spaces. Flow cytometry analysis of infiltrating cells in lungs showed that the numbers of Ly6G+CD11b+ neutrophils were significantly higher in the Clec4d<sup>-/-</sup> lungs at 3dp.i. in comparison with the WT (Fig. 10C). Interestingly, the Clec4d<sup>-/-</sup> mice showed a significantly increased number of neutrophils even at 2 p.i., a time when the bacterial burden was similar in the Clec4d<sup>-/-</sup> and WT lungs. In order to further determine if these neutrophils were defective in bacterial clearance in the absence of Clec4d, phagocytosis of GFP-labeled KPn was compared between WT and Clec4d<sup>-/-</sup> neutrophils by flow cytometry (as described in methods). As shown in Fig. 11A, Clec4d deficiency had no effect on phagocytosis of opsonized or non-opsonized bacteria. Furthermore, these neutrophils showed a similar bacterial killing ability as their WT counterparts (Fig. 11B). This indicated that Clec4d likely does not play a direct role in bacterial clearance and the increased neutrophil accumulation in pneumonic Clec4d<sup>-/-</sup>

**Figure 10. Pneumonic Clec4d<sup>-/-</sup> mice exhibit hyperinflammatory response and severe lung pathology characterized by massive neutrophil accumulation.**

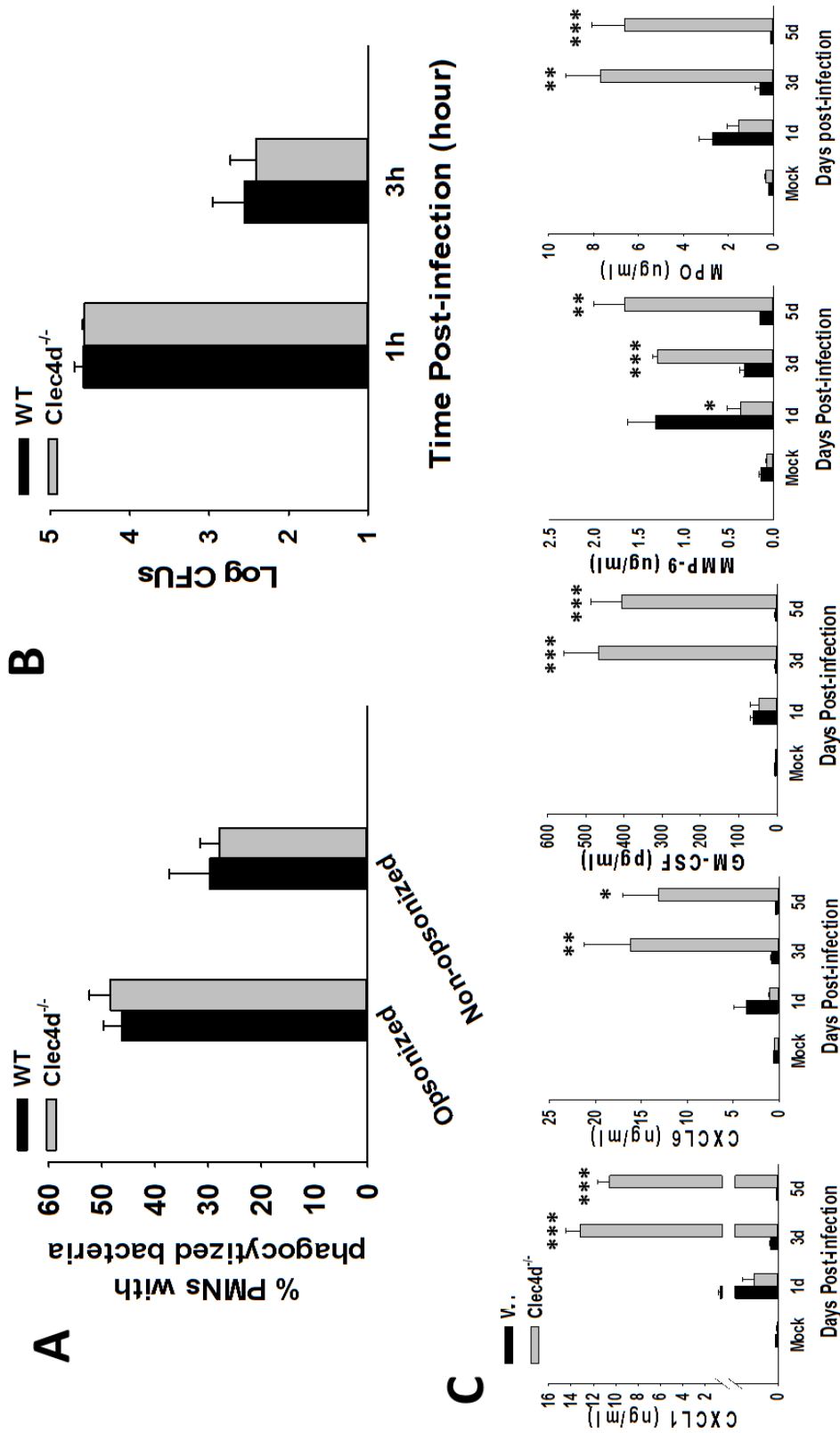
(A). The lungs from mock control and KPn infected WT and Clec4d<sup>-/-</sup> mice were harvested at indicated time points post-infection, homogenized in PBS with protease inhibitors and analyzed for rodent multi-analyte profile (Myriad<sup>TM</sup> Rules-Based Medicine, Austin, TX). Results shown are average of three infected and three mock control mice from 3 independent experiments. Significant differences are denoted by asterisks (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ , \*\*\* $p < 0.001$ ). (B). H&E staining of lung cryosections from mock control and KPn infected WT and Clec4d<sup>-/-</sup> mice isolated at indicated times post-infection. Magnification 100X. Inset shows a highly magnified area (1000X) of a lesion in infected Clec4d<sup>-/-</sup> depicting neutrophils as indicated by characteristic multilobed nuclear morphology. (C). Flow cytometry analysis of neutrophils in mock control (WT-M and Clec4d<sup>-/-</sup>-M) and KPn infected (WT-Inf and Clec4d<sup>-/-</sup>-Inf) WT and Clec4d<sup>-/-</sup> mice. Total lungs cells were isolated from mice by collagenase treatment at indicated times p.i.. The cells were stained with anti-Ly6G-APC and anti-CD11b-Pacific Blue antibodies as markers for neutrophils. Appropriate isotype matched negative controls were used to set the gates. The bar graph shows average of total number of neutrophils in lungs of three mock control and three KPn infected WT and Clec4d<sup>-/-</sup> mice from 3 independent experiments (total 9 mice per group). Dot plots shown on the right are representative of three mice per group in 3 independent experiments. Statistical significance between WT and Clec4d<sup>-/-</sup> mice are denoted by asterisks (\*\*,  $p < 0.005$ ).



**Figure 11. Clec4d deficiency does not impair neutrophil phagocytosis and killing of bacteria but leads to their unbridled accumulation and activation.**

(A). Peritoneal neutrophils from WT and Clec4d<sup>-/-</sup> 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. (B). Bacterial uptake and killing capacity of Clec4d<sup>-/-</sup> and WT neutrophils was determined at 1h and 3h by assessing intracellular CFUs in these cells as described in Methods. The experiment was repeated 3 times with similar results. (C). The lungs from mock control and KPn infected WT and Clec4d<sup>-/-</sup> mice were harvested at indicated time points post-infection, homogenized in PBS with protease inhibitors and analyzed commercially for neutrophil associated immune mediators using rodent multi-analyte profile (Myriad<sup>TM</sup> Rules-Based Medicine, Austin, TX). Results shown are average of three infected and three mock control mice from 3 independent experiments. Significant differences are denoted by asterisks (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ , \*\*\* $p < 0.001$ ).







mice is not a secondary effect resulting from a defective bacterial clearance in the absence of this CLR. On the other hand, neutrophil chemoattractants (CXCL1 and CXCL6), neutrophil survival mediator (GM-CSF) and neutrophil activation markers (MMP9, MPO) were significantly higher in *Clec4d*<sup>-/-</sup> mice, as compared to their WT counterparts (Fig. 11C). These results show that *Clec4d* is likely involved in neutrophil turnover and the absence of *Clec4d* results in greater and prolonged accumulation of neutrophils in lungs of mice. The persistent activation of these neutrophils is leading to greater lung pathology observed in *Clec4d*<sup>-/-</sup> 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 *Clec4d* does not impair infiltration, bacterial phagocytosis and activation of neutrophils, this CLR is required for resolution of inflammation by way of facilitating neutrophil turnover.

Resolution of inflammation is an active and tightly controlled process which is necessary for restoration and maintenance of tissue homeostasis. Deregulation of this process is the root cause of many diseases. Our results indicate that deficiency of *Clec4d* in an otherwise sublethal pulmonary KPn infection leads to a non-resolving, hyperinflammatory response which culminates in the death of infected KO animals. This report, for the first time shows that *Clec4d* likely plays a role in resolution of inflammatory response. 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 [126,128]. 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 KO mice and higher

amounts of their associated inflammatory mediators suggests that there is a defect in neutrophil turnover in these mice. Since Clec4d was found not to be directly involved in bacterial uptake and killing by neutrophils, it seems more likely that due to the widespread tissue damage owing to persistent neutrophil activity in the Clec4d<sup>-/-</sup> mice, other immune cells important for bacterial clearance are depleted leading to this increase in local bacterial burden as well a systemic spread. Thus an increased bacterial burden (secondary to the defect in neutrophil turnover) is likely a factor contributing to the increased mortality of these mice eventually. As the neutrophil turnover and resolution of neutrophil associated responses is a complex process involving apoptosis and clearance of their contents (efferocytosis) and class-switching of lipid mediators, we are currently investigating the role of Clec4d in these processes.

This study shows that Clec4d plays an important role in mitigating the inflammation during a pneumonic infection, possibly by facilitating neutrophil turnover. This study opens up new avenues of research on the role of Clec4d in resolution of inflammatory responses. This can have major implications in the therapeutic measurements of inflammation associated disorders.

### **Acknowledgement**

The authors thank Dr. Steven Clegg, University of Iowa for providing GFP-labeled *K. pneumoniae* and Dr. Umamahesh Gundra, New York University, for technical help with flow cytometry. This study was supported by grants 10BGIA4300041 from American Heart Association and 1R21AI101644-01 from the National Institutes of Health to JS.

## Conflict of Interest

The authors declare no financial conflict of interest.

# Alarmin function of Galectin-9 in murine respiratory tularemia

Running Title: Galectin-9 as novel alarmin

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## Abstract

Sepsis is a complex immune disorder that is characterized by systemic hyperinflammation. Alarmins, which are multifunctional endogenous factors, have been implicated in exacerbation of inflammation in many immune disorders including sepsis. Here we show that Galectin-9, a host endogenous  $\beta$ -galactoside binding lectin, functions as an alarmin capable of mediating inflammatory response during sepsis resulting from pulmonary infection with *Francisella novicida*, a Gram negative bacterial pathogen. Our results show that this galectin is upregulated and is likely released during tissue damage in the lungs of *F. novicida* infected septic mice. In vitro, purified recombinant galectin-9 exacerbated *F. novicida*-induced production of the inflammatory mediators by macrophages and neutrophils. Concomitantly, Galectin-9 deficient ( $\text{Gal-9}^{-/-}$ ) mice exhibited improved lung pathology, reduced cell death and reduced leukocyte infiltration, particularly neutrophils, in their lungs. This positively correlated with overall improved survival of *F. novicida* infected  $\text{Gal-9}^{-/-}$  mice as compared to their wild-type counterparts. Collectively, these findings suggest that galectin-9 functions as a novel alarmin by augmenting the inflammatory response in sepsis development during pulmonary *F. novicida* infection.

## Introduction

Systemic hyperinflammation is the underlying cause of many immune inflammatory diseases, including sepsis. Despite more than three decades of active research, severe sepsis and septic shock remain major healthcare challenges with a mortality rate of 20-50% and no effective therapy [5]. During sepsis, overwhelming and sustained release of pro- as well as anti-inflammatory cytokines, termed cytokine storm, causes extensive tissue damage and wide-spread cell death, eventually resulting in death. Despite the identification of cytokine circuitry as major determinants of mortality, pro-inflammatory cytokine blockade has been ineffective as therapy for sepsis. This indicates the involvement of additional mediators that are likely acting as regulators and/or perpetrators of this hyper inflammatory response.

Alarmins are evolutionarily conserved endogenous molecules that perform homeostatic functions when contained within cellular compartments [29]. However, under pathological conditions, these molecules can be released either passively from dead cells or actively via non-classical secretion pathways [84]. Once in the extracellular milieu, they exhibit immune modulatory properties such as induction of pro-inflammatory cytokines, immune cell chemotaxis, and regulation of cell death [29]. In fact, a sustained and excessive release of alarmins has been shown to contribute to pathogenesis of several sterile as well as infectious inflammatory conditions [129,130]. Pertaining to their ability to impact innate immune cells such as macrophages, dendritic cells and neutrophils, alarmins also represent a crucial link between innate and adaptive immune responses, and hence an attractive therapeutic target for complex disorders such as sepsis.

Francisella is a highly virulent bacterial pathogen that causes an acute lethal disease called tularemia in humans and mice. Although there are strain-dependent differences in the initial mechanisms involved [131,132], our studies have shown that pulmonary infection of mice with fully virulent *Francisella tularensis* as well as the murine model organism *F. novicida* (F.n.) leads to development of severe sepsis characterized by hyperinflammation, T cell depletion, and extensive cell death in systemic organs [73,77,78]. As pulmonary infections are a major cause of sepsis [76], we are using a murine inhalation model of F.n. infection to understand the mechanism/s responsible for pulmonary infection-induced sepsis development. A recent report of an F.n. outbreak in a correctional facility suggests that this strain may be more virulent to humans than initially surmised, supporting its relevance as a model strain to understand pathogenesis [133]. Furthermore, studies from our and other laboratories have shown that extensive tissue damage and wide-spread cell death is a hallmark of Francisella infection, regardless of the bacterial strain [78,80,81]. Additionally, our studies show that Francisella infected macrophages are defective in clearance of dead cells, a process termed efferocytosis, leading to accumulation of these dead cells and their progression to secondary necrosis [74]. It is thus likely that alarmins released from these dead or dying cells contribute to the inflammatory response culminating in sepsis development during respiratory infection with Francisella. We and others have reported an alarmin-mediated regulation of the inflammatory response during pulmonary infections [1,134,135]. However, in a complex immune disorder like sepsis which is an interplay of several host immune pathways such as the coagulation system, complement cascade and even the autonomic nervous system [26], several alarmins may be involved at the intersections of these

pathways. Thus, identification of additional alarmins will present therapeutic targets that may have more tangible translational potential when used in combination.

Galectins, mammalian  $\beta$ -galactoside binding lectins, are emerging as potent immune regulators in a variety of pathological processes including inflammation, autoimmunity, fibrosis, and cancer [88,121]. Curiously, some galectins (galectin-1 and -3) have been shown to be secreted in the extracellular milieu via a non-classical ER/Golgi-independent pathway, where they exert immune modulating effects on immune cells. This is a characteristic feature of alarmins [136,137]. However the contribution of galectins as alarmins to sepsis development is poorly understood. In this study we have investigated the role of galectin-9, in the F.n. induced inflammatory response and sepsis. Our analyses of galectin-9 expression, distribution, immune modulation and comparison of disease progression in F.n. infected galectin-9 sufficient and –deficient mice show that galectin-9 acts as an alarmin to exacerbate the inflammatory response in Francisella infection induced sepsis development.

## **Materials and Methods**

### Ethics Statement

The animal usage protocols were approved by the Institutional Animal Care and Usage Committee at the University of North Dakota (protocol no. 1108-3). All procedures strictly followed the institutional and federal guidelines and all efforts were made to minimize the animal suffering.

### Bacterial strains and Mice

The F.n. strain U112 was grown on Trypticase Soy Agar (TSA) medium supplemented with L-cysteine at 37°C. After overnight growth, the bacteria were harvested and



suspended in a freezing medium (250 mM sucrose, 10 mM sodium phosphate pH 7.2 and 5 mM glutamic acid). Aliquots of the stocks were frozen at  $-80^{\circ}\text{C}$  for further use. The animal usage protocols were approved by the Institutional Animal Care and Usage Committee at the University of North Dakota. All procedures strictly followed the institutional and federal guidelines and all efforts were made to minimize animal suffering. In vivo experiments were performed using 6-8 wk old female C57Bl/6 wild-type and Gal-9<sup>-/-</sup> mice. Gal-9<sup>-/-</sup> mice were kindly provided by Dr. Judy M. Teale, University of Texas at San Antonio (initially obtained from the Consortium of Functional Glycomics, Scripps, La Jolla) as F4 and were back-crossed for additional 6 generations. Sex- and age-matched Gal-9<sup>+/+</sup> mice with the same genetic background were used as controls.

#### Antibodies and Reagents

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. For detection of galectin-9 by immunofluorescence (IF) staining, a purified rat galectin-9 antibody (Abcam, San Diego, CA) followed by Alexa-546 conjugated chicken anti-rat antibody (Molecular Probes, OR) was used. A rat anti-mouse CD11b antibody conjugated to PE (BD Pharmingen) and a purified rat anti-mouse Ly-6G (Clone Accurate Chemical, Westbury, NY, USA) followed by chicken anti-rat Alexa488 (Molecular Probes, OR) were used for detection of activated neutrophils by IF staining. For flow cytometry Pacific Blue<sup>TM</sup> anti-mouse CD11b and APC anti-mouse Ly6G (Clone 1A8) antibodies (Biolegend, San Diego, CA) were used. The terminal deoxyribonucleotidyl transferase-mediated triphosphate (dUTP)-biotin nick end labeling (TUNEL) staining kit was purchased from Chemicon International, CA. Purified recombinant galectin-9 was

purchased from R&D Systems, MN. The endotoxin level was <1.0 EU per µg of protein. Ultrapure *E. coli* endotoxin was purchased from Invivogen and lactose was purchased from Sigma. For detection of reactive oxygen species, Fc OxyBURST assay reagent was purchased from Molecular Probes, Eugene, OR. Mouse IL-6 ELISA kits (BD OptEIA) were from BD Biosciences, San Diego, CA.

#### Infection of Mice, survival and bacterial burden

Mice were anaesthetized using a mixture of ketamine HCL and xylazine (30mg/ml ketamine, 4 mg/ml xylazine in PBS) and were infected intranasally with 50-70 CFUs of the F.n. strain U112 in 20 µl of PBS or with 20 µl of PBS alone. The mice were monitored daily. The survival of infected mice (15 mice each WT and Gal-9<sup>-/-</sup> in 3 independent experiments) was recorded for up to 2 weeks post-infection (p.i.). The death was recorded as tularemia induced mortality. Mice displaying severe signs of distress (labored breathing, non-responsiveness to cage tapping, failure of grooming and severe eye discharge) were humanely sacrificed by injecting a mixture of ketamine (90-120mg/kg) and xylazine (10mg/kg) followed by cervical dislocation. In some experiments, the mice were euthanized at indicated times p.i. and blood, lungs, liver and spleen were aseptically harvested. The organs were homogenized aseptically in cold PBS with Complete<sup>TM</sup> protease inhibitor cocktail (Roche Diagnostics, Germany). For the bacterial burden analyses, the homogenates and blood were serially diluted in PBS and plated on TSA. CFU counts per mouse were calculated after incubating the plates at 37°C overnight.

### Quantitative real-time PCR

Lungs from infected and mock control mice at various times p.i. were removed immediately after perfusion and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis of the samples was performed using SYBR green (Applied Biosystems, CA, USA) as described by us [1]. Transcript levels of the housekeeping ribosomal 18S and galectin-9 genes were measured in each sample by PCR amplification using specific primers: 18S (forward) 5'-CATGTGGTGTGAGGAAAGCA-3' and (reverse) 5'-GTCGTGGGTTCTGCATGATG-3'; Gal-9 (forward) 5'-TCAAGGTGATGGTGAACAAGAAA-3' and (reverse) 5'-GATGGTGTCCACGAGGTGGTA -3'. The target gene expression levels were normalized to those of the house keeping 18S gene in the same sample. Expression of galectin-9 in infected samples was determined as fold change over that in control samples calculated by using the formula  $2^{-(\Delta\Delta Ct)}$ .

### Histological and Immunofluorescence staining

For histological and immunofluorescence staining, frozen lung tissues were processed as previously described [77]. Lung cryosections thus obtained were stained with hematoxylin and eosin (H&E) for pathological analysis, or for the detection of galectin-9 by immunofluorescence staining, as previously described [89]. Semi-quantitative histopathology was performed as described previously in [138]. For the detection of cell death, TUNEL method was used according to manufacturer's instructions (Chemicon International, CA). The images were acquired using a Nikon Eclipse fluorescent

microscope with an attached SPOT II digital camera. The images were processed and analyzed using Adobe Photoshop 7.0 software (Adobe, Mountain view, CA).

#### Flow cytometry

Lungs were harvested from the infected and mock control mice at 3 days p.i. (dpi) after perfusion with PBS and were treated with collagenase to obtain single cell suspensions as previously described [77,78]. Cells were then co-stained with anti-CD11b and -Ly6G antibodies. FlowJo (Tree Star) software was used to analyze data.

#### 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 flow-based system: Mouse MAP™ (Multi-Analyte Profiles) analysis technology.

#### Neutrophil and macrophage activation

Cells were isolated from the peritoneal cavities of naïve C57BL/6 mice 12-14h after intraperitoneal injection of sterile 4% thioglycollate. Non-adherent neutrophils were plated at the density of  $1 \times 10^6$  cells and were infected with wild-type F.n. strain U112 at MOI 50 with or without pretreatment of the cells with 15µg/ml of purified recombinant galectin-9. Cells stimulated with galectin-9 alone or with 10ng/ml of phorbol myristate acetate (PMA) served as controls. One hour after stimulation, production of reactive oxygen species (ROS) was measured in the cells by flow cytometry using Fc OxyBURST reagent according to the manufacturer's instructions. A minimum of 20,000 events were read for each sample and all the cells fluorescing positive in FITC channel (excitation and

emission maxima of ~490 nm and ~520 nm, respectively) were gated to obtain the percentage of ROS producing cells.

Bone marrow was isolated from wild-type and Gal-9<sup>-/-</sup> mice and the cells were differentiated to macrophages as previously described [91]. On day 6 of culture, the cells were plated at  $8 \times 10^4$  cells / well in 96-well flat-bottom plates and were stimulated with galectin-9 with or without 25mM lactose. As a control for endotoxin contamination, recombinant galectin-9 or UltraPure LPS boiled for 45 min at 100 °C were used to stimulate cells. Culture supernatants were collected 24h after stimulation and IL-6 levels were measured by ELISA according to the manufacturer's instructions (BD OptEIA, BD Biosciences).

#### 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 two-way ANOVA with Tukey post-test.

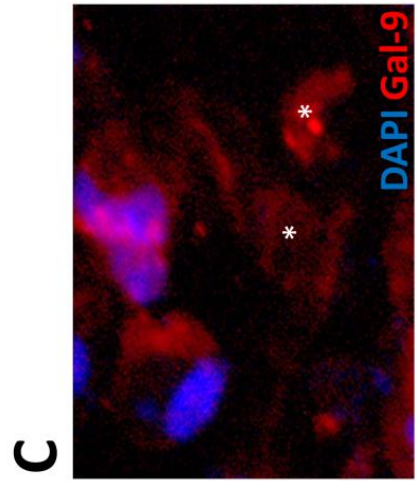
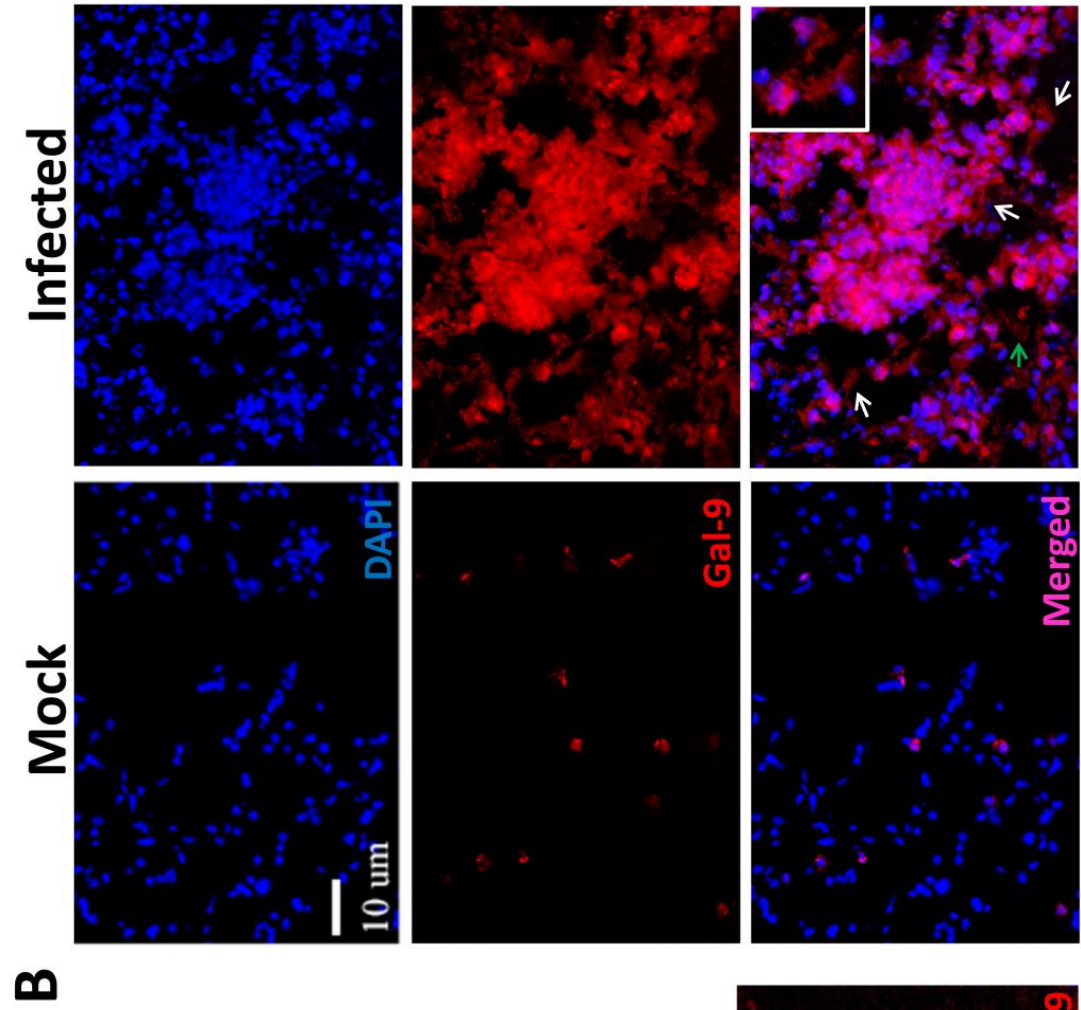
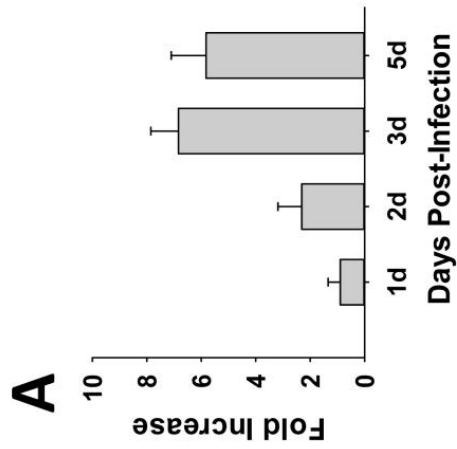
## Results

### Galectin-9 is upregulated and released during the septic phase of F.n. infection.

We and others have previously shown that extensive cell death is a hallmark of pulmonary Francisella infection, regardless of the bacterial strain used (8, 9, 21-23). Factors released from dead or dying cells, termed as alarmins, have been shown to exhibit inflammatory, chemoattractant and immune activating properties in various pathological conditions. To test our hypothesis that galectin-9 functions as an alarmin, we first analyzed the expression and distribution of galectin-9 in the lungs of mice undergoing pulmonary infection with F.n. As shown in Figure 12A, galectin-9 transcript levels progressively increased in the lungs of infected mice and reached the highest levels at 3dpi. This increase in galectin-9 expression at 3 dpi is consistent with the appearance of other sepsis features (extensive cell death, hyperinflammatory response, increased vascular injury) at this time, as reported in our previous studies with F.n. as well as the fully virulent *F. tularensis* [77,78]. Immunofluorescence (IF) analysis of galectin-9 protein expression in cryosections of the lungs harvested at this septic phase showed a low basal level of galectin-9 in mock control mice inoculated with PBS alone (Fig. 12.B). F.n. infected mice, on the other hand, exhibited very high galectin-9 expression in their lungs. Furthermore, galectin-9 could be detected in cell-free areas, especially in the lesions with massive cellular infiltration, indicating that it is likely released extracellularly (Fig. 12.B lower panel and inset). Together, these data suggested that galectin-9 likely exhibits a characteristic alarmin property of extracellular release during septic phase of pulmonary F.n. infection.

**Figure 12. Galectin-9 is upregulated and released in lungs of mice during respiratory *F. novicida* (F.n.) infection.**

(A) Total RNA was extracted by Trizol method from lungs harvested at the indicated times after infection with the F.n. strain U112. The mRNA levels of galectin-9 were analyzed by real-time PCR as described in Materials and Methods and are expressed as fold increase over the levels in mock control mice. Data shown are the averages of 3- 4 mice per group. (B) In-situ IF staining of frozen lung sections from mock control and U112 infected mice harvested at 3 dpi. The sections were stained for galectin-9 (red) using a purified rat galectin-9 antibody followed by Alexa-546 conjugated chicken anti-rat antibody. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindol-dilactate (DAPI). Magnification X 200. Inset depicts possible extracellular galectin-9 in infected mouse lungs. The area indicated by green arrow has been enlarged and shown in (C). Asterisks depict galectin-9 present in cell-free areas.



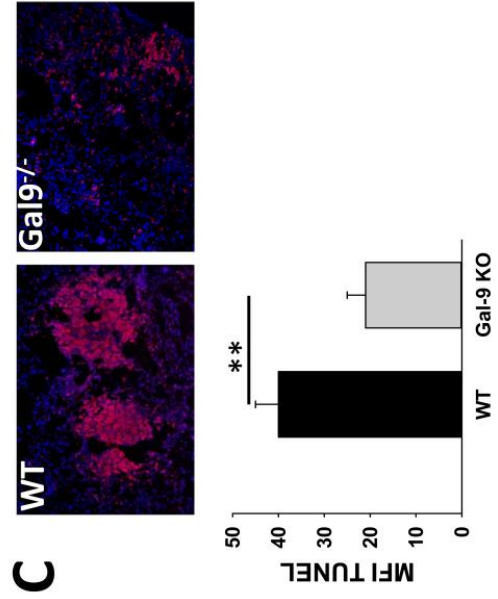
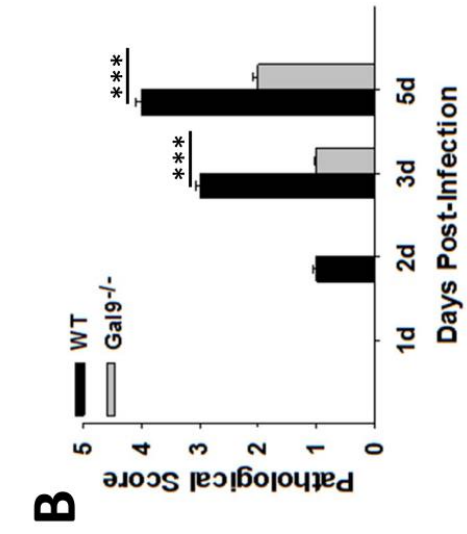
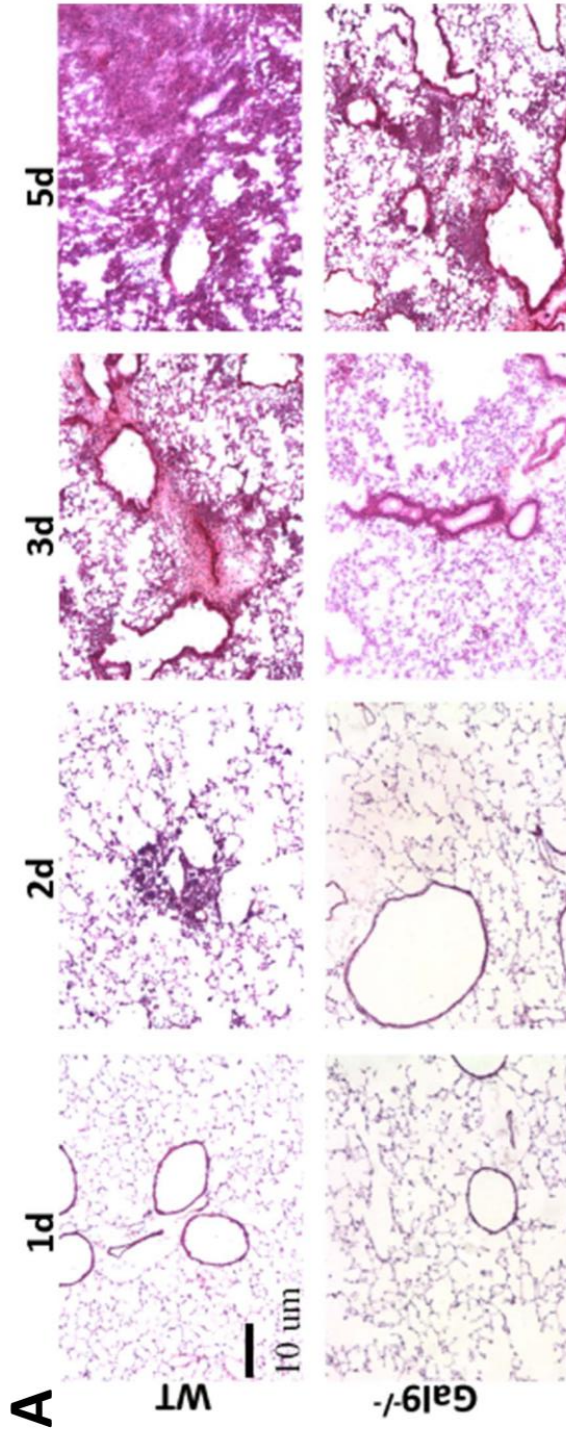


### Galectin-9 deficiency ameliorates lung pathology in F.n. infected mice

In order to assess the role of galectin-9 in overall disease severity during tularemia, we next examined H&E stained lung cryosections from F.n. infected wild-type (WT) and Gal-9<sup>-/-</sup> mice. Consistent with our previous studies, a progressive increase in cellular infiltration was observed in the F.n. infected WT mice with extensive pathology, severe bronchopneumonia and massive cell death, occurring in the center of large granuloma-like areas of infiltration at 3 dpi (Fig. 13.A upper panel). The lungs of Gal-9<sup>-/-</sup> mice, on the other hand, showed a substantial delay in the cellular infiltration with moderate peribronchial and perivascular infiltration by 3dpi (Fig. 13.A lower panel). Although these mice displayed an increased cellular infiltration at 5dpi, the lesions appeared much smaller in the lungs of Gal-9<sup>-/-</sup> mice as compared to those in the WT mice. Concomitantly, the Gal-9<sup>-/-</sup> mice exhibited significantly lower pathological score in comparison with their WT counterparts at all the time points tested (Fig. 13.B). Mock infected WT and Gal-9<sup>-/-</sup> mice exhibited similar normal lung architecture with minimal cellular infiltration and clear air spaces (data not shown). As alarmins are released passively from dead cells, we analyzed the extent of cell death in the lungs of infected WT and Gal-9<sup>-/-</sup> mice by TUNEL assay. As shown in Figure 13C, septic lungs of F.n. infected WT mice showed extensive cell death, indicated by a large number of TUNEL positive cells within perivascular and peribronchial lesions. In contrast, infected Gal-9<sup>-/-</sup> mice exhibited significantly fewer TUNEL positive cells in their lungs (Fig. 13.C). The improved lung architecture and reduced cell death in the absence of galectin-9 indicates a pathological role of this lectin during pulmonary Francisella infection.

**Figure 13. Gal-9<sup>-/-</sup> mice exhibit improved lung pathology and reduced TUNEL staining indicative of cell death upon pulmonary F.n. infection.**

**(A)** The lungs from F.n. infected wild-type (WT) or Gal-9<sup>-/-</sup> mice were harvested at indicated times post-infection, embedded in optimal-cutting-temperature (OCT) compound, and sectioned as described in Materials and Methods. The frozen sections were stained with Hematoxylin and Eosin (H&E). The images obtained are representatives of three experiments performed with 3 mice per group in each experiment. Magnification, ×200. **(B)** H&E sections were scored in blinded fashion according to the following scoring scale: 0, no inflammatory cells (macrophages or neutrophils) present in section; 1, <5% of section infiltrated by inflammatory cells; 2, 5–10% of section infiltrated; 3, 20% of section infiltrate; and 4, >20% of section infiltrated. **(C)** Frozen lung sections from F.n. infected WT or Gal-9<sup>-/-</sup> mice were processed for in-situ TUNEL staining for detection of DNA fragmentation (red) in nuclei. Nuclei (blue) were stained with 4',6'-diamidino-2-phenylindole dilactate. Bar graph shows Mean fluorescence intensity (MFI) quantified using Image J software. Magnification, ×100.



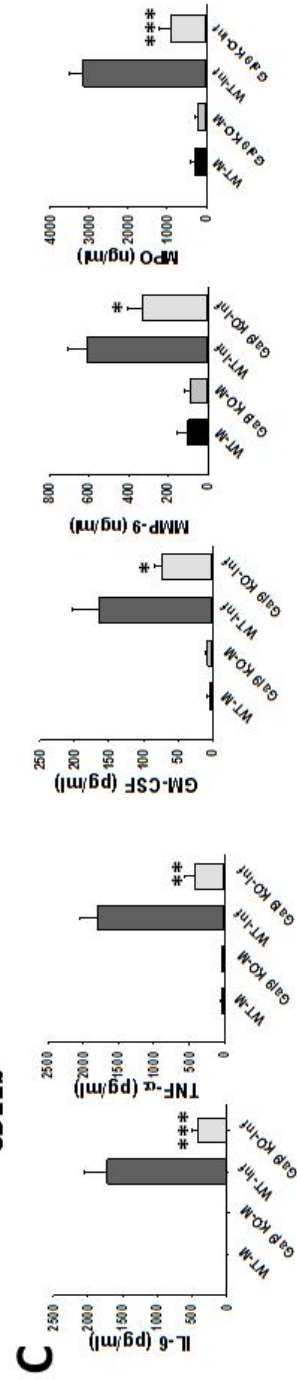
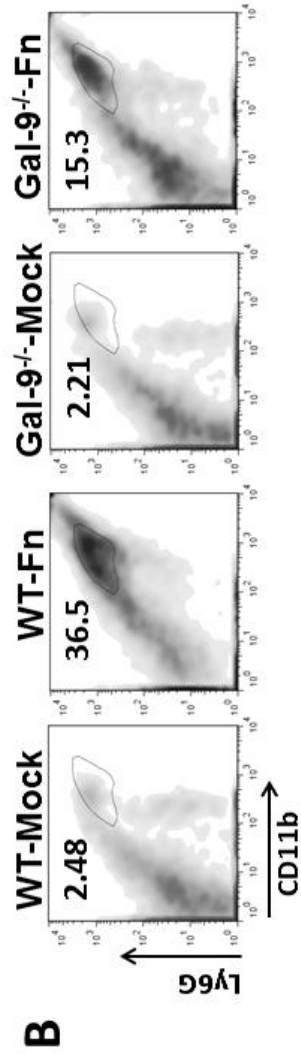
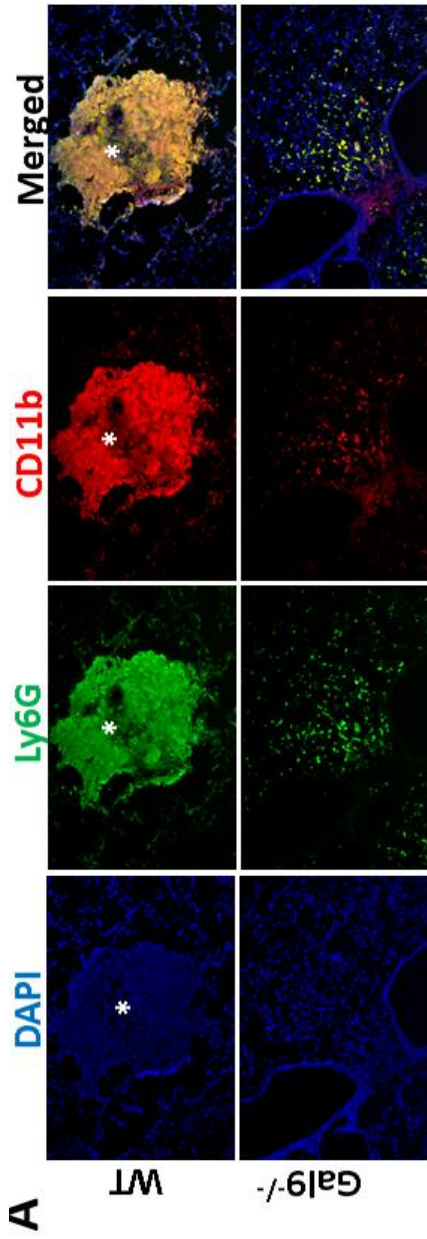
Galectin-9 regulates cellular infiltration and inflammatory response in vivo during F.n. infection

Alarmins exert their immune stimulatory effects by mediating immune cell infiltration directly or indirectly, and by predisposing the cells to produce increased levels of inflammatory mediators in response to infection. We hypothesized that; similar to the function of alarmins, increased expression and extracellular localization of galectin-9 may be contributing directly or indirectly to the increased cellular infiltration and hyperinflammatory response observed during pulmonary Francisella infection. Previous studies from our and other laboratories have shown that neutrophils are the predominant cell type infiltrating in F.n. infected WT mice [77,78,94,139]. In order to compare neutrophil infiltration in the lungs of Gal-9<sup>-/-</sup> and WT mice infected with F.n., IF staining and flow cytometry was performed. Consistent with our previous studies, lungs of the infected WT mice showed extensive infiltration of CD11b<sup>+</sup> Ly6G<sup>+</sup> neutrophils that accumulated largely in the lesions (Fig.14.A and B). The numbers of Ly6G<sup>+</sup>CD11b<sup>+</sup> cells in Gal-9<sup>-/-</sup> mice, on the other hand, were significantly reduced (Fig.14.A and B). These results suggested a role of galectin-9 in regulation of neutrophil accumulation in the lungs of mice during pulmonary F.n. infection. Furthermore, analysis of the levels of inflammatory cytokines and neutrophil-associated mediators in the lungs revealed significantly reduced levels of TNF- $\alpha$ , IL-6, myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9) and GM-CSF, in the infected Gal-9<sup>-/-</sup> mice (Fig.14.C). Together, these results suggested a putative role of galectin-9 in neutrophil accumulation and associated inflammation.

**Figure 14. Gal-9<sup>-/-</sup> mice display reduced accumulation of neutrophils and reduced inflammatory mediators in lungs during F.n. infection.**

**(A)** Frozen sections of lungs harvested at 3 dpi from F.n. infected WT or Gal-9<sup>-/-</sup> mice were co-stained with antibodies against myeloid cell markers CD11b (red) and Ly6G (green). A high co-expression of both markers is depicted by yellow color and indicates neutrophils. Nuclei (blue) were stained with 4'6' diamidino-2-phenylindol-dilactate (DAPI). Magnification X 200. Asterisks depict lesions in the lungs. **(B)** Flow cytometry analysis of neutrophils in mock control and F.n. infected WT and Gal-9<sup>-/-</sup> (WT-Fn and Gal-9<sup>-/-</sup>-Fn) mice. Total lungs cells were isolated from mice by collagenase treatment at 3 dpi as described in Methods. The cells were stained with anti-Ly6G-APC and anti-CD11b-Pacific Blue antibodies as markers for neutrophils. The plots are representative of three mice per group in 3 independent experiments. **(C)** Lungs from mock infected and F.n. infected WT or Gal-9<sup>-/-</sup> mice were harvested at 3dpi, homogenized with protease inhibitors in PBS and analyzed commercially for rodent multi-analyte profiles (Myriad Rules-Based Medicine, Austin, TX). Levels of inflammatory cytokines and neutrophil markers in lung homogenates are shown. Results shown are from 3-4 mice per group from 3 different experiments. MMP-9; matrix metalloproteinase 9, MPO; myeloperoxidase. \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.001$ . Comparisons were made between infected WT and Gal-9<sup>-/-</sup> groups.

5





### Galectin-9 exacerbates F.n. infection induced inflammatory response in vitro

To examine the direct effect of galectin-9 on activation of neutrophils and macrophages, two cell types playing a predominant role in tularemia pathogenesis, we next carried out in vitro stimulation of these cells with recombinant galectin-9 protein with or without F.n. infection. While stimulation with purified galectin-9 or U112 infection alone moderately induced ROS production in peritoneal neutrophils, pre-treatment of neutrophils with this lectin induced an increased amount of ROS in response to F.n. infection, which was significantly higher than that elicited by galectin-9 or F.n. infection alone (Fig.15.A). Stimulation of bone marrow derived macrophages (BMDMs) with galectin-9 alone or galectin-9 and U112 together elicited a significantly higher IL-6 production than U112 alone. (Fig. 15.B). This immune stimulatory effect of galectin-9 was abolished upon heat-denaturation or by addition of 25mM lactose to the cultures, confirming the specificity of this activity. These observations indicated that while galectin-9 by itself can stimulate myeloid cells, particularly macrophages, it can also augment Francisella infection-induced myeloid cell activation which likely has implications in exacerbation of inflammation culminating in sepsis development during this infection.

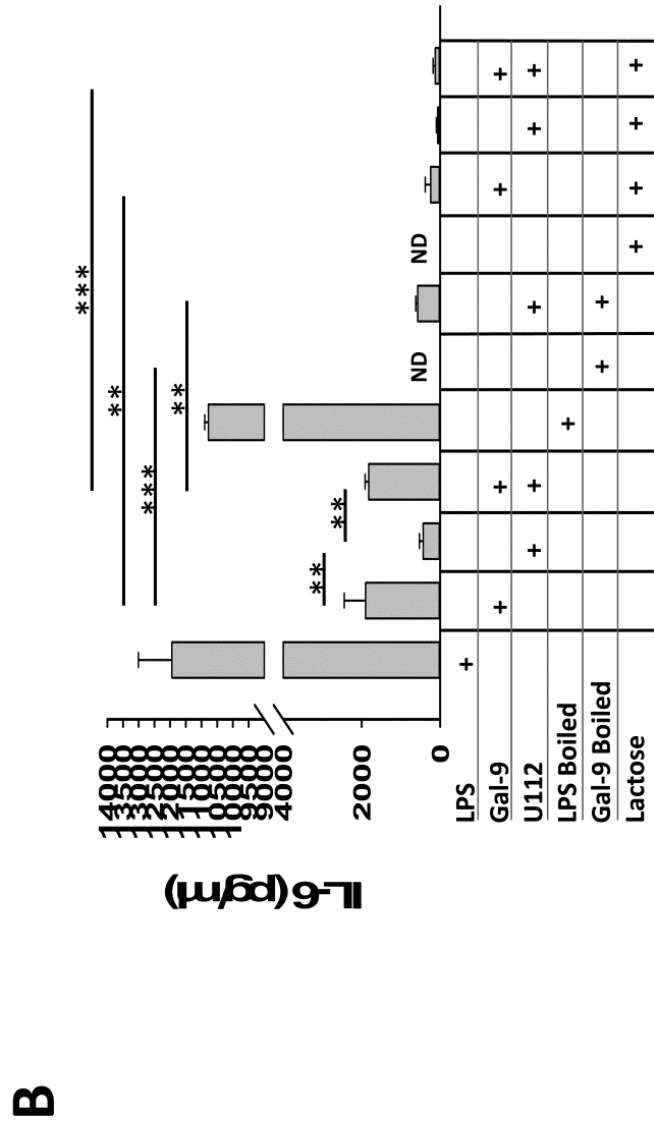
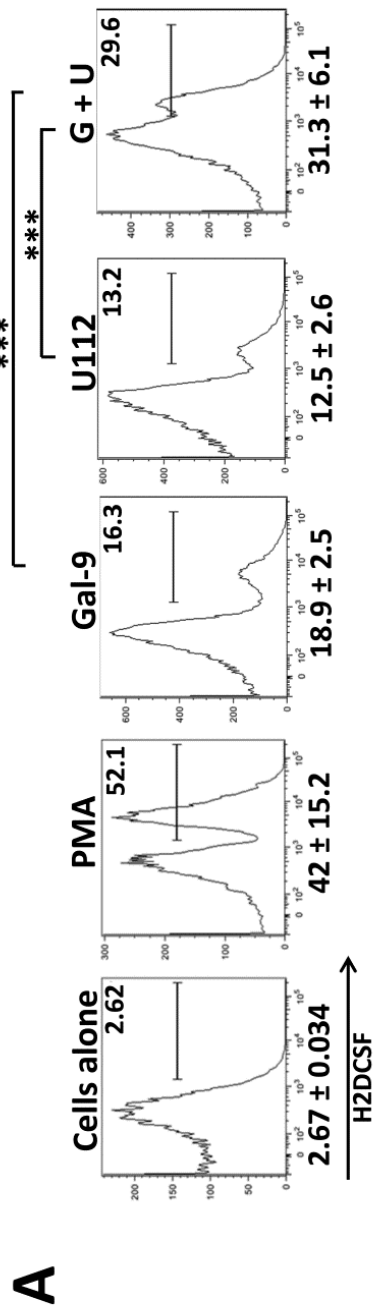
### Gal9<sup>-/-</sup> mice show improved survival following F.n. infection.

In order to correlate the improved lung pathology and reduced inflammatory responses with the disease outcome, overall disease severity and survival was compared in WT and Gal-9<sup>-/-</sup> mice infected with F.n. In the infected WT mice, visible signs of disease started to appear by 3 dpi which typically included piloerection, hunched gait, lethargy, and eye discharge. All of these mice succumbed to infection by day 6 p.i. (Fig. 16.A). By contrast

**Figure 15. Galectin-9 regulates F.n. infection induced inflammatory response in vitro.**

**(A).** Peritoneal neutrophils were isolated from mice 12-14h after injection with 4% thioglycollate and were stimulated with F.n. at an MOI 50 with or without pre-treatment with purified recombinant galectin-9 (15µg/ml). Stimulation with galectin-9 alone or phorbol myristate acetate (PMA, 10ng/ml) was used as a control. Production of reactive oxygen species (ROS) was measured one hour post-stimulation by flow-cytometry using Fc-OxyBURST dye following the manufacturer's instructions. Numbers below the plots depict average percent of ROS positive cells from 3 independent experiments. Plots from a representative of these 3 independent experiments are shown. **(B)** Bone marrow derived macrophages (BMDMs) from C57Bl/6 wild-type mice were stimulated with F.n. Strain U112 at an MOI of 50 with or without pretreatment with 15µg/ml of purified recombinant galectin-9. UltraPure E.coli LPS (10ng/ml) and galectin-9 with and without heat-denaturation (boiled at 100°C for 45 min) as well as competitive inhibition with lactose (25mM) were used as controls to test the specificity of galectin-9 effect, as described in methods. Culture supernatants were collected 24h after infection and the amount of IL-6 was measured by ELISA. The data shown is average of three independent experiments. Statistical analysis between the data sets was performed by Two-way ANOVA with Tukey post-test where \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.001$ .



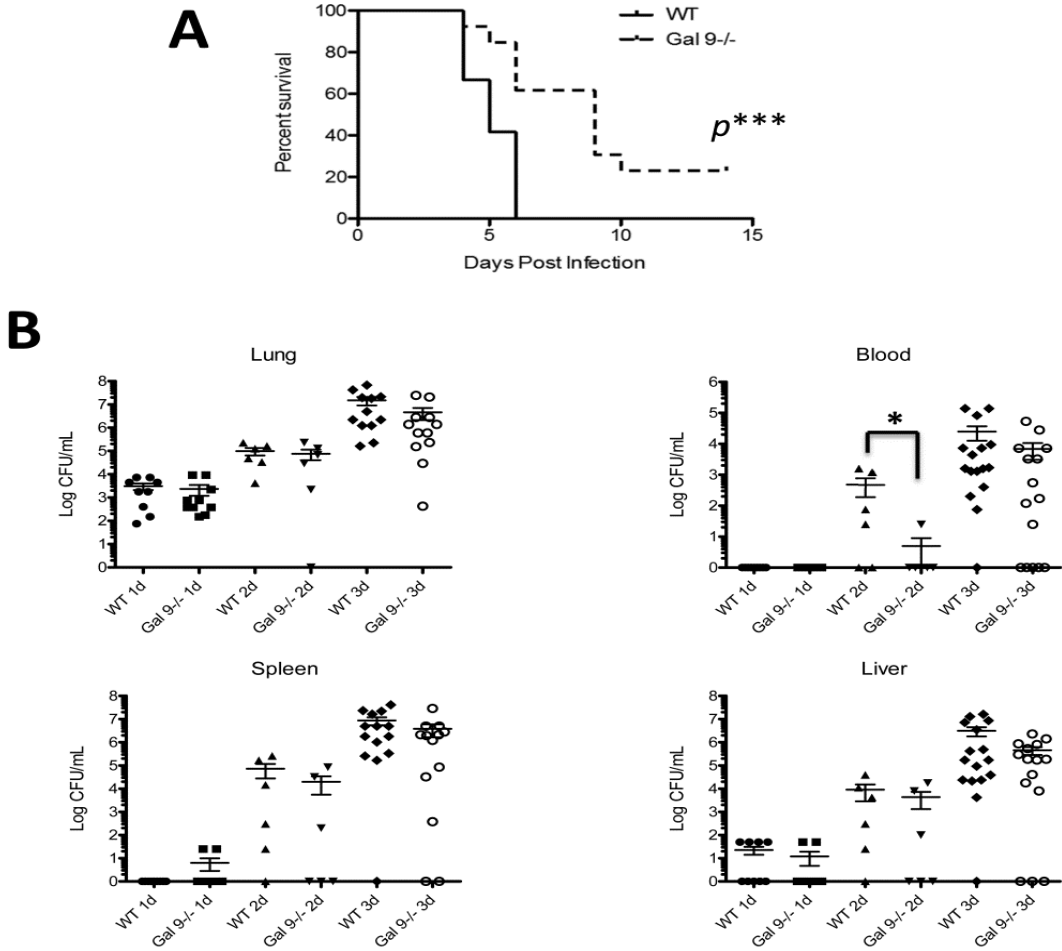


Gal-9<sup>-/-</sup> mice exhibited delayed appearance of disease symptoms and showed significantly improved survival as compared to the infected wild-type mice (Fig.16.A). The improved survival of Gal-9<sup>-/-</sup> mice, however, did not correlate with bacterial burdens in their lungs, spleen and liver since similar bacterial load was observed in WT and KO mice (Fig. 16.B). Intriguingly, Gal-9<sup>-/-</sup> mice exhibited significantly lower bacterial counts in their blood at 2dpi than their WT counterparts at that time point indicating a delayed development of bacteremia in the absence of galectin-9. However, the bacterial burden in blood was similar in infected WT and Gal-9<sup>-/-</sup> mice at later time points indicating that once hematogenous, bacteria replicated at similar rates in both strains.

## Discussion

In this study we examined the role of galectin-9, a mammalian  $\beta$ -galactoside binding lectin, in the development of sepsis during pulmonary infection with *F. novicida*. We report the upregulation and extracellular release of galectin-9 during F.n. infection and its ability to amplify Francisella infection-induced inflammatory response as well as concomitant improved disease severity of Gal-9<sup>-/-</sup> mice. These functions of galectin-9 are all consistent with characteristic properties of alarmins that have been described to play a pathological role in perpetuation of inflammation.

Sepsis is characterized by a complex, systemic inflammatory response to a traumatic or infectious insult, such as F.n. infection in the current study. Mechanisms of innate immune activation in sepsis are no longer thought to be induced exclusively by components of pathogen origin (pathogen associated molecular patterns, PAMPs). Alarmins, that are



**Figure 16. Gal-9<sup>-/-</sup> mice show improved survival during pulmonary F.n. infection.**

**(A)** Fifteen each C57Bl/6 WT and Gal-9<sup>-/-</sup> mice in 3 separate experiments (5 mice per experiment) were inoculated intranasally with F.n and were monitored daily for 2 weeks. The improved survival of Gal-9<sup>-/-</sup> mice compared to WT mice was statistically significant, as determined by Kaplan-Meier log-rank analysis (*P* value\*\*\* = 0.0003). **(B)** Bacterial burdens in lungs, blood, spleen and liver harvested from F.n. infected WT and Gal-9<sup>-/-</sup> mice at indicated times post-infection. Lung, liver and spleen homogenates prepared as described in Materials and Methods and blood were serially diluted and plated on TSA plates to enumerate bacterial burdens. Each symbol on the plots represents one mouse and data is from 2-3 independent experiments.

multifunctional host proteins, have been shown to regulate inflammatory response in many pathological conditions including sepsis, upon their release from dead/dying host cells [32,33,96]. High Mobility Group Box1 (HMGB-1), S100 family of proteins, and heat shock proteins are among the most well-characterized alarmins to date [84], although the list of alarmins has continued to grow over the past decade [140]. Indeed, in a complex immune disorder like sepsis, it is likely that several alarmins are involved at the intersections of various pathways. Our current study shows that galectin-9, a host lectin, plays a pathogenic role as an alarmin to exacerbate the inflammatory response during pulmonary infection with *Francisella* and contributes to sepsis development. Identification of novel alarmins such as galectin-9 may aide in understanding this complex disorder and may present additional targets for effective therapeutics.

Galectins are ubiquitously expressed  $\beta$ -galactose binding lectins. These constitute the only soluble mammalian lectins which are either passively released by dying cells or are actively secreted by inflammatory cells through a non-classical secretory pathway [66]. Although several galectins have been detected in the extracellular milieu [141], the precise mechanism of their secretion remains to be determined, since they lack a classical “leader” sequence. Galectins play homeostatic roles in regulation of cell cycle and apoptosis, phagosome formation, and stabilization of intracellular signaling when contained in intracellular compartments [63]. Some of these galectins such as galectin-1 and -3 exhibit inflammatory and T cell apoptotic activities upon extracellular release [64]. Moreover, the extracellular release of some galectins seems to correlate with the virulence of invading pathogen [65,137] as well as influences immune responses through chemotaxis and activation of innate immune cells [64,66]. Despite the progress in our

understanding of the inflammatory properties of galectins, the role of these lectins as alarmins in the development of sepsis is not defined. Our results described here show, for the first time that galectin-9, mostly studied for its function as modulator of T cell immunity, is likely released extracellularly and modulates innate immune responses by affecting immune cell infiltration and activation during a septic infection by F.n.

Although speculative at this stage, the reduced number of leukocytes in F.n. infected Gal-9<sup>-/-</sup> mice suggests that this lectin directly or indirectly affects cellular infiltration/accumulation in sepsis. To the best of our knowledge, there are no reports examining the impact of galectin-9 on innate cell infiltration during an acute pneumonic infection although, human galectin-9 has been identified as a potent eosinophil chemoattractant with implications in allergic airway inflammation [142]. Galectin-9 has also been shown to induce IL-8 production by bronchial epithelial cells in cystic fibrosis, influencing neutrophil infiltration and early neutrophil-dominated inflammation in the cystic fibrosis lung [143]. Reduced levels of neutrophil chemoattractant and activation markers in Gal-9<sup>-/-</sup> mice in our studies suggest that this lectin may exert its effect on cellular infiltration indirectly by modulating the levels of these chemokines. This observation is in line with the role of the prototypic alarmin HMGB1 in neutrophil migration by regulating the levels of chemoattractants such as IL-8 [99]. However it is also possible that galectin-9 influences neutrophil infiltration directly by binding to its receptor T cell Ig and mucin domain-containing molecule-3 (TIM-3) which was recently reported to be expressed by neutrophils [144]. Other proinflammatory alarmins such as S100 proteins have also been shown to directly promote migration of myeloid cells by binding to their cell surface receptors [101]. Further studies are needed to test these

hypotheses. Notwithstanding the mechanism, the reduced neutrophil accumulation in F.n. infected Gal-9<sup>-/-</sup> animals indicates a likely chemotactic function of this lectin in F.n. induced sepsis.

The role of galectin-9 in regulating T helper-1 responses via its interaction with TIM-3 has been extensively studied. Galectin-9 interaction with TIM-3 on T cells results in cell death causing down regulation of pro-inflammatory Th1 responses implicated in pathology of autoimmune diseases such as EAE and GVDH [145-147]. However, its role in myeloid cell activation is much less defined. Recent studies have reported involvement of TIM-3/galectin-9 interaction in activation of neutrophils and macrophages [144,148]. Another study showed galectin-9 induced secretion of pro-inflammatory cytokines from monocytes and macrophages, that could amplify immunopathology associated with HCV infection [149]. Additionally, the effects of galectin-9 on activation and maturation of antigen presenting cells have been shown [150-152]. More relevant to our study, a recent study reported that galectin-9 mediates potentiation of TLR-ligation induced TNF- $\alpha$  and IL-6 secretion from microglia [153]. This is similar to our observation of galectin-9 mediated potentiation of *F. novicida* induced inflammatory response. In our studies, consistent with the property of a typical alarmin, galectin-9 exhibited immune activating properties such as stimulation of the oxidative burst in neutrophils and inflammatory cytokine production in macrophages. Importantly, this lectin was able to augment F.n. infection induced inflammatory responses from neutrophils as well as macrophages, which can have important implications under in vivo conditions. Consistent with this pro-inflammatory activity of galectin-9, Gal-9<sup>-/-</sup> animals exhibited a reduction in the levels of inflammatory cytokines such as TNF- $\alpha$ , IL-6 and IL-1 during F.n. infection. Studies from

our laboratory have shown that pulmonary infection with F.n., as well as *F. tularensis*, results in extensive cell death [74]. It is likely that during pulmonary Francisella infection, galectin-9 released from dead cells primes the myeloid cells to produce heightened levels of inflammatory mediators in response to the bacteria. Furthermore, since galectin-9 can be upregulated and released from cells upon exposure to pro-inflammatory stimuli such as cytokines and TLR ligands [154], the activated cells likely release more galectin-9 resulting in a positive feed-back loop causing further tissue damage and ultimately organ failure, characteristic of sepsis. This is also supported by our unpublished observations showing that bone marrow derived macrophages from Gal-9<sup>-/-</sup> mice show impaired activation upon stimulation with TLR-4 ligand LPS. This immune stimulatory activity of galectin-9 in the context of Francisella infection is in line with a previous study showing the role of the prototype alarmin HMGB1 in promoting the inflammatory response of monocytes elicited by external stimuli [98].

As a result of mitigated inflammatory responses and tissue pathology, the survival of F.n. infected Gal-9<sup>-/-</sup> mice was significantly improved as compared to the WT mice which succumbed to infection by day 5-6. Interestingly, we observed similar bacterial burdens in the lungs of F.n. infected Gal-9<sup>-/-</sup> and wild-type mice. However, the bacteremia was delayed in Gal-9<sup>-/-</sup> mice with significantly lower burden in blood at 2dpi but the difference at later points as was not statistically significant as compared to the WT mice. Galectin-9 has been shown to mediate phagocytosis of *P. aeruginosa* by neutrophils thus contributing to bacterial clearance [144]. In contrast, a similar bacterial burden in WT and Gal-9<sup>-/-</sup> mice in our study indicates that galectin-9 does not play a role in F.n. clearance. Because of high bacterial burden, a majority of Gal-9<sup>-/-</sup> mice ultimately

succumbed to infection. These results are similar to our recently published studies of galectin-3 function in F.n. induced sepsis. In this scenario a combinatorial approach using blockade of galectin-3 and galectin-9 along with antibiotics could potentially treat Francisella infection induced sepsis. Additionally, in order to address any possible synergistic effect of galectin-3 and -9 (since we have found that these are the only two galectins, out of nine tested by us, which are upregulated and are likely released in Francisella infected lungs), we have already generated galectin-3/galectin-9 double knock-out mice and are currently analyzing them for sepsis development during bacterial infection. In light of a similar alarmin-like function of these galectins, the double knock-out mice may exhibit further improvement in the disease outcome.

Together our findings indicate that galectin-9 plays a pathogenic role as an alarmin to exacerbate the inflammatory response during pulmonary infection with Francisella and contributes to sepsis development. Galectin-9 thus may represent a potential target for treatment of sepsis during this infection.

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#### Author Contributions

Conceived and designed the experiments: BBM JS. Performed the experiments: ALS TJS SLS. Analyzed the data: BBM JS. Critically reviewed the manuscript: DWM. Wrote the paper: BBM JS.

#### Disclosure Statement

The authors have no financial conflict of interest.



# **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<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> 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.

## 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% [5,155]. Nosocomial infections with opportunistic pathogen *Klebsiella pneumoniae* (KPn) account for 5-20% of Gram-negative sepsis cases [117-119]. 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 [126,156-159], 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 [49,160]. It is an inducible receptor, expressed mainly by myeloid cells such as macrophages, neutrophils, myeloid dendritic cells as well as some B-cell subsets [160-162]. Functional analysis of this receptor in macrophages has received the most attention, where its association with FcR $\gamma$  activates downstream signaling cascades involving Syk kinases resulting in induction of protective inflammatory response [163-165]. 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 (Pattern Recognition Receptor) 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 [128,138]. 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 [166-168]. 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 [169,170], 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.

## 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<sup>-/-</sup> 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 ( $2.5 \times 10^4$  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 Complete<sup>TM</sup> 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 [1]). 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 [77,82]. Serial horizontal sections (10 µm thick) of frozen lung tissues thus obtained were stained with hematoxylin and eosin for pathological analysis as previously described [3,89] .

#### Flow Cytometry

Lungs or BAL cells were harvested from mice at 3 days p.i. and processed as previously described by us [77,78,82]. 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<sup>-/-</sup> 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<sup>-/-</sup> 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 s 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 17A). 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 17B). 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.

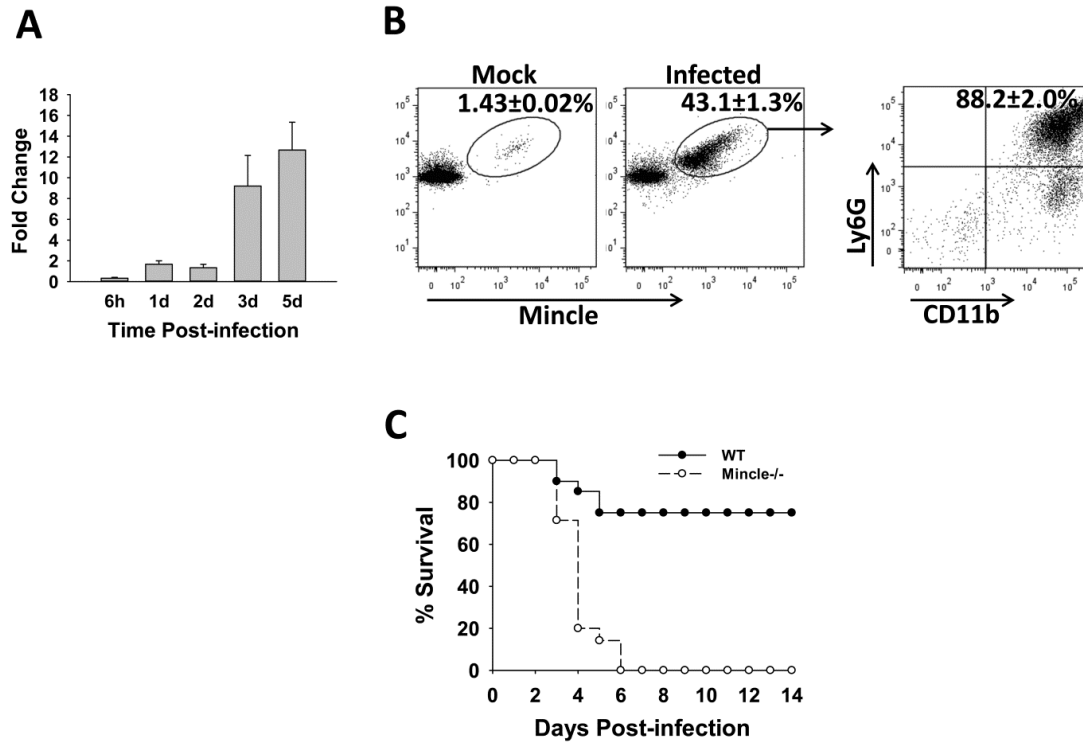
To examine the role of Mincle in disease development, overall disease severity and survival was compared in wild-type and Mincle<sup>-/-</sup> mice infected with a sub-lethal dose of KPn. This dose was experimentally determined at which the WT mice displayed minimal morbidity and mortality [3]. As shown in Figure 17C, 76% of WT mice infected with  $2.5 \times 10^4$  CFUs of KPn survived the infection with transient signs of disease (ruffled fur,

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<sup>-/-</sup> mice were intranasally infected with 2.5X10<sup>4</sup> CFUs of *Klebsiella pneumonia* (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).

lethargy) early during infection and appeared healthy later. The Mincle<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice clearly indicated a protective role played by this CLR during pneumonic KPn infection.

#### Mincle deficiency results in increased bacterial burden and systemic dissemination

In order to examine if increased susceptibility of Mincle<sup>-/-</sup> mice to KPn infection correlated with inefficiency to clear bacteria, homogenized lungs, liver and blood from infected Mincle<sup>-/-</sup> and WT mice collected at various times post infection were plated on LB agar. Up to 2dp.i., Mincle<sup>-/-</sup> and WT animals displayed similar bacterial burdens in their lungs (Figure 18A). By 3dp.i., however, lungs of Mincle<sup>-/-</sup> mice exhibited significantly higher bacterial counts as compared to their WT counterparts. The bacterial



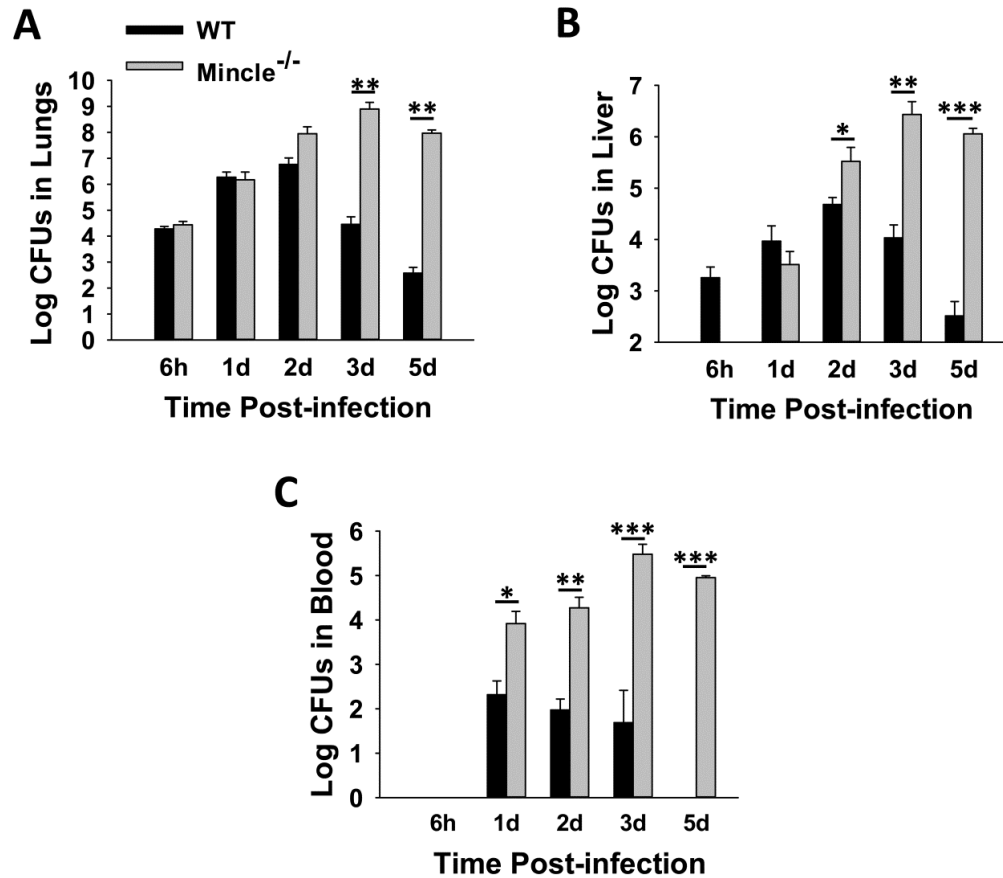
**Figure 17. Mincle is highly induced in the lungs of KPn infected pneumonic mice and Mincle deficiency increases susceptibility to the infection.**

**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  $2^{-\Delta\Delta Ct}$ . Data shown are the averages of 6-8 mice per group in two independent experiments. **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

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<sup>-/-</sup> mice also displayed a higher systemic dissemination of bacteria as depicted by significantly higher bacterial load in liver (Figure 18B) and a more severe bacteremia (Figure 18C). 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

#### Mincle<sup>-/-</sup> mice exhibit hyperinflammatory response

We next examined if the inability of Mincle<sup>-/-</sup> mice to clear the bacteria was due to a defect in mounting inflammatory response. In both WT and Mincle<sup>-/-</sup> strains, mock infected mouse lungs displayed similar low basal levels of inflammatory cytokines tested (Figure 19). 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 19). This was consistent with the reduced bacterial burden in these mice at these times p.i.. In contrast, infection of Mincle<sup>-/-</sup> 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 19). The levels of inflammatory cytokines and chemokines tested in lungs of these mice were significantly higher than WT mice at these infection with KPn.



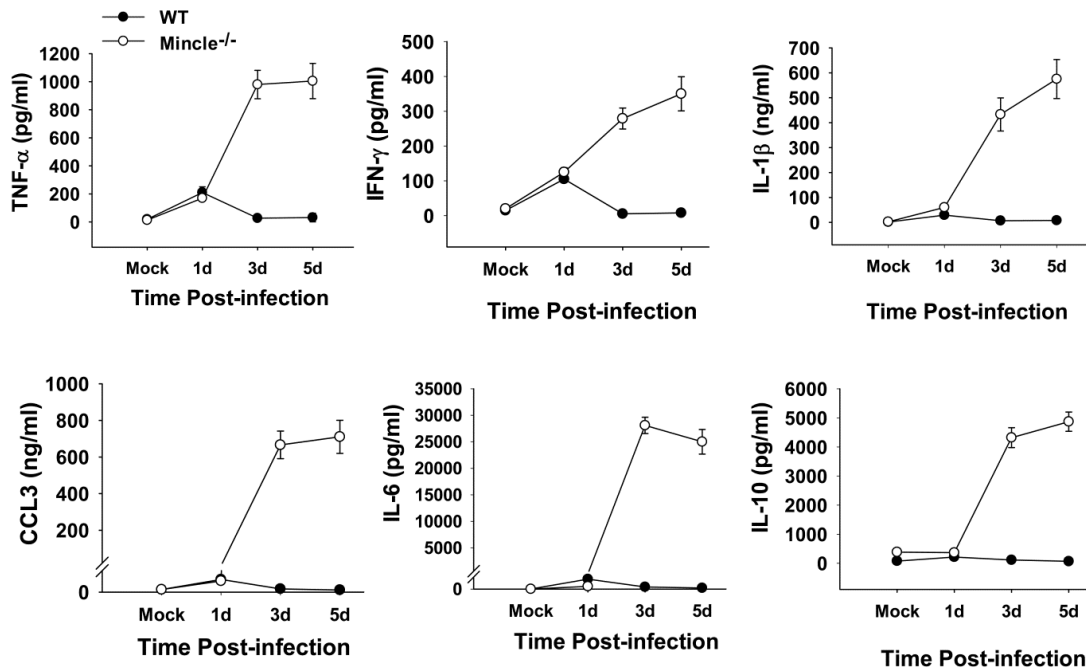
**Figure 18. Mincle<sup>-/-</sup> mice display increased bacterial burden and systemic dissemination during pneumonic KPn infection.**

WT and Mincle<sup>-/-</sup> were intranasally infected with  $2.5 \times 10^4$  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<sup>-/-</sup> are denoted by asterisks (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$ , \*\*\* $p < 0.001$ ).

time points. The levels of IL-10, an anti-inflammatory cytokine were also significantly higher in Mincle<sup>-/-</sup> 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 [171-173]. 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<sup>-/-</sup> mice undergoing pneumonic KPn infection.

#### Effect of Mincle deficiency on neutrophil infiltration and overall lung pathology

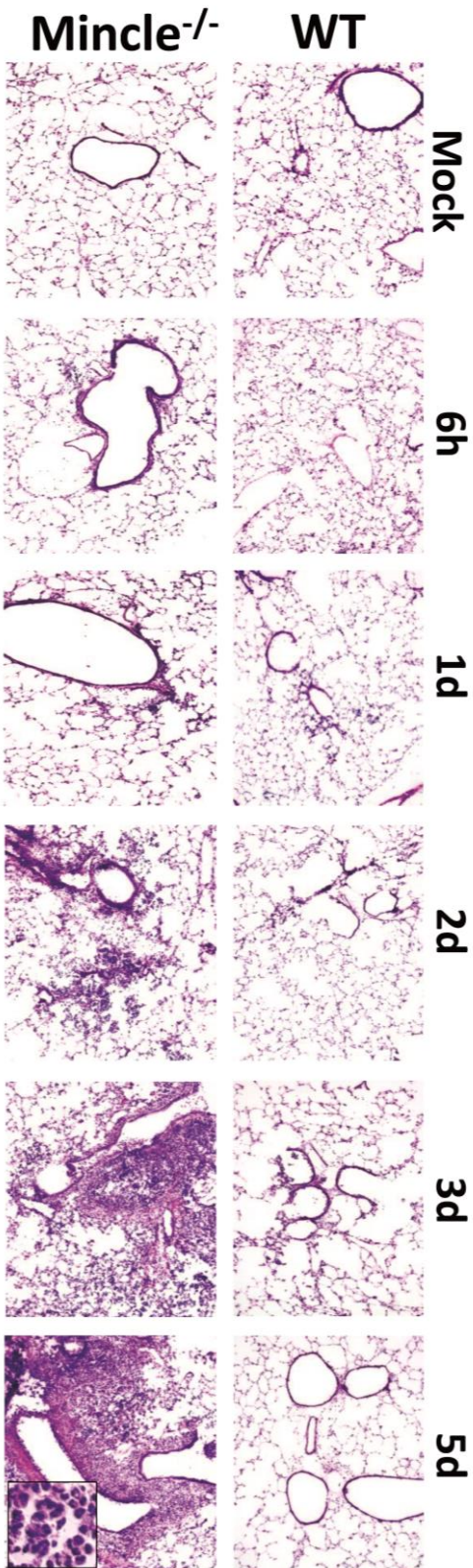
Since neutrophils are a key cell type involved in bacterial clearance and initiation of protective immune response during KPn pneumonia, we next compared neutrophil infiltration and gross immunopathological changes in KPn infected WT and Mincle<sup>-/-</sup> mice. The mock control mice of both strains displayed similar normal lung tissue morphology in H&E stained sections (Figure 20). A moderate transient infiltration of immune cells was observed substantially by 5dp.i. The overall architecture of the lungs was largely preserved in the WT animals throughout the infection. The Mincle<sup>-/-</sup> mice, on the other hand, displayed a progressive increase in immune cell infiltration, which were mainly neutrophils, based on characteristic multi-lobed nuclei (Figure 20 inset). By day 3 p.i. substantially increased in infected WT mice by day 3p.i. which was reduced



**Figure 19. Pneumonic Mincle<sup>-/-</sup> mice exhibit hyperinflammatory response.**

The lungs from mock control and KPn infected WT and Mincle<sup>-/-</sup> 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 (Myriad<sup>TM</sup> 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<sup>-/-</sup> mice at 3dp.i. and 5dp.i. in comparison with their levels in the infected WT mice at those time points tested.





**Figure 20. Pneumonic *Mincle*<sup>-/-</sup> 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*<sup>-/-</sup> mice isolated at indicated times post-infection. Magnification 100X. Inset shows a highly magnified area (1000X) of a lesion in infected *Mincle*<sup>-/-</sup> lung depicting neutrophils as indicated by characteristic multilobed nuclear morphology.



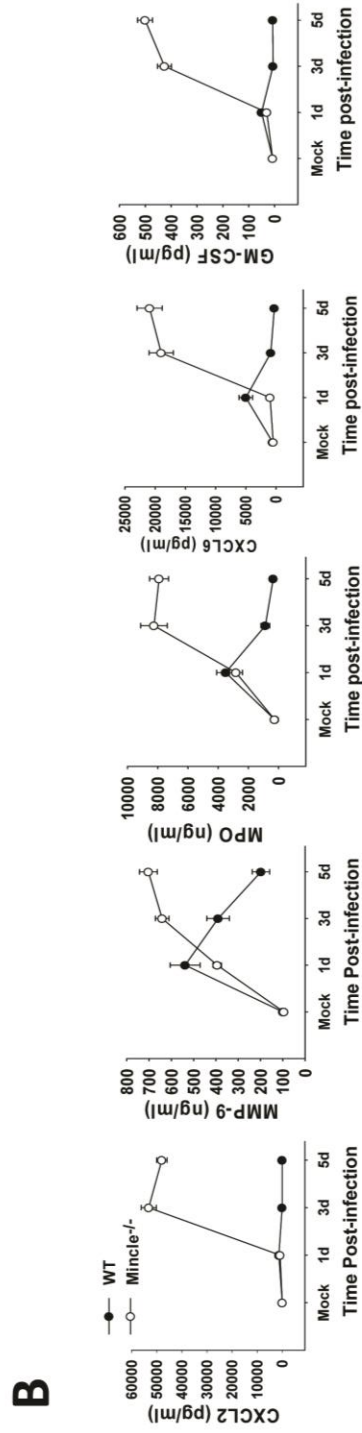
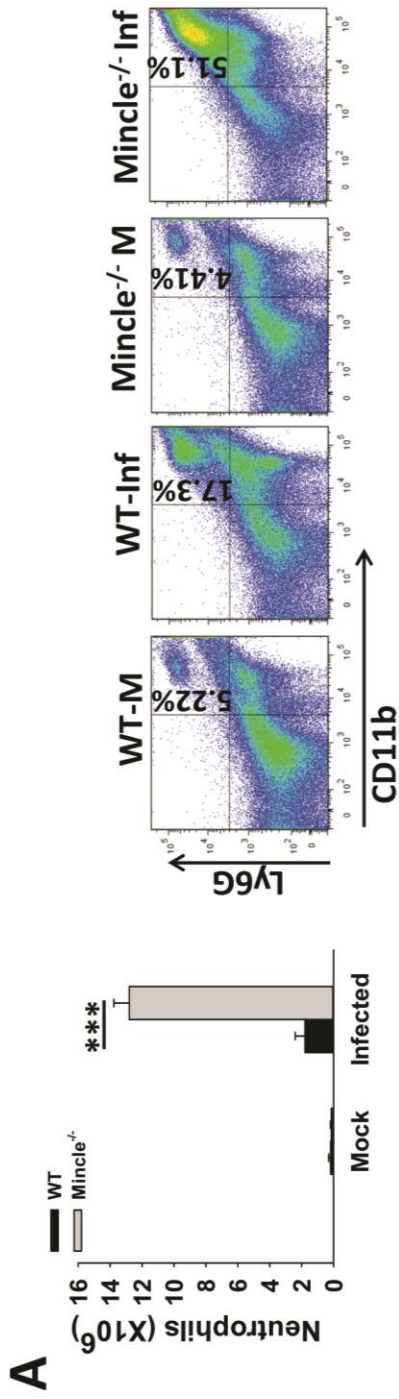
influx of cells was observed in large lesions and by 5 p.i., extensive foci of consolidation were visible with massive accumulation of neutrophils around alveolar spaces (Figure 20). Flow cytometry analysis of infiltrating cells in lungs confirmed that the majority of these cells were Ly6G+CD11b+ neutrophils (Figure 21A). The numbers of these cells were significantly higher in the infected *Mincle*<sup>-/-</sup> lungs than those in the WT mice (Fig. 21A, 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 21B).

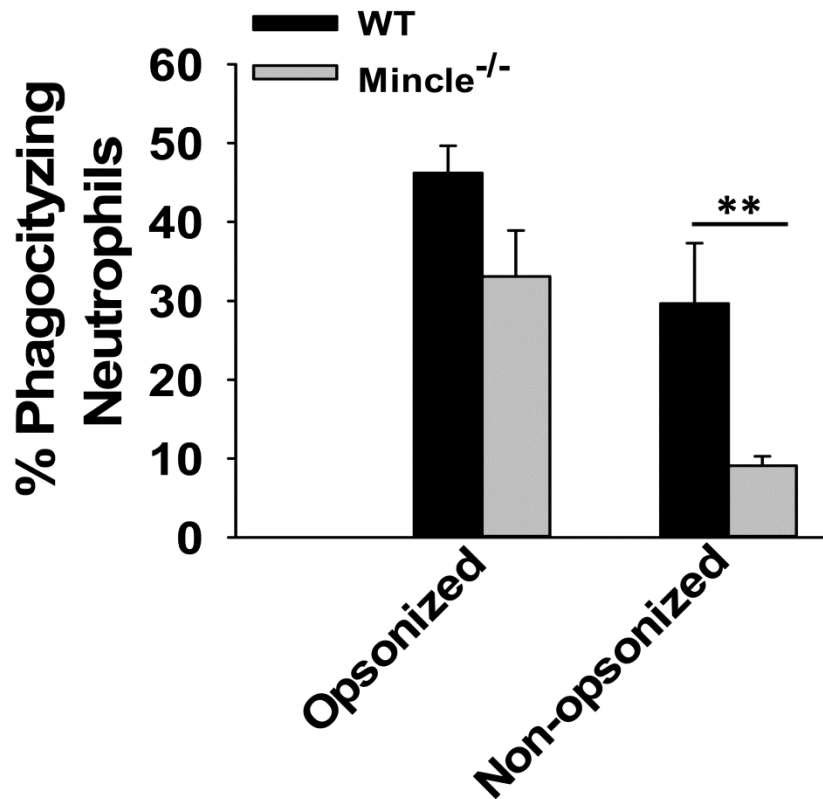
#### *Mincle*<sup>-/-</sup> neutrophils are defective in KPn phagocytosis

We next examined the bacterial uptake by *Mincle*<sup>-/-</sup> neutrophils, in light of an increased bacterial burden in *Mincle*<sup>-/-</sup> mice. For this, phagocytosis of GFP-labeled KPn was compared between WT and *Mincle*<sup>-/-</sup> neutrophils by flow cytometry. As shown in Figure 22, *Mincle* deficiency resulted in significantly reduced phagocytosis of non-opsonized bacteria by neutrophils. The uptake of opsonized bacteria was also reduced in *Mincle*<sup>-/-</sup> 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. Increased neutrophil accumulation coincides with elevated expression of neutrophil chemoattractant and activation markers in lungs of KPn infected Mincle<sup>-/-</sup> mice.**

**A.** Flow cytometry analysis of Ly6G+CD11b+ neutrophils in mock control (WT-M and Mincle<sup>-/-</sup>-M) and KPn infected (WT-Inf and Mincle<sup>-/-</sup>-Inf) WT and Mincle<sup>-/-</sup> 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<sup>-/-</sup> 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<sup>-/-</sup> mice were harvested at indicated time points post-infection and analyzed commercially for host immune mediators by rodent multi-analyte profile (Myriad<sup>TM</sup> 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<sup>-/-</sup> mice at 3dp.i. and 5dp.i. in comparison with their levels in the WT mice at those time points tested.





**Figure 22. Mincle deficiency impairs neutrophil phagocytosis of non-opsonized bacteria.**

Peritoneal neutrophils from WT and Mincle<sup>-/-</sup> 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).

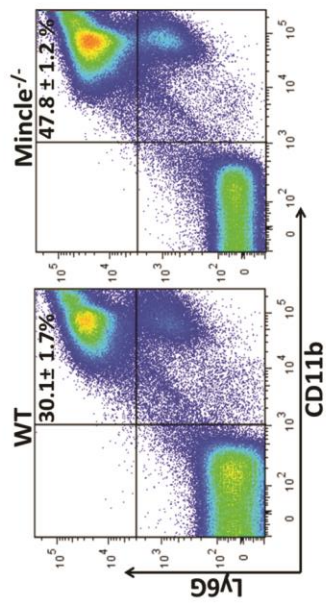
### Mincle<sup>-/-</sup> neutrophils are defective in NET formation

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 [156,174], 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<sup>-/-</sup> mice (Figure 23A). A quantitative comparison of BAL neutrophils showed that significantly higher numbers Mincle sufficient WT neutrophils produced NETs (Figure 23B) which stained positive for neutrophil-specific enzyme neutrophil elastase (Figure 23C), showing that these fibrillar structures originated mainly from neutrophils. Furthermore, the NETs observed in Mincle<sup>-/-</sup> neutrophils appeared dwarfed and lacked the web-like appearance as observed in the WT mice. This observation, together with phagocytic ability of Mincle<sup>-/-</sup> neutrophils shows that Mincle deficiency severely impairs neutrophil mediated bacterial uptake and clearance mechanisms in lungs during pneumonic KPn infection.

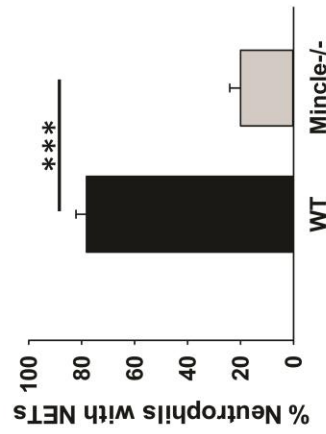
**Figure 23. Mincle deficiency causes a defect in formation of neutrophil extracellular traps (NETs) in the lungs of mice upon pneumonic KPn infection.**

**A.** Enumeration of CD11b+Ly6G+ neutrophils by flow cytometry in BAL isolated from KPn infected WT and Mincle<sup>-/-</sup> mice. **B.** Quantitation of neutrophils showing NETs in BAL isolated from WT and Mincle<sup>-/-</sup> mice infected with KPn. (\*\*\*p<0.001). **C.** Representative fluorescence images of the neutrophils isolated from BAL fluid of WT (upper panel) and Mincle<sup>-/-</sup> (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<sup>-/-</sup> 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.

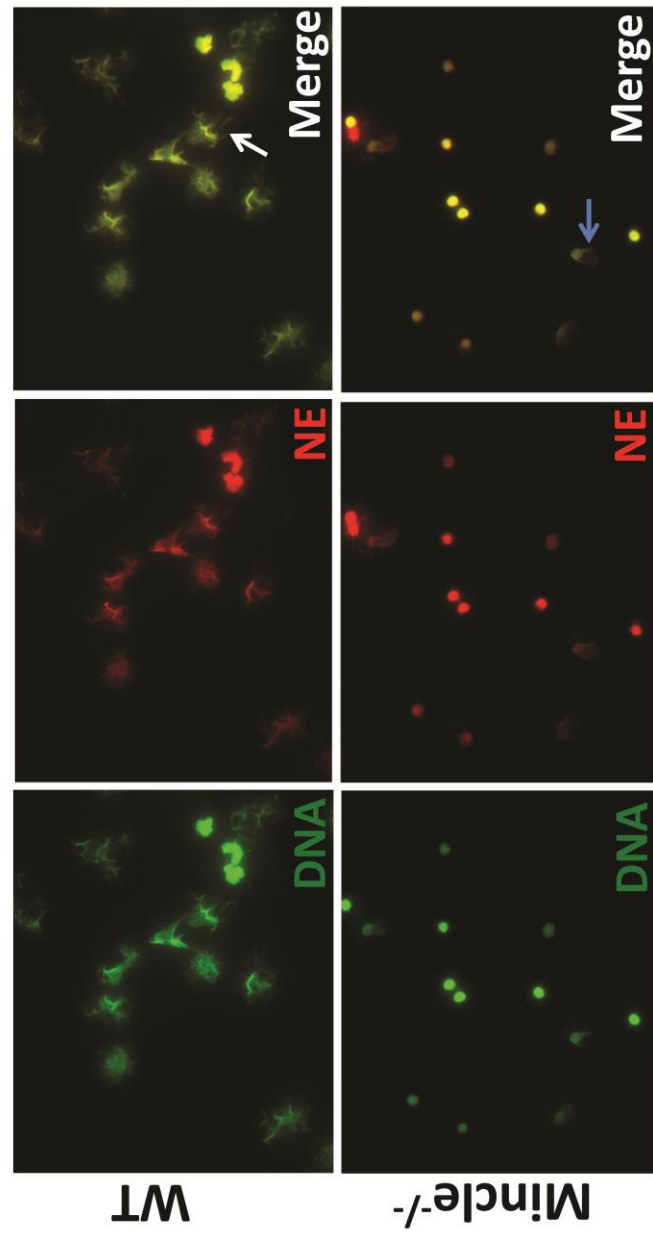
**Figure 7 A**



**B**



**C**



## DISCUSSION

Pneumonic sepsis is a major health care burden worldwide and *K. pneumoniae* (KPn) is the most frequent Gram-negative sepsis-associated opportunistic pathogen [155]. 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 [26,175,176]. 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<sup>-/-</sup> 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* [165,177-179]. In these infections, Mincle expressed on macrophages, upon recognition of its ligands triggers FcR $\gamma$ -Syk-Card9 pathway to induce production of protective Th1/Th17 responses as well as chemokines required for recruitment of



inflammatory cell types [164,178,180]. 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<sup>-/-</sup> 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<sup>-/-</sup> mice in our studies, exhibited overwhelming local as well as systemic bacterial burdens.

Concomitant to increased bacterial burden, Mincle<sup>-/-</sup> mice exhibited extensive neutrophil accumulation, the primary cell type shown to play an important role in mediating protective immune response against Kpn infection [117,126,138,181]. We thus examined if Mincle<sup>-/-</sup> neutrophils were defective in performing cellular functions such as internalization of bacteria via phagocytosis which would explain increased bacterial burden in Mincle<sup>-/-</sup> mice despite a heightened inflammation. Indeed, Mincle<sup>-/-</sup> 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 [182]). 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 [183]. 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-acetylglucosamine [184], 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 [166]. 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<sup>-/-</sup> 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 [156,174]. 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 that initially surmised.

## **Acknowledgements**

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# General Discussion

Sepsis is a complex immune disorder that results in 750,000 hospitalizations annually in the United States alone [185]. Pulmonary infections, in turn, are a major cause of sepsis [5]. In addition to posing an immense health hazard with a mortality rate of 20-50%, it is also a huge economic healthcare burden with the patients consuming half of the ICU resources in the country. In October 2011, the only FDA approved drug for the treatment of sepsis (Xigris) was withdrawn from market following the much anticipated results of the clinical trial (PROWESS-SHOCK) where it failed to show any survival benefit for severe sepsis and septic shock patients [186]. As a result we are now left with no effective preventive or treatment options for this deadly immune disorder. As clinical presentations of sepsis are known to be caused by an unbridled hyper-inflammatory responses causing massive tissue pathology leading to multiple organ failure [23,28], our major goal was to identify mechanisms responsible for pulmonary infection-induced sepsis development. In this regard, most of the sepsis research has been focused on initiation of inflammatory response upon pathogenic insult and the role of pathogen associated molecules or PAMPs [28]. However, our earlier studies with Francisella, a Gram-negative pathogen, demonstrated that pulmonary infection with this bacterium leads to development of severe sepsis and systemic organs failure despite absence of any bacterial toxins [73,78,80]. Moreover, LPS which is the major PAMP in Gram negative bacterial pathogens, is modified in Francisella and as a result, does not stimulate TLR4 and is thus hypo-inflammatory [187]. As our and other laboratories have clearly

shown that extensive tissue damage is a hallmark of sepsis, our initial hypothesis was that endogenous molecules released from the dead or dying cells contribute and/ or exacerbate the host inflammatory response culminating in sepsis development. Thus, our first goal was to identify novel self-molecules termed as alarmins that are released from dead/dying cells and their role to regulate induction and/ or exacerbation of host inflammatory response. An efficient recognition and clearance of the damaged cells by efferocytosis is essential to prevent inflammatory response due to PRR-alarmin interaction. Thus, efferocytosis is an important step for resolution of inflammation and maintenance of normal body physiology. Therefore, our second goal was to identify novel host cell surface receptors involved in efferocytosis. In this regard, the importance of the lectins in host immunity and homeostasis has been demonstrated in several recent studies in different clinical settings [55,56]. Lectins are involved in recognition of glycan structures on a wide range of antigens [188], including cryptic self-antigens that are exposed by cell death or are modified during disease conditions, leading to inflammation [189]. In contrast, studies have also shown that lectins mediated recognition and sampling of self-glycan antigens ensures a state of immune tolerance helping to maintain normal body physiology. Indeed, it has been established that lectins are important biological regulators in eliciting immune suppression [190-193]. *Owing to such dual functions of lectins in induction as well as resolution of inflammation in various clinical settings, here we focused to characterize lectins that can function as novel alarmin or alarmin receptor to regulate inflammatory pathology in pulmonary bacterial infection induced sepsis.*

Lectins as novel alarmins and their function in induction/ exacerbation of hyperinflammatory response in pneumonic sepsis: role of galectin -3 and -9

### **Extracellular presence and induction of inflammation**

Alarmins: a) are endogenous molecules typically perform homeostatic functions when contained within cellular compartments; b) can be found extracellularly despite lacking any signal sequence for active secretion; c) have chemoattractant and immune activating properties; and d) exhibit immune enhancing properties when injected in-vivo [26,29,30,84]. Galectins constitute the only soluble mammalian lectins which do not contain any secretion signal sequence [66]. Consistent with the property of alarmins, galectins play homeostatic roles [63]. We first analyzed the expression and distribution of galectins 1-13 in uninfected and Francisella infected lung tissues. Francisella infection was found to upregulate the expression of many galectins including galectin-3 and galectin-9. Interestingly, out of all galectins tested, only galectin-3 and galectin-9 exhibited extracellular presence. Such upregulated and extracellular expression suggested possible alarmin functions for these molecules in infection and pathological processes [63,64,66,137] associated with sepsis. As alarmins can induce and/or exacerbate inflammatory responses from myeloid cells such as macrophage and neutrophils that are typically associated innate immune responses, effect of galectin-3 and -9 on these cells was examined. Purified recombinant galectin-3 and -9 exacerbated F. novicida-induced production of the inflammatory mediators by macrophages and neutrophils. Notwithstanding the mechanisms involved in the disparity of inflammatory mediators induction by recombinant galectin-3 and -9 alone, clearly both these molecules augmented infection induced inflammatory response in myeloid cells. Similar studies

involving a prototype alarmin HMGB1 have also reported that while HMGB1 by itself failed to induce inflammatory mediators in macrophage, it exacerbated agonist-induced inflammatory mediator productions [31,32]. Increased extracellular release of galectin-3 and -9 in Francisella infected lungs, and their ability to increase production of inflammatory mediators likely is the primary contributor to the tissue damage observed during pulmonary Francisella infection-induced sepsis.

### **Inflammation and disease phenotype *in vivo*:**

In order to further establish a role for galectin-3 and -9 in murine sepsis, Francisella infected galectin-3<sup>-/-</sup> and galectin-9<sup>-/-</sup> mice were analyzed for overall disease pathogenesis. As both galectin-3 and -9 exposure enhanced development/ exacerbation of infection induced inflammation in myeloid cells *in vitro*, we speculated that in the absence of galectin-3 and galectin-9 Francisella-infected mice would display reduced systemic inflammation. Indeed these mice showed a reduced level of inflammatory mediators, including multitude cytokines and chemokines, many of them markers of sepsis. Additionally, a reduced number of neutrophils in lungs after Francisella infection was observed in both galectin-3<sup>-/-</sup> and galectin-9<sup>-/-</sup> mice. The results further suggest a key role for galectin-3 and galectin-9 as critical modulators of inflammatory response during bacteria induced murine sepsis. Since these mice also display decreased systemic tissue pathology, the results are consistent with alarmin properties playing a role in exacerbating inflammation induced tissue pathology which is detrimental to host in sepsis. Intriguingly, despite the fact that Francisella infected galectin-3<sup>-/-</sup> and galectin-9<sup>-/-</sup> mice exhibit improved survival, these infected mice eventually succumbed to infection which correlated with similar bacterial burdens in the knock-out and wild-type mice. It is



possible that, high bacterial burden contributes to increased cell death, albeit at a lower level as compared to the corresponding WT mice, leading to an increased accumulation of other alarmins which eventually mask the advantageous effect of the absence of galectin-3 and -9. Thus persistence of alarmins released by the damaged tissue and bacteria along with associated increased inflammation likely contributes to the mortality in infected galectin-3<sup>-/-</sup> and galectin-9<sup>-/-</sup> mice.

## **Conclusion**

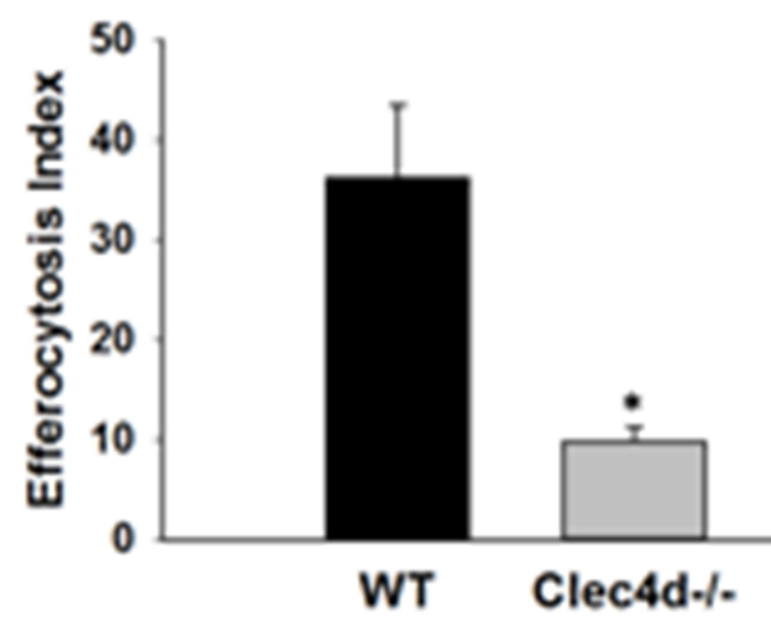
Our findings indicate that both galectin-3 and -9 function as novel alarmins and play a pathogenic role to exacerbate the inflammatory response during pulmonary infection with *Francisella* and contributes to sepsis development. Galectin-3 and -9 thus may represent combinatorial potential target/s for treatment of sepsis.

## **Lectins in resolution of inflammation: role of Clec4d**

Sepsis is now perceived as interplay of PAMPs (non-self) and alarmins (self) over-activating immune cells leading to an unbridled inflammatory response. As discussed in the introduction section, our laboratory and others have clearly demonstrated that massive cell death is a hallmark of sepsis [29,30,78]. Results from the present study discussed above together with other studies have established the pathogenic role of alarmins, that can be passively secreted from dead cells, in immunopathology of sepsis. In this regard, an understanding of the functioning of host innate immune components likely acting as receptors for alarmins released from dead cells or dead cells is critical. Here, our results clearly show that Clec4d deficiency, in an otherwise sublethal pulmonary bacterial infection, leads to a non-resolving hyperinflammatory response. Moreover, this was

correlated with increased mortality of the infected  $Clec4d^{-/-}$  (KO) mice. Interestingly, the KO mice displayed a massive accumulation of neutrophils in their lungs. Despite this, an increase in local as well as systemic bacterial burden was observed in these KO mice. This was not likely due to a dysfunction of neutrophils as bacterial uptake and killing by neutrophils was found to be unaffected in the KO neutrophils. Thus, we propose that persistent accumulation of neutrophils and their associated inflammatory response in lungs likely contributed to tissue destruction as well as inflammation inducing tissue pathology observed in the KO mice. Accumulation of large numbers of neutrophils in the lungs of KO mice and higher amounts of their associated inflammatory mediators suggest that there is a defect in neutrophil turnover in these mice. It is thus possible that  $Clec4d$  plays an important role in mitigating the inflammation during a pneumonic infection, by facilitating neutrophil turnover.

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. However, neutrophils that have infiltrated to the affected site are readily cleared to prevent bystander tissue destruction, owing to their tissue-destructive cargo. Indeed, to restore the quiescent state of homeostasis, clearance of infiltrating immune cells and host cell debris is very critical. This is achieved by a process called



**Figure 24. Impaired efferocytosis of uninfected Clec4d<sup>-/-</sup> neutrophils by murine J774 macrophages.**

CFSE-labelled dead neutrophils from WT and Clec4d<sup>-/-</sup> mice were incubated with J774 macrophages at a ratio of 10:1 for 1h at 37°C. Flow cytometry was performed and efferocytosis index calculated as the percent of CFSE positive macrophages. \*p<0.05.

efferocytosis which not only prevents the potential tissue damage caused by alarmins released from dead/dying cells but also leads to production of specialized lipid mediators by efferocytic cells which promote tissue repair [194-196]. Not surprisingly, a defective efferocytosis has been implicated in several acute and chronic inflammatory lung diseases (reviewed in [197]. We thus speculated that Clec4d plays a protective role in pulmonary KPn induced sepsis by mediating efferocytosis which resolves inflammation as well as aids in bacterial clearance. ). Indeed, our preliminary results (presented here) shows that Clec4d<sup>-/-</sup> neutrophils are not efferocytized efficiently (Fig.24). Furthermore, Clec4d-mediated uptake of KPn-infected neutrophils by macrophages aids in control of infection (data not shown). Thus, we propose that Clec4d-mediated clearance of infected neutrophils is critical for successful elimination of a pathological insult. Furthermore, Clec4d-mediated efferocytosis of infected neutrophils and dead neutrophils and their contents is important in mitigating the inflammation during a pneumonic infection. This study opens up new avenues of research on the role of Clec4d in the resolution of inflammatory responses. This can have major implications in the therapeutic measurements of inflammation-associated disorders.

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