THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) CHANNEL AS A HOST DETERMINANT OF INFLUENZA SEVERITY

DISSERTATION

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Abstract

Influenza A virus is a readily transmissible respiratory pathogen that remains a significant threat to human health. Annual influenza epidemics are responsible for roughly 3 to 5 million cases of severe illness and more than 300,000 deaths/year worldwide. Additionally, the emergence of novel pandemic strains has the potential to cause devastating loss of life. Treatments for influenza infection, including vaccination and antiviral therapy, have limited utility. Vaccination plays a pivotal role in preventing influenza infection, but several issues arise related to vaccine uptake, distribution, and production. Moreover, a recent meta-analysis determined that antiviral drugs do very little to prevent influenza-related hospitalizations. Thus, there is a need for new therapeutics that can treat late-stage, severe influenza infection.

In severe cases, primary influenza infection can lead the development of pulmonary edema and hypoxemia: key features of acute lung injury (ALI). Influenza infection gives rise to ALI via two mechanisms: 1) The disruption of normal ion transport in the distal lung leading to pulmonary edema; and 2) The induction of an over-robust immune response leading to tissue damage. We have previously shown that Influenza-induced ALI in C57BL/6 mice (WT mice)
was associated with increased Cl- secretion via the cystic fibrosis transmembrane conductance regulator (CFTR) Cl- channel expressed on cells of the distal lung. Interestingly, C57BL/6-congenic mice that are heterozygous for the F508del mutation in CFTR (HET mice), which exhibit a 50% reduction CFTR expression and CFTR-mediated Cl- transport, experienced a significant attenuation in ALI. Thus, the aim of these studies was to identify various factors within the HET model that dictate the beneficial phenotype. Attenuated ALI was alveolar macrophage (AM) dependent and was not linked to alterations in viral replication between strains. Also, HET AMs displayed an anti-inflammatory phenotype compared to their WT counterparts. Elevated levels of the cytokines, TGF-β and IL-6, were observed in the HET mice, and neutralization of these cytokines eliminated the beneficial phenotype. Lastly, using mice that were heterozygous for 2 additional CFTR mutations, we were able to determine that reduced CFTR expression, not CFTR-mediated Cl- secretion, may be most important in the attenuation of ALI. Taken together, these findings identify CFTR as a novel host determinant for influenza severity, and provide a rationale for modulating CFTR for therapeutic benefit.
Dedication

To all of my close friends and family

"May the roof above us never fall in,
and the friends gathered below it never fall out."

-Irish Blessing
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Chapter 1: Literature Review

1.1 Influenza

1.1.1 Influenza biology

Influenza viruses are single-stranded, negative-sense RNA viruses comprised of 7-8 gene segments, which code for 9-11 proteins depending on the genera. Influenza viruses belong to the viral family, Orthomyxoviridae. Within this family there are 3 different influenza genera: influenzavirus A, influenzavirus B, and influenzavirus C. These 3 genera are described by their antigenic differences in capsid and matrix proteins. Influenza A poses the most direct threat to public health through zoonotic transmission since its host range is comprised of humans, several other mammalian species, and birds (105; 144). In contrast, influenza B and influenza C viruses maintain a limited host range, made up of humans and seals, and humans and pigs, respectively. Thus, this thesis places emphasis on influenza A virus.

Influenza A viruses can further be classified into subtypes based on variations in the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, sixteen HA and nine NA subtypes have been identified. Other variations in influenza strains gives rise to a nomenclature system based on virus subtype,
year of isolation, and geographical location of the initial discover (i.e. Influenza A/WSN/33 (H1N1); where WSN stands for Wisconsin and 33 represents 1933).

Structurally, influenza A viruses are comprised of an 8 distinct gene segments coated by nucleoprotein (NP) and a host cell-derived envelop. Figure 1.1 provides a visual representation of influenza A virus (144). HA, NA, matrix protein 1 (M1), and matrix protein 2 (M2), along with the host cell plasma membrane, make-up the viral envelope. M2 is an ion channel that aides in uncoating of viral RNA. HA and NA are central to viral entry and egress, respectively. Within the virus particle, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) carry out genome replication, while PB1-F2 and non-structural protein 1 (NS1) are involved in host cell evasion. Lastly, nuclear export protein (NS2/NEP) and M1 aid in nuclear export of viral ribonucleoprotein complexes.

HA mediates viral binding to host cell sialic acid. Variability in HA composition determines influenza's preference for sialic acids with specific glycosidic bonds. For instance, human influenza HA (i.e. H1N1) binds preferentially to α2,6-linked sialic acid, while avian influenza HA (i.e. H5N1) is predominantly associated with α2,3-linked sialic acid (97; 158; 159). α2,6-linkages are primarily found on upper airway respiratory epithelial cells, whereas α2,3-linkages predominate on the surface of epithelial cells in the distal lung(60). It has been proposed that these binding relationships dictate influenza tropism and severity of disease pathogenesis.
Upon binding, HA functions as a fusion protein that fuses the viral envelope with the host endosomal membrane. Influenza M2 channel protein drives the acidification of the viral-endosomal complex by pumping hydrogen ions into the virus particle [reviewed in (132)]. Following acidification, viral ribonucleoprotein complexes are released into the cytoplasm and traffic to the nucleus by M1 protein where RNA replication and transcription are carried out by viral polymerases. For efficient transcription and trafficking of viral RNA, viral polymerase components initiate a process known as cap snatching. This involves cleaving the 5’ cap of host cell transcripts and adding said caps to newly transcribed, positive-sense, viral RNA (13; 155). Viral NEP/NS2 then mediates RNA export to the cytoplasm where translation of viral protein takes place(27). Throughout these processes influenza NS1 functions to shut down critical antiviral responses to allow for continual production of viral products (34; 101).

While HA is critical for viral entry, NA is central to viral egress from the host cell. As the newly produced virus begins to bud from the host cell, HA glycoproteins cling to sialic acid residues on the cell surface. NA hydrolyzes these sialic acid residues, which allows for active release (146). These viral progeny can now go on to infect other cells within the respiratory tract.

1.1.2 Influenza pathogenesis

Influenza virus infections can span a large spectrum of disease manifestations. Some individuals may experience an asymptomatic infection or an infection that results in no more than a sore throat and running nose. In contrast, others may experience a severe febrile illness characterized by cough, muscle aches,
headache, and fatigue that could progress into pulmonary complications or even death (103; 145). Symptoms and disease severity also vary based on age, with young children and the elderly typically experiencing the most significant morbidity and mortality (52; 110; 129; 145). These populations are more vulnerable to influenza infections due to immunological incompetence and underlying health conditions that contribute to disease exacerbations. For instance, individuals with cardiopulmonary disease or diabetes mellitus are more likely to develop viral pneumonia (79; 92; 95).

In contrast to the clinical manifestations of influenza infection, cellular pathology studies are more limited in their ability to describe the full spectrum of disease severity. Most influenza pathology studies have utilized autopsy material, thus late-stage, lethal disease has been most accurately described. Interestingly, the observed pathological changes remain well-conserved from year-to-year in these severe cases.

Influenza virus replication tops out at approximately 48 hours post exposure with the initial infection taking hold in the nasopharynx. As time progresses, influenza migrates down the respiratory tree where it predominantly replicates in the epithelial cells of the upper and lower respiratory tract. In the upper airway, viral replication induces structural changes in the epithelium in the absence of neutrophil infiltration and inflammation. These changes include ctyonecrosis and desquamation of the affected epithelium. Next, neutrophils migrate to the site of infection in response to epithelial cell necrosis followed by the predomination of resident macrophages and infiltrating monocytes. As infection progresses toward
the alveoli, the robust inflammatory response begins to lead to thrombosis of small vessels and the accumulation of edema in the airways. A severe influenza infection can result in the partial or complete disappearance of alveolar type II (ATII) cells (91). Influenza induces ATII cell proliferation and differentiation, which is initiated in an attempt to repair the damaged blood-air interface (57). Likewise, novel airway stem cell populations undergo proliferative expansion in response to influenza damage in an attempt to repopulate the alveolar epithelium (175).

There is some argument within the field for what disease process is the main driver of the pulmonary complications associated with fatal influenza infection. Several studies have proposed that excess pulmonary edema results from influenza-induced alterations in respiratory epithelial ion balance (3; 78; 80; 168). Influenza infection stimulates excess chloride secretion into the surrounding airways and inhibits sodium reabsorption into the cells. The net result leads to fluid accumulation as water travels down the concentration gradient toward the high ion content amassing in the lumen of the airway. Inability to clear this excess alveolar fluid from the airways is associated with poor outcomes (98).

A secondary school of thought with regards to severe disease pathology places emphasis on the robustness of the immune response, commonly referred to as the “cytokine storm.” This phenomenon often refers to the failure of the immune system to self-regulate upon the excess production of cytokines in response to viral antigen. This results in excess and detrimental inflammation that contributes to tissue damage and pulmonary edema. There is no firm definition of the cytokine storm, and thus it been used to describe disease processes induced by
other infectious agents and autoimmune disorders (11; 16; 68; 86). With regards to influenza, the term became common place in association with the high mortality rates documented in the 1918 and 2009 pandemics, where increased cytokine and leukocyte levels were observed in fatal cases (17; 31). Evidence supporting the cytokine storm theory has also been described in several animal models of severe influenza infection (24; 75; 139). This has lead researchers to investigate the potential for host-cell directed therapeutics that may serve to dampen the inflated inflammatory response (96; 163). On the other hand, others have shown that inhibition of the pro-inflammatory cytokine response fails to provide protection against lethal influenza infection (124). It is likely that both aberrant epithelial ion secretion and overproduction of inflammatory cytokines contribute to severe lung pathology following influenza infection.

1.1.3 Influenza Epidemiology

Influenza is a zoonotic infection that can be well-stabilized within the human host. This allows for efficient human-to-human transmission, which occurs via airborne transmission of respiratory droplets and droplet nuclei. All influenza A viruses can be traced back to an initial avian origin (166). Virtually all combinations of HA and NA subtypes have been characterized in waterfowl (ducks, geese, and swan). This suggests that a large pool of diverse viruses is circulating at any given time. Many of these viruses yield sub-clinical disease in birds, and thus may contribute to the co-circulation and co-infection with multiple strains and subtypes within bird populations.
Certain HA subtypes, specifically H5 and H7, may evolve toward a highly pathogenic state upon transmission into domestic poultry (51). These viruses are highly lethal within the avian population, and can be sporadically transmitted to humans, but are typically self-limiting epidemics. For instance, from 2003 to 2009, there were 433 documented cases of sporadic H5N1 infection in humans, with 262 (61%) of these cases resulting in death (156). These statistics highlight the low-incidence, yet high pathogenicity of these novel avian viruses.

In mammals, swine maintain the largest diversity of influenza A viruses, and can readily transmit swine-adapted influenza to humans. More importantly, swine can serve as mixing vessels for viruses of swine, avian, and human origin resulting in influenza “antigenic shift.” This phenomenon occurs when host cells are co-infected with two or more different virus strains. This can lead to the exchange of one or several gene segments from different viruses resulting in novel viral progeny packaged with gene segments of different origin. The 2009 H1N1 swine influenza pandemic strain contained gene segments from swine, avian, and human origin, and is one of the most prominent examples of antigenic shift (41). Influenza viruses can also obtain genetic diversity through the random accumulation of mutations throughout the genome (144). These mutation events are referred to as “antigenic shift.” The exact mechanism for these events has yet to be fully elucidated; however, a non-proofreading viral polymerase and immune selection within the host seem to play a pivotal role (15; 89). Influenza outbreaks in humans have been documented as early as the Middle Ages (143). Seasonal epidemics are responsible for thousands of
hospitalizations and deaths annually (149; 150). There is also the threat of pandemics, which sporadically occur every 40 years with some emerging as close as 10 years apart (104). The 1918 pandemic was the worst in recorded history resulting an estimated death toll of 50 million individuals worldwide (66; 142).

Reverse-transcriptase PCR is the most common molecular technique used to detect influenza within samples. The high sensitivity and cost-effectiveness of this method make it the most accepted screening tool used in influenza surveillance. Diagnostic tests such as serological tests, virus isolation, and rapid antigen tests can also be used to confirm influenza infection, but are often limited by cost and time (77). Unfortunately, there is no standardized method for detection that allows for efficient comparison of surveillance studies. This is unlikely to change in the immediate future given that resources and technical expertise are highly variable between countries and institutions (94).

1.1.4 Therapy and prophylaxis

The current mainstays of influenza treatment and prophylaxis are antiviral therapy and vaccination, respectively. While vaccination is paramount in curbing seasonal epidemics, there are many limitations in the developing an efficient and effective vaccine. Antigenic drift from year-to-year necessitates the annual production of a new influenza vaccine (19). Production is often based on predictions, which may lead to partial protection when vaccine components improperly match with the circulating strains. This was the case in 2014 where strain mismatch resulted in substantial reductions in vaccine effectiveness (39).
Moreover, vaccines may have limited utility even when a proper match has been made. A recent meta-analysis of influenza vaccine studies has suggested that the clinical efficacy and effectiveness of influenza vaccination is suboptimal and may only provide moderate protection against virologically confirmed influenza (111). Logistical complications associated with vaccine production and distribution further limit the effectiveness of annual vaccination programs (37; 47). Additionally, many developing nations lack proper access to influenza vaccines (38). Misconceptions of vaccine side effects contribute to the overall low vaccination rates in the United States. As a result, vaccination rates are too low to obtain herd immunity (73).

Antiviral drugs have been developed to treat influenza-infected individuals. The most common drugs are oseltamivir and zanamivir, which inhibit influenza NA activity to prevent viral cleavage and egress from the infected host cell. However, antiviral medications are limited in their effectiveness since they must be administered early in infection, often before any serious symptoms are presented (35; 64). There have also been documented cases of viral mutants exhibiting resistance to these drugs (151). Shockingly, recent reports have suggested that most antiviral influenza medications only serve to alleviate symptoms, and thus fail to provide the appropriate therapeutic benefit in life-threatening situations (25).

Taken together, the current mainstays of influenza therapy and prevention do little for individuals seeking treatment once severe infection has already been established. Non-specific supportive care in the ICU remains the current
treatment for patients presenting with end-stage influenza infections, which
demonstrates that a “therapeutic gap” in influenza therapy exists.

1.1.5 Animal models for influenza infection

1.1.5.1 Mice

There are multiple animal models that have been essential in furthering our
knowledge of influenza pathogenesis, with each model serving its own purpose
within the field. Murine models are the most commonly used mammal for
influenza studies and offer several advantages. It is relatively cheap to maintain a
mouse colony and reagents used in experimentation are commercially available.
Their short gestation period and time to adulthood allows for rapid generation of
transgenic mice for specialized studies. Moreover, the mouse genome has been
thoroughly characterized. In spite of these advantages, there are also several
limitations for this model system. Mice are not natural hosts for influenza, which
often necessitates the development of mouse-adapted viruses in order to
achieve productive infection and clinical disease. Yet, some highly pathogenic
viruses, such as the 1918 Spanish influenza and Avian H5N1, can yield a
productive infection in mice (139). Mice often fail to accurately mimic the exact
human inflammatory response to infection and other inflammatory stimuli (128).
Likewise, the clinical signs observed in mice deviate somewhat from a typical
human response. Mice present with pronounced anorexia resulting in an
exaggerated reduction in body weight due to their high metabolic rate. Unlike
humans, mice become hypothermic following infection rather than develop a
febrile response (147). Lastly, mice lack the ability to efficiently transmit infection
within a population so they cannot be used in models assessing influenza transmission (127).

While mice fail to fully replicate human disease, recent efforts have been made to more accurately assess influenza-induced pulmonary complications in mice. Murine influenza is primarily a lower respiratory tract infection, which make mice an effective model for studying primary viral pneumonia. Verhoeven et al. demonstrated that mouse adapted pulse-oximetry is a much better predictor of clinical disease and lung pathology than systemic weight loss measurements (161). Moreover, our lab has shown that influenza infection in mice results in impairment of gas exchange consistent with influenza-induced acute lung injury (ALI) in humans (152).

1.1.5.2 Non-human Primates

Non-human primates most closely resemble humans with regards to physiology and genomic sequence similarity; however, these animals are not frequently used in influenza studies due to high cost and ethical concerns. Limited use of rhesus, cynomolgus, and pigtailed macaques, and more recently, marmosets, has allowed for a more complete understanding of influenza pathology (7; 9; 102; 139). As with mice, non-human primate models also fail to fully mimic influenza disease observed in humans. Certain strains of human and avian influenza fail to infect the upper respiratory epithelium in cynomolgus macaques (160).

1.1.5.3 Ferrets

Ferrets readily transmit influenza from one animal to another, and are thus valuable models in transmission studies. Moreover, Ferrets are readily infected
by human influenza viruses, and they present with many of the human symptoms associated with seasonal, non-lethal influenza infection, such as fever, nasal congestion, and sneezing (134). In the same light, this makes ferrets a poor model for lethal influenza infection. The ferret model has also been instrumental in assessing pandemic potential of highly pathogenic viruses (53; 93; 169).

1.1.5.4 Other Animal Models
The use of guinea pigs has gained some momentum in influenza research, especially for the purpose of studying animal-to-animal transmission. They are highly efficient in transmitting unadapted human influenza viruses at distances up to three feet. Compared to ferrets, guinea pigs are easier to handle and house, lower in cost, and easier to obtain commercially. Additionally, guinea pigs have been well-characterized in other models of lung disease, such as asthma and chronic obstructive pulmonary disease (18).

Pigs are mainly used in the development of influenza vaccines (21). Pigs can be naturally infected by both avian and human influenza viruses, which is why pigs are often considered an intermediate host (90). Yet, few influenza studies utilize pigs mostly due to practical limitations related to husbandry requirements. While popular in some fields of pulmonary research, rats have been poorly characterized within the field of influenza pathogenesis (8). Much of this is due to rats’ inability to harbor a productive influenza infection. Thus, other than mice, cotton rats have been the most commonly used rodent. Proponents for the cotton rat model point to its ability to harbor both nasal and pulmonary infection (112). On the other hand, cotton rats are highly aggressive and the cotton rat genome
has yet to be fully sequenced (8). This, along with limited access to cotton rat-specific reagents, makes the model less than attractive.

Both dogs and cats have can be naturally infected with H5N1 influenza. This is of interest to public health given the close contact that domesticated dogs and cats have with humans (43; 121). Nonetheless, there have been no documented cases of influenza transmission from dogs or cats to humans.

1.2 Acute Lung Injury (ALI)

Acute lung injury (ALI) is a complex disease process with varying clinical presentations depending on the insult/stimuli. For instance, ALI can result from direct pulmonary insult, i.e. viral/bacterial pneumonia or gastric aspiration, or from an indirect insult, such as an acute systemic inflammatory response (12). Its broad characterization includes severe pulmonary edema, hypoxemia, and abnormal lung mechanics. ALI can be further characterized and categorized based on oxygenation readouts. For instance, acute respiratory distress syndrome (ARDS) is the most severe form of ALI, and can be distinguished from ALI by a distinct set of clinical parameters. To be considered ARDS, inflammation must be severe enough to induce a ratio of partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO₂:FiO₂) to less than 200mmHg, while ALI is defined by a PaO₂:FiO₂ < 300mmHg (12).

Like severe influenza, most of what has been described about the pathology of ALI comes from patients who have died from the disease. Similarly, consistent with severe influenza, fatal ALI pathology includes diffuse alveolar damage,
increased endothelial permeability, intravascular thrombosis, denudation of the epithelium, and heavy neutrophilic infiltration.

Treatment for ALI relies primarily on supportive care, and only one phase III trial have ever shown promise in reducing mortality associated with the disease (1). Clinically ALI is often embraced as a single entity irrespective of the disease etiology. Thus, it is highly desirable from a clinical perspective to develop a therapeutic that is effective regardless of the underlying cause. This is much of the same mindset in the clinical treatment of sepsis. However, ALI is a complex process with varying pathomechanisms depending on disease origin, and thus a “one for all” approach may be somewhat shortsighted.

1.2.1 Animal modeling of acute lung injury

Given its complexity, ALI cannot not be studied in cell culture alone. Animal models have been crucial in furthering the understanding of cellular mechanisms that drive the disease and for developing and testing novel therapeutics. Various animal models have been used and each offer advantages and disadvantages. Rodents have been the most widely used in studying individual pathways that contribute to disease. Large animals, such as primates, dogs, pigs, and sheep, allow for ventilator studies, but such studies become rather costly due to the need to create a retrofitted intensive care unit for animals (99). While all animal models have their shortcomings and the results should be interpreted with appropriate caution, such studies can provide information on key physiologic and cellular pathways that can be assessed in humans.
1.2.3 Influenza-induced ALI

The general overview of influenza-induced ALI was described earlier in the influenza pathogenesis section (Chapter 1.1.2). Thus, this section will focus more on cell-specific mechanisms that drive ALI, with emphasis on damage to the respiratory epithelium and endothelium. The inflammatory response, including cytokine secretion, reactive oxygen species generation, and immune cell recruitment, is necessary for the elimination of virus. However, these mechanisms of inflammation are also responsible for significant damage to the epithelium and endothelium during severe infection. For this reason, therapeutic development has focused on cellular repair/repletion and on the modulation of inflammation.

Damage to the alveolar-capillary barrier is a hallmark of severe influenza infection. In a study comparing low versus high pathogenic influenza, researchers noted infection was survivable until about 10% of the alveolar epithelium was lost (125). Notably, evidence of inflammation was observed in the surviving mice out to 30 days following infection, even though viral RNA was no longer detectable. This suggests that influenza-induced ALI is a prolonged process. Loss of alveolar epithelium is also accompanied by cellular proliferation and differentiation. Alveolar type II (ATII) epithelial cells differentiate into type I (ATI) epithelial cells in attempt to repair the alveolar-capillary barrier. However, it has yet to be determined whether this differentiation process is beneficial or contributes to influenza-induced ALI (57). ATII cells carry out seminal functions in the distal lung, such as surfactant production and fluid balance, thus too much
differentiation may only serve to enhance disease within the context of influenza. Intravenous mesenchymal stem cell (MSC) administration has been experimentally applied in an attempt to enhance alveolar-capillary barrier repair following severe influenza infection (29; 44). These studies found that mice were unresponsive to MSC therapy and only resulted in modest reductions in thrombosis. Moreover, porcine MSCs are able to support productive influenza replication, suggesting that MSC therapy could exacerbate disease (71; 148).

Keratinocyte Growth Factor (KGF) acts in a paracrine manner to trigger the proliferation of ATII cells \textit{in vivo} (154). Several studies have identified KGF as a key mediator in attenuating lung injury following bacterial or endotoxin exposure (62; 81; 82). Perhaps promoting ATII cell proliferation may be of therapeutic benefit within the context of influenza-induced ALI.

\textbf{1.2.4 Other models of inducible ALI}

Lipopolysaccharide (LPS), also referred to as endotoxin, is an outer membrane component of gram-negative bacteria that has been widely used to experimentally-induce ALI. These studies attempt to mimic ALI resulting from bacterial sepsis. Interestingly, severe sepsis is a risk factor in 79\% of all ALI cases, and 46\% of all ALI sepsis cases result from a pulmonary source (123). To mimic ALI, LPS is most commonly instilled intratracheally, and lung injury develops over several days. LPS can also be administered intravenously in an attempt to more appropriately mimic sepsis. In this case, the capillary endothelium is the initial site of injury followed by infiltrating tissue damage upon apoptosis of the endothelial cells (99). In general, LPS studies in mice fail to
mimic the severe endothelial and epithelial injury that occurs in human ALI, and by itself may fail to fully replicate the effects of live bacteria in the lung (167).

Acid-induced lung injury aims to model the clinical situation where aspiration of gastric juice damages the alveolar epithelium. Gastric acid aspiration can account for 11% of clinical ALI cases (123). Gastric acid is characterized by a low pH. To mimic the disease process hydrochloric acid (HCL) with a pH between 1 and 2 is instilled into the airways of experimental animals. This process is biphasic, with immediate damage resulting from physiochemical reactions to the acid and secondary damage resulting from infiltrating neutrophils (70). The exact mechanism by which acid triggers the innate immune response is poorly understood, but Toll-like receptor (TLR) 4 activation has been implicated (61). A major disadvantage to using HCL instillation in an experimental model is that it fails to completely mimic the complexity of human gastric contents. Gastric juice contains particulate matter, bacterial products, and cytokines that could also contribute to lung injury.

Ventilator-induced lung injury describes the phenomenon where mechanical ventilation produces lung trauma and inflammation. This involves activation of intracellular signaling pathways following mechanical stretch. Overstretching of the alveoli can lead to breaks in the endothelial-epithelial barrier leading to accumulation of edema fluid in the lungs (40). Furthermore, mechanical deformation of cells can be transformed into biochemical changes, including pro-inflammatory cytokine secretion by alveolar epithelial cells and macrophages (119; 162). The severity of lung injury is largely dependent on the ventilator
strategy that is applied in an experimental setting. Large tidal volumes lead to alveolar destruction, neutrophilic infiltration, and alterations in lung function. Smaller tidal volumes fail to elicit physical trauma or a pro-inflammatory response. For this reason, the complexity of the model itself is the major disadvantage of experimental ventilator-induced lung injury. Yet, the mechanical ventilation model is the only lung injury model that has had any positive impact on clinical practice by improving survival (1).

1.3 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

1.3.1 CFTR channel function and associated mutations

The cystic fibrosis transmembrane conductance regulator (CFTR) channel is a protein that conducts anions and other small molecules across epithelial cell membranes. The CFTR gene is approximately 189 kilobases in length and is composed of 27 coding and 26 noncoding regions. The channel is made up 1,480 amino acids that form 5 distinct protein domains. There are two membrane spanning domains (MSDs) that anchor the protein to the plasma cell membrane. Each MSD is associated with its own nucleotide binding domain (NBD), and the R (regulatory) domain links the entire complex together. In short, the MSDs form the selective channel pore through which anions travel (5; 130), the NBDs bind to and hydrolyze ATP to regulate channel gating (6; 59), and the R domain's phosphorylation status controls channel activity (10; 22). See figure 1.2 for a visual representation of CFTR's domain structure (131).

CFTR mainly functions to increase conductance of chloride ions down their electrochemical gradient. ATP binding to the NBD of CFTR induces conformation
changes, which allow for the passive transport of anions across the cell membrane. Thus, with regards to CFTR, ATP is not actively driving anion movement against electrochemical gradients, but rather it is opening the channel to allow for passive transport of anions.

CFTR is predominantly located on epithelial cells on organs throughout the body. Controlled regulation of chloride anions directs the movement of water within tissues allowing for the formation of an appropriate mucous composition that is thin and freely flowing. Mucous lubricates the lining of the respiratory, digestive, and reproductive tract to allow for proper movement of materials within the system. Within epithelial cells, CFTR also regulates the activity and expression of other ion channels, such as epithelial sodium channels (ENaCs), and thus can modulate cellular ion levels beyond its primary function of chloride transport (138). CFTR is also found on cells of the innate and adaptive immune system, yet much less is known about its function within this context.

Much of what is known about CFTR function has been derived from studying disease causing mutations in the protein that lead to cystic fibrosis (CF) disease. Over 1,000 mutations in the \textit{cftr} gene have been described in CF patients (See table 1.1 for the common classes of CF mutations). While mutations in CFTR impact normal function in multiple organ systems, this thesis will place emphasis on aberrant CFTR function in the lung. Disease-causing mutations in CFTR can impact the production, stability, expression, and conductance of the channel. Ultimately, anyone of these mutational defects can impair proper chloride channel activity resulting in abnormalities in water transport into and out of the
lung epithelium. As a result, epithelial cells lining the respiratory tract produce abnormally viscous mucous that obstructs airways and impedes normal lung processes. For instance, the abnormally thick mucous hinders clearance of foreign material, i.e. infectious agents and particulate matter, that enters the lung. Inability to clear material can lead to the recruitment of immune cells that mount a sustained inflammatory response in attempt to clear the foreign bodies. This chronic inflammation leads to airway remodeling and bronchiectasis. Consequently, a CF patient will experience a steady decline in lung function and will eventually need to undergo lung transplantation.

1.3.2 CFTR’s immune function

As mentioned earlier, CFTR functions mainly as a chloride channel and regulator of other ion channels, and many abnormalities associated with CF disease are directly related to chloride secretion. However, alterations in adaptive and innate responses are commonly observed in individuals with CF suggesting that CFTR has an immunomodulatory role. While some studies suggest that mutations in CFTR lead to a pro-inflammatory lung microenvironment, other lines of evidence propose that alterations in CFTR promote an anti-inflammatory immune response to pathogens. For example, CFTR deficiency leads to blunted epithelial type I interferon responses following bacterial challenge (114). Moreover, loss of CFTR results in increased basal activity of arginase1 (Arg1) and reductions in iNOS expression, which is characteristic of an anti-inflammatory phenotype (46; 69; 135). In contrast to these findings, others have described dysregulated pro-inflammatory transcription factor activity in CF airway epithelium (26; 55; 120).
Moreover, enhanced neutrophil and lymphocyte responses have been observed in the airways of CF patients (117; 141). The contradictory nature of these findings examining altered immune states in CF patients is often confounded by secondary complications. Variations also arise based on the immune stimulus used in each study (i.e. bacterial vs. viral stimulus), and thus results may be context dependent.

1.3.3 CFTR in influenza pathogenesis

Most of what is known of CFTR’s contribution to influenza-induced ALI is related to its role in ion transport. Influenza-induced ALI in mice is associated with increased Cl− secretion via CFTR expressed on cells of the distal lung (168). Our lab has shown that C57BL/6-congenic mice that are heterozygous for the F508del mutation in CFTR show reduced pulmonary edema and delayed mortality following influenza infection (2). These mice exhibit a 50% reduction in CFTR expression and CFTR-mediated Cl− transport, but are otherwise phenotypically normal. Others have shown that influenza infection leads to the degradation of CFTR (87; 88). However, these were in vitro studies, which fail to mimic the temporal nature of an in vivo system. It has yet to be determined whether CFTR degradation in this instance is an active process or one resulting from cellular necrosis following exposure to influenza.

1.4 Alveolar Macrophages

1.4.1 Normal macrophage function in the lung

Alveolar macrophages are long-lived resident cells that populate the distal lung at an approximate density of 7 cells per alveolus (137). There is roughly a 40%
turnover of the AM population in a single year. This is substantially longer than tissue-resident interstitial lung macrophages (IMs), which experience an almost complete turnover within 21 days. Initial macrophage colonization of the alveolus occurs only a few days after birth, and the self-renewing capacity of these cells will act as the primary source of AM replenishment throughout a lifetime (48). For instance, influenza infection leads to the partial exhaustion of the AM pool. While some infiltrating monocytes and IMs will remain in the lung following resolution of influenza infection, AMs predominate in replenishing the macrophage population within the airway lumen (45). The only circumstance in which AMs fail to repopulate the alveolus is following radiation-induced depletion. In this case, hematopoietic stem cell-derived monocytes will aid in repopulation of the airspaces (49).

Within the lung, macrophage function must be tailored in such a manner that is specific to both the cellular and physiological environment. Thus, AMs are highly specialized and are distinct from macrophages in the lung interstitium and monocytes in the circulation. They must maintain a high degree of plasticity in response to the constant barrage of environmental fluctuations experienced in the airway. In a healthy individual, the partial O₂ pressure (PO₂) in the alveolus is approximately 100-110 mmHg. PO₂ levels can be dramatically altered with exposure to infectious agents, environmental antigens, and in chronic inflammatory diseases. Moreover, hypoxic conditions occur frequently in normal airways and can often induce airway immune responses through induction of transcription factors, such as NF-κB and HIF-1α (28; 126). Lower airways are
also colonized with commensal bacteria that can fluctuate with alterations in the lung microenvironment (20). Hence, AMs must respond appropriately to these highly variable conditions in a way that not only prevents unwarranted inflammation, but also allows for clearance of disease causing agents.

Due to direct contact with external environment, AMs are well-equipped with mechanisms that inhibit unnecessary inflammatory responses. For example, murine AMs are poor at presenting antigens to T-cells, and human AMs induce T-cell unresponsiveness due to their lack of co-stimulatory molecules (14; 74). Additionally, AMs show marked reductions in phagocytic activity and reactive oxygen species (ROS) generation when compared to IMs (58).

AM activation is tightly regulated through direct interactions with the alveolar epithelium and microbiota. CD200 is expressed on the luminal aspect of alveolar type II cells (ATII) (65). Interestingly, CD200R is more highly expressed on AMs compared to other macrophage populations. This allows for direct interactions between AMs and the respiratory epithelium with overall inhibitory consequences. CD200R binding leads to the inhibition of kinases within AMs that are key to stimulating pro-inflammatory gene expression (100). Mannose receptor is another AM cell-surface molecule involved in lung homeostasis. The mannose receptor recognizes glycans on bacterial surface proteins. If the bacteria are unopsonized, such as in the case of commensal bacteria, then AMs remain in an immunologically inert state (173). However, if mannose receptors were to interact with opsonized bacteria then a proper immune response would be initiated. ATII cells can also influence AM homeostasis through secreted
products. Surfactant-associated protein A and D (SPA and SPD) are collectins secreted by ATII cells that function primarily in the opsonization of pathogens. When no pathogens are present, SPA and SPD are closely associated with TLRs in such a fashion that prevents unnecessary immune activation (171). Likewise, SPA and SPD can bind to signal-regulatory protein-α (SIRPα) on AMs. Surfactant protein-bound SIRPα suppresses AM phagocytosis and inhibits pro-inflammatory signaling within the cell (63).

1.4.2. Macrophages in influenza pathogenesis

Tissue-resident alveolar macrophages (AMs) are the initial immune cell population to respond to viral infection within the lung. They aid in viral clearance through phagocytosis of collectin-bound viral particles (opsonization) and infected apoptotic cells (efferocytosis) (50; 54). Moreover, AMs secrete a wide array of cytokines and chemokines in response to influenza to drive secondary immune responses (165). The production of cytokines by AMs can differ depending on the specific influenza strain. While macrophages can be infected with influenza, there is some argument regarding their ability to maintain productive replication. In vitro analysis suggests that AMs poorly support viral replication (122); however, influenza-infected monocyte derived-macrophages (MDMs) are readily susceptible to infection and can harbor productive replication. MDMs support productive replication of both seasonal and highly pathogenic influenza strains, but the highly pathogenic viruses induce a more robust pro-inflammatory response. This was not the case in AMs where direct infection with
highly pathogenic strains failed to generate a potent inflammatory response (23; 116; 157; 172). These studies highlight influenza's interactions with functionally distinct macrophage populations, and suggest that infected airway epithelial cells are paramount in stimulating AMs to respond to influenza infection.

It has been well-documented that AMs are critical mediators in controlling influenza infection. In animal models, depletion of AMs prior to influenza infection results in a higher viral load, increased lung pathology, and decreased survival(72; 108; 153). There is also evidence to suggest that the antiviral capabilities of AMs can be enhanced through manipulation of AM cell surface receptors prior to infection. For example, deletion of MARCO, a cellular receptor involved in lung immune homeostasis, primed AMs to be more effective in clearing virus and reduced influenza-induced ALI (42).

Pro-inflammatory cytokine release leads to the recruitment of monocytes into the lung. Upon recruitment, the lung microenvironment induces monocyte differentiation into AMs. Tissue-resident interstitial lung macrophages will also migrate to the alveolus following infection, but again, these cells will differentiate into AMs upon entering the airway lumen (58). All macrophage types responding to influenza infection appear to have the same goal, which is to clear virus. Yet, based on the depletion studies, AMs may be the most important in dictating the proper immune response and aiding in the attenuation on ALI. Other studies have even suggested that infiltrating macrophage populations are the predominant cause of immune pathology during influenza infection (30; 84; 85).

This is likely due to the fact that these infiltrating macrophage populations
release higher levels of pro-inflammatory cytokines than their resident AM counterparts (140). Upon stimulation with pro-inflammatory cytokines, macrophages will express inducible nitric oxide synthase (iNOS). iNOS is an enzyme that mediates nitric oxide production. Nitric oxide interacts with oxygen free radicals leading to the production of reactive oxygen species (ROS), which function to eliminate pathogens (36). However, ROS can also damage tissue when mounting an immune response against pathogens, and this appears to be the case with regards to influenza infection. For instance, iNOS inhibition attenuated pulmonary edema and reduced mortality following lethal influenza challenge (4). Likewise, global iNOS-knockouts failed to develop severe pneumonia after influenza infection even though higher viral titers were observed in these mice (67; 115). Taken together, these findings suggest that ROS generation is detrimental within the context of influenza infection and may only serve to exacerbate influenza-induced ALI.

1.4.3 Macrophage Polarization
The study of macrophage activation states, commonly referred to as macrophage polarization, has become an area of emphasis within the fields of tissue homeostasis, disease pathogenesis, and inflammation. Macrophages can be classified into two broad categories: classically-activated, pro-inflammatory macrophages (M1), and alternatively-activated, anti-inflammatory macrophages (M2). This concept first emerged in the early 1990s when researchers compared the differential effects of interleukin-4 (IL-4) to those of interferon γ (IFN-γ) on
macrophage function (136).

IFN-γ, known for its pro-inflammatory properties, promotes M1 polarization. M1-polarized macrophages are highly phagocytic and express high levels of inducible nitric oxide synthase (iNOS/nos2), which generates nitric oxide (NO) from arginine. As described earlier, NO can react with oxygen free radicals to generate ROS that aid in pathogen clearance, but ROS often have off-target effects leading to nucleic acid, lipid, and protein damage (36). In contrast, IL-4 promotes M2 polarization. M2-polarized macrophages express high levels of arginase 1 (Arg1). Arg1 catabolizes arginine to urea, which is immunologically inert (107). Arg1 and iNOS compete for the same substrate within macrophages, and, thus the relative ratio of these two enzymes can dictate polarization status. It should be noted, that M1 and M2 phenotypes are not discontinuous or mutually exclusive within the population of AMs responding to an inflammatory stimulus. Hence, individual macrophages can co-express iNOS and Arg1 and which ever enzyme dominates will often determine the polarized state.

A given polarization state can be beneficial or detrimental depending on the disease in question. For example, M2-polarized macrophages within the context of cancer biology are referred to as tumor-associated macrophages and can promote tumor persistence (109). Likewise, the reduced phagocytic capacity of M2 macrophages would be disadvantageous in controlling acute bacterial infections and could promote bacterial colonization (32). However, an excessive or prolonged M1 response to bacteria can be deleterious to the host in that it can
lead to tissue damage or even organ failure. M2-polarized macrophages are necessary for resolution of inflammation, and thus are considered favorable in disease states that promote sustained or severe inflammation, such as chronic inflammatory illnesses and acute viral infections.

NK- and T-cell-derived IFN-γ may be a central component of the “cytokine storm” and AM activation in the presence of IFN-γ promotes pro-inflammatory (M1) polarization. Excessive production of pro-inflammatory mediators by AMs has been correlated with the high mortality associated with infection by highly-pathogenic influenza strains (23). Inhibiting the recruitment of MDMs and IMs, which are typically associated with an M1 phenotype, via ablation of CCR2, reduces influenza-induced pulmonary pathology (30; 84; 85). Furthermore, influenza-induced ALI is attenuated in WT mice treated with an iNOS inhibitor and in nos2-knockout mice (67; 115). Influenza-induced ALI is also attenuated by priming the murine lung with bacteria that induce M2 polarization of lung macrophages (164). Taken together, these studies suggest that M1 polarization contributes to influenza disease pathogenesis, while M2 polarization is beneficial and can possibly be exploited for therapeutic benefit.

1.4.4 CFTR’s function in macrophage

CFTR is best known for its specialized role in epithelial cells. Nevertheless, CFTR is a widely distributed protein that is found on both the cell surface and intracellular compartments of various cell types. CFTR mRNA has been detected in macrophages, neutrophils, lymphocytes, and dendritic cells (33; 76; 113; 170); however, the functional role on these immune cells has yet to be fully elucidated.
It is well-documented that CF patients have reduced ability to clear bacterial infections (83; 106; 118; 133). Impaired bacterial clearance can only partially be explained through aberrant mucociliary transport, a process that traps and propels bacteria out of the lung (56). Thus, much emphasis has been placed on alveolar macrophages given their resident status within the lung and their bactericidal capacity. Anke et al. were the first to describe a functional role for CFTR in macrophages. They found that CFTR regulates acidification of the intracellular lysosome, and thus impacts bactericidal activity within the macrophage. More specifically, under normal conditions, CFTR-mediated chloride (Cl\(^-\)) transport functions as a charge shunt to balance out hydrogen (H\(^+\)) atoms being pumped into the lysosome. This creates an acidified environment, which allows for the optimal function of proteases that aid in bacterial killing. Conversely, in CFTR deficient macrophages, there is no charge shunt from Cl\(^-\) to counter H\(^+\) influx resulting in incomplete lysosomal acidification. Consequently, lysosomal proteases fail to function properly, which favors survival of the engulfed bacteria (33).

Macrophages from CF patients have been shown to have other defects in bactericidal activity beyond abnormal lysosomal acidification. TLR-5, a critical factor for phagocytosis, is reduced on CFTR deficient monocyte-derived macrophages (133). Moreover, AMs from CF mice exhibit reduced ROS production leading to increased bacterial survival (174).
1.5 Figures

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Figure 1.1: Diagrammatic representation of an influenza A virus

Figure 1.2: Domain structure of CFTR
### Table 1.1: Different classes of naturally occurring CFTR mutations, their prevalence within the United States Population, and their impact on cell surface protein expression

<table>
<thead>
<tr>
<th>Class</th>
<th>Defect</th>
<th>Example/ Prevalence of mutation in CF Patients (USA)</th>
<th>Reduced cell surface protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Truncated CFTR</td>
<td>G542X (5%)</td>
<td>YES</td>
</tr>
<tr>
<td>II</td>
<td>Aberrant folding &amp; ER stress</td>
<td>F508del (87%)</td>
<td>YES</td>
</tr>
<tr>
<td>III</td>
<td>Defective regulation by cAMP or ATP</td>
<td>G551D (4%)</td>
<td>NO</td>
</tr>
<tr>
<td>IV</td>
<td>Defective anion transport</td>
<td>R117H (3%)</td>
<td>NO</td>
</tr>
<tr>
<td>V</td>
<td>Dysregulated transcription of normal <em>cftr</em> gene</td>
<td>A455E (&lt; 3%)</td>
<td>YES</td>
</tr>
<tr>
<td>VI</td>
<td>Unstable CFTR protein</td>
<td>Q1412X (&lt; 3%)</td>
<td>YES</td>
</tr>
</tbody>
</table>
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Ref Type: Generic


Ref Type: Generic


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Chapter 2: **TGF-β-induced IL-6 prevents development of acute lung injury in influenza A virus-infected F508del CFTR-heterozygous mice**

### 2.1 Abstract

As the 8th leading cause of annual mortality in the USA, influenza A viruses are a major public health concern. In 20% of patients, severe influenza progresses to acute lung injury (ALI). However, pathophysiologic mechanisms underlying ALI development are poorly-defined. We reported that, unlike wild-type (WT) C57BL/6 controls, influenza A virus-infected mice that are heterozygous for the F508del mutation in the cystic fibrosis transmembrane conductance regulator (HETs) did not develop ALI. This effect was associated with higher IL-6 and alveolar macrophages (AMs) at 6 days post-infection (d.p.i.) in HET bronchoalveolar lavage fluid (BALF). In the current study, we found that HET AMs were an important source of IL-6 at 6 d.p.i. Infection also induced TGF-β production by HET but not WT mice at 2 d.p.i. TGF-β neutralization at 2 d.p.i. (TGF-N) significantly reduced BALF IL-6 in HETs at 6 d.p.i. Neither TGF-N nor IL-6 neutralization at 4 d.p.i. (IL-6-N) altered post-infection weight loss or viral replication in either mouse strain. However, both treatments increased influenza A virus-induced hypoxemia, pulmonary edema, and lung dysfunction in HETs to
WT levels at 6 d.p.i. TGF-N and IL-6-N did not affect BALF AM and neutrophil numbers, but attenuated the CXCL-1/KC response in both strains, and reduced IFN-γ production in WT mice. Finally, bone marrow transfer experiments showed that HET stromal and myeloid cells are both required for protection from ALI in HETs. These findings indicate that TGF-β-dependent production of IL-6 by AMs later in infection prevents ALI development in influenza A virus-infected HET mice.

2.2 Introduction

Influenza A viruses cause a highly contagious acute respiratory disease in man, which is the 8th leading cause of attributable annual mortality in the USA. It has been estimated that seasonal influenza infections result in 12 influenza-related deaths per 100,000 persons per year in the United States, most of which occur in the elderly (22; 48). The emergence of a novel H1N1 "swine flu" influenza A virus strain in 2009 resulted in a worldwide pandemic and caused an estimated 14,800 excess deaths in the United States alone (9). Emergent highly-pathogenic H5N1 and H7N9 avian influenza A strains pose a continuing danger to the human population, particularly as they have very high mortality rates (>60%) (45; 60). Vaccines and neuraminidase inhibitors are routinely used for influenza prophylaxis and treatment, respectively (11). However, there are significant limitations to vaccine development, uptake, and efficacy (41; 42; 50). Indeed, poor matching between the 2014 influenza vaccine strains and circulating
reduce influenza severity and transmission early in infection (32). However, development of viral resistance mutants is rapid and these drugs are increasingly poorly effective (12; 18).

Approximately 20% of patients with severe influenza develop acute lung injury (ALI), which is associated with poor prognosis (35). Once ALI has developed, the only treatment option is non-specific supportive management in an intensive care unit. This is often of limited efficacy and death from ALI in patients with severe influenza is common (49; 67). New drugs are needed to treat late-stage influenza (2). However, development of such drugs requires a better understanding of basic mechanisms underlying the pathogenesis of influenza-induced ALI (53).

Alveolar type II epithelial cells are the main infection target and site of replication for influenza viruses in the distal lung (30; 61). A primary physiologic function of these cells is to regulate the depth of the thin (8 to 10 μm) layer of fluid lining the bronchoalveolar space. This process is dependent upon the relative magnitude of active Na⁺ absorption via epithelial Na⁺ channels (ENaC) and Cl⁻ absorption or secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel (15). Compromised ion transport leads to pulmonary edema and hypoxemia, and is associated with poor outcomes in ALI patients (66).

We reported previously that increased CFTR-mediated anion secretion played a role in development of pulmonary edema in influenza A virus-infected mice (68). We have shown that, unlike in wild-type (WT) C57BL/6 controls, influenza A virus infection did not cause ALI in C57BL/6-congenic mice that are heterozygous for
the F508del mutation (a phenylalanine deletion at position 508) in CFTR (HETs) (1). This effect was associated with higher bronchoalveolar lavage fluid (BALF) IL-6 content and alveolar macrophage (AM) counts at 6 days post-infection (d.p.i.) in HETs. Notably, AM depletion in HETs increased ALI severity to WT levels at 6 d.p.i. and concomitantly reduced BALF IL-6 levels by almost 80%.

IL-6 can play both pro- and anti-inflammatory roles in ALI pathogenesis (17; 34; 44; 69). Given the strong correlation between increased BALF IL-6 and absence of ALI in influenza-infected HETs, the aim of this study was to define the source of this cytokine and its contribution to protection from ALI in HETs. By performing cytokine neutralization and bone marrow transfer experiments, we found that TGF-β produced early in the response to influenza infection prevents ALI development in HET mice by inducing AM production of IL-6 later in infection. These results indicate that alterations in CFTR expression and/or function significantly impact the innate immune response of AMs to influenza viruses, and further emphasize the role of CFTR as an important modulator of the host immune response (1; 2; 68).

2.3 Materials and Methods

Breeding and Genotyping of F508del mice. C57BL/6-congenic F508del CFTR-heterozygous mutants (HETs) and WT controls were generated by breeding B6.129S7-Cftr<sup>tm1Kth</sup> mice (70). All procedures were approved by the Institutional Animal Care and Use Committee at The Ohio State University. Ethical considerations precluded performance of survival studies.
**Infection of mice.** 8-12 week-old WT and HET mice were intranasally infected with 10,000 plaque-forming units (pfu)/mouse of egg-grown influenza A/WSN/33 (H1N1) in 50 μl PBS with 0.1% BSA (2; 63). In our hands, this inoculum induces ALI in WT mice by 2 d.p.i., and results in 100% mortality by 8 d.p.i. (median time to death: 7 days) (2; 68), without replication in the brain (3). At the time of infection, mice were individually marked then weighed every other day. Data for each experimental group were derived from a minimum of 2 independent infections.

**Immunohistochemistry.** Thin sections (3 μm) were prepared from formalin-fixed, paraffin-embedded lung tissue (31). IL-6 was detected using a goat polyclonal antibody (AB-406-NA; R & D Systems, Minneapolis, MN). Bound antibody was detected using biotinylated anti-goat immunoglobulin (Vector Laboratories, Burlingame, CA), the Vectastain ABC peroxidase system and 3,3′-diaminobenzidine substrate (Vector Laboratories). Sections were counterstained with Harris’ hematoxylin, scanned with a Scanscope® CS slide scanner (Aperio Technologies, Vista, CA), and visualized with ImageScope software (Aperio Technologies).

**Quantitative real-time PCR.** BALF AMs were isolated by adherence to polystyrene as in our prior studies (25). Isolated AMs were lysed and total RNA was extracted using the RNeasy system (Qiagen, Alameda, CA). RNA was reverse transcripted into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). Quantitative real-time PCR amplification of cDNA was performed using the TaqMan Gene Expression
system (Applied Biosystems). Expression of il-6 mRNA was determined by the
\( \Delta \Delta Ct \) method and normalized to the endogenous control 18s rRNA, which was not
altered by infection (26).

**Antibody-mediated cytokine neutralization.** To neutralize TGF-β, mice were
treated with a single dose of a polyclonal TGF-β neutralizing antibody (AB-100-
NA; 100 µg/mouse in 100 µl saline; R & D Systems) at 2 d.p.i. by intraperitoneal
(i.p.) injection (7). To neutralize IL-6, mice were treated i.p. with a single dose of
a polyclonal antibody to IL-6 (AB-406-NA; 0.5 µg/mouse in 100 µl saline; R & D
Systems) at 4 d.p.i. (56). Controls were treated i.p. with rabbit IgG at both
timepoints (100 µg/mouse in 100 µl saline; Southern Biotech, Birmingham, AL).

**Bone marrow transfer.** As in our previous studies, bone marrow recipient mice
were irradiated with 1000 cGy given in 2 doses by a \(^{137}\)Cs irradiator (3). 24 hours
later, donor mice were euthanized and hind limb long bones were flushed with
conditioned media to isolate low-density bone marrow. 5 x 10^6 fresh bone
marrow cells/mouse (in 0.5 ml PBS) were then transplanted by tail vein injection
into recipient mice, which were placed on Baytril antibiotic water bottles for 2-3
weeks peri-irradiation. This protocol resulted in no outward clinical signs.

**Additional methods.** Bronchoalveolar lavage and measurements of carotid
arterial O₂ saturation, heart rate, lung homogenate viral titers, lung wet:dry weight
ratios, lung mechanics, and BALF inflammatory mediators were performed as in
our previous studies (14; 68).

**Statistical Analysis.** Descriptive statistics were calculated using Instat 3.05
(GraphPad Software). Gaussian data distribution was verified by the method of
Kolmogorov and Smirnov. Differences between group means were analyzed by one-way ANOVA, with Tukey-Kramer multiple comparison post-tests. $P<0.05$ was considered statistically significant. All data are presented as mean ± SEM.

2.4 Results

**HET AMs produce more IL-6 than WT AMs in response to influenza A virus infection.** We reported previously that attenuation of cardiopulmonary dysfunction in HETs infected with the A/WSN/33 (H1N1) influenza strain (10,000 pfu/mouse) was associated with exaggerated AM and IL-6 responses at 6 d.p.i. (1). We also showed that HET BALF IL-6 was reduced by approximately 80% following depletion of AMs using clodronate liposomes, indicating that the exaggerated IL-6 response to influenza A virus in HETs was AM-dependent. We therefore wished to determine the extent to which IL-6 was AM-derived in infected HETs. IL-6 was not detected in lung tissue sections from WT mice at 6 d.p.i by immunohistochemistry (Fig. 1A). In contrast, the majority of AMs in HET lung tissues were IL-6-positive at 6 d.p.i. (Fig. 1B). No antigen-positive AMs were detected in tissues immunostained with a non-specific control antibody. Lastly, quantitative real-time PCR demonstrated that, relative to uninfected controls of the same strain, influenza A virus infection induced significantly higher $il-6$ gene expression in AMS isolated from HET mice than AMs from WT mice at 6 d.p.i. (Fig. 1C).

**The exaggerated IL-6 response of HETs to influenza A virus infection is TGF-β-dependent.** Given the known role of TGF-β as an anti-inflammatory
cytokine (55) and an inducer of IL-6 (4; 21), we extended these observations by determining effects of the HET genotype on the TGF-β response to influenza A virus infection. We found that BALF TGF-β levels were higher in influenza A virus-infected HETs at 2 d.p.i. (Fig. 2A). BALF TGF-β increased further in WT but not HET mice at 6 d.p.i., resulting in no difference between mouse strains at this timepoint.

To determine whether the exaggerated IL-6 response to infection in HET mice was TGF-β-dependent, influenza A virus-infected mice were treated with a single dose of a neutralizing antibody to TGF-β at 2 d.p.i. (TGF-N) (7). Control groups were untreated or treated with nonspecific IgG. TGF-N had no significant effect on BALF TGF-β at 6 d.p.i. in either WT or HET mice (not shown) but reduced BALF IL-6 by approximately 80% in HETs at this timepoint (Fig. 2B). This was comparable to that caused by AM depletion in HETs at 6 d.p.i. (1). Treatment with a single dose of a neutralizing antibody to IL-6 at 4 d.p.i. (IL-6-N) reduced BALF IL-6 at 6 d.p.i. in both mouse strains, although we cannot exclude the possibility that this effect was a result of competition between the neutralizing and ELISA antibodies to IL-6.

Neutralization of TGF-β or IL-6 increases severity of cardiopulmonary dysfunction in influenza A virus-infected HETs without significantly impacting viral replication. To determine whether altered TGF-β and IL-6 responses to infection contribute to protection from influenza A virus-induced ALI, we examined the effects of TGF-N and IL-6-N on weight loss, viral replication, and cardiopulmonary function in influenza A/WSN/33 virus-infected mice at 6
We selected this timepoint as it would allow us to directly compare the downstream effects of treatment with anti-TGF-β at 2 d.p.i. with those of treatment with anti-IL-6 at 4 d.p.i. Post-infection weight loss at 6 d.p.i. did not differ between untreated WT and HET mice (Fig. 3A). IgG treatment, TGF-N, and IL-6-N modestly accelerated weight loss in both strains. However, these effects were not statistically significant. Likewise, viral replication was not significantly affected by IgG treatment or IL-6-N in either strain (Fig. 3B). Viral titers in HET mice were significantly higher than in WT controls following TGF-N, although the difference in titers was less than 1 log and thus is unlikely to be of biological significance. Importantly, both TGF-N and IL-6-N increased the severity of influenza A/WSN/33 virus-induced hypoxemia (Fig. 3C) and bradycardia (Fig. 3D) in HET mice but not WT controls at 6 d.p.i. IgG treatment had no effect on carotid arterial oxygen saturation or heart rate in either strain at this timepoint.

**Neutralization of TGF-β or IL-6 in HETs increases severity of influenza A virus-induced pulmonary edema to WT levels.** In the absence of treatment, lung water content (wet:dry weight) remained normal (comparable to uninfected values) in HET mice at 6 d.p.i. but was significantly elevated in WT controls (Fig. 4). Both TGF-N and IL-6-N significantly increased lung water content at 6 d.p.i. in HETs but not WT controls, resulting in equally severe pulmonary edema in both strains at this timepoint. IgG had no such effect.

**TGF-β and IL-6 are necessary to maintain normal lung function in influenza-infected HETs.** In the absence of treatment or after treatment with IgG, static and dynamic lung compliance were both significantly higher (and essentially
normal) in infected HETs at 6 d.p.i. (Fig. 5A and not shown, respectively). Likewise, total lung resistance was not higher in untreated or IgG-treated HETs than uninfected WT mice at 6 d.p.i., but was greatly increased in influenza A virus-infected WT controls at this timepoint (Fig. 5B). Protective effects of the HET genotype on static lung compliance, dynamic lung compliance, and airway resistance were completely abrogated by TGF-N and IL-6-N, which resulted in comparable values to WT mice.

**Neutralization of TGF-β or IL-6 does not impact leukocyte infiltration of the lung in response to influenza infection.** As in our earlier studies, BALF from influenza A/WSN/33 virus-infected HET mice contained far greater numbers of AMs than WT controls at 6 d.p.i. (Fig. 6A). However, BALF neutrophil counts did not differ between strains at this timepoint (Fig. 6B). Interestingly, IgG treatment, TGF-N, and IL-6-N had no effect on BALF AM and neutrophil numbers in either mouse strain at 6 d.p.i.

**Neutralization of TGF-β and IL-6 alter bronchoalveolar lavage fluid chemokine and cytokine responses to influenza infection.** We previously reported that BALF IFN-γ, IL-10, CCL-2/MCP-1, CCL-5/RANTES, and CXCL-10/IP-10 content did not differ between WT and HET mice at 6 d.p.i. (1). However, HET BALF contained larger amounts of the neutrophil chemoattractant CXCL-1/KC. In the current study, we also found similar levels of IFN-γ, IL-10, CCL-2/MCP-1, and CCL-5/RANTES in untreated infected animals from both strains at 6 d.p.i. (not shown). However, CXCL-1/KC levels did not differ between untreated HETs and WT controls in these experiments. Interestingly, we also
found that BALF IL-12 content (which we had not previously measured) was almost 4-fold higher in untreated HETs at 6 d.p.i.

TGF-N and IL-6-N did not alter BALF IL-10 and CCL-2/MCP-1 levels at 6 d.p.i. in either WT mice or HETs (not shown). However, both treatments significantly reduced BALF CXCL-1/KC content in both mouse strains. In WT mice, both TGF-N and IL-6-N also significantly reduced BALF IFN-γ, but increased IL-12 at 6 d.p.i. TGF-N, but not IL-6-N also reduced BALF CCL-5/RANTES in WT mice at this timepoint. In contrast, neither treatment impacted BALF IFN-γ or CCL-5/RANTES in HET mice, and only IL-6-N significantly increased BALF IL-12 at 6 d.p.i. Treatment with non-specific IgG had no effect on CXCL-1/KC, IFN-γ, IL-12, or CCL-5/RANTES in either mouse strain.

**Both stromal and myeloid cells from HET mice are necessary for protection from ALI and exaggerated secretion of IL-6.** We previously demonstrated that HET AMs were necessary for protection from influenza A virus-induced ALI in HET mice (1). To determine the extent to which interactions between AMs and respiratory epithelial cells were necessary for induction of IL-6 production at high levels by HET AMs at 6 d.p.i., we performed reciprocal bone marrow transfers between WT and HET mice. We found that, relative to WT controls, transfer of WT bone marrow to WT mice had no effect on lung water content (Fig. 7A), static lung compliance (Fig. 7B), dynamic lung compliance (not shown), and total lung resistance (not shown). WT to WT bone marrow transfer also had no effect on BALF IL-6 content (Fig. 7C) or viral replication (Fig. 7D). These data indicate that the irradiation and bone marrow transfer procedures had no effect on the
response of WT mice to influenza A virus infection. In contrast, both transfer of WT bone marrow to HET mice and transfer of HET bone marrow to WT mice increased lung water content to WT levels. This effect was accompanied by a reduction in BALF IL-6 to WT levels. Viral replication did not differ between groups, with the exception of HET recipients of WT bone marrow, whose lung homogenate viral titers were approximately 1 log higher than WT controls. Due to limited availability of HET mice, HET to HET control bone marrow transfer experiments were not performed.

2.5 Discussion

Despite a 50% reduction in cell-surface CFTR expression and anion transport (20), we and others have reported that uninfected HETs have normal lung function and exhibit no immune abnormalities (1; 13). However, we have demonstrated that, unlike WT controls, influenza A virus-infected HET mice did not develop ALI, despite comparable viral replication kinetics and weight loss in both strains (1). This protective phenotype was associated with exaggerated AM and IL-6 responses at 6 d.p.i. In the current study, we extended these observations by investigating the role of IL-6 in protection from ALI in infected HET mice. We found that AMs were a major source of IL-6 in HETs at 6 d.p.i. Infection also induced TGF-β secretion by HET but not WT mice at 2 d.p.i. Importantly, treatment with a single dose of a TGF-β-neutralizing antibody at 2 d.p.i. significantly reduced BALF IL-6 in HETs at 6 d.p.i. This indicated that IL-6 production by HET AMs at this timepoint was TGF-β-dependent. Neither TGF-N
nor treatment with a single dose of a neutralizing antibody against IL-6 at 4 d.p.i. significantly altered the rate of post-infection weight loss or the magnitude and kinetics of viral replication in either mouse strain. In contrast, both treatments increased the severity of hypoxemia, pulmonary edema, and lung dysfunction in HETs to WT levels. TGF-N and IL-6-N did not affect BALF AM and neutrophil numbers in either strain. However, both treatments attenuated the CXCL-1/KC response (which is important for neutrophil recruitment to the lung) in both mouse strains. TGF-N and IL-6-N also reduced IFN-γ production in WT mice. Finally, bone marrow transfer experiments showed that HET stromal and myeloid cells are both required for protection from ALI in HETs. Together, our data show that TGF-β-dependent production of IL-6 by AMs later in influenza infection prevents ALI development in HETs.

Previous investigators have reported that an attenuated AM response contributes to increased influenza severity (29; 59; 64). We showed previously that AMs were required for protection from ALI in infected HETs, which supports these earlier findings (1). We also showed that AM depletion reduced BALF IL-6 to WT levels at 6 d.p.i., which suggested that IL-6 production was AM-dependent in HETs. However, because IL-6 can be produced by multiple cells, these data did not definitively indicate whether macrophages were the actual source of IL-6 or simply necessary for induction of IL-6 production by other cells in the distal lung. Likewise, we could not determine whether AMs, IL-6, or both were necessary to prevent development of ALI in HETs. In the current study, we found that TGF-N and IL-6-N did not change BALF AM and neutrophil levels in HETs at 6 d.p.i., yet
both treatments reduced BALF IL-6 and increased the severity of ALI to WT levels at this timepoint. Importantly, TGF-N and IL-6-N did not alter ALI severity in WT mice at 6 d.p.i., which indicates that the effects of cytokine neutralization were specific. Taken together, these data indicate that, while increased AM recruitment to the lungs of HET mice is not TGF-β- or IL-6-dependent, AMs are necessary for protection from development of ALI in HETs because they release large amounts of IL-6 at 6 d.p.i. in response to TGF-β produced at 2 d.p.i. However, because bone marrow transfer experiments show a role for stromal cells in protection from ALI in HETs, we cannot exclude an additional role for alveolar epithelial cells in IL-6 production.

We found no differences in viral replication kinetics and BALF neutrophil counts between WT mice, untreated or IgG-treated HETs, and antibody-treated HETs at 6 d.p.i. This indicates that, in our model, influenza-induced ALI is independent of these factors. Moreover, we did not find that development of ALI in HETs following TGF-N and IL-6-N was associated with development of a so-called “cytokine storm” (10; 16; 33; 62). For instance, both TGF-N and IL-6-N decreased BALF IFN-γ in WT mice, yet neither treatment had any effect on ALI severity in this mouse strain. In contrast, both TGF-N and IL-6-N induced ALI in HET mice without altering BALF IFN-γ content.

Some investigators have reported an association between high BALF IL-6 and increased influenza mortality (37; 39; 56). However, others have found that disease severity is not reduced in IL-6-knockout mice (43; 54; 58), or that IL-6 is protective (6; 17; 65). Our data support this latter conclusion, as do our earlier
studies which showed a correlation between increased BALF IL-6 at 6 d.p.i. and amelioration of ALI following treatment of influenza-infected WT mice with the *de novo* pyrimidine synthesis inhibitor A77-1726 (2). Hence, we find that increased IL-6 production is associated with protection from ALI in both genetic and therapeutic models. However, the specific cell type producing IL-6 was not defined in our earlier experiments. AMs have been shown to produce IL-6 in response to *in vitro* infection with highly-pathogenic influenza viruses (46). Likewise, AM production of IL-6 has been inferred from clodronate liposome-mediated AM depletion experiments (1; 39), although the underlying mechanisms by which AM production of IL-6 was induced were not investigated.

Greater influenza severity has also been linked to inadequate anti-inflammatory responses. TGF-β is a potent inflammatory regulator (55) and increased lung levels of active TGF-β have been linked to reduced influenza morbidity and mortality (7; 8; 19; 40). We found previously that attenuation of influenza A virus-induced ALI in WT mice treated with A77-1726 was temporally associated with elevated BALF TGF-β (2). Importantly, TGF-β has been shown to induce IL-6 (4; 21), which is consistent with our data. Unfortunately, however, because both TGF-N and IL-6-N resulted in comparable effects on the infected HET lung, we were unable to determine whether TGF-β can also contribute to ALI attenuation in influenza-infected HETs in an IL-6-independent fashion. Moreover, we have yet to define the cellular mechanism(s) by which a 50% reduction in CFTR expression and function in HETs leads to earlier TGF-β production after influenza
infection, although studies in cystic fibrosis patients suggest that reduced CFTR expression and/or function results in increased TGF-β production (24).

We have yet to determine the pathophysiologic processes that underlie protection from ALI by TGF-β and/or IL-6 in influenza. Alveolar type II respiratory epithelial cells are the main infection target and site of replication for influenza viruses in the distal lung (30; 61). In addition to synthesizing surfactant lipids and proteins, a primary function of these cells is to regulate the depth of the bronchoalveolar lining fluid. ENaC and CFTR channels expressed on the apical surface of bronchoalveolar epithelial cells play a central role in this process (5; 15; 38). Importantly, TGF-β can down-regulate epithelial CFTR expression and function (27; 28; 47; 52; 57). Moreover, IL-6 can stimulate ENaC activity (36). We have previously shown that influenza infection results in activation of A1-subtype adenosine receptors, which induces increased CFTR-mediated Cl⁻ secretion (68). Hence, we propose that ALI does not develop in influenza-infected HETs as a result of three factors. First, the capacity for CFTR-mediated Cl⁻ secretion by HET respiratory epithelial cells in response to A1-adenosine receptor activation is inherently reduced as a result of a 50% decrease in CFTR expression (20). Second, higher TGF-β production early in infection will further inhibit CFTR-mediated Cl⁻ secretion by HET respiratory epithelial cells and also induces IL-6 production by HET AMs. Third, higher levels of IL-6 in the HET lung at 6 d.p.i. will result in greater stimulation of ENaC-mediated Na⁺ absorption compared to WT mice. Together, these effects will result in reduced alveolar edema and improved lung function. However, we cannot yet exclude the possibility that IL-6 acts
upstream of some other protective mediator.

In conclusion, our findings indicate that IL-6 secreted by AMs in response to epithelial TGF-β prevents development of ALI in influenza-infected HETs. By extension, our data imply that inadequate AM, TGF-β, and/or IL-6 responses may contribute to the development of ALI following infection by highly-pathogenic influenza strains. These results indicate that CFTR and IL-6 are both important host determinants of influenza severity, and suggest that CFTR may have potential as a target for development of novel treatments for influenza-induced ALI.
Figure 2.1: HET AMs produce more IL-6 than WT AMs in response to influenza A virus infection. (A) Representative immunohistochemistry for IL-6 at 6 d.p.i. in formalin-fixed, paraffin-embedded lung tissue from a WT mouse intranasally infected with influenza A/WSN/33 (10,000 pfu/mouse; original objective lens magnification 40x); (B) Representative immuno-histochemistry for IL-6 in formalin-fixed, paraffin-embedded lung tissue from a HET mouse at 6 d.p.i. (original objective lens magnification 40x; inset shows tissue stained with non-specific polyclonal goat antibody); and (C) Fold-change in il-6 gene expression in AMs isolated from WT mice (n=11) and HET mice (n=13) at 6 d.p.i., relative to uninfected WT mice (n=4) and HET mice (n=3). WT: wild-type C57BL/6 mice. HET: C57BL/6-congenic F508del CFTR heterozygotes. **P<0.005, vs. WT mice. Data are presented as mean ± SEM.
Figure 2.2: The exaggerated IL-6 response of HETs to influenza infection is TGF-β-dependent. (A) Effect of influenza A/WSN/33 infection on bronchoalveolar lavage fluid (BALF) TGF-β in WT controls (n=10-14) and HETs (n=10-12); and (B) Effect of intraperitoneal treatment with nonspecific IgG (IgG; 100 μg/mouse), treatment with a neutralizing antibody to TGF-β at 2 d.p.i. (TGF-N; 100 μg/mouse), or treatment with a neutralizing antibody to IL-6 at 4 d.p.i. (IL-6-N; 0.5 μg/mouse) on BALF IL-6 in WT mice (n=8) and HETs (n=8) at 6 d.p.i. WT: wild-type C57BL/6 mice. HET: C57BL/6-congenic F508del CFTR heterozygotes. UNTx: untreated animals. #P<0.005, vs. WT mice at the same timepoint. **P<0.005, #P<0.001, vs. WT mice in the same treatment group. Data are presented as mean ± SEM.
Figure 2.3: Neutralization of TGF-β or IL-6 increases severity of cardiopulmonary dysfunction in influenza-infected HETs without significantly impacting viral replication. Effects of systemic treatment with nonspecific IgG (IgG), antibody-mediated neutralization of TGF-β at 2 d.p.i. (TGF-N), and antibody-mediated neutralization of IL-6 at 4 d.p.i. (IL-6-N) on: (A) Body weight (BWT; % change from day 0; n>10 per group); (B) Viral titers in lung homogenates at 6 d.p.i. (log pfu/g; n=5-8 per group); (C) Carotid arterial oxygen saturation (SaO₂) at 6 d.p.i. (n>10 per group); and (D) Heart rate at 6 d.p.i. (n>10 per group). WT: wild-type C57BL/6 mice. HET: C57BL/6-congenic F508del CFTR heterozygotes. UNTx: untreated animals. Dotted line indicates mean value for each parameter in uninfected WT mice. *P<0.05, **P<0.005, #P<0.001, vs. WT mice in the same treatment group. Data are presented as mean ± SEM.
Figure 2.4: Neutralization of TGF-β or IL-6 in HETs increases severity of influenza A virus-induced pulmonary edema to WT levels. Effects of systemic treatment with nonspecific IgG (IgG), antibody-mediated neutralization of TGF-β at 2 d.p.i. (TGF-N), and antibody-mediated neutralization of IL-6 at 4 d.p.i. (IL-6-N) on lung water content (wet:dry weight ratio) at 6 d.p.i. WT: wild-type C57BL/6 mice. HET: C57BL/6-congenic F508del CFTR heterozygotes. UNTx: untreated animals. Dotted line indicates mean value for each parameter in uninfected WT mice. n≥8 per group. *P<0.05, #P<0.001, vs. WT mice in the same treatment group. Data are presented as mean ± SEM.

Figure 2.5: TGF-β and IL-6 are necessary to maintain normal lung function in influenza-infected HETs. Effects of systemic treatment with nonspecific IgG (IgG), antibody-mediated neutralization of TGF-β at 2 d.p.i. (TGF-N), and antibody-mediated neutralization of IL-6 at 4 d.p.i. (IL-6-N) on: (A) Static lung compliance (C_{ST}; ml/cmH₂O, x 10) at 6 d.p.i.; and (B) Baseline total lung resistance (R_{BASAL}; cmH₂O.s/ml) at 6 d.p.i. WT: wild-type C57BL/6 mice. HET: C57BL/6-congenic F508del CFTR heterozygotes. UNTx: untreated animals. Dotted line indicates mean value for each parameter in uninfected WT mice. n=6-8 per group. #P<0.001, vs. WT mice in the same treatment group. Data are presented as mean ± SEM.
Figure 2.6: Neutralization of TGF-β or IL-6 does not impact leukocyte infiltration of the lung in response to influenza infection. Effects of systemic treatment with nonspecific IgG (IgG), antibody-mediated neutralization of TGF-β at 2 d.p.i. (TGF-N), and antibody-mediated neutralization of IL-6 at 4 d.p.i. (IL-6-N) on: (A) BALF alveolar macrophage counts (AMs; x 10^6) at 6 d.p.i.; and (B) BALF neutrophil counts (PMNs; x 10^6) at 6 d.p.i. WT: wild-type C57BL/6 mice. HET: C57BL/6-congenic F508del CFTR heterozygotes. UNTx: untreated animals. n=5-7 per group. **P<0.005, #P<0.001, vs. WT mice in the same treatment group. Data are presented as mean ± SEM.
Figure 2.7: Both stromal and myeloid cells from HET mice are necessary for protection from ALI and exaggerated secretion of IL-6. Effects of reciprocal bone marrow transfer on: (A) Lung water content (wet: dry weight ratio) at 6 d.p.i.; (B) Static lung compliance ($C_{ST}$; ml/cmH$_2$O, x 10) at 6 d.p.i.; (C) BALF IL-6 (ng/ml) at 6 d.p.i.; and (D) Viral titers in lung homogenates at 6 d.p.i. (log pfu/g). WT: wild-type C57BL/6 mice. HET: C57BL/6-congenic F508del CFTR heterozygotes. WT TO WT: transfer of WT donor bone marrow to WT recipient mice. WT TO HET: transfer of WT donor bone marrow to HET recipient mice. HET TO WT: transfer of HET donor bone marrow to WT recipient mice. Dotted line indicates mean value for each parameter in uninfected WT mice. $n=5$-7 per group. **$P<0.005$, #$P<0.001$, vs. WT mice. Data are presented as mean ± SEM.
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Chapter 3: **Heterozygosity for the F508del mutation in CFTR promotes alternative macrophage activation in mice following influenza challenge**

3.1 Abstract

Influenza A viruses cause a highly contagious respiratory disease that poses a significant risk to public health. Severe primary influenza infection can result in development of pulmonary edema and hypoxemia: key features of acute lung injury (ALI). We have shown that influenza-induced ALI in C57BL/6 (WT) mice is associated with increased bronchoalveolar epithelial cell Cl- secretion via the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel. C57BL/6-congenic mice that are heterozygous for the F508del mutation in CFTR (HET mice) exhibit a 50% reduction in both CFTR expression and CFTR-mediated Cl- transport. We subsequently showed that HET mice did not develop ALI following influenza A virus infection. Consequently, we hypothesized that: 1) HET AMs undergo “alternative” activation (M2 polarization) in response to infection; and 2) this M2 polarized AM response is critical to amelioration of influenza-induced ALI in this mouse strain. Compared to WT AMs, Arginase 1 (Arg1) protein expression was higher in HET AMs at 6 d.p.i. HET AMs also expressed less inducible nitric oxide synthase (iNOS) protein at this timepoint, which is characteristic of a M2 phenotype. Additionally, Arg1 enzymatic activity
was higher in HET AM lysates. BALF from HET mice contained higher levels of urea and lower levels of nitrate/nitrite (end-products of Arg1 and iNOS metabolism, respectively) than WT BALF at 6 d.p.i. Lower levels of oxidized protein were also evident in HET BALF suggesting minimal production of reactive oxygen species by HET AMs. Interestingly, there were no observed differences in secondary markers used to define macrophage polarization states. These findings suggest that the anti-inflammatory nature of HET AMs contributes to attenuation of influenza-induced ALI in HET mice. Hence, we propose that, by promoting M2 polarization of AMs, short-term inhibition with CFTR inhibitors may be a novel approach to preventing influenza-induced ALI.

3.2 Introduction
Influenza A viruses are readily transmissible respiratory pathogens that remain a significant threat to human health. Influenza is the second leading cause of community-acquired pneumonia hospitalizations in the United States (13). In 2009, the emergence of the H1N1 “swine flu” influenza A virus was responsible for an estimated 500,000 deaths worldwide. Moreover, the vast majority of deaths during the “swine flu” pandemic were reported in people 18-64 years of age (7). These findings are of great concern considering 80-90% of seasonal influenza deaths are typically observed in people 65 years of age and older. Novel avian influenza strains, such as H5N1 and H7N9, also pose a great risk to the general population in that they are highly pathogenic and have unusually high mortality rates (24; 27).
Vaccines and antiviral therapy, specifically neuraminidase inhibitors, are the current mainstays of influenza prophylaxis and therapy, respectively. While vaccines are valuable in preventing influenza infection, their efficacy is often limited by difficulties in the vaccine development process and issues with efficient uptake by the recipient (22; 23). Neuraminidase inhibitors rely on early administration in order to alleviate severe influenza symptoms and prevent excess viral shedding. Furthermore, recent reports suggest that most antiviral influenza drugs only serve to relieve minor symptoms of influenza infection, and thus fail to provide the appropriate therapeutic benefit in life-threatening situations (5; 9).

In severe cases, uncontrolled influenza infection can rapidly progress into the lower respiratory tract leading to acute lung injury (ALI) and life-threatening respiratory failure (25). Influenza-induced ALI occurs in 20% of severe influenza cases, and associated with poor outcomes. Unfortunately, treatment for influenza-induced ALI is limited to non-specific supportive care in the intensive care unit. Hence, there is great need for the development of novel therapeutics that can treat late-stage influenza infections, but to due so requires a better understanding for the fundamental mechanisms underlying influenza-induced ALI.

Alveolar Macrophages (AMs) play a critical role in the clearance of pathogens within the lung. Upon influenza infection, AMs become classically-activated (M1-polarized), and produce various inflammatory mediators and reactive oxygen species (ROS) that, if left unchecked, can contribute significantly to ALI and
mortality (16; 17). An M1 macrophage is universally characterized by high inducible nitric oxide (iNOS) expression and enhanced phagocytic activity (12). (30). Alternatively-activated macrophages (M2-polarized) are phenotyped based on high expression of arginase 1 (Arg1): an enzyme that competes with iNOS for the same substrate (arginine), but metabolizes arginine into immunologically inert products (12). Although activated AMs have been tied to the development of lung pathologies following influenza infection, various studies have shown that AM depletion enhances respiratory epithelial damage and mortality (15; 21; 29). Additionally, pretreating murine lungs with S. aureus attenuates influenza-induced ALI by shifting AMs toward an M2-polarized phenotype. Thus, alveolar macrophages, in the appropriate context, are essential in reducing influenza disease severity.

We previously demonstrated that influenza-induced ALI was highly-attenuated in C57BL/6-congenic mice heterozygous for the F508del mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel (HET mice) (2). This effect was associated with an exaggerated AM response late in infection, while neutrophil and lymphocyte responses remained comparable between the two strains. Additionally, clodronate liposome depletion of AMs abrogated the beneficial phenotype observed in HET mice, yet had no effect on WT mice, indicating that the observed reductions inflammation are AM-dependent. Given these observations, the goal of this investigation was to determine how altered CFTR expression/activity promotes a favorable AM phenotype following influenza infection.
3.3 Materials and Methods

**Animals.** Pathogen-free C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6-congenic mice heterozygous for the F508del mutation in CFTR were bred in-house from mice generously provided by Dr. Amal Amer (The Ohio State University) (34).

**Preparation of viral inoculum.** Mouse-adapted influenza A/WSN/33 was prepared by standard protocol in embryonated chicken eggs. Viral titers were obtained via routine plaque assay and stocks were tested for *Mycoplasma pulmonis* (MycoAlert™ Mycoplasma Detection Kit; Lonza; Basel, Switzerland) and endotoxin (Limulus amebocyte assay; Lonza; contamination.

**Mouse inoculation.** 8-12 week old mice of either genotype were anesthetized by intraperitoneal injection of ketamine (8.7 mg/kg)/xylazine (1.3 mg/kg) and infected intranasally with 10,000 PFU/mouse of influenza A/WSN/33 H1N1 50 μl PBS/0.1% BSA (3; 28). Mice were individually marked and weighed daily to ensure successful infection.

**Alveolar Macrophage (AM) Isolation.** AMs were isolated by adherence to polystyrene. In brief, mice were euthanized and their trachea cannulated. AMs were collected by injecting 1ml of sterile saline into lungs and subsequently aspirating the saline into a collecting vessel. This process was repeated 10 times per mouse, and lavages from 2 mice were used to obtain an N of 1. The lavage cells were then pelleted, resuspended in 2ml of RMPI media, and plated on polystyrene. AMs were given 2 hours to adhere. After appropriate adhesion,
plates were washed with warm PBS to remove non-adherent cells (non-AMs and dead cells), and the purified AMs were processed for downstream applications.

**Western Blot.** AMs were scraped directly into Cell Lysis buffer (Cell Signaling Technology (CTS), Danvers, MA) following adherence to polystyrene. Lysates were centrifuged at high speed to clear cellular debris and protein concentration was determined using Pierce’s BCA Protein Assay. Equal amounts of protein were loaded onto a 4-12% polyacrylamide gel and subjected to SDS-PAGE. Western Blotting was performed by a standard protocol. iNOS (CTS #2982) and GAPDH (sc-25778) were detected using rabbit polyclonal antibodies. Arg1 was detected using a goat polyclonal antibody (ab118884; Abcam, San Francisco, CA). Primary antibodies were exposed to the appropriate HRP-conjugated secondary antibody (anti-rabbit IgG CTS #7074 and anti-goat IgG Millipore) and immunoreactive bands were visualized using ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL). Densitometry was performed on scanned film images using ImageJ software. Data were normalized to GAPDH.

**Quantitative realtime-PCR.** Freshly isolated AMs were lysed in Buffer RLT and processed for RNA isolation using RNeasy spin column technology (Qiagen). Quality and concentration of RNA was assessed via spectrophotometry (Nanodrop2000). Equal amounts of total RNA was converted to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY, USA) The TaqMan Gene Expression system (Applied Biosystems) was utilized for qRT-PCR analysis of cDNA. *arg1* and *nos2* expression was calculated by the ΔΔCt method and normalized to the endogenous control *18S rRNA*. 

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Colormetric Assays. The initial 1ml saline lavage was collected for bronchoalveolar lavage fluid (BALF) analysis, and commercially available kits were used to quantify BALF urea (Bioassay Systems DIUR-500), nitrate/nitrite (Cayman Chemical 78001), and protein carbonyl content (Cayman Chemical 10005020). Arginase enzymatic activity was assessed in freshly isolated AMs using an assay kit produced by BioVision (K755-100).

Statistical analyses. Descriptive statistics (mean and standard error) were calculated using Instat software (GraphPad, San Diego, CA). Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. An unpaired Student’s t-test was used when comparing 2 groups. Statistical analyses of datasets containing more than 2 groups were made by ANOVA, with a post hoc Tukey-Kramer multiple comparison post-test. All data are presented as mean ± S.E.M. P<0.05 was considered statistically significant. Data undergoing statistical analysis was derived from no less than 2 separate infection groups.

3.4 Results

AM iNOS and Arg1 protein and gene expression differs between WT and HET mice following influenza A infection. We had previously demonstrated that HET AMs were essential in reducing influenza-induced lung pathology. Thus, we assessed the polarization status of HET AMs following influenza challenge to determine if these AMs manifested anti-inflammatory properties. AMs isolated from HET mice at 6 days post infection (d.p.i.) with influenza A/WSN/33 (10,000 pfu/mouse) had 19-fold lower levels of inos protein relative to
WT AMs at the same timepoint. In contrast, Arg1 protein expression was 5-fold higher in HET AMs at 6 d.p.i. (Fig. 3.1A and 3.1B). In agreement with the protein expression data, nos2 gene expression was significantly lower in HET AMs compared to WT AMs at the same timepoint. There was no difference in AM arg1 gene expression between the two groups (Fig. 3.1C). Taken together, the expression profile of HET AMs is indicative of an M2 polarized phenotype.

**AMs from influenza-infected HET mice exhibit higher Arg1 enzymatic activity.** Given the observed expression profile of Arg1 and iNOS in HET AMs, we next assessed the AM metabolism of arginine via Arg1 enzymatic assays. Arg1 activity was roughly two-fold higher in AMs obtained from HET mice infected with influenza (fig. 3.2A). Additionally, HET BALF contained significantly more urea (end-product of Arg1 metabolism) and significantly less nitrate/nitrite (end products of iNOS metabolism) than WT BALF (Fig.3. 2B). This suggests that Arg1 metabolism of arginine predominates over iNOS metabolism in HET AMs leading to reduced ROS generation.

**Reduced Oxidative Stress is observed HET BALF following influenza A Infection.** M1 macrophages generate highly reactive ROS that can oxidize and damage proteins, lipids, and DNA. The most universally accepted and general indicator of protein oxidation is protein carbonyl content (6). BALF fluid collected from HET mice contained significantly lower protein carbonyl content than WT BALF following influenza A infection (Fig.3.3); indicating reduced ROS damage within the HET respiratory tract.
No significant differences are observed in secondary macrophage polarization markers. Macrophages polarization phenotypes can be described by a plethora of secondary markers beyond Arg1 and iNOS (19). Thus, qRT-PCR was carried out to assess gene expression of the secondary M1 marker, cd86, and the secondary M2 Markers, chitinase-3-like protein 3 (chi3l3/ym1) and resistin-like molecule alpha1 (retnla/fizz1). Interestingly, there were no differences in secondary polarization marker gene expression between HET and WT AMs (Fig 3.4).

AMs from uninfected HETs display intrinsic differences in Arg1 protein expression and NOx levels. Arg1 protein expression was significantly higher in AMs collected from uninfected HETs compared to their WT counterparts (iNOS was undetectable in AMs isolated from uninfected mice of either strain) (Fig. 3.5A and 3.5B). arg1 gene expression was slightly higher in HET AMs, but the difference was not statistically significant (Fig. 3.5C). Furthermore, there were no differences in AM Arg1 enzymatic activity or BALF urea content between to two strains. In contrast, baseline BALF nitrate/nitrite levels were significantly lower in uninfected HET mice (Fig. 3.5D and 3.5E).

3.5 Discussion

Although therapeutic options exist to combat infection, influenza still remains a leading cause of attributable annual mortality the United States (10). Thus, we must develop a more comprehensive understanding of the basic mechanisms underlying the pathogenesis of influenza-induced lung injury. We have previously
shown that HET mice fail to develop ALI compared to WT controls (2). This phenomenon was independent of viral replication and weight loss. However, this protective effect was associated with a robust macrophage response late in the infection time course. In this current study, we expanded our observations by characterizing the functional phenotype of HET AMs. We found that HET AMs at 6 d.p.i. express higher levels of Arg1 protein and lower levels of iNOS protein compared to WT control AMs. This protein profile is highly reflective of an anti-inflammatory (M2) phenotype. *nos2* gene expression was lower in HET AMs than in their WT counterparts at 6 d.p.i., but no differences in *arg1* gene expression were observed. Moreover, at 6 d.p.i., HETs possessed higher AM Arg1 activity, more BALF urea, and less BALF NOx, all of which are indicative of an M2-polarized macrophage. Less oxidative stress was observed in HET BALF protein, yet there were no gene expression differences in secondary macrophage polarization markers. Interestingly, uninfected HET AMs expressed higher Arg1 protein, and uninfected HET BALF contained lower levels of NOx. Taken together, these data suggest that M2 polarization, specifically alterations in arginine metabolism leading to reduced ROS production, aides in the attenuation of influenza-induced ALI in HETs.

Several studies have highlighted the protective effects of AMs in reducing influenza disease severity (15; 21; 29). As stated earlier, we have shown that AMs are central to the protective effects observed in HETs following influenza challenge. However, our earlier studies only identified differences in the magnitude of the AM response, and thus failed to describe the AM phenotypic
characteristics responsible for attenuating ALI. In the present study, we found that HET AMs exhibit an alternatively-activated, anti-inflammatory phenotype in response to influenza. HET AMs exhibited higher Arg1 protein expression and activity while maintaining lower iNOS/nos2 gene expression levels. Interestingly, there was no correlation between Arg1 protein and gene expression in HET or WT AMs. This suggests that Arg1 protein levels are regulated at the post-transcriptional level. This becomes apparent upon western blot analysis where a second, higher molecular weight Arg1 band predominates in the HET AMs at 6 d.p.i. Researchers have identified potential alternate translation initiation sites in the arg1 transcript that offer a possible explanation for the observed phenomenon (11). Likewise, the higher molecular weight Arg1 band, while more highly expressed in uninfected HET AMs compared to WT controls, was poorly expressed prior to influenza challenge. This suggests that that the lower molecular weight Arg1 isoform predomnates under steady-state conditions, while translation of the higher molecular weight isoform protein is induced in response to external stimuli such as influenza. Apparently, the stimulus must be robust and prolonged given that there was little evidence of the higher molecular weight Arg1 isoform in AMs at 2 d.p.i. (data not shown).

We found no differences in gene expression of cd86 or chi3l3/ym1 and retnla/fizz1, secondary M1 and M2 polarization markers, respectively. While these findings are perplexing, they are not entirely unprecedented. M1 macrophage polarization has been thoroughly characterized and is defined by a narrower set of parameters than M2 macrophages. For instance, M2
macrophages can be further divided into 4 different subclasses, each expressing various secondary markers and maintaining differential cytokine secretion profiles (20). On the other hand, high Arg1 and low iNOS expression remain conserved across all M2 subclasses. This is a critical observation in that arginine metabolism provides a more functional description for macrophage polarization in that relative ratios of Arg1 and iNOS determine ROS output (1; 18; 33). Thus, M2-polarized macrophages with high Arg1 and low iNOS levels produce lower amounts of ROS, which makes them more anti-inflammatory/immunologically inert in nature.

Previous studies have demonstrated that iNOS inhibition and/or ablation is protective within the context of influenza-induced ALI (14; 26). However, these studies took a global approach rather than one that was macrophage-specific. Given our data and that macrophages are one of the most potent producers of ROS, it is likely that targeted inhibition/ablation of iNOS solely in macrophages may provide the most therapeutic benefit in attenuating ALI. More recently, a group found that priming the murine lung with *Staphylococcus aureus* shifted macrophages toward an M2 polarized state, which in turn significantly attenuated influenza-mediated lung immune injury (30). Moreover, several studies have demonstrated that inhibiting airway infiltration of non-resident macrophage populations, which are typically M1 in nature, reduces influenza-induced pulmonary pathology (8; 16; 17). Taken together, these studies, along with our own, highlight beneficial effects of M2 macrophage polarization within context of influenza-induced ALI.
We have yet to determine the mechanism by which F508del mutation in CFTR promotes M2 polarization of murine AMs following influenza challenge. Heterozygosity for the F508del mutation not only results in a 50% reduction in CFTR-mediated Cl⁻ transport and total cell surface CFTR expression, but also may lead to increased proteasomal degradation of nascent CFTR protein resulting in induction of ER stress (4; 31; 32). Thus, F508del mutation offers three plausible mechanisms that may be inducing M2 polarization. This investigation is outside the scope of our current study, but will be an area of interest to our lab in the future.

In summary, our findings demonstrate that HET AMs are M2-polarized, which aids in the attenuation of influenza-induced ALI. By extension, this suggests that macrophages exhibiting M2 properties can reduce influenza-induced pulmonary pathology that is often attributed to the “cytokine storm.” These results imply that dampening the ROS response through direct manipulation of CFTR, iNOS, or Arg1 may offer potential therapeutic benefit in reducing influenza-induced ALI.
3.6 Figures

Figure 3.1: AM iNOS and Arg1 protein and gene expression differs between WT and HET mice following influenza A infection.

Effects of influenza infection on iNOS and Arg1 protein/gene expression at 6 d.p.i. (A) Two representative Western blots evaluating Arg1 and iNOS protein expression in AMs from a total of 3 WT and 3 HET mice at 6 d.p.i. Probing for GAPDH demonstrated comparable loading conditions between groups; (B) Relative protein expression of Arg1 and iNOS is normalized to GAPDH; (C) Fold-change in nos2 and arg1 gene expression in AMs isolated from WT and HET mice at 6 d.p.i.; nos2 and arg1 expression is normalized to AMs isolated from uninfected mice* P<0.05; **P<0.005; #P<0.001 vs. WT mice the same timepoint. Data are presented as means ± SEM.
Figure 3.2: AMs from influenza-infected HET mice exhibit higher Arg1 enzymatic activity.

Effects of influenza infection on: (A) Arg1 activity in AMs collected from WT (n=6) and HET (n=7) mice at 6 d.p.i.; and (B) NOx (n=5) and Urea levels (n=5) in BALF collected from WT and HET mice at 6 d.p.i. *P<0.05; **P<0.005; #P<0.001 vs. WT mice the same timepoint. Data are presented as means ± SEM.
Figure 3.3: Reduced Oxidative Stress is observed HET BALF following influenza A Infection.
Effects of influenza infection on BALF protein carbonyl content at 6d.p.i. in WT controls (n=8) and HET (n=9). **P<0.005 vs. WT mice at same the same timepoint. Data are presented as means ± SEM.

Figure 3.4: No significant differences are observed in secondary macrophage polarization markers.
Effects of influenza infection on fold-change in cd86, chitinase-3-like protein 3 (chi3l3/ym1) and resistin-like molecule alpha1 (retnla/fizz1) gene expression in AMs from WT mice (n=6) and HET mice (n=5) at 6 d.p.i. relative to uninfected WT (n=3) and HET AMs(n=3). Data are presented as means ± SEM.
Figure 3.5: AMs from uninfected HETs display intrinsic differences in Arg1 protein expression and NOx levels.

(A) Western blot analysis of Arg1 in uninfected AMs from 3 WT and 3 HET mice. Probing for GAPDH demonstrated comparable loading conditions between groups; (B) Relative expression of Arg1 normalized to GAPDH; (C) Fold-change in arg1 gene expression in uninfected WT (n=5) and HET (n=5) AMs; (D) Arg1 activity in AMs from uninfected WT (n=3) and HET (n=3) mice; (E) NOx (n=14-15) and Urea levels (n=9-10) in BALF collected from uninfected WT and HET mice. **P<0.005; #P<0.001 vs. WT mice the same timepoint. Data are presented as means ± SEM.
3.7 References


Chapter 4: Reduced CFTR protein expression, not reduced CFTR-mediated Cl⁻ secretion or ER Stress, protects against Influenza-induced lung injury

4.1 Abstract

Seasonal Influenza A infections result in significant hospitalizations and deaths annually. Moreover, the emergence of a novel, pandemic influenza strain could have devastating consequences. Influenza infection stimulates the hypersecretion of Cl⁻ via the cystic fibrosis transmembrane conductance regulator (CFTR) channel, and this excess Cl⁻ accumulation at the air-liquid interface contributes to influenza-induced acute lung injury (ALI). Influenza-induced ALI is highly-attenuated in C57BL/6-congenic mice heterozygous for the F508del CFTR mutation (F508del HET mice). These mice exhibit a 50% reduction in CFTR channel expression and CFTR-mediated Cl⁻ secretion, but are otherwise normal upon physiological evaluation. It has also been reported that the F508del mutation in CFTR results in endoplasmic reticulum (ER stress) due to increased proteasomal degradation. To understand the exact mechanism by which mutations in CFTR aid in the attenuation of ALI, we used mice that are heterozygous for 2 additional CFTR mutations. Cftr<sup>tm1CAM</sup> (null) HETs maintain a 50% reduction in normal CFTR expression, while heterozygotes for the Cftr<sup>tm1G551D</sup> (G551D) mutation express 50% normal CFTR and 50% G551D missense mutant CFTR protein that lacks Cl⁻ transport capacity. As in F508del
HETs, Cl- transport is reduced by 50% in both strains. However, neither mutation results in induction of ER stress resulting from mis-folding of F508del mutant CFTR. Thus, from these mice we were able to determine whether reduced CFTR-mediated Cl- transport, reduced total cell surface CFTR expression, or increased proteasomal degradation of nascent CFTR protein resulting in induction of ER stress impedes influenza-induced ALI. Interestingly, hypoxemia, pulmonary edema, and airway resistance was attenuated in the F508del and null HET mice. Influenza-induced ALI was not attenuated in G551D HETs, which were more comparable to WT controls. Moreover, there was no correlation in bronchoalveolar lavage fluid (BALF) leukocyte counts and protective phenotype between strains. These data suggest that reduced CFTR protein expression, not reduced CFTR-mediated Cl- secretion or ER Stress, is protective following influenza challenge.

4.2 Introduction

Influenza A viruses remain a threat to public health in that they are highly contagious and can cause a severe, acute respiratory illness. Despite access to antiviral drugs and vaccines, influenza-related disease accounts for approximately 200,000 hospitalizations and more than 36,000 excess deaths per year in the United States (U.S.) alone (11). Furthermore, the 2009 H1N1 “swine influenza” pandemic resulted in the infection of roughly 61 million individuals in the U.S. (10). While not particularly lethal compared to other pandemics in recent history, the 2009 pandemic demonstrated the ease and swiftness in which
influenza can spread in our global society (23; 24). Future emergence of a novel, highly-pathogenic influenza strains could yield a pandemic with devastating consequences. This was the case with the 1918 “Spanish influenza” pandemic where an estimated 50 million individuals died (16; 25). Most recently, public health officials have become concerned with sporadic human infections with highly pathogenic bird influenza strains, such as H5N1. While incidence remains low, these influenza infections have a mortality rate greater than 60% (29). If these highly pathogenic bird influenza strains were to acquire mutations leading to increased infectivity in humans, then a global health crisis would emerge.

Severe, primary influenza pneumonia can progress to acute lung injury (ALI). Approximately 20% of patients hospitalized with severe influenza infection develop ALI (17). Other than supportive care in the intensive care unit, no therapeutic options are available to directly treat ALI (22; 34). Thus, in order to develop new drugs that can fill this "therapeutic gap," we must first gain a better understanding of the pathophysiological mechanisms that contribute to influenza-induced ALI.

The alveolar epithelium is the primary site for influenza infection within the respiratory tract (13; 26). A seminal function of these epithelial cells is to maintain proper fluid balance at the air-liquid interface, which allows for proper gas exchange (7). This process is dependent on epithelial Na⁺ channel (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR) activity. ENaC inhibition and/or stimulation of CFTR-mediated Cl⁻ secretion leads to
accumulation of fluid in the alveolar space, impaired gas exchange, and hypoxemia (32).

We have shown that increased Cl⁻ secretion via CFTR contributes to pulmonary edema in mice following influenza challenge (35). Moreover, we reported that C57BL/6-congenic mice heterozygous for the F508del CFTR mutation (F508del HETs), which exhibit a 50% reduction in CFTR-mediated Cl⁻ secretion and CFTR channel expression, experience significant attenuation in influenza-induce ALI (2). These studies highlight CFTR's contribution to lung injury within the context of influenza infection.

Given the protective phenotype observed in F508del HETs, the aim of this study was to determine the mechanism by which alterations in CFTR aid in the attenuation of influenza-induced ALI. Heterozygosity for the F508del mutation not only results in a 50% reduction in CFTR-mediated Cl⁻ transport and total cell surface CFTR expression, but also may lead to increased proteasomal degradation of nascent CFTR protein resulting in induction of ER stress (3; 30; 31). To determine whether reduced CFTR-mediated Cl⁻ secretion, reduced CFTR channel expression, or enhanced ER stress dictate disease benefit, we utilized mice heterozygous for the Cftr^{tm1CAM} (null HETs) and Cftr^{tm1G551D} (G551D HETs) mutations in CFTR (9; 21). Null HETS exhibit a 50% reduction in CFTR channel expression, while G551D mice exhibit a 50% reduction in CFTR-mediated Cl⁻ transport. As in F508del HETs, Cl⁻ transport is reduced by 50% in both strains (See Table 1.1 for mutational overview). By comparing the three heterozygous CFTR mutants and WT mice following influenza challenge, we found that the
F508del and null HET mice experienced attenuated ALI. Influenza-infected F508del and null HET mice experienced attenuations in hypoxemia, pulmonary edema, and airway resistance compared to G551D HETs and WT controls. Interestingly, there was no correlation in disease severity with bronchoalveolar lavage fluid (BALF) leukocyte counts, lung viral titers, or weight loss between strains. These data suggest that reduced CFTR protein expression, not reduced CFTR-mediated Cl- secretion or ER Stress, is protective following influenza challenge.

4.3 Methods

Breeding and Genotyping of CFTR mutants mice. C57BL/6-congenic F508del CFTR heterozygous mutants and WT controls were generated by breeding B6.129S7-Cftrtm1Kth mice (37). C57BL/6-congenic G511D heterozygous mutants were generated by breeding Cftr tm1G551D mice. C57BL/6-congenic null heterozygous mutants were kindly donated by our colleague Dr. Estelle Cormet-Boyaka. All procedures were approved by the Institutional Animal Care and Use Committees at both The Ohio State University and the University of Alabama at Birmingham.

Infection of mice. Eight to 12 week-old mice of each genotype were infected intranasally with 10,000 focus-forming units (FFU)/mouse of egg-grown H1N1 influenza A/WSN/33 in 50 μl PBS with 0.1% BSA (12; 27). Mice were individually marked and weighed daily. Data for each experimental group were derived from a minimum of 2 independent infections.
Measurement of lung mechanics. Mechanical properties of the mouse lung were assessed in valium/ketamine-anesthetized, tracheotomized mice using the forced-oscillation technique (14) as in our previous studies (28). Mice were mechanically ventilated on a flexiVent computer-controlled piston ventilator (SciReq, Montreal, Canada), with 8 ml/kg tidal volume, at a frequency of 150 breaths/minute, against 2-3 cmH2O PEEP. Total lung resistance and static lung compliance at baseline were calculated using the single-compartment model.

Other methods. Measurements of carotid arterial O2 saturation, lung homogenate viral titers, and lung wet:dry weight ratios were performed as in our previous studies (1; 6; 36).

Statistical Analysis. Descriptive statistics were calculated using Instat 3.05 (GraphPad Software, San Diego, CA). Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. Differences between group means were analyzed by one-way ANOVA, with Tukey-Kramer multiple comparison post-tests. P<0.05 was considered statistically significant. All data are presented as mean ± SEM.

4.4 Results

Influenza-infected F508del and null HETs exhibit improved arterial O2 saturation. We have previously reported that F508del HET mice present with higher arterial O2 saturation (SaO2) compared to WT controls following influenza challenge. There were no observed differences in between mice strains at 0, 2, or 4 days post infection (d.p.i.) (data not shown). However, at 6 d.p.i., SaO2
remained relatively high in both F508del and null HETs, while G551D HETs and WT mice experienced severe hypoxemia (Fig. 4.1A). Viral titers and the rate of post infection weight loss did not differ between strains (Fig.4.1B and 4.1C).

**Detrimental effects of influenza infection on airway resistance and lung compliance are attenuated in F508del and null HETs.** In past studies, we have found F508del HETs experience limited perturbations in lung function following influenza infection. Baseline airway resistance to airflow did not differ between any strains of uninfected mice (data not shown). In WT mice, influenza challenge induces a progressive increase in total airway resistance throughout the infection time course. Increases in airway resistance were observed in all mice following influenza infection; however, this effect was attenuated in F508del and null HETs. In contrast, G511D HET mice experienced increases in airway resistance that were more comparable to WT mice (Fig. 4.2A).

Static lung compliance is measured at a fixed lung volume and serves as an indicator of lung stiffness to inflation on inspiration. Thus, it is a key parameter in assessing normal breathing dynamics. All mice strains experience decreases in static lung compliance following influenza challenge. Yet, this effect was attenuated in all Heterozygous CFTR Mutants. Interesting, static lung compliance was highest in F508del HETs. Null and G551D HETs experienced greater perturbations in static lung compliance compared to F508del HETs, but compliance readouts remained significantly higher than WT mice (4.2B).

**Heterozygosity for the F508del or null CFTR mutation prevents influenza-induced pulmonary edema.** Influenza infection in WT mice results in the
progress development of pulmonary edema. This can be evaluated via lung water content (wet:dry weight ratio). At 6 d.p.i., mean lung water content increased by 150% above baseline values in WT and G551D HETs. On the other hand, there were little to no pulmonary edema observed in F508del and Null HETs following influenza challenge (Fig. 4.3).

**BALF leukocyte counts differ between the various heterozygous CFTR Mutants.** Prior to infection, over 95% of the cells within the BALF of all the mice strains are alveolar macrophages (AMs), and total cell counts did not differ between strains (data no shown). Likewise, there were no significant differences in BALF AM or neutrophil counts at 2 d.p.i. in any strain (Fig 4.4). Conversely, BALF AM counts were significantly higher in F508del HET mice at 6 d.p.i. compared to all other strains. BALF AM counts were similar between WT and G551D HET mice at 6 d.p.i. Null HET mice had a lower number of AMs at 6 d.p.i, but this effect was not statistically-significant. Likewise, BALF neutrophil counts, while lacking in statistical significance, were lower null HETs compared to all other strains. BALF neutrophil counts were comparable in F508del HET, G551D HET, and WT mice at 6 d.p.i.

**4.5 Discussion**

F508del HET mice exhibit a 50% reduction in CFTR channel expression and Cl⁻ transport, yet these mice are experience no physiologically abnormalities under steady-state conditions (5). Some investigators have reported that F508del mutant CFTR undergoes increased proteasomal degradation of nascent CFTR
protein resulting in induction of ER stress (4; 30; 31). It is thought that this ER stress may alter cellular processes and enhance disease under certain immune stimuli (18), but this theory of an altered cellular state due to mutant CFTR has yet to be thoroughly proven (15; 33).

We have shown that, unlike WT control mice, F508del HET fail to develop severe influenza-induced ALI despite no differences in viral replication between strains (2). This protective phenotype, i.e. reductions in pulmonary edema and minimal cardiopulmonary dysfunction, was associated with an exaggerated macrophage response and differential BALF cytokine profile. In this current study, we aimed to tease out the underlying mechanism by which mutations in CFTR are involved in the attenuation of ALI in mice challenged with influenza. We did this by using mice heterozygous for 2 additional CFTR mutations: G551D HETs (50% reduction in CFTR anion secretion) and null HETs (50% reduction in CFTR channel expression). This allowed us to determine whether CFTR-mediated Cl- transport, reduced total cell surface CFTR expression, or increased proteasomal degradation of the CFTR protein resulting in induction of ER stress was responsible for the protective HET phenotype. We found that G551D HETs exhibited no significant attenuation in influenza-induced ALI and were comparable to WT controls in almost every readout. In contrast, influenza-infected F508del and null HET mice experienced minimal alterations in arterial O2 saturation, lung water content, and pulmonary function. No differences in viral replication kinetics were observed between any of the mouse strains in this study, and only F508del HET mice had significantly higher BALF AM counts.
Altogether, our data suggest that a 50% reduction in CFTR expression, not reductions in Cl⁻ secretion or increased ER stress, is responsible for the protective HET phenotype following influenza challenge.

There is evidence, although controversial, supporting various mechanism by which loss-of-function CFTR mutations alter lung processes. It has been reported that mutations in CFTR lead to cellular ion imbalances, altered submucosal gland fluid secretion, intrinsic hyperinflammation, and aberrant immune cell function, as well as other mechanisms. Considering that over 1000 mutations in CFTR have been identified to cause Cystic Fibrosis (CF) disease, these findings are not unexpected. Moreover, different mutations in CFTR lead to CF disease of varying severity (8; 19), and thus mutation-specific therapeutics have had the greatest success in treating the clinical illness (20).

On the other hand, some investigators have reported that that different mutations in CFTR result in similar functional consequences. For example, macrophages isolated from mice homozygous for F508del, G551D, or null mutations in CFTR exhibited reduced reactive oxygen species generation (38). This in vitro study was novel and highly mechanistic, but failed to determine if these CFTR mutations, albeit altering biochemical pathways in a single cell type, had any in vivo implications. In our current study, we found that different heterozygous mutations in CFTR offer varying levels of protection against influenza-induced ALI. Given that the G551D heterozygous mutation offered no protection against influenza-induced ALI, one may speculate that reduced Cl⁻ secretion does not play a role in reducing disease severity. Unlike G551D HETs, F508del and null
HETs both exhibit a 50% reduction in CFTR cell surface and a protective phenotype following influenza challenge. This suggests that reduced CFTR expression cell surface expression attenuates influenza-induced ALI.

In our previous studies, we found that the protective phenotype in F508del HETs was dependent upon an exaggerated macrophage response to influenza infection compared to WT controls (2; 36). Null HETs did not display a similar macrophage response following influenza challenge, but still experienced attenuation in disease severity. These findings are somewhat confounding in that a 50% reduction in CFTR cell surface expression offers a protective phenotype against influenza infection, but does not necessarily alter macrophages responses. As mentioned earlier, different CFTR mutations often lead to perturbations in different cellular pathways. Hence, although F508del and null HETs both experience reductions in CFTR expression, the genotypic manifestations of their CFTR mutations are quite different. Thus, it may be that these distinct mutations offer protection against influenza-induced ALI via entirely different cellular mechanisms.

In conclusion, our findings indicate that reduced CFTR-mediated Cl\(^-\) secretion does not offer protection against influenza-induced ALI. Instead, there appears to be a correlation between reduced CFTR protein expression and the attenuation of influenza disease. These findings suggest that CFTR is an important host determinant of influenza severity and provide a rationale for targeting CFTR in attempt to combat influenza-induced ALI.
4.6 Figures

**Figure 4.1: Influenza-infected F508del and Null HETs exhibit improved arterial O₂ saturation**

Effect of intranasal infection with H1N1 influenza A/WSN/33 (10,000FFU/mouse) on: (A) arterial O₂ saturation (SaO₂; n=10-15 per group) (B) body weight (BWT; % change from day 0; n=10-15 per group) (C) Log viral titers in Day 6 lung homogenates (log FFU/g; n=2-10 per group). WT: wild-type C57BL/6. F508del HET: mice heterozygous for the F508del CFTR mutation. Null HET: mice heterozygous for the null CFTR mutation. G551D HET: mice heterozygous for the G551D CFTR mutation. *P<0.05; **P<0.005; #P<0.001 vs. all mice at the same timepoint. Data are presented as means ± SEM.
Figure 4.2: Detrimental effects of influenza infection on airway resistance and lung compliance are attenuated in F508del and Null HETs

Effect of influenza infection at 6 d.p.i. on: (A) Baseline total lung resistance ($R_{\text{BASAL}}$; cmH$_2$O.s/ml; n = 6-10 per group); and (B) Static lung compliance ($C_{\text{ST}}$; ml/cmH$_2$O x 10; n = 6-10 per group). WT: wild-type C57BL/6. F508del HET: mice heterozygous for the F508del CFTR mutation. Null HET: mice heterozygous for the null CFTR mutation. G551D HET: mice heterozygous for the G551D CFTR mutation. *P<0.05; **P<0.005; #P<0.001 vs. all mice at the same timepoint. Data are presented as means ± SEM.

Figure 4.3: Heterozygosity for the F508del and Null CFTR mutation prevents influenza-induced pulmonary edema.

Effect of influenza infection at 6 d.p.i. on lung water content (wet:dry weight ratio; n=4-10 per group). WT: wild-type C57BL/6. F508del HET: mice heterozygous for the F508del CFTR mutation. Null HET: mice heterozygous for the null CFTR mutation. G551D HET: mice heterozygous for the G551D CFTR mutation. *P<0.05; **P<0.005; #P<0.001 vs. all mice at the same timepoint. Data are presented as means ± SEM.
Figure 4.4: BALF leukocyte counts differ between the various Heterozygous CFTR Mutants.
Effects of influenza infection at 2 and 6 d.p.i. on: (A) Bronchoalveolar lavage fluid alveolar macrophages (AMs; n=5-15 per group); (B) BALF PMNs (n=5-15 per group). WT: wild-type C57BL/6. F508del HET: mice heterozygous for the F508del CFTR mutation. Null HET: mice heterozygous for the null CFTR mutation. G551D HET: mice heterozygous for the G551D CFTR mutation. *P<0.05; **P<0.005; #P<0.001 vs. all mice at the same timepoint. Data are presented as means ± SEM.

4.1 Tables

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Table 4.1 Cellular phenotype resulting from the heterozygous F508del, null, or G551D CFTR mutations
4.8 References


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Chapter 5: Future Directions

5.1 The future of influenza prophylaxis and treatment

Vaccination will forever remain an important tool in protecting the general population against seasonal influenza infection. However, vaccine uptake remains rather low within certain subpopulations, such as the immunocompromised and elderly (35). The development of more potent and novel adjuvants may perhaps provide one solution to poor vaccine uptake (16). Moreover, due to influenza’s high mutation rate, vaccines must be reconfigured each season in order to offer protection. The 2014-2015 influenza vaccine proved to be a poor antigenic match for the predominant circulating stains, and thus was limited in preventing infection (11; 12). Beyond proper strain matching, vaccine production faces challenges with timely production, efficient distribution, and a limited shelf life (10). Unfortunately, vaccination rates remain low due to common misconceptions associated with vaccine safety and side-effects. Low vaccination rates fail to provide “heard immunity,” which would allow for the protection of unvaccinated individuals (13; 20). Until some of these challenges are met, vaccines will fail to fully protect the population from influenza epidemics. Antiviral drugs, such as oseltamivir, can be given to influenza-infected individuals in an attempt to limit viral dissemination from cell-to-cell. While some studies
suggest these drugs can significantly decrease viral load, a recent meta-analysis would suggest otherwise. The Cochrane Collaboration determined that antiviral drugs do little to prevent hospital admissions or severe complications of influenza infection (17; 18). Thus, these drugs have really only proven to shorten influenza symptoms, i.e. fever and joint pain, by half a day. This may be attributed to the development of several drug-resistant virus strains that have emerged since these drugs have come to market (44). Even if antiviral drugs were more effective in treating severe influenza infection, they must be administered early in the infection time course. Many influenza-infected patients fail to show severe complications until several days after initial exposure, and thus antiviral drugs offer little clinical utility under these circumstances.

The shortcomings associated with our current options to prevent and treat severe influenza infections highlight the need for more specific therapies. Non-specific supportive care remains the standard treatment regimen for individuals experiencing influenza-induced acute lung injury (24; 47). This regimen relies heavily on mechanical ventilation to keep individuals alive. Roughly 95% of mechanical ventilators are in use during a typical influenza season, thus there would be a shortage of ventilators with the emergence of a highly novel virus (34). Taken together, all of these findings suggest that a “therapeutic gap” exists. Future research efforts should place more emphasis on the treatment of end-stage, severe influenza infections.
5.2 Re-evaluation of influenza-induced ALI

Many researchers have placed emphasis on attenuating the robust inflammatory response associated with influenza-induced ALI (42). Unfortunately, the sole targeting of the immune response has been mostly unsuccessful in impeding severe influenza complications (45). *In vitro* and *in vivo* studies have identified several immunomodulatory pathways that could potentially be exploited for therapeutic benefit; however, these have yet to be thoroughly tested in human patients (5; 30; 51). This is not to say that the “cytokine storm” does not contribute to influenza pathogenesis, but suggests that we must take more holistic measures in an attempt to treat the disease.

Our lab group and others have shown that dysregulated ion transport contributes to influenza-induced ALI (3; 22; 23; 48). Excess accumulation of ions in the respiratory tract resulting from influenza infection leads to pulmonary edema. These studies argue that therapeutic restoration of normal ion balance within the distal lung may provide clinical benefit. Thus, therapeutics that have the ability to simultaneously target the immune response and ion imbalances may provide a significant degree of therapeutic benefit.

5.3 Targeting CFTR: A dual approach

As mentioned in the previous section, targeting the cytokine storm has failed to provide any breakthrough treatments for influenza-induced ALI (45). Yet, our studies suggest that these pathways are still worth exploring (42). Using mice heterozygous for the F508del mutation in CFTR, we demonstrated that cytokine
kinetics play a critical role in attenuating pulmonary edema. Specifically, high levels of TGF-β early in infection that induces high levels of IL-6 late in infection (49). It is well accepted that TGF-β is anti-inflammatory in nature (26). Moreover, IL-6 has been implicated in anti-inflammatory processes within the lung (33). Initially, IL-6 was thought to contribute to influenza pathogenesis; however, IL-6 knockout mice experienced no attenuation in influenza disease (41). More recently, studies suggest that IL-6 may actually bind to and inhibit TNF-α and IL-1, which are potent pro-inflammatory cytokines (27; 50). Beyond their immunomodulatory roles, TGF-β and IL-6 exert other effects within the lung. TGF-β can downregulate CFTR channels on the surface of epithelial cells (14; 15; 39; 40; 43). IL-6 can also impact ion transport by stimulating ENaC activity (25). Hence, high levels of TGF-β early in infection may inhibit influenza-stimulated, CFTR-mediated Cl⁻ secretion by the respiratory epithelium. Moreover, high levels of IL-6 late in infection may stimulate ENaC-mediated Na⁺ absorption from the extracellular environment. These processes will result in the net accumulation of ions within the alveolar epithelium, and thus pull water from the respiratory lumen and into the cells. TGF-β’s and IL-6’s effects on ion transport, along with their anti-inflammatory properties, provide a dual approach for reducing pulmonary edema and improving lung function. While we cannot rule out that these cytokines can be attenuating influenza-induced ALI through some unknown mechanism, these studies highlight the importance of cytokine kinetics in the course of disease. Simply blocking a single cytokine or administering a
single recombinant cytokine will likely fail in treating severe influenza infection. Studies like ours highlight the importance in understanding how the differential expression and the kinetics of cytokines may be exploited to treat disease.

Our F508del HET murine model once again sheds light onto the importance of AMs in the influenza disease process. These mice mount an alternatively-activated (M2) macrophage response to influenza infection. M2-polarized macrophages are likely beneficial within the context of influenza infection due to their anti-inflammatory properties (1; 31). Specifically, M2-polarized macrophages produce significantly less reactive oxygen species than the classically-activated (M1) macrophages associated with a typical influenza response (6; 28; 29). Others have shown that limiting ROS generation can reduce influenza disease severity (19; 36). While we are not the first group to show that M2-polarized macrophages attenuate influenza-induced ALI (46), we are the first to show that alterations in CFTR can induce an M2 phenotype. Again, this brings up the possibility for the therapeutic modulation of CFTR. Ideally, administration of a CFTR modulator to an influenza-infected patient could provide therapeutic benefit by two mechanisms: 1) The inhibition of influenza-stimulated, CFTR-mediated Cl− secretion by the alveolar epithelium; and 2) The promotion of M2-polarized macrophages to reduce ROS lung damage. The exact mechanism by which alterations in CFTR promote M2-polarization remains to be determined. However, future studies utilizing mice heterozygous for various mutations in CFTR may allow for the elucidation of the specific mechanism.
5.4 Nucleotide Signaling: Targets upstream of CFTR

We have shown that influenza A virus stimulates the release of nucleotides into the respiratory lumen (4). Extracellular nucleotides bind to purinergic receptors on the alveolar epithelium and alter steady-state ion levels within the cell by the concomitant inhibition of ENaC-mediated Na⁺ absorption and CFTR-mediated Cl⁻ secretion (9; 32). Thus, targeting nucleotide signaling pathways within the context of influenza infection may provide similar therapeutic benefit to the targeting of CFTR. Moreover, extracellular nucleotide signaling functions in the activation and recruitment of immune cells that may contribute to influenza-induced lung pathology (8; 21).

Activation of A₁-adenosine receptors (A₁R) occurs upon the excess release of nucleotides into the extracellular environment. A₁R activation leads to excess CFTR-mediated Cl⁻ secretion, increased pulmonary edema, and impaired alveolar fluid clearance (9). Our lab has shown that A₁R ablation or inhibition improves cardiopulmonary function, attenuates pulmonary edema, and reduces immune cell infiltration to the lung following influenza challenge (4). Interestingly, it appeared that A₁R activation was equally as important as KC, the principal neutrophil chemoattractant in mice, in the recruitment of leukocytes. Taken together, these findings suggest that targeting A₁R yields similar results to the direct targeting of CFTR in that A₁R inhibition dampens the immune response and blocks CFTR-mediated pulmonary edema.

We have also explored the role of nucleotide metabolism in influenza-induced lung injury. We hypothesized that the inhibition of ecto-5’-nucleotidase (CD73),
an enzyme that metabolizes AMP to adenosine, would act in a similar fashion as A₁R inhibition. However, CD73 ablation or inhibition only modestly prevented neutrophil recruitment to the lung and failed to attenuate pulmonary edema (2). This has lead us to explore another nucleotide metabolizing enzyme, tissue non-specific phosphatase (TNAP), in an attempt to attenuate inflammation and prevent altered ion transport. CD73 is anatomically located on epithelial cells of the proximal airway, while TNAP is located on epithelial cells of the distal lung making it a more promising candidate for preventing fluid accumulation within the alveolus (38). Moreover, CD73 is a high-affinity, low-capacity enzyme that specifically metabolizes AMP to adenosine, and is thus likely to regulate adenosine levels under normal conditions (7). In contrast, TNAP is a low-affinity, high-capacity enzyme that breaks down nucleotides in a non-specific manner, and thus is more likely to breakdown nucleotides in situations of robust nucleotide release (37). Our preliminary data suggest that influenza infection induces the upregulation of TNAP protein expression and activity. Furthermore, TNAP inhibitor administration reduces hypoxemia, pulmonary edema, and immune cell infiltration in WT mice. TNAP inhibition, much like the targeting CFTR and A₁R, is likely attenuating influenza-induced ALI by inhibiting alterations in ion transport and blunting the immune response.

5.5 Final Thoughts
The studies described in this thesis highlight the contributions of both ion transport and inflammation in influenza-induced ALI. Thus, future therapeutic
development should place emphasis on a dual approach that targets both mechanisms in an attempt to attenuate disease. Modulating CFTR and upstream processes, such as nucleotide signaling/metabolism, appear to meet this dual mechanism targeting approach. In the future, a more thorough investigation into these pathways may offer new insights into how to effectively treat severe influenza illness in humans.

5.6 References


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