The Role of Lung Epithelial IL-17RC signaling during Influenza, MRSA, and Influenza-MRSA Super-Infection

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Influenza is a common cause of respiratory illness contributing significantly to morbidity and mortality each year. Secondary-bacterial infections following influenza illness are less common but increase the likelihood of developing severe infection complications further increasing morbidity and mortality. IL-17, an immune system signaling molecule, induces cell signaling that drives proinflammatory activation of the immune response including the recruitment of immune cells to infection sites and inducing changes in gene expression. These actions have been shown to increase morbidity associated with influenza infections by increasing damage to host tissues. During bacterial infections, such as methicillin-resistant *Staphylococcus aureus* (MRSA), IL-17 signaling helps with bacterial clearance, reducing morbidity. Understanding the roles IL-17 signaling has in the lungs during different infections can help provide new avenues for therapeutic interventions. IL-17 is thought to activate immunity via signaling through its receptor on lung epithelial cells. Club cells of the lung epithelium, identified by club cell secretory protein, are known as immune activators. In this study, we sought to understand what effects IL-17 signaling in club cells has using three infection models: primary influenza, primary MRSA, and influenza-MRSA super-infection of the lungs. Mice were generated using cre-lox recombination to remove the gene for IL-17RC, a receptor subunit needed for IL-17 signaling initiation, specifically in club cells. In all three infections, no significant changes were observed between the knockouts and control mice. This suggests that

IL-17A and IL-F signaling in club cells does not greatly affect the immune response in this setting.

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Preface

This project could not have been completed without the help of those around me and I want to thank each of them for their roles. Saran Kupul maintained and screened the mice to ensure the correct genotypes were present. Shuxia Wang assisted with the murine infections and organ collection and processing. Nick Constantinesco synthesized cDNA and ran RT-PCR plates . Lacee Richwalls helped with differential cell counts.

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1.0 Introduction

1.1 Global Impact of Influenza

Influenza infections in the US contribute significantly to morbidity, mortality, and costs relating to treatment and lost earnings. Each year, millions of people become infected with influenza which results in millions of outpatient visits, hundreds of thousands of hospitalizations, and tens of thousands of deaths in the US alone. The mortality rate of influenza underestimates the disease toll associated with the infection. Influenza associated costs for treatment and loss work days is estimated to be tens of billions of dollars annually, during non-pandemic years [1]. During the most recent pandemic in 2009, it is estimated that up to 24% of the world, over 1.6 billion people, became infected with the virus [2]. Despite its low mortality rate; influenza remains a top 10 cause of death in Americans aged 1-24 and 65 and older [3]. Secondary bacterial infections, with pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), associated with influenza infections contribute to an increase in rates of mortality and morbidity [4].

1.2 Influenza Viral Cycle

Influenza virions begin the viral cycle by entering the respiratory tract of humans. The virions have hemagglutinin proteins protruding from their cell membrane. These proteins bind to sialic acid receptors on host cells initiating endocytosis of the virion. Upon acidification of the

endosome and cleavage of hemagglutinin by host proteases, the membranes of the virion and endosome fuse allowing for the release of the viral RNA along with viral proteins into the cytoplasm. These all traffic to the nucleus of the host cell where viral RNA can be replicated, snatch 5' caps from host mRNA, and return to the cytoplasm for translation. As virions are formed, they can exit the host cell via budding from the host cell membrane after being cleaved from the membrane via neuraminidase and infect new cells [5].

1.3 Immune Response to Influenza

When a pathogen infects a host, the immune system must be able to respond to quell the infection before it causes significant damage. Pathogens have conserved structural components known as pathogen-associated molecular patterns (PAMPS) made of carbohydrates, lipids, nucleic acids, or proteins. These PAMPS can be detected by pattern recognition receptors on host cells that can initiate the innate immune response [6, 7]. There are a number of ways for the host to recognize influenza.

One mechanism involves identifying the viral genome. After influenza is taken into an endosome, its RNA genome can be detected by toll-like receptors 7 and 9 found in the endosome [5]. Toll-like receptor 7 sees single-stranded RNA while toll-like receptor 9 detects unmethylated CpG-DNA from viruses or bacteria [8]. Activation leads to initiation of the transcription factors NF-κB or interferon-regulated factor causing expression of proinflammatory cytokines and type I interferons. RIG-I in the cytoplasm can recognize newly replicated viral RNA and causes the production of type I interferons through the mitochondrial antiviral signaling protein [5].

During an influenza infection, type I (interferon-α and β), type II (interferon-γ), and type III (interferon-λ) interferons are released by infected cells and act as an alarm system for nearby cells, warning them of the viral infection. Type I interferons bind to transmembrane receptors leading to the formation of heterodimers made of signal transducer and activator of transcription 1 and 2 (STAT1-STAT2). Type II interferons lead to STAT1-STAT1 homodimers. These dimers act as transcription factors and move into the nucleus to induce expression of interferon-stimulated genes [5, 9-14]. Interferon-stimulated genes reduce viral protein production. Common examples include: protein kinase R, which prevents translation initiation 2'-5'-oligoadenylate synthetase activates RNase L in the cytoplasm to destroy viral genomes; Mx1 protein, a GTPase, destroys nucleocapsid proteins of the virus [15]. Interestingly, type I interferon gene expression has been positively correlated with influenza burden and weight loss [16].

Neutrophils and macrophages have both been implicated in assisting in influenza clearance during infection. Dessing et. al. used an influenza model with mice deficient in the macrophage chemokine monocyte chemoattractant-1 (MCP-1). They showed that MCP-1 is critical in macrophage recruitment during influenza infection and that reduced macrophage recruitment caused increased viral burden and weight loss in MCP-1 deficient mice [17].

Like macrophages, neutrophils are recruited during an influenza infection. Neutrophil depletion has been shown to increase viral burden and mortality to sublethal doses of influenza infections [18]. Prolonged neutrophil chemokines and recruitment have been associated with increased viral burden and weight loss most likely from immunopathology. Lung damage during an influenza infection is caused by both direct killing of lung cells, but also from excessive immune responses damaging lung cells [16].

Natural killer cells are important in lysing virally infected cells contributing to reduced viral proliferation and spread. Type I interferons have been shown to activate natural killer cells during influenza infection, but there may be other indirect activators as well. STAT1 activity, induced by type I interferons, increased granzyme B which increases apoptosis of host cells by natural killer cells [19]. The number of natural killer cells in the lungs increases four-fold during influenza infection [20]. Depleting natural killer cells or inhibiting their lysis induction of host cells has been shown to increase morbidity and mortality from influenza infection [20, 21].

Adaptive immune cells contribute to influenza clearance and immunity following infection. Type I interferons are important ligands for T and B cell activities. Kohlmeier et. al. experimented with cytotoxic T cells ($CD8^+$) to determine how their lytic capabilities were influenced during influenza infection. Type I interferon signaling in these cells was required for increased expression of granzyme B and lyse cells presenting the antigen-specific ligand. This response enables better clearance of influenza early on in the infection [22].

Type I interferons can directly activate B cells early on during influenza infection. Type I interferons are both needed and sufficient for B cell activation. Type I interferon induced activation enhanced B cell antibody production and likely contributes to influenza control and clearance [23].

Proinflammatory cytokines released during influenza infection can contribute to causing or preventing host damage. IL-33 can cause innate lymphoid cells in the lung to initiate repair of damaged tissues by secreting amphiregulin. This action does not affect viral burden. In some circumstances, the innate lymphoid cells can secrete IL-13 inducing damage to the airways from mucus production and tissue remodeling. Tissue growth factor-β (TGF-β) can be activated by proteolytic cleavage from the influenza neuraminidase protein. It can increase the viral burden

but reduce inflammatory pathology. IL-17A and F released from γδ T cells and tumor necrosis factor-α promote immunopathology, damaging host tissues without contributing to viral clearance [5].

T helper cells ($CD4_+$) change from a naïve T cell to an activated T cell after an antigen-T cell receptor sustained interaction mediated by antigen presenting cells. Antigen presenting cells, most commonly dendritic cells, macrophages, and B cells, present digested, protein fragments from exogenous pathogens on the surface protein MHC class II. If the antigen presented can form a sustained interaction with the T cell receptor, the T cell can become activated and differentiate into five main subtypes: T_H1, T_H2, T_H17, Treg, and T_{FH} [24, 25]. Each of these serve a different role in host defense.

T_H1 cells release antiviral cytokines including interferon-γ and tumor necrosis factor-α which help activate macrophage cells in the lungs and induce antiviral states. T_H2 cells bind to digested influenza antigens presented on antigen-presenting cells via MHC class II molecules. If the antigen matches the receptor on the T_H2 cell, the cytokines interleukin-4 (IL-4) and IL-13 can be produced to activate B cells [26].

T_H17 cells release IL-17 cytokines including IL-17A, IL-17F, and IL-22 [25]. These appear to alter the cytokine production of other cells altering the cellular response and promote mucus production. IL-17A promotes T_H2 cytokine release while IL-17F likely inhibits it. IL-17A can increase neutrophil infiltration into the lungs. These responses, particular the mucus production and IL-17A induced neutrophil recruitment, can induce immunopathology [27].

Regulatory T cells (Treg) release the immunosuppressive cytokines IL-10 and transforming growth factor-β. This action can help the host by reducing damage to host tissues as a result of the immune response (immunopathology). However, the suppression can also be

detrimental to the host by reducing pathogen clearance. In fact, some pathogens take advantage of this system by secreting proteins homologous to IL-10 or transforming growth factor-β and inducing the same suppression mechanisms [28]. During influenza infections, Treg cells have been shown to play important roles in reducing immunopathology by reducing monocyte and macrophage recruitment to the lungs early on by delaying chemokine production [29].

T follicular helper (T_{FH}) cells help create germinal centers that support B plasma cells for antibody production and secretion. Influenza induces T_{FH} populations to increase primarily in draining lymph nodes, the spleen, and to a lesser extent, the lungs. These cells induce B cell activating and germinal center formations by releasing IL-21, IL-4, and interferon-γ [30]. These actions are critical to the immune response as the innate immune system cannot fend off influenza by itself forever. Failure of the adaptive immune system to respond to the virus proves fatal. Although the immune response needs to be robust enough to eliminate influenza, it needs to be regulated to reduce damage to host tissues [29].

1.4 Methicillin Resistant *Staphylococcus Aureus* (MRSA)

MRSA is a gram-positive bacterium commonly found to be the causative agent of hospital-acquired infections. Like influenza, MRSA can enter the respiratory tract and begin infection in the epithelial region of the lungs. MRSA uses various compounds to latch onto epithelial cells lining the mucous membrane of the respiratory tract including clumping factor b and teichoic acids from the cell wall [31, 32]. An important difference between MRSA and influenza is that MRSA can survive and replicate extracellularly. However, it has also been shown that *S. aureus* can survive intracellularly after being engulfed by macrophages.

Macrophages can clear the intracellular bacteria if they were primed with interferon-γ before phagocytosis [33]. *S. aureus* has also been shown to be able to infect neutrophils and promote apoptosis in endothelial cells given the right gene cassettes [34, 35]. Because of MRSA's growth cycle, the immune system must be able to recognize the bacteria extracellularly and intracellularly as well as respond to the bacteria in these different environments.

1.5 MRSA Recognition and Immune Response

Like influenza, there are multiple pattern recognition receptors that can identify motifs on MRSA. Toll-like receptor 2, as a dimer complexed with toll-like receptor 1 or 6, can recognize gram positive bacterial cell wall components including lipoproteins and lipoteichoic acids. Toll-like receptor 9 can recognize the genome of phagocytosed MRSA that has undergone degradation in the endosome [8]. It has also been found that toll-like receptor 9 recognition of MRSA can trigger the type I interferon cascade [36]. C-type lectins can detect carbohydrate recognition domains found on the surface of MRSA [37]. Nod-like receptors can also recognize peptidoglycan on MRSA [38]. Recognition leads to the activation of transcription factors such as NF-κB or STAT1-STAT2 heterodimers and the release of antibacterial compounds as well as chemokines to attract immune cells [39, 40]. MRSA infections alone present many challenges to humans especially those who are patients in hospitals [41, 42]. However, pneumonia caused by MRSA infections are often associated with preceding viral infections [43].

1.6 Influenza Induced Secondary Bacterial Infections

Influenza-associated mortality is greatly increased by secondary bacterial infections most commonly from *Streptococcus* and *Staphylococcus* bacterium [43]. In fact, during the Spanish influenza pandemic, most of the 50 million deaths were caused by secondary bacterial infections [44]. The increased risk is generally during the end of the first week of influenza infection [43]. There are a few immunologic mechanisms that describe the increased risk. It has been shown that influenza can induce secondary necrosis processes resulting in 90% of alveolar macrophages to be depleted at the one-week mark. The macrophages that remained exhibited signs of necrosis. This suggests that the lack of viable macrophages in the alveolar space compromises clearing of the bacteria [45]. Another proposed mechanism is that influenza infection decreases the action of the T-helper cell 17 (T_H17) signaling pathway. Type I interferons produced in response to an influenza infection have been shown to inhibit the T_H17 signaling pathway. It does so by inhibiting the production of IL-23, a cytokine needed to activate $T_H17$pathway. This pathway is responsible for increasing the production of antimicrobial proteins and lipocalin-2. Without this pathway, the production of antimicrobial proteins decreases. Lipocalin-2 is responsible for sequestering iron, needed by MRSA to survive, away from the extracellular space in the lungs. Without antimicrobial compounds and lipocalin-2, the clearance of MRSA becomes more difficult. Other mechanisms involving the $T_H17$pathway likely exist, but more research is needed to elucidate these [43, 46, 47].

2.0 Interleukin-17

Interlleukin-17 (IL-17), originally named CTLA-8, was first discovered in 1993 by researchers looking for new molecules in immune responses [48]. More than a decade later, a new subset of T-helper (Th) cells was discovered that produces IL-17 as its primary effector cytokine [49]. By this time, researchers had already discovered that IL-23 induces Th cell IL-17 production as well as pro-inflammatory actions induced by IL-17 [50, 51]. IL-17 cytokines are also produced from innate immune cells including γδ T cells, natural killer cells, and Paneth cells [52]

2.1 IL-17 Cytokines

The IL-17 cytokine family has 6 members: IL-17A (originally CTLA-8), IL-17B, C, D, E, and F. IL-17A and F are the most studied interleukins and also the most closely related. They share about half of their amino acids and are both coded on the same chromosome [53]. The IL-17 cytokines are all capable of inducing proinflammatory effects in various tissues. This inflammation can help during infections, but if prolonged can contribute to chronic inflammation in autoimmune diseases [54].

2.1.1 IL-17A

IL-17A is secreted as a homodimer, held together by disulfide bonds, by activated T cells [55]. IL-17A can increase proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6 through the transcription factors NF-κB and AP-1 in macrophages [56]. Another study indicated that the increased IL-6 levels was not due to IL-17A increasing mRNA transcription. Instead, it's thought that IL-6 levels increase from higher IL-6 mRNA stability allowing for increased translation of IL-6 [57]. IL-17A has also been shown to play an important role in controlling infections. Hamada et. al. demonstrated that IL-17A plays an important role in early liver innate immune response to *Listeria monocytogenes* infection in the liver. Mice deficient in IL-17A had a higher bacterial burden in the liver and more liver damage than wild-type mice [58]. Huang et. al. showed that IL-17A signaling is important for fungal control with *Candida albicans* infection. Mice deficient in the IL-17A receptor had reduced neutrophil recruitment and uncontrolled fungal growth. This group of mice also died quicker than the wild-the mice [59]. IL-17A has also been found to have increased actions in tissues affected by autoimmune diseases.

Hueber et. al. studied the effects of AIN457, an IL-17A antibody that inhibits IL-17A actions in three autoimmune diseases: plaque psoriasis, rheumatoid arthritis, and uveitis. In all of these diseases, patients treated with AIN457 showed decreased inflammation and reduced symptoms [60]. In 2015, the US Food and Drug Administration approved AIN457, trade name Cosentyx, as a treatment for plaque psoriasis [61]. The same drug was tried to improve the conditions of patients with Crohn's disease. Unlike with the previous autoimmune diseases, patients receiving AIN457 did not show improvement in their symptoms, In fact, these patients experienced higher adverse effects than placebo including increased infections than the placebo

groups and worsening of symptoms [60]. Together, these studies show that IL-17A induced inflammation can be detrimental to individual's health, such as in plaque psoriasis and arthritis, but also beneficial, as seen in Crohn's disease.

2.1.2 IL-17B and IL-17C

IL-17B and C were discovered together in 1999 by researchers screening expressed sequence tags from public databases. IL-17A, B, and C share about 27% of their amino acids. IL-17B was found to be expressed in pancreas, small intestine, and adult stomach tissues. IL-17C was found in smaller quantities in adult prostate tissue and fetal kidney tissue. Li et. al. assayed the expression of TNF-α and IL-6 from different tissues to determine how the three (IL-17A, B, and C) molecules acted on different tissues. IL-17B and C, in contrast to IL-17A, did not induce IL-6 production from fibroblast cells. In a human leukemic monocytic cell like, IL-17B and C induced TNF-α in a time and dose dependent manner with higher TNF-α following a higher dose or longer time. This effect was not seen with IL-17A [62].

IL-17B is found to be increased in patients with pneumonia. It influences the release of the proinflammatory cytokine IL-8. The IL-17B receptor (IL-17RB) was found in both lung fibroblasts and bronchial epithelial cells. Upon IL-17B binding, only the bronchial epithelial cells expressed IL-8 and also in dose and time dependent manner. The amount of IL-8 expressed could be influenced by the presence of other signals. When both IL-17B and TNF-α were present, IL-8 expression increased. When interferon-γ was present, IL-8 expression decreased [63].

Song et. al. demonstrated the crucial need for IL-17C signaling during mouse intestinal infections with *Citrobacter rodentium*. This infection exhibits similar pathogenesis to human infections with *Escherichia coli*. The researchers disrupted IL-17C signaling by using mice deficient in the receptor for IL-17C (IL-17RE). Following infection, the IL-17RE deficient mice had uncontrolled bacterial growth, up to 100x more bacteria than wildtype, in the spleen, colon, and feces. This increased bacterial burden was coupled with decreased antibacterial peptides, proinflammatory cytokines, and chemokines. As a result, IL-17RE deficient mice lost more weight and died from the infections by day 13 in contrast to wildtype mice who lost no weight and remained alive [64].

Both IL-17B and C have been implicated as serving roles in human disease. Huang et. al. demonstrated that IL-17B signaling through its receptor, IL-17RB, can induce breast cancer. It does so by activating NF-κB to increase transcription of the antiapoptotic factor Bcl-2. By interrupting the signaling cascade with IL-17B antibodies or IL-17RB antibodies, tumor growth could be disrupted [65]. By examining paws of mice with arthritis, Yamaguchi et. al. demonstrated IL-17B can elicit inflammation seen in arthritis by increasing the proinflammatory cytokines of TNF-α, IL-1β, IL-6, and IL-23 in macrophages .IL-17C increased the same cytokines in macrophages except IL-6. Blocking IL-17B could halt the progression of arthritis [66].

2.1.3 IL-17 D

IL-17D, the last member of the IL-17 cytokine family to be discovered, was found by Starnes et. al. They discovered the gene after conducting rapid amplification of cDNA ends (RACE) PCR. It is most homologous with IL-17B (27% similar amino acid sequence). The

researchers found it to be expressed at high levels in tissues of skeletal muscle, lung, pancreas, heart, brain, and adipose. They also found low levels in bone marrow, leukocytes, lymph nodes, thymus, spleen, kidney, and fetal liver. Interestingly, it was expressed by resting $CD4^+$ T cells (Helper T cells)and resting $CD19^+$ B cells, but not when these cell types become activated. It was not expressed by $CD8^+$ T cells (Cytotoxic T Cells) [67].

In endothelial cells, IL-6 and IL-8 proinflammatory cytokines were increased following IL-17D incubation. IL-8 expression increased as a result of increased NF-κB activity. IL-17D was also shown to promote the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) by human umbilical vein endothelial cells (HUVEC) which do not normally release it [67].

O'Sullivan et. al. demonstrated IL-17D could be used to reduce cancer growth or rejection of a tumor in mouse lines. The effect was seen greatest in smaller tumors (25 mm^2) and had no effect on larger tumors (100 mm^2). The antitumor effect is believed to be from the recruitment of natural killer (NK) cells. Mice treated with anti-NK1.1 no longer rejected tumors in response to IL-17D [68].

IL-17D has been shown to increase in response to viral infections as a result of the transcription factor Nrf2. IL-17D serves a protective role that reduces scarring of host tissue and reduces weight loss. IL-17D did not directly protect cells from viral infection [69].

2.1.4 IL-17E

Lee et. al. discovered IL-17E in 2000 following examination of the human genome in GenBank . It shares about 18% of amino acids with IL-17A-C. IL-17E expression was found in low levels in many tissues including the kidney, lung and brain [70]. IL-17E, now renamed as

IL-25, has been shown to induce the production of the proinflammatory cytokines CCL-5, CCL-11, CXCL-8, and GM-CSF. When coupled with TNF-α, expression of CXCL-8 and GM-CSF increased more than with IL-17E alone. This effect was not observed with CCL-5 and CCL-11. In asthma patients' bronchial biopsies, IL-17E was elevated and so was eosinophil infiltration [71]. Considering CCL-5 and CCL-11 are both chemokines for eosinophils, this implicates an indirect role of IL-17E on the recruitment of eosinophils [72, 73]. These findings are also consistent with other IL-17E investigations that demonstrated after direct inhalation of IL-17E or IL-17E expression mediated by an adenovirus, caused eosinophil recruitment [74, 75]. IL-17E has been found to be released by T helper type 2 cells and by mast cells [76, 77]. Blocking IL-17E was shown to prevent hyperresponsiveness in allergic asthma [78].

Benatar et. al. demonstrated IL-17E as having antitumor effects reducing not only how large the tumor grows, but also decreases the rate of growth. It's believed the antitumor effects are a result of B cell responses induced by NF-κB activation that also promotes eosinophil infiltration into the tumor [79]. The effects of IL-17E are also thought to be why the cancer drug Virulizin, which triggers the release of IL-17E, causes tumor destruction [80].

Another role of IL-17E is with parasitic infections. Expulsion of parasitic helminth worms is mediated in part by T helper type 2 cell activity. IL-17E induces this activity contributing to parasite clearance [81, 82].

2.1.5 IL-17F

Starnes et. al. discovered IL-17F by examining RACE PCR on cDNA from the GenBank. After elucidating its structure, the researchers noted that IL-17F has a third pair of cysteines in a disulfide bridge, unlike the other members who contain 2 pairs of cysteines and 2 disulfide

bridges. Expression was found in activated CD4+ T cells and activated monocytes. There was no expression in resting CD4+ T cells, resting monocytes, CD8+ cells (both resting and activated), B cells or leukocytes. IL-17F also inhibited vascular formation in a dose dependent manner. IL-17F increased gene expression of transforming growth factor beta 1 (TGFβ1), TGFβ2, monocyte chemoattractant protein-1, IL-2, and lymphotoxin-β [83]. IL-17F shares about 50% of its amino acids with IL-17A. IL-17F is also secreted as a homodimer held by disulfide bonds [84].

Ishigame et. al. showed the effects IL-17F had on infection susceptibility. When both IL-17A and F were knocked out, mice exhibited larger abscess and higher bacterial burden of *Staphylococcus aureus* from mucocutaneous tissue than wildtype, IL-17A$^{-/-}$ alone, and IL-17F$^{-/-}$ alone. There was no difference in bacterial control between these groups during systemic infection with *S. aureus*. These results show that IL-17A and F play a role in controlling opportunistic infections. When challenged with *C. rodentium*, all knockouts (IL-17A$^{-/-}$, IL-17F$^{-/-}$, and IL-17A$^{-/-}$IL-17F$^{-/-}$) exhibited higher peak bacterial burdens, but eventually returned to wildtype level given enough time (day 28). Both the IL-17F$^{-/-}$ and IL-17A$^{-/-}$IL-17F$^{-/-}$ groups had more enlarged colons and spleens than wildtype and IL-17A$^{-/-}$ mice suggesting IL-17F is more important in the inflammatory response than IL-17A in this infection model [85].

During viral infection in the liver, IL-17F$^{-/-}$ had less liver damage accompanied with lower pathology scores than wildtype and IL-17A$^{-/-}$. However, there were no differences in viral burden between the groups meaning that the increased inflammatory action of IL-17F has more detrimental effects. Interestingly, IL-17A has a negative feedback loop with IL-17F production, but IL-17F does not exhibit this same effect with IL-17A [86].

A clinical study has shown that the drug bimekizumab, which neutralizes both IL-17A and IL-17F, reduced inflammation associated with psoriatic arthritis. When IL-17A was blocked, proinflammatory cytokines were reduced. IL-17F blocking had no significant changes in the proinflammatory cytokines. However, when both IL-17A and F were neutralized, the reduction in inflammation was more pronounced than IL-17A blocking alone [87].

2.2 IL-17 Receptors

The IL-17 receptor (IL-17R) family includes 5 members: IL-17RA-E. The receptors are found on the cell membrane and are found as heterodimers [50]. IL-17RA is found as one subunit in most of the IL-17 receptors while the second subunit determines receptor identity and function [88].

2.2.1 IL-17RA

The first IL-17 receptor was located after Yao et. al. found that the herpesvirus saimiri encodes a viral IL-17 protein that has a similar structure to IL-17A (57% identical) during infection. A fusion protein made up of the viral IL-17 and the Fc portion of human IgG, allowed the researchers to identify the receptor for IL-17A [89]. Toy et. al. demonstrated that for IL-17RA to respond to IL-17A, it must form a heterodimeric receptor with a second IL-17 receptor subunit, IL-17RC [90]. IL-17RA is expressed on most body tissues enabling for IL-17A signaling to impact a profound number of systems [89]. Because of their shared structure, IL-17F, like IL-17A, has been shown to bind to IL17RA, albeit with less binding affinity (1000 fold

difference) [91]. It has also been discovered that IL-17A and IL-17F can exist as heterodimers with one another changing the binding affinity to IL-17RA to be an intermediate affinity and subsequently, an intermediate cytokine release than IL-17A or IL-17F alone [92]. IL-17A binds the strongest to IL-17RA; IL-17F has the weakest binding affinity, and the IL-17A/F heterodimer has an intermediate binding affinity [93].

2.2.2 IL-17RB

IL-17RB has been shown to bind to IL-17B as well as IL-17E [70, 94]. IL-17RB forms a heterodimeric receptor with IL-17RA for IL-17E. What role each subunit contributes to downstream signaling remains unknown [95]. It's not known yet what IL-17RB interacts with to form the receptor responsible for binding with IL-17B. The receptor is expressed in a variety of tissues, but most highly in the kidney, liver, and small intestine. Binding of IL-17E led to the activation of NF-κB [70]. NF-κB activation relies on TRAF6 involvement. TRAF6 is bound to a cytoplasmic region of the IL-17E receptor and induces NF-κB when IL-17E binds [96]. The activation of NF-kB has been shown to require the adaptor protein Act1[74]. Act1 acts upstream of the NF-kB signaling cascade and is required to IKK. IKK phosphorylates and deactivates the NF-κB inhibitory molecule, IκBα, enabling NF-κB to move into the nucleus and begin transcription activation [97].

2.2.3 IL-17RC

IL-17RC expression appears to be highest in non-immune cells, including the liver, prostate, and thyroid, but lower in immune cells and hemopoietic tissues [91]. It has been shown

to only associate with IL-17RA [90]. Unlike IL-17RA, IL-17RC appears to bind both IL-17A and IL-17F with similar affinities. These data suggests that IL-17F signals through the IL-17RC subunit and therefore primarily has effects only on tissues expressing IL-17RC [91]. IL-17RA,RB, and RC all share a SEFIR domain that binds to Act1 to mediate the NF-κB activation pathway [98]. IL-17RB and RC do not have a CBAD domain found in IL-17RA. This domain can interfere the binding between Act1 to IL-17RA which can affect I-17RA signaling. This difference highlights why the receptors are heterodimers [99].

IL-17RA can bind IL-17A, E, and F, while IL-17RC only binds IL-17A and F. In this study, IL-17RC was knocked out as opposed to IL-17RA as it has fewer signaling ligands [99]. More information regarding the knockout is explained below in section 4.

2.2.4 IL-17RD

Rong et. al. found IL-17RD after examining the National Center for Biotechnology Information (NCBI) database based off of the IL-17RA's intracellular domain. They were able to show that IL-17RD interacts with IL-17RA independently of IL-17A binding. It can also interact with IL-17RB. Like IL-17RA, IL-17RD can interact with TRAF6 and may be responsible for helping to transmit IL-17 signaling. The intracellular domain of IL-17RD proved to be required for signal transduction. The exact order of interactions and subsequent signaling cascades are not known. The ligand for IL-17RD is not known either so its biological role remains unknown [100].

2.2.5 IL-17RE

IL-17RE forms a heterodimeric dimer with IL-17RA to act as a receptor for IL-17C. Song et. al. demonstrated that IL-17RE proved critical for mucosal immunity against *C. rodentium* as it enables the production of antibacterial compounds as well as proinflammatory cytokines. IL-17C signaling caused an increase in NF-kB pathways and mitogen-activated protein kinase pathways [64].

3.0 Lung Epithelium

The lung epithelium is a mechanical barrier that comprises a diverse number of cell types and functions to impede the entry and proliferation of pathogens. The main broad classes of cells are ciliated and secreting cells [101]. The predominant cells include goblet cells, serous cells, type I and type II cells, multiciliated cells, basal cells, and club cells [102]. Rig-like and Toll-like receptors are the primary means of pathogen recognition in the epithelium initiating the production of host immune responses [103]. Changes to cells encompassing this region from environmental conditions, including smoking and infections, have been attributed to the development of respiratory diseases including asthma and COPD [104, 105].

3.1 Goblet Cells

In healthy adult mice, goblet cell populations in the lung epithelium are very small [106]. In humans, the population is much more abundant. Their numbers decrease as you progress deeper into the respiratory tract. They make up about 11% and 10% in the bronchi and bronchioles, respectively. In the terminal bronchioles, they make up about 2% [107]. Goblet cells contribute to the pathogenesis of obstructive airway diseases including COPD and asthma in both humans and mouse disease models [108].

Mucous lines the lung epithelium aiding in pathogen defense and homeostasis of the lungs. Mucous is a gel-like liquid that contains water, ions, and macromolecules including proteins and carbohydrates. The mucous traps pathogenic microbes that can be destroyed by

antimicrobial peptides found in the mucous [109]. The mucous can also be cleared out of the system by ciliated cells that push the mucous into the nasopharynx where it is then swallowed [110].

Goblet cells, along with cells of the submucosal glands, provide these mucous secretions [111]. Although this function serves to protect the host from pathogens, it also contributes to the development of obstructive air diseases [109].

COPD patients experience a persistent inability to move airflow properly resulting from the accumulation of mucous. This excess mucous occurs in response to infections, inflammation, or inhalation of irritants including those found in cigarette smoke. These irritants cause remodeling of the lung airways and increase the amount of goblet cells present and therefore the amount of mucous secreted [112].

In asthmatics, an increase in goblet cell populations and subsequent mucous secretions also facilitates the pathogenesis of asthma [113]. Progenitor cells for goblet cells include basal and club cells. These cells induce the transcription factors SAM-pointed domain-containing ETS-like factor (SPDEF) and forkhead ortholog A3 (FOXA3) that facilitate goblet cell differentiation in response to infections, cytokines, and irritants [114]. In addition to increasing mucous secretions, the goblet cells also release proinflammatory cytokines that may help with asthma pathogenesis [115].

3.2 Brush Cells

Brush cells make up only 1-7% of the lung epithelium. They contain about 120-140 microvilli on their cell surface and are found throughout the lung epithelium with varying

densities. Their highest presence are in the first bifurcation of the alveolar ducts and the trachea [116]. Saunders et. al. determined the lineage for brush cells. Brush cells likely differentiate from basal cells and terminate differentiation as a brush cell. New brush cells can be regenerated following injury; however, their population size is relatively static in healthy adults [117]. Krasteva et. al. discovered the chemosensory function of brush cells. Brush cells contain receptors for bitter taste transduction. When a noxious substance triggers the receptor, brush cells release acetylcholine decreasing respiration [118].

During pulmonary infections or inflammatory reactions, such as those observed in asthmatics, inflammatory cells can generate lipid mediating leukotrienes. Cellular sources of leukotrienes include neutrophils, macrophages, and mast cells. When leukotrienes bind to their receptors, either the leukotriene B_4 or cysteinyl leukotriene receptors, inflammation is induced [119, 120]. The reaction includes recruitment of neutrophils, monocytes, lymphocytes, and eosinophils. As well as capillary leakage and edema [120]. Bankova et. al. demonstrated that brush cells are the dominant sources of IL-25 (IL-17E) in the tracheal epithelium making up 93% of IL-25 expressing cells of naïve mice. When the brush cells were exposed to leukotriene E_4, they responded through the cysteinyl leukotriene 3 receptor and increased IL-25 production. The authors believe this IL-25 production acted in an autocrine loop that expanded the brush cell population. This expansion could also be triggered by exposure to the common fungal aeroallergen *Alternaria*. This response lead to the development of type 2 inflammation suggesting a role for brush cells in the pathogenesis of allergen induced responses [121].

3.3 Alveolar Type I and II Cells

The epithelium of the alveolar space is dominated by type I and type II cells. Type I cells are flat making up 95-97% of the total surface area of the lung periphery and make up 8-10% of total lung cells [122, 123]. They are believed to differentiate from type II cells and are the last stage of differentiation [122]. Lack of type I cell development in new born mice results in respiratory failure, usually within minutes after birth [124]. Type II cells make up about 15% of total lung cells. They are cuboidal in shape and capable of self-renewal [125]. They produce surfactants to reduce surface tension within the alveoli and prevent alveolar collapse. Both cell types express pattern recognition receptors including toll-like and nod-like receptors. Type I cells express a pattern recognition receptor for damage-associated molecular patterns, the receptor for advanced glycation end products [111].

3.4 Multiciliated Cells

Multiciliated cells are column shaped cells that differentiate from basal cells and make up much of the human epithelium. They comprise 46% of the cells in the trachea and 73% in the small airway epithelium [126].

Their primary function is to clear mucous out of the airways and into the digestive tract with the actions of their cilia. Mucous traps pathogens in a sea of antimicrobial peptides including lysozyme and lactoferrin, as well as antiproteases and antioxidants to aid in host tissue protection. Clearing mucous either through ciliary movement or coughing are critical in maintaining the health of the host. Severe, and sometimes fatal, consequences develop from an

inability to clear the mucous that can create a breeding ground for pathogens as seen in cystic fibrosis patients [101].

Cystic fibrosis patients have a mutation in the chloride channel cystic fibrosis transmembrane conductance regulator that pumps chloride ions into the mucous. The increase in ion concentration in the mucous attracts water thereby hydrating the mucous and allowing for it to be cleared by ciliated cells. When this hydration becomes impaired, the mucous becomes too thick to move by cilia, decreasing mucous clearance. Patients have chronic lung infections and increased inflammation aiding in damage to the lungs and reducing lung function [101].

3.5 Basal Cells

Basal cells in human lungs are found throughout the respiratory system up to the terminal bronchiole while in mice, the cells are confined to the trachea [127, 128]. In humans, basal cells make up 34%, 27%, and 10% of cells in the trachea, large airways, and small airways, respectively [129]. In mice, the distribution of tracheal basal cells is between 5-10% [106]. Basal cells of both human and mouse lungs are stem cells that can both self-renew and differentiate into club and ciliated cells. Rock et. al. demonstrated that murine basal cells serve as progenitor cells for the development of the mouse trachea after birth, and for repair following injury [127].

Damage to basal cells from inhalation of irritants, specifically those found in cigarette smoke, may lead to the development of COPD. These irritants can lead to a change in normal gene expression that diminishes the capability of the basal cells to differentiate into a normal epithelium. In addition, basal cells contain the epidermal growth factor receptor that can respond to epidermal growth factor and amphiregulin. These two signaling molecules increase in smokers

causing consistent activation. This activation results in a change in the normal distribution of epithelial cells including an increase in basal cells and mucous cells as well as a decrease in cilia length. Together, these changes can act to damage the epithelium and lead to the development of COPD [129].

3.6 Club Cells

In humans, club cells are absent from the bronchi and increase as one travels further towards the respiratory bronchioles. In the bronchioles, club cells make up less than 1% of epithelial cells. In the terminal bronchioles, the population increases to about 11%. The highest concentration of club cells are is in the respiratory bronchioles where they make up about 22% of epithelial cells [107]. In mice, a larger percentage of epithelial cells are club cells. Karnati et.al. found that 64% of bronchiolar cells were club cells [130]. Fanucchi et. al. found an even higher percentage, 77%, of bronchiolar epithelial cells were club cells [131]. Zuo et. al. described a thorough list of genes expressed by club cells. Genes expressed include anti-inflammatory genes, chemokines for dendritic cells and neutrophils, complement and pattern recognition receptors, antibacterial compounds, protease inhibitors, and proteins for barrier function. Club cells also have viral receptors including those for influenza, adenovirus, and measles [132, 133]. Heaton et. al. demonstrated that influenza-infected club cells can survive viral replication and mediate increased proinflammatory cytokine expression contributing to immunopathology [132]. Further, club cells express genes whose mutations can result in a number of hereditary lung diseases including cystic fibrosis suggesting that club cells may be involved in the pathogenesis

of some lung diseases [133]. Club cells are major secretory cells with a dome shaped appearance along with many mitochondria and smooth and rough endoplasmic reticula [134].

One of the primary proteins secreted by club cells is secretoglobin 1a1(Scgb1 a1) or club cell secretory protein (CCSP). CCSP is thought to be an anti-inflammatory protein. Mice lacking CCSP have been shown to have increased total leukocytes, macrophages, and neutrophils in bronchoalveolar lavage fluid compared to wildtype mice after both groups are exposed to air after just one month. CCSP knockouts also had higher levels of apoptotic bronchial epithelial cells and alveolar septal cells along with an increase in small airway remodeling. Together, these data support the anti-inflammatory and protective roles of CCSP [135].

4.0 Cre-Recombination

The P1 bacteriophage is a virus commonly found to infect *E. coli* bacteria. Upon infection of a bacterium, P1 integrates its DNA genome into that of the bacterium with the use of a virally encoded integrase. This process allows the virus to be replicated each time the bacterium replicates and will continue until environmental conditions force P1 to induce lysogeny. At which point, P1 will be rapidly replicated and force the lysis of the bacterium.

P1 encodes the Cre recombinase protein. This enzyme catalyzes site specific recombination events on DNA between two *loxP* sites. *LoxP* sites are 34-bp sequences that contain two 13-bp inverted repeats where Cre binds. Strand exchange happens between these sequences in an 8-bp spacer region [136]. If the *loxP* sites are oriented in the same direction flanking a target gene, the target gene can be excised from the DNA and permanently removed from the genome. The excised DNA, including one *loxP* site, is circularized, and the original DNA strand is ligated together. Alternatively, if the *loxP* sites are facing different directions, the target gene is inverted. This inversion is not permanent, and can be reversed as two *loxP* sites remain in place [137].

Cre recombinase can be used to create gene changes that do not induce a total (global) gene knockout in an organism, but instead target the deletion to a particular organ, tissue, or specialized cell.

4.1 Cre-Recombination in this Study

This study used mice generated with cre-recombination. The Cre recombinase DNA segment was inserted into exon 1 of CCSP. By doing so, Cre expression would only occur when CCSP is being expressed as the two now share the CCSP promoter. Therefore, the Cre recombinase will only be expressed in club cells of the lung epithelium [138].

LoxP sites were inserted and flanked the gene encoding IL-17RC in the same direction. In cells expressing Cre (i.e. Club cells), a segment of the IL-17RC gene would be removed from the genome along with one of the *loxP* sites permanently. These cells, and their daughter cells, would no longer encode a gene for IL-17RC. IL-17RC was chosen as it only forms receptor complexes with the IL-17RA subunit and therefore only assists in transmitting IL-17A and IL-17F signaling [90]. Most work in the past have used IL-17RA$^{-/-}$ models. However, IL-17RA can associate with IL-17RB,IL-17RC and IL-17RE receptor subunits. IL-17RA is therefore used to transmit IL-17E (IL-25)IL-17A, IL-17F and, IL-17C signaling [64, 76, 90]. IL-17E mediates the T_H2 pathway and by choosing an IL-17RA$^{-/-}$ model, both T_H17 and T_H2 pathways would be affected [76]. Choosing the IL-17RC receptor subunit would only impact the T_H17 signaling pathway, minimizing disruption of other signaling pathways, and enabling clearer conclusions to be made. This genetic manipulation will allow us to determine what role IL-17RC has in club cells using the three infection models: primary influenza, primary MRSA, and influenza-MRSA super-infections.

4.2 Hypotheses

4.2.1 Primary Influenza

IL-17 has been shown to increase inflammation in the lungs leading to increased damage to host tissues increasing morbidity and mortality. One of the most damaging consequences of immunopathology in the respiratory tract is the development of acute lung injury or acute respiratory distress syndrome (a more severe form of acute lung injury) which have mortality rates as high as 20% and 45%, respectively. They are characterized by inflammatory cytokines, particularly from neutrophils, and fluid buildup leading to damage that compromises gas exchange in the alveoli [139].

IL-17RA signaling has been attributed to the development of acute lung injury. Crowe et. al. did extensive work on the role of IL-17 during primary influenza infections. They found IL-17A and IL-17F protein and gene expression levels to rise as early as 2 days post influenza infection. They determined that the increase in IL-17 was a result of γδ T cells, not αβ T cells. Not only did the number of γδ T cells increase, but also the percentage of γδ T cells that expressed IL-17. The percentage of IL-17 expressing αβ T cells did not change during the infection. Mice deficient in γδ T cells had reduced IL-17A mRNA expression at the 6 days post infection mark and decreased IL-17F mRNA expression as early as 2 days post infection. Together, this information demonstrates that the influenza infection induces an increase in IL-17A and IL-17F expression levels in a growing γδ T cell population [140].

Next, they characterized the effects of IL-17 signaling through the IL-17RA subunit by comparing the immune responses to influenza in wildtype and IL-17RA$^{-/-}$ mice. IL-17RA$^{-/-}$ mice

had reduced weight loss than wildtype mice and also lower mortality rates (80% for IL-17RA$^{-/-}$ mice and 20% for wildtype, at 2 weeks post infection). Viral burden at day 6 was higher in IL-17RA$^{-/-}$ mice than wildtype mice despite reduced morbidity factors [140].

IL-17RA$^{-/-}$ mice compared to wildtype mice experienced reduced inflammation determined from lower pathology scores. IL-17RA$^{-/-}$ also had reduced IL-17 signaling downstream cytokines, including IL-6, and chemokines, including the neutrophil attracting chemokines CXCL-1 and granulocyte-colony stimulating factor. Neutrophil recruitment decreased in IL-17RA$^{-/-}$ mice [140]. IL-6 has been suggested to reduce lung injury during influenza infection as a result of increased macrophage recruitment and activation along with a reduction in influenza induced epithelial cell apoptosis [141]. IL-17RA$^{-/-}$ did not have reduced macrophage recruitment. Further, when wildtype mice were infected and treated with an IL-6 neutralization antibody, there were no significant differences in disease severity indicators such as weight loss and total protein in bronchoalveolar lavage fluid [140].

In addition, neutrophils have been attributed to increasing acute lung injury. Neutrophils can induce damage to host tissue through the formation of neutrophil extracellular traps. Narasaraju et. al. showed that reactive oxygen species found in neutrophils, such as myeloperoxidase, can increase the production of neutrophil extracellular traps [142]. Neutrophils also increase oxidized phospholipids which can trigger toll-like receptor 4 signaling and contribute to acute lung injury [143]. Li et. al. showed that acute lung injury could be reduced in mice deficient in IL-17A or in mice treated with an IL-17A monoclonal antibody. These mice had higher survival rates and reduced weight loss than mice with active IL-17A [144]. Together, these data support that the decreased neutrophil recruitment observed in IL-17RA$^{-/-}$ mice reduces injury to host tissue [140].

In another study, Gopal et. al. demonstrated the increased immunopathology observed from IL-17 signaling during an influenza infection. Mice were treated with PBS or one of two mucosal TH17-inducing adjuvants: type II heat-labile enterotoxin (LT-IIb) and cholera toxin (CT). After treatment, the mice were infected with the highly virulent H5N1 influenza strain. Those receiving the adjuvants had higher morbidity characterized by an increase in weight loss, neutrophil accumulation, and lung inflammation than mice treated with PBS. This effect from the adjuvants was attenuated when the mice were treated with an antibody blocking IL-17RA or in IL-17RA knockouts [145].

Together, these data indicate that the damage observed during influenza infections can be attributed to the host IL-17 signaling immune response. Inhibiting this mechanism can reduce immunopathology to the host. Therefore, in my study, I expect to see reduced inflammation in IL-17RC-cre+ mice compared to IL-17RC-cre- mice following influenza infection indicated by a reduction in proinflammatory molecules. I also expect to see reduced morbidity factors indicated by less weight loss in IL-17RC-cre+ mice than wildtype mice. Lastly, the viral burden in IL-17RC-cre+ mice are expected to be higher than in IL-17RC-cre- mice, but the damage should be reduced in IL-17RC-cre+ mice indicated by reduced pathology scores.

4.2.2 Primary MRSA Infection

Job's syndrome, also known as Hyper IgE syndrome, affects patients who have mutations in *STAT3* that results in a deficiency of TH17 cells and subsequently IL-17. They frequently experience opportunistic infections including *Candida albicans* in the mouth, skin, or lungs. They also have infections with *Staphylococcus aureus* on the skin or in the lungs. Because IL-17 signaling induces the recruitment of neutrophils through granulocyte-colony stimulating factor.

This relationship suggests a potential protective role for neutrophils during fungal and bacterial infections mediated by IL-17 [146].

Kudva et. al. demonstrated the need for IL-17 signaling during pulmonary bacterial infections with *S. aureus* by infecting wildtype and IL-17RA$^{-/-}$ mice. 24 hours later, the lung bacterial burden was determined and IL-17RA$^{-/-}$ mice had significantly higher bacterial burdens. Neutrophilic chemokine granulocyte-colony stimulating factor and the proinflammatory cytokine IL-6 were decreased in IL-17RA$^{-/-}$ mice than wildtype mice [147]. The increased bacterial burden is consistent in data collected in our lab. Wildtype and IL-17RC$^{-/-}$ mice were infected with *S. aureus* and 24 hours later, the lung bacterial burden was measured. IL-17RC$^{-/-}$ mice had significantly higher, approximately four-fold, bacterial burden than wildtype mice (Supplemental Figure 1).

I expected IL-17RC-cre^{+} mice to have higher bacterial burdens in the lungs than IL-17RC-cre^{-} mice 24 hours post infection. I expect IL-17RC-cre^{+} mice to have reduced inflammation characterized by decreases in proinflammatory cytokines as well as reduced number of cells, particularly neutrophils, in the bronchoalveolar lavage fluid than IL-17RC-cre^{-} mice. IL-17RC-cre^{+} will have increased lung damage than IL-17RC-cre^{-} mice.

4.2.3 Influenza-MRSA Super-infection

Influenza is known to increase the risk of developing secondary bacterial infections [43]. Robinson et. al. have described a mechanism responsible for the increased risk and reduced ability to clear secondary bacterial infections.

The bacterial burden of wildtype mice with influenza-*S. aureus* super-infections have not only significantly higher bacterial burdens than *S. aureus* alone, but also reduced clearance of

bacteria. Whereas *S. aureus* alone (10_8 cfu) infected mice will clear the infection within 4 days, those with preceding influenza infections still have a high bacterial burden 5 days post infection [47].

The IL-17 protein level in mice following *S. aureus* infection lasts as long as 5 days post infection. When measuring the IL-17 protein level in mice with influenza preceding the *S. aureus* infection, the IL-17 protein levels disappear by day 3 post bacterial infection and remains at 0 as long as 5 days post bacterial infection [47].

Kudva et. al. demonstrated why the IL-17 levels do not increase as greatly following *S. aureus* infection in wildtype mice that initially had an influenza infection. IL-23 is a cytokine released by antigen presentation cells to induce T_H17 polarization and T_H17 cytokine production and release. When comparing the IL-23 levels of wildtype mice during *S. aureus* alone and influenza- *S. aureus* super-infections, IL-23 is significantly reduced during the super-infection. Interferon-a signaling, which is induced in response to influenza infections, is responsible for the decrease in IL-23. In mice deficient in the interferon-α receptor, IL-23 protein levels can increase during the influenza-*S. aureus* to levels observed in *S. aureus* alone infected mice. In addition, bacterial clearance during the super-infection in interferon-a receptor deficient mice returns to levels observed in *S. aureus* alone infected wildtype mice [147].

Robinson et. al. demonstrated that the inhibition of the IL-17 signaling pathway causes a reduction in antimicrobial peptide mRNA expression of RegIIIβ, lipocalin 2, and S100 A8. Each of these peptides contribute to bacterial clearance and control. Because of the suppression of the IL-17 signaling pathway by influenza-induced interferon-α signaling, these antimicrobial peptide levels during the super-infection are decreased compared to mice infected with *S. aureus* alone.

This suppression of antimicrobial peptides contributes to the decreased bacterial burden observed in super-infected mice than *S. aureus* alone infected mice [47].

Given that suppression of the IL-17 signaling mechanism during influenza infections increases morbidity factors following bacterial infection, I expect IL-17RC-cre^{+} mice will have increased bacterial burdens than IL-17RC-cre^{-} mice. Without the IL-17RC subunit, IL-17 signaling will be reduced causing a reduction in antimicrobial peptides and likely a reduction in inflammation. I also expect IL-17RC-cre^{+} mice will have increased lung damage than IL-17RC-cre^{-} mice following the super-infection.

5.0 Methods

5.1 Mice

The IL-17RC-ccsp-cre mice on C57BL/6 background were a kind gift from Dr. Jay Kolls, Tulane University, LA, and colonies were subsequently maintained under specific pathogen-free conditions. *In vivo* studies were performed on age matched adult male littermate mice that were either positive or negative for the ccsp-cre gene, unless otherwise indicated. All experiments were approved by the University of Pittsburgh IACUC.

Prior to use, all mice were genotyped by collecting tail-snips. These were lysed and DNA was extracted using the DirectPCR Lysis Reagent (Tail) kit (Viagen Biotech, Los Angeles, CA). The DNA was used to conduct PCR to identify that the *loxP* sites were placed flanking the IL-17RC gene and that the cre recombinase gene was present. The presence of l*oxP* sites flanking the IL-17RC gene was determined by using IL-17RC flox primers (Forward: GGA AGG CAT GAG GAT TGC AGA CT; Reverse: CAG ACT TTC CAG CTT CTT CAG GCT). The presence of cre recombinase in the CCSP gene was determined by using CCSP-cre primers (Forward: CGG TCG ATG CAA CGA GTG ATG AG; Reverse: ACG AAC CTG GTC GAA ATC AGT GCG. The DNA can then be southern blotted (2% agarose gel, 80-150 V, 1-1.5 hours). In homozygous floxed mice, a band should be observed at 287 bps while CCSP-cre+ mice should have a band observed at 400 bps.

5.2 Infections

5.2.1 Influenza A PR/8/34 H1N1 infection

Mice were infected with 100 PFU of Influenza A/PR/8/34 (influenza H1N1) in 50 µl of sterile PBS from a frozen stock. Infections were administered on isoflurane-anesthetized mice using oropharyngeal aspiration. Infected mice were incubated for 7 days. On the 7_{th} day, mice were sacrificed and bronchoalveolar lavage (BAL) fluid, lungs, and serum were collected. Quantitative real-time RT-PCR was used to determine viral burden on lung RNA based on the amount of viral RNA (M protein) [16]. The primers and probe used were:

Forward primer: 5' GGACTGCAGCGTAGACGCTTT 3'

Reverse primer: 5' CATCCTGTTGTATATGAGKCCCAT 3'

Probe: 5' 6FAM-CTMAGYTATTCWRCTGGTGCACTTGCC-BHQ 3'

5.2.2 MRSA USA300

Mice were infected with $1x10_8$ cfu (during primary infection) or $5x10_7$ cfu (during secondary infection) of MRSA USA300 in 50 µl of sterile PBS from a frozen stock. A lower dose of MRSA was used during secondary infections as mice are more susceptible to bacterial infections if they have an influenza infection. Infections were administered on isoflurane-anesthetized mice using oropharyngeal aspiration. In primary bacterial infections, infected mice were incubated for 24 hours. 24 hours was chosen as previous work in our lab has shown that wildtype mice can clear the bacteria within 48 hours. At 24 hours, mice were sacrificed and bronchoalveolar lavage fluid, lungs, and serum were collected. Lung homogenate was used to

grow bacteria on *Staphylococcus aureus* growth plates and incubated over night to determine bacterial burden. In secondary bacterial infections, mice were infected with influenza as described before. On day 6, mice were then infected with MRSA as described before. 24 hours post MRSA infection mice were sacrificed and samples collected as described before [148].

5.3 Lung inflammation measurement

5.3.1 Bronchoalveolar lavage fluid measurement

After the mice were sacrificed, bronchoalveolar lavage (BAL) fluid was collected by flushing the lungs of mice with 1 mL of sterile PBS. The BAL was used to make slide smears using a cytospin. The slides were stained with Protocol Hema 3 staining (Fisher Scientific, Kalamazoo, MI) and used for differential cell counts. Stained slide smears were observed under light microscope and the cell types (neutrophils, lymphocytes, and macrophages) were determined based off of cell size, nuclear shape, and staining pattern.

5.3.2 Gene expression via RT-PCR

Middle and accessory lobes of the right lung were collected and snap frozen with liquid nitrogen. These were homogenized mechanically, and RNA was extracted using the Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, Ca). Gene expression was measured using RT-PCR with Taqman primer and probe steps (Applied Biosystems, Foster City, Ca). The data was calculated using the delta-delta CT method, as described before, and were normalized

to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as specified in the figure [149].

5.4 Statistical Analysis

All analyses were performed using GraphPad Prism Software. Two-tailed Student's t-test was used for analysis and differences were deemed significant if $p \leq 0.05$. All figures represent data as mean±SEM.

6.0 Results

6.1 Primary Flu Infection

6.1.1 No difference in weight loss or viral burden in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

The first infection model we conducted our tests on were primary influenza infections. IL-17RC-cre+ and IL-17RC-cre- mice were infected with 100 pfu of influenza in 50 mL of sterile PBS (day 0) and were sacrificed on day 7. Based off of other works that show IL-17 signaling contributes to immunopathology during viral infections, I first analyzed the weight loss and viral burden between the IL-17RC-cre+ and IL-17RC-cre- [86, 140]. Weight loss can serve as an indicator of infection severity. Those who lose more weight tend to not be controlling the infection as well as those who lose less weight. Viral burden was measured using RT-PCR based off of viral gene expression levels of influenza matrix (M) protein. There were no differences observed between the two groups in terms of weight loss or viral burden at the one-week mark (Figure 1 A and B).

6.1.2 IL-17A levels and downstream proinflammatory cytokine expression levels in the lungs are the same between IL-17RC-cre+ and IL-17RC-cre- mice

Next, we wanted to see if there were any changes in IL-17A production in the lungs and if there were any effects from downstream IL-17 signaling at the one-week mark. First cytokine

we looked at was IL-23, which induces the production of IL-17 [51]. There were no differences between the IL-17RC-cre+ and IL-17RC-cre- mice for IL-23 expression in the lungs(Figure 2A). Likewise, the amount of IL-17A trends higher in IL-17RC-cre+ but was not significantly different with IL-17RC-cre- mice levels (Figure 2B). IL-17A is known to increase proinflammatory cytokines such as TNF-α and IL-6 [56, 57]. Both of these inflammatory markers were expressed equally in IL-17RC-cre+ and IL-17RC-cre- mice (Figures 2C and 2D).

6.1.3 No change in neutrophil chemokines or neutrophilic activity associated gene expression levels in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

Previous studies have shown that IL-17RA signaling increases neutrophil recruitment during a fungal infection and a primary influenza infection [59, 140]. The neutrophil attractant chemokine CXCL-1 did not differ between IL-17RC-cre+ and IL-17RC-cre- mice (Figure 3A). Two neutrophil activity markers, neutrophil elastase and cathepsin G, did not differ between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 3B and 3C) [150, 151].

6.1.4 No difference in cellular makeup of bronchoalveolar lavage fluid between IL-17RC-cre+ and IL-17RC-cre- mice

Bronchoalveolar lavage (BAL) fluid was collected from the mice one-week post infection and used to develop cell smears to measure the number and type of cells present in the lung alveoli. The cellular composition of the BAL fluid did not differ between IL-17RC-cre+ and IL-17RC-cre- mice as shown by no changes in the percentage of neutrophils, lymphocytes, or macrophages (Figures 4A-E).

6.2 Primary MRSA Infection

6.2.1 No difference in lung bacterial burden or weight loss between IL-17RC-cre+ and IL-17RC-cre- mice

The next infection model we studied was a primary MRSA infection. IL-17RC-cre+ and IL-17RC-cre- mice were infected with 1 x 10^8 cfu of MRSA in 50 µl of sterile PBS (day 0) and sacrificed 24 hours later. Previous studies have shown that decreased IL-17A signaling results in higher bacterial burden the liver following to *Listeria monocytogenes* [58]. Other studies have observed reduced bacterial clearance in *S. aureus* when IL-17A and F signaling is blocked (as a result of knocking out both IL-17A and F) and higher bacterial burdens in response to *C. rodentium* when IL-17A or IL-17F were knocked out [85]. Based off of this information, we hypothesized that the IL-17RC-cre+ mice would have increased bacterial burden and potentially more weight loss.

Following the MRSA infection, there were no differences in bacterial burden between IL-17RC-cre+ and IL-17RC-cre- mice after incubation of MRSA collected from the upper right lung lobe (Figures 5B). There were also no differences between IL-17RC-cre+ and IL-17RC-cre- mice weight loss (Figure 5A).

6.2.2 No difference in gene expression levels of IL-17A and downstream proinflammatory cytokines in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

The next gene expression levels examined were those of IL-17A and downstream signaling induced genes. IL-17A expression levels were measured to see if IL-17RC-cre+ and IL-

17RC-cre- mice both were able to induce the same amount of IL-17A. The proinflammatory cytokines released after IL-17A signaling were also measured to see if there were any defects in production from a lack of IL-17A signaling in IL-17RC-cre+ mice. Both groups had the same expression level of IL-17A and the proinflammatory cytokine IL-6 (Figures 6A and 6C). IL-17RA and TNF-α trended to be lower in IL-17RC-cre+ mice, but were not significantly different (Figures 6B and 6D; TNF-α p-value=0.0807).

6.2.3 No difference in recruitment of neutrophils, lymphocytes, or macrophages to the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

Using BAL fluid, slide smears of the immune cells were made and the number of total cells and number of neutrophils, lymphocytes, and macrophages were enumerated. Reduced IL-17A signaling has been shown to cause less CXCL-5, neutrophil chemokine, and impair neutrophil recruitment [152]. There were no differences in the number of total cells, neutrophils, lymphocytes, or macrophages between IL-17RC-cre+ and IL-17RC-cre- mice following infection (Figures 7A-D). The cellular composition of the BAL fluid was the same IL-17RC-cre+ and IL-17RC-cre- mice (Figures 8A-E). No difference in the neutrophil activity markers neutrophil elastane and cathepsin g, nor any differences in the neutrophil chemokine CXCL-5 (Figures 9A-C).

6.2.4 IL-17RC-cre+ have reduced mannose receptor c type 2 gene expression in the lungs, but no changes in other M2 macrophage markers or M1 macrophage marker Nos2

There are two broad classes of macrophages: M1 and M2 macrophages. M1 macrophages mainly phagocytose microbes while M2 macrophages are involved in host tissue repair. Nitric oxide synthase 2 (Nos2) is used as a marker for M1 macrophages. Mannose receptor c type 2 (MRC2), arginase 1 (Arg1), and macrophage receptor with collagenous structure (MARCO) are M2 macrophage markers [153]. There is no difference in Arg1 or MARCO gene expression levels between IL-17RC-cre+ and IL-17RC-cre- mice following MRSA infection (Figures 10B and D). However, IL-17RC-cre+ mice have less expression of the M2 macrophage marker, MRC2 (Figure 10C, p=0.0176). The M1 macrophage marker Nos2 expression trended to be lower (Figure 10A, p-value= 0.0669).

6.2.5 No differences in antimicrobial peptide expression in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

The last set of gene expression levels tested were those of the antimicrobial peptides lipocalin 2 (Lcn2), calprotectin subunit S100A8, and regenerating islet derived protein 3β (Reg3β) that are produced to aid in bacterial clearance [154]. No differences in the expression levels of these proteins were seen between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 11A-C).

6.3 Influenza-MRSA Super Infection

6.3.1 No differences in weight loss, viral burden, or bacterial burden between IL-17RC-cre+ and IL-17RC-cre- mice

The last infection model used is an influenza-MRSA super-infection. IL-17RC-cre+ and IL-17RC-cre- mice were infected with 100 pfu of influenza in 50 μL of sterile PBS (Day 0). On day 6, the mice were infected with 5 x 10_7 cfu of MRSA in 50 μL of sterile PBS. The mice were sacrificed 24 hours later (Day 7). This model mimics the secondary bacterial infection patients can experienced superimposed on their current influenza infection. The risk of developing a secondary bacterial infection is greatest one week after influenza infection [43].

Like before, weight loss, viral burden, and bacterial burden were measured to determine if the knockout had any effect on these disease severity indicators. It's been shown that interrupting IL-17 signaling diminishes bacterial clearance [58, 85]. It's also been shown that influenza infection before a bacterial infection diminishes bacterial clearance. Interferons produced in response to influenza can inhibit IL-23 release, which promotes the release of IL-17 from γδ T cells, which attenuates the IL-17 signaling mechanism [43]. With this in mind, we hypothesized the IL-17RC-cre+ mice would experience greater morbidity. When weight, lung viral burden, and lung bacterial burden were measured, no significant differences were observed between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 12A-C).

6.3.2 No differences in IL-17A or TNF-α expression in the lungs, nor any differences in total cells in BAL between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17A expression did not differ between IL-17RC-cre+ and IL-17RC-cre- mice nor did the downstream TNF-α expression differ (Figures 13A and B). The total number of cells enumerated from the BAL did not differ between IL-17RC-cre+ and IL-17RC-cre- mice either (Figure 13C).

6.3.3 No differences in the macrophage and neutrophil marker expressions in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

The M1 macrophage marker, Nos2, did not have different expression levels in the lungs between the groups following infection (Figure 14A). There were no differences in expression levels of the M2 macrophage markers MARCO, MRC2, or Arg1 in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 14B-D). There were no differences in expression levels of the neutrophil chemokine CXCL5 and no difference in expression of the neutrophil marker ELANE in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 15A and 15B).

6.3.4 No difference in the expression of interferons or downstream interferon induced gene expression levels in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

Interferon-β and λ expression levels in the lungs did not differ between IL-17RC-cre+ and IL-17RC-cre- mice nor did the interferon-stimulated gene Mx1 expression in the lungs differ between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 16A-C).

6.3.5 No differences in antimicrobial peptide expression in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

The antimicrobial Lcn2 expression levels in the lungs trended to be lower in IL-17RC-cre+ mice, but was not statistically different (Figure 17A, p-value= 0.0969). Reg3β expression levels in the lungs did not differ between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 17B).

6.3.6 Influenza-MRSA super-infection increases total number of cells in BAL fluid in IL-17RC-cre- mice compared to primary MRSA infection in IL-17RC-cre- mice

When comparing the total number of cells in BAL fluid from Il-17RC-cre- mice in primary MRSA and influenza-MRSA super-infections, the influenza-MRSA super-infection resulted in a greater number of cells in BAL fluid (Figure 18; p-value = 0.0011). There were no changes in total BAL cell count between IL-17RC-cre+ mice (Figure 18).

7.0 Discussion

This study sought to understand what effects IL-17RC signaling has on club cells of the lung epithelium during three infection models: primary influenza, primary MRSA, and influenza-MRSA superinfection. The experimental mice had the IL-17RC receptor removed via cre-recombination in cells that express club cell secretory protein-16 (CCSP). This knockout is confined strictly to club cells of the lung epithelium inhibiting IL-17A and IL-17F signaling effects on the club cells.

IL-17A has been shown to induce inflammation that results in immunopathology increasing damage to host tissues. In three autoimmune diseases: plaque psoriasis, rheumatoid arthritis, and uveitis, IL-17A inhibition has reduced the damaging effects of inflammation to the tissues affected by these diseases [60]. IL-17F increases inflammation in concert with IL-17A signaling to produce the immunopathology observed in psoriatic arthritis [87] .

During viral infections, these two cytokines have shown to increase inflammation that may result in enhanced viral clearance but increases tissue damage. These data suggests that the inflammatory actions resulting from the signaling cascades hurt's the host more than the pathogen [86, 140]. Based on these data, we expected IL-17RC-cre^{+} mice to exhibit less signs of morbidity. Surprisingly, no differences in morbidity were seen between IL-17RC-cre^{+} and IL-17RC-cre^{-} mice. Weight loss and viral burden were the same as were downstream IL-17 proinflammatory molecules (Figures 2A-D). Previous studies showed that IL-17A signaling enhances neutrophil recruitment [59, 140]. When the neutrophil attractant chemokine CXCL-1 was measured, its expression levels were not different between IL-17RC-cre^{+} and IL-17RC-cre^{-} mice one week after influenza infection (Figure 3A). Likewise, there were no changes in

neutrophil activity markers neutrophil elastase and cathepsin G, nor differences in the cellular composition of collected BAL fluid (Figures 3B-C, 4A-C). These data suggests that IL-17RC mediated signaling in club cells does not significantly affect the immune response to influenza nor any effects resulting from influenza infection.

A possible explanation for no changes is that other cells present in the lungs can secrete proinflammatory cytokines making up for the decreased expression levels from club cells. Mast cells and eosinophils can both secrete CXCL-1 allowing for chemotaxis of neutrophils to the lungs [155, 156]. Once in the lungs, these neutrophils can induce the immunopathology associated with their activity [142, 143].

Unlike during influenza and other viral infections, IL-17A and IL-17F signaling proved to aid the host in bacterial control. IL-17A and IL-17F both reduced inflammation during *C. rodentium* intestinal infections [85]. IL-17A signaling has also been shown to improve host tissue protection during Crohn's disease, an autoimmune disease that has been correlated with a dysregulation of the intestinal microbiome [60, 157]. In *K. pneumonia* infections, IL-17RA and IL-17RC signaling in the club cells garnered better bacterial control then without the signaling [152]. Based off of these results, we predicted that IL-17RC-cre^{+} mice would have increased morbidity and bacterial burden than IL-17RC-cre^{-} mice during a primary MRSA infection.

Both groups had similar weight loss 24 hours after infection (Figure 5A). This result was not entirely surprising as the infection window was short: 24 hours. However, there were no differences in MRSA bacterial burden in the lungs between IL-17RC-cre^{+} and IL-17RC-cre^{-} mice (Figures 5B). IL-17A and IL-17RA expression levels were the same 24 hours after infection as were the downstream proinflammatory molecules IL-6 and TNF-α (Figures 6A-D).

The number and types of cells recruited to the lungs did not change when examining the cellular composition of BAL fluid (Figures 8A-E). Previous studies indicated IL-17A signaling increases expression of the neutrophil chemokine CXCL-5 [152]. IL-17RC-cre+ mice did not have changes in this chemokine or neutrophil markers (Figures 9A-C). The M1 macrophage marker Nos2 did not differ between IL-17RC-cre+ and IL-17RC-cre- mice nor did the M2 macrophage markers MARCO and Arg1 (Figures 10A,B, and D). IL-17RC-cre+ mice did have decreased expression of the M2 macrophage marker MRC2 (Figure 10C, p=0.0176). Antimicrobial peptide production did not differ between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 11A-C). Decreased MRC2 could impair host tissue repair, however with no other differences observed in the morbidity indicators, this difference likely does not matter at the 24-hour time point. Similar antimicrobial production as well as phagocyte activity markers correlate with a similar bacterial burden as there are no overt differences in bacterial clearance mechanisms. The phagocytes, macrophages and neutrophils, both can produce S100A8 during bacterial infections and neutrophils can generate Lcn2 [158-160]. With no differences in cell numbers and composition, the expression of these antimicrobials is not surprisingly the same.

Influenza infections have been shown to cause a deficit in the immune response to bacterial infections in the lung increasing the likelihood of a secondary infection [43]. One particular mechanism is the inhibition of the TH17 signaling pathway brought on by the suppression of IL-23 by type I interferons. This inhibition causes a deficit in antimicrobial production and compromises bacterial clearance [43, 46, 147]. Given that the IL-17 pathway is important in bacterial clearance, we expected the IL-17RC-cre+ mice to have increased bacterial burden and greater weight loss. However, there were no differences in viral burden, bacterial burden, or weight loss between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 12A-C). IL-17A

and downstream proinflammatory cytokine TNF-α all had equal expression between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 13A-B). The total number of cells in the BAL fluid were not significantly different (Figure 13C).The macrophage markers for both M1 and M2 macrophages did not significantly differ between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 14A-D). The neutrophil chemokine CXCL5 and the neutrophil activity marker ELANE did not differ between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 15A and 15B). These data indicate that IL-17A and F signaling in club cells does not significantly impact the recruitment or activity of immune cells to the lung. The amount of interferon expression levels and downstream interferon induced Mx1 expression are not different between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 16A-C). Expression of the antimicrobial Lcn2 and Reg3β were not different between IL-17RC-cre+ and IL-17RC-cre- mice (Figures 17A and 17B). Together these data indicate that IL-17A and F signaling in club cells does not significantly impact the production of antimicrobials in the lungs.

Furthermore, when comparing the total number of cells in BAL fluid between the MRSA and influenza-MRSA super-infections, there was a significant increase in total number of cells in IL-17RC-cre- mice during influenza-MRSA super-infection than in IL-17RC-cre- mice (Figure 18). There were no changes in total number of cells in BAL fluid between IL-17RC-cre- mice during MRSA or influenza-MRSA super-infection (Figure 18).

Altogether, this study has shown that IL-17A and IL-17F signaling in club cells of the lung epithelium likely does not contribute significantly during primary influenza, primary MRSA, or influenza-MRSA superinfections. In all infection models, there were no changes in the immune responses between IL-17RC-cre+ and IL-17RC-cre- mice. Immune cell recruitment was not affected nor were immune cell activity markers following each infection. Different

signaling mechanisms including interferons, IL-17A expression levels, and proinflammatory cytokines did not differ between IL-17RC-cre+ and IL-17RC-cre- mice . Most importantly, morbidity factors such as weight loss, viral burden, were bacterial burden were not different between IL-17RC-cre+ and IL-17RC-cre- mice. Affecting IL-17A or IL-17F signaling in club cells will most likely not prove to have therapeutic outcomes in these infections.

However, there are important caveats to these interpretations that extend beyond concluding that IL-17RC signaling in club cells does not significantly affect the host immune response. There are a number of potential reasons why no differences were observed between IL-17RC-cre+ and IL-17RC-cre- during these infection models.

The first involves the data collection and sample size. The data collection of this study was severely impacted from the Covid-19 pandemic. It forced the premature shutdown of the study. Although I had more infection studies planned and more data to collect from previous studies, no more data could be collected. The variation in samples sizes between and within infection models of this study resulted from the shutdown. Given the low numbers, it's possible that the small sample size does not truly reflect the general population. IL-17RC-cre+ mice had trends towards reduced proinflammatory cytokine TNF-α, M1 macrophage marker Nos2, during primary MRSA infections (TNF-a p= .0807; Nos2, p= 0.06600) and reduced antimicrobial compound Lcn2 during influenza-MRSA super-infection (p= 0.0969). It's possible that these may have reached significantly different if given more data. Further studies will need to be done to truly determine whether or not IL-17 signaling in the club cells impacted these factors.

It's possible that the gene deletions did not work as effectively as we thought. There are no direct data that support the gene deletions were not as expected; however, there are couple of tests that can be done to determine if the gene deletion worked. The IL-17RC mRNA expression

can be determined using RT-PCR and compare the IL-17RC expression rates between IL-17RC-cre+ and IL-17RC-cre- mice. If the gene deletion worked as expected, IL17RC-cre+ should have reduced IL-17RC expression compared to IL-17RC-cre- mice. Immunohistochemistry could be used with antibodies that bind to CCSP, therefore labeling club cells, and IL-17RC [161]. When the club cells are observed, cells from IL-17RC-cre+ should have no IL-17RC antibody staining on the cell membrane while IL-17RC-cre- should have IL-17RC antibody staining on the cell membrane.

Another possibility is that IL-17 signaling may be occuring in the club cells despite the absence of the IL-17RC subunit. Recently, Goepfert et. al. discovered that IL-17RC can bind to IL-17A and IL-17F in the absence of IL-17RA. Whether or not the interaction leads to intracellular signaling is unknown [162]. Although no studies have pointed to the ability of IL-17RA to bind to IL-17 cytokines independently of other receptor subunits, it raises the possibility that IL-17RA may share this ability. However, even if IL-17RA could effectively transmit a signaling cascade from IL-17 activation, it's not known if this signaling has significant effects on the immune response. Numerous studies have been conducted exploring the effects of IL-17 receptor subunit deletions and they have shown that there are significant changes in the immune response. These suggest that even if there is independent signaling occuring through one IL-17 subunit (IL-17RA or IL-17RC), it cannot act as a substitute for whole receptor, IL-17RA/RC, signaling.

To determine if the club cells of IL-17RC-cre+ mice have changes in IL-17 signaling response, club cells from these mice and IL-17RC-cre- mice can be selected for with flow cytometry based off of CCSP immunoreactivity [161]. These cells can then be cultured in the presence of IL-17 and antibodies for IL-17 signaling induced genes. If the deletion is correct and

the signaling is disrupted in IL-17RC-cre^{+} mice, their club cells should have reduced protein production, and therefore reduced antibody binding than IL-17RC-cre^{-} mice.

In addition, these studies should be repeated by introducing exogenous IL-17 or a combination of IL-1β and IL23 (this combination triggers IL-17 release from γδ T cells) intranasally [152]. Any differences between the mice (IL-17RC-cre^{+} and cre^{-}) would be attributed to lack of IL-17 response, as a result of the receptor missing, and not IL-17 production.

8.0 Figures

8.1 Primary Flu Infection

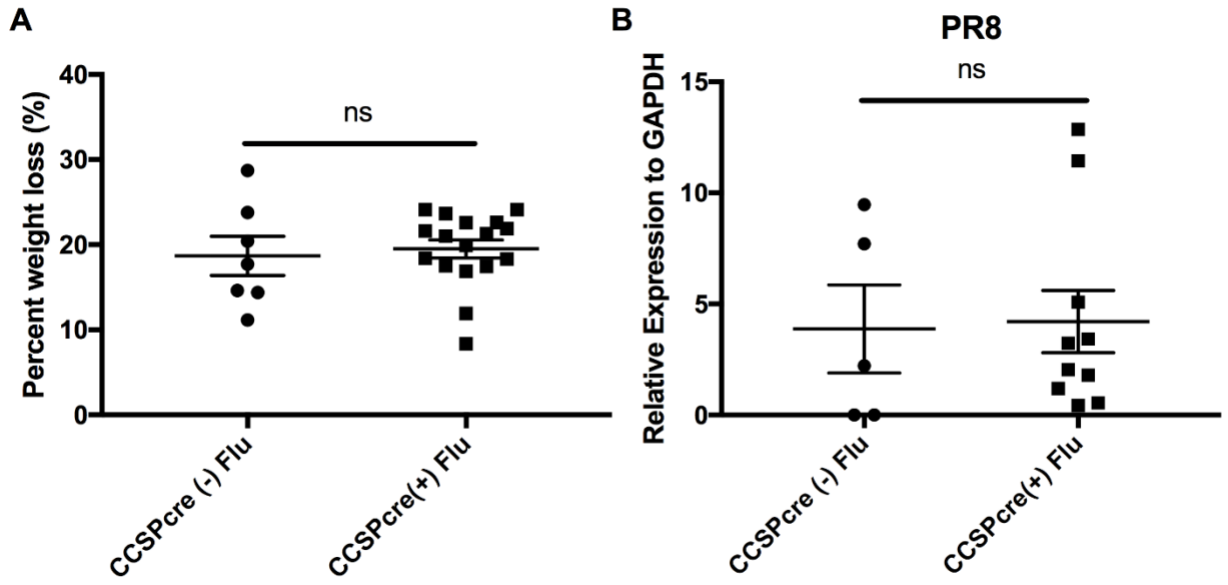


Figure 1. No difference in weight loss or viral burden in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, , 6-8 weeks old mice were infected with 100 pfu of influenza (N=10 per group). (A) Weight loss was determined from comparing weight at time of infection (Day 0) and on day of sacrifice (Day 7) n= 7 and 17 for IL-17RC-cre- and IL-17RC-cre+ groups respectively. (B) Viral burden was measured by influenza M protein expression in lung by RT PCR, N=5 and 10 for IL-17RC-cre- and IL-17RC-cre+ groups respectively. Data are represented as mean±SEM, two tailed Student's *t* test, ns- not significant

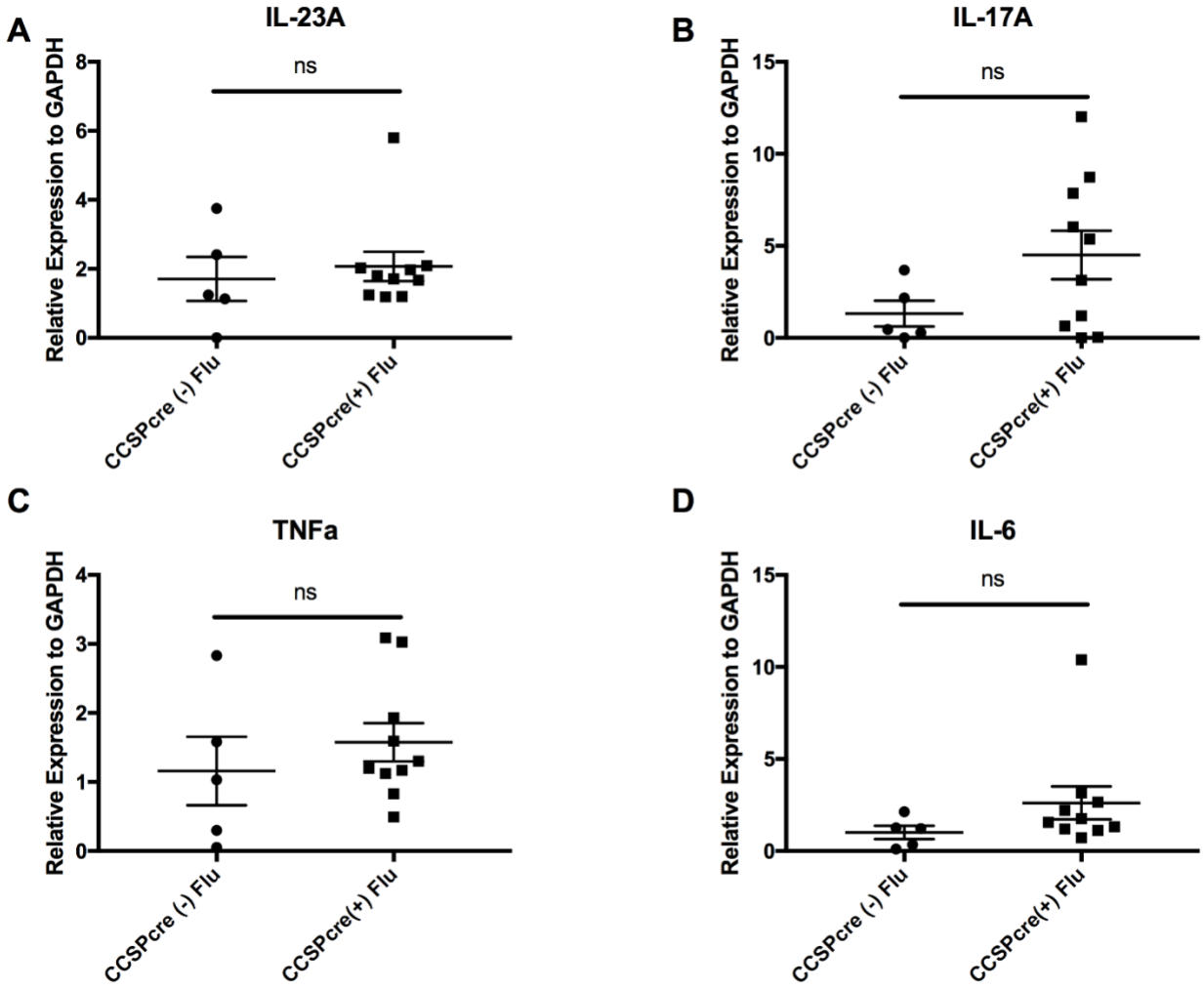


Figure 2. IL-17A levels and downstream proinflammatory cytokine expression levels in the lungs are the same between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with 100 pfu of influenza. (A-D) IL-23A, IL-17A, TNF-α, IL6 expression was measured in whole lung by RT-PCR, N=5 and 10 for IL-17RC-cre- and IL-17RC-cre+ groups respectively. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

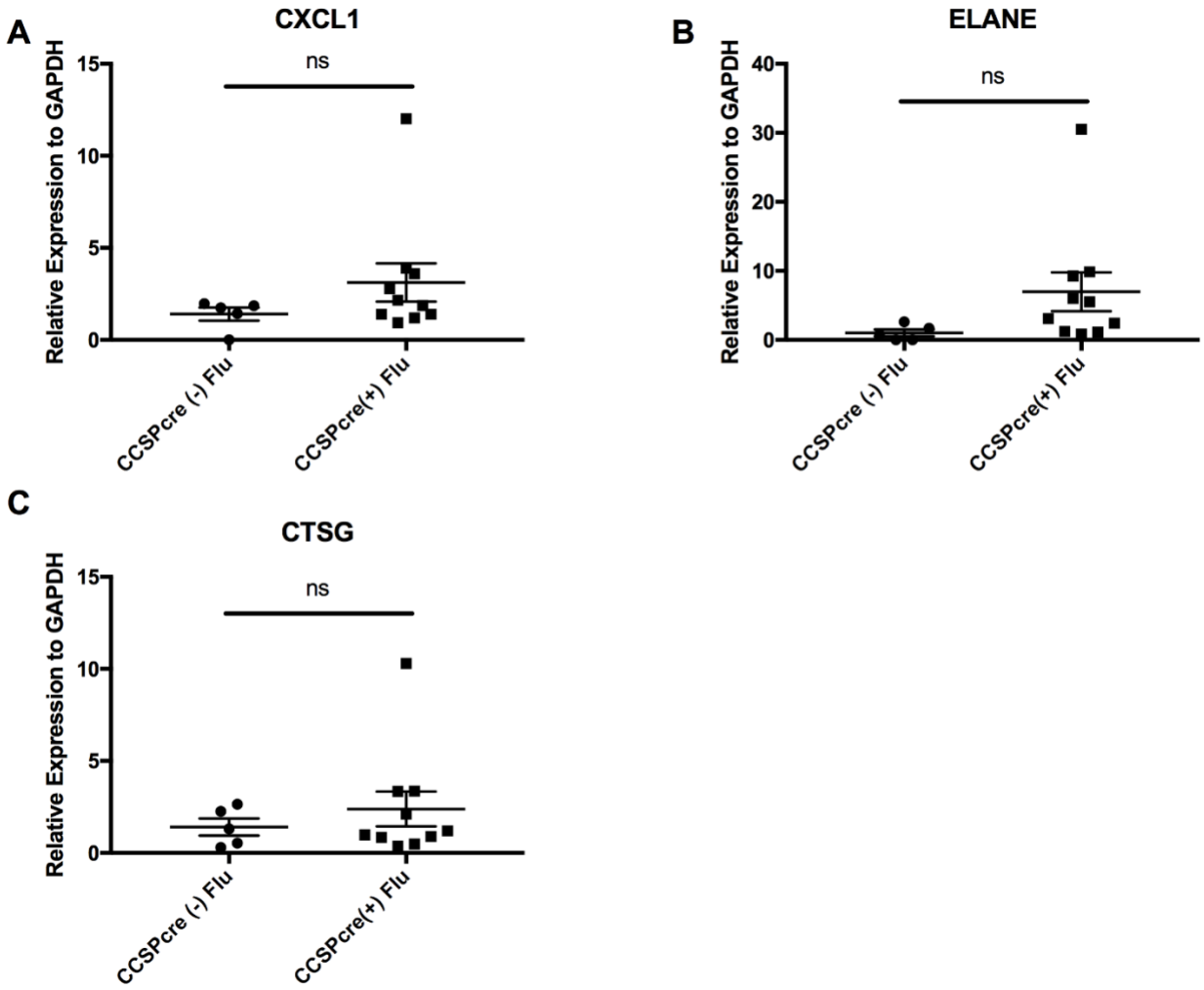


Figure 3. No change in neutrophil chemokines or neutrophilic activity associated gene expression levels in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with 100 pfu of influenza (N=10 per group). (A-C) CXCL1, neutrophil elastane (ELANE), cathepsin g (CTSG) expression was measured in whole lung by RT-PCR, N=5 and 10 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

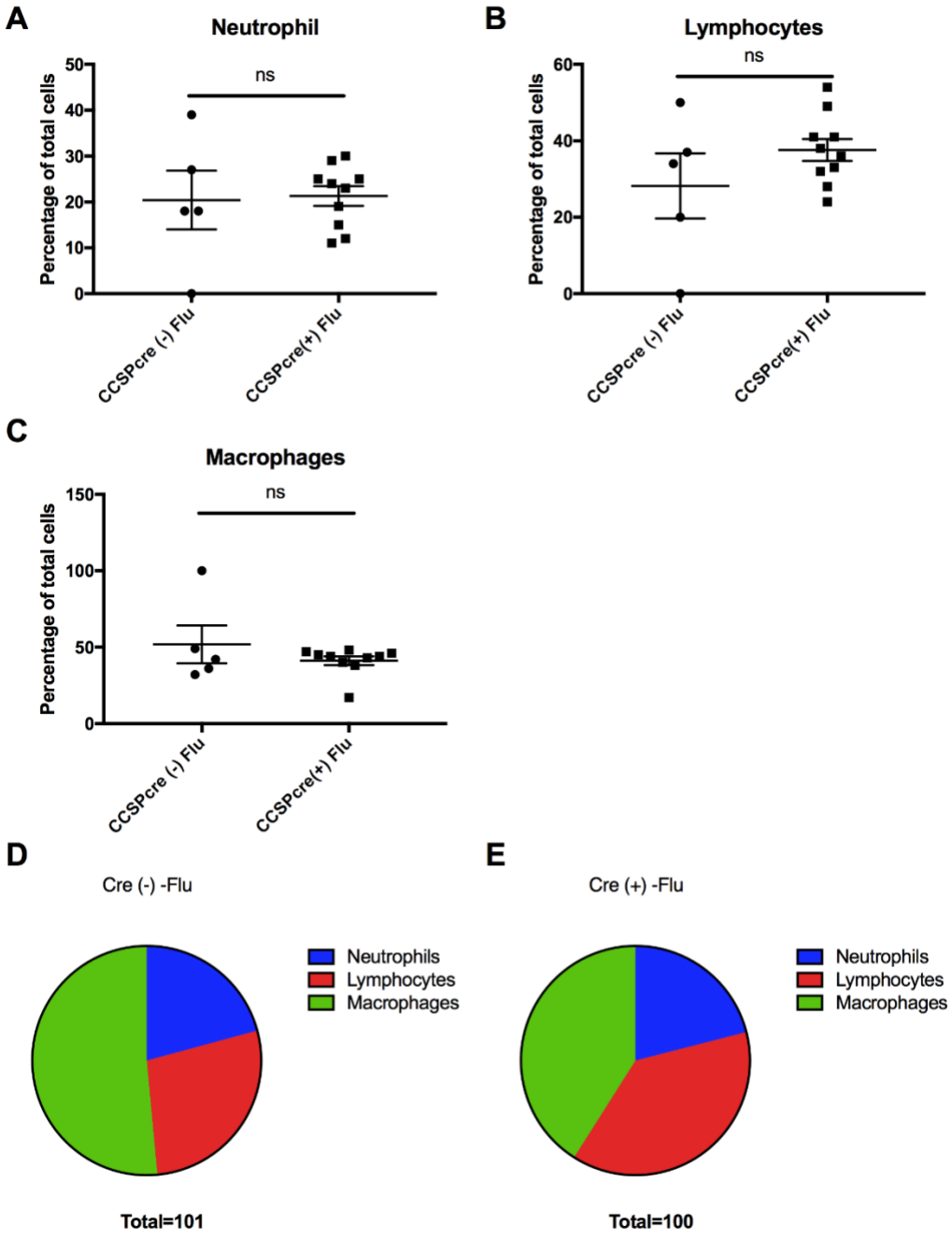


Figure 4. No difference in cellular makeup of bronchoalveolar lavage fluid between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with 100 pfu of influenza. The BAL cells were stained, and differential cells were counted as described in methods. (A) Percentage of neutrophils, (B) macrophages, (C) and lymphocytes were measured in BAL. (D) IL-17RC-cre-, (E) IL-17RC-cre+ BAL cell composition. N=5 and 10 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively. Data are represented as mean±SEM, two tailed Student's *t* and χ_2 test, ns-not significant.

8.2 Primary MRSA Infection

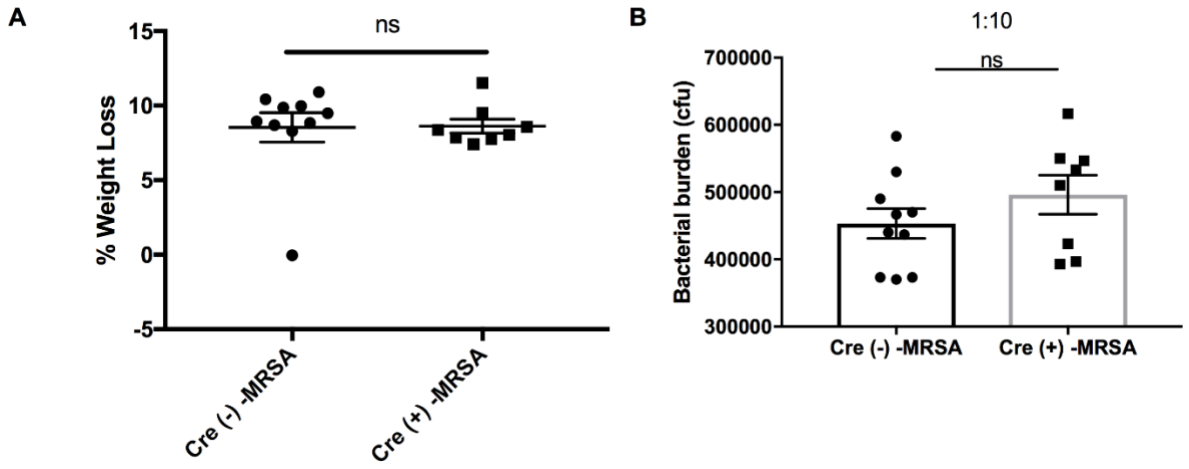


Figure 5. No difference in weight loss or lung bacterial burden between IL-17RC-cre+ and IL-17RC-cre- mice
IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with $1x10^8$ CFU of MRSA. (A) Weight loss was determined from comparing weight at time of infection (Day 0) and on day of sacrifice (Day 1) (B) Bacterial burden measured by amount of MRSA growth from homogenized lungs in 1:10 dilutions. N=10 and 8 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

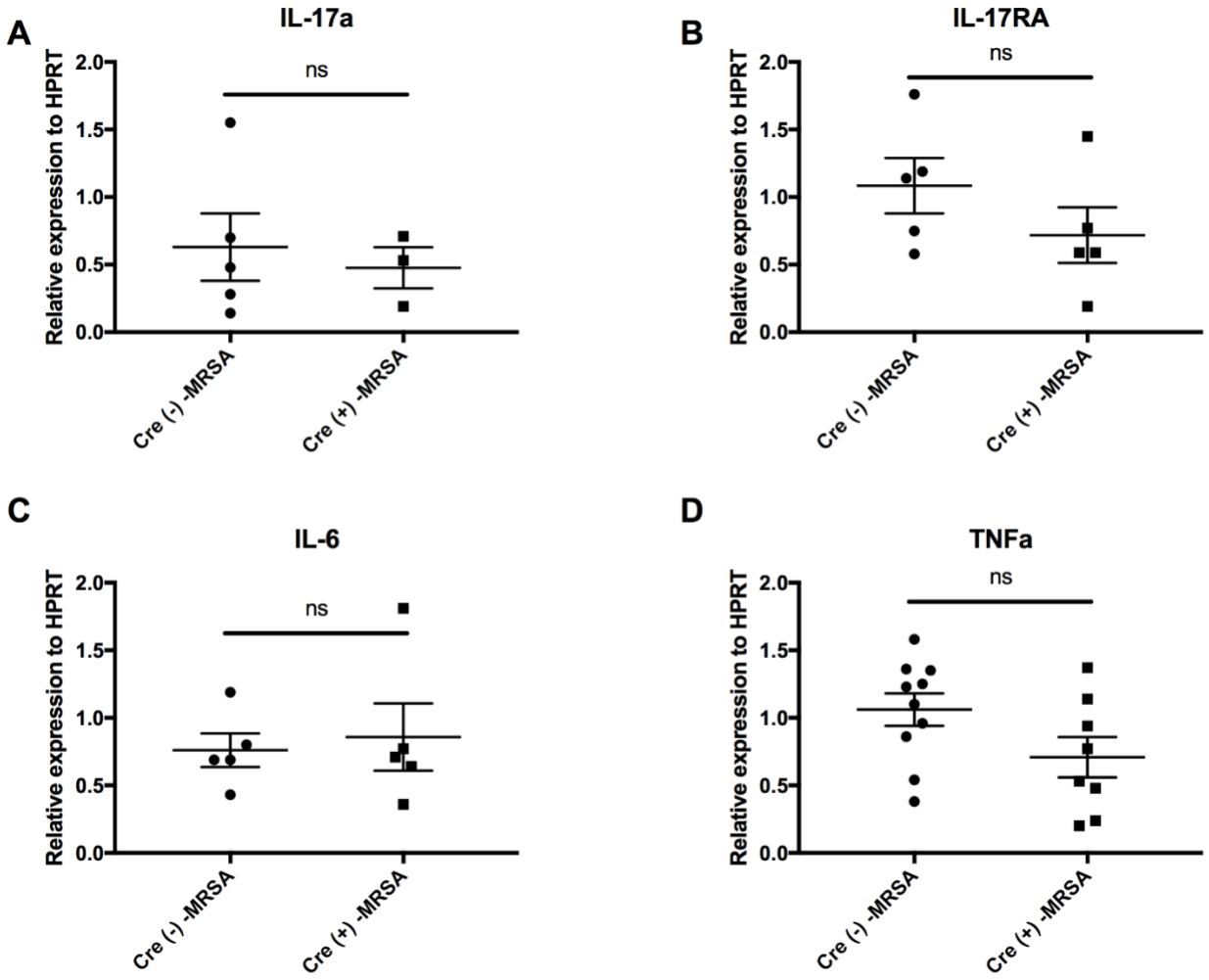


Figure 6. No difference in gene expression levels of IL-17A and downstream proinflammatory cytokines in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with $1x10^8$ cfu of MRSA.(A-D) IL-23A (N=5 and 3 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively), IL-17A (N= 5 per group), IL-6 (N= 5 per group), TNF-α (N= 10 and 7 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively), expression was measured in whole lung by RT-PCR, Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

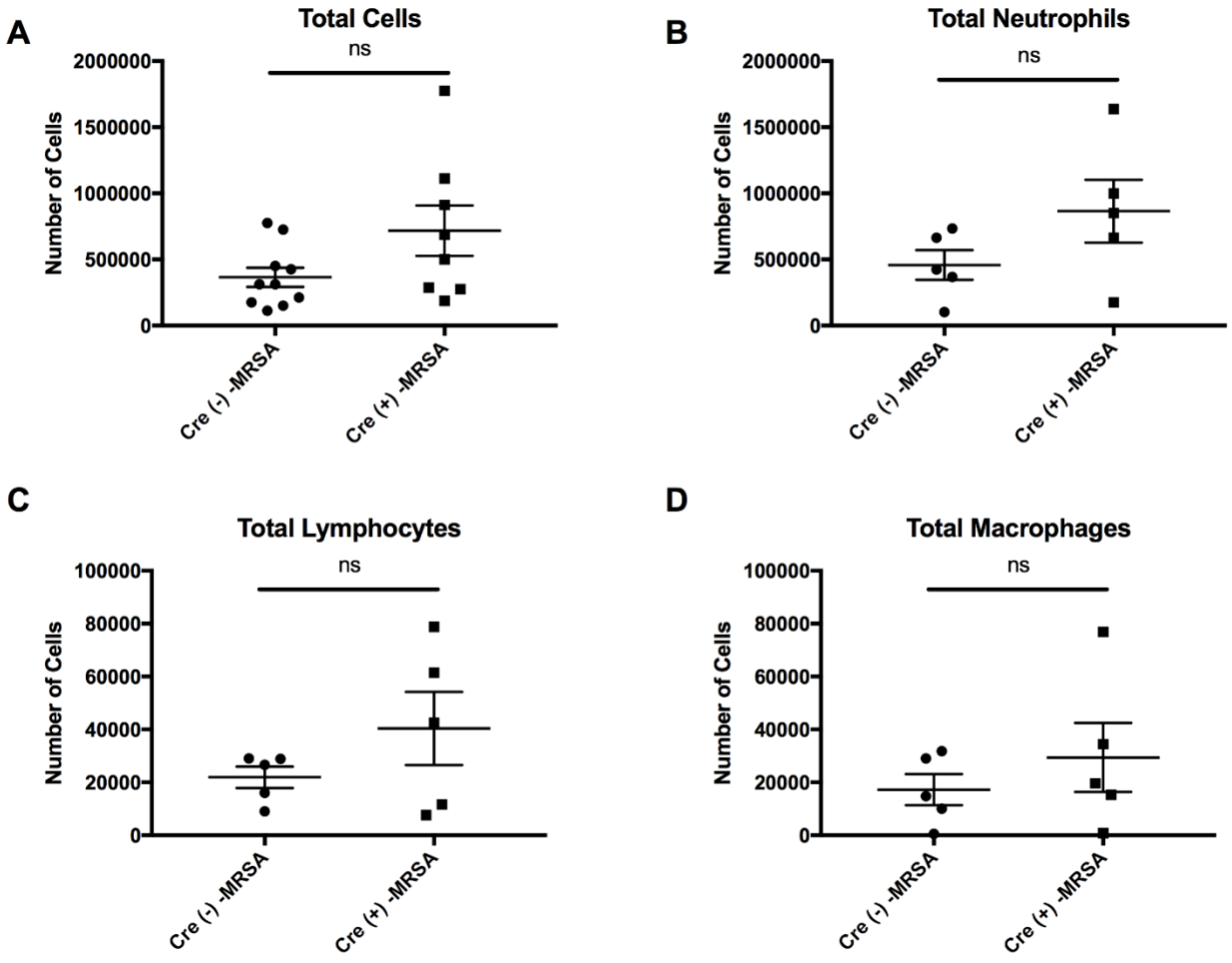


Figure 7. No difference in recruitment of neutrophils, lymphocytes, or macrophages to the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with $1x10^8$ cfu of MRSA. N=5 per group unless otherwise noted. The BAL cells were stained, and differential cells were counted as described in methods. (A) Total number of cells (N=10 and 8 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively), (B) neutrophils, (C) lymphocytes (D) and macrophages were measured in BAL. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

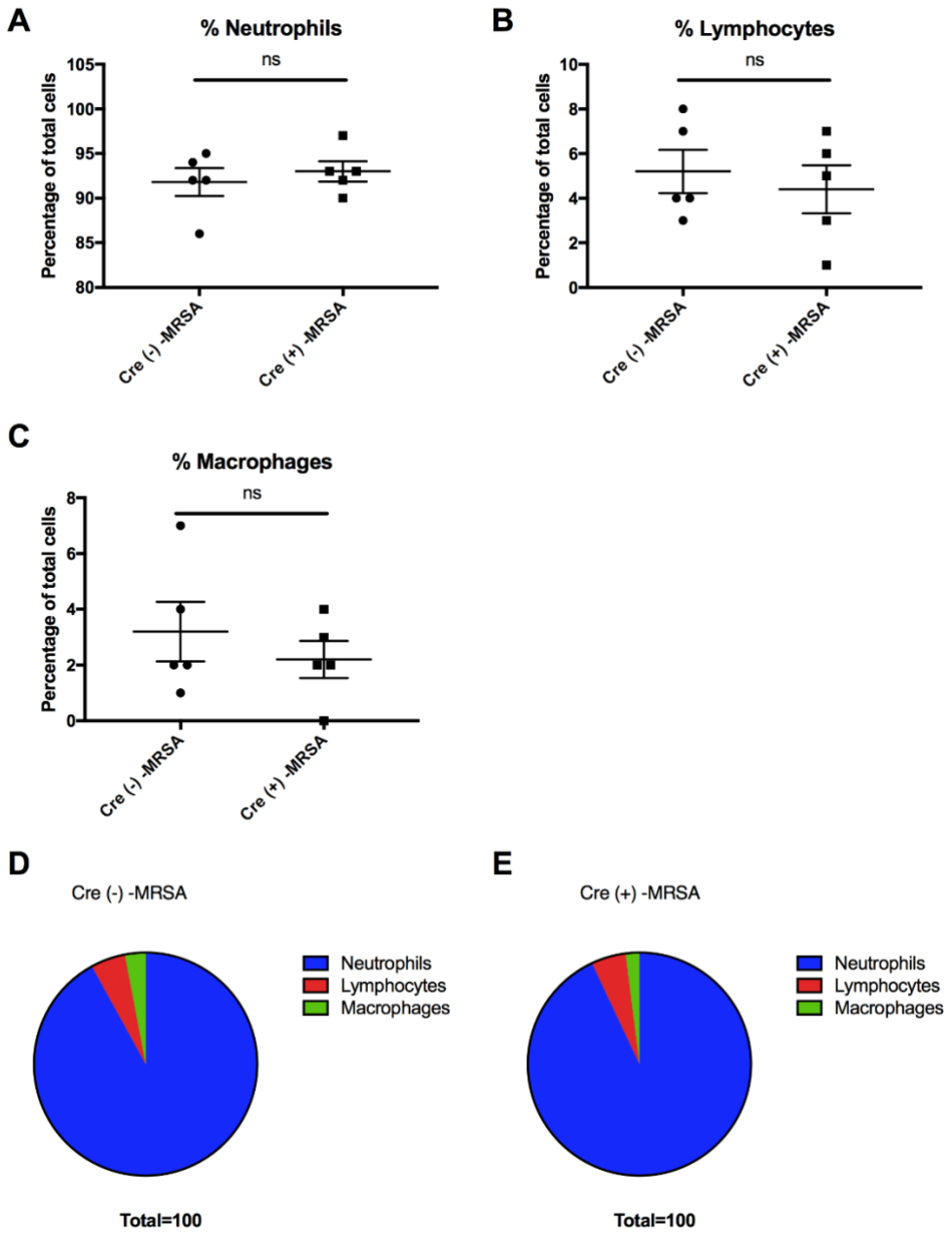


Figure 8. No difference in cellular composition of BAL fluid between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with $1x10^8$ cfu of MRSA. The BAL cells were stained, and differential cells were counted as described in methods. (A) Percentage of neutrophils, (B) macrophages, (C) and lymphocytes were measured in BAL. (D) IL-17RC-cre-, (E) IL-17RC-cre+ BAL cell composition. N=5 per group. Data are represented as mean±SEM, two tailed Student's *t* and χ^2test, ns-not significant.

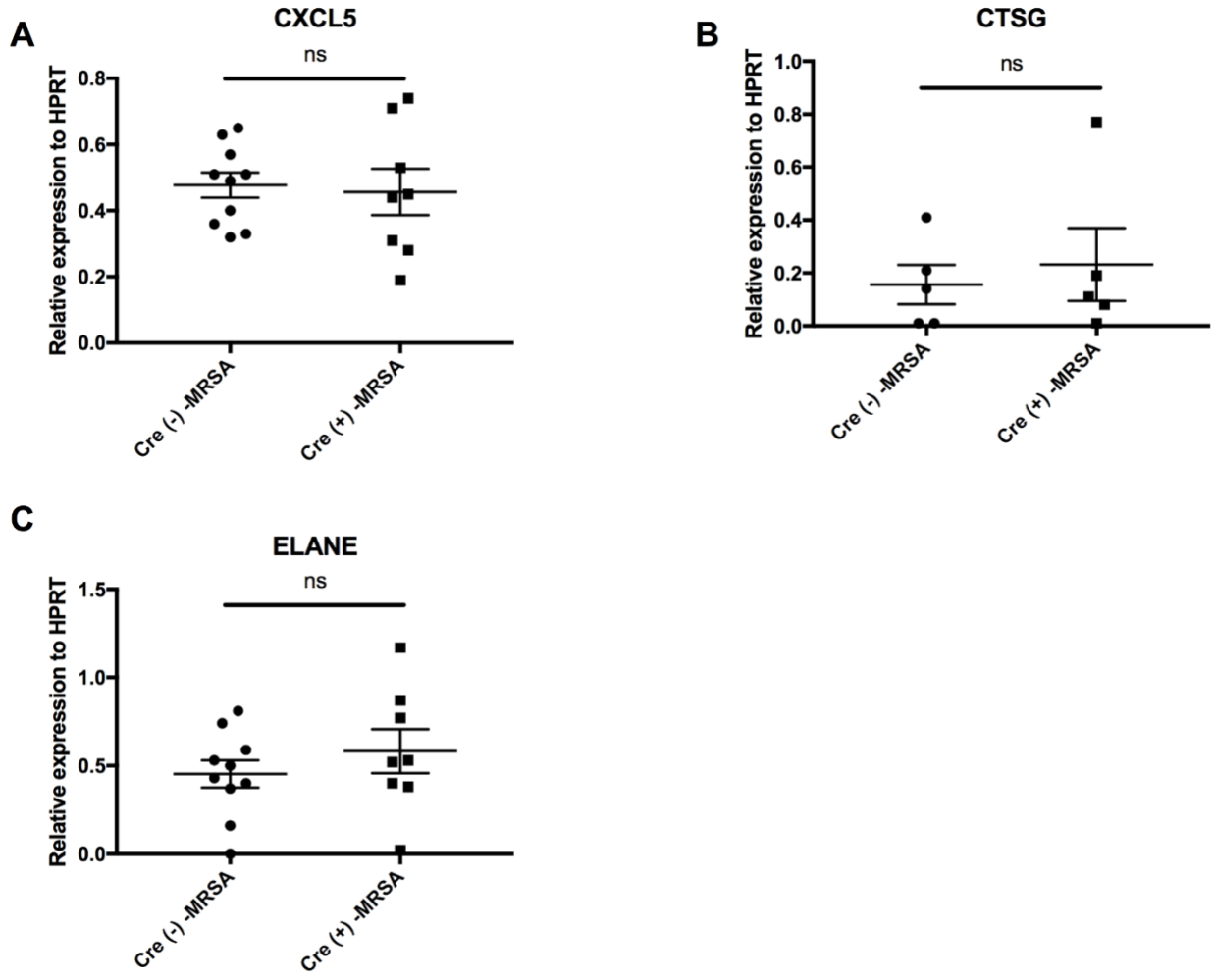


Figure 9. No difference in neutrophil chemokine or neutrophil activity markers' expression levels between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with $1x10^8$ cfu of MRSA (N=10 and 8 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively unless otherwise noted.). (A-C) CXCL5, cathepsin g (CTSG) (N=5 per group), neutrophil elastane (ELANE), expression was measured in whole lung by RT-PCR. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

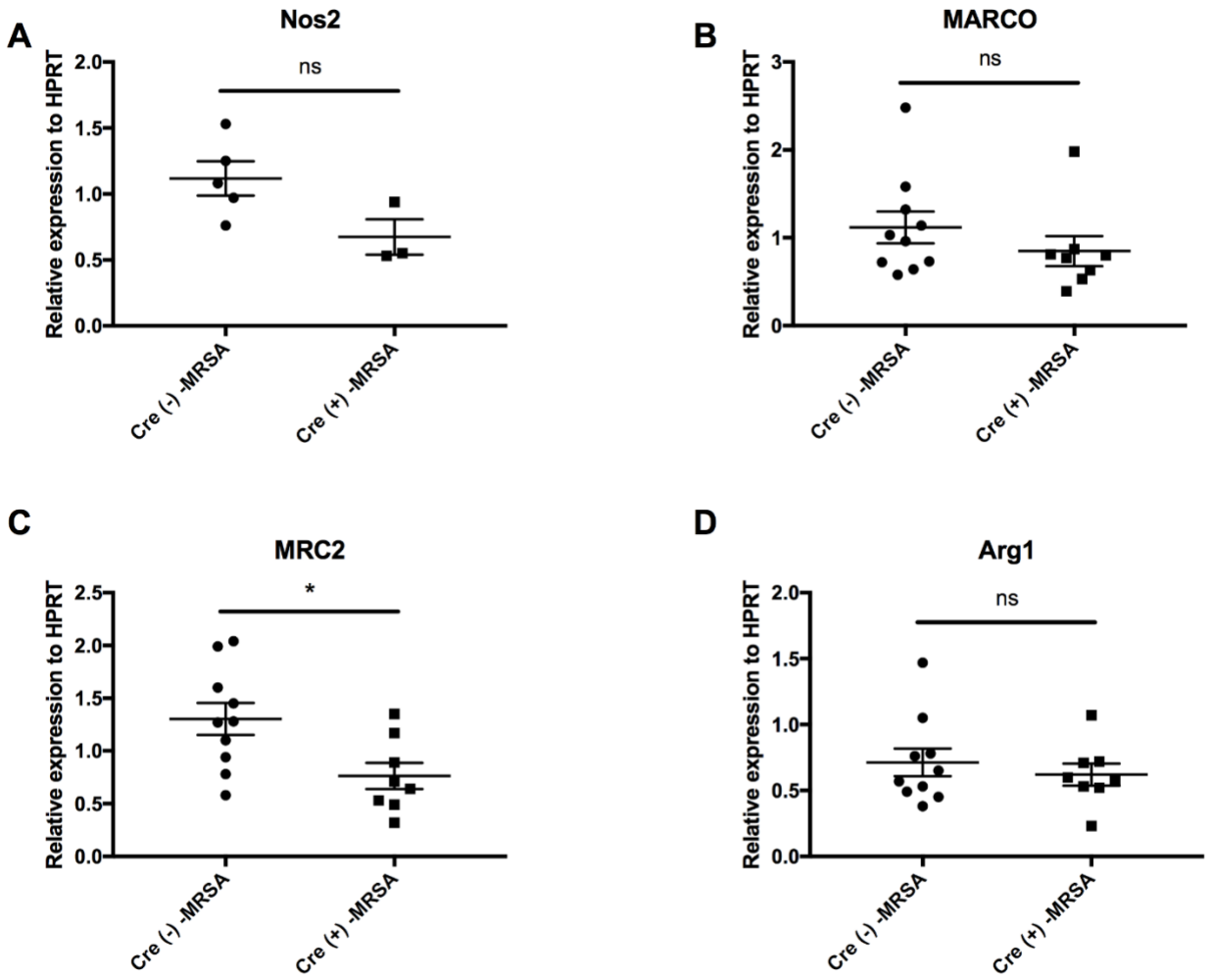


Figure 10. IL-17RC-cre+ have reduced mannose receptor c type 2 gene expression in the lungs, but no changes in other M2 macrophage markers or M1 macrophage marker Nos2

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with $1x10^8$ cfu of MRSA (N=10 and 8 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively unless otherwise noted.). (A-D) Nos2 (N=5 and 3 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively), MARCO, MRC2, Arg1 expression was measured in whole lung by RT-PCR. Data are represented as mean±SEM, two tailed Student's *t* test, *= p= 0.0176, ns-not significant.

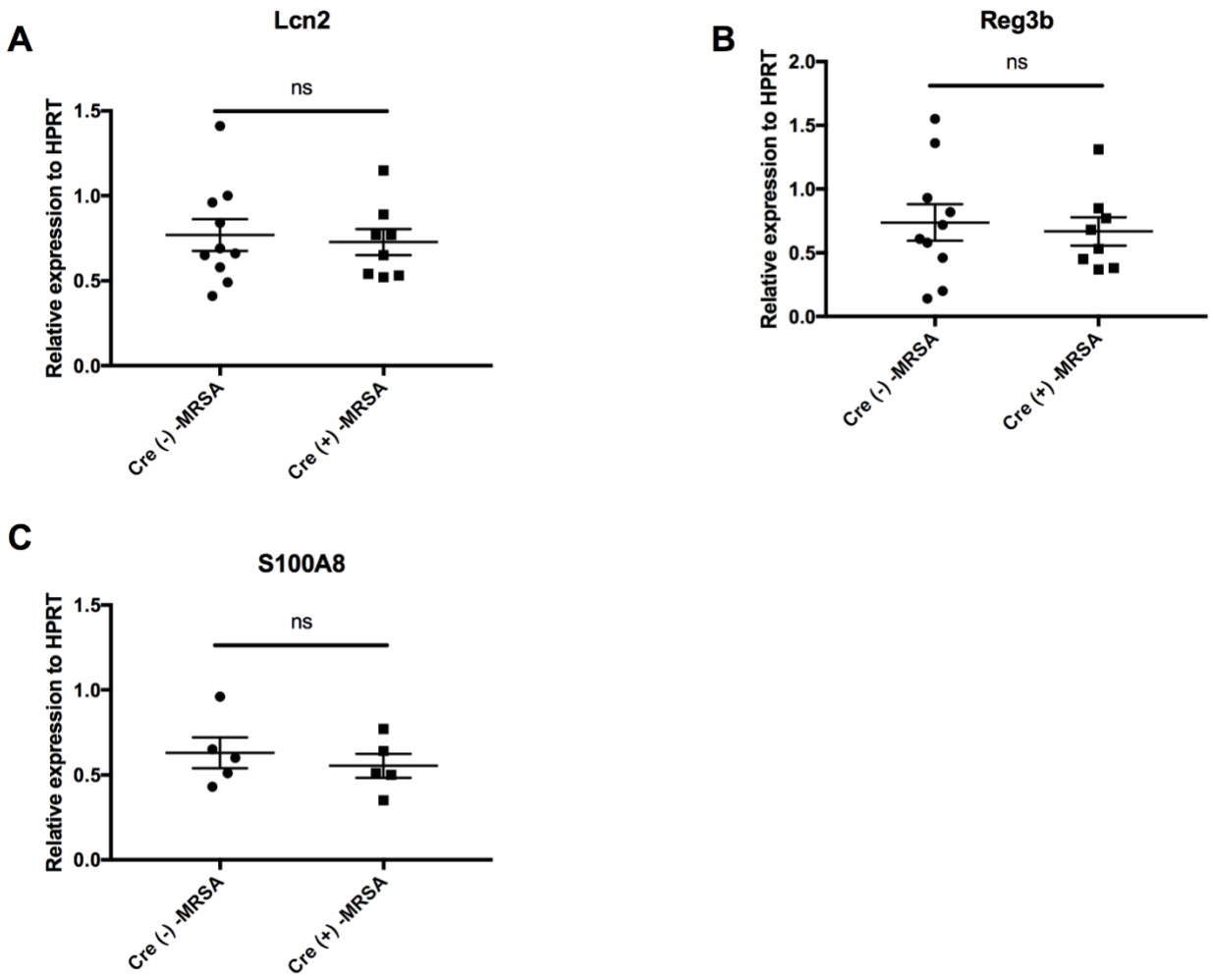


Figure 11. No differences in antimicrobial peptide expression in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with $1x10^8$ cfu of MRSA (N=10 and 8 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively unless otherwise noted.). (A-C) Lcn2, Reg3β, S100A8 (N=5 per group), expression was measured in whole lung by RT-PCR. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

8.3 Influenza-MRSA Super-infection

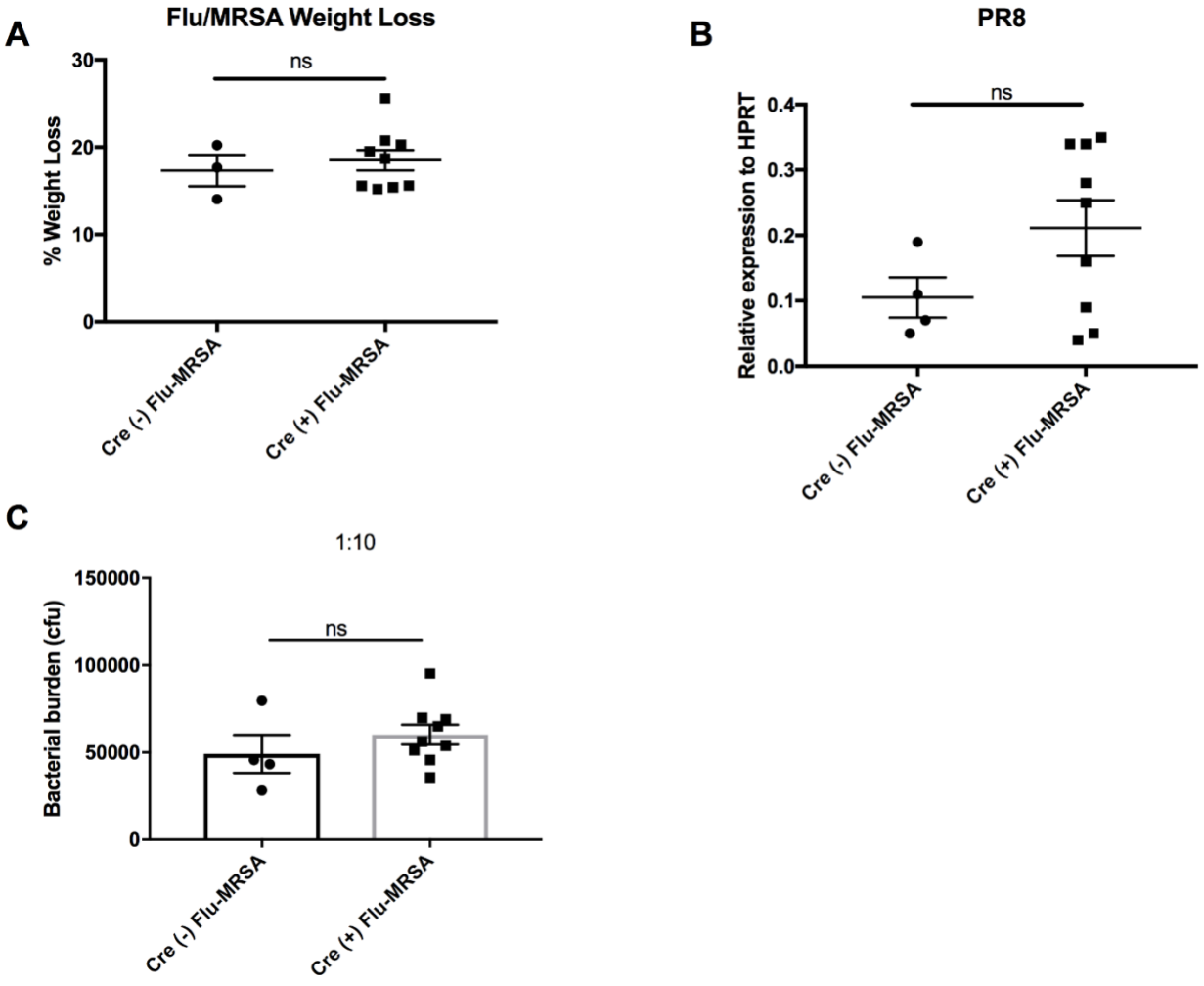


Figure 12. No differences in weight loss, viral burden, or bacterial burden between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were first infected with 100 pfu of influenza and then 5 x 10_7 CFU of MRSA 6 days later. (N=4 and 9 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively). (A) Weight loss was determined from comparing weight at time of infection (Day 0) and on day of sacrifice (Day 7) n= 7 and 17 for IL-17RC-cre- and IL-17RC-cre+ groups respectively. (B) Viral burden was measured by influenza M protein expression in lung by RT PCR (C) Bacterial burden measured by amount of MRSA growth from homogenized lungs in 1:10 dilution. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

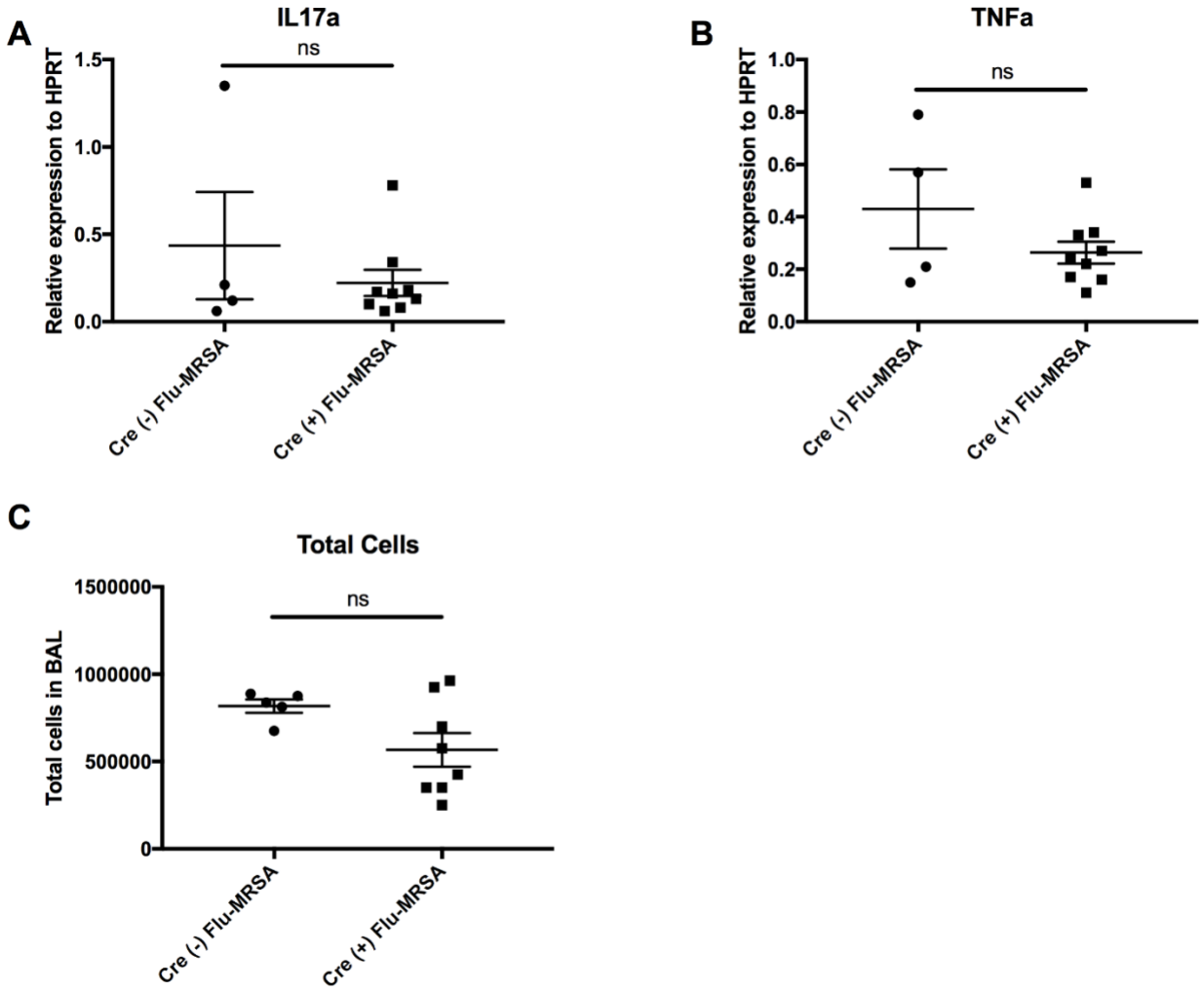


Figure 13. No differences in IL-17A or TNF-α expression in the lungs, nor any differences in total cells in BAL between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were first infected with 100 pfu of influenza and then 5 x 10^7 CFU of MRSA 6 days later. N=4 and 8 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively unless otherwise noted, (A-B) IL-17A,TNF-α expression was measured in whole lung by RT-PCR. The BAL cells were stained, and differential cells were counted as described in methods. (C) Total number of cells (N=5 and 8 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively) .Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

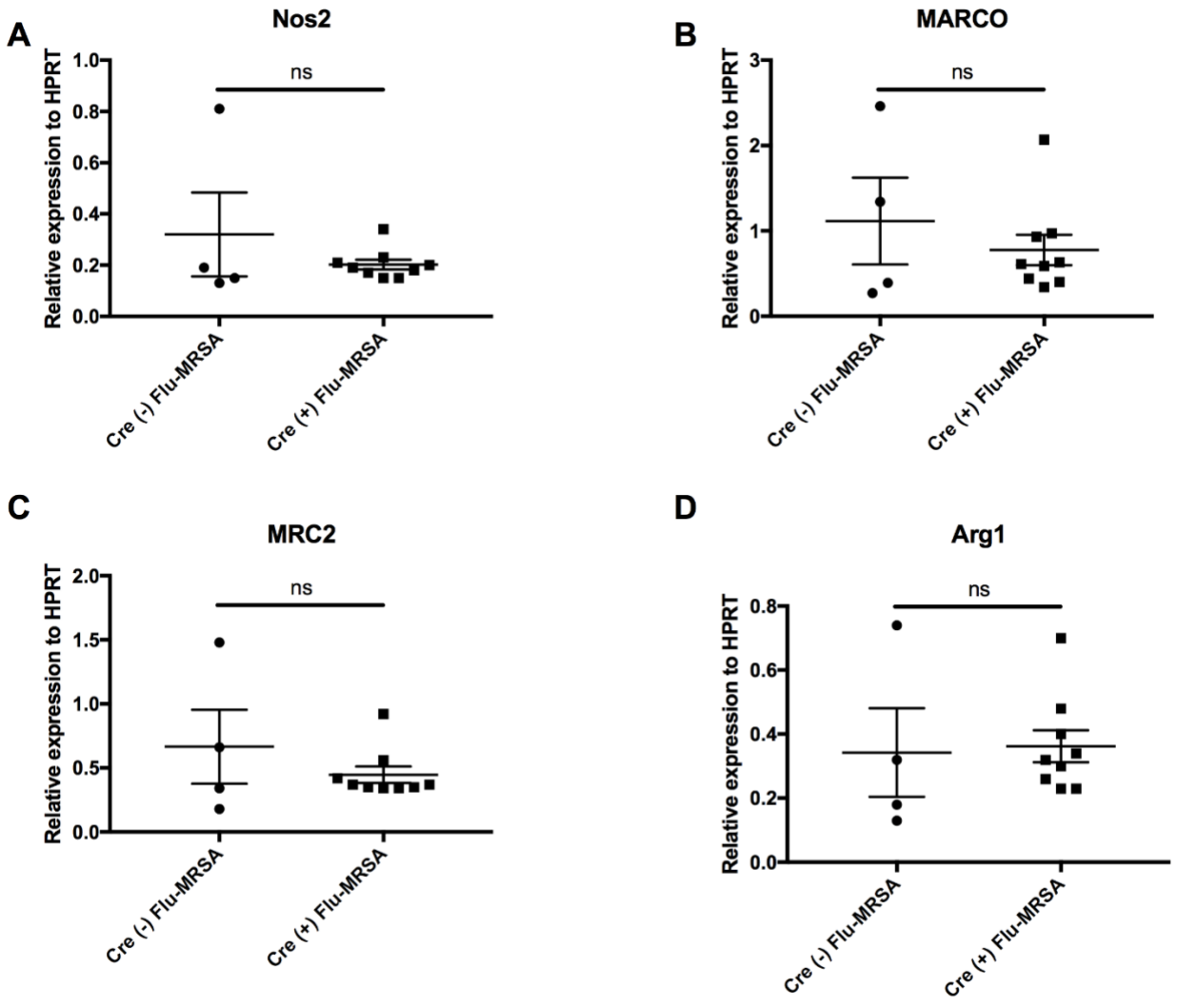


Figure 14. No differences in the macrophage marker expressions in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre$_{-}$ and IL-17RC-cre$_{+}$ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were first infected with 100 pfu of influenza and then 5 x 10_{7} CFU of MRSA 6 days later. N=4 and 9 for IL-17RC-cre$_{-}$ and IL-17RC-cre$_{+}$ groups, respectively unless otherwise noted, (A-D) Nos2, MARCO, MRC2, Arg1 expression was measured in whole lung by RT-PCR. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

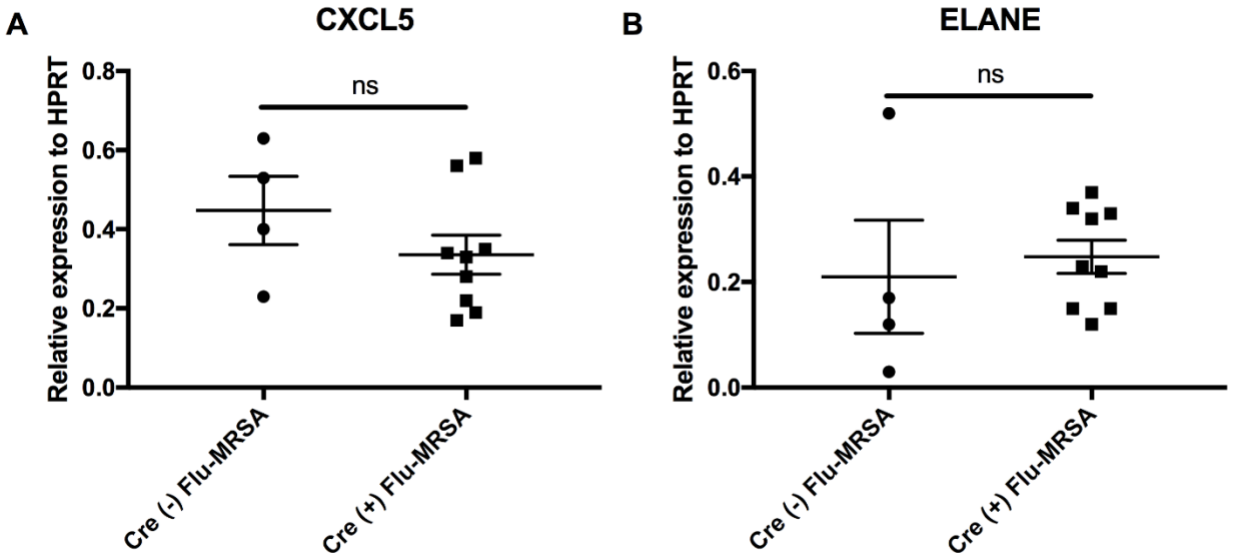


Figure 15. No differences in the neutrophil marker expressions in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were first infected with 100 pfu of influenza and then 5 x 10^7 CFU of MRSA 6 days later. N=4 and 9 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively unless otherwise noted, (A-B) CXCL5, ELANE expression was measured in whole lung by RT-PCR. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

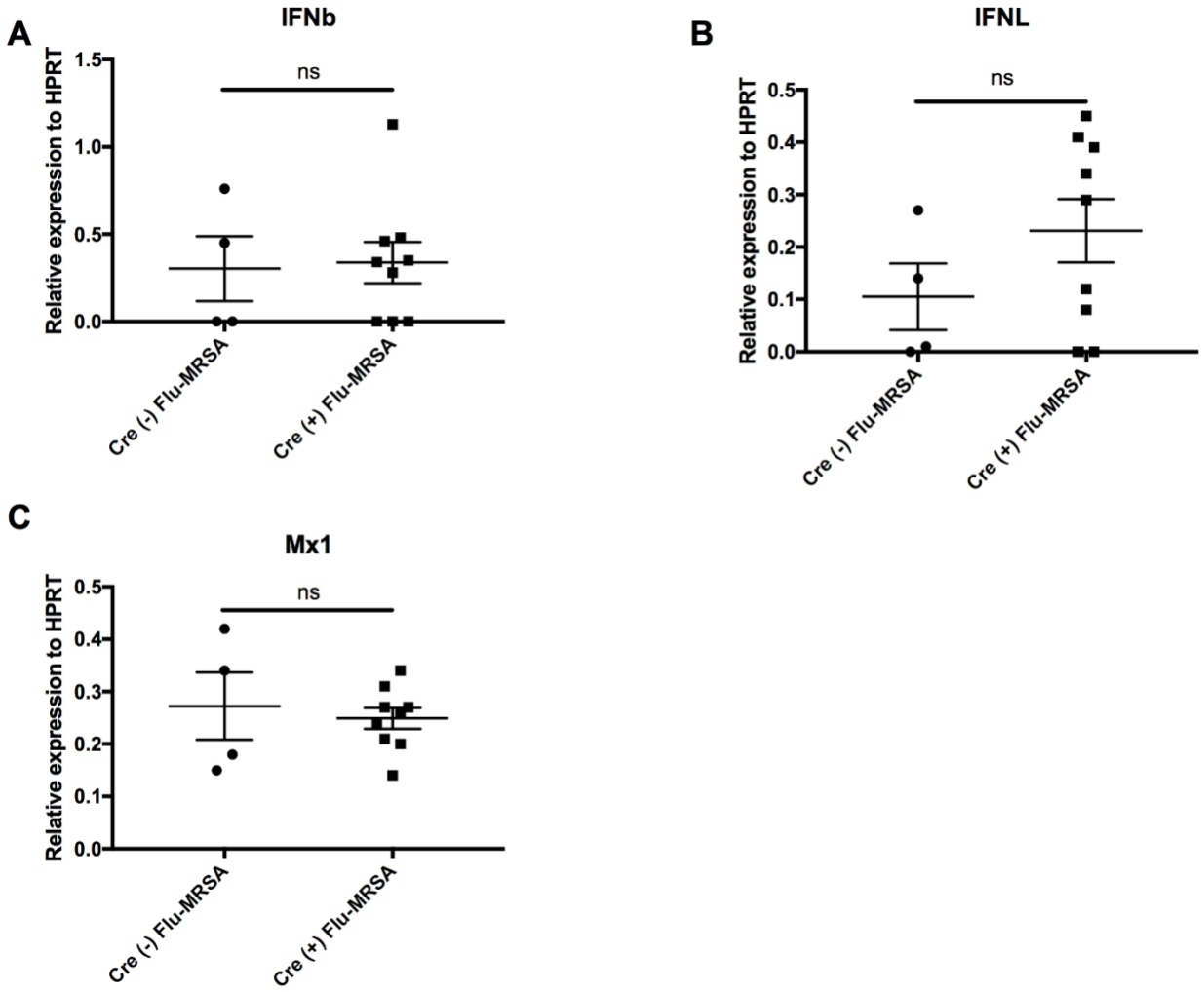


Figure 16. No difference in the expression of interferons or downstream interferon induced gene expression levels in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were first infected with 100 pfu of influenza and then 5 x 10^7 CFU of MRSA 6 days later. N=4 and 9 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively unless otherwise noted, (A-C) IFNβ, IFNλ, and Mx1 expression was measured in whole lung by RT-PCR. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

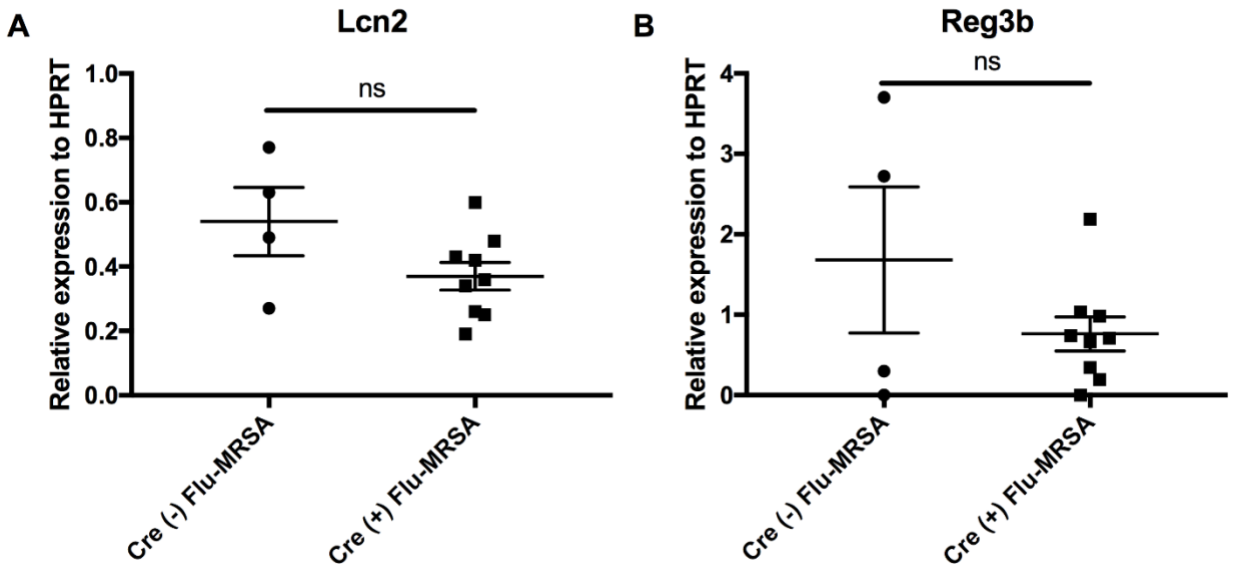


Figure 17. No differences in antimicrobial peptide expression in the lungs between IL-17RC-cre+ and IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were first infected with 100 pfu of influenza and then 5 x 10^7 CFU of MRSA 6 days later. N=4 and 9 for IL-17RC-cre- and IL-17RC-cre+ groups, respectively unless otherwise noted, (A-B) LCN, and Reg3β expression was measured in whole lung by RT-PCR. Data are represented as mean±SEM, two tailed Student's *t* test, ns-not significant.

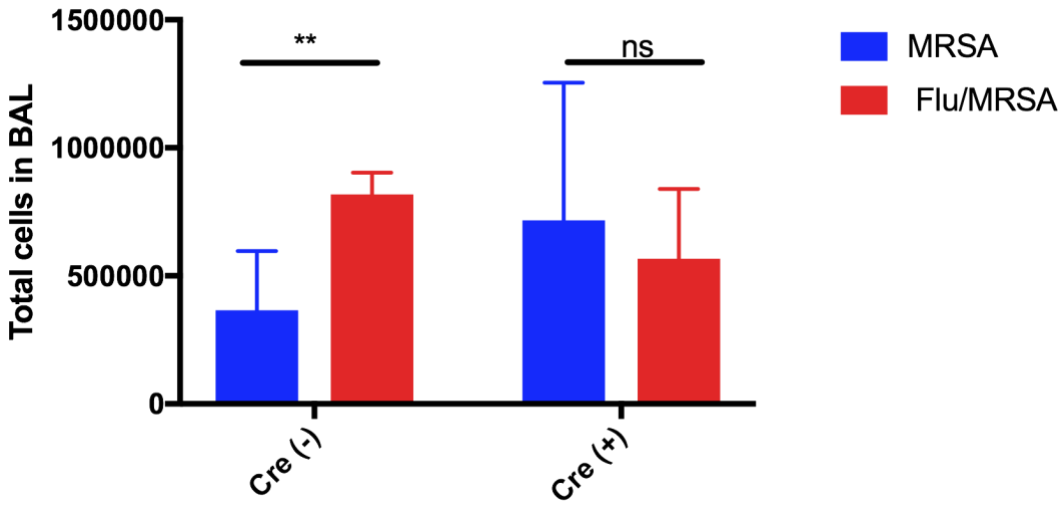
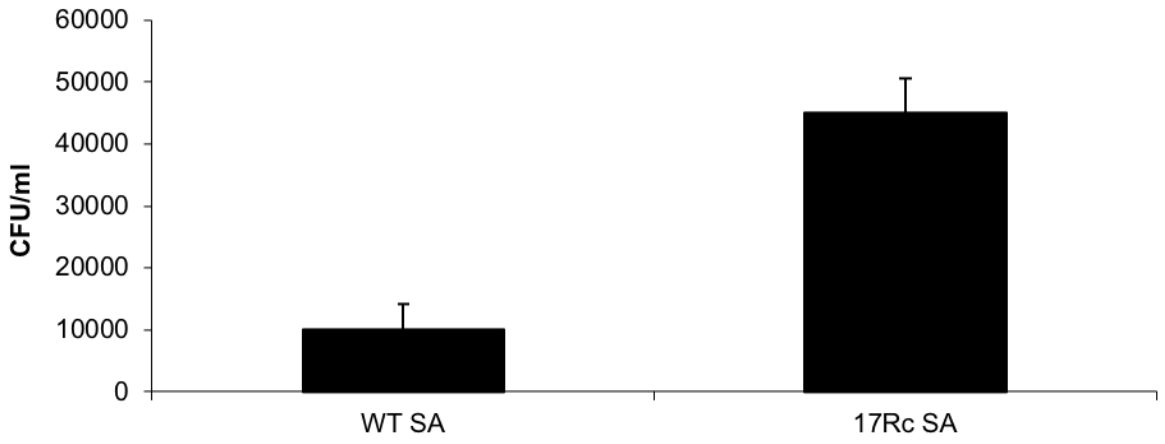


Figure 18. 6.3.6 Influenza-MRSA super-infection increases total number of cells in BAL fluid in IL-17RC-cre- mice compared to primary MRSA infection in IL-17RC-cre- mice

IL-17RC-cre- and IL-17RC-cre+ (CCSPcre (-) and CCSPcre (+)) female, 6-8 weeks old mice were infected with either $1x10^8$ cfu of MRSA or with 100 pfu of influenza and then 5 x 10^7 CFU of MRSA 6 days later. N=8 for CCSPcre (+) mice in each infection model. N= 10 and 5 for CCSPcre (-) mice in primary MRSA and influenza-MRSA super-infection models, respectively. The BAL cells were stained, and differential cells were counted as described in methods. Total number of cells in BAL fluid. Data are represented as mean ± SEM, two tailed Student's *t* test, **p-value = 0.001l, ns-not significant.

9.0 Supplemental Figure



Supplemental Figure 1. Global IL-17RC knockout mice have increased bacterial burden

Wildtype and IL-17RC knockout mice male, 6-8 weeks old were infected with 1 x 10^8 cfu *of S. aureus* for 1 day. N=4 in both groups. Bacterial burden measured by amount of *S. aureus* growth from homogenized lung in 1:10 dilution. Data are represented as mean ± SEM, two tailed Student's t test, *p-value= 0.0028509.

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