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*University of Nebraska Medical Center*

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**Inflammatory Monocytes in Post-Influenza Methicillin-Resistant  
*Staphylococcus Aureus* Pneumonia**

By

**Karl Jacob Fischer**

A THESIS

Presented to the Faculty of  
The University of Nebraska Graduate College  
In Partial Fulfillment of the Requirements  
For the Degree of Master of Science

Pathology & Microbiology  
Graduate Program

Under the Supervision of Professor Keer Sun

University of Nebraska Medical Center  
Omaha, Nebraska

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career. I cannot wait to see where life takes us and to start our lives together. I love you and dedicate this thesis to you.

# **Inflammatory Monocytes in Post-Influenza Methicillin-Resistant *Staphylococcus Aureus* Pneumonia**

Karl Jacob Fischer, M.S.

University of Nebraska Medical Center, 2016

Advisor: Keer Sun, PhD

## **ABSTRACT**

Secondary bacterial infections following influenza can lead to poor clinical outcomes and mortality. It is widely accepted that susceptibility to secondary bacterial infections is attributable to a suppressed innate antibacterial immunity. In contrast, a dysregulated host inflammatory response may also contribute to disease severity. Inflammation induced by viral infection alone significantly affects lung pathology, potentially exacerbating the destruction of the respiratory tract. Interestingly, the role of inflammatory mediators such as inflammatory monocytes have been extensively studied during influenza infection alone; however, their role during secondary bacterial infection are still not fully established. The objective of this study was to analyze the contribution of inflammatory monocytes during secondary *S. aureus* infection and their effect on lung pathology.

Based on the negative impact of inflammatory monocytes during influenza infection alone, and their little recruitment during *S. aureus* infection alone, we hypothesized that inflammatory monocytes contribute to increased mortality and lung pathology during secondary MRSA pneumonia. In order to study the possible effects of inflammatory monocytes, we developed post-influenza MRSA pneumonia murine models, with and without antibiotic treatment, using mice deficient in the chemokine receptor, C-C chemokine receptor type 2 (CCR2). Interestingly, we found that CCR2-deficient (CCR2<sup>-/-</sup>) mice, which are unable to

sufficiently recruit inflammatory monocytes to the airways, survive significantly better compared to WT mice after post-influenza MRSA infection. Furthermore, we show, mechanistically, that inflammatory monocytes may impair the phagocytic bacterial killing function of alveolar macrophages, leading to decreased bacterial clearance and increased mortality. Future studies will evaluate the effect of inflammatory monocytes on lung damage during post-influenza MRSA pneumonia.

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## **LIST OF ABBREVIATIONS**

ssRNA	single-stranded ribonucleic acid
dsRNA	double-stranded ribonucleic acid
HA	hemagglutinin
NA	neuraminidase
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
PAMPs	pathogen associated molecular patterns
PRRs	pattern recognition receptors
TLRs	toll-like receptors
NLRP3	NOD, LRR, and PYD domains-containing protein 3
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
LRR	leucine rich repeat
PYD	pyrin domain
RIG-1	retinoic acid-inducible gene 1
DC	dendritic cell
aDC	alveolar dendritic cell
iDC	interstitial dendritic cell
pDC	plasmacytoid dendritic cell
tipDC	TNF $\alpha$ /iNOS-producing dendritic cell
NK	natural killer cell
TRIF	TIR-domain-containing adaptor-inducing interferon $\beta$
NF- $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells

ISGs	interferon stimulating genes
IFN	interferon
IAV	influenza A virus
MyD88	myeloid differentiation primary response gene 88
IRF	interferon regulatory factor
ASC	apoptosis-associated speck-like protein containing a CARD
CARD	caspase recruitment domain
CCL	chemokine (C-C motif) ligand (e.g. CCL2)
RANTES	regulated on activation, normal T cell expressed and secreted (CCL5)
CXCL	chemokine (C-X-C motif) ligand (e.g. CXCL5)
MIP	macrophage inflammatory protein (e.g. MIP-2)
MCP	monocyte chemoattractant protein (e.g. MCP-1)
KC	keratinocyte-derived chemokine (CXCL1)
APC	antigen presenting cell
TNF $\alpha$	tumor necrosis factor alpha
IL	interleukin (e.g. IL-2)
NCR	natural cytotoxicity receptor
NETs	neutrophil extracellular traps
HGF	hepatocyte growth factor
MHC	major histocompatibility complex
iNOS	inducible nitric oxide synthase
MAVS	mitochondrial antiviral-signaling protein
CTL	cytotoxic T lymphocyte
NADPH	nicotinamide adenine dinucleotide phosphate
PFU	plaque forming unit
CFU	colony forming unit

PBS	phosphate-buffered saline
BALF	bronchoalveolar lavage fluid
ELISA	enzyme-linked immunosorbent assay
BCA	bicinchoninic acid
LDH	lactate dehydrogenase
mAb	monoclonal antibody

## **CHAPTER 1: INTRODUCTION**

Throughout the past century, influenza virus has been closely associated with morbidity. It is also known that acquiring influenza respiratory infection can lead to increased susceptibility to secondary bacterial infection. Recent studies have shed new light on an adaptive antiviral immune response impairing innate immune defenses against secondary bacterial infections following influenza [1]. This review aims to highlight past pandemics, innate and adaptive immune responses to influenza virus infection, influenza-suppressed innate antibacterial immunity, and secondary bacterial infections. Better analyses of these mechanisms, may reveal approaches to manage secondary bacterial infection disease progression.

### ***Clinical Scenario of Influenza Pandemics***

The 1918 “Spanish flu” pandemic claimed more than 50 million lives worldwide. There is overwhelmingly convincing data that a majority of the deaths were caused by secondary bacterial infections [2-7]. A recent study conducted on 58 autopsies and 8398 postmortem examinations from 1918 patients, concluded that influenza A virus with bacterial infection led to lethal outcomes in the vast majority of these cases [6]. The most prominent pathogens that were recovered from patients with secondary bacterial infections were *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* with *S. pneumonia* being the most prevalent [2]. It was also unique in the 1918 pandemic that the largest age demographic most severely affected by these infections were young adults without previous medical conditions [8].

Similarly, the majority of deaths in the 1957-58 “Asian flu” pandemic showed evidence of secondary bacterial infection. However, in this pandemic, *Staphylococcus aureus* emerged as a leading cause of secondary bacterial pneumonia [9]. Upon autopsy of these patients, severe pulmonary edema and hemorrhage were found with death resulting in seven days or fewer [10],

and bacterial pneumonia accounted for about 44% of deaths despite the availability of antibiotics [10].

The H1N1 influenza virus pandemic in 2009, intensively focused researchers on how to prevent, respond, and treat the next influenza pandemic [1, 2]. This influenza pandemic, the first in roughly 40 years, consisted of a novel, triple-reassortment influenza A (H1N1) virus infection originating in North America then spreading world-wide [11]. In approximately 25%-50% of severe or fatal cases, evidence of secondary bacterial infection was found with *S. pneumoniae* and *S. aureus* being the most frequent bacterial pathogens [10, 12].



### ***Innate Immune Recognition of Influenza Virus Infection***

The influenza virus is an enveloped virus that contains a genome of seven to eight pieces of negative-sense single-stranded RNA (ssRNA). The major viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) coat the envelope, and can be detected by antibodies [13]. HA is involved in viral attachment to host cells and NA has a role in viral penetration into the host cells [14]. There are 18 HA proteins and 11 NA proteins, in total, that define the subtype of the virus. However, there are typically only 3 HA and 2 NA proteins that are associated with human disease [14]. The innate immune system is able to detect viral infections through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Specifically, influenza virus is recognized through at least three classes of toll-like receptors (TLRs), the NOD-like receptor family member NOD-, LRR- and pyrin domain-containing 3 (NLRP3), and retinoic acid-inducible gene I (RIG-1) [13].

Multiple cell types express TLRs such as macrophages, dendritic cells (DC), natural killer (NK) cells, T and B cells, and even non immune cells like epithelial cells [13]. TLR3 is constitutively expressed by human epithelial cells and recognizes double-stranded RNA (dsRNA) in endosomes [13]. Due to the activity of RNA helicase UAP56, influenza virally-infected cells do not generate dsRNA [13, 15]. So, it is likely that TLR3 recognizes currently unidentified RNA structures that are present in dying influenza virally-infected cells that have been phagocytized [13, 16]. TLR3 signaling occurs through TIR-domain containing adapter-inducing interferon-B (TRIF), which leads to the expression of nuclear factor- $\kappa$ B (NF- $\kappa$ B)-dependent pro-inflammatory cytokines [13]. Signaling through TRIF also leads to the production of type I interferons (IFNs) and IFN-stimulated genes (ISGs) downstream of IFN-regulatory factor 3 (IRF3) [13]. Due to the production of pro-inflammatory cytokines mediated by TLR3 sensing, it was found that this may also cause damage to the host [13, 17, 18]. Following lethal influenza virus infection, TLR3<sup>-/-</sup> mice show prolonged survival compared to wild-type mice controls despite containing higher

viral loads in the lungs [18]. Also, TLR3<sup>-/-</sup> mice had decreased CD8<sup>+</sup> T lymphocyte infiltration leading to reduced lung injury [18]. So, interestingly, although TLR3 recognition is needed to induce signals that are able to restrict viral replication, it also induces signals that recruit innate and adaptive immune effectors that cause pathological damage to the host [13, 18].

TLR7 and TLR8 recognize single-stranded RNA (ssRNA) molecules that have been taken up into the endosomes of innate immune cells such macrophages and plasmacytoid DCs (pDC) [19]. Downstream signaling through the adaptor, myeloid differentiation factor 88 (MyD88), also leads to the expression NF- $\kappa$ B-dependent pro-inflammatory cytokines [13]. Along with signaling through MyD88, it can also occur through IRF7 which leads to the production of type I IFNs and ISGs [13]. In one study with mice deficient in TRIF and MyD88 or MyD88 alone, that were challenged with high doses of influenza virus, the MyD88<sup>-/-</sup> mice were more susceptible to influenza virus infection and displayed increased mortality [20]. Another study utilizing a high-dose challenge model of influenza virus did not find a necessity for TLR7 or MyD88 [18]. Contrasting the high-dose challenge model of influenza virus, mice infected with sublethal doses of influenza virus elicited a robust antibody response to the virus mediated through TLR7 signaling, but not T cell responses [13, 21, 22]. It was found that mice deficient in MyD88 had increased levels of IgG1 and Th2 cytokines, but lower levels of Th1 cytokines [20]. It is then possible that the Th2-skewed environment typical of mice deficient in MyD88 could be involved in the induction of normal humoral immune responses despite minimal levels of CD4<sup>+</sup> T cell responses [20]. This shows a unique antiviral role for TLR7 signaling, by inducing B cells to elicit appropriate antibody production [13, 22]. Human monocytes and macrophages express TLR8, and through the stimulation of its ligand, ssRNA, results in the production of the cytokine IL-12 [13, 23]. However, this does not result in the production of the type I interferon- $\alpha$  (IFN $\alpha$ ) [23]. The exact roles of TLR8 in influenza virus infection have yet to be clearly defined [13].

RIG-1 detects 5'-triphosphate RNA generated during viral replication [13]. Once RIG-1 is activated, it binds to its adaptor, mitochondrial antiviral signaling protein (MAVS), which initiates NF- $\kappa$ B and IRF3 signaling that leads to pro-inflammatory cytokine production and type I IFN production, respectively [13, 24]. RIG-1 is necessary for viral detection and for type I IFN production in infected epithelial cells, alveolar macrophages, and conventional DCs [25]. Indeed, mice deficient with RIG-1 are highly susceptible to influenza A virus (IAV) infection [26].

The NLRP3 inflammasome recognizes an array of stimuli and its formation is generally triggered by host cell membrane damage, infection, or stress [27]. This multiprotein inflammasome complex consists of an NLRP, the adaptor associated speck-like protein containing CARD (ASC), and pro-caspase I [13]. Once activated, this results in the autocatalytic processing of pro-caspase I into its active form, which goes on to cleave pro-IL-1 $\beta$  and pro-IL-18 into IL-1 $\beta$  and IL-18, respectively, leading to their secretion [13]. NLRP3 is expressed by multiple myeloid cell types including monocytes, macrophages, DC, and neutrophils [28]. The importance of NLRP3 inflammasomes during influenza virus infection involves an increase in disease tolerance and not antiviral resistance [13]. In one study using a high-dose viral challenge with the A/PR8 strain of influenza A virus, it was found that NLRP3 was crucial for the recruitment of leukocytes into the lungs [29]. Also, mice deficient in NLRP3, caspase I, or IL-1R suffer from reduced respiratory function and increased pulmonary necrosis leading to increased mortality [13, 29, 30]. Unlike type I IFNs, IL-1 $\beta$  and IL-18 do not induce direct antiviral resistance, which indicates that the susceptibility to influenza virus infection in mice that are deficient in NLRP3 is independent of an inability to control viral replication [13, 30].

This initial ability of the innate immune system to detect influenza virus infection and virally-infected cells is clearly needed to elicit the proper immune response. This insures proper recruitment and activation of innate and adaptive immune cells and the production of pro-

inflammatory cytokines. Furthermore, this will produce the proper antiviral state which will help the host combat viral replication and viral-induced inflammation.

### ***The Role of Innate Immune Cells to Influenza Virus Infection***

In humans and mice, the greatest susceptibility to secondary bacterial infection is at day seven following influenza infection [1]. Clinically, this occurs at a time when the virus is being cleared from the airways through an elicited adaptive immune response with T cell recruitment and the patient is entering the recovery stage [1]. The restoration of lung immune homeostasis, characterized by a general anti-inflammatory state with increased IL-10 production, may lead to multiple mechanisms involved in the suppression of pathogen recognition and clearance [10, 31, 32]. Influenza virus is highly associated with pneumonia and acute respiratory disease, and until recent times, it was widely thought that influenza-induced airway damage was the main mechanism for bacterial adhesion in order to establish a secondary bacterial infection [3, 33]. Influenza infection alone leads to epithelial damage in the airways, along with decreased ciliary beat frequency, decreased oxygen exchange, etc. which all limit pulmonary function and can facilitate secondary bacterial infection [10, 34]. Influenza neuraminidase is able to cleave epithelial sialic acids, which exposes cryptic receptors on host cells and disrupts sialylated mucins that function as decoy receptors enabling bacterial adhesion [35]. Despite influenza-induced lung damage occurring at a time which is greatest for bacterial susceptibility, viral strains that cause minimal epithelial damage still enhance susceptibility to bacteria in mice [1, 36, 37]. To complicate the matter further, in the 1918 pandemic, virus attack rates (defined as susceptible individuals exposed to a particular pathogen who become infected) showed no correlation to human mortality, so even with viral-mediated lung damage, it alone isn't sufficient to induce secondary bacterial pneumonia [1, 8]. Therefore, it is possible that different cell types in lung involved in the innate immune response to influenza virus infection may have an active role in

enhancing susceptibility and progression of secondary bacterial infections. Some of these cells and their roles during influenza virus infection alone will be reviewed below.

### ***Alveolar Macrophages***

As the first line of defense against invading respiratory pathogens in the airways during steady-state conditions, alveolar macrophages play an important role in controlling influenza virus infection. Along with recruiting other cells such as inflammatory monocytes and “exudate” macrophages to the site of infection, the phagocytic activity of these cells is important to help clear virally-infected cells [13]. Once activated, alveolar macrophages become highly phagocytic and virally-infected macrophages are potent producers of cytokines and chemokines that contribute to lung pathology [38, 39]. This cytokine/chemokine secretion mechanism can further stimulate an inflammatory response and recruit adaptive immune cells.

Macrophages along with monocytes are highly susceptible to influenza A virus infection [40]. Following influenza virus infection, macrophages and monocytes die by apoptosis within 24-48 hours [40, 41]. However, despite the onset of early apoptosis, as described earlier, alveolar macrophages and monocytes will significantly contribute to the lung cytokine/chemokine environment and influenza virus-associated immunopathology [40]. These infected macrophages may also release the pro-inflammatory cytokines: TNF $\alpha$ , IL-1 $\beta$ , IL-6, and type I IFNs [40, 42]. Furthermore, these pro-inflammatory cytokines are known to promote the upregulation of CCL2, CCL7, and CXCL10 [40]. Importantly, there is a strong correlation between higher TNF- $\alpha$  production following H3N2 influenza virus infection leading to increased mortality in elderly patients. [40, 43, 44]. Thus, there is a delicate balance in mediating lung homeostasis following influenza virus infection with the robust production of cytokines/chemokines. On one hand, alveolar macrophages produce cytokines/chemokines to increase lung inflammation in order to

recruit other innate immune phagocytes and adaptive immune cells. However, this also results in more pathologic damage in the airways further contributing to disease severity and mortality.

### *Dendritic Cells*

Dendritic cells play an important role during influenza virus infection by bridging the innate and adaptive immune responses. Previously, these cells were thought to be a homogenous population; however, it is now known that lung-resident DC are a heterogeneous population [45]. In the naïve lung, the predominant DC are airway and alveolar DC (aDC), characterized as being CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> [46]. Interstitial DC (iDC) are characterized as being CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>hi</sup>CD4<sup>-</sup>CD8<sup>-</sup> [46]. Following pulmonary infection additional subsets of DC are also recruited into the lungs such as inflammatory monocyte-derived DC, plasmacytoid DC (pDC), and CD8 $\alpha$ <sup>+</sup> DC [13].

Dendritic cells are able to recognize influenza virus infection through various PRRs. Upon activation in the lung, they are able to process viral peptides and display them to IAV-specific T cells, which initiates an adaptive immune response [45]. Another major role that dendritic cells play is, once activated, they are able to release multiple pro-inflammatory cytokines and chemokines to help limit viral spread and replication, including: RANTES, IL-6, IL-8, IL-12, MIP-1 $\beta$ , TNF $\alpha$ , and type I IFNs [45]. Importantly, type I IFNs are able to enhance rDC maturation and antigen cross-priming by CD8 $\alpha$ <sup>+</sup> DC [45]. Along with other innate immune cells this robust pro-inflammatory response can enhance disease severity [45].

### ***Neutrophils***

Neutrophils are an abundant population of granulocytes known for their phagocytic capability. They are a vital component of the innate immune response to primary influenza infection and recent studies have shown that neutrophils help with viral clearance [47]. Despite the possible role of aiding in viral clearance, many studies have focused on other important innate immune cells in response to influenza virus infection alone.

Neutrophils are also known for the prominent role during bacterial infections. It has been shown that neutrophils significantly contribute to the reduced susceptibility to secondary bacterial infections early after influenza virus infection (around day three following influenza infection [48, 49]). It has been suggested that enhanced neutrophil recruitment during secondary bacterial infections, at day six or seven following influenza virus infection, may contribute to lung pathology increasing disease severity, possibly through the formations of neutrophil extracellular traps (NETs) [48, 50]. Despite this, neutrophils are a necessary component in controlling secondary bacterial infections, but their role in susceptibility to secondary bacterial infections following influenza virus infection at later time points (day six and seven) remains unclear.

### ***Natural Killer Cells***

Natural killer (NK) cells are innate lymphocytes that play an important role in innate immunity against many pathogens including bacteria, intracellular parasites and viruses. In the first few days of influenza infection, NK cells are recruited to the lungs [51]. Along with DC, NK cells are required for activation of the cytotoxic T lymphocyte (CTL) response [51]. Once activated, NK cells can directly target and kill virus-infected cells through the use of cytolytic granules and are potent producers of IFN $\gamma$  [51]. However, their role in controlling IAV infection remains largely undefined.

NK cells can be activated through the Fc receptor CD16 and NKG2D. They also utilize the natural cytotoxicity receptors (NCR) NKp46, in humans, and NCR-1, in mice and NKp44. [45, 51]. These receptors are able to recognize influenza hemagglutinins on virally-infected cells. [51]. Recently, a study has shown that mice deficient in NCR-1 exhibit increased mortality and morbidity following influenza infection [52]. This implicates an important role for NKp46 expression on NK cells to recognize and control viral infection [52]. Furthermore, studies depleting NK cells prior to and during influenza infection renders mice more susceptible to mortality. [51, 53, 54]. Mechanistically, studies need to be performed to evaluate whether it is due to the direct involvement of NK cells toward virally-infected cells or indirectly through a diminished involvement with other immune cell types such as DC and adaptive T-cell responses [45].

### ***Inflammatory Monocytes***

Inflammation is a major contributor to pulmonary immune pathology. Highly pathogenic influenza strains are known to induce severe cytokine-mediated immune pathology resulting in high mortality rates [55]. Neutrophils, NK cells, dendritic cells, and inflammatory monocytes are heavily recruited to the airways during influenza infection [13]. They are thought to be trafficked to the site of inflammation through recognition of the chemokine (C-C motif) ligand 2 (CCL2), also called monocyte chemoattractant protein-1 (MCP-1) through the receptor, C-C chemokine receptor type 2 (CCR2) [56]. Alveolar macrophages are thought to be the predominant cell type in the recruitment these monocytes. However, recently, it has been suggested that much of the recruitment of inflammatory monocytes results from alveolar epithelial cells that produce high levels of CCL2 following infection [45].

In mice, the current understanding of monocytes is that there are two major subsets: “classic” inflammatory monocytes which express high levels of CCR2 and Ly6C (in humans, CD14<sup>hi</sup>CD16<sup>-</sup>) and monocytes that express low levels of CCR2 and Ly6C [56, 57]. Ly6C<sup>low</sup>



monocytes have been shown to patrol the vasculature to clear damaged endothelial cells and participate in tissue remodeling at the latter stages of inflammation [57]. In contrast, Ly6C<sup>hi</sup> monocytes are recruited to sites of inflammation [57]. Monocyte accumulation at the site of inflammation has been shown to enhance the immunopathology of influenza infection [58]. This is partially due to these cells producing pro-inflammatory cytokines, chemokines, and stimulating the expression of inducible nitric oxide synthase [58]. Along with pro-inflammatory cytokine production, recruited monocytes ingest apoptotic leukocytes poorly, thus further contributing to lung inflammation and pathology [59].

The impact that monocytes have on the course of influenza infection has been well studied. During influenza infection, mice deficient in CCR2 are unable to sufficiently recruit monocytes to infected airways. Under a low-dose of influenza infection, Wareing *et al.*, report that mice deficient in CCR2 did not result in higher viral titers and morbidity compared to WT mice [60]. Dawson *et al.*, report similar findings that despite of their higher viral loads, the survival of CCR2<sup>-/-</sup> mice was comparable to WT controls after influenza infection [61]. Multiple studies show that mice deficient in CCR2 have less lung pathology due to the reduction of monocyte/macrophage accumulation and pro-inflammatory cytokine production despite increased numbers of neutrophils [60-63]. CCR2 deficiency also results in increased CCL2 and CXCL10 in the airways of influenza-infected mice [60, 61]. In contrast, treatment of influenza-infected WT mice with anti-CCL2 antibody actually promotes lung injury while treatment with CCL2 reduces overall lung injury, due to macrophage production of hepatocyte growth factor (HGF) [58].

Under higher influenza doses, CCR2<sup>-/-</sup> mice were reported to have an even greater reduction in lung pathology and mortality [63]. Gunn et al, pinpoint CCR2<sup>+</sup> monocytes as a predominant cause of inflammation during influenza infection [63]. [1].

### ***Adaptive Immune Response to Influenza Virus Infection***

An adaptive immune response to influenza virus infection is necessary to clear the virus. The pro-inflammatory cytokine environment produced by the innate immune system favors the development of Th1-type immune response [42]. Naturally, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells carry out important function to aid in this type of cellular response.

#### ***B and T cells***

Influenza virus infection induces the production of virus-specific antibodies particularly directed against the HA and NA viral proteins. Binding to the HA receptor can block viral attachment to host cells and can inhibit receptor-mediated endocytosis [64]. Importantly, most antibodies directed against HA are influenza-virus strain specific and fail to neutralize other subtypes of influenza viruses [65]. Antibodies generated against NA do not neutralize the virus like those generated against HA [14, 66]. NA-specific antibodies are able to slow viral spread and may limit the duration of influenza virus infection [66].

Activation of naïve CD4<sup>+</sup> T cells, through recognition of viral epitopes associated with MHC class II and interaction with co-stimulatory molecules on APCs, can result in the differentiation into CD4<sup>+</sup> T helper 1 cells (Th1) or CD4<sup>+</sup> T helper 2 cells (Th2) based on the expression of cytokines [14, 67]. Influenza-specific Th1 CD4<sup>+</sup> T cells are the predominant adaptive immune cell found during the time of viral clearance and are known to secrete high levels of IFN $\gamma$  and IL-2 [46]. Th1 cells are essential for the induction of memory CD8<sup>+</sup> T cells and play an important role promoting CTL responses [46]. In addition, they are extremely important in the activation and recruitment of macrophages into the area of inflammation in a Type IV hypersensitivity response to tissue damage [68]. Although efficient viral clearance is not solely dependent on CD4<sup>+</sup> T cells (in the murine model), the adoptive transfer of influenza-

specific Th1 CD4<sup>+</sup> T cells clones in BALB/C mice following influenza infection was shown to protect the host and decrease immunopathology in the lungs [40].

Th2 cells are known to produce IL-4, IL-5, IL-10, and IL-13. These cells aid in the activation of B cells leading to antibody production [40, 46]. In contrast to the adoptive transfer of influenza-specific Th1 CD4<sup>+</sup> T cell clones, adoptive transfer of influenza-specific Th2 CD4<sup>+</sup> T cell clones was associated with delayed viral clearance failing to show protection against influenza virus infection [69].

Adaptive immunity is essential to viral clearance. The delay in macrophage recruitment, in turn, impairs trafficking of CD4<sup>+</sup> T cells to the lungs [60, 61]. CCR2 deficiency in mice has also been shown to promote the development of Th1-type immune responses in various infection models [70-72]. The complete diminishment of CCR2-monocyte derived cells, such as TNF $\alpha$ /inducible nitric oxide synthase (iNOS) producing DCs or tipDCs, has been shown to be detrimental to survival of influenza infection due to their ability to recruit CD8<sup>+</sup> T cells [73]. This is important because CD8<sup>+</sup> T cells have a vital role in clearance of influenza [73].

Activation of naïve CD8<sup>+</sup> T cells occurs through recognition of viral epitopes associated with MHC class I molecules on APCs in the draining lymph nodes (DLN) [46]. They are then able to migrate to the site of infection and lyse virally-infected cells, through the release of perforin and granzymes [46]. These cells also produce pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$  [40, 46]. Much of our understanding of CD8<sup>+</sup> T cell responses come from mouse models; however, few studies have investigated the role of this adaptive immune cell population in humans in response to highly pathogenic influenza [46].

## ***Post-Influenza Bacterial Infections***

### ***Immunity and Susceptibility to Secondary Bacterial Infections***

The most frequent bacteria associated with secondary bacterial infections following influenza are *Streptococcus pneumoniae* and *Staphylococcus aureus*, including methicillin-resistant strains. The innate immune system is able to recognize these bacteria through pathogen-associated molecular patterns (PAMPs) on the bacterial surface that interact with the pathogen recognition receptors (PRRs) on innate immune cells to initiate a pro-inflammatory response. Although monocytes and adaptive immune cells are recruited to the site at the later stage of infection, other phagocytes such as alveolar macrophages and neutrophils are critical to clearing bacteria and controlling bacterial outgrowth. Since it appears that the greatest susceptibility to secondary bacterial infection occurs approximately seven days following influenza virus infection, multiple studies have examined the viral-bacterial synergy by infecting mice with bacteria at this time point. Recently, it has been demonstrated that a dysfunctional innate immune response induced by influenza that includes the suppression of phagocytic activity and an enhanced pro-inflammatory response can potentially lead to secondary bacterial infection (ref).

All three IFN types, i.e.,..., contribute to innate and adaptive immune responses to influenza virus infection [13]. However, their activities appear to increase the susceptibility of the host to secondary bacterial infection [48]. For instance, it has been shown that type I IFN signaling can inhibit IL-17-mediated neutrophil recruitment by perhaps targeting  $\gamma\delta$ T cells [1, 74, 75]. Furthermore, mice deficient in the type I IFN receptor, IFNAR1, were more resistant to *S. pneumoniae* infection at day 7 following influenza infection and demonstrated increased neutrophil recruitment with improved pneumococcal clearance [48, 76]. Type I IFNs have also been found to inhibit the normal phagocytic activity of macrophages and neutrophils [76]. The type II IFN, IFN- $\gamma$ , has been shown to inhibit the phagocytic capability of alveolar macrophages

following influenza infection. For example, a recent study has implicated IFN- $\gamma$  in inhibiting alveolar macrophage phagocytosis of *S. pneumoniae* [77]. Similarly, another study reported decreased phagocytosis of *S. aureus* by alveolar macrophages due to the role of IFN- $\gamma$  [78]. Traditionally, low levels of IFN- $\gamma$  are thought to enhance intracellular killing of bacteria [48]. However, higher levels of IFN- $\gamma$  have been shown to downregulate the expression of scavenger receptors necessary to uptake bacteria, such as MARCO and the mannose receptor, used by alveolar macrophages [77].

In addition, it has been shown that the suppression of NADPH oxidase-dependent phagocytic bacterial clearance by influenza leads to increased susceptibility to secondary methicillin-resistant *Staphylococcus aureus* (MRSA) infection [79].

### ***Immunomodulation of Secondary Bacterial Infections***

Characteristic of secondary bacterial infection is widespread bronchopneumonia with infiltration of neutrophils in the bronchioli, with neutrophils filling the airspaces of surrounding alveoli, and fibrin deposition in airway spaces [6]. In severe to fatal cases of the 1918 pandemic, capillary thrombosis, necrotic areas of bronchiolar damage, and vasculitis all occurred following secondary bacterial invasion [6]. Clinical management of excessive inflammatory responses following the establishment of a secondary bacterial infection comprises yet another difficulty in ensuring positive outcomes in patients.

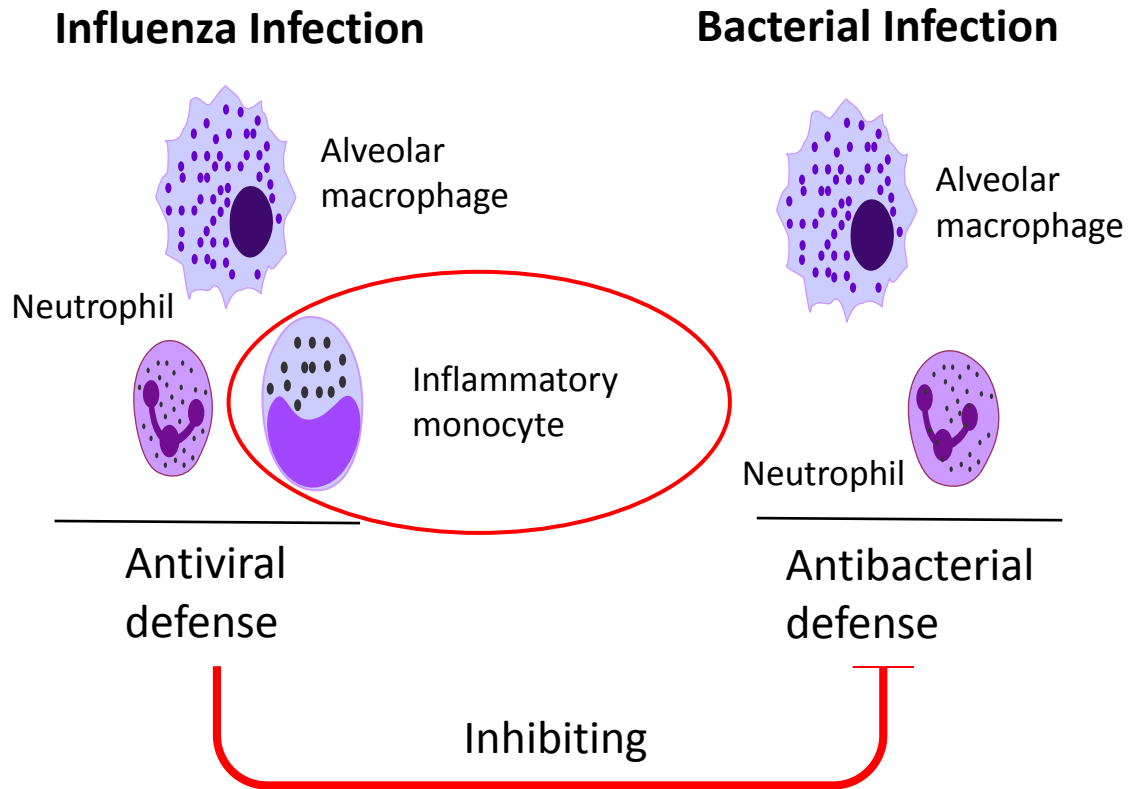
Even with effective antibiotics, significant mortality is observed in patients with severe pneumonia [80]. The host inflammatory response is needed to control bacterial infections; however, excessive inflammation leads to increased pathology and death [81]. Alternative strategies, besides antibiotics and antivirals, used to treat post-influenza bacterial pneumonia are gaining more attention and this includes immunomodulation [81]. During secondary bacterial pneumonia following influenza, large levels of pro-inflammatory cytokines are produced [81].

However, neutralizing or inhibiting particular cytokines lead to increased disease and mortality in mice [81]. Other immunomodulation treatment strategies such as the use of steroids to combat the intense inflammation following secondary bacterial infection have not produced consistent results [81].

As described earlier, inflammatory monocytes are recruited to the airways during influenza infection but little during bacterial infection alone. They have been demonstrated to contribute to influenza virus infection disease severity. Thus, it would seem that this cell population might have a negative impact during secondary bacterial pneumonia. This could be due to their function in contributing to an exacerbated inflammatory response or their interaction with other innate immune cells [Figure 1]. Analyzing their role during secondary bacterial infection post-influenza is necessary to further establish immunopathogenesis.

### ***Inflammatory Monocytes During Secondary MRSA Pneumonia***

Highly pathogenic and seasonal influenza virus continue to pose a serious public health threat. Just within the past century, four influenza pandemics have claimed the lives of millions of people throughout the world. Influenza infection leads to the heavy recruitment of inflammatory monocytes into the airways. Through CCR2-deficient murine models, this cell population has been shown to contribute to lung pathology during influenza infection alone. Besides influenza-induced inflammation, excessive inflammatory immune pathology is a hallmark of post-influenza bacterial infections. In the present study, we investigated the recruitment of inflammatory monocytes during post-influenza MRSA pneumonia and the impact they have on disease pathogenesis. This can hopefully shed light on new approaches to clinically manage severe diseases due to bacterial super-infection following influenza.



**Figure 1: Model proposed for the role of inflammatory monocytes during post-influenza bacterial infection.** Inflammatory monocytes during influenza infection alone and their implicated role during secondary bacterial infections.

## **CHAPTER 2: MATERIAL AND METHODS**

### ***Murine Model of Viral and Bacterial Infection***

Specific pathogen-free, 6- to 8-wk-old C57BL/6 wild-type (WT) and CCR2<sup>-/-</sup> mice were initially purchased from The Jackson Laboratory and bred at University of Nebraska Medical Center following Institutional Animal Care and Use Committee guidelines.

For viral challenge, 8- to 10-week-old-sex- and age-matched mice were anesthetized and infected intranasally with a sublethal dose (0.25LD<sub>50</sub>), i.e., 25 PFU/female and 50 PFU/male of A/PR/8 virus in 50  $\mu$ L of sterile PBS. Titers of virus stocks and viral levels in the bronchoalveolar lavage fluids (BALF) and lungs were determined by plaque assay on Madin-Darby canine kidney (MDCK) cell monolayers. Secondary methicillin-resistant *Staphylococcus aureus* challenge was performed seven days following influenza virus infection [79]. To induce bacterial pneumonia, anesthetized mice were intranasally infected with 50  $\mu$ L of PBS containing 0.025 mg to 0.5 mg (10<sup>7</sup> to 6 x 10<sup>8</sup> CFU/mouse) of ATCC MRSA strain BAA-1695 (a pvl- isolate from patient sputum). Bacterial burdens in the BALFs and lungs were measured by sacrificing mice at the indicated time points following plating serial 10-fold dilutions of each sample onto blood agar plates. The plates were then incubated at 37°C overnight and CFUs were enumerated 24 hours later.

### ***Antibiotic Treatment***

Mice were intraperitoneally (i.p.) injected with a therapeutic dose (100 mg/kg) of gentamicin at 4 hours after MRSA infection and then followed by 50 mg/kg/day [82]. Control mice received sterile PBS. Antibiotic treatments and control injections continued through indicated time points or until day 10 after MRSA infection in survival studies.



### ***Plasmid Expressing DsRed***

The plasmids pCM29 and pVT1 were transduced into BAA-1695 using  $\phi$ 11 phage [83, 84]. In order to construct the plasmid pVT1, a 5.86-kb vector backbone devoid of sGFP gene was initially PCR amplified from pCM29 using primers pCM29 forward (5'-GAATTCGTAATCATGTCATAGC-3') and pCM29 reverse (5'-AAATAATCATCCTCCTAAGGTAC-3'). The optimized DsRed insert (677 bp) was PCR amplified from plasmid pDM4 using primers DsREDOpt forward (5'-CTTAGGAGGATGATTATTTATGGATAATACAGAAGATGTTATTAAG-3') and DsREDOpt reverse (5'-TGACATGATTACGAATTCTTATAAAAACAAATGATGACGACC-3'). The two fragments were assembled using the NEBuilder high-fidelity DNA assembly cloning kit (New England BioLabs, Ipswich, MA) according to the manufacturer's instructions, and the resulting plasmid (pVT1) was electroporated into Escherichia coli ElectroTen-Blue (Stratagene). Clones were verified by colony PCR and sequencing.

### ***Bronchoalveolar Lavage (BAL) Cell Analysis***

BALFs were collected by making a longitudinal incision on the ventral side of the neck exposing the trachea and lavaging the lung twice with 0.8 mL of PBS (pH 7.4). Total leukocyte counts were determined by using a hemocytometer.

For flow cytometric analysis, BAL cells were incubated with 2.4G2 mAb against mouse Fc $\gamma$ RII/RII. The cells were then stained with allophycocyanin-conjugated anti-CD11c (BD Biosciences), BUV395-, PE-, or FITC-conjugated anti-CD11b (BD Biosciences), FITC-, PE-, or PE-Cy7-conjugated anti-Ly6G mAb (clone 1A8, BD Biosciences), PerCP-Cy5.5-conjugated anti-Ly6C mAb (BioLegend), and BV421-conjugated anti-F4/80 (BioLegend). The stained cells were analyzed on a BD FACSCanto using a BD FACSDiva and Flowjo software.

### ***Determination of Cytokines/Chemokines by Enzyme Linked Immunosorbent Assay (ELISA)***

BALFs and lung homogenates were harvested and assayed for IL-6, IL-1 $\beta$ , TNF $\alpha$ , IFN $\gamma$ , MCP-1, MIP-1 $\alpha$ , MIP-2, and KC by ELISA using commercially available kits from BD Biosciences and R&D Systems (Minneapolis, MN).

### ***Neutrophil Depletion***

Neutrophils were depleted by injecting C57BL/6 mice intraperitoneally (i.p.) with 0.1 mg using anti-Gr-1 mAb RB6-8C5 (Bio X Cell) or with rat IgG as a control immediately following MRSA infection. The efficiency of neutrophil depletion in bacterial-infected mice was confirmed by flow cytometry.

### ***Live/Dead Bacteria Immunostaining Analysis***

For cytospin analysis, BAL cells were harvested from mice 24 hours after super-challenge of day seven PR8-infected WT and CCR2<sup>-/-</sup> mice with MRSA. All mice were treated with gentamicin four hours post-secondary MRSA infection. The cells were then incubated with lysostaphin for 10 minutes at 37°C to lyse extracellular bacteria. Next, the cells were then cytocentrifuged onto microscope slides at 1000 rpm for 5 min using 100  $\mu$ L cell suspension containing  $1.0 \times 10^6$  cells/mL in PBS ( $\sim 1 \times 10^5$  cells/slide). The slides were prepared using a Cytopro Cytocentrifuge 7620 (Wescor, Inc., Logan, Utah).

To assess live/dead bacteria within alveolar macrophages, the LIVE/DEAD® BacLight™ Bacterial Viability Kit L13152 (Molecular Probes, Invitrogen, UK) was used. Stocks of SYTO 9 (green, live bacteria) and propidium iodide (red, dead bacteria) were prepared by adding 5 mL of ultrapure water to each plastic pipette containing the solid stains. A 1:1 mixture of SYTO 9 and propidium iodide was made followed by adding 50  $\mu$ L to each sample and incubated in the dark,

at room temperature for 15 minutes. The slides were washed and examined using an EVOS FL microscope with and without oil immersion (Thermo Scientific).

### ***Lung Histology Analysis***

Mice were sacrificed 6 days after secondary bacterial MRSA infection and the lungs were removed for histological analyses. Paraffin-embedded tissues were sectioned to a thickness of 5  $\mu$ m and stained with hematoxylin and eosin by standard methods. The samples were analyzed with a double blind setup.

### ***Statistical analyses***

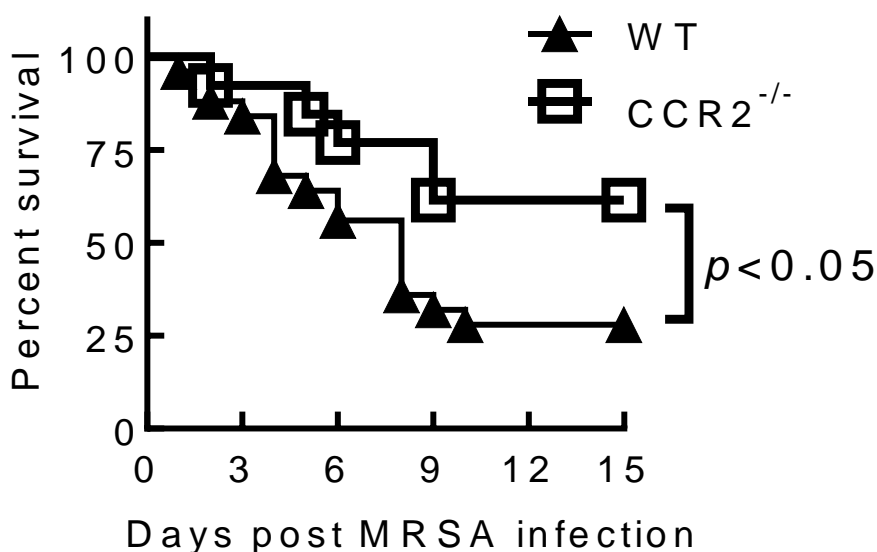
Results are expressed as means + SD. Significant differences between experimental groups were determined using a two-tailed Student *t*-test (to compare two samples), an ANOVA analysis followed by Tukey's multiple comparisons test (to compare multiple samples), or a Mann Whitney test (nonparametric test) in GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Survival analyses were performed using the log-rank test. For all analyses, a *p* value <0.05 was considered to be significant.

## CHAPTER 3: RESULTS

### *Section I – Post-Influenza MRSA Pneumonia Murine Model*

#### *CCR2<sup>-/-</sup> Mice Survive Significantly Better During Post-Influenza MRSA Pneumonia*

Inflammatory monocytes are recruited to the site of infection in a CCR2-dependent manner. It has been demonstrated in mouse models with influenza virus infection, that genetic ablation of CCR2 will significantly reduce the recruitment of inflammatory monocytes to the airways. In order to investigate the possible contribution of inflammatory monocytes to coinfection disease outcome, we first compared the survival between CCR2<sup>-/-</sup> and WT mice infected with MRSA on day seven post-PR8 infection (Figure 2). Interestingly, CCR2<sup>-/-</sup> mice survived significantly better compared to WT mice (Figure 2). Although both WT and KO mice showed early mortality around day three following secondary MRSA infection, CCR2<sup>-/-</sup> mice showed significantly increased survival rates.

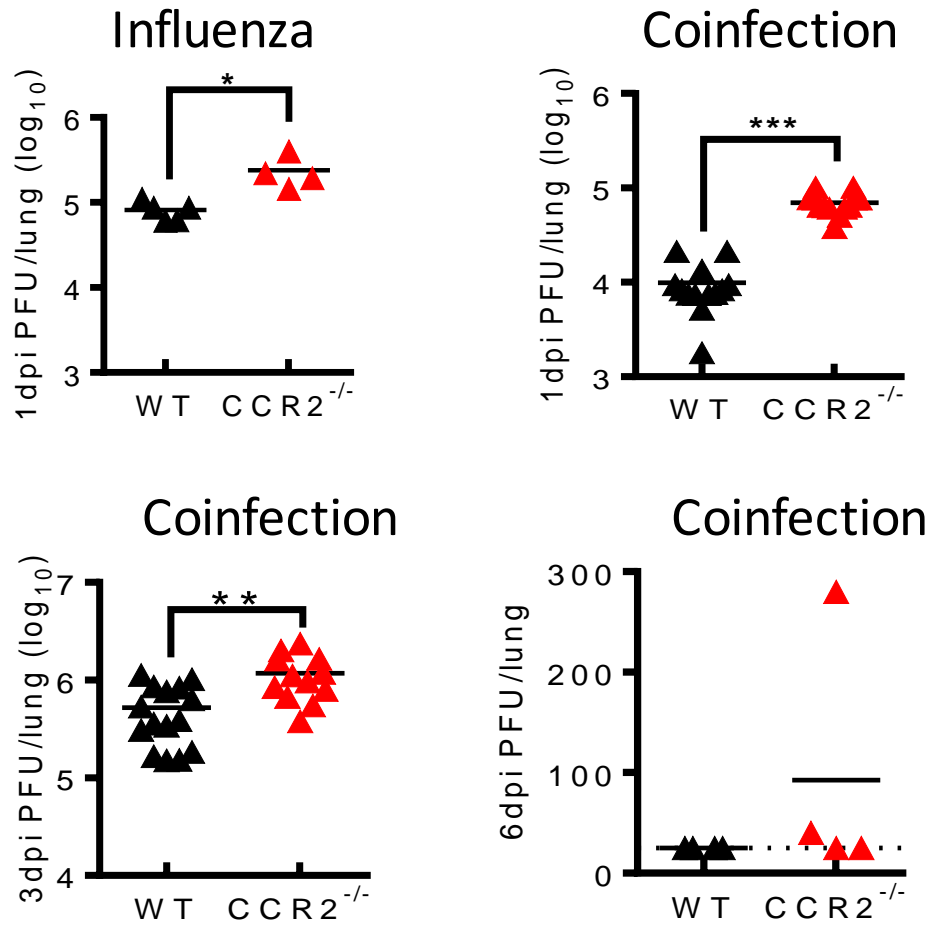


**Figure 2. CCR2<sup>-/-</sup> mice survive significantly better than WT mice following post-influenza MRSA infection.** Survival of C57BL/6 WT, and CCR2<sup>-/-</sup> mice super-challenged with MRSA on day seven after PR8 virus infection. Survival analyses were performed using the log-rank test.

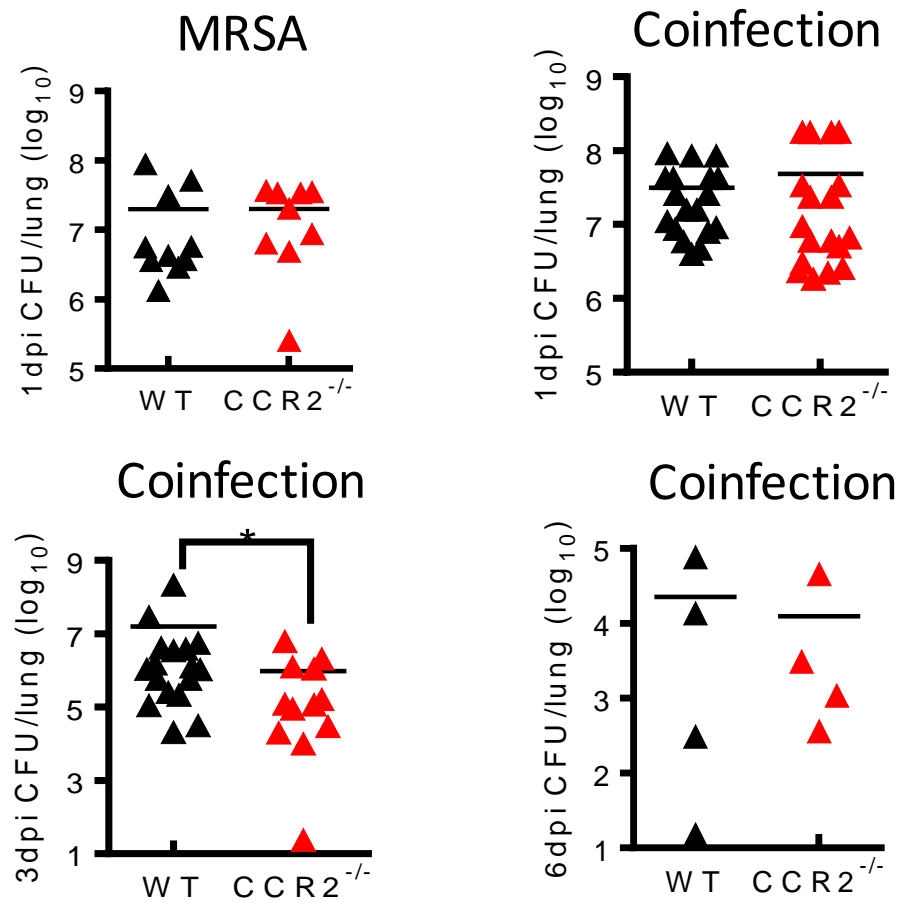
***CCR2<sup>-/-</sup> Mice Exhibit Increased Viral Titers and Decreased Bacterial Titers During Post-Influenza MRSA Infection***

In order to determine the mechanisms for the increased survival observed in CCR2<sup>-/-</sup> mice, we analyzed the viral and bacterial burdens following coinfection. Specifically, we examined the viral and bacterial burdens at days one, three, and six following secondary MRSA infection. It was found that CCR2<sup>-/-</sup> mice had higher viral titers in the lungs compared to WT mice during the course of secondary MRSA infection (Figure 3). On day one after secondary MRSA infection, coinfecting CCR2<sup>-/-</sup> mice had similar viral titers compared to CCR2<sup>-/-</sup> mice infected with PR8 alone (Figure 3). However, compared to CCR2<sup>-/-</sup> mice, WT mice exhibited better clearance of the virus during the course of coinfection (Figure 3). Nonetheless, both CCR2<sup>-/-</sup> and WT mice exhibited effective viral control around day six after secondary MRSA infection.

Next, we assessed the bacterial burdens following secondary MRSA infection. At day one after MRSA super-infection, both CCR2<sup>-/-</sup> and WT mice showed similar bacterial loads compared to mice infected with MRSA only (Figure 4). However, at days three and six, coinfecting CCR2<sup>-/-</sup> mice showed significantly decreased burdens compared with WT controls. In conclusion, we found that despite delayed viral clearance in the CCR2<sup>-/-</sup> mice, these mice exhibited improved bacterial control after coinfection, which is consistent with their increased survival compared with WT controls.



**Figure 3. CCR2<sup>-/-</sup> mice display decreased viral clearance during coinfection.** Lung viral titers in WT and CCR2<sup>-/-</sup> mice following MRSA super-challenge on day seven after PR8 virus infection. All mice were harvested 24 hours following coinfection. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , unpaired  $t$ -test.



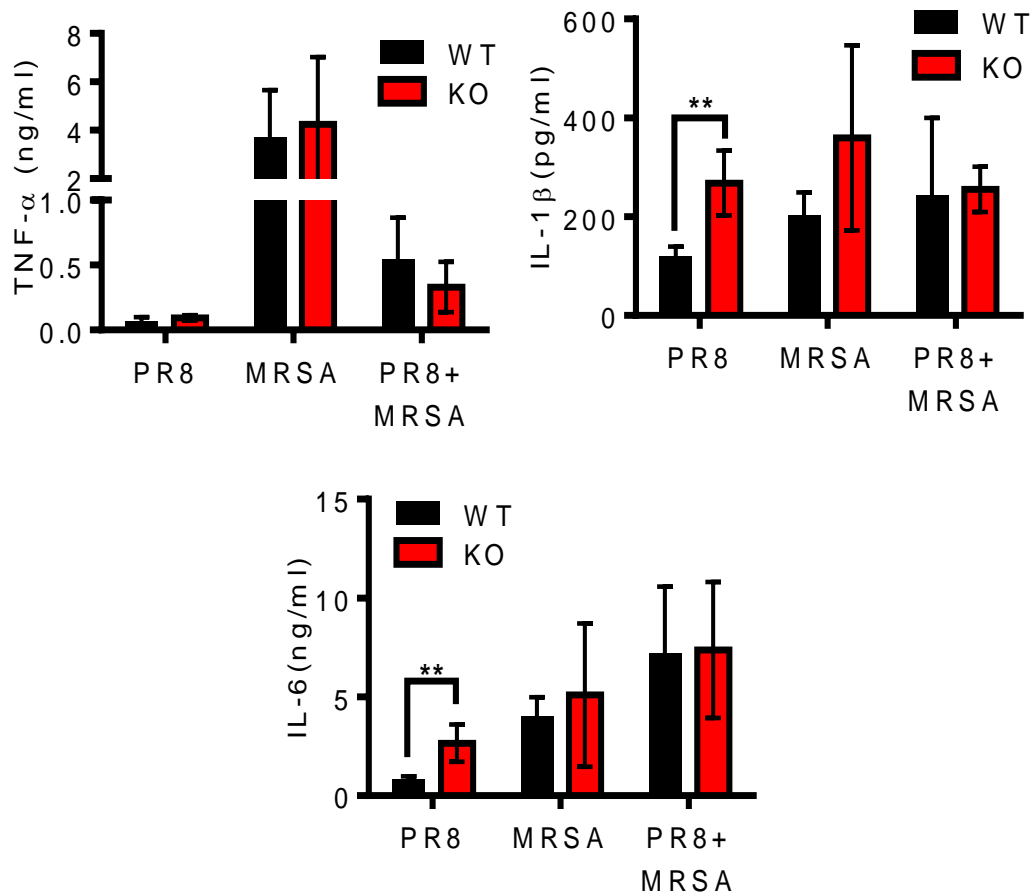
**Figure 4. CCR2<sup>-/-</sup> mice display increased bacterial clearance during coinfection.** Lung bacterial titers in WT and CCR2<sup>-/-</sup> mice following MRSA super-challenge on day seven after PR8 virus infection. All mice were harvested 24 hours following coinfection. \**P* < 0.05, unpaired *t*-test.

### ***Pro-Inflammatory Chemokine and Cytokine Response***

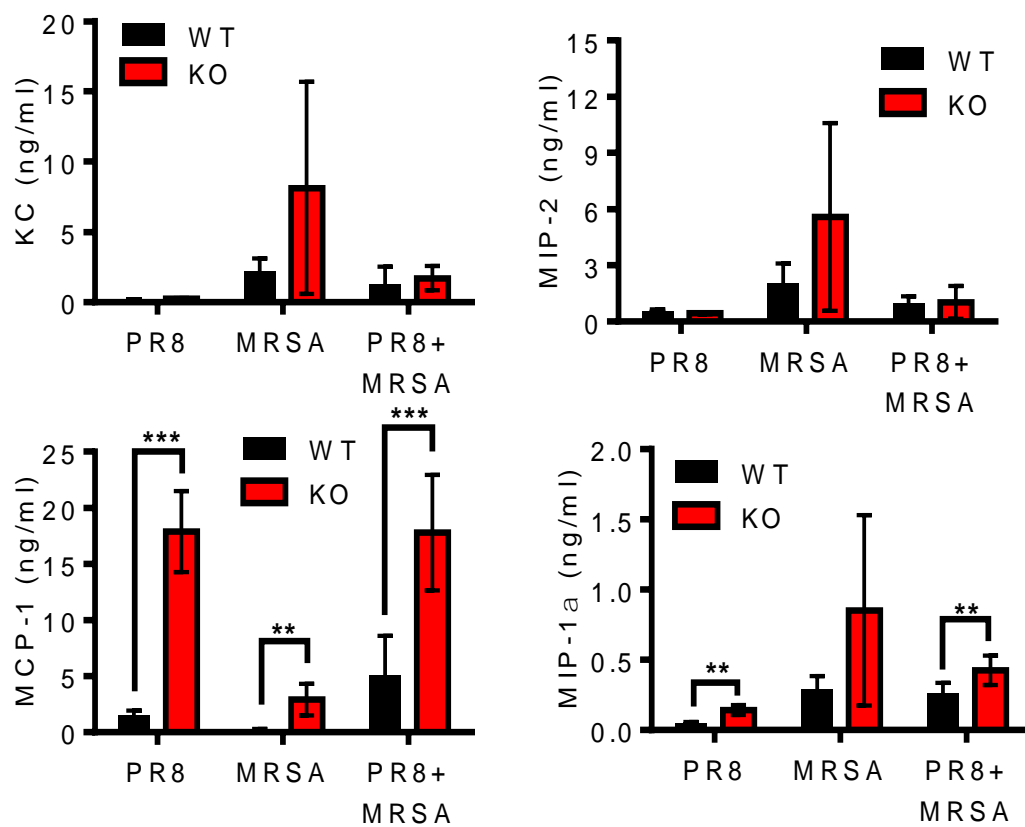
Pro-inflammatory cytokines and chemokines are known to contribute to influenza infection immunopathology and their elevation can also be seen during bacterial infection. They also contribute to the recruitment of innate immune cells to the airways. Thus, we examined the pro-inflammatory cytokine and chemokine response in CCR2<sup>-/-</sup> mice airways at 24 hours following MRSA super-infection. Compared to WT mice, CCR2<sup>-/-</sup> mice exhibited similar levels of pro-inflammatory cytokine production during secondary MRSA infection (Figure 5). During influenza infection alone, CCR2<sup>-/-</sup> mice showed increased production of IL-1 $\beta$  and IL-6; however, there was little production of TNF- $\alpha$  (Figure 5). Although TNF- $\alpha$  production was increased for both WT and CCR2<sup>-/-</sup> mice during MRSA infection alone, there were little differences in their IL-1 $\beta$  and IL-6 production during coinfection or MRSA infection alone (Figure 5).

We also assessed the pro-inflammatory chemokines: KC, MIP-2, MCP-1, and MIP-1 $\alpha$ , in airways at 24 hours following secondary MRSA infection. As expected, production of MCP-1 was significantly increased in CCR2<sup>-/-</sup> mice compared to WT mice during influenza infection alone, MRSA infection alone, and secondary MRSA infection (Figure 6). Production of MIP-1 $\alpha$  was significantly increased in CCR2<sup>-/-</sup> mice during influenza alone and post-influenza MRSA infection, but not during MRSA infection alone (Figure 6). There was no significant difference in the levels of chemokines KC and MIP-2 between coinfecting CCR2<sup>-/-</sup> and WT mice (Figure 6).





**Figure 5. No difference in peak pro-inflammatory cytokine response in CCR2-deficient mice.** TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 production in airways after super-challenge of day seven PR8-infected WT and CCR2<sup>-/-</sup> mice with MRSA. Mice were sacrificed 24 hours later. \*\* $P < 0.01$ , unpaired t-test.



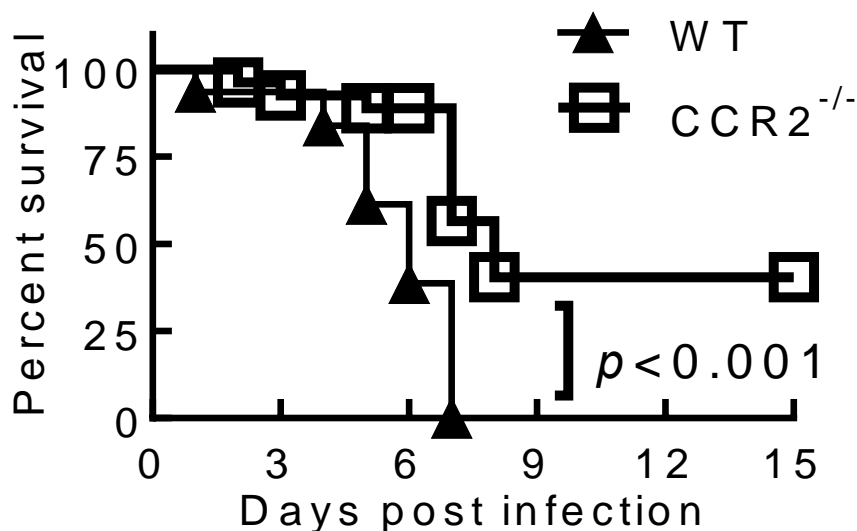
**Figure 6. Peak pro-inflammatory chemokine response in CCR2-deficient mice.** KC, MIP-2, MCP-1, and MIP-1a production in airways after super-challenge of day seven PR8-infected WT and CCR2<sup>-/-</sup> mice with MRSA. All mice were sacrificed 24 hours later. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , unpaired  $t$ -test.

## ***Section II – Post-Influenza MRSA Pneumonia with Antibiotic Treatment***

### ***Murine Model***

#### ***Antibiotic-Treated CCR2<sup>-/-</sup> Mice Survive Significantly Better During Post-Influenza MRSA Pneumonia***

In order to characterize the role of inflammatory monocytes during post-influenza MRSA pneumonia, we developed a similar infection model with addition of antibiotic treatment. Specifically, antibiotic gentamicin was used to control extracellular bacterial outgrowth. Mice were infected with PR8 and then on day 7 MRSA , followed by daily gentamicin treatment starting at 4 hours after MRSA super-infection. Interestingly, coinfecting CCR2<sup>-/-</sup> mice survived significantly better than WT controls (Figure 7). Although the majority of mice died around day 7 following secondary MRSA infection, CCR2<sup>-/-</sup> mice were able to survive past this time point (Figure 7). As with the infection model without antibiotics, this model with the use of antibiotics shows that CCR2<sup>-/-</sup> mice are able to survive post-influenza MRSA pneumonia better.



**Figure 7. CCR2<sup>-/-</sup> mice survive significantly better than WT mice following secondary bacterial MRSA infection post-influenza.** Survival of C57BL/6 WT, and CCR2<sup>-/-</sup> mice super-challenged with MRSA on day seven after PR8 virus infection. Coinfected mice were treated daily with the antibiotic gentamicin. Survival analyses were performed using the log-rank test.

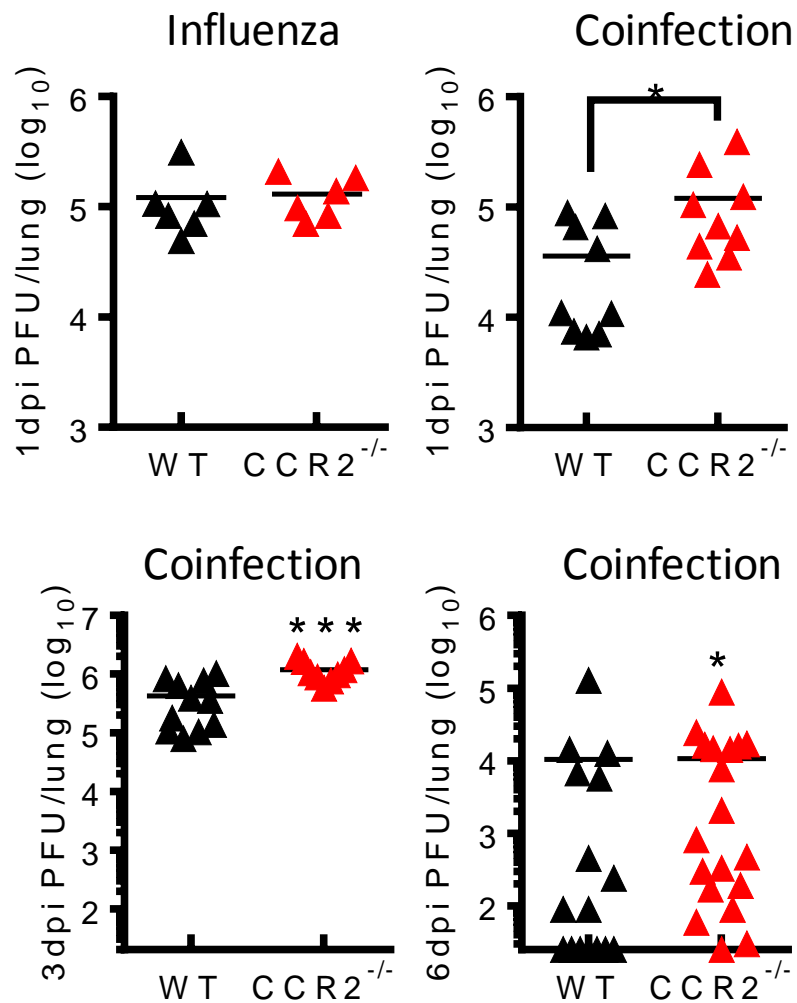
### *Antibiotic-Treated CCR2<sup>-/-</sup> Mice Exhibit delayed Viral clearance but Improved*

#### *Bacterial Control During Post-influenza MRSA Infection*

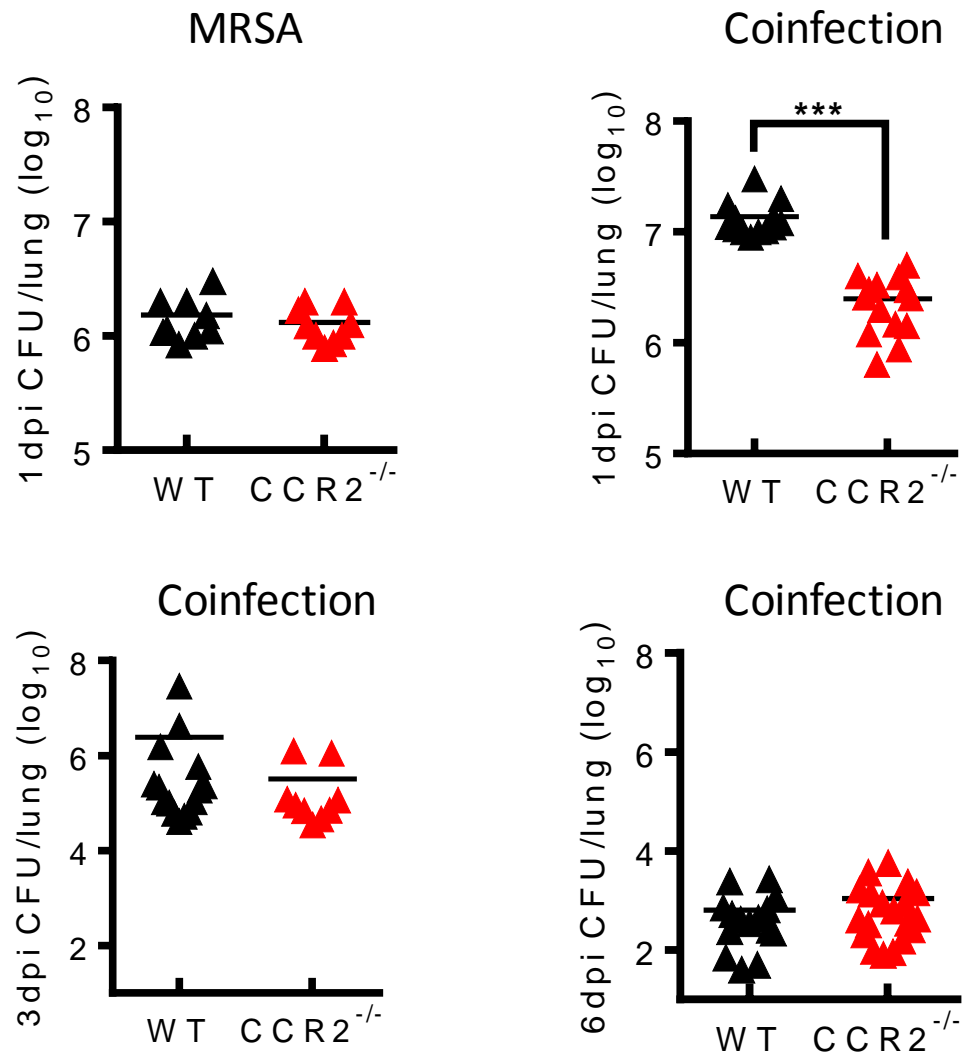
Along with the previous infection model, we assessed the viral and bacterial burdens in CCR2<sup>-/-</sup> mice at days one, three, and six after MRSA super-infection. Significantly higher viral burdens were found in the lungs of CCR2<sup>-/-</sup> mice compared to WT mice (Figure 8). There was little difference in the viral titers in CCR2<sup>-/-</sup> mice during influenza infection alone compared to coinfection at day one (Figure 8). However, compared with WT controls, CCR2<sup>-/-</sup> mice exhibited delayed viral clearance during -post-influenza MRSA infection (Figure 8).

We then assessed the bacterial burden in CCR2<sup>-/-</sup> and WT mice. At day one after MRSA super-infection, CCR2<sup>-/-</sup> mice had a significantly decreased bacterial burden compared to WT

mice (Figure 9). However, both CCR2<sup>-/-</sup> and WT mice surviving at day six were able to control bacterial burden (Figure 9). The improved bacterial control in CCR2<sup>-/-</sup> mice at day one is consistent with their increased survival after secondary MRSA infection.



**Figure 8. CCR2<sup>-/-</sup> mice display increased viral burdens during coinfection.** Lung viral titers in WT and CCR2<sup>-/-</sup> mice following MRSA super-challenge on day seven after PR8 virus infection. All mice were treated with gentamicin until 24 hours before harvesting the samples. \* $P < 0.05$ , \*\*\* $P < 0.001$ , unpaired  $t$ -test.

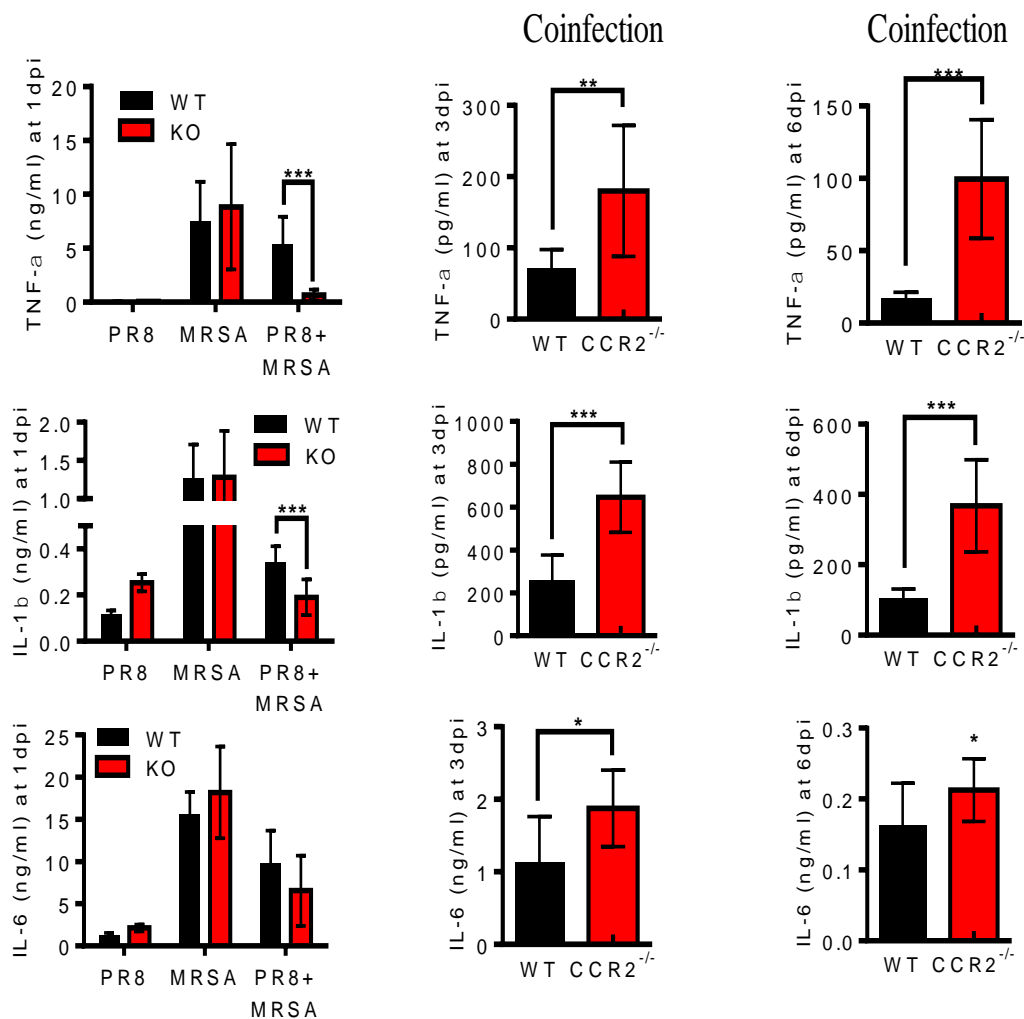


**Figure 9. CCR2<sup>-/-</sup> mice display decreased bacterial burdens during coinfection.** Lung bacterial titers in WT and CCR2<sup>-/-</sup> mice following MRSA super-challenge on day seven after PR8 virus infection. All mice were treated with gentamicin until 24 hours before harvesting the samples. \*\*\* $P < 0.001$ , unpaired  $t$ -test.

***Antibiotic-Treated CCR2<sup>-/-</sup> Mice Exhibit Decreased Peak Pro-Inflammatory Chemokine and Cytokine Response***

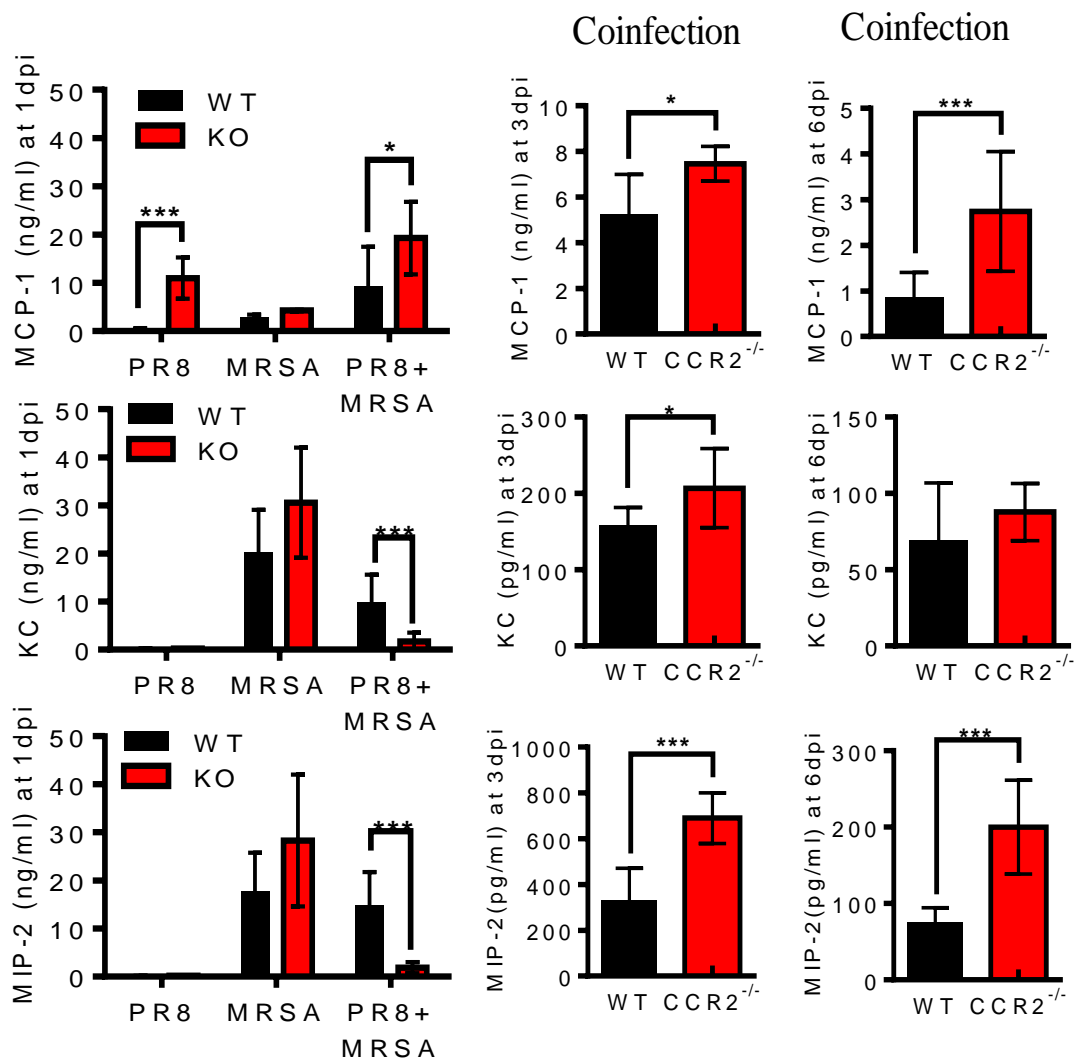
Pro-inflammatory chemokines and cytokines have been shown to contribute to increased lung immunopathology following influenza infection alone and post-influenza bacterial infection. Thus, we further characterized this infection model by analyzing the pro-inflammatory cytokine and chemokine production at days one, three, and six after MRSA super-infection. At day one after MRSA super-infection, CCR2<sup>-/-</sup> mice had decreased production of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Figure 10). Despite the overall decreased concentration at later days after coinfection, CCR2<sup>-/-</sup> mice had significantly increased levels of these pro-inflammatory cytokines. (Figure 10).

We also assessed the production of pro-inflammatory chemokines MCP-1, KC, and MIP-2. MCP-1, i.e. CCL2, is the main ligand for CCR2, while KC and MIP-2 are generally responsible for neutrophil recruitment. Significantly increased levels of MCP-1 were found in the lungs of CCR2<sup>-/-</sup> mice at days one, three, and six following MRSA super-infection (Figure 11). Compared with WT mice, CCR2<sup>-/-</sup> mice had decreased production of KC and MIP-2 at day one; but increased levels at days three and six after MRSA super-infection (Figure 11).



**Figure 10. CCR2-deficient mice exhibit decreased peak pro-inflammatory response.** Kinetics of (A) TNF- $\alpha$ , (B) IL-1 $\beta$ , and (C) IL-6 production in airways after super-challenge of day seven PR8-infected WT and CCR2<sup>-/-</sup> mice with MRSA. All mice were treated with gentamicin until sacrificed at indicated time points. \* $P < .05$ , \*\* $P < 0.01$ , \*\*\* $P < .001$ , unpaired t-test.





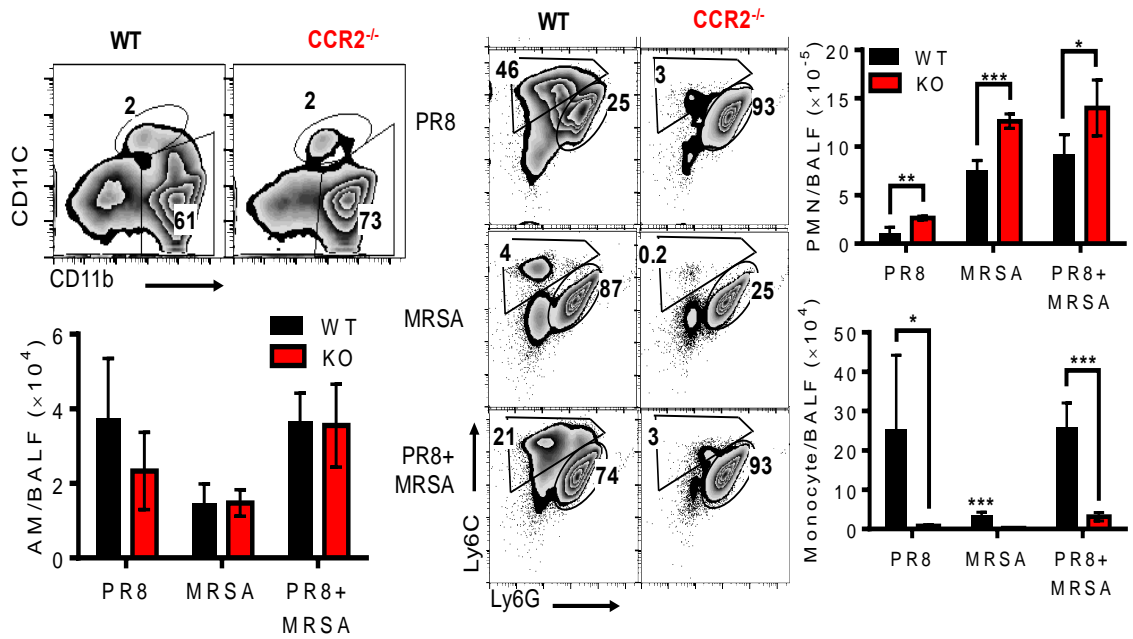
**Figure 11. Pro-inflammatory chemokine response in CCR2-deficient mice.** Kinetics of MCP-1, KC, and MIP-2 production in airways after super-challenge of day seven PR8-infected WT and CCR2<sup>-/-</sup> mice with MRSA. All mice were treated with gentamicin until sacrificed at indicated time points. \* $P < .05$ , \*\*\* $P < .001$ , unpaired t-test.

***Recruitment of inflammatory cells in CCR2<sup>-/-</sup> and WT Mice During Post-Influenza  
MRSA Infection***

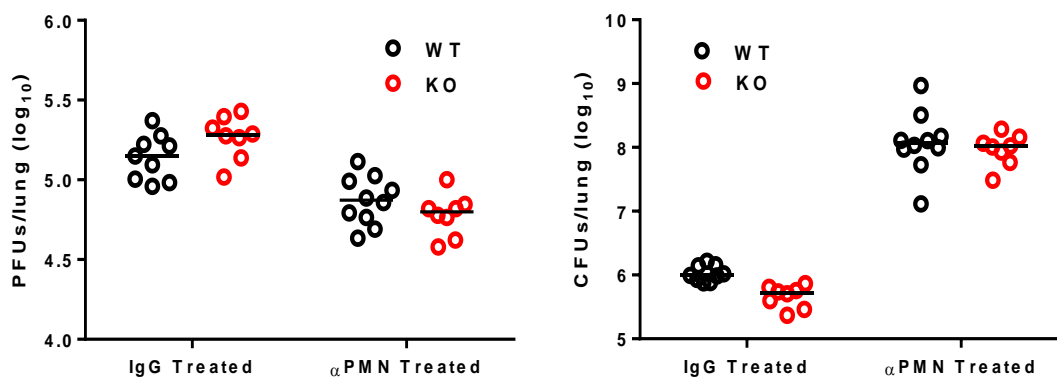
To further investigate the mechanism for decreased bacterial burdens in coinfecting CCR2<sup>-/-</sup> mice, we assessed the numbers of phagocytes during infection, primarily: alveolar macrophages (AM), neutrophils (PMNs), and inflammatory monocytes. It was found that there were similar numbers of alveolar macrophages (CD11c<sup>+</sup>) in CCR2<sup>-/-</sup> mice compared to WT mice during the monoinfections and coinfection (Figure 12).

We further gated the CD11b<sup>+</sup>CD11c<sup>-</sup> cells into Ly6G<sup>+</sup> or Ly6C<sup>+</sup> cells. Increased numbers of neutrophils (CD11b<sup>+</sup>CD11c<sup>-</sup>Ly6G<sup>+</sup>) were found in the airways of CCR2<sup>-/-</sup> mice compared to WT mice, while there were significantly increased numbers of inflammatory monocytes (CD11b<sup>+</sup>Ly6C<sup>+</sup>) in WT mice compared to CCR2<sup>-/-</sup> mice (Figure 12). Our results indicate that CCR2<sup>-/-</sup> mice are indeed defective in recruiting inflammatory monocytes to the site of infection.

The increased numbers of neutrophils seen in the airways of CCR2<sup>-/-</sup> mice could mediate increased bacterial clearance. In order to assess the contribution of neutrophils to bacterial control, we depleted neutrophils with antibody treatment immediately after a nonlethal dose of MRSA super-infection. Interestingly, antibody-mediated depletion of neutrophils in mice decreased viral burdens compared to Rat IgG treated controls (Figure 13). Although bacterial burdens were significantly increased in neutrophil-depleted mice, they were similar between neutrophil-depleted CCR2<sup>-/-</sup> and WT mice (Figure 13), indicating that neutrophils are critical for bacterial control and necessary for the beneficial effect of CCR2 deficiency to be observed.



**Figure 12.** CCR2-deficient mice display similar numbers of phagocytes compared to WT mice. Numbers of alveolar macrophages (CD11c<sup>+</sup>), and neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>) and inflammatory monocytes (CD11b<sup>+</sup>Ly6C<sup>+</sup>) in airways at one dpi after super-challenge of day seven PR8-infected WT and CCR2<sup>-/-</sup> mice with MRSA. Mice were treated with gentamicin 4 hours post-MRSA infection. \* $P < 0.05$ , \*\*\* $P < 0.001$ , unpaired  $t$ -test.



**Figure 13.  $CCR2^{-/-}$  mice display similar bacterial burdens during neutrophil depletion.** Lung viral and bacterial titers in WT and  $CCR2^{-/-}$  mice following a sublethal dose of MRSA super-challenge on day seven after PR8 virus infection. All mice were treated i.p. with rat IgG or anti-Gr-1 mAb RB6-8C5 immediately following secondary MRSA infection and samples were harvested 24 hours later. Unpaired  $t$ -test.

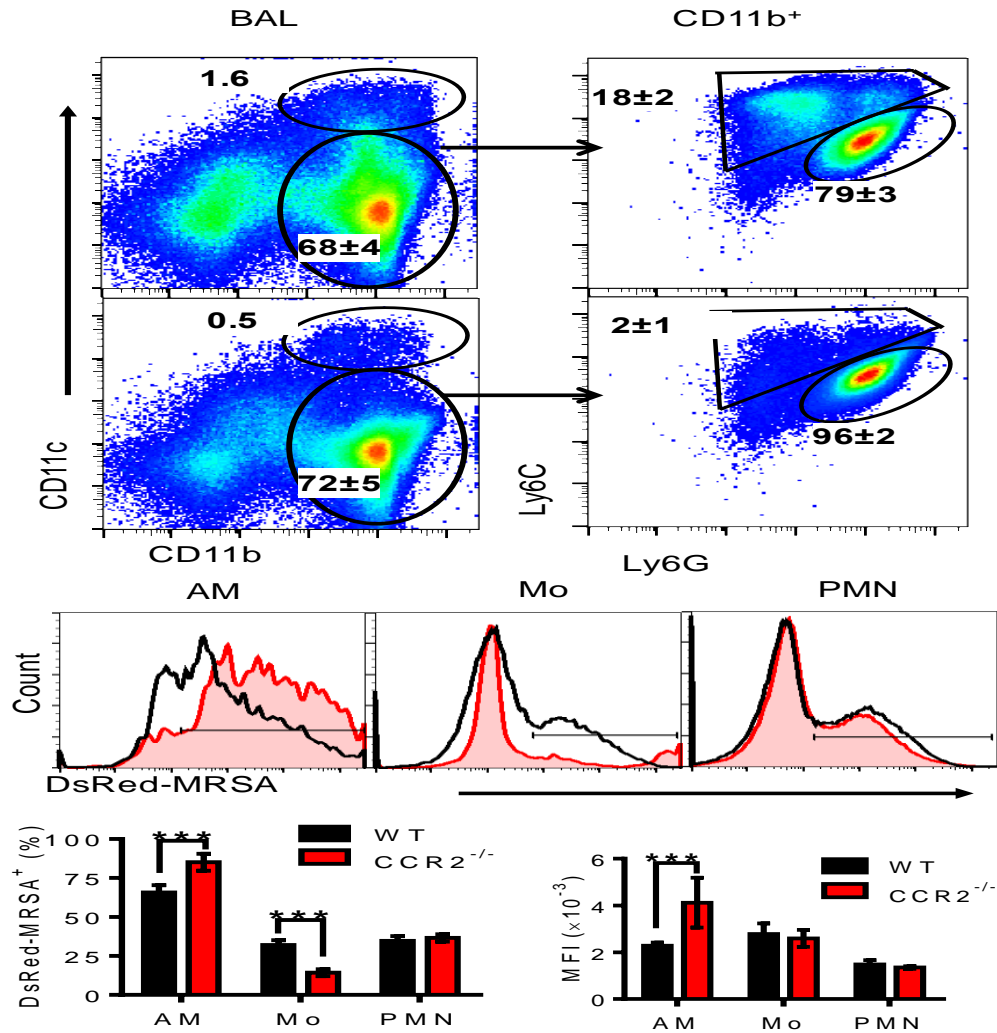
### ***The Phagocytic Capability of $CCR2^{-/-}$ Alveolar Macrophages is Increased During Post-Influenza MRSA Infection***

Our results indicate that  $CCR2^{-/-}$  mice have decreased bacterial burdens compared to WT mice, despite diminished monocytes recruitment into the airways during infection. Therefore, we sought to assess a possible defect of phagocytic ability of alveolar macrophages or neutrophils during secondary MRSA infection. In order to examine the uptake of bacteria in these phagocytes, we used a DsRed-expressing MRSA strain and confirmed bacterial uptake with flow cytometry (Figure 14). As shown also in figure 12,  $CCR2^{-/-}$  mice have decreased frequencies of inflammatory monocytes recruited into the airways following secondary MRSA infection (Figure 14).

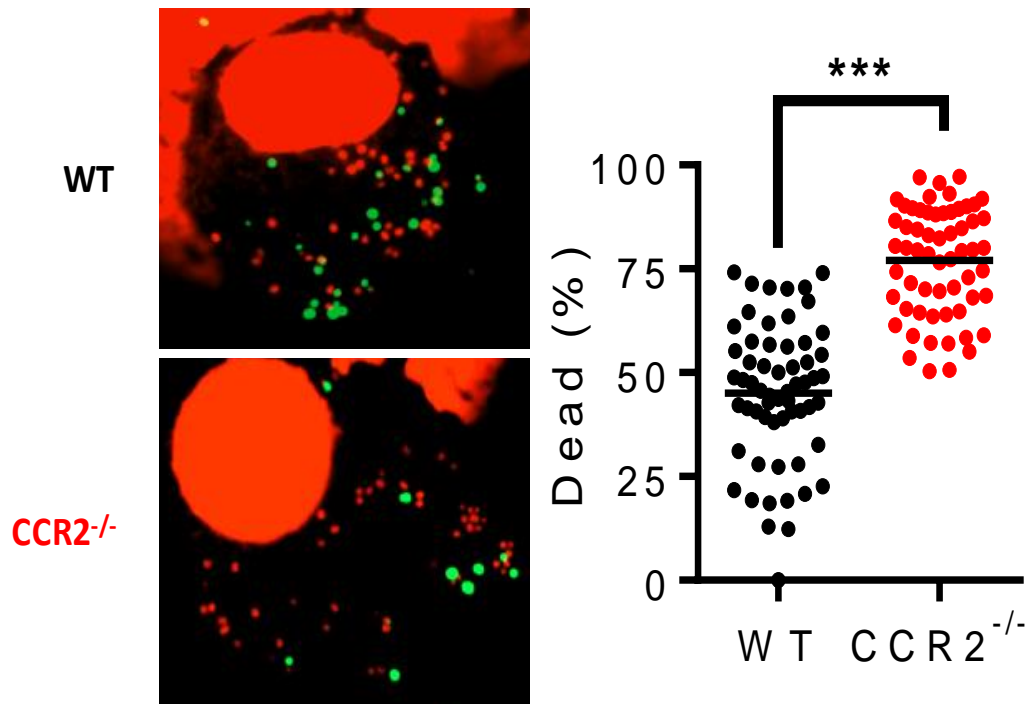
The representative histograms for alveolar macrophages, neutrophils, and inflammatory monocytes indicate the uptake of the DsRed-MRSA (Figure 14).  $CCR2$ -deficient alveolar macrophages show increased MRSA uptake compared to WT alveolar macrophages (Figure 14). WT mice displayed an increase in the percentage of DsRed<sup>+</sup> inflammatory monocytes compared to  $CCR2^{-/-}$  mice, due to the inability of  $CCR2^{-/-}$  mice to recruit inflammatory monocytes (Figure

14). Interestingly,  $CCR2^{-/-}$  and WT neutrophils showed similar bacterial uptake (Figure 14). In addition to quantify the numbers of phagocytes able to uptake bacteria, we compared the relative amount of bacteria phagocytized by alveolar macrophages, inflammatory monocytes, and neutrophils, respectively, with the mean fluorescence intensity (Figure 14). Our results indicate that  $CCR2^{-/-}$  alveolar macrophages have improved phagocytosis ability compared to WT controls during secondary MRSA infection (Figure 14).

Although we show that  $CCR2^{-/-}$  alveolar macrophages were able to uptake more bacteria compared to WT alveolar macrophages, their intracellular killing ability remains unclear. In order to examine this, BALF cells were harvested from day one coinfecting- $CCR2^{-/-}$  and WT mice and incubated with lysostaphin to lyse the extracellular bacteria (Figure 15). The cells were then stained with SYTO9 (green) and propidium iodide (Red) to examine live and dead bacteria, respectively, inside individual alveolar macrophages (Figure 15). Relatively more dead bacteria were found within  $CCR2^{-/-}$  alveolar macrophages compared to WT alveolar macrophages (Figure 15). Thus, we conclude that  $CCR2^{-/-}$  alveolar macrophages have improved intracellular bacterial killing ability compared to WT alveolar macrophages during post-influenza MRSA infection.



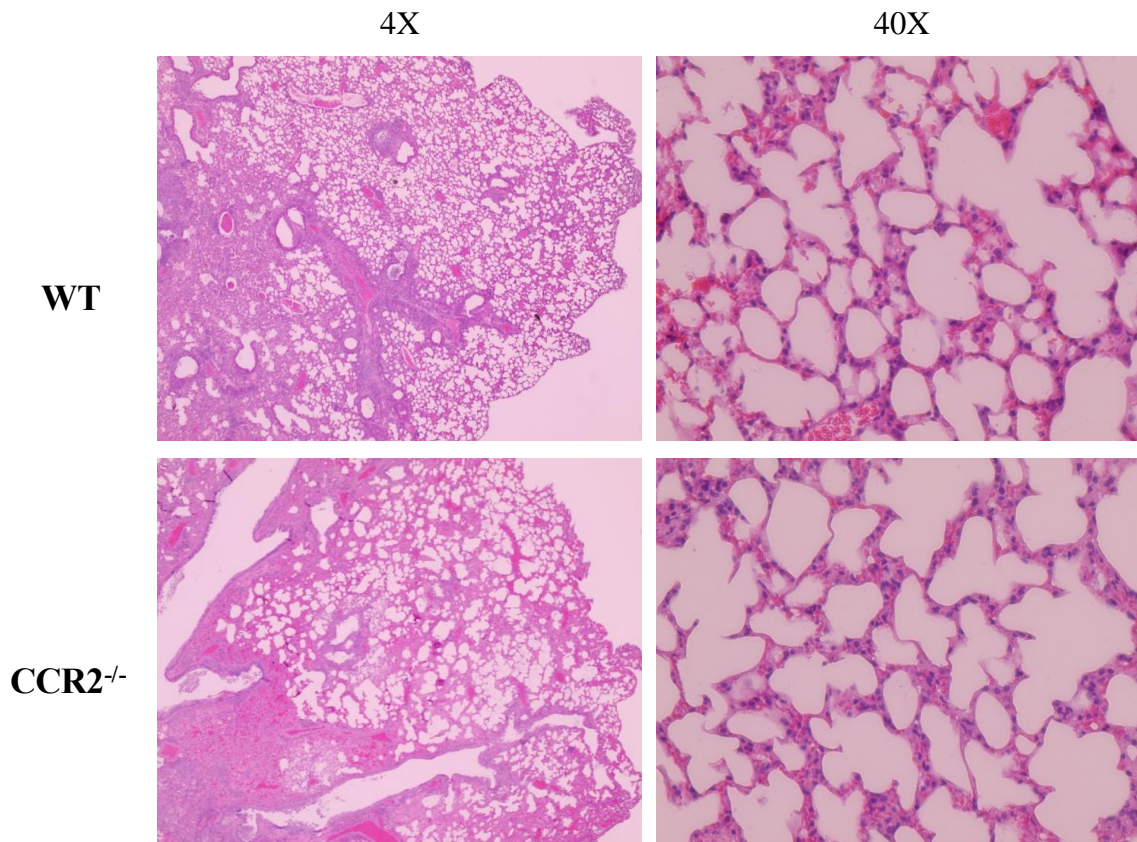
**Figure 14. *CCR2*<sup>-/-</sup> mice alveolar macrophages exhibit increased bacterial uptake.** Airway inflammatory cell profiles, representative histograms and quantification of *S. aureus* phagocytosis by alveolar macrophages (AM), monocytes (Mo) and neutrophils (PMN) at day one after super-challenge of day seven PR8-infected WT and *CCR2*<sup>-/-</sup> mice with DsRed-MRSA., respectively. Mice were treated with gentamicin 4 hours post-MRSA infection. \*\*\**P* < 0.001, unpaired *t*-test.



**Figure 15. CCR2<sup>-/-</sup> display increased intracellular killing of bacteria within alveolar macrophages.** Microscopy analysis and quantification of live (SYTO9, green) and dead cells (propidium iodide, red) within alveolar macrophages 24 hours after super-challenge of day seven PR8-infected WT and CCR2<sup>-/-</sup> mice with MRSA. Before immunostaining, extracellular bacteria were lysed by lysostaphin. Mice were treated with gentamicin 4 hours post-MRSA infection. \*\*\* $P < 0.001$ , unpaired t-test.

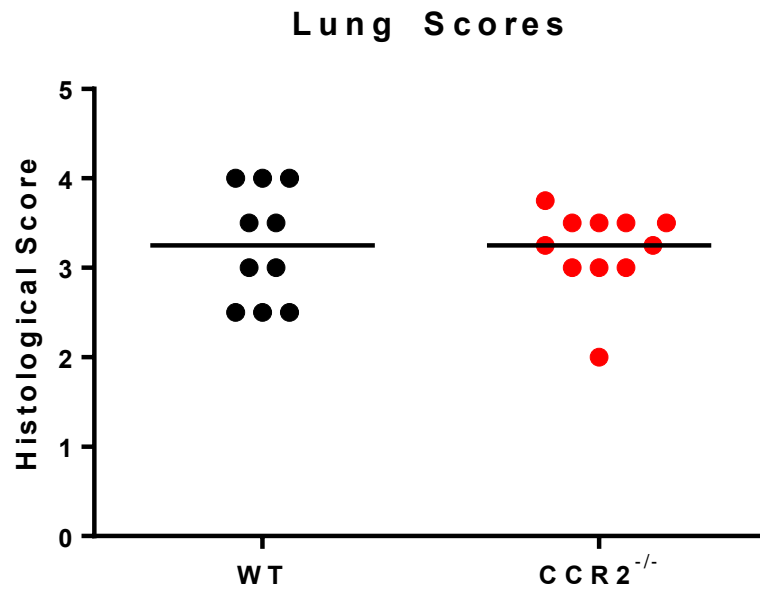
### Lung histopathology

Finally, we assessed lung histopathology after MRSA super-infection of CCR2<sup>-/-</sup> and WT mice. Lungs were harvested from CCR2<sup>-/-</sup> and WT mice at day seven following secondary MRSA infection and then stained and scored as described in the Material and Methods section. The lungs of CCR2<sup>-/-</sup> mice and WT mice displayed similar levels of histopathology caused by secondary MRSA infection (Figures 16). Despite their increased bacterial clearance and survival, lung histological scores of coinfecting CCR2<sup>-/-</sup> mice are similar to those of WT controls (Figure 17). Further studies are necessary in order to effectively evaluate the extent of lung inflammation in CCR2<sup>-/-</sup> mice.



**Figure 16: Immunopathology is similar in the lungs of CCR2-deficient and WT mice.** Lungs were harvested from CCR2<sup>-/-</sup> and WT mice at day seven (?) following secondary MRSA infection. Representative images of each sample are shown at 4X and 40X. Samples were stained as described in the Materials and Methods section.





**Figure 17. Histological scores of CCR2-deficient and WT mice lungs.** Mice were sacrificed at day seven (?) following secondary MRSA infection. Lung samples were stained and scored as described in the Material and Methods section.

## **CHAPTER 4: DISCUSSION**

The innate immune response during post-influenza bacterial infections still remains understudied. It has been acknowledged that a dysregulated innate immune response induced by influenza virus infection leads to increased susceptibility and disease severity to secondary bacterial infections. Specifically, the role of inflammatory monocytes during secondary bacterial infections had remained unclear. Therefore, the objective of this thesis was to characterize the role inflammatory monocytes during secondary MRSA pneumonia, possibly providing a therapeutic target for future treatments. The hypothesis tested in these studies was that inflammatory monocytes contribute to increased mortality and lung pathology during post-influenza MRSA pneumonia. By utilizing a CCR2-deficient murine infection model with and without antibiotics, we were able to examine the possible contribution of these cells to disease progression. The initial objectives included analyzing the survival, viral and bacterial burdens, and pro-inflammatory cytokine and chemokine response of CCR2-deficient mice during secondary MRSA infection. Lastly, the final objectives of this thesis were to elucidate a possible mechanism that inflammatory monocytes contribute to a dysregulated innate antibacterial immune response.

### **Post-influenza MRSA Pneumonia Murine Model**

Inflammatory monocytes have been shown to regulate pathologic responses in multiple infection models [85]. Specifically, Ly6C<sup>hi</sup> monocytes are a dominant recruited cell type during acute inflammation [86]. These Ly6C<sup>hi</sup> monocytes are generally recruited to the site of inflammation in a CCR2-dependent manner. In addition, during influenza infection alone, mice deficient in CCR2 have shown increased survival and decreased lung pathology. In order to understand the possible role of inflammatory monocytes during secondary MRSA infection, we established a post-influenza MRSA infection model using CCR2-deficient mice.

Initially, we examined the survival from coinfection between WT and CCR2<sup>-/-</sup> mice and interestingly, the CCR2<sup>-/-</sup> mice displayed increased survival compared to WT mice. Although a simple conclusion, it was intriguing that inflammatory monocytes may have a negative impact on secondary MRSA infection disease outcome. This led us to further examine the coinfection model by analyzing the viral and bacterial burdens and their contributions to disease progression. Influenza virus infection alone can lead to cytokine storm, increased lung damage, and increased mortality [58]. However, despite significantly increased viral burdens in CCR2<sup>-/-</sup> mice throughout coinfection, these mice displayed increased survival, which is consistent with improved bacterial control. Of note, at day one post-MRSA infection, both coinfecting WT and CCR2-deficient mice showed similar bacterial burdens compared to mice infected with MRSA-only. This could be due to the overwhelming dose of bacterial challenge. Indeed, CCR2<sup>-/-</sup> mice showed decreased bacterial burdens compared to WT mice at 24hr after a sublethal dose of MRSA super-infection I

## **Post-influenza MRSA Pneumonia Murine Model with Antibiotic**

### **Treatment**

To further investigate the role of inflammatory monocytes during secondary MRSA infection, mice were treated with the antibiotic, gentamicin, following secondary MRSA infection. Due to the oversaturation of bacteria in the model described above, antibiotic treatment controls bacterial outgrowth and allows for evaluation under inflammatory conditions. Under the post-influenza MRSA pneumonia antibiotic treatment model, CCR2<sup>-/-</sup> mice displayed significantly increased survival when compared to WT mice. Consistent with previous results, CCR2<sup>-/-</sup> mice exhibited increased viral burdens. Although inflammatory monocytes may be necessary for antiviral defense, when coinfecting with MRSA, higher viral titers are not seen as associated with increased mortality. Unlike the previous model, CCR2-deficient coinfecting mice

exhibit similar levels of bacterial titers at day one post-MRSA infection compared to controls infected with MRSA only . Whereas, coinfecting WT mice exhibited increased bacterial burdens compared to MRSA-infected WT mice at day one post-MRSA infection. Histological samples indicated that lung inflammation was similar in both CCR2<sup>-/-</sup> and WT mice. We thus concluded inflammatory monocytes exacerbate the disease outcome mainly through inhibiting bacterial clearance during coinfection.

Interestingly, our results indicated that at day one-post secondary MRSA infection, CCR2<sup>-/-</sup> mice exhibited decreased pro-inflammatory cytokine and chemokine levels. With the diminished presence of inflammatory monocytes, bacterial burdens are reduced along with the peak pro-inflammatory and chemokine and cytokine levels. Inflammatory monocytes are known to be potent producers of cytokines and chemokines leading to increased lung pathology [58]. This initial immunosuppressive state observed in CCR2<sup>-/-</sup> mice, may allow for crucial containment of the inflammatory environment in the airways leading to reduced lung damage. With significantly decreased chemokine and cytokine concentrations on days three and six post-secondary MRSA infection, it appears that CCR2<sup>-/-</sup> mice have increased pro-inflammatory cytokine and chemokine levels. This may indicate that along with decreased bacterial burdens that CCR2<sup>-/-</sup> mice have entered a healthy pro-inflammatory state leading to better control of disease pathogenesis. It should be noted that, generally, increased pro-inflammatory cytokine and chemokine recruitment enables increased innate immune phagocytic ability [43]. Consistent with our results, increased bacterial clearance in the CCR2<sup>-/-</sup> mice is associated with increased survival. Therefore, controlling this peak pro-inflammatory response with the reduced recruitment of inflammatory monocytes may be necessary to control secondary MRSA disease progression. It was expected to find elevated levels of MCP-1 (CCL2) in CCR2<sup>-/-</sup> mice, as this is the main ligand for CCR2. Two possible explanations may exist for this observation: since CCR2 is mostly diminished, MCP-1 cannot bind to its main receptor leaving elevated levels or more of this chemokine is being produced to overcompensate for not being able to recruit monocytes.

## **Phagocytic Capability of Alveolar Macrophages May Be Inhibited by Inflammatory Monocytes**

In order to further characterize the role that inflammatory monocytes during lethal post-influenza MRSA infection, we evaluated the number of phagocytes recruited during secondary MRSA infection. The increased bacterial clearance observed in coinfecting  $CCR2^{-/-}$  mice could be due to increased phagocyte recruitment. As confirmed by flow cytometry,  $CCR2^{-/-}$  mice exhibited significantly decreased numbers of inflammatory monocytes ( $CD11b^{+}Ly6C^{+}$ ) during influenza virus infection alone, MRSA infection alone, and coinfection when compared to WT mice. Although numbers of alveolar macrophages were similar to WT controls, coinfecting  $CCR2^{-/-}$  mice had increased numbers of neutrophils. In order to evaluate the contribution of neutrophils in  $CCR2^{-/-}$  mice bacterial clearance, we depleted the neutrophils with anti-Gr1 antibody treatment immediately following secondary challenge with MRSA bacteria. Depletion of neutrophils facilitated comparable bacterial outgrowth in both WT and  $CCR2^{-/-}$  mice. This indicates that neutrophils are critical in controlling the bacterial load and are necessary for the beneficial effect observed in  $CCR2^{-/-}$  mice. Furthermore, we show that  $CCR2^{-/-}$  alveolar macrophages have improved phagocytic capability compared with WT controls. Together, the increased neutrophil numbers and alveolar macrophage bacterial killing ability facilitate bacterial control in coinfecting  $CCR2^{-/-}$  mice.

## **Conclusion**

Inflammatory monocytes have been implicated in contributing to lung pathology during influenza virus infection alone. To our knowledge, little is known about the role of inflammatory monocytes during post-influenza MRSA pneumonia. Although multiple cell types and factors are critical in controlling secondary bacterial infection, in this study, we show that inflammatory monocytes contribute to increased lung pathology leading to increased mortality. In conclusion, our results indicate that inflammatory monocytes may inhibit the phagocytic ability of alveolar macrophages contribute to increased bacterial burdens and pro-inflammatory cytokine/chemokine environment. Future studies will continue to explore the roles that inflammatory monocytes have in secondary MRSA disease progression.

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