CRITICAL ROLE OF INTERLEUKIN-17 RECEPTOR SIGNALING IN THE IMMUNOPATHOLOGY OF INFLUENZA INFECTION

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Interleukin-17 (IL-17) is a cytokine produced mainly by T cell lineages that plays a key role in regulation of neutrophil responses. Given the importance of neutrophils in the immune response directed against extracellular pathogens, it is no surprise that IL-17 is important in host defense against a multitude of pathogens. Importantly, however, neutrophils also have been shown to play a role in several immunopathological conditions, including acute lung injury. In this dissertation, we evaluate the role that IL-17 plays in the immunopathology of influenza infection. We show here that IL-17 is produced as early as day 2 following influenza challenge, and that this expression is sustained throughout the first week of infection. Further, we identify γδ T cells as at least one important source of IL-17 in response to influenza. We also demonstrate that loss of IL-17 receptor A (IL-17RA) signaling results in a profound decrease in neutrophil recruitment to the lung following influenza challenge. This decrease in neutrophils results in substantially less inflammation and lung injury, as well as higher survival rates. Additionally, there is only a moderate impact on viral clearance and T cell responses. Further, we detail similar findings in a non-infectious aspiration model of acute lung injury. Taken together, this data suggests that IL-17 signaling may be a key event, and intriguing therapeutic target, in the pathogenesis of acute lung injury.

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PREFACE

My graduate school experience has been a time of not only scientific discovery, but also great personal discovery. For this, I owe a debt of graditude to many people who have helped to shape my experiences these past four years. First, I would like to thank my mentor Jay Kolls. Jay's seemingly endless optimism and enthusiasm for science is something that I will always remember and hope to emulate as I move on in my training and throughout my career. I want to thank you, Jay, for the opportunities you have given me, the ideas and insight you have provided, and for the advice, encouragement and wisdom you have shared.

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1. Introduction

1.1 Acute Lung Injury and Acute Respiratory Distress Syndrome

1.1.1 Definition and Epidemiology

Acute lung injury (ALI) is a syndrome characterized by increased vascular permeability resulting in persistent lung inflammation, and can afflict people of all ages. The term ALI covers a spectrum of severity, with the most severe injury being referred to as acute respiratory distress syndrome (ARDS). A diagnosis of ALI is determined by four clinical features: acute onset, bilateral infiltrates, hypoxemia (PaO2/FiO2 ratio between 201 and 300 mmHg), and no evidence of an elevated left atrial pressure (to distinguish it from cardiogenic pulmonary edema). ARDS is distinguished from ALI by a more severe hypoxemia (PaO2/FiO2 ratio < 200 mmHg), but otherwise shares the same definition (1, 2).

ALI is a significant burden on the healthcare system. One multi-center cohort study of 1131 patients demonstrated that the incidence and mortality of ALI increases with age. Extrapolating that data, it is estimated that there are roughly 190,600 cases of ALI in the United States each year with nearly a 40% mortality rate, meaning 74,500 patients die of ALI each year in this country (3). Other studies have suggested that 10-15% of patients admitted to intensive care units meet the diagnostic criteria of ALI, and that this number increases to 20% when looking only at patients on mechanical ventilation (4-7).

ALI can be precipitated by a number of causes. Although more than 60 causes have been identified, the majority of cases of ALI are due to a much shorter list of causes, both pulmonary and extrapulmonary (7, 8). The most common cause is sepsis (8-10), and the risk of ARDS may be especially elevated in septic patients with a history of alcohol abuse (11, 12). In one study of 220 patients with septic shock, 70% of the patients with a history of alcohol abuse developed ARDS while 31% of the septic patients who were non-alcoholics developed ARDS (13).

Infectious pneumonias are another major cause of ALI. Among the most common pathogens are *Streptococcus pneumoniae*, *Legionella pneumophila*, *Staphylococcus aureus*, enteric gram negative organisms, and respiratory viruses such as influenza. While community-acquired pneumonias are very common (in fact, pneumonia is the most common cause of ARDS that arises outside of the hospital), it is also important to note that nosocomial pneumonias, including those caused by *Pseudomonas aeruginosa* can progress to ARDS as well (14, 15).

Yet another common cause of ALI is the aspiration of stomach contents. It has been estimated that as many as 33% of patients who suffer a gastric aspiration develop severe lung injury (9, 16). This may also be seen in patients who develop a tracheoesphageal fistula. Gastric contents can be damaging in several ways. The pH of the stomach acid may contribute to injury, but gastric enzymes and small food particles may also play a role (17).

Other common causes of ALI include severe trauma or burns (18), massive blood transfusions (8), drugs (including aspirin, cocaine, opioids, phenothiazines, and some chemotherapeutic agents) (19, 20), fat or air emboli in the pulmonary vascular bed (21, 22), and graft failure following lung or bone marrow transplant (23-25).

1.1.2 Respiratory viral infections and ALI

As mentioned previously, infectious pneumonias are among the most common causes of ALI. Of particular interest is the role of ALI in the pathogenesis of respiratory viral infections. In 2003, an outbreak of a novel respiratory coronavirus, the severe acute respiratory syndrome (SARS) virus, began in Asia and rapidly spread as far as Canada. Within six months of the index case, over 8,000 cases had been documented worldwide, the majority of which were in China. Among patients with SARS, nearly 25% progressed and developed severe respiratory failure. One study of 199 SARS patients reported a mortality rate at 4 weeks post-infection of 10%, but that jumped to 52% at 13 weeks when looking specifically patients who were admitted to an intensive care unit. Of those who died in the ICU, 80% had a proximate cause of death given as ARDS or complications related to ARDS (26).

Even more recently, cases of avian influenza A (H5N1) have been documented among humans. Between December, 2003 and August, 2005, 112 cases of H5N1 influenza were documented in southeastern Asia, mostly among poultry workers or their contacts (27). While human-to-human spread has been documented, it is highly inefficient, which explains the low infection rate (28). However, manifestations of ARDS are common, with rates of respiratory failure ranging from 70-100% (28-31). This is also reflected in the high mortality rates. Morality tends to peak on an average of 9-10 days after onset of illness with progressive respiratory failure being the cause of death (28-31).

Historically, influenza pandemics have been even more severe. Perhaps the worst was the 1918 “Spanish” Influenza A (H1N1) pandemic which killed nearly 50 million people worldwide (32). While our insights are limited due to the lack of tissue, pathologists at the time

described severe destruction of the lung tissue and noted that this was unlike what was normally seen in more typical pneumonias (33, 34). Further, researchers have recently been able to use plasmid-based reverse genetics to reconstruct the 1918 influenza strain and study it in animal models (35). Non-human primates infected with the 1918 influenza demonstrated severe alveolar damage, extensive pulmonary edema, and hemorrhagic exudates consistent with findings associated with ARDS (36, 37). Mouse models of the 1918 influenza virus also confirm the severe lung pathology described in human patients (38, 39). Given influenza's ability to rapidly mutate (discussed later), it is reasonable to believe that a similar pandemic, perhaps even stemming from current circulating H5N1 strains, may strike again. For that reason, it is important to understand the mechanisms by which ALI and ARDS develop in response to viral infections.

1.1.3 Molecular and Cellular Mechanisms of ALI

Regardless of the cause, much of the molecular and cellular pathology observed in ALI is the same. There is generally damage to the alveolar epithelium, edema formation, an accumulation of inflammatory cells (especially neutrophils), and a strong increase in proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6, and IL-8. The inflammation results in worsening edema, resulting in hemorrhagic, proteinaceous fluid filling the air spaces (40-45).

Of the two separate barriers that make up the alveolar-capillary barrier- the vascular endothelial cells and the alveolar epithelium- the alveolar epithelium is much less permeable. Therefore, loss of epithelial integrity is a key component to alveolar flooding (46). The

epithelium is made up of two cell types. Type I cells are squamous and comprise approximately 90% of the alveolar surface area. Type II cells are cuboidal, and while they make up only 10% of the surface area, they are functionally very important, as they are a major source of surfactant production and ion transport. Subsequently, loss of type II alveolar cells may impair removal of edema fluid by affecting epithelial fluid transport, as well as reducing the production of surfactant (40, 47-49). Importantly, in the case of infectious pneumonias, the alveolar epithelium also serves to limit spread of the infectious organism to the bloodstream. Therefore, damage to the epithelium may lead to septic shock in these cases (50).

The production of proinflammatory cytokines by epithelial cells, fibroblasts, and inflammatory cells may contribute to the worsening immunopathology observed in ALI. In particular, production of IL-8 by fibroblasts and alveolar macrophages serve to recruit large numbers of neutrophils (51, 52). Additionally, IL-1β, TNF-α, CXCL2 (macrophage inflammatory protein, or MIP-2), and multiple other chemokines are produced by resident pulmonary cell populations (44, 53). However, it is also important to note that in addition to the production of proinflammatory cytokines, one must also consider the balance between proinflammatory cytokines and the production of anti-inflammatory cytokines such as IL-10 and endogenous anti-inflammatory mediators, including IL-1 receptor antagonist (IL-1RA), soluble TNF-α receptor, and autoantibodies against IL-8 (54).

The neutrophils that are recruited to the lung by production of these proinflammatory cytokines and chemokines have been implicated as a major player in ALI (40, 51). In fact, neutrophils are the dominant cell found in the edema fluid and bronchoalveolar lavage fluid (BALF) in ALI patients (54, 55). The neutrophils serve as another major source for cytokines and chemokines, especially IL−1β, TNF-α, CXCL1 (KC) and CXCL2 (MIP-2).

Further, neutrophils are a source of reactive oxygen intermediates (ROI), which are capable of activating NF-κB and CREB, leading to even more production of proinflammatory cytokines (56, 57). These ROI may also play a role in oxidative damage to the cells of the airway and the oxidation of phospholipids in the airway (58). A recent study has demonstrated that oxidized phospholipids (OxPLs) are capable of serving as an endogenous signal for Toll-like Recepter (TLR)- 4. This signaling is TRIF/TRAF6 dependent (MyD88-independent), and blocking this signaling lessens the degree of lung injury, suggesting another mechanism by which neutrophils could contribute to the immunopathology of acute lung injury. These authors also reported that large concentrations of OxPLs are present in the lungs of all severe cases of lung injury that they examined, which included human cases of H5N1 influenza and SARS (59). Other studies have confirmed a role for TLR 4 signaling in ALI (60-62), as well as demonstrated a role TLR 2 signaling (60).

Additionally, neutrophils are a source of proteases, including elastase, collagenase, and protease 3. These proteases are beneficial and necessary in helping the neutrophil degrade extracellular matrices as they leave the vasculature and move through connective tissue to the site of infection. However, in the context of ALI, the excessive neutrophil recruitment results in excessive production of proteases, which then in turn results in damage to the lungs' connective tissue matrix (58, 63).

Following this acute phase there is a resolution of lung injury. In some patients, this is uncomplicated and rapid (64). In other patients, there is progression to fibrosing alveolitis (41), which is marked by the accumulation of mesenchymal cells in the alveolar space (65), along with increased production of collagen and fibronectin (66). Fibrosis results in an increased risk of death (67), and is thought to begin during the acute phase of lung injury, promoted by IL-1 (68).

1.1.4 Treatment of ALI and ARDS

Managing a patient with severe lung injury first involves searching for the underlying cause, and in the case of treatable infections such as pneumonia or sepsis, treatment with the appropriate antimicrobial agents (69, 70). Beyond that, no specific therapies exist for the treatment of ALI. However, supportive care- including ventilation strategies- can play a big role in the outcome of ALI, and due to advances in our understanding of supportive care, the mortality rates of ALI have dropped from 67% in 1990 (71) to roughly 25-30% in 2006 (72, 73).

Appropriate use of mechanical ventilation in ALI and ARDS patients has been disputed. Because there is extensive damage to the lung tissue in these patients, they are predisposed to barotraumas, particularly at high tidal volumes and pressures while on mechanical ventilation. A recent study has suggested that ventilation with lower tidal volumes (6 mL per kg of predicted body weight versus a normal volume of 12 mL per kg) can result in a 22% reduction in mortality (74). In addition to low tidal mechanical ventilation, some studies have confirmed a benefit for restricting fluids in patients with ALI, presumably by decreasing the pulmonary edema (75). While improvements in supportive care may serve to lower the mortality rates, it is important to note that patients who recover from ARDS may have long-term sequelae, including abnormalities in pulmonary function and exercise endurance as well as an impaired quality of life (76, 77).

Treatments targeting the immunopathology of ALI have not shown much benefit. In particular, studies of anti-inflammatory agents and antioxidant therapies have shown no benefit (2, 78, 79). It may be, though, that these treatments may be beneficial in some subset of ALI patients, but that this benefit is masked when looking at a heterogenous study population. For

instance, anti-inflammatory agents might be useful in curtailing excessive neutrophil activity, but patients with bacterial pneumonias may do worse because neutrophils are a key component to clearing the bacterial infection. In contrast, studies have shown a benefit to the use of corticosteroids in preventing the fibroproliferation seen following ALI (80, 81).

Other therapies that have failed to show a benefit in ALI include surfactant therapy (82) and administration of inhaled nitric oxide and other vasodilators (83, 84). Studies have also been aimed at accelerating the removal of the pulmonary edema using beta-agonists, which may also increase surfactant secretion and even have an anti-inflammatory effect (85). Also, experiments have shown that treatment with keratinocyte growth factor can protect against lung injury by increasing the proliferation of type II alveolar epithelial cells and increasing the clearance rate of alveolar fluid (86). Interestingly, an antioxidant effect was also observed (87). These experiments suggest that future clinical studies with beta-agonists and keratinocyte growth factor may be useful.

1.1.5 Acute Lung Injury: Summary

Acute lung injury is a serious clinical condition, characterized by an acute onset of hypoxia, with bilateral infiltrates and no evidence of increased left atrial pressures. The most severe form of acute lung injury is referred to as acute respiratory distress syndrome and is characterized by more severe hypoxia. Over 60 causes of acute lung injury have been identified, but most cases tend to result from just a handful of causes, including sepsis, bacterial or viral pneumonias, aspiration of gastric contents, severe trauma or burns, high volume blood transfusions, or drugs.

Regardless of the cause, the underlying mechanism of lung injury is fairly conserved. Damage to the alveolar epithelium and leakiness of the vascular endothelium contribute to flooding of the airspace. Additionally, loss of type II pneumocytes results in a loss of surfactant production and impairs fluid reabsorbtion. Dysregulation of the proinflammatory cytokine response both by resident cells such as fibroblasts and epithelial cells, as well as by inflammatory cells is observed, as is an excessive neutrophil response. This neutrophil infiltration is both in response to and contributes to the production of proinflammatory cytokines. Further, neutrophils produce reactive oxygen species that damage the cells of the lung and proteases that are able to destroy connective tissues.

Unfortunately, treatment options are limited. In addition to treating the underlying cause of the lung injury, treatment mainly consists of supportive mechanical ventilation. Using lower tidal volumes during mechanical ventilation has been shown to result in lower mortality rates. While our insights into ventilation strategies have advanced, the mortality rate for acute lung injury is still near 30%. There exists a need to develop therapies targeted at the lung injury process.

1.2 Influenza

1.2.1 Influenza Epidemiology

Influenza viruses circulate in the human population and generally cause an acute respiratory infection. Seasonal influenza outbreaks occur every winter and generally have attack rates of 10-

20% of the population. Global pandemics occur far less often, at variable intervals, and may have attack rates exceeding 50%. An important consideration in determining the incidence of influenza infection is that many cases go unreported, as most seasonal strains are self-resolving in otherwise healthy individuals. Seasonal outbreaks generally peak over a 2-3 week period and can last as long as 2-3 months. Often, the first indication of a seasonal influenza outbreak is an increase in the number of children presenting with febrile respiratory illness, and is then followed by increases in presentation of flu-like symptoms in adults (88-90).

The degree of morbidity and mortality of each influenza outbreak depends largely on the pathogenicity of the circulating strain of the virus, with age and immune status of the infected host playing a big role in outcome. In general, influenza B outbreaks tend to be less widespread, and the disease less severe, than influenza A outbreaks (91). The elderly and those with other underlying co-morbidities tend to experience higher levels of morbidity and higher mortality rates (92). Over a period of 20 years (1972-1992) there were 426,000 deaths from influenza in the United States (93). More recent numbers suggest that there was an average of 226,000 hospitalizations per year due to influenza in the United States between 1979-2001 (88). Costs have been estimated to be as high as $12 billion per year for the management and treatment of influenza cases each year in the United States. It is estimated that a pandemic with attack rates between 35-50% would cost the United States $71-167 billion (94).

Clinical manifestations of influenza infection can vary, particularly in severity. Generally patients experience an acute onset of systemic symptoms, including headaches, fevers, chills, myalgia, and malaise. This is accompanied by respiratory symptoms, including a sore throat and cough. As previously mentioned, in milder cases of influenza, patients may not seek care, as the symptoms are similar to a common cold. In other cases, patients may experience

severe symptoms and have fevers as high as 41°C (105.8°F). In uncomplicated cases, symptoms resolve over 2-3 days, though a sore throat or persistent cough may last for a week or more (88).

In elderly patients, influenza infection carries a risk of pneumonia (92). This can be broken down into two main categories: primary influenza viral pneumonia and secondary bacterial pneumonia. Primary influenza viral pneumonia is less common, but more severe. In the case of secondary bacterial pneumonia, the most common pathogens are *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*. The patient generally shows improvement over 2-3 days before suddenly becoming febrile again and developing symptoms of pneumonia, including a productive cough (88, 92, 95-97).

Other complications from an influenza infection can include exacerbation of chronic obstructive pulmonary disease (COPD) or asthma, sinusitis, or otitis media (middle ear infection). Rarely are there extrapulmonary complications, including myositis, rhabdomyolysis, myocarditis, pericarditis, or encephalitis (88, 97).

1.2.2 Influenza Virus Biology

The influenza viruses are divided into 3 genera: influenza A, influenza B, and influenza C. These viruses are members of the Orthomyxoviridae family. The virions are spheres that are approximately 100nm in diameter and are surrounded by a lipid envelope. Two surface proteins-hemagglutinin (HA) and neuraminidase (NA) protrude from this envelope to interact with the surface of host cells. The influenza genome is unique in that it consists of 8 single-stranded negative-sense RNA segments which encode for 10 viral proteins.

The three largest genome segments encode the proteins PB2 (759 amino acids), PB1 (757 amino acids), and PA (716 amino acids), all of which are subunits for the viral RNA polymerase. The next largest segment encodes the HA protein (566 amino acids), a surface protein that exists as trimers and binds to host cells (described in greater detail later). Nucleoprotein (NP, 498 amino acids) is encoded by the 5th RNA segment and encapsidates each RNA segment. The 6th RNA segment encodes the NA protein (454 amino acids) which is the other surface protein, expressed as a tetramer and critical to the release of viral progeny. Genome segment 7 encodes 2 proteins due to alternative splicing: matrix (M1) protein (252 amino acids) which is an important structural component of the virion, and M2 (97 amino acids), which forms a tetramer and serves as an ion channel. The 8th and final segment of the genome also encodes for two proteins: NS1 (230 amino acids), which plays a role in inhibiting the interferon response to the virus, and NS2 (121 amino acids), which is involved in export of viral RNA to the host cell nucleus (98-100).

Viral replication takes place in respiratory epithelial cells. The HA trimers on the virion surface bind to sialic acid residues on the host cell and the virus enters via endocytosis. Once inside the endosome, the M2 protein serves as a channel for protons, resulting in the acidification and uncoating of the virion. The viral membrane fuses to the endosomal membrane and allows for the export of viral RNA- bound to NP and NS2 proteins- into the cytoplasm. These ribonucleocapsids enter the nucleus through nuclear pores and are transcribed in the nucleus. One unique feature of influenza RNA transcription involves “cap stealing”, whereby the PB2 protein binds to the caps of host mRNA and uses them to prime transcription of viral mRNAs. These viral mRNAs are then translated to make viral proteins, and also serve as template for the generation of negative-sense viral RNA. Virion assembly occurs in the cytoplasm, and newly assembled virons bud from the host cell. HA proteins on the new progeny virions also bind to

the sialic acid residues of the host cell; therefore, cleavage by NA is necessary for release of the virion (98).

1.2.3 Genetic Reassortment

Influenza viruses are further subtyped based on their surface (HA and NA) antigens. By convention, each HA and NA subtype is numbered, and viral strains are referred to as HxNx (for example, H1N1 or H3N2). While there are 16 different HA subtypes, only H1, H2, H3, and recently H5 have been shown to cause outbreaks in humans. Similarly, though there are 9 distinct N subtypes, only N1 and N2 have been identified in human outbreaks (88).

New strains of influenza result from two distinct processes of genetic variation: antigenic drift and antigenic shift. Antigenic drift is a gradual process that, in essence, is the accumulation of point mutations within a virus over multiple generations. This is particularly apparent in the HA and NA proteins; the other viral proteins are comparatively highly conserved. Antigenic drift is helped by the fact that influenza's RNA polymerase is rather error prone. The amino acid substitutions in HA range from 1.9-3.0 x 10^{-3} per year, whereas for the more highly conserved M1 protein, amino acid substitutions average 3 x 10^{-5} per year. Overall, the influenza genome averages 0.5 x 10^{-3} per year (101, 102).

Antigenic shift is a process unique to influenza's segmented genome. Antigenic shift occurs when two viral subtypes co-infect the same organism. When progeny virions of two influenza subtypes are replicating and being packaged within the same cell, it is possible that segments of one subtype's genome get packaged with another subtype, and was first demonstrated over 50 years ago (103). For example, in a cell infected with both H1N1 subtypes

and H3N2 subtypes, it would be possible for the HA and NA genomic segments to get "switched", resulting in not only H1N1 and H3N2 progeny, but also H1N2 and H3N1 progeny. This resulting "shift" explains how new subtypes of virus are able to rapidly emerge in the population.

1.2.4 Animal models of influenza infection

For the sake of brevity, only the three most commonly used models of influenza will be discussed: mice, ferrets, and nonhuman primates. Of these, the mouse is perhaps the most commonly used model of influenza infection. The advantages of using mice are that they are inexpensive, easy to house, and many reagents are available to evaluate the immune response. Mice are not natural hosts for influenza, however, which necessitates adapting the virus by several passages. Additionally, mice are generally inbred, a fact that must be considered when translating data to heterogenous human populations (104).

One of the most commonly used mouse adapted strain is influenza A/Puerto Rico/8/34 (H1N1). Innoculation with mouse adapted strains of influenza results in an illness generally similar to human disease, with similar histopathology and reduced blood oxygen saturation levels. However, mice generally do not exhibit fever, sneezing, coughing, or dyspnea. Useful outcome measures to determine disease severity include weight loss, viral titers, average time to death, lung pathology scores, cellular recruitment to the airway, and markers of lung injury, including total protein and LDH in the bronchoalveolar lavage fluid (104-106).

In contrast to the mouse, the ferret is an animal model that displays many symptoms similar to human disease. Influenza virus-infected ferrets exhibit nasal discharge, fever, and

watery eyes, along with anorexia. Also unlike mice, ferrets are able to spread the virus among themselves, even in separate- but connected- cages (104, 107). Ferrets are used less frequently than mice for many logistical reasons. First, they are more aggressive and difficult to work with, they are more expensive to house, it can be difficult to obtain specific pathogen-free animals, and reagents for evaluating the ferret immune response are not as extensive as those for mice (108).

Nonhuman primates are also used as a model for human influenza infection, with rhesus macaques and cynomolgus macaques being the most commonly used species (36, 109). The chief benefit to this model is that they are more closely related to humans than other small mammals. Drawbacks are similar to those of the ferret model, specifically costs and difficulties associated with obtaining and housing the animals and availability of reagents (108).

Other less studied models of influenza infection include rats, pigs, cats, and dogs. Additionally, development of vaccines for avian influenza has included studies in several avian species (108).

1.2.5 Influenza: Immune Response- Innate Mechanisms

The immune response to the influenza virus, like those in response to most pathogens, is complex and multifaceted. Both innate and adaptive mechanisms are important in viral clearance. While neither "arm" of the immune system works independently, for the sake of clarity, the description of the immune response will be broken down into innate mechanisms, T cell roles, and B cell and antibody roles.

The innate response to influenza begins with the cells that are the primary targets of infection: the respiratory epithelial cells. Influenza virus stimulates epithelial cells through Toll-

like receptor (TLR)-3, whose primary ligand is dsRNA. TLR-3 is expressed intracellularly in human pulmonary epithelial cells, and is upregulated upon infection with influenza (110). TLR3, unlike other TLR's, signals through TRIF as opposed to MyD88. While TLR3 plays a role in activating epithelial cells, it is interesting to note that mice lacking TLR3 have less inflammation and higher viral burdens, but perhaps paradoxically have a survival advantage (111).

Upon infection with influenza virus, these epithelial cells activate a number of transcription factors that induce cytokine and chemokine production, including nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs), signal transducers and activators of transcription (STATs), and activating protein (AP)-1 (112, 113). This leads to the production and release of a variety of proinflammatory mediators, including IFN-α, IFN-β, IL-6, CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), and CCL5 (RANTES) (114, 115).

Perhaps most importantly, these epithelial cells upregulate type I interferons (IFN-α and IFN-β). These type I interferons subsequently induce the expression of Mx proteins, which inhibit the transcription of influenza genes by complexing with the viral NP protein. *Mx1*-null mice have in fact been shown to be more susceptible to influenza infection (116, 117). It is also important to note that the influenza NS protein is capable of antagonizing type I interferons, thereby promoting viral replication (118, 119).

Alveolar macrophages also play an important role in the innate response to influenza. Like epithelial cells, alveolar macrophages can be infected by influenza virus. Also like epithelial cells, these alveolar macrophages are an important source of proinflammatory mediators, particularly TNF-α, IL-1β, IL-6, type I interferons, CCL4 (MIP-1), CCL5 (RANTES), and CXCL10 (IP-10) (113, 120-122). In contrast to epithelial cells, macrophage

death is almost exclusively apoptotic (123). The apoptotic and necrotic death of epithelial cells and alveolar macrophages also serves as another trigger for the release of proinflammatory cytokines and chemokines. In fact, caspase-1 activation- which is associated with apoptosis- appears to be important in achieving efficient production of both IL-1β and IL-18 (124).

In vivo, the release of the cytokines and chemokines by epithelial cells and macrophages leads to the recruitment of monocytes and neutrophils- as well as T cells at later time points- into the lung (122). Given the role of neutrophils in ALI, their role in influenza infection is of particular interest. Their ability to bind and take up influenza virus in the absence of antibody opsonization (125) is important to their role in early control of viral infection.

It has been shown that influenza is capable of activating the neutrophil, resulting in the production of multiple antimicrobial products. One such example is α-defensins- a group of small cationic antimicrobial peptides that have shown to be active against a wide spectrum of gram-negative and gram-positive bacteria, fungi, parasites, and viruses. While α-defensins may be capable of binding to and disrupting the lipid envelop of viruses (including influenza), it appears that these peptides may also exert their anti-viral activity by affecting the target cell and inhibiting viral replication (126). Additionally, α-defensins increase neutrophil uptake of influenza virus and may play a role in the respiratory burst response (125). Further, defensins may modulate the immune response, as they are chemotactic for human T cells, monocytes and immature dendritic cells (127).

In addition to the production of defensins, influenza also stimulates an oxidative burst in neutrophils. Both reactive oxygen intermediates (ROI) and reactive nitrogen intermediates have been implicated in lung damage following influenza infection. The primary product of the neutrophil's respiratory burst in response to influenza is hydrogen peroxide (H_2O_2) (128).

Production of superoxide anion (O_2^-) is less defined, with some reports suggesting neutrophils do make superoxide anion when stimulated with influenza (129, 130) and others reporting they do not (128). The latter is consistent with other reports that demonstrate defects in neutrophil chemotactic, oxidative, and bacterial killing functions following influenza infection (131, 132). Additionally, stimulation with influenza accelerates neutrophil apoptosis (133). The neutrophil dysfunction induced by influenza may partially explain why influenza-infected hosts are more susceptible to secondary bacterial infections (131, 132, 134, 135).

1.2.6 Influenza: Immune Response- T cells

The T cell response to influenza involves both CD4+ T helper (Th) cells and CD8+ cytotoxic T lymphcytes (Tc or CTL), with both types of effector cells playing key roles in resolution of infection. In general, CD4+ and CD8+ T cells do not arrive at the site of infection in the lung until approximately 4 days post-infection, and these populations peak around 10 days post-infection, which coincides with clearance of sublethal infections (136, 137). In terms of magnitude, the CD8+ T cell response is larger than the CD4+ T cell response (138). Further, while roles for both CD8+ T cells and CD4+ T cells have been established in viral clearance, mice lacking one subset or the other still clear infection, albeit with some delay. In contrast, mice lacking both CD4+ and CD8+ T cells do not survive influenza infection (139).

CD4+ T cells recognize antigen in the context of major histocompatibility complex (MHC) class II molecules on the surface of antigen presenting cells. Because there is no viral replication in lymphoid tissue, antigen presentation to CD4+ T cells is dependent on dendritic cell trafficking from the lung. This begins within 36 hours of viral infection, and antigen

presenting cells (APCs) are detected throughout the resolution of viral illness (10-14 dpi) (140). Studies in CD4+ T cell receptor (TCR) transgenic animals have even suggested that APC's are present and capable of stimulating T cells as late as 3 weeks post-infection (141).

CD4+ T cells are further subdivided into multiple subsets based on their cytokine production, including Th1 cells (producing IFN-γ, IL-2, and TNF-α), Th2 cells (IL-4, IL-5, IL-13), Th17 cells (IL-17, IL-22), and Treg (IL-10, TGF-β) among others. Th1 cells tend to promote activation of CD8+ T cells and macrophages as well as influence B cell differentiation towards production of IgG2a, whereas Th2 cells tend to influence B cell differentiation towards IgG1 an IgE, as well as activate eosinophils. Th17 cells tend to result in neutrophil recruitment, and while Tregs generally dampen the immune response (reviewed in (142-145).

Using HA-specific TCR transgenic CD4+ T cells, it has been demonstrated that the Th1 response is dominant in response to influenza infection (146, 147). Further, while adoptive transfer of influenza-specific Th1 cells has been shown to be protective, adoptive transfer of influenza-specific Th2 cells is not protective, and in fact resulted in delayed viral clearance and excessive congestion of the lung (148). The role of Th17 cells in influenza infection remains undescribed.

Because the predominant cytokine of a Th1 response is IFN-γ, further studies examined IFN-γ null mice, and found that loss of IFN-γ results in increases in Th2 cytokine production. However, cytotoxic T cell responses were similar to wild-type mice. Further, CD4+ T cells from these IFN-γ knockout mice were still protective, and in fact demonstrated a cytolytic ability (149). Additional studies have also shown that IFN-γ is dispensable for the cytolytic ability of these cells. More recent studies seeking to understand the mechanism by which CD4+ T cells

can exert cytotoxicity have shown that CD4+ effector T cells that are primed *in vitro* are capable of acquiring perforin-mediated cytotoxicity, and that this ability is maintained *in vivo* (150).

As alluded to previously, in addition to this direct cytotoxic effect, CD4+ T cells also play other roles in the clearance of influenza. CD4-depleted mice are still capable of clearing influenza, suggesting that CD4+ T cells are not required for survival (151). Further, mice lacking both CD8+ T cells and B cells do not survive influenza challenge, suggesting that CD4+ T cells alone are also not sufficient for viral clearance (152). However, there is ample evidence for the role of CD4+ T cells in helping B cells and CD8 T cells. Mice lacking CD8+ T cells can survive influenza challenge, but that ability is compromised by depletion of CD4+ T cells, providing evidence that B cells alone, without CD4+ T cell help, are not sufficient (139, 152, 153). Similarly, mice lacking B cells are able to clear influenza, but when depleted of CD4+ T cells cannot, suggesting that the CD8+ T cell response also requires CD4+ T cell help (151).

Multiple studies have confirmed the role of CD4+ T cells in helping to promote the B cell response to influenza. Adoptive transfer of CD4+ T cells results in antibody titers that are 10- to 50-fold higher than control mice (150). More specifically, titers of IgG2a are increased, reflecting the skewing towards a Th1 response. In contrast, mice receiving CD4+ T cells that are IFN-γ – deficient produce higher levels of IgG1, reflecting a Th2 predominance. This CD4+ T cell-dependent antibody response is important in viral clearance, as mice lacking B cells were not protected by transfer of CD4+ T cells (150). Other studies have shown that influenza-specific antibody titers are decreased in thymectomized mice or mice lacking T cells (154, 155).

CD8+ T cells have been demonstrated to be protective, principally through cytolytic mechanisms. CTLs recognize antigen in the context of MHC class I molecules, which can be expressed by nearly all nucleated cells, including the respiratory epithelium. Virus-specific

CD8+ T cells are capable of killing infected cells both through Fas/FasL and perforin-dependent mechanisms (156, 157). The CTL response is generally directed towards conserved epitopes of internal proteins, including NP and matrix protein, which allows for a degree of heterosubtypic immunity (158). Similar to CD4+ T cells, CD8+ T cells are subclassified into analogous Tc1 and Tc2 subtypes based on their cytokine profiles. Interestingly, Tc1 and Tc2 cells are both protective, but Tc1 cells are more effective at reducing viral load, and are predominantly localized at the respiratory epithelium. In contrast, Tc2 effector cells are found predominantly in the interstitial space of the lung (137, 159).

A recent report has established a third subtype of CD8+ T cells: Tc17 cells. These cells are analogous to Th17 cells in terms of cytokine profile. Tc17 cells generated *in vitro* are protective against influenza challenge when adoptively transferred. Interestingly, these cells lack perforin or granzyme B and do not display cytolytic activity. Protection is assumed to be through the recruitment of neutrophils by production of IL-17 (160).

It is also important to note that CD8+ T cells have been implicated in the immunopathology of influenza infections. This immunopathology is due, at least in part, to the antigen-specific production of TNF-α by these CD8+ T cells. Antibody neutralization of TNF-α or loss of TNF receptor-1 (TNFR-1, or p55) is able to eliminate this immunopathology. In response to membrane-bound TNF-α on the CD8+ T cell, alveolar epithelial cells produce MCP-1 and MIP-2, resulting in increases in the number of inflammatory cells recruited to the lung. Interestingly, it is also suggested that TNF-α is dispensable for CD8+ T cell antiviral activity, raising the possibility of targeting TNF-α to protect against immunopathology without interfering with viral clearance (161, 162).

One additional class of T cells is the γδ T cells. These differ from CD8+ and CD4+ T cells in multiple ways, the first being that their TCR is composed of γ and δ chains, as opposed to α and β. Their receptor diversity is much more limited, and thus these cells are often thought of as innate-like lymphocytes. Additionally, these cells are capable of recognizing target antigens directly, as opposed to bound to MHC molecules. Not much literature exists in describing the role of these cells in influenza infection (163), though it has been reported that these cells may respond to endogenous antigens such as heat shock proteins, which are upregulated during influenza infection (164).

1.2.7 Influenza: Immune Response- B cells and Antibodies

B cells contribute to the immune response to influenza in several distinct ways. First, it has been shown that B cells can produce a number of cytokines, including IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ (165). Further, B cells are capable of presenting antigen via MHC class II (166) and providing costimulation to T cells (167). However, their major role in most immune responses is production of antibody. Antibodies are capable of neutralizing and opsonizing antigen, and are therefore a key player in antigen clearance (139, 168).

Studies of B cell deficient (μMT) mice have produced mixed results. Some studies have reported that these mice have difficulty clearing virus and increased mortality (151, 169) while others show minimal differences from wild-type controls (170-172). However, adoptive transfer of influenza-specific IgG antibodies to SCID mice is protective in the influenza model, indicating a beneficial role for a B cell response. In contrast, influenza-specific IgM antibodies do not provide protection to SCID mice, indicating that only isotype-switched antibodies are

effective in viral clearance (173). Antibody class switching requires T cell help, and in mice lacking T cells, influenza-specific antibody titers are much lower (154, 155). However, there are reports demonstrating that there is some protective CD4+ T cell-independent antibody response (174, 175).

Both influenza-specific IgA, found mainly in mucosal surfaces, and systemic IgG are capable of recognizing and neutralizing influenza virus (176, 177). However, there are two weaknesses to the antibody response. First, antibodies are generally directed to the surface proteins of influenza, HA and NA. As discussed above, these antigens are highly variable, and due to genetic shift and genetic drift, differ between different strains of the virus. As a result, antibodies to one strain may not have much cross-reactivity, and therefore offer little protection, against a different strain. However, reports have suggested that preexisting antibodies are able to offer early control over viral replication even if there is only partial cross-reactivity (176).

The second weakness to the antibody response to influenza is the fact that there is a delay between the time of infection and the time when antibody titers are able to be measured. Significant titers of class-switched antibodies generally do not appear until late in the infection, not peaking until 10 days post-infection or later, after the virus has been cleared (178, 179). This implies that antibody production is not a significant factor in the clearance of a primary influenza challenge. Instead, the value of the antibody response appears to be secondary protection against re-infection. Indeed, virus-specific IgG can be detected in the serum for years following infection. Studies in the mouse have demonstrated that these antibodies come from plasma cells in the bone marrow that are generated in response to viral infection and are long-lived (180).

1.2.8 Influenza: Immune Response- Memory

Virus-specific antibody titers are detectable for years after infection (139, 168, 180), and indeed, adoptive transfer of IgG from animals that have been infected with influenza is protective in naïve animals, including SCID mice (173). This demonstrates that the humoral memory response is capable of providing protection against re-infection (139). This humoral response is the main mechanism by which current vaccines confer protection. However, as previously discussed, this is limited by the fact that the antibody response is directed against the HA and NA surface proteins of the influenza, and that these proteins are highly variable between different strains of the virus. Thus, current vaccines would not be protective in the case of the emergence of a reassortant virus (181).

Because the CD8+ and CD4+ T cell response is directed towards internal viral proteins that are more highly conserved, it stands to reason that T cell memory is capable of providing broader protection against a wider variety of influenza strains (182). The effector CD4+ T cell response rapidly transitions to a resting population with memory characteristics following viral clearance (136, 183). Though the frequency of CD4+ memory cells is lower than that of CD8+ memory cells (138), they are still detectable both in the lung and in secondary lymphoid tissues, including spleen and lymph nodes. CD4+ memory cells in the lung express high levels of CD43 and low levels of CD62L and CCR7, whereas CD4+ cells in the spleen and lymph node are more typical of central memory cells, expressing high levels CD62L and CCR7. These phenotypical differences suggest that there may be functional differences between different memory populations that is site-specific (184).

The CD8+ T cell response does not undergo the same magnitude of contraction that the CD4+ T cell response does; therefore, CD8+ memory T cells are generally detectable at higher frequencies (138). Similarly, CD8+ memory cells can be found both in the lung and lymphoid tissue (185). Upon secondary challenge, there is rapid proliferation of the CD8+ T cell population. Interestingly, both the size of the resting CD8+ memory T cell pool and the magnitude of the recall response are diminished in mice lacking CD4+ T cells, illustrating a role for CD4+ T cells in supporting CD8+ T cell memory responses (186).

1.2.9 Highly virulent strains of influenza

While seasonal outbreaks of influenza occur every year, it is important to note that strains capable of producing global pandemics circulate at irregular intervals. Perhaps the worst of these was an outbreak of an H1N1 strain that occurred in 1918 in what is commonly referred to as the Spanish Flu, despite the fact that there is no evidence that it originated in Spain. It has been estimated that anywhere from 20-50 million people died from this pandemic (32, 187). In contrast to most seasonal strains, where mortality is highest among the elderly, the 1918 pandemic saw significant mortality among young adults (188). Re-creation of the 1918 pandemic strain (189) has allowed for the study of differences between this highly pathogenic strain and more typical, interpandemic strains.

One major difference observed between the 1918 H1N1 strain and interpandemic strains is an increased rate of viral replication (190). This may be one trigger that results in an early and sustained abberant innate immune response that is associated with the increased pathology. In what is commonly referred to as a “cytokine storm”, there are marked elevations of several

proinflammatory cytokines, including TNF-α, IL-1, and IL-6 (38, 191). In mice, it has been reported that there is an excessive infiltration of macrophages and neutrophils in the lung (38, 39). Similarly, studies in nonhuman primates revealed increases in inflammation, with higher levels of CCL2, CCL5, IL-8, and IL-6 (36).

Strains of H5N1 influenza currently circulating in avian populations- though also capable of causing fatal disease in humans- also display highly pathogenic characteristics. Similar to the 1918 H1N1 strain, H5N1 influenza infection in mice results in excessive macrophage and neutrophil recruitment to the lung (39) as well as higher levels of proinflammatory cytokines (39, 192, 193). High levels of TNF-α, IFN-γ, and IL-6 were also described in serum from patients infected with H5N1 in Hong Kong during outbreaks in 1997 (194). Additionally, H5N1 strains may also be capable of disrupting immunity beyond the innate response, as infection of dendritic cells has been noted in H5N1 infection (39).

Fortunately, human to human transmission of H5N1 strains is rare, and generally requires intimate contact (28). This is thought to be due to receptor binding specificities of the H5 molecule. This form of hemagglutinin predominantly recognizes α2-3-bound sialic acids, whereas α2-6-bound sialic acids are dominant in the human upper respiratory tract. There are, however, α2-3-bound sialic acid residues in the lower respiratory tract, meaning that if the innoculum is heavy enough and deep enough, it will cause disease (195, 196). There is fear that through antigenic drift and antigenic shift that H5N1 strains could adapt to recognize α2-6 residues and subsequently evolve into a serious pandemic.

1.2.10 Current treatment and vaccination strategies

In the case of uncomplicated influenza, treatment options are generally supportive- hydration and symptom-based therapy with acetaminophen. In the case of severe cough, codeine-containing cough suppressants are often used (88). Specific antiviral therapy is available in the form of neuraminidase inhibitors zanamivir and oseltamivir. Use of these agents can decrease the duration of symptoms by as much as two days if begun within 48 hours of onset of symptoms (197, 198).

In the past, amantadine and rimantadine have been used to treat influenza. The presumed mechanism of action was interference with the viral M2 protein, which blocked viral uncoating and replication. However, resistance to these drugs is very high (>90% of H3N2 viral isolates in 2005-2006 were resistant to amantadine) and therefore they are no longer recommended for treatment. In addition to antiviral drugs, antibiotics should be used for the management of secondary bacterial infections. The use of a specific antibiotic should be determined by the bacterial species causing disease (88).

Influenza vaccination is perhaps the best weapon we have against the virus. Vaccination strategies against influenza are unique among all other vaccination strategies in that a new vaccine must be given each year. This is because antigenic drift and antigenic shift result in strains that may not be covered by the antibody response to previous vaccines. This also requires epidemiologists to accurately identify the predominant circulating strains of influenza prior to the "flu season" for the vaccine to be effective (88, 199). Ongoing research seeks to develop better vaccines that prime both a humoral and cellular immune response. This includes development of both DNA-based vaccines (182) and non-infectious influenza virus-like particles (200-202).

Currently, both inactivated and live viral vaccines are available. In both cases, the vaccine is a mixture of influenza A and B strains thought to predominate, and they are highly effective if the vaccine strains closely match the circulating strains. Vaccination is recommended for children, elderly, and immunocompromised patients, as well as patients who have any chronic pulmonary disorders that may predispose them to infection. Additionally, vaccination is recommended for health care workers and people who live with or care for high-risk patients (199).

1.2.11 Influenza: Summary

Influenza is an enveloped, negative-sense segmented RNA virus capable of causing infection in the respiratory tract by infecting respiratory epithelial cells. Severity of influenza infection can range from subclinical to fatal. Outbreaks of seasonal strains of influenza occur annually in the winter months, while more severe pandemic strains occur at irregular intervals over periods of decades. Seasonal outbreaks tend to affect the elderly and immunocompromised disproportionately, whereas pandemic strains have historically had higher mortality rates, including mortality among young adults. The immune response is multifaceted and complex, involving contributions from alveolar epithelial cells and macrophages, neutrophils, CD4+ T cells, CD8+ T cells, and B cells (and antibodies). Dysregulation of this immune response, particularly the neutrophil and CD8+ T cell response, may be responsible for much of the immunopathology observed in severe cases of influenza.

Currently, vaccination is the most useful method of combating the spread of influenza. Unfortunately, the virus is continually changing its antigenic epitopes- through

genetic shift and genetic drift- necessitating updated vaccines each year. Treatment of influenza is generally supportive and consists of hydration and symptom-dependent use of acetaminophen. The only virus-specific treatment available is the use of neuraminidase inhibitors, as influenza has become resistant to amantadine.

1.3 Interleukin-17 and Interleukin-17 Receptor A Signaling

1.3.1 Discovery and Cellular Sources of Interleukin-17

IL-17, also known as IL-17A, was first cloned in 1993 and identified as cytotoxic T-lymphocyte-associated antigen (CTLA)-8. It was found to have high similarity with open reading frame 13 of *Herpesvirus saimiri*, a virus capable of inducing T cell lymphomas in several species of primates. In humans, the gene for IL-17 was mapped to chromosome 2q31 (203, 204). However, subsequent studies have identified the gene on chromosome 6 (205, 206). Mouse and human IL-17 exhibit 63% amino acid homology (207). Since the discovery of IL-17, several other homologous proteins have been identified, resulting in an IL-17 cytokine family in which the members are designated IL-17A through IL-17F (206, 208-210). IL-17A and IL-17F share the greatest homology and are perhaps the best characterized cytokines in the family (206). These two molecules may also heterodimerize (211). In contrast, IL-17E- also referred to as IL-25- is the most divergent member (212).

IL-17 was first shown to be produced by activated CD4+ T cells (213). Since that discovery, it has been determined that CD4+ T cells producing IL-17 constitute a distinct

lineage, referred to as Th17 cells (in contrast to Th1 or Th2 cells) (reviewed in (214-216). Th17 cells produce both IL-17A and IL-17F (along with other cytokines), though subsets producing only IL-17A or IL-17F have also been described (217). These cells are described in more detail below.

Other cell types have also been described to produce IL-17. These include invariant NKT cells (218) and CD8+ T cells (219). In fact, a recent report has identified a subset of CD8+ cells that produces IL-17 and termed them Tc17 cells (160), following the convention of CD4+ T cell lineages. γδ T cells have also been described as an important source of IL-17 in multiple models (220-223). IL-17 mRNA has also been measured in neutrophils in response to challenge with lipopolysaccharide (LPS) (224), though production and secretion of IL-17 protein by these cells has not been described.

Other isoforms of IL-17 have different expression patterns. In the case of IL-17B, mRNA has been detected in a wide variety of tissue, including the gastrointestinal tract, prostate, ovary, testis, and spinal cord (208). In contrast, IL-17C expression is much rarer, having only been identified in prostate and fetal kidney (208). IL-17D is expressed in muscle and adipose tissue as well as brain, lung and pancreas (210, 225). IL-17E (IL-25), like IL-17A and F is expressed most abundantly by T cells, especially Th2-polarized CD4+ T cells (226).

1.3.2 Regulation of IL-17 Production

Of the various cell types that are capable of producing IL-17, CD4+ T cells are the best characterized producers. Production of IL-17 by these cells is dependent upon polarization to a Th17 phenotype upon antigenic stimulation. For example, polarization to the Th1 subset occurs

in the presence of IL-12 and results in a T cell population that produces IFN-γ and TNF-α. Polarization to the Th2 subset generally requires the presence of IL-4, and results in a cell population that produces IL-4, IL-5, and IL-13 (reviewed in . In the presence of TGF-β alone, T cells polarize to regulatory T cells, producing TGF-β and IL-10 (228, 229). In mice, the presence of TGF-β and proinflammatory cytokines IL-1β and IL-6, T cells polarize to Th17 cells and produce IL-17A and IL-17F, along with IL-21 and IL-22 (229-231). Other studies have identified IL-21 as being capable of driving Th17 differentiation along an alternative pathway (232, 233).

Initially, it was believed that Th17 polarization in mice also required IL-23. However, it was shown that receptors for IL-23 were not present on naïve T cells (234), and subsequently demonstrated that IL-23 is not responsible for polarization of naïve T cells to a Th17 phenotype (230, 231). Despite this, it is clear that IL-23 is important for Th17 cells, as loss of IL-23 results in decreased production of IL-17. It has been shown that IL-6 and TGF-β synergize to induce expression of IL-23 receptor, and that subsequently, IL-23 is responsible for the expansion and maintenance of Th17 cells, as well as the expression of chemokine receptors on these cells (235-237). Further, loss of IL-23 results in a decrease of IL-7 receptor alpha (IL-7Rα), which is necessary for survival of memory Th17 cells, suggesting that IL-23 also supports Th17 memory (237).

In humans, Th17 development can be driven by IL-1β and IL-23 (238, 239) and enhanced by IL-6 (240). The role of TGF-β in human Th17 development is unclear, as some reports suggest TGF-β may actually suppress the generation of human Th17 cells (238-240). However, other studies have shown that TGF-β is necessary (241, 242). Further, one study has shown that TGF-β plus IL-21 can drive human Th17 polarization (243), while another study

shows no IL-17 production under those conditions (242). Reasons behind these discrepancies must be further explored, but may be due to differences in culture media (serum vs. serum free) and time of the culture (244).

Induction of Th17 cells requires signaling through signal transducer and activator of transcription (STAT) 3 (245, 246), and activation of transcription factors RORγt and RORα (242, 247). STAT3 is activated both by IL-6 and IL-23. Additionally, aromatic hydrocarbon receptor (AhR) has been shown to be capable of stimulating Th17 differentiation (231). This cytosolic receptor, once bound to an aromatic hydrocarbon ligand, translocates to the nucleus and serves as a transcription factor that works synergistically with RORγt and RORα. Even more recently, it has been shown that prostaglandin E2, acting through prostaglandin EP2 and EP4, regulates Th17 cell differentiation (248).

Another family of transcription factors important in T cell differentiation is the family of interferon regulatory factors (IRFs). IRF1 and IRF2 have both been established to play roles in Th1 differentiation (249, 250). IRF4, on the other hand, has been shown not only to promote development of Th17 cells but to antagonize IRF1. IRF4-deficient mice exhibit decreased levels of IL-17 and IL-21. Under conditions favoring Th17 differentiation (TGF-β and IL-6), these mice instead polarize towards a regulatory T cell phenotype (251).

Production of IL-17 by γδ T cells similarly requires polarization. Subsets of IL-17-producing γδ cells have been identified that are distinct from those producing IFN-γ. Further, these IL-17 producers are CD25+ CD122-, whereas IFN-γ producing γδ are CD25- CD122+. In contrast to the αβ T cells described above, which leave the thymus as naïve cells and polarize in the periphery, the γδ T cells are capable of undergoing polarization in the thymus (222). It is hypothesized that this allows them to be able to rapidly respond to antigen in the periphery.

The precise factors determining γδ polarization are not as well described as those for Th17 cells. One report describes the role of thymic selection in polarization of these cells, demonstrating that γδ cells that are "antigen-naïve" during development produce IL-17, while those that encounter antigen during thymic selection instead produce IFN-γ (252). Other studies have demonstrated that, similar to Th17 cells, IL-17 production by γδ cells is supported by IL-23, and that this IL-23 signal is mediated through Tyk2 signaling (223).

1.3.3 The IL-17 Receptor Family and IL-17 Signaling

The first known IL-17 receptor, now known as IL-17 receptor A (IL-17RA), is ubiquitously expressed. Both IL-17A and IL-17F are capable of binding IL-17RA, but at low affinities (affinity for IL-17F is less than that for IL-17A) (253). However, a second IL-17 receptor, IL-17RC, has been identified that has a much higher affinity for both IL-17A and IL-17F. Further, IL-17RC, when co-transfected with IL-17RA, results in optimal signaling by both IL-17A and IL-17F. Additionally, these two proteins co-immunoprecipitate, providing evidence of association (254). The precise stoichiometry of this association is not determined.

IL-17RA appears to be essential for both IL-17A and IL-17F signaling, as loss of this protein blocks the ability of cells to respond to these cytokines (255). The cytoplasmic portion of this receptor contains a sequence similar to the TIR domains common to TLR's and IL-1 receptors (256). This region of the protein has been termed a "SEFIR" domain, and deletions of this region impair IL-17 signaling (257). It has further been reported that the signaling protein Act1, which also encodes a SEFIR domain, interacts with IL-17RA and results in activation of NF-κB and TAK1 (258). However, IL-17RA also has been shown to activate other signaling

pathways, including activation of ERK (258), suggesting that other signaling motifs remain to be described.

In response to IL-17RA signaling, cells produce several proinflammatory chemokines, cytokines, and growth factors. Among the chemokines that are upregulated are CXCL1 (KC), CXCL2 (MIP-2), CXCL5 (LIX) and CXCL8 (IL-8), all of which are chemoattractive for neutrophils (205, 259, 260). Additionally, G-CSF and GM-CSF are upregulated, promoting granulopoeisis, including neutrophil production (205, 261). Other chemokines that are responsive to IL-17 signaling include CXCL9 (MIG), CXCL10 (IP-10), CCL2 (MCP-1), and CCL20 (MIP3α) (262-264). Other proinflammatory cytokines produced in response to IL-17 signaling include TNF-α and IL-6 (204, 265). Additionally, IL-17 upregulates several acute phase response proteins and anti-microbial proteins, either alone or synergistically with IL-22. These proteins include SAA, C-reactive protein, lipocalin 2, β-defensins, S100 proteins, and mucins (266-271).

1.3.4 Roles of IL-17 in the Lung

Given the role of IL-17 in the regulation of growth factors that promote granulopoeises and chemokines that recruit neutrophils, it plays an important role in the neutrophil response in the lung. Coupled with the production of proinflammatory cytokines and antimicrobial peptides, this results in important consequences for lung host defense. Additionally, the IL-17 signaling pathway may play an important role in airway hypersensitivity and other chronic lung diseases, including chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF).

As mentioned before, IL-17RA is ubiquitously expressed. In the lung, IL-17RA expression has been described on the basolateral surfaces of well-differentiated airway epithelial cells, suggesting that these cells can serve as a target for IL-17 (272). Mice lacking either IL-17 or IL-17RA have demonstrated increases in susceptibility to Gram-negative bacteria, including *Klebsiella pneumoniae* (273) and *Mycoplasma pneumoniae* (274). In the *K. pneumoniae* model, IL-17RA knockout mice showed a decrease in neutrophil response and higher bacterial burdens in the lung as well as more dissemination to the spleen. All of this correlated with increased mortality (273). IL23p19 deficient mice, which have decreased levels of IL-17 exhibit a similar phenotype. In these mice, treatment with recombinant IL-17 restores the normal immune response and results in reduced bacterial burden (275). Results in the *M. pneumoniae* model are similar: lower levels of IL-17 correlate with decreased neutrophil recruitment and higher bacterial burden (274).

In contrast, reports suggest that IL-23 and IL-17 are dispensable for protection against primary *Mycobacterium tuberculosis* infection. Loss of IL-17RA does not decrease clearance of *M. tuberculosis* (276). On the other hand, IL-23 is induced by *M. tuberculosis,* and use of IL-23 as a vaccine adjuvant in *M. tuberculosis* vaccination results in a more robust antigen-specific T cell response (262, 277, 278). Use of IL-23, while decreasing mycobacterial burden, increases both Th1 cells and Th17 cells (277, 278). It has further been demonstrated that γδ T cells are the dominant source of IL-17 in the tuberculosis model (220).

Aside from the aforementioned report on Tc17 cells in the setting of influenza infection (160), there is little data on the role of IL-17 in host defense against viral pneumonias. However, there is ample evidence that fungal host defense in the lung is also affected by IL-17 signaling. In a model of *Pneumocystis carinii* pneumonia (PCP), mice with reduced levels of IL-17 have

higher fungal burdens, suggesting a beneficial role for IL-17 in fungal clearance (279). However, in apparent contrast to this, decreases in IL-23 results in more efficient clearance of *Aspergillus fumigatus*. Further studies suggested this was because IL-17 may actually inhibit fungicidal activity of neutrophils (280). This apparent dichotomy in protective vs. detrimental roles of IL-17 in fungal clearance is reflected in other extrapulmonary models of fungal infection, including *Candida albicans* (protective role in intravenous infection, detrimental role in intragastric infection) (280-282) and *Crytococcus neoformans* (protective role) (283). Further studies are needed to clarify the factors that determine whether IL-17 is protective or detrimental in the antifungal immune response.

Further evidence for the role of IL-17 in host defense is apparent in Job's syndrome (also known as hyper-IgE syndrome) patients. These patients have a defect in STAT3, which is important in both IL-6 and IL-23 signaling, and therefore key to the polarization towards Th17 cells (284). As a result, there is a decrease in Th17 cells and IL-17 production in these patients (285, 286). These patients come to medical attention with chronic, recurrent infections with *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and other pathogens (284-286).

Importantly, the neutrophil response is not the only mechanism by which IL-17 aids in host defense. As mentioned previously, IL-17 results in the induction of several antimicrobial peptides. These include β-defensins and S100 proteins (S100A7, S100A8, S100A9). The expression of these antimicrobial peptides is enhanced by the synergistic expression of IL-17 and IL-22, another Th17 cytokine (266-271). Like IL-17, IL-22 has been shown to play an important role in host defense against bacterial pneumonia as loss of IL-22 results in increased susceptibility to *K. pneumoniae* (268).

It is also important to note, however, that host defense is not the only role that IL-17 has in the lung. IL-17 also plays a role in upregulating mucus production in the airway. MUC5B and MUC5AC, two mucin proteins that constitute much of the mucus in respiratory secretions, are both inducible by IL-17 (271). Several chronic lung diseases are characterized by hypersecretion of mucus, including CF, COPD, and asthma; therefore, it is important to understand what role, if any, IL-17 might play in these disease states.

The role of IL-17 in allergic airway disease and asthma models is somewhat less clear than its role in host defense. It had been widely thought that asthma was due to Th2 cytokine production driving an eosinophilic response in the airways, resulting in mucus hypersecretion, bronchoconstriction, and airway hyperresponsiveness. This could be attenuated by the use of glucocorticoids (287, 288). However, it has been suggested that there are also steroid-resistant forms of asthma which are mediated not by eosinophils, but by neutrophils (289). Multiple studies have demonstrated that neutralization or loss of IL-17 reduces neutrophil recruitment to the lung in response to an OVA-induced asthma murine model (290-293). However, some studies have shown no effect on eosinophil recruitment (291, 293) while others suggest that these mice also have lower numbers of eosinophils in the airways and lower serum IgE levels (290, 292). Interestingly, transfer of Th17 cells that have been polarized *in vitro* results in a neutrophilic inflammation of the airways in response to the OVA-induced asthma model, whereas transfer of Th2 cells results in an eosinophilic inflammation. In both cases, the airways were hyperresponsive to methacholine challenge, but only the Th2 response was attenuated by glucocorticoid treatment (294). This suggests that Th17 may play a role in the pathogenesis of steroid-resistant asthma.

CF is another serious, chronic inflammatory disease of the lung in which patients have an excess of mucus in the airway. CF is attributed to mutations in an ion channel- cystic fibrosis transmembrane conductance regulator (CFTR)- that results in decreased amounts of airway surface liquid. This in turn prevents normal function of cilia and the accumulation of mucus, which leads to recurrent pulmonary infections (reviewed in (295). CF patients undergoing pulmonary exacerbations were found to have increases in IL-17 in their sputum. These IL-17 levels correlated with colonization by *Pseudomonas aeruginosa*, and declined with antibiotic treatment (255). This suggests that IL-17 plays a role in host defense under chronic inflammatory conditions. However, it has also been shown *in vitro* that IL-17 is capable of inducing bicarbonate secretion in human bronchial epithelial cells (296). This may have important consequences in regulating the volume of the airway surface liquid, and combined with the ability of IL-17 to induce mucus production (271), suggests that IL-17 may play an important role in mucociliary clearance in both the CF and healthy lung.

1.3.5 Roles of IL-17 Outside of the Lung

Interleukin 17 has been studied in several settings outside of the lung. Aside from roles in host defense, it has been shown to play a role in models of several autoimmune diseases, including psoriasis, rheumatoid arthritis, colitis, and experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis.

Similar to the lung, IL-17 has been identified as playing a protective role in several other bacterial infection models. Enteric *Citrobacter rodentium* infection is worsened in IL-23p19-deficient mice (230). Neutralization of IL-17 results in decreased clearance of *Escherichia coli*

following intraperitoneal infection (297). IL-17RA knockouts are more susceptible to *Porphyromonas gingivalis* (298) and have higher bacterial burdens in both the spleen and liver following *Salmonella enterica* infection (299). These receptor knockout mice, when challenged with *Listeria monocytogenes* do not demonstrate increased bacterial load in the spleen (268), but other studies have demonstrated that in the absence of IL-17, the bacterial burden in the liver is higher, and granuloma formation is impaired (300, 301). This suggests that IL-17 may play a role in clearance from the liver but not in dissemination to, or clearance from, the spleen. Interestingly, in both the *S. enterica* and *L. monocytogenes* models, γδ T cells appear to be an important early cellular source of IL-17 (299, 300).

As in the lung, there is little data available concerning IL-17's role in other viral infections. Virus-specific IL-17-producing CD4+ T cells have been detected early in HIV infection, but these cells disappeared throughout the duration of chronic infection. Further, there was no correlation between the number of IL-17-producing cells and viral load (302). In a vaccinia virus-infection model, IL-23 induced protection, and this protection was at least partly mitigated by neutralization of IL-17 (303). In a corneal model of herpes simplex virus (HSV)-1 infection, IL-17 is induced as early as 24 hours and persists at low levels. Early in the infection, IL-17RA deficient mice display decreased neutrophil recruitment to the eye, and this correlates with decreased corneal opacity, a measure of tissue pathology. However, this effect was temporary, as the receptor-deficient mice had similar levels of neutrophil recruitment and corneal pathology later in the infection (304).

IL-17 has also been extensively studied in different autoimmune models. In the EAE model of MS, it was initially believed that Th1 cells were responsible for the autoimmune pathology, though loss of Th1 cytokines (including IFN-γ and IL-12) did not affect disease

progression (305, 306). It was later shown that IL-23, not IL-12, was responsible for development of EAE (307), leading to the view that Th17 cells were responsible for the pathology. Indeed, adoptive transfer of Th17 cells resulted in the induction of autoimmunity (308, 309). Adoptive transfer of myelin-specific T cells from IL-17 knockout mice resulted in milder disease, implicating the cytokine in disease pathogenesis (310). However, a recent report has demonstrated that mice lacking either IL-17A or IL-17F (or both) are still susceptible to developing EAE. Further, mice overexpressing IL-17A do not display a difference in disease development compared to wild type controls (311). This suggests that while Th17 cells may be pathogenic, EAE is likely not entirely IL-17-dependent, but there are also contributions from IL-17-independent mechanisms. In support of this, others have shown that transfer of Th1 cells also results in disease, though the pathology is slightly different (namely, Th1-induced infiltrates were mainly macrophages whereas Th17 infiltrates were mainly neutrophils) (312, 313), again suggesting that disease development is not entirely dependent upon IL-17.

Th17 cells, though perhaps not IL-17, may also be implicated in psoriasis, a chronic inflammatory skin disease. Both IL-23 and IL-17 have been identified in psoriatic lesions (314, 315). T cells taken from the lesions and stimulated *in vitro* demonstrate an ability to produce IL-17 (315). However, levels of these cytokines in non-lesional skin or in the periphery are undetectable (316). Further, no causative effect of IL-17 on the development of psoriasis was established. Instead, reports identified the Th17 cytokine IL-22 as playing a role in the pathogenesis of the disease. This may be due, in part, to the induction of tissue repair and wound healing genes in keratinocytes in response to IL-22. Not only is IL-22 elevated in psoriatic lesions, it is also present in the serum of psoriatic patients, and these levels correlate with disease severity (317, 318). A subsequent report has likewise identified IL-22, as well as IL-20, as a key

mediator of the epithelial changes seen in psoriasis, and that IL-17, while increasing levels of neutrophil-attracting chemokines, does not induce psoriatic changes in the epithelium (319).

Th17 cells have also been shown to be involved in autoimmune arthritis. IL-17 is found at higher levels in the joint spaces of rheumatoid arthritis (RA) than in healthy patients or in osteoarthritis patients (320). Higher levels of IL-17 are predictive for worse disease outcomes (321). There are multiple mechanisms by which IL-17 may play a role in disease pathogenesis. IL-17 synergizes with IL-1 to increase osteoclast differentiation, resulting in bone erosion (322). It has further been demonstrated that IL-17 upregulates production of matrix metalloproteinases (MMPs) by synovial fibroblasts, and that these MMPs are capable of directly breaking down cartilage (323). This is in addition to IL-17's role in recruiting both monocytes and neutrophils to the joint space (324, 325).

In the gut, the IL-23-Th17 pathway has been implicated in not just host defense, but has been heavily studied in several models of colitis as well. Because the gut is a site of bacterial colonization, IL-23 and IL-17 are constituitively expressed (326). However, in multiple models of colitis there are increased levels of IL-23, IL-17, and RORγt (327-330). Further, overexpression of IL-23p19 results in severe inflammation of the small and large intestine (as part of a systemic inflammatory phenotype) (331). Additionally, transfer of Th17 cells from colitic mice into SCID mice leads to the development of colitis that is treatable by blocking IL-23p19 (332). However, like other diseases previously described, the role of IL-17 in colitis is not clearly defined. Data from these same models of colitis have shown tissue inflammation and injury in the absence of IL-17 and T cells, suggesting that IL-23 has other effects in causing disease (333). Perhaps even more contradictory, IL-17 neutralization increases the severity of dextran sodium sulfate (DSS)-induced colitis (334), whereas IL-17RA-deficient mice are

protected in a different model (atrinitrobenzenesulfonic acid (TNBS)-induced colitis) (335). Taken together, all of this suggests that regulation of the IL-23/IL-17 response in the gut is important in the development of colitis, though the exact role and mechanisms may not be fully understood.

1.3.6 Interleukin-17: Summary

IL-17 is a cytokine that plays a role in many disease states and organ systems. The IL-17 family consists of 6 members (IL-17A through IL-17F) that are capable of homo- or heterodimerizing. Of these, IL-17A and IL-17F share the highest degree of homology, while IL-17E (also known as IL-25) is the most disparate member of the family. IL-17A and IL-17F are expressed mostly by activated T cells. Indeed, a distinct subset of CD4+ T cells that produces IL-17A, IL-17F, and other cytokines including IL-22, has been characterized and termed Th17 cells. A recent report describes a similar subset of CD8+ T cells, referred to as Tc17 cells. In addition to these subsets, IL-17 can also be produced by γδ T cells and NKT cells.

Of the cellular sources of IL-17, Th17 cells have been characterized most extensively. In murine models, these cells differentiate in the periphery in response to TGF-β, IL-6, and IL-1β. IL-23 supports the expansion and maintenance of these cells. Aryl hydrocarbon receptor (AhR) has also been reported to play a role in polarization towards IL-17Differentiation requires STAT3 signaling and activation of the RORγt transcription factor. In contrast to αβ CD4+ and CD8+, it has been reported that γδ T cells differentiate into IL-17 producers during thymic selection, allowing them to quickly produce IL-17 in the periphery upon stimulation.

IL-17A and IL-17F both signal through IL-17RA. The receptor-ligand affinity is rather low, but recent studies have suggested an interaction between IL-17RA and IL-17RC which increases binding affinity, though the stoichiometry of these interactions are not known. IL-17RA signals via activation of NF-kB and ERK pathways. This results in the induction of several genes, including proinflammatory cytokines, chemokines, and growth factors that promote granulopoeisis and neutrophil chemotaxis. Additionally, IL-17 upregulates mucin expression as well as several acute phase proteins, and can synergize with IL-22 to upregulate production of several antimicrobial effector molecules.

The IL-23/IL-17 signaling axis has been studied in multiple disease models. This includes many autoimmune diseases, including psoriasis, rheumatoid arthritis, colitis, and the EAE model of multiple sclerosis. In many of these models, the precise role of IL-17, or the mechanism by which it affects disease progression is not fully understood. In the lung, IL-17 has been implicated in the pathogenesis of steroid-resistant asthma as well as other chronic inflammatory diseases, including CF. IL-17 also plays a key role in host defense, primarily through recruitment of neutrophils and induction of antimicrobial peptides. However, the effect of IL-17 on protection seems to depend on the infectious organism and model system being studied. This perhaps emphasizes the point that IL-17 is but one part of a complex and intricate immune response, whether in an autoimmune or host defense role.

1.4 Statement of Hypothesis and Specific Aims

It is well-established that one endpoint of IL-17 signaling is neutrophil production and recruitment. This is important in the control of bacterial pneumonias, due to the antimicrobial properties of neutrophils. However, the role of IL-17 signaling and neutrophil responses in the setting of viral pneumonias is not well established. In the influenza model, neutrophils have been shown to exert some antiviral effects, including the production of reactive oxygen species and α-defensins. Further, in highly pathogenic influenza models, depletion of neutrophils using anti-Gr1 results in decreased viral titers.

Unfortunately, neutrophils have also been implicated in acute lung injury in response to a wide variety of insults. This includes models of highly pathogenic influenza, in which there is an excessive neutrophil infiltration associated with increased lung injury. We therefore have chosen to investigate the role of IL-17 signaling in a model of influenza pneumonia to identify its role in neutrophil recruitment and the effects on lung injury and viral clearance. **We hypothesized that interrupting IL-17 signaling would result in decreased neutrophil recruitment to the airway, associated with decreased damage to the lung, and with minimal effects on viral clearance.**

Aim 1: Identify and describe the phenotype of IL-17RA-/- mice in response to challenge with influenza A. IL-17RA-/- and wild-type control mice are to be challenged with a lethal dose of influenza A/PR/8/34 (H1N1). Outcome measures will include morbidity and mortality, viral burden, lung histopathology, neutrophil recruitment in the BALF, proinflammatory cytokine production, and lung injury as measured by total protein and LDH in the BALF. We hypothesize that the IL-17RA-/- mice will have less lung injury, less

inflammation, and lower levels of proinflammatory cytokines, resulting in decreased morbidity and mortality. We further expect minimal differences in viral clearance.

Aim 2: Determine the role(s) that neutrophils, CD8+ T cells, and proinflammatory cytokines may play in the phenotype described in Aim 1. To assess potential mechanisms for differences in lung injury and/or viral clearance, we propose to examine three aspects of the immune response: CD8+ T cell recruitment, differences in cytokine production- especially TNF-α and IL-6, and differences in neutrophil recruitment and oxidative damage to the lung.

Aim 3: Identify the time course and cellular source of IL-17 production in response to influenza. The time course of IL-17 production will be measured at the level of mRNA by semi-quantitative real time PCR and at the protein level by ELISA. The cellular source of IL-17 production will be determined by ELISpot, intracellular cytokine staining, and use of IL-17A-GFP reporter mice.

2.0 CRITICAL ROLE OF IL-17RA SIGNALING IN THE IMMUNOPATHOLOGY OF INFLUENZA INFECTION

2.1 ABSTRACT

Acute lung injury due to influenza infection is associated with high mortality, an increase in neutrophils in the airspace, and increases in tissue myeloperoxidase (MPO). Because IL-17A and IL-17F, ligands for IL-17RA, have been shown to mediate neutrophil emigration into the lung in response to LPS or gram negative bacterial pneumonia, we hypothesized that IL-17RA signaling was critical for acute lung injury in response to pulmonary influenza infection. IL-17RA was critical for weight loss and both neutrophil migration and increases in tissue MPO after influenza infection. However IL-17RA was dispensable for the recruitment of CD8+ T-cells specific for influenza hemagglutinin or nucleoprotein. Consistent with this, IL-17RA was not required for viral clearance. However, in the setting of influenza infection, IL-17RA-/- mice showed significantly reduced levels of oxidized phospholipids, which have previously been shown to be an important mediator in several models of acute lung injury, including influenza infection and gastric acid aspiration. Taken together, these data support targeting IL-17 or IL-17RA in acute lung injury due to acute viral infection.

2.2 INTRODUCTION

Acute lung injury (ALI) is a severe clinical state, characterized by non-cardiogenic pulmonary edema, capillary leak, and hypoxemia. It can be triggered by both infectious and non-infectious stimuli. The most severe form of ALI is acute respiratory distress syndrome (ARDS)(51). Most patients who died in the severe acute respiratory syndrome (SARS) virus outbreak in 2003 developed ARDS (26). Likewise, the Spanish influenza pandemic of 1918 and current outbreaks of H5N1 influenza have both been documented to cause ARDS in mice and primates (27, 36, 39, 189). Neutrophils play an important part in the pathogenesis of ALI, as they rapidly infiltrate the lung and serve as an important source of proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α as well as reactive oxygen intermediates (ROI) (56, 336-338).

Recent studies have shown that oxidized phospholipids have many pro-inflammatory properties (339) and are an important signal in the development of ALI. Upon oxidation, airway phospholipids are capable of signaling through Toll-like receptor (TLR) 4. This signal is MyD88-independent and involves the TRIF-TRAF6 signaling pathway, and blocking this signal can prevent ALI. This role for oxidized phospholipids in the pathogenesis of ALI has been shown for both infectious (influenza) and non-infectious (acid aspiration) models (59). Given the ability of neutrophils to generate ROI's, it is possible that these cells are responsible for the oxidation of airway phospholipids.

The role of neutrophils in severe influenza infections has been debated. Studies using the reconstructed 1918 virus show that highly virulent strains of influenza result in an overwhelming neutrophilic infiltrate that is associated with worsening lung injury (36, 38, 39). However, neutrophils have also been shown to play a role in control and clearance of influenza virus in

experimental models (125, 126, 340-342), and neutrophil depletion resulted in an uncontrolled and ultimately lethal viremia in the reconstructed 1918 influenza model (38). It is important to note, though, that neutrophil depletion using the monoclonal antibody RB6-8C5 is complicated by the concurrent depletion of other monocytes, dendritic cells, and even some CD8+ T cells (343-345).

IL-17 is a potent regulator of the neutrophil response. Both IL-17A and IL-17F are capable of signaling through the IL-17 receptor A (IL-17RA) to induce granulopoetic factors such as granulocyte-colony-stimulating factor (G-CSF) and stem cell factor (SCF) which leads to expansion of neutrophil progenitors in the bone marrow and spleen, as well as expansion of mature neutrophils in peripheral blood (346, 347). Further, IL-17RA signaling upregulates production of neutrophilic-chemokines such as CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8, which in turn regulate neutrophil emigration to mucosal sites (260, 348, 349). Due to this role in neutrophil expansion and recruitment, IL-17RA signaling is an important part of the host response to extracellular bacterial infections. It has previously been demonstrated that defects in the IL-17 axis results in a decreased neutrophil response associated with higher bacterial burdens and decreased survival when mice are challenged with the gram negative bacterium *K. pneumoniae* (273, 348).

Given IL-17RA's critical role in neutrophil recruitment and the ability of neutrophils to generate ROI's, along with the important role oxidized phospholipids play in the pathogenesis of ALI, we wanted to determine if blocking IL-17RA signaling conferred protection against lung injury associated with influenza infection. Using an acute infection model- influenza A/PR/8/34 (H1N1) (PR/8)- we sought to identify a pathway by which the neutrophilic infiltrate is able to be regulated in order to reduce lung injury.

2.3 METHODS

2.3.1 Animal Infections and HCl Challenge

IL-17ra on a BALB/cJ background (350) and B6 background (348), and *p55/p75* on a B6.129 background (351), have been previously described. Wild-type controls included BALB/cJ, B6.129, and C57BL6 mice. Mice were challenged intranasally with an LD_{90} of influenza A/PR/8/34 (H1N1) in phosphate-buffered saline (PBS). Following infection, mice were monitored daily for weight loss and signs of clinical illness (352). Mice were sacrificed at indicated time points. For HCl challenge, mice were given 100 μL of HCl (pH 1.5) intratracheally and sacrificed at 24 hours post-challenge (353). Control mice received equivalent volumes of PBS.

2.3.2 Lung harvest and bronchoalveolar lavage

At indicated time points, mice were euthanized with an overdose of inhaled isofluorane. The abdomen was opened and the animals were exsanguinated by cutting of the renal artery. The trachea was then exposed and cannulated. 1 mL of sterile PBS was injected into the airway and then collected. Animals were then perfused with PBS via injection in the right ventricle to flush blood vessels in the lung. Left lungs were collected in TRIzol reagent (InVitrogen) and homogenized using an electric tissue grinder. RNA was purified according to manufacturer's instructions. Right lungs were collected and homogenized in PBS for use in plaque assays and

ELISA. Lavage fluid was spun down at 500 x g for 5 minutes to pellet cells. BAL cells were counted using a Coulter Counter (Beckton-Dickinson) and 10^5 cells were spun onto a glass slide, then fixed and stained for counting.

2.3.3 IL-6 neutralization studies

A monoclonal antibody capable of neutralizing IL-6 (clone MP5-20F3) was administered to mice via intravenous injection at a concentration of 3 μg/g of body weight at the time of influenza challenge (354). A second dose was administered at 3 dpi. Mice were monitored daily and sacrificed at 6 dpi. To ensure sufficient neutralizing antibody was delivered, lung homogenates were used in a direct ELISA. Briefly, 96 well ELISA plates were coated with 100 ng of recombinant IL-6 at room temperature for 4 hours. Plates were washed 4x, blocked overnight with 1% bovine serum albumin (BSA) at 4 degrees Celsius, then washed 4x again. 100 μL of lung homogenates were added per well, incubated for 2 h at room temp, then washed 4x, followed by detection with α-IgG1-HRP. Plates were developed using TMB substrate reagents (BD).

2.3.4 Real time PCR

RNA collected from the left lung was used as template to generate cDNA using iScript reagents and protocol (BioRad). cDNA was then used in fast real-time PCR using the Applied Biosystems 7900HT. Reaction conditions were 95° Celsius for 1 second and 60 degrees for 20 seconds, repeated for 40 cycles, with a 20 second hot start at 95° C. Primer and probe sequences

were as follows: Influenza M Protein Forward- 5'-GGA CTG CAG CGT AGA CGC TT-3', Influenza M Protein Reverse- 5'-CAT CCT GTT GTA TAT GAG GCC CAT-3', Influenza M Protein Probe- 5'-CTC AGT TAT TCT GCT GGT GCA CTT GCC A-3'. 18s RNA was used as a housekeeping gene with the following primer/probe sequences: Forward- 5'-GAT CCA TTG GAG GGC AAG TCT-3', Reverse- 5'- GCA GCA ACT TTA ATA TAC GCT ATT GC-3', Probe- 5'-TGC CAG CAG CCG CGG TAA TTC-3'. Fold-change in mRNA was quantified using the ΔΔCT method.

2.3.5 Measurement of cytokines

IL-17A and IL-17F in lung homogenate were measured by ELISA (R&D DuoSet) according to manufacturer's instructions. All other cytokines were measured by multiplex analysis using Millinex (Millipore) reagents according to manufacturer's instructions and analyzed on the Bioplex reader (BioRad).

2.3.6 Assessment of lung injury

Total protein in the BALF was measured using the BCA Protein Assay Kit (Pierce). LDH activity was measured using the lactate dehydrogenase assay (Sigma). Both assays were performed in a 96 well plate for 30 minutes according to manufacturer's instructions and analyzed using the Benchmark Plus plate reader (BioRad).

2.3.7 Lung fixation and histologic examination

Animals were sacrificed at the indicated time points, the trachea was exposed and cannulated. Lungs were inflated with 10% neutral buffered formalin (Sigma) for 10-15 minutes, removed from the animal and placed in fresh 10% neutral buffered formalin for at least 24 hours at 4° Celsius before processing and embedding. Sections were cut (5μm) and stained with Hematoxylin and Eosin (H&E) for histopathologic evaluation (355).

Slides were blinded and scored in a semiquantitative manner according to the relative degree of inflammatory infiltration (356). Inflammation was scored as follows: 0= no inflammation, 1= perivascular cuff of inflammatory cells, 2= mild inflammation, extending throughout <25% of the lung, 3= moderate inflammation covering 25-50% of the lung, 4= severe inflammation involving over half of the lung.

2.3.8 Measurement of oxidized phospholipids

Oxidized phospholipids were detected by direct ELISA. Briefly, BALF was adsorbed onto 96-well ELISA plates, normalized for 100 ng total protein per well, overnight at 4 degrees Celsius. Plates were washed 4x with PBS + 0.5% Tween-20 before blocking with 1% BSA for 1 h at room temperature. Plates were then washed 4x again and probed with 100 μL of the monoclonal antibody EO6 (a generous gift of J.L. Witztum) (357), diluted to 5 μg/mL in PBS. Plates were incubated for 2 h at room temperature, then washed 4x. Anti-IgM conjugated to HRP (Southern Biotech) was used as a secondary antibody, diluted 1:2,000, for 2 h at room temperature. Plates were washed one more time before being developed with TMB substrate

reagents (BD). Absorbance was read at 450nm, with a wavelength correction at 540nm subtracted out.

2.3.9 Plaque assay

Influenza titers were measured by traditional plaque assay (358). Briefly, lungs were homogenized and homogenates were diluted before being applied to 95% confluent MDCK cells. Virus was adsorbed onto the cells for 1hr at room temperature before being washed off 4x with serum-free DMEM. Cells were then covered with serum-free DMEM containing trypsin and 0.8% agarose and incubated for 48h at 37° Celsius with 5% carbon dioxide. Agarose was then removed and the monolayer was stained with 1% Genetian Violet to allow for the visualization and counting of plaques.

2.3.10 Depletion of neutrophils

Depletion of neutrophils was accomplished by the administration of monoclonal antibodies RB6-8C5 (anti-Gr1, R&D) (342) or 1A8 (anti-Ly6G, BioXCell) (359) where indicated. Antibodies were given by intraperitoneal injection 24 hours prior to challenge, and every 48 hours thereafter. Both antibodies were given at a dosage of 0.3mg per animal. Neutrophil depletion was confirmed by analysis of peripheral blood smears at the time of challenge.

2.3.11 Statistics

All data is presented as the mean ± s.e.m. Significance was tested using unpaired t-tests, ANOVA, or, in the case of survival curves, Kaplan-Meier analysis. All statistics were analyzed using GraphPad Prism 4 software.

2.4 RESULTS

2.4.1 Decreased Morbidity and Mortality among IL-17RA knockout mice

To examine the effects of IL-17 signaling during influenza infection, we challenged IL-17RA knockout mice on a BALB/cJ background (350) and BALB/cJ controls with 100 pfu of PR/8. Following viral challenge, we noted that the IL-17RA-deficient mice experienced less weight loss throughout the course of infection (Figure 1A). This correlated well with higher survival rates among the knockout mice (Figure 1B), suggesting that IL-17RA signaling may be detrimental in the response to influenza virus challenge. It is important to note that the differences in weight loss were significant from days 2 through 8, beyond which the data is subject to survival bias. To rule out the possibility that the morbidity and morality differences that we observed were due to differences in viral clearance, we measured viral burden by real-time PCR, using primers and probe for the influenza M1 gene. Somewhat surprisingly, we found that despite their decrease in morbidity and mortality, the IL-17RA knockout mice had a trend toward a higher viral burden at 6 dpi (Figure 1C). This data trend was also supported by plaque

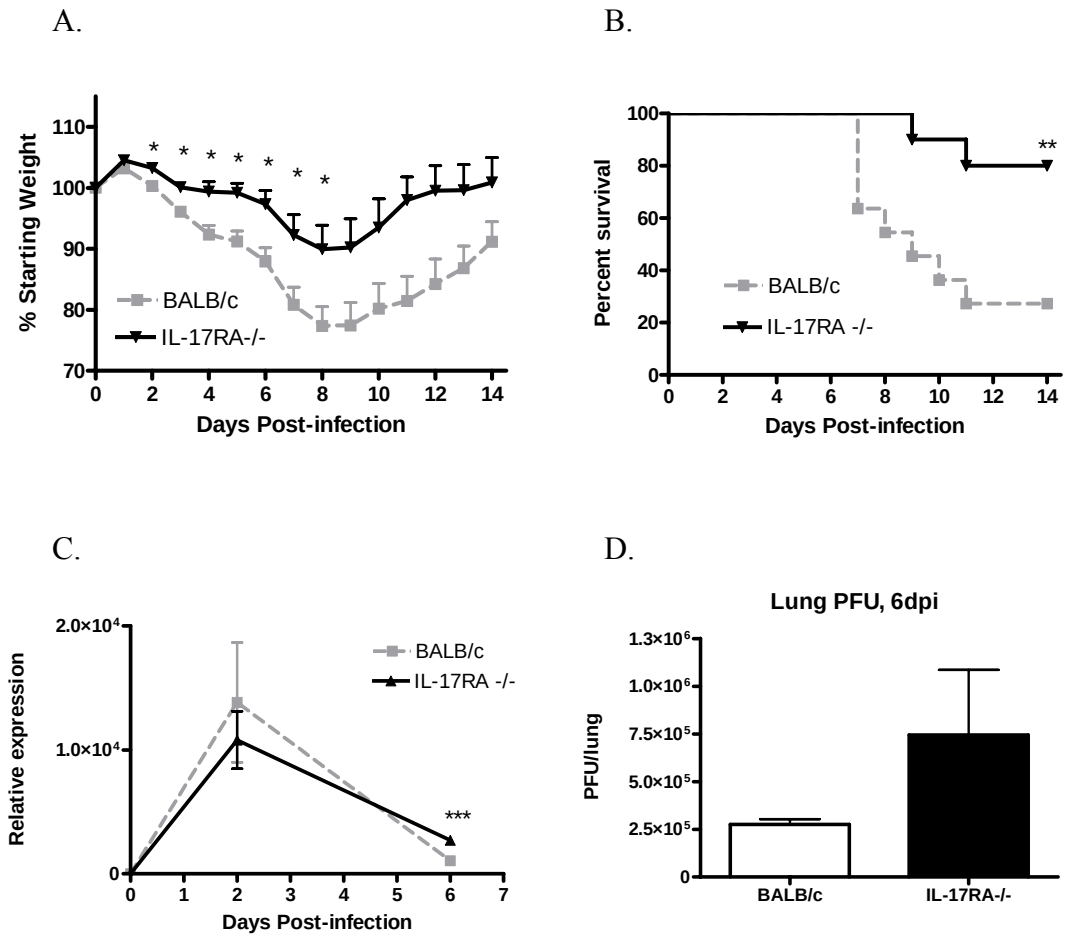


Figure 1: Weight loss, mortality, and viral burden following challenge with influenza A/PR/8 IL-17RA-/- mice exhibit less weight loss (A) and mortality (B) following viral challenge (n=10/group). Mice were monitored daily for weight loss and clinical signs of illness. Viral burden was determined by quantitative PCR (C) for the influenza M1 protein (n=4/group/time point), or by viral plaque assay (D). *p<0.05, **p=0.0067, ***p=0.0093

assay (Figure 1D). Taken together, these data show that despite a trend toward higher viral burdens, IL-17RA knockout mice are able to better tolerate influenza challenge, as they ultimately clear the virus and recover from the challenge. This suggests that these mice may have decreased immunopathology that would explain the observed phenotype.

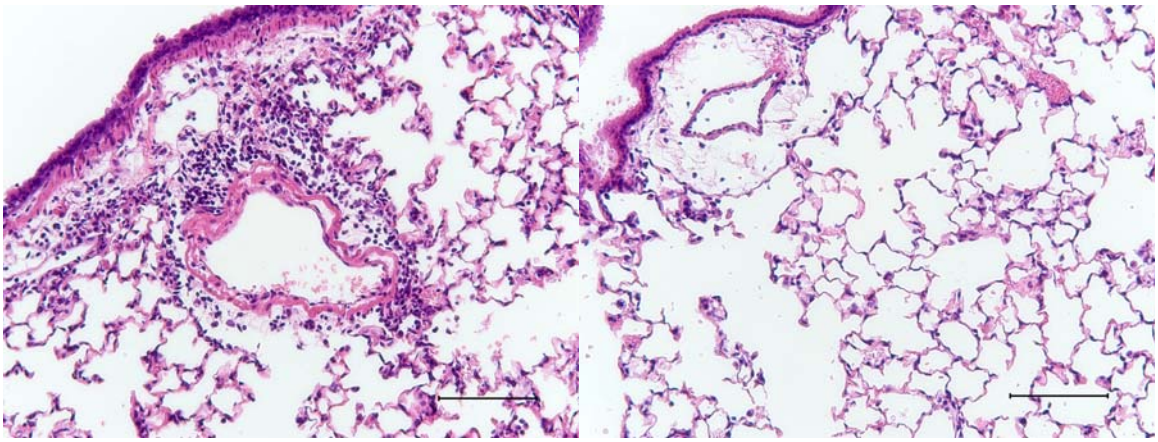
2.4.2 IL-17RA knockout mice have lower levels of inflammation

Histological examination of lungs at 6 dpi showed dramatic decreases in inflammation among the IL-17RA knockout mice (Figures 2A and B). Lungs were scored on the basis of the degree and extent of inflammation (356). By day 2, both the wild-type and knockout mice exhibit limited inflammation, mainly restricted to the perivascular areas (Figure 2C). However, by day 6, wild-type mice showed heavy perivascular inflammatory infiltrates, with inflammation extending out into the parenchyma (Figures 2A and 2C). In contrast, the IL-17RA knockout mice had only mild inflammation mainly limited to the perivascular bed, with the lung parenchyma relatively unaffected (Figures 2B and 2C).

Given these histological findings, we sought to measure the levels of inflammatory cytokines in lung homogenates. TNF-α (Figure 3A) and IL-1β (Figure 3B), both of which can be produced by neutrophils in ALI (337), were observed to be lower in IL-17RA/- mice. Additionally, IL-6 was greatly reduced in the IL-17RA knockout mice compared to wild-type controls (Figure 3C). Furthermore, levels of the IL-17-responsive chemokines and cytokines, including CXCL1 (KC) and G-CSF (Figures 3D and E) were lower in the IL-17RA knockout mice, as would be expected. Interestingly, there was a decrease in IFN-γ at 6 dpi among the IL-

A. BALB/c

B. IL-17RA-/-



C.

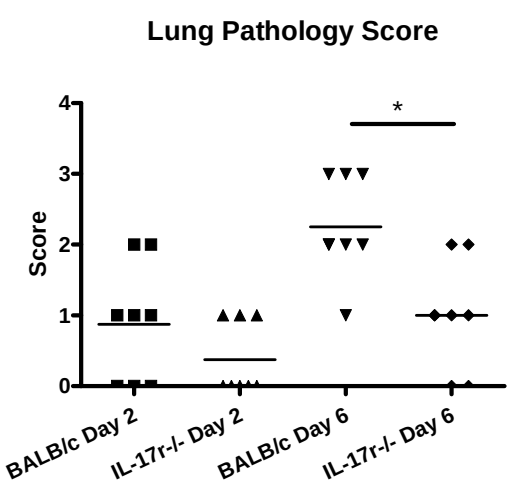


Figure 2: **Differences in inflammation following influenza challenge** BALB/c (A) and IL-17RA -/- (B) lungs harvested at 6 dpi. Inflated with 10% formalin, paraffin-embedded, and H&E stained. Calibration bar is 100um. Pathology scored (C) as follows: 0= no inflammation, 1= perivascular cuff of inflammatory cells, 2= mild inflammation, extending throughout <25% of the lung, 3= moderate inflammation covering 25-50% of the lung, 4= severe inflammation involving over half of the lung. *p=0.004

17RA knockout mice (Figure 3F). These cytokine data support the histological findings of less inflammation in the IL-17RA-deficient mice.

We next sought to determine if this decrease in inflammation resulted in lower levels of lung injury. Measurement of total protein in the bronchoalveolar lavage fluid (BALF) suggests decreased capillary leak in the IL-17RA knockout mice (Figure 4A). Moreover, consistent with less lung injury, levels of lactate dehydrogenase in the BALF was significantly lower in IL-17RA knockout mice (Figure 4B). In the context of lower levels of inflammation, these data suggest that the protection seen in the IL-17RA knockout mice may be due to lower levels of immunopathology.

2.4.3 Protection is independent of TNF–α and IL-6

Previously published studies have identified a role for TNF-α in the pulmonary immunopathology caused by influenaza infection, as mice lacking TNF receptors (p55 and p75) had decreased mortality and weight loss (161, 162). Because we observed a decrease in TNF-α levels in our knockout mice, we sought to determine if reduced TNF production was responsible for the protective phenotype we observed in IL-17RA -/- mice. To investigate this, TNF receptor p55/p75 double knockout mice (351) were challenged with an LD_{90} of PR/8. These mice are commercially available, but have only been backcrossed four times; therefore, B6.129 mice were

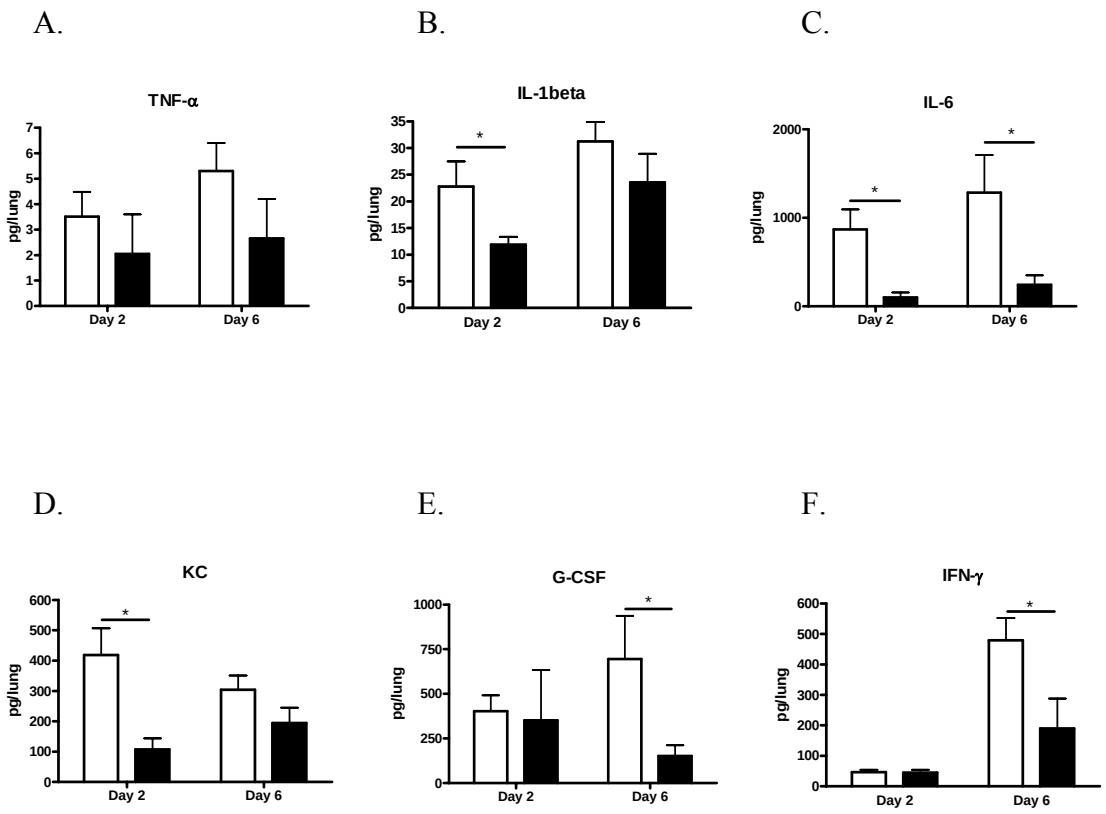


Figure 3: Cytokine response to influenza challenge. Cytokines measured at 2 and 6 days post-infection. BALB/c (white bars) vs. IL-17RA -/- (black bars) *p<0.05 (n=4/group/timepoint) (A). TNF-α (B). IL-1β (C). IL-6 (D). KC (E). G-CSF (F). IFN-γ

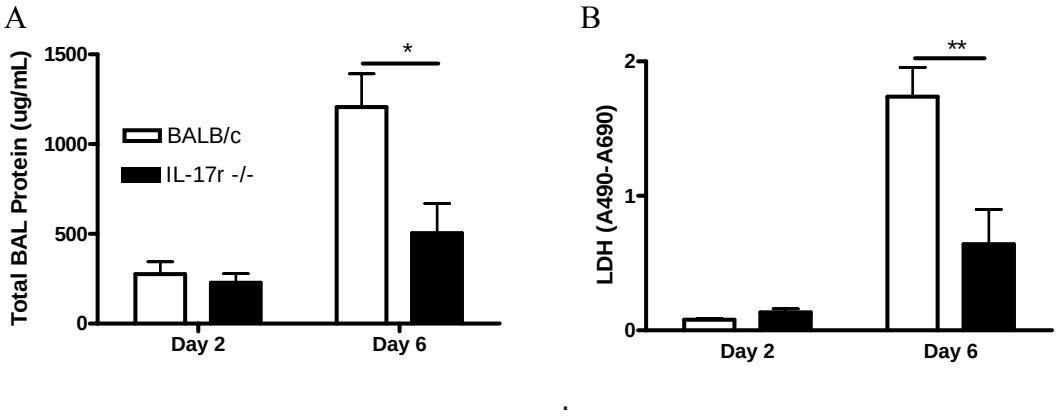
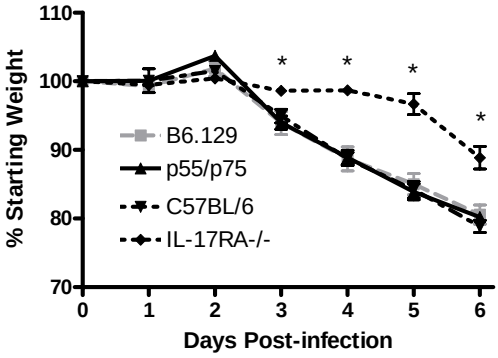


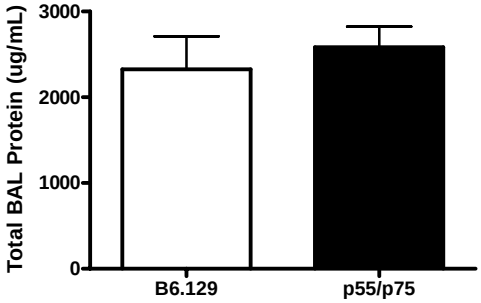
Figure 4: Markers of Lung Injury. Total protein (A) and LDH activity (B) in the bronchioalveolar lavage fluid. White bars represent BALB/c mice, black bars show IL-17RA -/- mice. *p=0.01, **p=0.01 (n=4/group/timepoint).

used as controls. Additionally, we infected IL-17RA-/- mice on a C57/B6 background (348) along with wild-type controls to demonstrate that the phenotype described was not strain-specific. In contrast to the IL-17RA -/- mice, TNF receptor p55/p75 double knockout mice showed significant weight loss which was similar to wild-type mice (Figure 5A). Moreover, in contrast to IL-17RA-/- mice, TNF receptor p55/p75 double knockout mice had similar levels of total protein in the BALF (Figure 5B), compared to wild-type control mice. Additionally, levels of LDH were unchanged (Figure 5C). This suggests that the phenotype observed in the IL-17RA knockout mice is independent of TNFR signaling. It is also important to note that G-CSF was unchanged (Figure 5D) and KC was even elevated in the double knockouts (Figure 5E), indicating that the reductions in these growth factors and chemokines among IL-17RA-/- mice was not TNF-dependent.

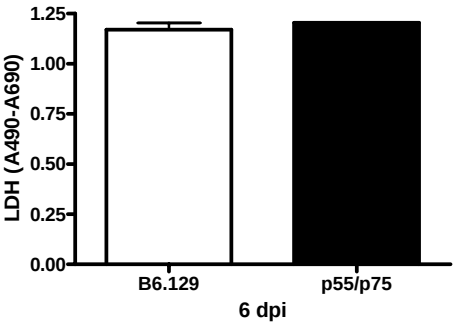
A.


110
100
90
80
70
% Starting Weight
0
1
2
3
4
5
6
Days Post-infection
B6.129
p55/p75
C57BL/6
IL-17RA-/-
*
*
*
*

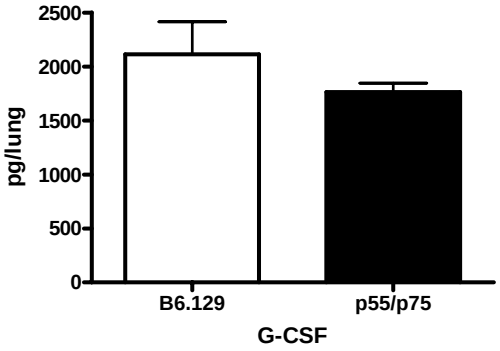
B.


3000
2000
1000
0
Total BAL Protein (ug/mL)
B6.129
p55/p75

C.


1.25
1.00
0.75
0.50
0.25
0.00
LDH (A490-A690)
B6.129
p55/p75
6 dpi

D.


2500
2000
1500
1000
500
0
pg/lung
B6.129
p55/p75
G-CSF

E.

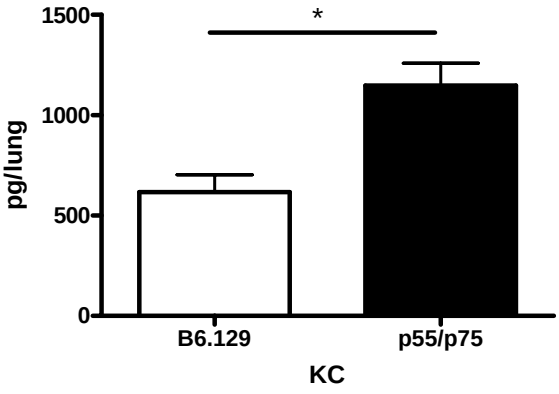
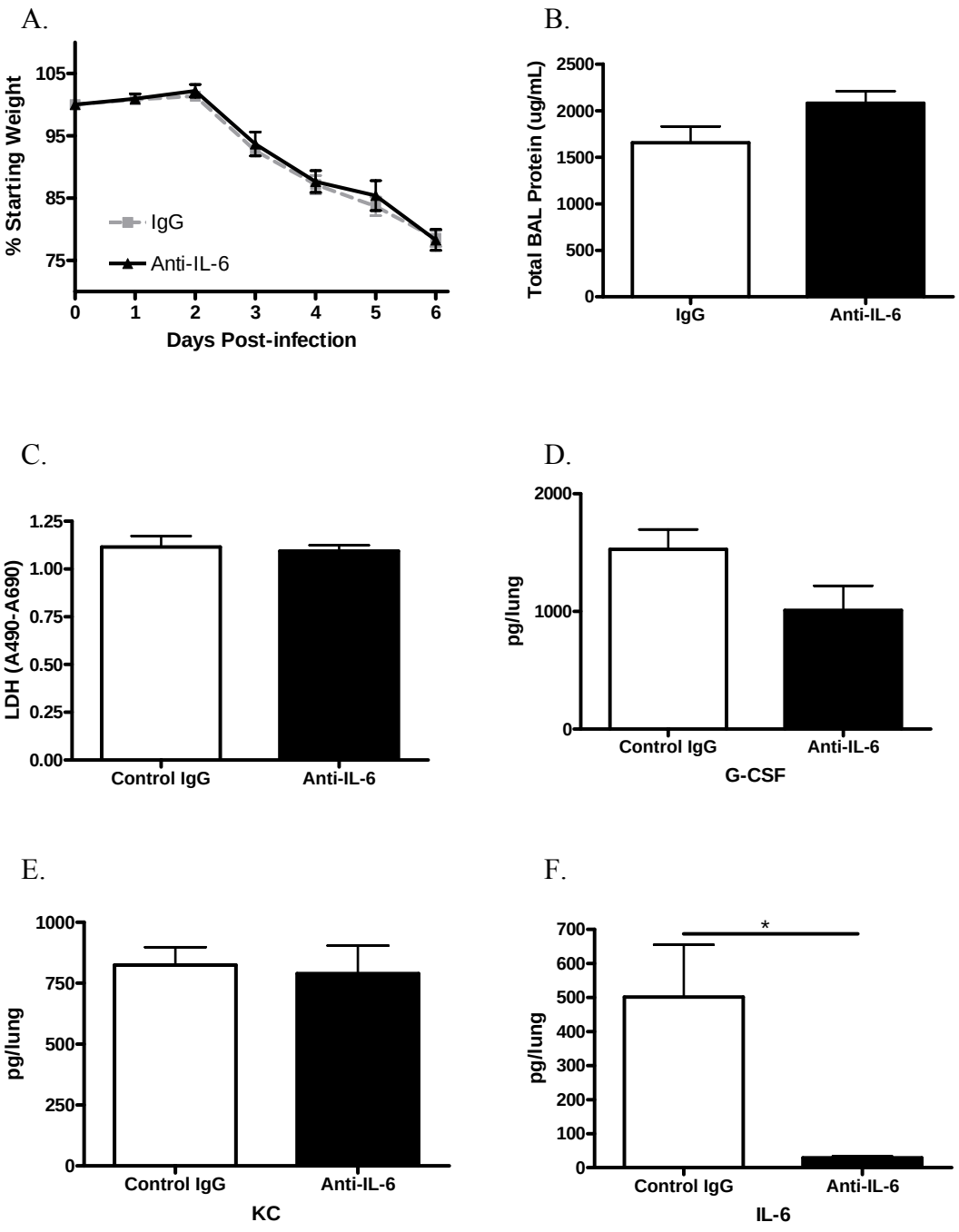

1500
1000
500
0
pg/lung
*
B6.129
p55/p75
KC

Figure 5: Protection is not dependent on TNF-α (A) Weight loss among B6.129 and p55/p75 (n=6/group) following influenza challenge. For comparison, IL-17RA-/- on C57/BL6 background and wild-type controls (n=4/group) are also provided (B) Lung injury assessed by total protein in the BALF on day 6. (C) LDH activity in the BALF at 6 dpi was unchanged compared to wild-type controls (D). Levels of G-CSF were unchanged, and (E) levels of KC were elevated in the lung homogenate at 6 dpi. *$p<0.05$


A.
% Starting Weight
105
95
85
75
IgG
Anti-IL-6
0
1
2
3
4
5
6
Days Post-infection
B.
Total BAL Protein (ug/mL)
2500
2000
1500
1000
500
0
IgG
Anti-IL-6
C.
LDH (A490-A690)
1.25
1.00
0.75
0.50
0.25
0.00
Control IgG
Anti-IL-6
D.
pg/lung
2000
1000
0
Control IgG
Anti-IL-6
G-CSF
E.
pg/lung
1000
750
500
250
0
Control IgG
Anti-IL-6
KC
F.
*
pg/lung
700
600
500
400
300
200
100
0
Control IgG
Anti-IL-6
IL-6

G.

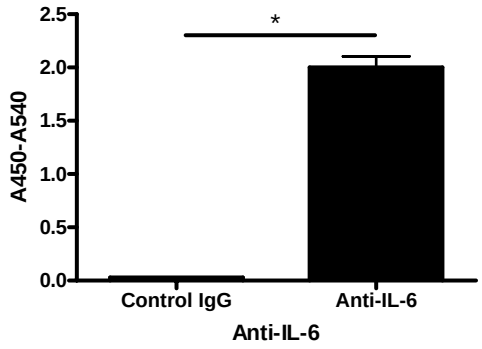


Figure 6: Protection is not dependent on IL-6 (A) Weight loss among mice treated with an IL-6 neutralizing antibody or isotype control (n=6/group) following influenza challenge. Lung injury assessed by total protein in the BALF (B) and LDH activity in the BALF(C) on day 6 was unchanged compared to isotype controls. Levels of G-CSF (D) and KC (E) were unchanged. Levels of IL-6 (F) were drastically reduced by antibody neutralization, and an excess of anti-IL-6 in the lung (G) confirms that a sufficient amount of neutralizing antibody was present. *p<0.001

Similarly, we chose to test if the decrease in IL-6 levels in the IL-17RA mice was responsible for the protective phenotype. Anti-IL-6 or control IgG was administered to BALB/c mice at the time of influenza challenge and again at 3 dpi. The anti-IL-6 treated mice showed no differences in weight loss compared to controls (Figure 6A), nor did they show any differences in total protein in the BALF at 6 dpi (Figure 6B). Likewise, levels of LDH were unchanged (Figure 6C). There were no differences in G-CSF or KC after IL-6 neutralization (Figure 6D and E), illustrating that production of these proteins are also independent of IL-6. To confirm the effectiveness of IL-6 antibody neutralization, IL-6 was measured in lung homogenate at 6 dpi and found to be drastically reduced (Figure 6F). Additionally, free anti-IL-6 was measured in the lung homogenates, and it was found that free concentrations of neutralizing antibody were present in excess of IL-6 (Figure 6G), implying that the dosage of antibody was sufficient. These data indicate that the decrease in IL-6 levels observed in the IL-17RA knockout mice is not responsible for the reduced cachexia.

2.4.4 IL-17RA-/- mice have reduced neutrophil migration and lipid oxidation

IL-17 is responsible for the induction of several granulopoetic factors and chemokines that can result in an influx of neutrophils (260, 348). Examination of the cells recovered in the BALF demonstrated that the IL-17RA knockout mice had a dramatic reduction in the number of neutrophils recruited to the airway (Figure 7A). In contrast, BAL macrophages and

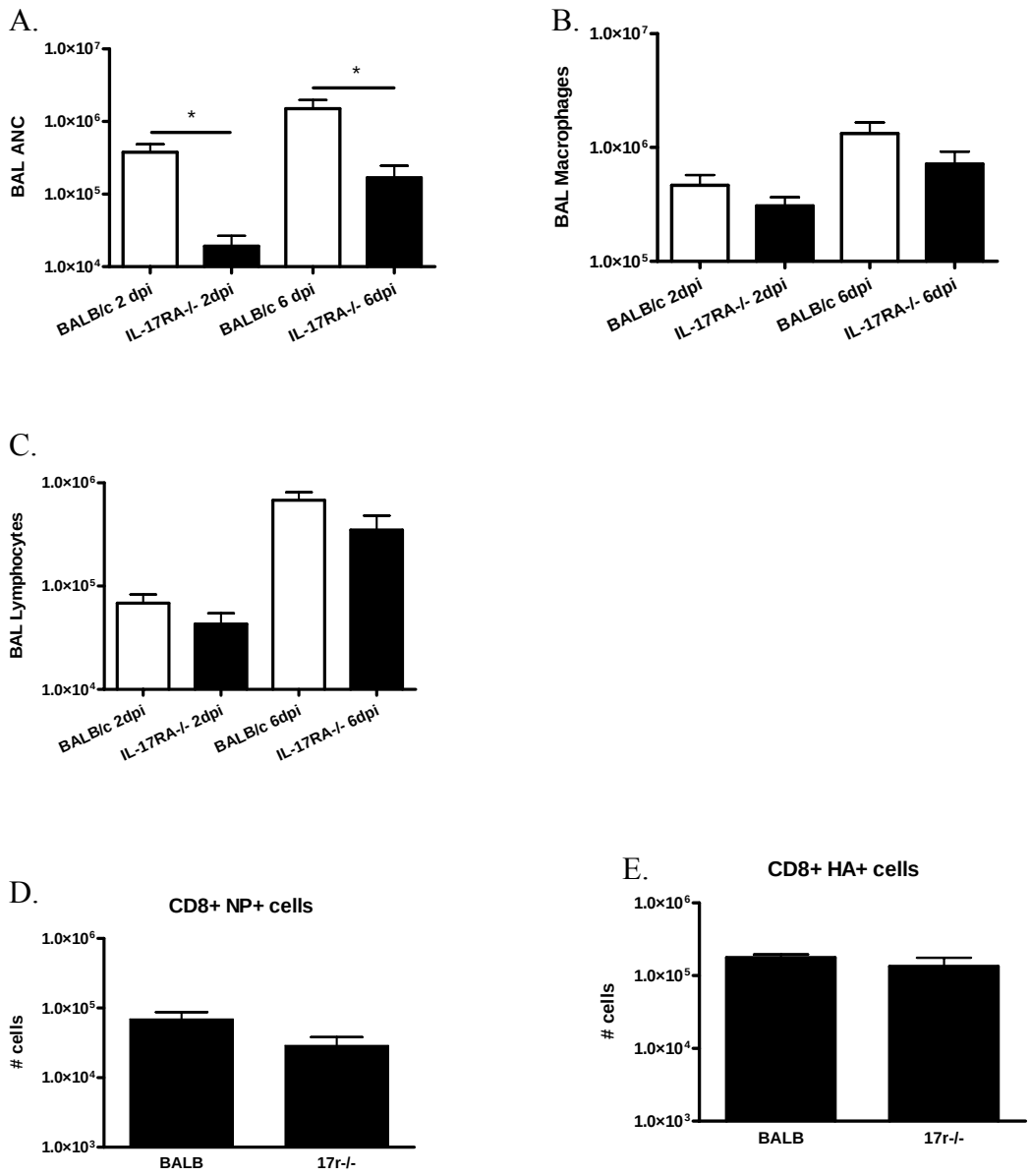


Figure 7: Differences in cell recruitment to the airway. IL-17RA-/- mice (black bars) show decreases in the number of neutrophils (A.) in the BAL compared to BALB/c controls (white bars), but no differences in macrophages (B.) or lymphocytes (C.) (n=4/group/timepoint). Pentamer staining at 10 dpi show that there is no difference in the numbers of NP-specific (D.) or HA-specific (E.) CD8+ T cells (n=4/group) *p=0.03

lymphocytes were not significantly reduced (Figures 7B and 7C). Further, examination of the hemagglutinin- and nucleoprotein-specific CD8+ T cell population revealed no differences at 10 dpi, indicating that the recruitment of these cells is unaffected in the IL-17RA knockout animals (Figures 7D and E). At 6 dpi, there were also no differences in the nucleoprotein-specific CD8+ T cells (BALB/c: $8.6x10^4 \pm 1.0x10^4$, IL-17RA-/-: $6.9x10^4 \pm 1.5x10^4$), and hemagglutinin-specific cells were not detected.

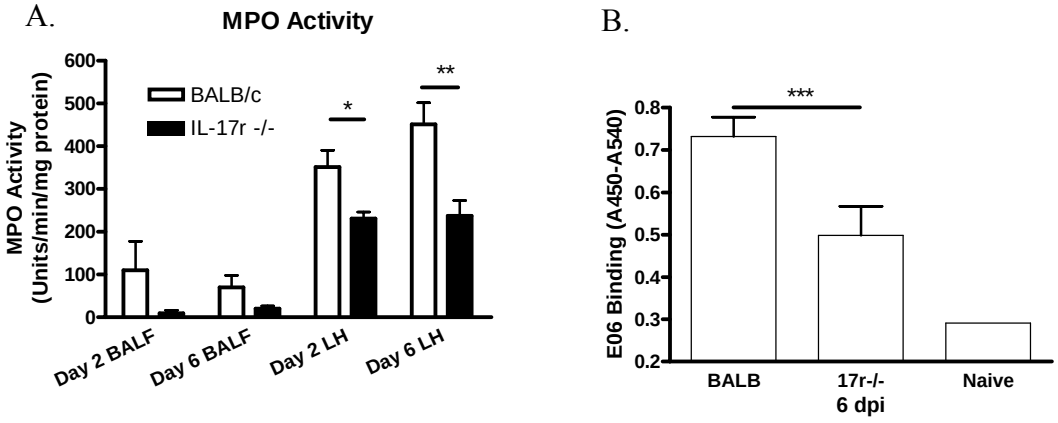


Figure 8: Differences in oxidative stress. Myeloperoxidase activity is decreased in the lung homogenates, and trends lower in the BALF, of the IL-17RA-/- mice compared to wild-type controls (n=5/group/timepoint).*p=0.03, **p=0.01 (A). Levels of oxidized phospholipids- capable of signaling through TLR4 to induce acute lung injury- as measured by ELISA, are lower in IL-17RA-/- mice than BALB/c controls (B.). (n=5/group/timepoint) ***p=0.02

Excessive neutrophil recruitment has been demonstrated in models of severe influenza infections, including H5N1 and a reconstructed 1918 virus, and is associated with worse immunopathology (36, 38). Because of this, we chose to study the differences in neutrophils more closely. Myeloperoxidase (MPO) activity, an enzyme found most abundantly in neutrophils and responsible for generating reactive oxygen species that can lead to oxidative damage (360), was decreased in lung homogenates of the IL-17RA- deficient mice when compared to BALB/c controls. Further, MPO activity trended to be lower in the BALF cell pellets of these animals as well (Figure 8A). Based on these findings and the established role of oxidized phospholipids in the pathogenesis of acute lung injury (59), we hypothesized that the IL-17RA-/- mice might be accumulating fewer oxidized phospholipids, which would in turn explain the decrease in lung injury seen in these mice during influenza infection.

To measure oxidized phospholipids, we adsorbed BALF onto 96-well ELISA plates normalized for the amount of protein in the sample. We then probed with EO6, a monoclonal antibody that specifically recognizes the phosphocholine head of phospholipids that contain an oxidized sn2 side chain, but does not recognize nonoxidized phospholipids (357). It has been previously demonstrated that oxidized phospholipids that are recognized by EO6 are pro-inflammatory (361). We found that the IL-17RA knockout mice had significantly decreased levels of oxidized phospholipids (Figure 8B) in BAL fluid compared to wild-type mice at 6 dpi.

To confirm the role of neutrophils in generating the oxidized phospholipids, we used an anti-Ly6G antibody (clone 1A8) to deplete neutrophils. In contrast to the anti-Gr1 antibody (RB6-8C5) which recognizes both Ly6G and Ly6C, clone 1A8 reduces neutrophils but not Gr1+ monocytes (359). This antibody was given by intraperitoneal injection every 48 hours, starting

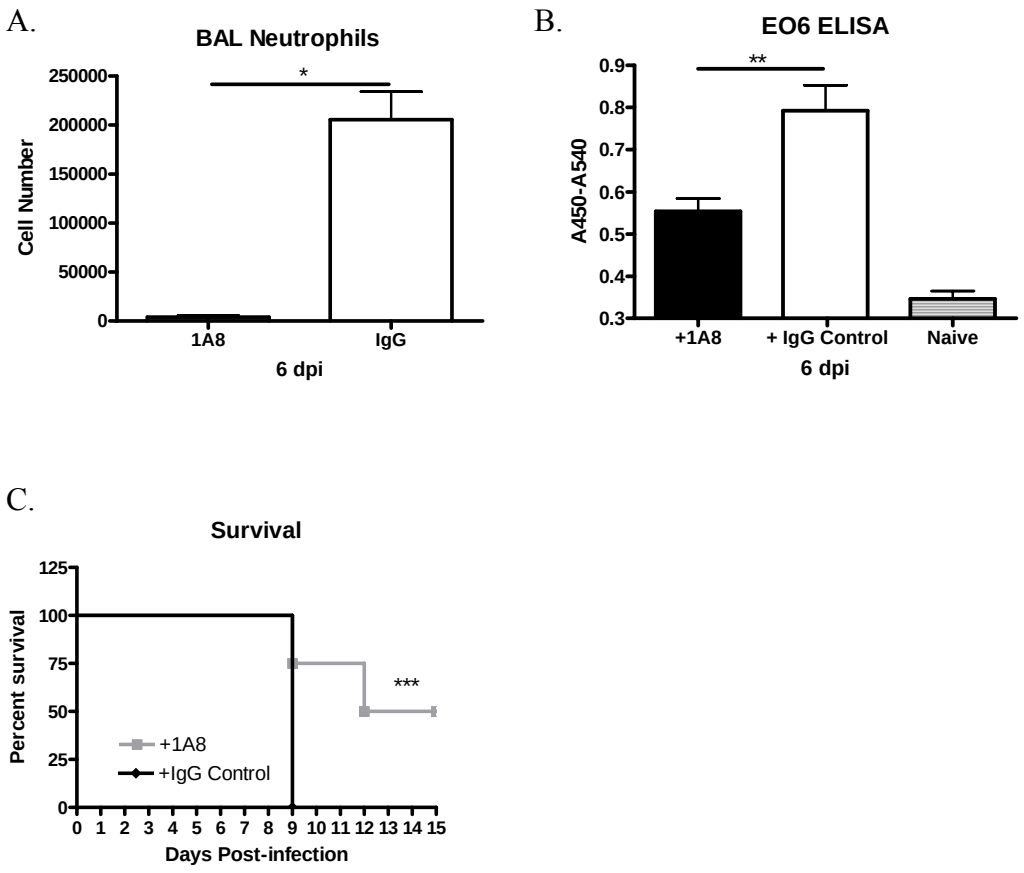


Figure 9: Depletion of neutrophils decreases oxidized phospholipids and promotes survival

Administration of monoclonal antibody 1A8 results in depletion of neutrophils, reflected by decreased neutrophils in the BALF (A.). Mice receiving 1A8 also exhibited a decrease in the amount of oxidized phospholipids in the BALF (B.) Administration of 1A8 also conferred a survival benefit (C.). *$p<0.001$, **$p=0.01$, ***$p=0.04$

24 hours prior to influenza challenge. Neutrophil depletion was confirmed by analysis of peripheral blood smears at the time of influenza challenge (data not shown). At day 6, mice receiving 1A8 exhibited a paucity of neutrophils in the BALF (Figure 9A), as would be expected. Furthermore, these mice demonstrated decreased levels of oxidized phospholipids in the BALF compared to mice receiving isotype control antibody (Figure 9B), suggesting that the difference in neutrophil recruitment in the IL-17RA-/- mice is sufficient to explain the decrease in oxidized phospholipids in that model. Most importantly, the mice receiving anti-Ly6G exhibited a survival advantage compared to the isotype control group (Figure 9C). This is in apparent contrast to other studies which have shown a detrimental effect to depleting neutrophils during an influenza challenge (38, 341, 342). The reason for this difference may be due to differences in antibody specificity, as the other studies used RB6-8C5, thereby depleting other Gr1+ cells.

Because oxidized phospholipids have previously been shown to play a critical role in acute lung injury in both acute viral infections as well as in acid aspiration, we investigated if IL-17RA signaling was also critical for the generation of oxidized phospholipids and lung injury in acid aspiration. To test this hypothesis, we chose to examine a model of aspiration pneumonia wherein hydrochloric acid is instilled into the airway. Following HCl challenge in the airway, IL-17RA-/- mice had a significant decrease in neutrophil influx at 24 h post-challenge (Figure 10A) as well as a decrease in the amount of oxidized phospholipids (Figure 10B) in BAL fluid.

We next decided to specifically test the role of neutrophils in lipid oxidation and lung injury by depleting neutrophils in the HCl aspiration model using anti-Gr1. As stated above, anti- Gr1 has been shown to also deplete a subpopulation of dendritic cells as well as CD8+ T

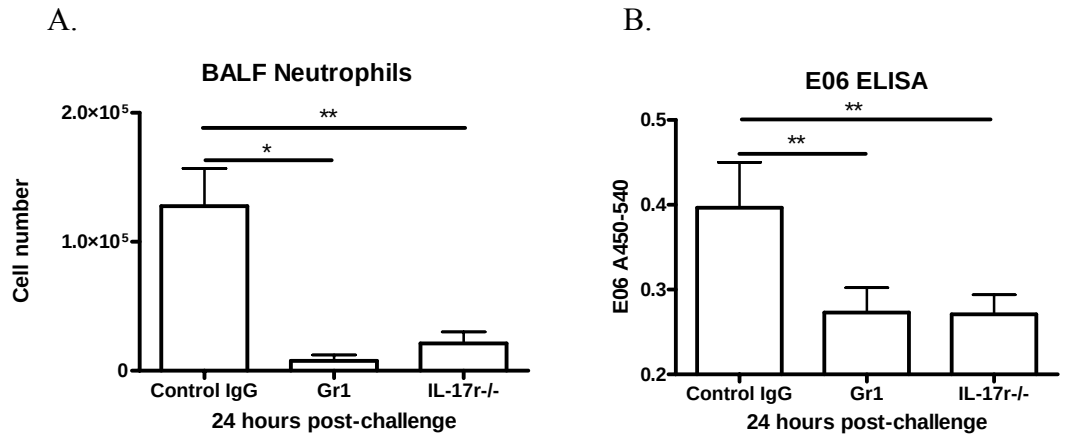


Figure 10: Decrease in neutrophils and oxidized phospholipids in response to HCl. BALB/c mice were treated either anti-Gr1 (n=4) or control IgG (n=4). These mice, plus IL-17RA-/- mice (n=4) were then challenged 24 hours later with 100uL of HCl (pH 1.5) and lavaged at 24 hours after challenge to assess (A). neutrophil recruitment and (B). levels of oxidized phospholipids in the BALF. *p=0.006, **p=0.01

cells (344, 345), making it difficult to interpret data in the influenza model; however due to the short time course of HCl model, depletion of other subsets would less likely play a role apart from depletion of neutrophils. Administration of anti-Gr1 24 h prior to HCl challenge resulted in a highly significant decrease in neutrophils in the BALF at 24 h post-challenge similar to the IL-17RA knockout mice (Figure 10A). Importantly, anti-Gr1 also resulted in a significant decrease in the level of oxidized phospholipids in the BALF (Figure 10B), indicating that neutrophils are important in generating oxidized phospholipids, and that the decrease in neutrophils in our model is sufficient to explain the decrease in the accumulation of oxidized phospholipids in the airways.

2.5 DISCUSSION

The data presented in this report provides support for the concept that IL-17RA signaling plays a key role in the process of lung injury. We observed that IL-17RA knockout mice recruited fewer neutrophils to the airway in response to challenge with either influenza A virus or hydrochloric acid, and that this decrease in neutrophils results in lower amounts of oxidized phospholipids. Others have previously shown that oxidized phospholipids play a key role in acute lung injury by serving as an endogenous ligand for TLR 4 and signaling through the TRIF-TRAF6 pathway (59). Our data shows that IL-17RA signaling regulates the amount of oxidized phospholipids in the airway, likely by regulating tissue burdens of neutrophils, which are critical sources of reactive oxygen species.

IL-17RA-deficient mice are more susceptible to certain extracellular bacterial infections of the lung (273, 274), in part due to defective neutrophil migration into the lung. However, the role of IL-17RA in primary pulmonary influenza host response has not been evaluated. As IL-17 has been shown to regulate ligands for CXCR2 such as KC and LIX (critical for neutrophil recruitment) as well as the ligands for CXCR3, MIG and IP-10 (important for Th1 cell recruitment) (262), it was important to determine if IL-17RA signaling was critical for viral clearance and virus specific CD8+ T-cell recruitment. Additionally, CD8+ T cells are a contributor to lung damage in response to influenza challenge (362, 363). Although IL-17RA-/- mice showed a trend towards higher viral burdens on day 6, these mice ultimately cleared the virus and recovered. Consistent with these findings, there was no significant differences in recruitment of virus specific CD8+ T-cells either for nucleoprotein on day 6 or 10 or hemagglutinin on day 10. For these latter experiments, it was necessary to challenge mice with a

sublethal dose (so as to avoid survival bias) and examine the antigen-specific population at 10 dpi since CD8+ T cells do not peak until 10 dpi.

The slightly higher viral titers in the IL-17RA-/- mice was not unexpected, as it has previously been shown that neutrophil depletion has a deleterious effect on clearance of influenza (38, 341, 342). However, this seemed to be in apparent conflict with the fact that they had decreased morbidity and mortality. This can be explained by studies that have demonstrated that during highly virulent influenza infections, excessive neutrophil infiltrates were associated with more immunopathology (38). This led us to investigate differences in inflammation and lung damage as a mechanism for the survival phenotype we observed.

We observed that the IL-17RA knockout mice did indeed have less lung injury, as indicated by less total protein and LDH activity in the BALF. Upon histological examination, it was apparent that the IL-17RA knockout mice had a dramatic decrease in the amount of inflammation in the lung. This correlated well with decreases in many inflammatory cytokines. Importantly, three pro-inflammatory cytokines that are generally elevated in severe influenza infection as part of a cytokine storm, TNF-α , IL-1β , and IL-6 (36, 364), all either trended lower or were significantly reduced. One potential explanation for the reduced levels of TNF-α and IL-1β is that neutrophils are a source of both TNF-α and IL-1β (336, 337, 365). Because the cytokine storm has been hypothesized to play a role in the immunopathology of the more virulent influenza infections, and the fact that in certain models of arthritis, IL-17 has a TNF-α– and IL-1β– independent role in tissue inflammation (366, 367), we chose to examine whether TNF-α or IL-6 could account for the reduced lung injury and cachexia we observed in IL-17RA knockout mice.

Neutralization of IL-6 or genetic ablation of TNF p55/p75 receptor signaling failed to reduce acute lung injury or cachexia associated with influenza infection, demonstrating that the reduced lung injury in IL-17RA -/- mice is independent of the differences observed in the levels of these cytokines. The lack of a role for TNFR signaling is in apparent contrast to experiments that have previously shown that immunopathology is driven by TNF signaling (161, 162). This may be due to differences in our viral infection model compared to transgenic models used in the other reports. Regardless, or data suggests that the differences in immunopathology that we observed in IL-17RA-/- mice were not due to the decreases in TNF-α or IL-6.

Having eliminated the possibility of a decreased cytokine storm and decreased CD8+ T cells as causes of the protection, we next investigated the differences in neutrophil recruitment. IL-17 plays a key role in neutrophil proliferation and migration, primarily by induction of granulopoietic factors and chemokines (260, 346-349, 368). Further, it has been demonstrated that genetic ablation of CXCR2 results in decreased neutrophil migration to the lung in response to influenza (369), and IL-17 is responsible for the regulation of some CXCR2 ligands (348). Consistent with this, we observed reduced lung neutrophils following influenza infection in the IL-17RA knockout mice. Considering the mechanisms by which neutrophils are capable of causing tissue damage, one likely mechanism of lung injury is through the generation of reactive oxygen species and specifically, the oxidation of phospholipids (59).

In support of that hypothesis, we found reduced MPO activity in both the BAL cell pellet and lung homogenate in IL-17RA knockout mice as well as a significantly lower level of oxidized phospholipids in the BALF. The combined decrease in neutrophil emigration and oxidized phospholipids is a likely explanation for the decrease in lung injury in the setting of influenza infection.

To confirm the role of IL-17RA signaling and the role of neutrophils in the generation of oxidized phospholipids, we used a monoclonal antibody specific for Ly6G to deplete neutrophils during influenza infection. Neutrophil depletion was observed in both the peripheral blood and BALF. Importantly, this resulted in a decrease in oxidized phospholipids in the BALF, which therefore suggests that the decrease in neutrophils that we observed in the IL-17RA-/- mice was sufficient to explain the decrease in oxidized phospholipids in that model. Additionally, this conferred a survival advantage among the anti-Ly6G-treated mice, further demonstrating the protective effect in decreasing oxidized phospholipids. Interestingly, this is in apparent contrast to other reports that describe a detrimental effect to depletion of neutrophils (38, 341, 342). This may be due to differences in viral strains used, or due to differences in antibody specificity, as the other reports have used anti-Gr1, which not only recognizes Ly6G but also Ly6C, resulting in depletion of other cell subsets in addition to neutrophils.

We have also examined a non-infectious model of acute lung injury-acid aspiration- to determine if the role of IL-17 signaling is common to different causes of lung injury. Again, in this model IL-17RA was required for neutrophil recruitment, generation of oxidized phospholipids, and ultimately, higher levels of lung injury. The specific role of neutrophils in the generation of oxidized phospholipids in this model was shown using anti-Gr1 depletion of neutrophils. Taken together these data strongly implicate IL-17RA signaling in neutrophil recruitment and the generation of oxidized phospholipids in both influenza and acid aspiration. Moreover, in the setting of influenza infection, lung injury due to IL-17RA signaling is independent of TNF-α or IL-6. These data support the potential therapeutic manipulation of IL-17 or IL-17RA in acute lung injury.

The role for oxidative damage in lung injury is supported by another report (370) examining production of reactive oxygen species in influenza-challenged mice. Similar to our model, Cybb tm1 mice (lacking the gp91phox subunit of phagocyte NADPH oxidase) demonstrate less lung injury than wild-type control mice as measured by both total protein and LDH in the BALF following influenza challenge. Additionally, these mice exhibited better lung function by whole body plethysmography. However, in contrast to our model, these mice exhibited increased weight loss and airway cellularity, as well as improved viral clearance, suggesting a role for ROS in regulating the immune response. It is possible, then, that in that model, the decrease in lung injury may not only be due to a direct role for ROS in causing injury, but also due to more efficient viral clearance (370).

Other studies have also suggested roles for ROS in regulating lung inflammation. Mice lacking the p47phox gene, which also leads to a non-functional NADPH oxidase, produce fewer ROS in response to intratracheal administration of lipopolysaccharide (371) or hydrochloric acid. However, in response to acid aspiration, these mice have more neutrophils recruited to the airway and fewer macrophages, leading to an increase in lung injury (372). This suggests a beneficial role for ROS in driving a less injurious monocytic response and limiting a more damaging neutrophilic response. However, in our IL-17RA knockout model, this is less of a concern due to the fact that the neutrophil response is already attenuated.

If the signaling of oxidized phospholipids via TLR-4 were the only mechanism behind our phenotype, one would expect that TLR-4 null mice would be protected from lung injury in the setting of influenza challenge. While that question has not been explicitly examined, multiple groups have demonstrated that TLR-4 knockout mice have no differences in viral clearance compared to wild-type controls, nor do they exhibit any deficits in the recruitment of

inflammatory cells to the airway or production of proinflammatory cytokines (373, 374). Further, no differences in histopathology are noted (374), suggesting that these mice have just as much inflammation as the wild-type controls, even though oxidized phospholipids signaling through TLR-4 would presumably be ablated. This would seem to suggest that in our IL-17RA knockout model, while it is apparent that a decrease in oxidized phospholipids contributes to the phenotype we observe, it may not be the only explanation.

Aside from any role a reduction in ROS might have in regulating the inflammatory response in the lung, there may be additional mechanisms that were not explored here. In addition to the production of ROS, which in turn result in the production of oxidized phospholipids and direct oxidative damage to cells, neutrophils are also an important source of proteases that are capable of degrading the extracellular matrix of the lung and thereby contributing to lung injury (58, 63). Interestingly, production of proteases by the neutrophil may contribute to a "feed-forward" loop to recruit more neutrophils. Elastase production can result in the cleavage of CXCR1 on the surface of neutrophils, and the resulting CXCR1 fragment stimulates further IL-8/MIP-2 production, driving neutrophil recruitment (375). Therefore, by reducing neutrophils in the IL-17RA knockout mice, we are also reducing the effects of neutrophil-derived proteases, which may help to lessen the lung injury.

CCR2-mediated recruitment of monocytes has been shown to contribute to TRAIL-mediated epithelial cell death following influenza, and lung injury is reduced in mice lacking CCR2 (376). In addition to the differences in neutrophils, we also saw a trend towards fewer macrophages in the BALF, supported by a trend towards lower MCP-1 in the IL-17RA knockout mice (data not shown); therefore, there may be neutrophil-independent mechanisms that are also contributing to the phenotype we describe here.

Somewhat related, it has also previously been shown that TLR3-deficient mice have a similar phenotype with decreased inflammation, decreased weight loss, and decreased mortality despite slightly higher viral titers (111). Similar to oxidized phospholipids signaling through TLR4, TLR3 signals through TRIF and not MyD88 (110). This provides further evidence that TRIF signaling may play a key role in ALI.

IL-17 signaling has not been extensively studied in viral models, despite the fact that the cytokine has been detected in the settting of viral infection (302, 303). A recent report has described the generation of IL-17+ CD8+ T cells- termed Tc17 cells- in response to influenza. These cells, when generated *in vivo* and transferred to mice prior to influenza challenge were protective, though these cells lacked granzyme B and perforin. Additionally, in apparent contrast to our study, antibody neutralization of IL-17 reduced protection from heterosubtypical challenge (160). This may be due to a role for IL-17 in the memory response, something that we do not examine in the present study.

While our mouse model of influenza A/PR/8 infection is well-characterized, it is important to note that it may differ from infection with other strains or from human influenza. However, our observations are similar to those made by others in a corneal HSV-1 model (304), in which it was shown that IL-17RA knockout mice have decreased neutrophil infiltration that correlated with a decrease in corneal opacity- suggesting less tissue damage- at 24 hours post-challenge. In contrast to our model, this effect was transient, with the IL-17RA knockouts having similar levels of neutrophils and corneal pathology by 4 dpi. These data seem to suggest that therapeutic modulation of IL-17 signaling may be beneficial not only in ALI, but also in treating immunopathology associated with viral infections of other organs.

2.6 ACKNOWLEDGEMENTS

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3.0 TIMECOURSE AND CELLULAR SOURCE OF IL-17 PRODUCTION IN RESPONSE TO INFLUENZA CHALLENGE

3.1 ABSTRACT

Interleukin-17 is a cytokine produced in response to a variety of infectious (and non-infectious) agents, and is critical in regulating the host neutrophil response through upregulation of granulopoetic factors and chemokines. Production of IL-17 has been described by several cell types, including CD4+ and CD8+ T cells, γδ T cells, and NK1.1neg invariant NKT cells. We have previously shown that mice lacking IL-17RA are protected from lung injury during influenza challenge, but the timecourse and cellular source(s) of IL-17 production in this model remain undescribed. Here we demonstrate that IL-17 mRNA and protein is detectable in the lung as early as 48 hours post-challenge and persists throughout the course of infection. The cell population responsible for IL-17 is small but detectable, and seems to be predominated by γδ T cells. We therefore conclude that γδ T cells are a critical source of IL-17 in response to influenza challenge.

3.2 INTRODUCTION

Multiple studies have demonstrated that IL-17 is produced in response to a variety of infectious pathogens. This includes both bacterial pathogens such as *E. coli*, (223) *L. monocytogenes* (300,

301), *K. pneumoniae* (273), and *M. pneumoniae* (274) as well as fungal pathogens, including *P. carinii* (279), *C. albicans* (280), *A. fumigatus*, and *C. neoformans* (283). IL-17 has also been detected in the setting of viral infections such as Human Immunodeficiency Virus (HIV) (302) and Herpes Simplex Virus (HSV) (304). Further, we have already demonstrated that mice lacking IL-17 signaling are protected from the lung immunopathology associated with severe influenza infection (see section 2.0), implying that IL-17 is also made in response to this virus. However, the timecourse and cellular source of IL-17 in this model remain incompletely described.

Multiple cell types have been demonstrated to be capable of producing IL-17. Perhaps the best described are CD4+ effector T cells. CD4+ T cells capable of producing IL-17 comprise a specific subset of cells, termed Th17 cells. In the mouse model, polarization of CD4+ T cells towards a Th17 phenotype is dependent upon IL-6, TGF-β, and IL-1β (229–231). IL-23 is also involved, playing a role in the expansion and maintenance of the Th17 population (236, 237). An alternative pathway of Th17 polarization, dependent upon IL-21, has also been described (232, 251).

In addition to CD4+ T cells, CD8+ T cells have also been shown to be capable of IL-17 production (160, 219). A recent report by Hamada, *et. al.* describes a CD8+ T cell subset analogous to Th17 cells that is capable of producing IL-17, terming them Tc17 cells. Interestingly, these cells are present during influenza infection and are capable of providing protection when adoptively transferred 24 hours prior to influenza challenge (160).

Several studies have also identified γδ T cells as a source of IL-17 (222, 223, 252, 297, 300). In an *E. coli* challenge model, γδ T cells in the peritoneal cavity are capable of producing IL-17 (297). At least *in vitro*, the ability to produce IL-17 is enhanced by exogenous IL-23

(223). Subsequent studies have demonstrated that these γδ T cells differentiate into IL-17 producers while still in the thymus (222). Further, others have demonstrated that γδ cells differentiate into IL-17 producers or IFN-γ producers during thymic selection, with antigen-naïve cells becoming IL-17 promoters and antigen-experienced cells becoming IFN-γ producers (252). Differentiating during thymic selection would allow these cells to rapidly produce IL-17 in response to antigenic stimulation once in the periphery.

A recent report has also identified a distinct population of invariant NKT (iNKT) cells capable of producing IL-17 and driving neutrophilia in the lung. These cells lack the NK1.1 marker, but are stimulated by CD1d-bound α-galactosylceramide to produce IL-17. In contrast, NK1.1 positive cells are poor producers of IL-17 (218).

In addition to these cell populations, IL-17mRNA has been detected in neutrophils, though IL-17 protein secretion by these cells has not been demonstrated (224). Another study has shown production of IL-17 by macrophages in the setting of allergic lung inflammation. Mediators released by mast cells upregulated IL-17 production by the macrophages, whereas IL-10 inhibited IL-17 production (377).

In the present study, we sought to identify when IL-17 was produced in response to influenza challenge, and what cells were producing it. We show here that IL-17 is upregulated as early as two days post-infection, and is detectable throughout the first week of infection, at which point the mice succumb to the lethal infection. Further, we demonstrate that the number of IL-17 producing cells is rather small, but still detectable. This IL-17+ population is predominantly γδ T cells. In support of this, mice lacking γδ T cells have a decrease in IL-17, suggesting that these cells are a critical source of IL-17A and F.

3.3 METHODS

3.3.1 Animal Infections

IL-17ra on a BALB/cJ background (350) and $\gamma\delta$ knockout mice on the B6 background (378) have been previously described. Wild-type controls included BALB/cJ, and C57BL6 mice. IL-17-IRES-GFP reporter mice used in these experiments have not been previously described. Briefly, A BAC clone consisting of IL-17A genomic DNA derived from C57BL/6 mice was purchased from BacPac (Oakland, CA). An 8-kb BamHI-MluI fragment comprising exons 1, 2 and 3 for *IL-17A* gene was cloned into pEasy-Flox vector adjacent to the thymidine kinase selection marker. The IRES-eGFP cassette was linked to a LoxP-flanked neomycin (Neo) selection marker to obtain the IRES-eGFP-Neo cassette. The targeting construct was generated by cloning the IRES-eGFP-Neo cassette into a SacII site between the translation stop codon (UGA) and the polyadenylation signal (A2UA3) of the *IL17A* gene. The targeting construct was linearized by *Cla*I cleavage and subsequently electroporated into Bruce4 C57BL/6 ES cells. Transfected ES cells were selected in the presence of 300 μg/ml G418 and 1 μM ganciclovir. Drug resistant ES cell clones were screened for homologous recombination by PCR. To obtain chimeric mice, correctly targeted ES clones were injected into BALB/c blastocysts, which were then implanted into CD1 pseudopregnant foster mothers. Male chimeras were bred with C57BL/6 to screen for germ-line transmitted offspring. Germ-line transmitted mice were bred with germline Cre transgenic mice (Tet-Cre mice) to remove the neomycin gene. Mice bearing the targeted IL-17A allele were screened by PCR.

Mice were challenged intranasally with a lethal dose of influenza A/PR/8/34 (H1N1) in phosphate-buffered saline (PBS). Following infection, mice were monitored daily for weight loss and signs of clinical illness (352). Mice were sacrificed at indicated time points. Control mice received equivalent volumes of PBS.

3.3.2 Real time PCR

RNA collected from the left lung was used as template to generate cDNA using iScript reagents and protocol (BioRad). cDNA was then used in fast real-time PCR using the Applied Biosystems 7900HT. Reaction conditions were 95° Celsius for 1 second and 60° for 20 seconds, repeated for 40 cycles, with a 20 second hot start at 95°. Primer and probe sequences were as follows: IL-17A Forward- 5'-GCT CCA GAA GGC CCT CAG A-3', IL-17A Reverse- 5'-CTT TCC CTC CGC ATT GAC A-3', IL-17A probe- 5'-ACC TCA ACC GTT CCA CGT CAC CCT G-3', IL-17F Forward- 5'AGG GCA TTT CTG TCC CAC GTG AAT-3', IL-17F Reverse- 5'-GCA TTG ATG CAG CCT GAG TGT CT-3', IL-17R Probe- 5'-CAT GGG ATT ACA ACA TCA CTC GAG ACC C-3'. 18s RNA was used as a housekeeping gene with the following primer/probe sequences: Forward- 5'-GAT CCA TTG GAG GGC AAG TCT-3', Reverse- 5'- GCA GCA ACT TTA ATA TAC GCT ATT GC-3', Probe- 5'-TGC CAG CAG CCG CGG TAA TTC-3'. Fold change in RNA levels was calculated using the ΔΔCT method.

3.3.3 IL-17A and IL-17F ELISA

Right lungs were collected in 1mL of PBS at the time of euthanasia and homogenized using an electronic tissue homogenizer. Cellular debris was centrifuged out at 12,000 x g for 10 minutes. Supernatants were collected and concentrations of IL-17A and IL-17F were measured by ELISA (R&D) according to manufacturer's instructions.

3.3.4 IL-17A ELISpot

For ELISpot, the lungs of each animal were collected in PBS and dissected into 1mm pieces under sterile conditions. They were then digested with collagenase and DNAse for 45 minutes at 37° Celsius. The digest was passed through a 40 μm filter, red blood cells were lysed, and cells were washed 3x with PBS. Cells were plated at $1x10^5$ cells per well on an IL-17A ELISpot plate (eBioscience) in DMEM and stimulated with 50 ng/mL PMA and 750 ng/mL ionomycin overnight. Plate was developed according to manufacturer's instructions.

3.3.5 Intracellular Cytokine Staining

Lungs were collected and digested as described in section 3.3.4. Cells were plated at a concentration of $1\text{-}2x10^6$ cells/well in a sterile 96-well round-bottom plate and stimulated overnight in DMEM with 50 ng/mL PMA and 750 ng/mL ionomycin at 37 degrees Celsius. Brefeldin A was added during the last 6 hours of incubation. Cells were then washed and resuspended in PBS + 2% fetal bovine serum (FBS), and Fc receptors were blocked. Cells were

then stained with fluorochrome-conjugated monoclonal antibodies for CD4, CD8, TCR β chain, TCR δ chain, and α-galcer-CD1 tetramer in the dark, on ice, for 30 minutes. Following staining, cells were washed prior to fixation and permeabilization, which was performed using the Cell Fixation and Permeabilization Kit (Beckton, Dickinson and Company) according to manufacturer's instructions. Intracellular staining was performed with fluorochrome-conjugated monoclonal antibody directed towards IL-17A.

3.4 RESULTS

3.4.1 IL-17 is induced during influenza infection

Among IL-17 family members, IL-17A and IL-17F share the greatest homology, and both are capable of signaling through IL-17RA, though IL-17F binds with a much lower affinity (254). BALB/c mice were challenged with 100 pfu of influenza A/PR/8/34 (H1N1), and following challenge we were able to detect an increase in IL-17A (Figure 11A) and IL-17F (Figure 11B) mRNA in lung tissue compared to baseline as early as 2 days post-infection (dpi).

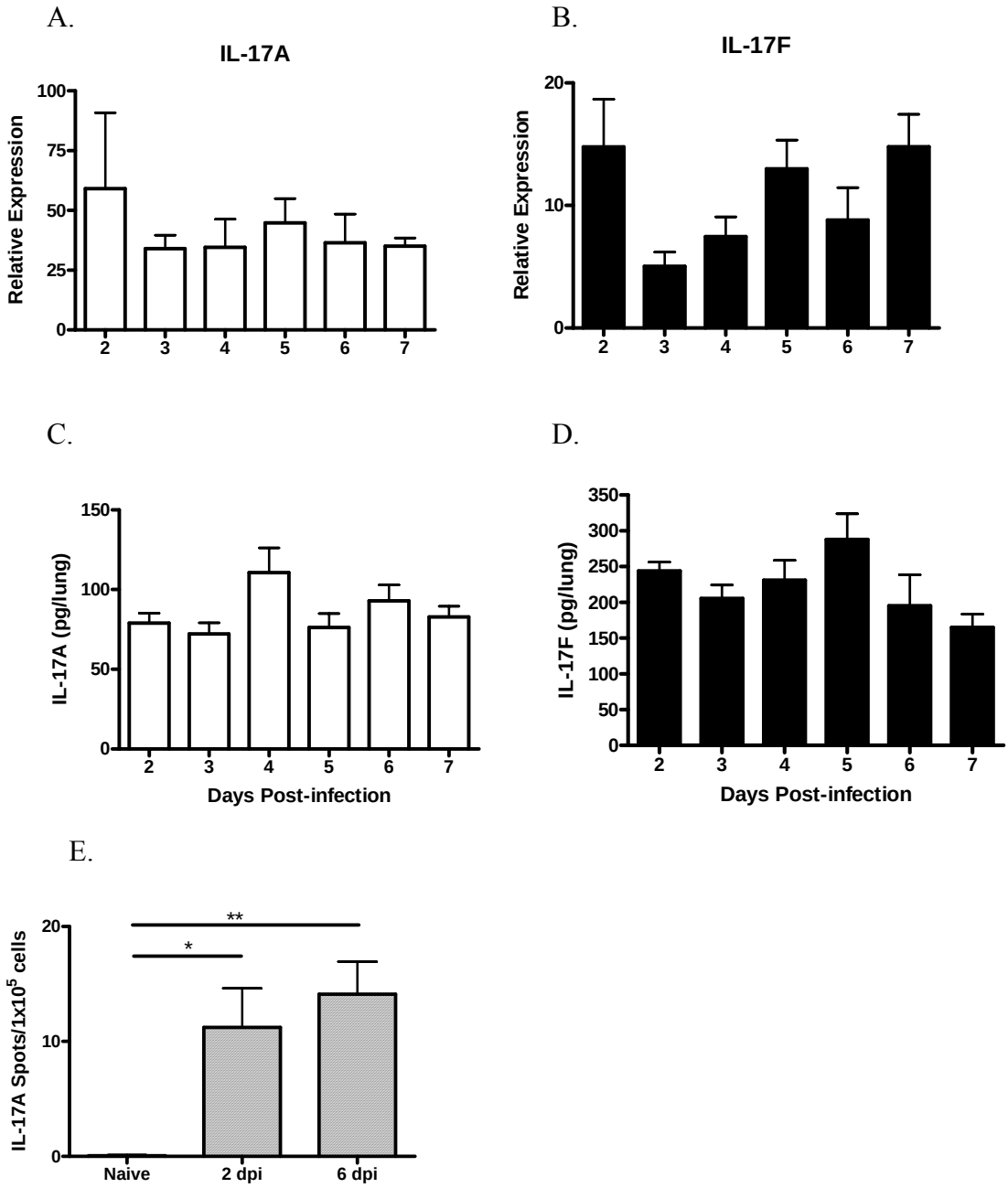

Figure 11: IL-17A and IL-17F are expressed in response to influenza. Time course of IL-17A (A and C) and IL-17F (B and D) following influenza challenge. mRNA was detected by quantitative real-time PCR. Fold change in mRNA was calculated using the ΔΔCT method, is expressed relative to naïve levels, with all timepoints having a p value of <0.05 compared to naïve controls. Protein levels were detected by ELISA, and all timepoints had a p value of <0.05 compared to naïve controls. (E) The number of IL-17A-producing cells was quantified by ELISpot *p=0.016, **p=0.003.

This increase in mRNA remains detectable through 7 dpi. We sought to confirm this increase in mRNA by measuring protein levels. IL-17A (Figure 11C) and IL-17F (Figure 11D) were detectable by ELISA from 2 dpi through 7 dpi. Beyond 7 dpi, there was significant mortality among BALB/c mice. Thus, to avoid survival bias in data analysis, we chose to use an early time point- 2 dpi- and a late time point- 6 dpi- for our studies. In order to gauge the number of cells producing IL-17, we performed an ELISpot on lung digests at 2 and 6 dpi. We found that the population of IL-17A producing cells was relatively small, at 0.01-0.02% of the lung digest, and that the size of this population was not statistically different between the early and later timepoint (Figure 11E).

3.4.2 γδ T cells as a Source of IL-17

The detection of IL-17 as early as 2 days post-infection is important due to the fact that CD4+ and CD8+ T cells are typically not recruited to the lung until 4 dpi or later in response to influenza (379). This implies that IL-17 production must also involve resident cells, including γδ T cells, as recruited CD4+ and CD8+ cells would not yet be in the lung. Therefore, our efforts were aimed at identifying which cells are producing IL-17, with a focus on resident cell populations.

As illustrated in Figure 11E, the size of the IL-17-producing cell population was relatively small. Therefore, to identify what cells were producing IL-17, we chose to perform intracellular cytokine staining. Cells were restimulated with PMA/ionomycin prior to staining. In addition to staining for IL-17 intracellularly, we also co-stained for a variety of surface markers, including CD4, TCR δ chain, and TCR β chain. Additionally,

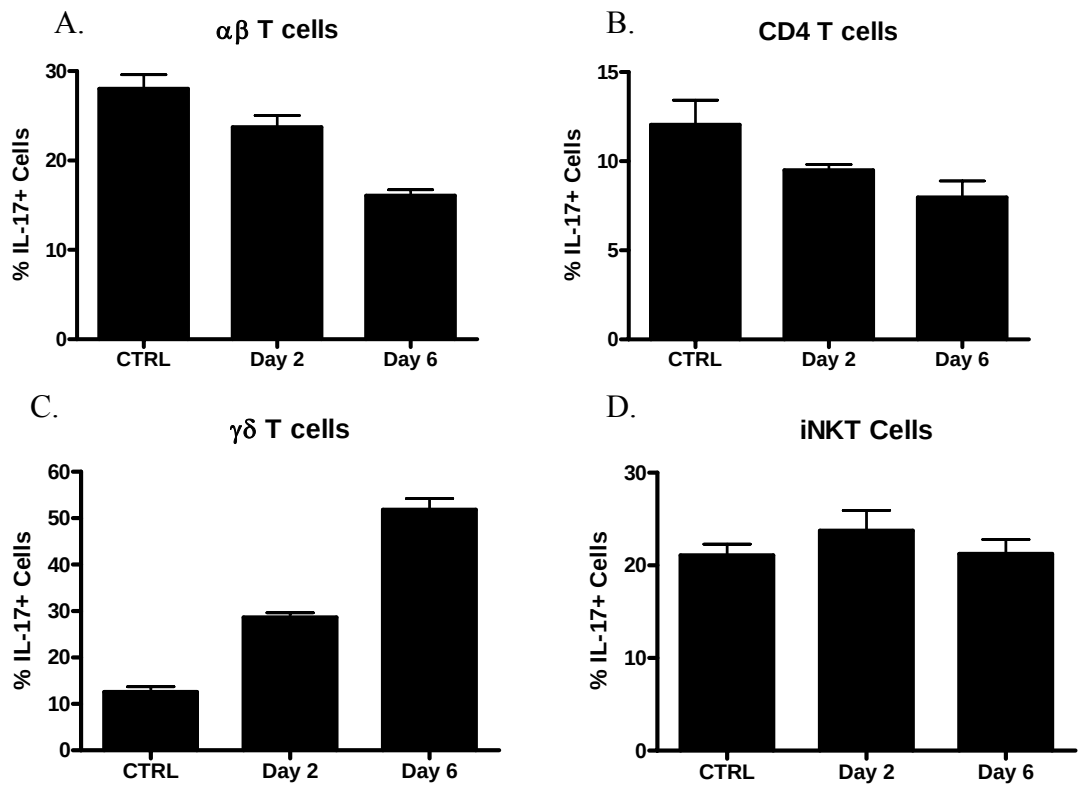

Figure 12: Majority of IL-17+ cells are γδ T cells. Lung digests from naïve or influenza-infected mice were stimulated overnight with PMA/Ionomycin and used for intracellular cytokine staining. Cells were analyzed by flow cytometry, gating on the IL-17+ population. Surface markers stained include T cell-receptor β chain (A), CD4 (B), or T cell-receptor δ chain (C). iNKT cells were stained using tetramer containing α-gal-cer bound to CD1d (D).

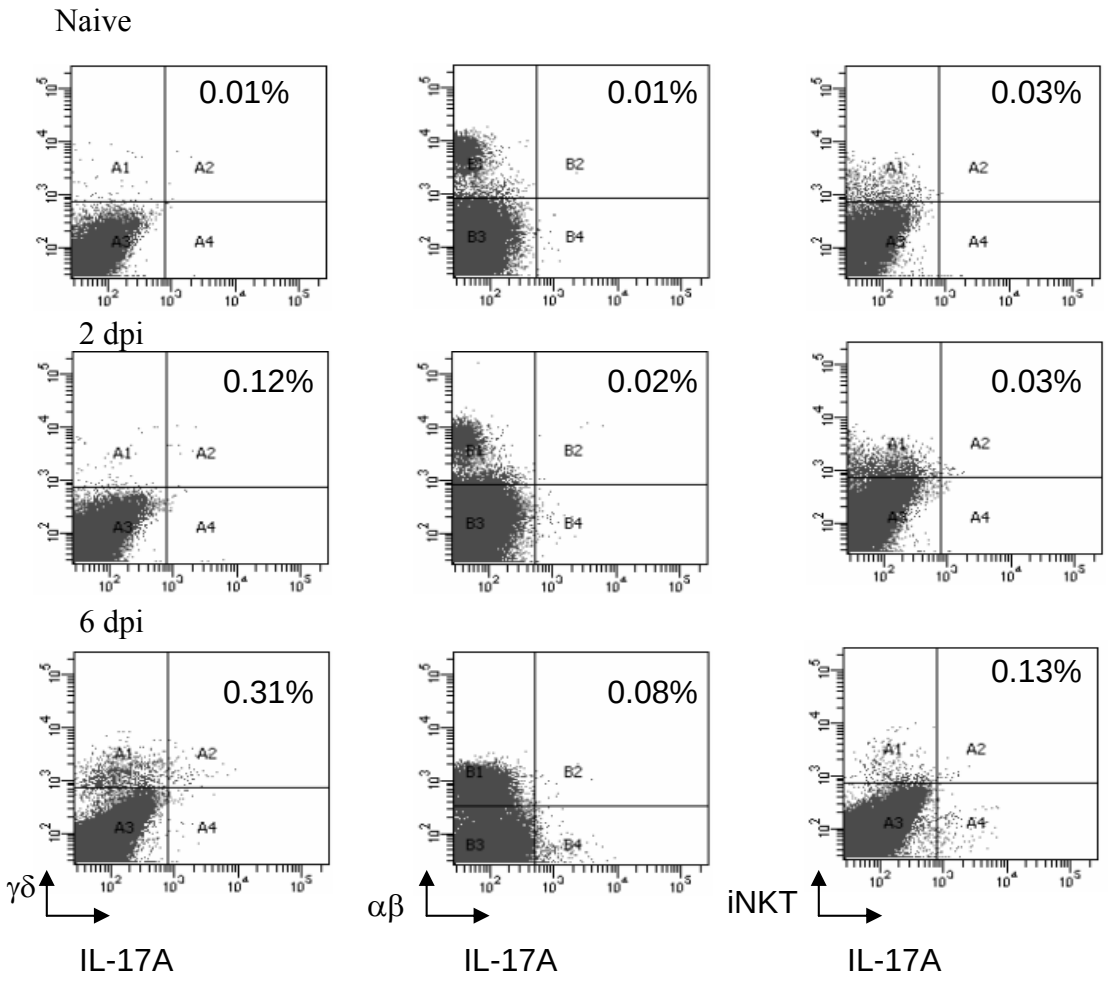

Figure 13: IL-17-GFP is produced by γδ T cells. Challenge of IL-17A-GFP reporter mice demonstrates that there is a population of IL-17+ cells that costain as gd T cells, and that this population grows from 2 to 6 dpi. In contrast, there are nearly no IL-17+ cells in the ab T cell population, and a small iNKT IL-17+ population at 6dpi, though the majority of IL-17+ cells do not stain as iNKT cells.

we used tetramer consisting of α-gal-cer bound to CD1d to identify iNKT cells. Upon analysis, we gated on the IL-17+ population and analyzed the surface staining profile of these cells. Following this treatment, the IL-17-producing cells from naïve mice were approximately 20% iNKT cells, 10% γδ T cells, and 30% αβ T cells (Figure 12). In mice challenged with influenza, the γδ+ IL-17+ population expanded to constitute approximately 30% of the IL-17+ cells by day 2 and over 50% by day 6 (Figure 12C) while the αβ T cells made up a correspondingly decreasing percentage of the IL-17+ population (Figure 12A). This indicates that γδ T cells are a major source of IL-17 in response to influenza.

One major weakness of intracellular cytokine staining is that it requires the use of PMA/ionomycin to restimulate the cells. To avoid this, we also challenged IL-17A-GFP reporter mice with influenza, and analyzed the IL-17A-GFP+ population at 2 and 6 days post-infection. In doing so, we observed nearly no IL-17 expression in cells from naïve lungs, and nearly all IL-17+ cells following influenza challenge stained as γδ T cells, further confirming these cells as the source of IL-17 in our model. Further, the size of this population increases from 2 to 6 dpi, and there are essentially no IL-17+ αβ T cells at this later timepoint, suggesting that the γδ T cells continue to be the predominant source of IL-17 even after the expansion of CD4+ and CD8+ T cells in the lung (Figure 13).

3.4.3 γδ T cell-Deficient Mice Have Decreases in IL-17

Based on the fact that γδ T cells have been identified as a major source of IL-17 in other models, and the fact that γδ T cells made up an increasingly large percentage of IL-17+ cells in our influenza model, we hypothesized that mice lacking γδ T cells would have a deficit in IL-17

production following influenza challenge. To test this hypothesis, we challenged TCR δ -/- mice, which have deficient γδ T cell receptor expression, with influenza. We then examined both IL-17A and IL-17F production at 2 and 6 dpi, looking at mRNA by semi-quantitative real-time PCR. In contrast to the BALB/c mice examined in figure 11, the change in IL-17A and IL-17F mRNA among B6 controls at 2 dpi is less than 10-fold compared to naïve mice. However, despite that, we detect a significant decrease in IL-17F message in the γδ knockout mice as early as 2 dpi, and at 6 dpi, both IL-17A and IL-17F mRNA is significantly decreased (Figure 14). This data further confirms the role of γδ T cells as a major source of IL-17 in the lung following influenza challenge.

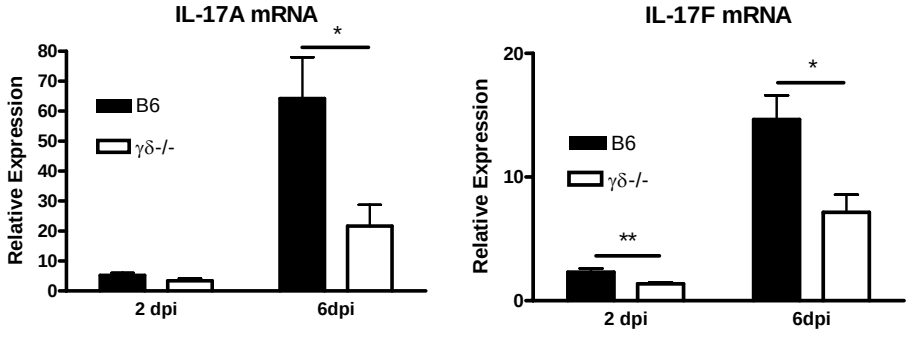

Figure 14: γδ T cell-deficient mice make less IL-17. Challenge of γδ knockout mice results in lower levels of IL-17A and IL-17F message, expressed as fold change from naïve, at 6dpi. *p=0.03, **p=0.02.

3.5 DISCUSSION

There is considerable interest in understanding the role of individual cytokines- including IL-17- in host defense against infectious pathogens. IL-17 has been shown to play a role in host defense against several pulmonary pathogens (for a complete discussion, refer to section 1.3.4). In response to influenza challenge, IL-17 appears to play a role in the immunopathology, specifically through the regulation of the neutrophil response and subsequent oxidative damage (section 2.0). However, to fully understand the role IL-17 plays, it is important to understand the source of the IL-17 and when it is produced. This is even more essential if one hopes to use IL-17 neutralization as a therapeutic strategy in treating acute lung injury.

Th17 cells, a subset of CD4+ T cells, are perhaps the most well-described producers of IL-17 (307, 309, 380-383). However, in the influenza model, antigen-specific CD4+ T cells don't appear in the lung until 4 dpi or later (379, 384). Because we observed IL-17A (and IL-17F) as early as 2 dpi (Figure 11), it is likely that there is another cellular source of these cytokines in the early part of the infection. In other models, CD8+ T cells (160, 219), γδ T cells (223), iNKT cells (218), macrophages (377) and neutrophils(224) have all been shown to produce IL-17 (though in the case of neutrophils, only mRNA has been measured). Of these, CD8+ T cells have similar kinetics to the CD4+ T cell response in that they do not arrive in the lung until at least 4 dpi (379). We hypothesized, therefore, that the IL-17 being produced early in the infection must be from some resident population, possibly γδ T cells. ELISpot results demonstrated that the population of IL-17+ cells was very small (Figure 11). Through intracellular cytokine staining, we were able to demonstrate that much of the influenza-induced

IL-17 production was due to γδ T cells (Figure 12), though contributions from other cellular sources should not be considered insignificant.

To follow up the results of our intracellular staining, we also challenged IL-17A-GFP reporter mice with influenza. The primary advantage to this system is that there is no need to restimulate the cells *in vitro*. By restimulating *in vitro*, it is reasonable to believe that you may be activating some cells that are capable of producing IL-17 that are not actually producing the cytokine *in vivo*. Use of the reporter mouse system eliminates this potential variable. Our data in these reporter mice confirms what we saw by intracellular staining, namely that IL-17+ cells are mostly γδ T cells, not αβ T cells. Further, challenge of γδ knockout mice demonstrated a decrease in IL-17A and IL-17F message, providing additional support for the claim that γδ T cells are the primary source of IL-17 following influenza challenge.

While it was expected that γδ T cells would be the primary producer of IL-17 early in the infection, it is somewhat suprising that they remain the primary producer of IL-17 at 6 dpi, a timepoint by which CD4+ and CD8+ T cells are more prominent in the lung. A report has demonstrated IL-17+ CD8+ T cells in the lung following influenza infection, but those cells do not seem to significantly expand until 7 dpi or later (160). Therefore, it is possible that other cell types, including CD8+ and CD4+ T cells, contribute to IL-17 production later in the influenza model, but at timepoints beyond the first week of infection. This makes it unlikely that the production of IL-17 by these sources is contributing to the phenotype described in Chapter 2 of this report, in which mice succumb to the infection prior to the peak of the CD4+ and CD8+ T cell responses.

While knowledge of the cellular source of IL-17 is critical to gaining an understanding of how the cytokine affects the response to influenza, it is also important to recognize that cells

producing IL-17 also produce other inflammatory mediators, and therefore may not make optimal therapeutic targets. Further, the production of IL-17 may be necessary for other aspects of host defense. In the case of γδ T cells, it has been shown that γδ T cell deficient mice have immune defects that result in worse outcomes in response to bacterial infections and other neutrophil-dominated inflammatory responses (385, 386). Further, γδ T cells may play a role in resolution of lung injury in the influenza model, as well as heterosubtypic immunity (164, 387). Because of this, it may be preferential to not target the cells producing IL-17, but rather IL-17 itself and its downstream mediators in an effort to mitigate the immunopathology observed in response to influenza.

3.6 ACKNOWLEDGEMENTS

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4.0 SUMMARY AND CONCLUSIONS

In this dissertation, we have described our efforts to understand the role of IL-17 signaling in response to influenza infection. A summary of our results is as follows:

1. IL-17 is produced early following influenza challenge- as soon as 2 days post-infection- and expression continues throughout the infection. Resident cell populations- most notably γδ T cells, are at least one source of the IL-17 early, as CD4+ and CD8+ T cells are not recruited to the lung until day 4 or later. However, this does not rule out the possibility of CD4+ or CD8+ T cells contributing to the IL-17 response later in the infection. Due to the lethality of our model, we did not evaluate time points beyond 6 days post-infection. Other reports have, however, demonstrated IL-17 production by both CD4 and CD8 T cells in sublethal infections.
2. IL-17RA signaling plays a key role in neutrophil recruitment to the lung, and this neutrophil recruitment is responsible for increased immunopathology. We demonstrate that IL-17RA knockout mice have far fewer neutrophils in the airway. This is associated with decreased morbidity- as measured by weight loss and signs of clinical illness- and mortality. Specifically, these IL-17RA-deficient mice display far less inflammation and lung damage.
3. Loss of IL-17RA signaling had a minimal but measurable impact on viral clearance. The knockout mice had higher levels of virus at 6 days post-infection than their wild-type counterparts. However, these animals were ultimately able to clear the infection and survive.

4. Decreases in neutrophil recruitment resulted in decreases in oxidative damage. As expected, mice lacking IL-17RA had less myeloperoxidase in the lung. We were also able to detect lower levels of oxidized phospholipids in these animals. Oxidized phospholipids are capable of serving as an endogenous signal through Toll-like Receptor (TLR) 4 to induce acute lung injury. Therefore, this serves as a probable mechanism for why IL-17RA knockout mice are protected from lung injury. We were further able to demonstrate, using monoclonal antibodies to deplete neutrophils, that the differences in neutrophil numbers were sufficient to explain the differences in oxidized phospholipids.
5. Decreases in neutrophil recruitment and production of oxidized phospholipids were also detected in IL-17RA mice treated with hydrochloric acid in the airway, compared to wild-type controls. Because our findings are similar in this model of aspiration pneumonia, it suggests that IL-17 may play a key role in acute lung injury in response to a variety of stimuli. This suggests that IL-17 may be an important therapeutic target for the treatment of acute lung injury.

Based upon the findings detailed in this report, we therefore conclude that IL-17, produced mainly by γδ T cells in response to both infectious (influenza) and non-infectious (acid aspiration) insults to the airway, plays a key role in the pathogenesis of acute lung injury. This is due, at least in part, to the recruitment of neutrophils to the lung, production of reactive oxygen species, and accumulation of oxidized phospholipids in the airway. Therefore, therapies aimed at blocking this pathway may be useful in the treatment of acute lung injury.

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