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Role of CD38 in Pulmonary Host Defense against Gram-negative Pneumonia

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ROLE OF CD38 IN PULMONARY HOST DEFENSE AGAINST GRAM-NEGATIVE PNEUMONIA

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Abbreviations

WT	Wild type
BALF	Bronchoalveolar lavage fluid
MPO	Myeloperoxidase
PAMPs	Pathogen associated molecular patterns
DAMPs	Damage-associated molecular patterns
NF-κB	Nuclear Factor kappa B
MAPKs	Mitogen associated protein kinases
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
NLRs	NOD-like receptors
TNF-α	Tumor necrotic factor alpha
IL-1β	Interleukin 1 beta
IL-17A	Interleukin 17A
IL-6	Interleukin 6
IL-18	Interleukin 18
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-γ	Interferon gamma
FBS	Fetal bovine serum
PBS	Phosphate buffered saline

Abstract

The Gram-negative bacterium, *Klebsiella pneumoniae*, is a major cause of hospital-acquired pneumonia in the U.S. In particular, the dramatic increase in carbapenem-resistant *K. pneumoniae* infections poses a serious threat to the public health both in the United States and worldwide. Clearance of bacteria in the lungs depends on effective pulmonary immune response. It may be possible to design improved therapies that augment host immune responses while attenuating excessive pulmonary inflammation through modulation of key innate immunity molecules during pneumonic infections. Cluster differentiation CD38 (CD38) has been detected on the surface of many immune cells or intracellular compartments, acting as an enzyme or a receptor. Although deficiency of CD38 has been shown to attenuate neutrophil recruitment and bacterial clearance in mice with Gram-positive pathogens, its role in Gram-negative bacterial pneumonia is largely unknown. In the present study, we determined the role of CD38 in pulmonary host defense against the Gram-negative bacterium Carbapenem-resistant *K. pneumoniae* (CRKP) in a model of bacteria-induced pneumonia. Both wild-type (WT) and CD38-deficient mice ($CD38^{-/-}$) mice were infected with CRKP (1×10^8 CFU/mouse) by oropharyngeal aspiration. We monitored survival and determined the phenotype of the bronchoalveolar lavage fluid (BALF) cells and bacterial burden in the pulmonary and extrapulmonary organs following infection. We quantified the level of cytokines in the BALF, as well as the level of myeloperoxidase (MPO) in the lung homogenates following CRKP infection. We also examined the intracellular bacterial killing of CRKP by WT and CD38-deficient bone marrow-derived neutrophils (BMDN). In addition, we conducted bone marrow transplantation studies to determine the contribution of CD38 from hematopoietic cells versus non-hematopoietic tissue to host defense following CRKP infection.

Our results indicated that $CD38^{-/-}$ mice showed enhanced survival and increased bacterial clearance in lungs and extrapulmonary organs as compared with WT counterparts during CRKP-induced pneumonia. Augmented host protection in $CD38^{-/-}$ mice was associated with the increased neutrophil influx and increased level of cytokines in BALF, as well as accumulated neutrophil (MPO) in the lung parenchyma following CRKP infection. Furthermore, CD38-deficient neutrophils demonstrated enhanced intracellular bacterial killing ability against CRKP. Interestingly, CD38 deficiency in non-hematopoietic tissue contributes to host protection against CRKP infection. Collectively, our study clearly demonstrates the detrimental role of CD38 in pulmonary host protection against CRKP-induced pneumonia. These findings suggest that neutralization of CD38 could be a potential target for therapeutic intervention of Gram-negative bacterial pneumonia.

Chapter 1. Introduction

Bacterial pneumonia remains a leading cause of morbidity and mortality [1]. The first line of host protection against microbial invasion is the evolutionarily conserved innate immune system, and clearance of bacteria in the lungs depends on an effective innate immune response. Thus, better understanding of the mechanisms underlying innate immunity against bacterial infections is critical for therapeutic augmentation of host defense. The Gram-negative bacterium, *Klebsiella pneumoniae*, is the most common cause of nosocomial bacterial infections in the U.S. Unfortunately, this pathogen has developed resistance to the carbapenems, and effective therapeutic strategies are limited. Thus, this underscores the importance of modulating innate immunity for treatment of CRKP-induced pneumonia.

Neutrophils are the primary defenders of innate immunity, and their importance in the immune response against bacterial infections has been demonstrated through: (i) their depletion, which impairs bacterial clearance, and (ii) their repletion, which restores host protection in the lungs of neutropenic mice [2-4]. Bacteria-induced neutrophil infiltration into the lungs and eventual lung inflammation is a complex cascade of events involving many molecules and multiple steps, which have been researched for many years [5]. Understanding the fundamental mechanisms underlying the modulation of neutrophil infiltration is crucial for the design of novel therapeutic strategies to augment innate immunity while attenuating detrimental pulmonary inflammation in inflammatory diseases.

CD38 is a multifunctional plasma membrane protein with dual enzymatic and receptor functions [6]. Widely expressed on neutrophils, macrophages, B cells, and other immune cells [7], CD38 is markedly induced during microbial infections and the ensuing inflammatory response in

both mice and humans [8]. Its robust induction suggests an active role for CD38 in the immune response to bacterial infections. Recent studies demonstrated that CD38 impacts a wide range of processes, from regulation of cell differentiation to effector functions during infection-induced inflammatory responses, where CD38 appears to modulate cytokine/chemokine release, cell adhesion and migration toward inflamed tissues [9-11]. Although CD38 plays an important role in inflammatory processes, knowledge of the role of CD38 in pulmonary host protection during CRKP-induced pneumonia is limited.

Based on our preliminary studies, we hypothesize that CD38 regulates host protection during CRKP-induced pneumonia. We tested the hypothesis with the following aims: 1) To determine the role of CD38 in pulmonary host protection against CRKP; 2) To examine the contribution of CD38 in hematopoietic versus non-hematopoietic cells to host defense following CRKP infection. Specifically, we combined *in vivo* and *in vitro* experiments, including using CD38 gene-deficient mice, bone marrow chimeras, cell phenotyping, and neutrophil killing assays. Our studies demonstrate that $CD38^{-/-}$ mice exhibit increased survival and enhanced bacterial clearance in lungs and extrapulmonary organs as compared with their WT counterparts during CRKP-induced pneumonia. Augmented host protection in $CD38^{-/-}$ mice results from increased neutrophil recruitment and increased cytokine levels in BALF along with the accumulation of neutrophils in the lung parenchyma using myeloperoxidase (MPO) assay following CRKP infection. Interestingly, using bone marrow chimeras we found expression of CD38 in the non-hematopoietic compartment is more detrimental for bacterial clearance than its expression in hematopoietic compartments during CRKP-induced pneumonia.

Our study clearly demonstrates the deleterious role of CD38 in pulmonary host defense against CRKP-induced pneumonia. Therefore, inhibition of CD38 could be an attractive therapeutic target

for pneumonic infections by Gram-negative bacteria. Moreover, elevated levels of IL-17A were detected in the BALF and lung homogenates of $CD38^{-/-}$ mice. Thus, it is crucial to identify whether pulmonary inflammation is mediated via IL-17A in $CD38^{-/-}$ mice and to identify the primary source of IL-17A following CRKP infection. To better decipher the roles of CD38 in different cells and tissues, approaches using conditional knockout mice, such as myeloid cell-specific CD38-deficient mice, will be required. This knowledge will enhance our understanding of how CD38 regulates immune response during Gram-negative bacterial infections and can be widely adapted to other infectious diseases, including other Gram-negative bacterium, such as *E. coil*. In the long term, this work has the potential to facilitate the development of antimicrobial therapeutic interventions.

Chapter 2. Literature Review

2.1. Bacterial pneumonia is a public health crisis

Pneumonia is an infection that affects one or both lungs and leads to symptoms including cough, fever, and shortness of breath. Approximately 450 million people are affected by pneumonia worldwide every year with severity ranging from mild to deadly. In fact, pneumonia is the main cause of death from infectious diseases worldwide [12]. In 2015 alone, pneumonia killed more than 920,000 children under the age of 5 years [13]. Moreover, while pneumonia can be caused by infection with viruses, bacteria, or fungi, greater than 50% of pneumonia cases are caused by bacteria.

Based on Gram staining, most bacteria can be broadly grouped into Gram-positive and Gram-negative bacteria. Gram-negative bacteria have a more structurally complex cell wall than Gram-positive bacteria, with two membranes (an outer membrane and an inner membrane) that sandwich a thin layer of peptidoglycan and an outer lipid membrane with lipopolysaccharide attached. As a result, it is more difficult for antibiotics to penetrate the cells and effectively target these pathogens. Therefore, even without being drug-resistant, Gram-negative bacteria are more difficult to treat with antibiotics.

K. pneumoniae, the most common Gram-negative bacterium, is an opportunistic pathogen associated with various infections, including bloodstream and urinary tract infections [14]. Particularly, *K. pneumoniae* has been found to cause an increasing number of hospital-acquired invasive pneumonia cases in the United States. *K. pneumoniae* induces lung abscesses, empyema, and septicemia, especially in immunocompromised patients. The mortality rate has been observed to be as high as 50%, even with antimicrobial treatment [15]. Unfortunately, *K. pneumoniae* has

become carbapenem-resistant, resulting in a dramatic increase in its prevalence worldwide [16-19]. Carbapenems are members of the beta lactam class of antibiotics that are commonly used to treat severe life-threatening infections by targeting penicillin binding proteins (PBPs) to inhibit cell wall synthesis, and they are last resort drugs for the eradication of multi-drug resistant *superbug* bacterial infections. Carbapenem-resistant *K. pneumoniae* (CRKP) resistance to carbapenems is mediated by the production of an enzyme known as carbapenemase [20]. Thus, carbapenems cannot work to kill the bacteria and effective treatment strategies are limited. The increasing prevalence of antimicrobial resistance, as seen in CRKP, accentuates the need to design improved host-targeted immune therapies for bacterial pneumonia.

2.2. Neutrophils are the primary defenders of innate immunity

The immune system is made up of two types of defense systems: innate immunity and adaptive immunity. Innate immunity is at the first line of host protection and comprises of molecular sensors and effectors that can be rapidly activated when detecting invading microbial components or tissues damage are detected. It generates fast and non-specific inflammatory immune responses during the first critical hours or days of exposure to microbial infections. Effective innate immune activation and responses in the lungs are critical for the initial containment of invading microbial pathogens. In addition, efficient neutrophil infiltration is essential to induce successful adaptive immunity during host defense. Upon bacterial infection, professional phagocytes, like neutrophils and macrophages, are recruited to inflamed tissues to engulf and kill invading microbes. Due to their early response and rapid increase, neutrophils are considered primary defenders of innate immunity[21] and account for 50% to 70% of all circulating leukocytes. Further, a stable basal neutrophil level is maintained through steady-state granulopoiesis in the BM (where their daily production can be up to 2×10^{11} cells) [22]. Under homeostatic conditions, short-lived neutrophils

are constantly released from the BM reserve pool, while senescent neutrophils that have entered tissues become apoptotic and are removed by macrophages through efferocytosis. However, during emergency granulopoiesis increasing numbers of neutrophils are generated in the BM and are rapidly released into the circulation. They then migrate to inflamed tissues at the site of infection during microbial invasion. Daily neutrophil production can be boosted to reach up to 10^{12} cells and turnover rates are also accelerated under these conditions [23]. In addition, neutrophil longevity is enhanced significantly following their activation during inflammatory responses [24, 25]. Moreover, neutrophils are the most numerous leukocytes trafficking to the site of infection through migration and diapedesis. There, they recognize microbial pathogens and perform significant antibacterial functions, including phagocytosis, production of ROS, degranulation, and the formation of neutrophil extracellular traps (NETs) [26]. Following the resolution of inflammation, neutrophils undergo apoptosis and are then cleared by professional phagocytic macrophages in order to restore tissue integrity and function.

We and others have discovered multiple steps of neutrophil recruitment under inflammatory conditions [27]. Recognition of microbial pathogens is the first step of a cascade of events that lead to neutrophil accumulation. This function primarily relies on the signals from pattern recognition receptors (PRRs), which sense damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) [26]. So far, several major groups of PRRs have been extensively researched, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [27]. Binding of PRR ligands to their receptors triggers downstream signaling cascades that result in transcription factor activation and pro-inflammatory cytokine/chemokine induction. These cytokines/chemokines in turn upregulate the expression of adhesion molecules on the endothelium resulting in neutrophil adhesion, rolling, arrest, and subsequent migration toward the chemo-

attractants. Gradually, this cascade of events leads to neutrophil infiltration during inflammatory responses in the host. However, it remains poorly understood how these neutrophil recruitment cascades are regulated to provide an effective immune response while attenuating unwanted tissue injury caused by the excess neutrophil influx.

2.3. Cluster of differentiation 38 (CD38)

2.3.1. Distribution and function of CD38

CD38, a 42 kDa membrane glycoprotein, is widely expressed in the immune system, including on the cell surface of many immunocytes, including B cells, neutrophils and bronchial epithelial cells [28-30], and it is also found in many intracellular organelles, such as the endoplasmic reticulum, mitochondria, and nuclear membrane [31-34]. CD38 is somewhat unique among molecules because it is a multifunctional protein with dual receptor and enzymatic functions [6].

CD38 is predominantly expressed on the surface of immune cells and acts as an ectoenzyme with its active sites outside the cells. As a multifunctional ectoenzyme, CD38 catalyzes different enzymatic activities: 1) synthesis of adenosine diphosphate ribose (ADPR) from nicotinamide adenine dinucleotide (NAD^+) via NAD^+-glycohydrolase activity; 2) synthesis of cyclic ADPR (cADPR) from NAD^+ via cyclase activity; 3) hydrolysis of cADPR to ADPR via hydrolase activity; 4) synthesis of nicotinic acid adenine dinucleotide phosphate (NAADP) from $NADP^+$ (the phosphorylated equivalent of NAD^+) via NAADP-synthase activity; 5) hydrolysis of NAADP to ADPR.P via NAADP- hydrolase activity [6]. Notably, all the final enzymatic reaction products are second messengers essential for the regulation of cytoplasmic $Ca2^+$ in a number of cells [35]. Furthermore, intracellular Ca^{2+} mobilization is a fundamental signaling mechanism that plays a crucial role in cell proliferation, differentiation, migration, and death [36]. However, some of the

inflammatory responses mediated by CD38 are dependent on its enzymatic activity, while others seem to occur independently of this function [37].

In addition to its enzymatic activities, CD38 can also serve as the signaling receptor for CD31, which is a major cell adhesion molecule present on endothelial cells involved in transendothelial migration [35]. Through their interaction, hematopoietic cells expressing CD38 can bind to endothelial cells and transmigrate into inflamed tissues [38]. Moreover, recent studies have shown that the adhesion of lymphocytes to endothelial cells is blocked using CD38-specific antibodies [39-41]. Similarly, anti-CD38 monoclonal antibodies inhibit migration of human mature DCs [41]. In addition, CD38-deficient DCs exhibit defective migration from the skin to local lymph nodes following antigenic stimulation [40].

2.3.2. CD38 regulates inflammatory responses in response to infection

CD38 is associated with the pathogenesis of various inflammatory diseases. In this regard, *in vivo* studies have revealed that deficiency of CD38 in mice results in increased susceptibility to infection by a number of bacterial pathogens, including *Streptococcus pneumoniae* [37, 42], *Mycobacterium avium*[43], and *Listeria monocytogenes* [44]. These findings clearly show that CD38 is a positive regulator of infectious inflammation.

2.3.2a. CD38 is upregulated in inflammation

It has been demonstrated that CD38 is robustly induced in many immune cells during bacterial infection and the ensuing inflammatory response. In addition, the expression level of CD38 on immune cells, such as NK cells, lymphocytes, neutrophils and macrophages, varies with their maturation and/or activation status [35]. For example, the expression of CD38 is relatively low on

BM myeloid precursors, granulocytes, and DCs; however, it is markedly increased on neutrophils and macrophages isolated from sites of inflammation [42]. Similar results have also been reported with human monocytes and granulocytes [45-47]. Further, LPS induces the expression of CD38 in RAW 26.4 cells in a dose- and time-dependent manner [48]. The expression of CD38 in DCs is upregulated upon LPS and *E. coli* challenge [49]. Likewise, CD38 is upregulated on the surface of neutrophils, inflammatory monocytes, and DCs in the liver and spleen following *Listeria monocytogenes* infection [44]. In addition, stimulation with thioglycolate results in significantly increased expression of CD38 on both peripheral and peritoneal neutrophils in WT mice [40]. These findings suggest an active role for CD38 in the inflammatory response to infections. Furthermore, the expression of CD38 is controlled by a promoter region containing binding sites for LXR, RXR, NF-κB, and STAT, implying its prominent role in the inflammatory response [50].

2.3.2b. CD38 regulates cellular migration

The enzymatic activities of CD38 that function as calcium-mobilizing second messengers are important for leukocyte transmigration to the site of infection. Furthermore, CD38 may act as a plasma membrane signaling receptor in cooperation with chemokine receptors, including CXCR4 and CCR7, to induce the transmigration of monocytes and neutrophils to inflamed tissues [44]. In fact, attenuated neutrophil recruitment has been reported in the lungs of CD38-deficient mice following *S. pneumoniae* infection [37, 42]. Likewise, impaired accumulation of neutrophils and inflammatory monocytes was observed in the spleens of *Listeria*-infected CD38-deficient mice [44]. However, no differences in the proportions of these cells in the BM and blood were found between WT and CD38-deficient mice, suggesting that mobilization of neutrophils and inflammatory monocytes from BM is CD38-independent, whereas their migration to the spleen is CD38-dependent following *Listeria* infection [44]. Interestingly, it has been shown that, due to the

loss of CD38, the arrival of neutrophils is delayed in the liver in response to parasite infections. Briefly, during *Entamoeba histolytica*-induced hepatic amoebiasis the number of neutrophils reaches the maximum at 6 hours and then decreases over time in WT mice, while neutrophils gradually increase until reaching a similar maximum peak at 48 hours in $CD38^{-/-}$ mice [51]. A similar delay in the host inflammatory response in CD38-deficient mice has been reported in amoebic meningoencephalitis [52]. These two studies revealed that additional signaling pathways involved in neutrophil accumulation might be activated as compensatory mechanisms in CD38-deficient mice at later time points during inflammatory infections.

Recent studies have also focused on the role of CD38 in leukocyte chemotaxis. Deficiency of CD38 in immature and mature DCs results in impaired chemotaxis toward chemokines CCL2, CXCL12, and CCL19 [40]. In addition, CD38 regulates the chemotaxis and transendothelial migration of human monocyte-derived dendritic cells (MDDCs) towards chemokine CCL21 [40, 42]. Also, upregulation of CD38 in human chronic lymphocytic leukemia (CLL) cells results in increased migration in response to chemokines *in vitro* [30]. Likewise, bone marrow-derived CD38-deficient neutrophils exhibit defective chemotaxis toward a gradient of bacterial chemoattractant fMLP *in vitro*, but not the potent neutrophil activator, IL-8 [37].

2.3.2c. CD38 modulates signaling transduction cascades

Invading microbial pathogens contain conserved molecular structures that can be recognized by TLRs resulting in the initiation of innate immune responses during bacterial infection. Upon stimulation, TLRs trigger downstream signaling cascades and sequentially activate transcription factors, an important feature of inflammation [53]. Among these transcription factors, NF-κB is a critical mediator of neutrophil accumulation at sites of infection. In this regard, intratracheal LPS

administration results in NF-κB translocation into the nucleus and subsequent neutrophil accumulation in the alveoli [54]. Recent studies imply an active role for CD38 in the regulation of TLRs and/or downstream signal transduction during inflammation. For example, CD38 deficiency enhances the expression of TLR4 and nuclear translocation of NF-κB p65 in the kidneys of LPS-treated septic mice [55]. Likewise, the expression of TLR4 and phosphorylated NF-κB p65 are upregulated in the liver of $CD38^{-/-}$ mice as compared to WT and $CD38^{-/-}$ $TLR4^{mut}$ mice during *E. coli*-induced sepsis [56]. However, knock down of CD38 by small interfering RNA (siRNA) in RAW 264.7 cells decreased their polarization toward M1 through inhibiting NF-κB signal transduction upon LPS challenge [48]. Collectively, these studies point to a supporting role of CD38 in the regulation of PRRs and/or downstream signaling transduction in the immune response to infections.

2.3.2d. CD38 regulates cytokine/chemokine induction

Engagement of PRRs by pathogen components initiates downstream signaling cascade events that result in translocation of transcription factors and production of pro-inflammatory mediators. In this sense, pro-inflammatory cytokines and chemokines are released to facilitate the recruitment of immune cells and sequential elimination of pathogens during host defense against bacterial infections. Our laboratory has demonstrated that BMDMs produces a higher level of CXCL1 chemokines when stimulated with LPS through activation of TLR4 and NF-κB compared with PBS-treated controls [57]. However, the contribution of CD38 signaling to cytokine/chemokine release is controversial, as opposite results have been reported in various murine infectious models. Several studies have demonstrated that deficiency of CD38 promotes the release of pro-inflammatory mediators in inflammatory-related diseases. In the absence of CD38 cytokines/chemokines are still produced that are responsible for neutrophil activation and

recruitment, such as IL-1β, MCP-1 and TNF-α, following *S. pneumonia* challenge [37]. Blocking CD38 promotes the production of the pro-inflammatory cytokines, like IL-6, TNF-α, and IFN-γ, in the serum of LPS-induced septic mice [58]. Similarly, CD38-deficient mice showed aggravated liver injury accompanied by elevated induction of IL-6, IL-18, and IL-1β following E. coli-induced sepsis [59]. Moreover, knockdown of CD38 in RAW 264.7 macrophages promotes the secretion of pro-inflammatory mediators, such as IL-1α, IL-6, and G-CSF, through the Sirtl/NF-κB/TLR2 signaling pathway [60]. However, opposite behaviors of CD38 have also been reported in murine models. As a receptor, CD38 can attach to T cells expressing CD31 on the surface and then activate these cells to produce a variety of cytokines , such as IL-10 and IFN-γ in lamina propria T cells [61]. CD38 modulates the production of type I and III IFNs in host responses caused by respiratory syncytial virus (RSV), as a CD38 antagonist was found to decrease the expression of IFN-β in human DCs *in vitro* [47]. Further, the inflammatory response was impaired in CD38-deficient macrophages during *S. typhimurium* infection, with reduced levels of IL-6, TNF-α, and IL-1β [60], and genetic blockade of CD38 was found to impact the secretion of IL-12 and IL-1β in human macrophages under inflammatory conditions [8]. Upon stimulation with the anti-CD38 monoclonal antibody, cytotoxic responses of activated NK cells were triggered resulting in release of granzymes and cytokines by CD38 [62]. Similar results were obtained with respect to adaptive immunity showing that CD38 enhances the expression of IL-6, IFN, and GM-CSF in human T and B lymphocytes. In addition, the kinetic expression of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, in the liver of WT and $CD38^{-/-}$ mice was compared following *E. histolytica* challenge. At 6-12-hrs post-infection, WT mice displayed higher levels of cytokines, while at 48-hrs post infection, these two strains of mice showed similar expression. In contrast, at 72-hrs post-infection, CD38-deficient mice showed higher pro-inflammatory cytokine expression than their

WT counterparts [51]. Together, the variation in these study results may be explained by the differences in cell types and infection models used. Nevertheless, these results support the concept that CD38 modulates cytokine/chemokine production in inflammatory diseases.

Based on the above studies, we conclude that CD38 impacts the pathogenesis of various inflammatory diseases. Although recent studies have determined the role of CD38 in host defense in certain Gram-positive bacterial infections, including *S. pneumoniae* [37, 42] and *Listeria monocytogenes* [44], the role of CD38 in host protection against Gram-negative bacterium CRKP has not been explored. Thus, the overall goal of this work is to explore the role of CD38 in host defense following CRKP infection. We proposed our hypothesis that CD38 regulates host defense against CRKP-induced pneumonia. We tested the hypothesis with the following aims: 1) To determine the role of CD38 in pulmonary host protection against CRKP; 2) To examine the contribution of CD38 in hematopoietic versus non-hematopoietic cells to pulmonary host protection following CRKP infection. This knowledge will not only enhance our understanding of molecular mechanisms of innate immunity against bacterial pneumonia, but also identify novel targets for future therapeutic treatment.

Chapter 3. Materials and Methods

3.1. Animals

Eight to twelve-week-old female wild-type (C57BL/6J genetic background, purchased from Jackson Laboratory) and CD38 genetically deficient mice were used in our study. Mice were raised in specific pathogen-free conditions with a 12 h light/dark cycle. The CD38 knockout mice develop normally, are healthy and fertile, and exhibit no significant differences in body size and weight compared with WT littermates. All animal experiments were performed with the approval Institutional Animal Care and Use Committee (IACUC) at Louisiana State University (LSU).

3.2. Pneumonia model

Carbapenem-resistant Klebsiella pneumoniae (CRKP) (ATCC BAA-1705) was used to induce pneumonia. WT and $CD38^{-/-}$ mice were anesthetized using 150 µL of ketamine and xylazine mixture by intraperitoneal injection (IP) and inoculated with CRKP suspension by oropharyngeal aspiration. Tongues of mice were gently pulled out using sterile forceps and then 50 µL of bacterial suspension containing 1×10^{8} CFUs/ mouse of CRKP was inoculated into the oral pharynx for the mice to inhale by reflexive aspiration. At 24- and 48- hrs post-infection, mice were euthanized to determine the phenotype of the bronchoalveolar lavage fluid (BALF) cells and the bacterial burden in the pulmonary and extrapulmonary organs. For survival experiments, mice were infected with a lethal dose of CRKP (1.7×10^{8} CFUs/mouse) and were monitored for up to 10 days for a survival study.

3.3. BALF collection, cell count, and bacterial burden

BALF and organ samples were collected as described earlier [63, 64]. Briefly, the mice were euthanized using ketamine/xylazine mixture and trachea was cannulated with a 20-gauge catheter. BALF was collected by four times lung lavage with 0.8 ml of PBS (containing heparin and dextrose) and approximately a volume of 3 ml was retrieved per mouse [63, 64]. Total white blood cells (WBCs) in BALF were enumerated using a hemocytometer. Differential leukocyte subtype counts were performed using cytospin slides stained with the Diff-Quick method. Organ homogenates and BALF samples were plated in serial dilutions onto MacConkey agar plates for bacterial enumeration.

3.4. Cytokine/Chemokine assays

BALF samples were centrifuged, and the supernatants were aliquoted and frozen at -80° C. Different pro-inflammatory cytokines in the BALF and lung homogenates were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF-α, IL-6, IL-1β, IL-18, GM-CSF, IL-17A and IFN-γ (eBioscience). Based on the experimental procedure, the capture antibody was coated in appropriate number of wells of the microplates. Standards and samples were then added into these wells to bind to the capture antibody. After the washing step, the detection antibody was added to form the sandwich. After washing off unbound antibodies, a second enzyme-conjugated antibody (avidin-HRP) was added to react with the specific substrate (TMB) to produce measurable signals.

3.5. Myeloperoxidase activity (MPO)

Neutrophil migration into the lung parenchyma was quantitated by myeloperoxidase assay with lung homogenates from CRKP-infected WT and CD38-deficient mice as described earlier [65]. In

brief, the lung samples were weighted and homogenized in 50 mM potassium phosphate buffer supplemented with 0.5% hexadecyltrimethylammonium bromide and then centrifuged. Seven microliters of the supernatant were transferred into a 96-well microplate. Immediately after adding hydrogen peroxide/O-dianisidine hydrochloride buffer, an increment of the absorbance was measured using a spectrophotometer at 460 nm.

3.6. Intracellular neutrophil killing assay

Bone marrow-derived neutrophils (BMDNs) from both WT and CD38-deficient mice were purified using the EasySep™ Mouse Neutrophil Enrichment Kit (STEMCELL™ Technologies) [66]. Then 0.25×10^6 neutrophils/0.25 ml DMEM (supplemented with 10% FBS) were allocated in 96-well culture plates and infected with CRKP (MOI: 10). At the designated time points of 1.5- and 4-hrs, cells were treated with Gentamycin (250 ug/ml) for 30 mins, washed thrice with sterile PBS and lysed with 120 μL of 0.1% triton X. The lysates were serially diluted and plated onto MacConkey agar plates and incubated overnight to estimate intracellular bacterial burden.

3.7. Extracellular neutrophil killing assay

First, 0.25×10^6 neutrophils/0.25 ml DMEM (containing 10% FBS) were allocated in culture plates and treated with cytochalasin D (10 mg/ml; Sigma-Aldrich) for 20 mins to block phagocytosis prior to infection. Then these cells were infected with CRKP (MOI: 1). At the designated time points of 1-, 2- and 4-hrs, the cells were centrifuged, and the supernatants were collected, serially diluted with PBS, and plated onto MacConkey agar plates to enumerate the extracellular bacterial loads.

3.8. Chimeras

We performed bone marrow transplantation to generate bone marrow chimeras in which the genetic deletion of CD38 was limited to either the myeloid cells ($CD38^{-/-}$→WT) or stromal cells (WT→$CD38^{-/-}$). Recipient WT and $CD38^{-/-}$ mice were lethally irradiated with two 525 Rads doses 3 hours apart. During the time in between irradiation, donor naïve WT and $CD38^{-/-}$ mice were sacrificed to prepare donor BM cells. The femur and tibia were flushed, RBCs were lysed, and BM cells were centrifuged and resuspended in sterile HBSS. Fresh donor BM cells (8×10^6 cells/200 μL HBSS) were transfused into recipient mice via tail vein injection. Four groups of chimeras were generated: WT→$CD38^{-/-}$ (irradiated $CD38^{-/-}$ mice reconstituted with WT donor cells), $CD38^{-/-}$→ WT (irradiated WT mice reconstituted with $CD38^{-/-}$ donor cells), $CD38^{-/-}$→$CD38^{-/-}$ (irradiated $CD38^{-/-}$ mice reconstituted with $CD38^{-/-}$ donor cells), and WT → WT (irradiated WT mice reconstituted with WT donor cells). Then the chimeric mice were treated with 0.2% neomycin sulfate for 2 weeks. After 8 weeks of reconstitution, pneumonia was induced with a sub-lethal dose of CRKP (1×10^8 CFUs/ mouse) by oropharyngeal aspiration. At 48-hrs post-infection, mice were euthanized to determine the phenotype of the BALF cells and bacterial burden in pulmonary and extra-pulmonary organs.

3.9. Flow cytometry

The granulopoiesis experiments were performed using flow cytometric analysis as described previously[67]. At 48-hrs post-infection with CRKP, single-cell suspensions were obtained from the BM of WT and $CD38^{-/-}$ mice. RBCs were lysed, and BM cells were stained with antibody cocktails. The following fluorescence-conjugated antibodies were used: Ly6G (1A8), c-Kit (2b8), CD34 (RAM34, eBioscience), CD3 (145-2C11), CD4 (RM4.5), CD8a (53-6.7), CD19 (6D5),

B220 (RA3-6B2) and TER119 (Ter119) (Biolegend) monoclonal antibody. Data were analyzed using FlowJo 10 (Treestar, Sanford, CA).

3.10. Data analysis

Data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were done using GraphPad Prism 8.4. Unpaired t-tests or one-way ANOVAs (followed by Bonferroni's multiple comparison) were used to compare differences between groups as appropriate. Log-rank test was used to analyze survival data. Significant differences are defined as $*p<0.05$, $**p<0.01$, $***p<0.001$.

Chapter 4. Results and Discussion

4.1. Results

4.1.1. Deficiency of CD38 enhances host protection during CRKP-induced pneumonia

To assess the role of CD38 in host defense against CRKP infection, WT and $CD38^{-/-}$ mice were inoculated with a lethal dose of CRKP (1.7×10^8 CFU/mouse) by oropharyngeal aspiration, and the survival of mice was monitored up to 10 days. Compared to WT mice, $CD38^{-/-}$ mice displayed decreased mortality in response to CRKP infection, with 70% of $CD38^{-/-}$ mice surviving for up to 10-days, whereas 80% of WT mice died within 5 days (Figure 4.1A). To examine whether the increased host survival in $CD38^{-/-}$ mice was caused by decreased bacterial burden, we infected WT and $CD38^{-/-}$ mice with a sublethal dose of CRKP (1×10^8 CFUs/mouse) and assessed the bacterial burden in the pulmonary and extra-pulmonary organs at both 24 and 48-hrs post-infection. As anticipated, $CD38^{-/-}$ mice displayed a decreased bacterial burden in the lungs and BALF together with diminished bacterial dissemination to the liver and spleen at 48-hrs post-infection (hpi) (Figure 4.1B-E).

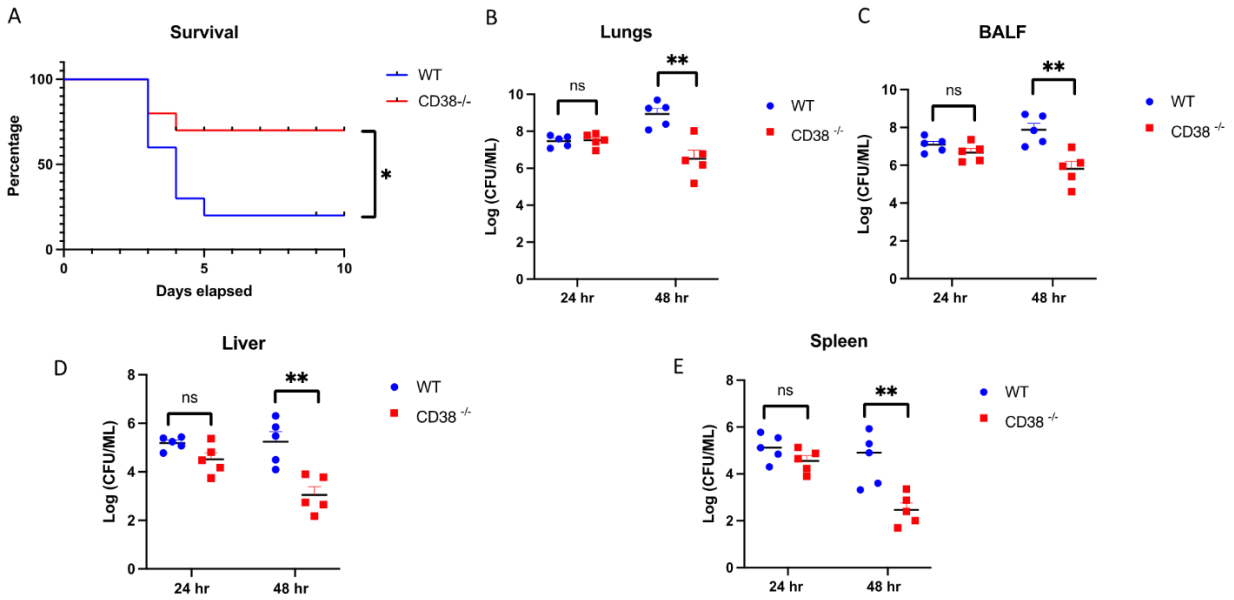

Figure 4.1. Deficiency of CD38 contributes to host protection against CRKP-induced pneumonia. (A)WT and CD38$^{-/-}$ mice (10 mice/group) were infected with a lethal dose of CRKP (1.7 ×10^{8} CFUs/ mouse) by oropharyngeal aspiration and survival was recorded for up to 10 days. WT and CD38$^{-/-}$ mice were infected with a sublethal dose of CRKP (1 ×10^{8} CFUs/ mouse). Bacterial burden (CFU) in the lung (B), BALF (C), liver (D), and spleen (E) was enumerated at 24- and 48-hrs post-infection.

4.1.2. CD38 deficiency enhances the pulmonary inflammatory response following CRKP infection

Because the enhanced bacterial clearance in CD38$^{-/-}$ mice likely results from increased leukocyte recruitment into the lungs, we assessed the recruitment of leukocytes in the BALF at 24-hrs and 48-hrs after infection with CRKP. As expected, augmented host defense in CD38$^{-/-}$ mice was associated with enhanced accumulation of total white blood cells (WBCs) and neutrophils in the BALF (Figure 4.2A-B). However, we did not observe a significant difference in macrophage populations between WT and CD38$^{-/-}$ mice (Figure 4.2C). Furthermore, a myeloperoxidase (MPO) assay was performed to measure neutrophil accumulation in the lungs of WT and CD38$^{-/-}$ mice at 48-hrs post-infection with CRKP. Consistent with increased neutrophil recruitment in the BALF, our results show that the MPO levels were increased in the lungs of CD38$^{-/-}$ mice infected with CRKP (Figure 4.2D).

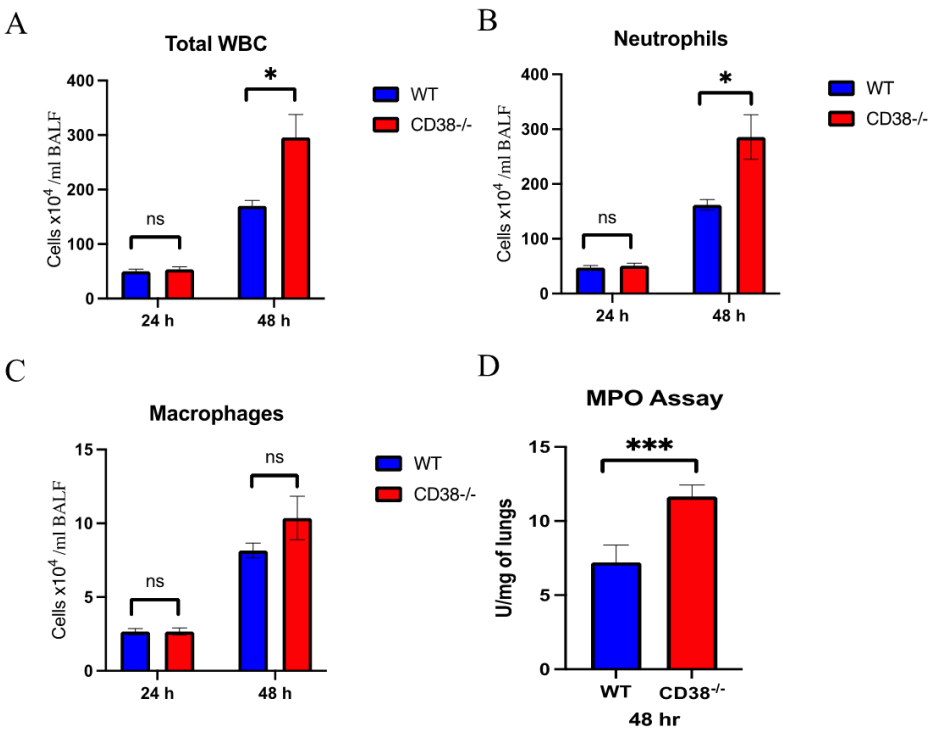

Figure 4.2. CD38 deficiency promotes inflammatory cell recruitment to the lungs of CRKP-infected mice. Total WBCs (A), neutrophils (B), and macrophages (C) were quantitated in the BALF of the mice at both 24-hrs and 48-hrs post infection with CRKP. (D) Myeloperoxidase assay was performed in the lungs at 48-hrs post-infection.

4.1.3. CD38 deficiency enhances cytokine production during CRKP infection

Neutrophil infiltration is primarily controlled by proinflammatory cytokines and chemokines in the lungs [68]. The enhanced neutrophil recruitment in CD38$^{-/-}$ mice likely reflects increased expression of inflammatory mediators. Therefore, we quantitated the pro-inflammatory cytokines in the BALF of both WT and CD38$^{-/-}$ mice at 24 and 48-hrs post-infection. As anticipated, the levels of TNF-α, IL-6, IL-1β, IL-18, GM-CSF, and IL-17A were increased in the BALF of CRKP-infected CD38$^{-/-}$ mice (Figure 4.3A-F). Furthermore, the level of IFN-γ and IL-17A was increased in the lung parenchyma of CRKP-infected CD38$^{-/-}$ mice at 48-hrs post-infection (Figure 4.3G-H).

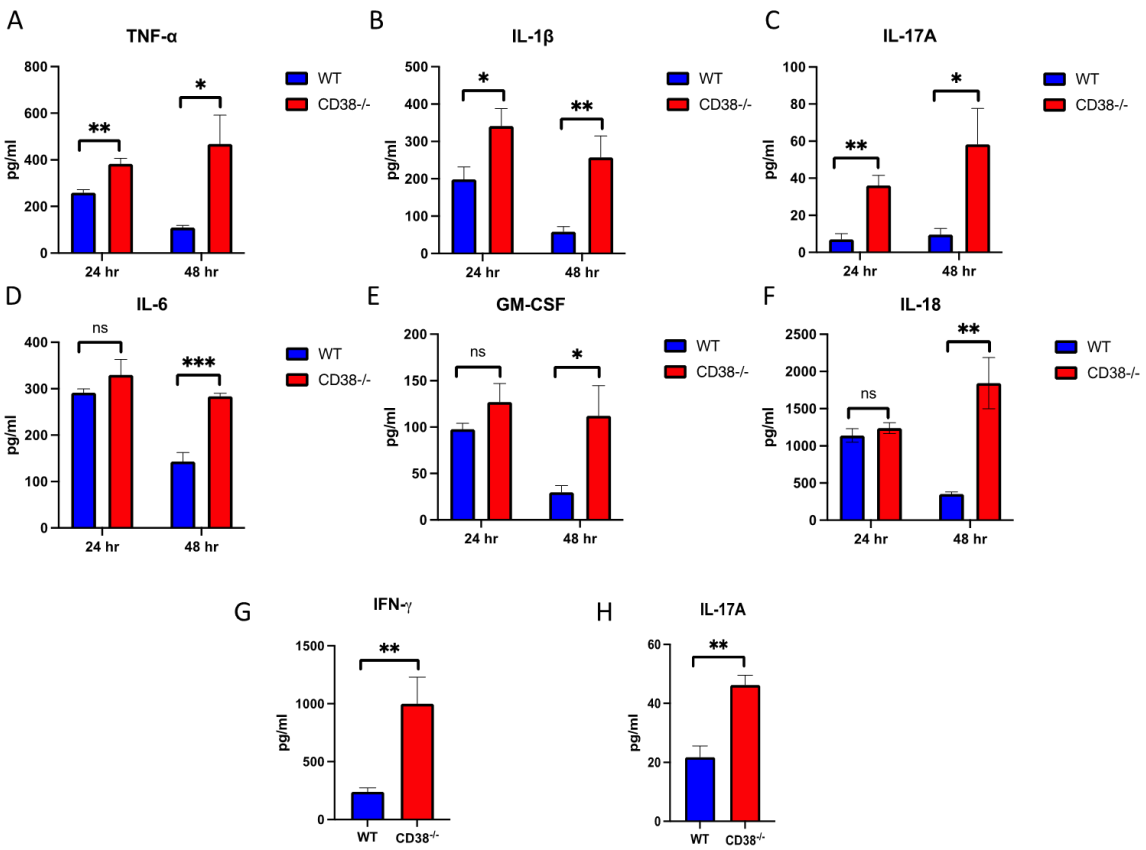

Figure 4.3. Increased cytokine production in the lungs of CRKP-infected CD38-deficient mice. Levels of TNF-α (A), IL-1β (B), IL-17A (C), IL-6 (D), and GM-CSF (E), IL-18 (F) in the BALF were measured using ELISA. Levels of IFN-γ (G) and IL-17A (H) in the lung homogenates at 48-hrs post-infection were measured using ELISA.

4.1.4. CD38 impairs the intracellular killing abilities of neutrophils against CRKP

In addition to sufficient neutrophil recruitment, the effective function of neutrophils is also critical for pulmonary host defense against bacterial infection. Since CD38 has been shown to be upregulated in human and murine pneumonic neutrophils, we sought to examine whether deficiency of CD38 impacts the function of neutrophils. BMDNs from both WT and $CD38^{-/-}$ mice were isolated to compare their intracellular killing ability in response to infection with CRKP (MOI:10). CD38-deficient neutrophils exhibited enhanced bacterial killing ability in comparison to WT counterparts as delineated by the higher intracellular bacterial survival in WT BMDNs (Figure 4.4A). However, CD38 is dispensable for the extracellular killing of BMDNs (Figure 4.4B).

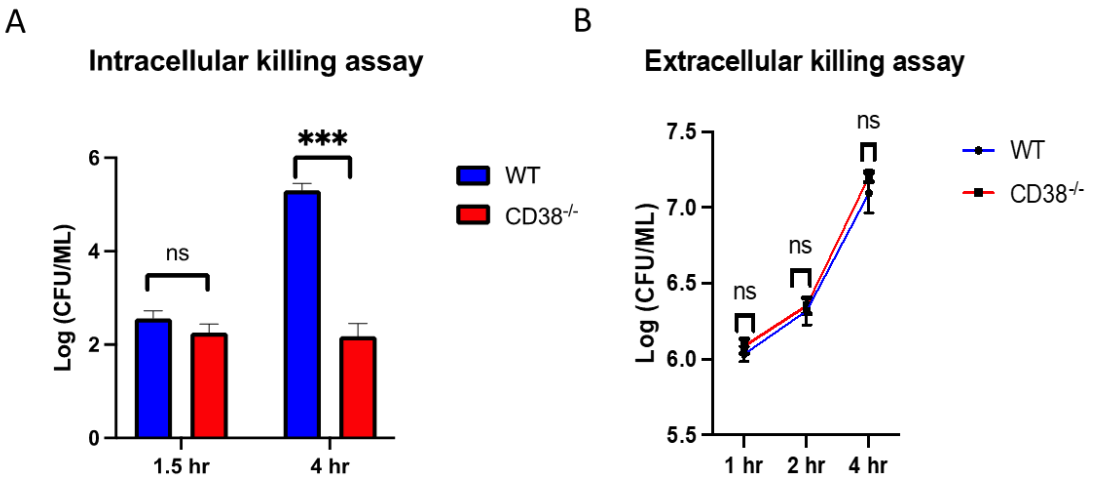

Figure 4.4. CD38$^{-/-}$ neutrophils exhibit enhanced intracellular killing ability. (A) Intracellular killing assay with BMDNs isolated from WT and CD38$^{-/-}$ mice at designated time points after infection with CRKP (MOI:10). (B) Extracellular killing assay with BMDNs isolated from WT and CD38$^{-/-}$ mice at designated time points after infection with CRKP (MOI:10).

4.1.5. CD38 deficiency in non-hematopoietic cells confers host protection against CRKP pneumonia.

Bacteria initially interact with alveolar cells, including neutrophils, macrophages, and epithelial cells. Furthermore, CD38 has been shown to be expressed on hematopoietic and non-hematopoietic cell types [30, 44]. Thus, using bone marrow chimeras, we assessed the contribution of CD38 from myeloid cells versus stromal cells to host defense against CRKP-induced pneumonia. Chimeric groups harboring the loss of CD38 in non-hematopoietic tissue (WT→CD38$^{-/-}$), hematopoietic cells (CD38$^{-/-}$→WT), or both compartments (CD38$^{-/-}$→CD38$^{-/-}$) had enhanced total WBCs and neutrophil accumulation in the BALF in comparison with WT→WT group at 48-hrs post-infection (Figure 4.5A-B). Interestingly, the WT→CD38$^{-/-}$ (deficiency of CD38 in non-hematopoietic tissue), CD38$^{-/-}$→CD38$^{-/-}$ (deficiency of CD38 in both compartments), but not CD38$^{-/-}$→WT (deficiency of CD38 in hematopoietic cells) group, had reduced bacterial burden in lungs in comparison with WT→WT group at 48-hrs post-infection (Figure 4.5C). Furthermore,

there was no significant difference in bacterial burden in the spleen among these four chimeric groups (Figure 4.5D). Taken together, our results suggest CD38 in both compartments serves to impair neutrophil recruitment, but CD38 in the non-hematopoietic compartment impedes bacterial clearance to a greater extent than that in hematopoietic compartments during CRKP-induced pneumonia.

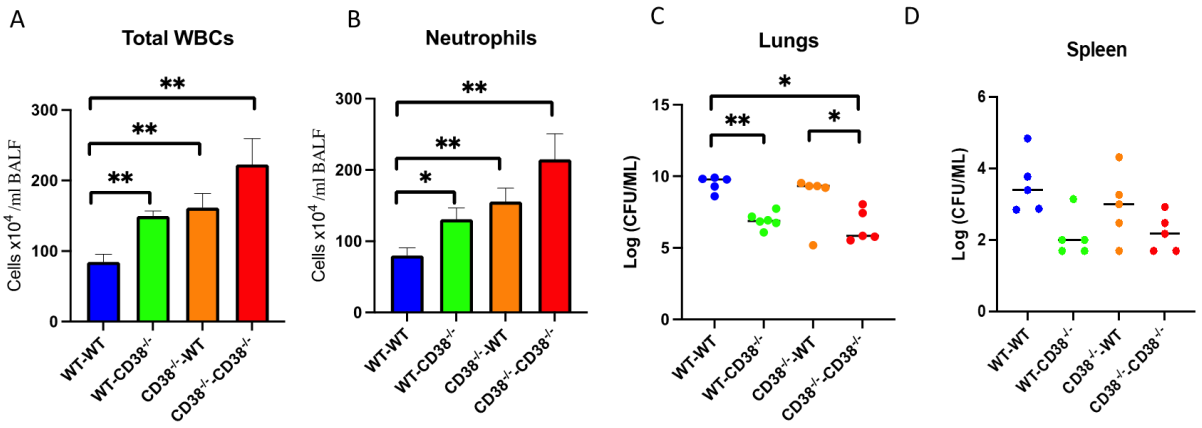

Figure 4.5. Expression of CD38 in non-hematopoietic tissue is detrimental to host protection against CRKP infection. Bone marrow-reconstituted mice were infected with a sublethal dose of CRKP (1×10^8 CFUs/ mouse) and euthanized at 48-hrs post-infection to analyze total WBCs (A) and neutrophils (B) in the BALF and the bacterial burden in the lung (C) and spleen (D) following infection.

4.1.6. CD38 does not regulate CRKP-induced emergency granulopoiesis

Sufficient granulocytes for the demands of host protection are continuously produced via emergency granulopoiesis following microbial infection. Therefore, we determined whether the enhanced inflammatory response in the lungs of CRKP-infected $CD38^{-/-}$ mice was due to increased emergency granulopoiesis. We assessed the granulopoietic compartments using a flow cytometric method, as previously described [67]. During the differentiation of granulocytes, neutrophils gradually lose the expression of c-Kit and acquire the expression of Ly6G. Thus, subpopulation 1 ($c\text{-}Kit^{high}Ly6G^{-}$) cells are early granulocytic precursors and subpopulation 5 ($c\text{-}Kit^{low}Ly6G^{high}$) cells are mature neutrophils that are ready to be released into the circulation (Figure 4.6A). As

anticipated, both WT and CD38$^{-/-}$ mice displayed a significant decrease in subpopulation 5 at 48-hrs post infection with CRKP as compared to uninfected control mice (Figure 4.6B). This suggests that an increasing number of mature neutrophils were released from the BMs into the circulation following infection of CRKP. However, the distribution of granulocytic compartments (P1 to P5) was identical between WT and CD38$^{-/-}$ mice during emergency granulopoiesis (Figure 4.6C). These results indicate that CRKP-induced emergency granulopoiesis is independent of CD38.

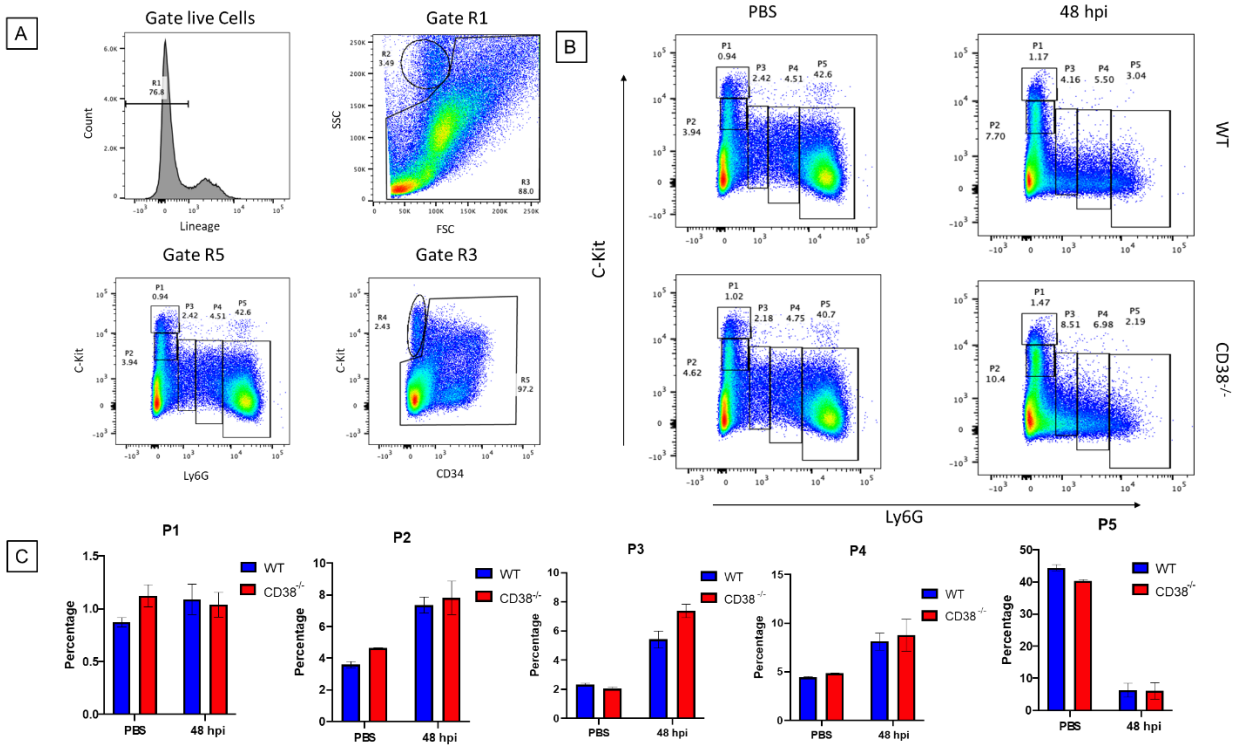

Figure 4.6. CRKP-induced emergency granulopoiesis does not require CD38. WT and CD38$^{-/-}$ mice were infected with a sublethal dose of CRKP (1 ×10^8 CFUs/ mouse). Mice were euthanized to collect BM cells at 48-hrs post infection. (A) Staining and gating strategy of BM cells to analyze emergency granulopoiesis. First, BM cells that have lost the potential to give raise to granulocytes were excluded, including lineage positive T lymphocytes, B lymphocytes and erythroid cells. Discrete populations R2 (Eosinophils) and R4 (megakaryocyte-erythroid progenitors, MEPs) were also removed. Cells in R5 were then divided into five subpopulations based on c-Kit and Ly6G expression. (B) Flow cytometric analyses of WT and CD38$^{-/-}$ mice were presented. (C) Percentages of subpopulations P1 to P5 within granulopoietic compartments were shown.

4.2. Discussion

It is projected that antibiotic-resistant infectious diseases will lead to 10 million deaths annually by the year 2050. The increasing prevalence of antimicrobial resistance, as seen with CRKP, and

a lack of efficacious vaccines underscore the need for novel treatments for pneumonia. Thus, the cellular and molecular mechanisms responsible for neutrophil recruitment in bacterial pneumonia have long been a research focus. Thus far, we and others have investigated innate immune molecules, primarily in the context of TLRs, NLRs, cytokines, and chemokines, which are crucial regulators of neutrophil recruitment in the pulmonary immune response against bacterial pneumonia. CD38 is a cell surface protein constitutively or inducibly expressed in immune cells. It is established that CD38 regulates a wide variety of inflammatory processes, ranging from cell differentiation, cell activation, and cell apoptosis to adaptative immune responses. However, the importance of CD38 in the immune response in certain disease settings and disease models and the underlying mechanisms remain to be defined. Thus, we used a murine model of CRKP to delineate the role of CD38 in pulmonary host protection against Gram-negative pneumonia.

Several *in vivo* investigations have reported the protective role of CD38 in pulmonary host defense against Gram-positive pathogens, such as *Listeria monocytogenes* [44], *Mycobacterium avium*[43], and *Streptococcus pneumoniae*. In contrast, our current study shows that CD38 is detrimental to the immune response to Gram-negative bacteria, as $CD38^{-/-}$ mice display enhanced survival following infection with CKKP. The controversial findings between Gram-positive and negative studies might be explained by the differences in virulent factors and their interaction with PRRs after microbial infections. For example, Gram-negative bacteria are detected by TLR4 via LPS whereas Gram-positive bacteria are recognized by TLR2 via lipoproteins. Augmented host protection in $CD38^{-/-}$ mice occurs through decreased bacterial burden in the lungs and BALF and diminished dissemination to the liver and spleen at 48-hrs post-infection. The pulmonary host protection regulated by CD38 is likely associated with 1) sufficient neutrophil recruitment to the lung; 2) enhanced bacterial killing ability of CD38-deficient neutrophils against CRKP. Our results

clearly show that deficiency of CD38 not only affects neutrophil recruitment, but also their function.

The inflammatory response to bacterial infections is always accompanied by increased induction of pro-inflammatory cytokines/chemokines. Our study demonstrates increased levels of TNF-α, IL-6, IL-1β, IL-18, GM-CSF, and IL-17A in the BALF of $CD38^{-/-}$ mice following CRKP infection. Especially, $CD38^{-/-}$ mice showed significantly elevated levels of IL-17A in the BALF and lung homogenates at 48-hrs post-infection. Accumulated evidence implicates the crucial role of IL-17A in host protection against a number of microbial infections, such as *Streptococcus pneumoniae, Klebsiella pneumoniae,* and *Mycobacterium tuberculosis* [68]. IL-17A elicits protective inflammation by inducing pro-inflammatory cytokines/chemokines, recruiting immune cells (e.g., neutrophils), and activating T cells [1]. IL-17A was demonstrated to recruit neutrophils both directly and indirectly by inducing the production of cytokines/chemokines such as KC (CXCL1) and G-CSF [65, 69]. Therefore, future studies are required to measure the level of KC and G-CSF in the BALF of both WT and $CD38^{-/-}$ mice following CRKP infection.

Although deficiency of CD38 is essential for leukocytes recruitment, its role in emergency granulopoiesis has not been investigated. Sufficient neutrophils are continuously produced in the BM and are then be released to the circulation or sites of infections on demand following microbial infection. The process of emergency granulopoiesis is important for clearance of CRKP in the host. Surprisingly, we did not observe any significant difference in the proportion of granulocytic compartments in the BM. These findings suggest that CD38 is dispensable during CRKP-induced emergency granulopoiesis. The enhanced inflammatory response in $CD38^{-/-}$ mice might be due to increased neutrophil mobilization in the BM. Therefore, we will further examine whether the

expression of adhesion molecules, such as CD62L and CD49d, on neutrophils in the BM is altered in these mice during CRKP-induced pneumonia.

Recent studies have indicated the important role of both myeloid and stromal cells in regulating neutrophil infiltration during bacterial lung inflammation [63, 64]. CD38 has been shown to be expressed on both myeloid and stromal cells [30, 44]. Further, it has been reported that increased susceptibility to *S. pneumoniae* infection in CD38 knockout mice results from the deficiency of CD38 in bone marrow-derived myeloid cells [42]. However, we have limited knowledge on the contribution of CD38 expressed on myeloid versus stromal cells in host defense against CRKP-induced pneumonia. Commonly used WT and $CD38^{-/-}$ recipient mice can tolerate two times irradiation with the dose of 525 Rads 3 hours apart. After bone marrow transplantation, mice were maintained on antibiotic water for 2 weeks to prevent infection. According to our previous work, more than 78-86% of leukocytes in the circulation originate from the donor mice at 8 weeks post-transplantation when experiments are conducted [1]. In contrast, irradiated mice with ineffective transplantation die between days 18 and 20 after irradiation [1]. Control mice that did not receive donor cells died at 18 days after irradiation while all the other experimental mice survived until the CRKP infection experiment.

Our bone marrow chimeras showed that WT→$CD38^{-/-}$ group had increased bacterial clearance in the lungs in comparison with WT→WT group. In addition, $CD38^{-/-}$→$CD38^{-/-}$ group had increased bacterial clearance in the lungs as compared with the $CD38^{-/-}$→WT group. These findings suggest that the loss of CD38 in stromal cells of the WT→$CD38^{-/-}$ (non-hematopoietic) group may have enhanced the bacterial killing ability of these cells. However, the loss of CD38 in myeloid cells of the $CD38^{-/-}$→WT (hematopoietic) group was dispensable for bacterial clearance as no significant difference was found between $CD38^{-/-}$→WT and WT→WT group. The

findings contrast with our previous results that CD38-deficient BMDNs are more efficient than WT BMDNs at clearing bacterial infections; however, there were several possible reasons for this. A recent report has demonstrated that there is a significant cross talk between hematopoietic and non-hematopoietic cells during microbial infections and the ensuing inflammatory response [70]. In particular, airway epithelial cells (non-hematopoietic cells in the lung) have been shown to be critical in controlling *L. pneumophila* infection by acting as sensors (PRRs) and amplifiers of pulmonary inflammation [70]. Therefore, it is conceivable that loss of CD38 in non-hematopoietic cells contributes to bacterial control through enhanced recruitment of migratory leukocytes during CRKP infection. Alternatively, it is possible that the killing ability of CD38-deficient BMDNs was dampened by other alveolar cells or inflammatory mediators in the lung following infection with CRKP. A wide range of immune cells play an essential role in host protection against respiratory infections, such as neutrophils, macrophages, epithelial cells, dendritic cells (DCs) and T cells in the alveoli. It should be noted that different alveolar cells can interact either synergistically or antagonistically in the pulmonary immune responses against bacterial infections. Moreover, it likely that the life span of $CD38^{-/-}$ BMDNs may be shorter than that of WT BMDNs, resulting in the phenotype that deficiency of CD38 in myeloid cells is dispensable for host protection at 48-hrs post infection with CRKP. We, therefore, propose that myeloid and stromal cells may interact sequentially to amplify maximal immune responses against CRKP infections. For example, myeloid cells display enhanced bacterial killing during the early stage of infection, whereas stromal cells are indispensable for bacterial killing during the late stage of CRKP infection (or vice versa). In the future, we will determine the contribution of CD38 from hematopoietic versus non-hematopoietic cells to host defense at different time points of infection with CRKP. In addition,

future studies are needed to determine whether there is any difference in the number of viable neutrophils between WT and $CD38^{-/-}$ mice over time after CRKP infection.

The cytokine IFN-γ is a key effector in the innate and adaptive immune responses against vial and some bacterial infections. Besides its ability to inhibit viral replication, it is an important activator of phagocytic cells [71]. Our previous studies have shown that IFN-γ enhances the bacterial killing ability of BMDNs against *S. aureus* through increased ROS production [63]. In our current study, $CD38^{-/-}$ mice displayed an elevated level of IFN-γ in the lung homogenates at 48-hrs post-infection. In our model, it is possible that deficiency of CD38 increases bacterial clearance by neutrophils through increased IFN-γ. Likewise, neutrophils play a crucial role in pulmonary host protection against *L. pneumophila* infection via immunomodulation (production of IFN-γ) instead of direct bacterial killing [72, 73]. Similarly, another study reported that induction of IFN-γ by neutrophil-derived IL-17 enhances host protection following *L. pneumophila* infection [1]. Therefore, future studies are required to elucidate the precise mechanism through which increased bacterial killing occurs in CD38-deficient mice following CRKP infection.

Collectively, we identified a detrimental role of CD38 in pulmonary host protection in response to CRKP lung infection. These findings suggest that neutralization of CD38 could be a potential therapeutic strategy to augment pulmonary host defense against pneumonia exclusively induced by Gram-negative bacteria.

4.3. Future Directions

Future studies are required to further characterize the underlying pathogenic mechanisms by which CD38 regulates host defense in bacterial CRKP infection.

Although neutrophil recruitment and activation are critical in pulmonary host protection against invading pathogens, other immune cells, including macrophages, epithelial cells, NK cells, and T cells, also contribute to bacterial clearance. Macrophages are the professional phagocytic cells that play an essential role in bacterial killing. Our previous studies demonstrated that any defects in the function of alveolar macrophages results in ineffective containment of infection and ultimately impaired host defense [74]. Although we have displayed that CD38 affects neutrophil killing ability, we are not clear whether deficiency of CD38 affects the function of macrophages. Future studies are needed to determine the role of CD38 in the phagocytic uptake and bacterial killing ability of bone marrow-derived macrophages (BMDMs) from CD38 WT and CD38-deficient mice.

We and others have investigated the early events leading to bacteria-induced neutrophil recruitment to the lungs in the context of TLRs and NLRs. Of our interest is TLR4 because it has been implicated in sensing the components of *K. pneumoniae*. In the future, we will perform western blot to determine whether CD38 regulates TLR4 expression and NF-κB activation during CRKP-induced pneumonia. Furthermore, we will investigate the other possible signaling pathway downstream of TLR4, such as MAPK which is also an inflammatory-mediated signal pathway.

Additionally, if the change of TLR4 is not observed during CRKP infection, we will further examine the expression of Nod-like receptors (NLRs), another crucial family of PRRs. NLRs has been extensively studied in the context of inflammatory immune responses during both Gram-

positive and negative bacterial pneumonia. CD38 is implicated in NLRP3 inflammasome activation in coronary artery smooth muscle cells (CASMCs). Increased expression of NLRP3, cleaved caspase-3, IL-18, and IL-1β were observed in $CD38^{-/-}$ septicemia mice [55]. In addition, we detected elevated levels of IL-1β and IL-18 in the BALF of $CD38^{-/-}$ mice at 48-hrs post-infection with CRKP, suggesting the activation of NLRs in the immune responses. A more recent study has demonstrated that NOD-dependent signaling transduction can also activate transcription factors NF-κB. Furthermore, it is possible that different PRRs can interact either sequentially or synergistically to amplify maximal immune responses against bacterial infections. For example, it has been shown that both TLR5 and NLRC4 signaling pathways can be triggered by bacterial flagellin [75]. Cooperative inflammasome signaling between NLRP3 and AIM2 has also been implicated following *Plasmodium* infection [76]. If required, mice containing double KO, such as TLR5/NLRC4-double KO (T5/N4-DKO) mice, would be helpful for further study.

$CD38^{-/-}$ mice displayed increased levels of IL-17A in the BALF and lung homogenates at 48-hrs post-infection. IL-17A has been widely demonstrated to play critical roles in pulmonary host defense against invading microbial pathogens. Interestingly, recent evidence has revealed that a wide range of immune cells secrete IL-17A besides TH17 T cells, such as neutrophils, γδ T cells, and innate lymphoid cells (ILCs) [77-79]. Therefore, it is critical to examine whether pulmonary inflammation is mediated via IL-17A in $CD38^{-/-}$ mice and identify the primary source of IL-17A during CRKP-induced pneumonia. So, it will be interesting to administer mouse anti-IL-17A antibody 24 h prior to CRKP infection to investigate whether pulmonary inflammation is mediated via IL-17A in $CD38^{-/-}$ mice. To further identify the primary source of IL-17A, we can proceed toward the intracellular Flow cytometry with different cell marker antibodies, including γδ T cell,

neutrophil, and NK/NKT cell markers. Then the number and percentage of IL-17A positive cells in the lungs of CRKP infected WT and $CD38^{-/-}$ mice will be analyzed.

In the current study, chimera mice were created by using bone marrow transplantation to determine the role of CD38 in myeloid versus stromal cells in host defense following CRKP infection. Comprehensive studies using conditional knockout mice would be useful to delineate the role of CD38 in different cells and tissues in a conclusive manner. We have successfully generated CD38-floxed mice in our lab. Myeloid cell-specific $CD38^{-/-}$ mice would be generated by crossing CD38-floxed mice with LysM-Cre mice [80]. The findings would improve our understanding of the role of CD38 in pulmonary immunity and guide therapeutic strategies to target specific cells (hematopoietic or non-hematopoietic cells) during CRKP-induced pneumonia.

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