PATHOPHYSIOLOGIC EFFECTS OF INFLUENZA INFECTION ON THE MURINE LUNG AND EVALUATION OF NOVEL THERAPEUTIC TARGETS

DISSERTATION

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Abstract

Influenza viruses are a major public health concern. Seasonal influenza causes a significant number of deaths worldwide each year and sporadic pandemics have resulted in the loss of up to 3% of the human population (e.g., 1918 “Spanish flu”). Vaccination can prevent influenza infection, but slow vaccine production and low rates of compliance by the general public are problematic. Although antiviral drugs are available to treat influenza virus pneumonia, many viral strains are drug-resistant. Additionally, antiviral therapeutics are ineffective if administered late in the disease progression. Thus, novel therapies are urgently needed to prevent late-stage respiratory failure in patients with influenza.

Since severe influenza infection in humans oftentimes leads to acute respiratory distress syndrome (ARDS), here we re-define the existing animal model of ARDS by adapting several functional readouts that are commonly employed in human patients for use in a mouse model of ARDS induced by H1N1 influenza infection. This includes the incorporation of variety of disciplines, including viral pathogenesis, innate immunity, cell biology, lung physiology, airway mechanics, and pulmonary critical care medicine and presents a more functionally based and meaningful assessment of potential drugs.
After establishing this re-defined mouse model of ARDS, it was put to use to evaluate the treatment effects of the *de novo* pyrimidine synthesis inhibitor A77-1726 on H1N1 infected mice. We have previously shown that nucleotide signaling is important to development of lung dysfunction in influenza-infected mice. Nucleotide signaling can be prevented by the drug A77-1726, which blocks nucleotide synthesis. A77-1726 is an attractive therapeutic candidate because it is stable, inexpensive and suitable for administration via nebulization. We found that a single treatment with aerosolized A77-1726 significantly improved pulmonary function for at least 5 days after influenza virus infection and thereby prevented respiratory failure and providing prolonged survival. Perhaps the most promising finding was that treatment late in infection, after the onset of lung disease, significantly reduced the severity of ongoing respiratory failure. Hence, we have identified a novel and inexpensive therapeutic strategy that prevents respiratory failure and improves survival after influenza virus infection.

The same work that pointed in the direction of investigating nucleotide signaling had also highlighted a potential role of the chloride channel CFTR (cystic fibrosis transmembrane conductance regulator anion channel) in the development of pulmonary failure after H1N1 influenza infection. This work demonstrated that *heterozygosity* for this mutation reduces severity of influenza-induced lung dysfunction and significantly alters the innate immune response to infection. These findings suggest that CFTR is a potential therapeutic target in non-cystic fibrosis patients with influenza.
Specifically the role of adenosine and its signaling through the A1-adenosine receptor in the development of pulmonary pathology was investigated by utilizing previously developed techniques in the A1-adenosine receptor knockout mouse. We have been able to show that these mice display better disease outcomes and less pulmonary injury, mainly by preventing neutrophil influx and tissue destruction. Similar outcomes were seen when wild-type mice were treated with the A1-adenosine receptor antagonist DPCPX.
To John

“Promise me you will not spend so much time treading water and trying to keep your head above the waves that you forget, truly forget, how much you have always loved to swim.”
Tyler Knott Gregson
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In loving memory of Ari Chaim Booty.
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Chapter 1: Literature Review

1.1 Influenza

1.1.1 Influenza virology

Influenza viruses belong to the family *Orthomyxoviridae* and are enveloped negative-sense RNA viruses containing 7-8 gene segments (reviewed in 1;2). Within the family of *Orthomyxoviridae* there are 5 genera: influenzavirus A, influenzavirus B, influenzavirus C, thogotovirus, and isavirus (infectious salmon anemia virus) (1). Influenza A and B viruses are similar in structure, while C is more divergent, however they appear to have a common ancestor virus and split up several thousand years ago. Only Influenza A viruses pose a significant risk of zoonotic infections in humans (1). Therefore, the following overview focuses on Influenza A viruses exclusively.

Influenza A viruses are characterized by their surface glycoproteins (Fig. 1). Currently there are 16 different hemagglutinin (HA) and 9 neuraminidase (NA) proteins known. These can all be found in the avian population. Theoretically 144 HA-NA combinations are possible and so far at least 116 of these have been isolated from birds (3;4).

The envelope of Influenza A viruses is host derived and contains eight RNA gene segments that encode at least 11 open reading frames (2). Table 1.1 contains an
overview of all known proteins and their function. Three proteins project outward from the virus envelope: HA, NA, and the matrix 2 protein (M2). The matrix 1 protein is localized to the submembranous compartment (1).

HA functions as a viral receptor-binding protein as well as a fusion protein. It recognizes sialic acids on host cells and different HA proteins have variable specificities for dissacharides containing sialic acids with different glycoside bond isomerization. Influenza A viruses that are adapted to avian species bind specifically to α2-3 sialic acid (SAα2-3), while adaptation to humans is connected to a higher specificity for α2-6 sialic acid (SAα2-6). In order for HA to function as a fusion protein, it needs to be proteolytically cleaved into the HA1 and HA2 subunits (activation) (1). This is usually performed by an exogenous serine protease, more specifically in the human respiratory tract the tryptase produced by Clara cells (5). Influenza viruses of the H5 and H7 subtypes can acquire mutations creating polybasic cleavage sites with broader protease specificity. This enables systemic replication, resulting in highly pathogenic and potentially pandemic strains (1).

NA is a membrane protein that is required for cleavage of both host cell and viral sialic acids, allowing for release of new virions and preventing agglutination of viral particle in close proximity. Both HA and NA are major antigenic targets (1).

Inside the virion, viral RNA is wrapped around nucleoproteins and packaged into ribonucleoprotein complexes, together with three polymerase proteins (polymerase basic protein 1, polymerase basic protein 2, polymerase acidic
protein). Polymerase basic protein 1 is a RNA-dependent RNA polymerase, while polymerase basic protein 2 is involved in mRNA synthesis by binding host mRNA caps (1).

After the HA binds its receptor on the host cell, the virus is internalized and the acidic pH in the endosomes lead to conformational changes in the HA. This leads to the fusion of viral and endosomal membranes and allows for the release of viral ribonucleoproteins into the host cell cytoplasm (1). These are then transported into the nucleus for RNA replication and transcription. Viral mRNA segments translocate back into the cytoplasm for translation (6). The newly formed viral proteins M1, NP and NS2 are transported into the nucleus once more to facilitate viral RNA transport into the cytoplasm and virion assembly (7;8). In contrast, HA, NA and M2 migrate to the apical cell surface to become part of the viral envelope once the virion buds from the cellular membrane (9). The NA protein is then required to cleave the sialic acids as described above to release the budding virus (10).

Influenza viruses have a low-fidelity viral RNA polymerase that lacks an exonuclease proofreading function and has a high error rate. Due to the resulting high mutation rate, Influenza A viruses have been called a “quasispecies” (11). Selection pressure induced by novel host environments (12), responses to pre-existing immunity (13) or antiviral drugs (14) can favor certain mutations over others in a rapid fashion.
1.1.2 Influenza disease and pathogenesis

Influenza viruses can cause severe respiratory infections in humans with significant morbidity and mortality (1). However, infections can vary in severity from asymptomatic to severe febrile illness characterized by headache, sore throat, cough, runny nose, muscle aches, fatigue, and sometimes gastrointestinal symptoms (15). Symptoms are usually more severe in children (15). Complications are most common in the young and elderly and oftentimes consist of exacerbation of underlying health conditions as well as progression to viral and secondary bacterial pneumonia (16;17). Patients with underlying pulmonary or cardiac disease, obesity or diabetes mellitus are at high risk for severe complications. These can include hemorrhagic bronchitis, pneumonia and death (18).

Fatal cases with severe pneumonia have been characterized by development of multi-organ failure and acute respiratory distress syndrome (ARDS) before death (19;20).

In humans, initial viral replication occurs in the nasopharynx and peaks approximately 48 hours after inoculation. It then extends into the lower respiratory tract, where replication occurs in epithelial cells (18).

With regards to morphologic changes, early in the infection there is a striking absence of neutrophilic infiltration in areas of necrotic ciliated respiratory epithelium of larger conducting airways. Then neutrophils migrate in, later mononuclear cells predominated. Alveoli are commonly flooded by edema,
inflammatory cells including neutrophils, and hyaline membranes. Necrosis of alveolar walls can be common. Capillaries and small vessels thrombose and the interstitium is extended by edema. Later stages show organizing diffuse alveolar damage, fibrosis, epithelial regeneration (alveolar type II cell hyperplasia) and squamous metaplasia. However, histologic changes in influenza infection are nonspecific and therefore insufficient to make a specific diagnosis. In addition they can be complicated or masked by secondary bacterial infections (reviewed in 18). A diagnosis typically requires PCR, viral isolation and/or serology (18).

With regards to disease mechanism, many have postulated that the so-called “cytokine storm” is responsible for fatalities in influenza infection. While a detailed definition of what constitutes a cytokine storm is missing, it is generally viewed as a scenario in which mechanisms that modulate the immune response fail and result in an excessive innate and adaptive immune response (21). While this term was first coined in graft-versus-host disease, it has since then been applied to various infectious and noninfectious conditions (21;22) and in 2005 it was first applied to influenza infection (23). In this scenario, pathologic tissue injury during influenza infection is not only caused by the virus’ virulence, but also by the intensity of the immune response (24). This theory is based upon the observations that infected individuals displayed high mortality with increased cytokine concentrations, leukocyte inflammation and pulmonary edema during the 1918 Spanish flu as well as during the 2009 pandemic (25-27). In addition, animal models (non-human primates, swine and mice) show similar
characteristics (28-31). Investigators observed for example equal viral load in lungs of human patients, independent of whether the infection was fatal or not. In the same study, mortality was however correlated with higher cytokine and chemokine levels (27). Based on these findings, some researchers are turning their focus to the task of calming the hosts’ exaggerated cytokine response to influenza infection as a means to decrease morbidity and mortality. Because there seems to be a certain susceptibility of individuals to the cytokine storm, there has been interest in identifying the underlying genetic mechanisms as well (32).

However, others have been critical of the proposed cytokine storm, stating that while there has been a documented correlation, it has not been proven that the cytokine storm is the cause of death in fatal influenza patients. Human studies especially rely heavily on intermittent sampling from one compartment (peripheral blood), while many changes in cytokine levels and signaling likely happen at a more localized level. In this case, the sampling only assesses the spillover of cytokines and chemokines into the systemic circulation (21). In addition, different influenza strains seem to develop different cytokine profiles (33).

1.1.3 Epidemiology and threats

Influenza A viruses can cause zoonotic infection by stably adapting to humans leading to person-to-person transmission. Several stable host switch events have been reported (34).
Molecular techniques are widely and most commonly used to detect influenza viruses. These include reverse-transcriptase PCR, real time reverse-transcriptase PCR, microarrays and pyrosequencing. Molecular methods have a high sensitivity and allow for high throughput screening at relatively low costs. However, there is currently no standardized universally-accepted influenza detection assay that allows for easy comparison of surveillance studies around the globe (2). The World Health Organization (WHO) lists recommended techniques in its website, including different real time PCR protocols for different influenza strains.

While selective mutations of the antigenic domains of HA and NA are called genetic drift, influenza A viruses are also capable of genetic shift (35;36). This is possible in the situation of a coinfection of one host cell with two different strains. An exchange of one or several of the eight RNA segments can result in viral progeny that can contain genetic information from both viruses. However, it is important to specify that the term antigenic shift only applies to the reassortment of the segments encoding the HA and/or NA genes (35-37). These mechanisms play a crucial role in the evolution of the virus as well as in host switch events (35;36;38;39). Homologous recombination however is not common in negative-sense RNA viruses (40).

The major natural host species of influenza A viruses include aquatic waterfowl and shorebirds (41). However, the virus has been able to adapt to several avian and mammalian species, with the most recent discovery being an influenza virus
in bats (42). Novel human-adapted viruses have emerged several times during the last 100 years, causing several severe pandemics (43), including the most recent “swine flu” pandemic. Each of these pandemic viruses had emerged in a different way; therefore a general statement about the adaptation of Influenza A viruses to the human is not possible (1). However, aquatic birds are the likely reservoir of all known influenza A subtypes and probably the ultimate source of all human pandemic strains (41).

It appears that Influenza outbreaks in humans have occurred at least since the Middle Ages (43). Pandemics have emerged every 8-41 years for the last centuries resulting in a total of 14 pandemics in the last 500 years (1;43). In general up to 50% of the world’s population can be infected in one pandemic year (1). During the worst recorded pandemic in 1918, approximately 50 million people died worldwide (43-45).

Most recently, the first case of human infection with an H7N9 influenza strain has been reported (46). Within only two months, at least 143 people were infected and 32 died. To date no human to human transmission has been reported. There is no available vaccine against H7N9 at this point (47). A significant problem in the surveillance of this strain is that infected birds show no symptoms of disease, while humans infected with the same strain develop rapid and severe flu-like symptoms and ARDS (48).
1.1.4 Treatment options

As prophylactic measure, influenza vaccines are available. However, since circulating strains need to be predicted, antigenic mismatch can result in only partial protection (49-52). To circumvent these problems, much effort has been invested lately into the development of universal influenza vaccines with focus on highly conserved proteins across virus subtypes (49). While there has been some discussion questioning the overall efficacy of the currently available vaccines (53), one of the main problems of disease preventions is the lack of vaccine uptake by the general population. Vaccine rates throughout the United States are low, including amongst healthcare workers as well as high-risk populations (54). More specifically, the general vaccine rate is below the threshold of herd/communal immunity that would protect even unvaccinated individuals (54;55). In addition, it has been estimated that 85% of developing countries have no access to vaccines during influenza pandemics (56).

Antiviral drugs to treat influenza infection are available. The NA protein on the surface of the Influenza A viral membrane is a target for the antiviral drugs oseltamivir and zanamivir. In contract, the small M2 protein, which is a proton channel crucial for viral replication, is the target of the adamantine class of antiviral drugs (1;2). Unfortunately, the development of viral mutants that are resistant to these therapeutic are more and more common (57). In addition, like vaccines, antiviral agents have a limited national and global availability, can be
costly, and may be of only limited use, even in the early stages of infection (58;59).

Awareness of the copathogenesis of influenza and secondary bacteria infections has led to early antibiotic treatment of 2009 pandemic influenza patients who might have succumbed to fatal secondary bacterial pneumonia (60).

1.1.5 Animal models of influenza

While there is no one perfect animal model for studying influenza infection, all animal models together have been instrumental in furthering our knowledge of influenza virus biology and pathogenesis. Each model has its advantages and disadvantages that should be carefully considered before making a selection (61).

1.1.5.1 Mice

Animal models have been critical for influenza research, with mice being the most commonly used mammal (61).

The advantages of using mice are that the individual animal is relatively inexpensive, the genetics are well characterized, and inbred strains as well as reagents are readily available (61). Amongst the disadvantages are the facts that mice are not natural hosts for influenza, that the virus is not transmitted from mouse to mouse and that the ability to infect varies from strain to strain. In addition, mice commonly show hypothermia instead of fever after infection, and the clinical signs displayed during the course of disease correspond poorly with those seen in human influenza patients (61;62).
In general human influenza strains have to be adapted to mice to replicate efficiently in the mouse. This adaptation is achieved by serial passage of the virus in mice that results in mutations in influenza gene segments that will increase the virus’s replication and virulence (62). However, some high- as well as low-pathogenic flu strains including the 1918 Spanish flu and the 2009 Swine flu can infect mice without adaptation (63;64).

The need for virus adaptation is in part believed to be caused by the different distribution of SAα2,3 and SAα2,6 receptors, which can even differ between mouse strains. It is widely accepted that commonly used mouse strains express both receptor types. However, it has recently been shown that while BALB/c mice express both receptors in multiple tissues, C57BL/6 seem to lack SAα2,6 receptors in their lungs (65;66).

In addition, genetics are important in determining a mouse strain’s susceptibility. Mice can express the Mx1 protein, which is a nuclear protein that is both necessary and sufficient to protect from influenza infection. Mx1 is a member of a family of interferon-induced proteins that inhibits influenza replication by inhibiting mRNA synthesis. However, most inbred strains carry defective Mx1 alleles and are therefore not protected (67;68). Humans express a homologous protein termed MxA, however this is not sufficient to prevent influenza infection (68).

1.1.5.2 Ferrets

Ferrets have long been considered a valuable model for influenza research. They are susceptible to many human and avian virus strains and share similarities to
humans in terms of clinical signs, airway morphology and lung physiology. In addition, their receptor distribution appears to resemble that of humans (69). However, these findings have recently been challenged (70). In addition, ferrets only display mild symptoms and influenza infection rarely causes mortalities (71). The main advantage of utilizing ferrets in influenza research is their ability to effectively transmit virus from one animal to another. In general, this has been shown to happen via direct contact with respiratory droplets (72).

1.1.5.3 Non-human primates
While non-human primates are the closest animal model to the human, especially in terms of respiratory physiology and genomic sequence, this animal model is not as frequently used due to high costs and other difficulties. Several non-human primate species have been utilized to investigate human and avian influenza viruses. These include both Old World and New World monkeys (61;73;74).

However, the pattern of viral attachment differs significantly between human and non-human primates. For example, viral attachment was not observed in the trachea, bronchi, or bronchioles in cynomolgous macaques (69). This effect might be due to differences in receptor distribution between species.

1.1.5.4 Other animal models
Despite their common use in research in general, the rat model of influenza infection has not been well characterized (62). Like mice, rats can only be infected with species-adapted virus. While there are strain-specific differences,
influenza infection usually only results in mild symptoms and few pulmonary pathologic abnormalities (75). More recently, Brown Norway rats have been established as a model to evaluate influenza vaccines (76).

The influenza A cotton rat model utilizes nasal as well as pulmonary infection (77). Because their immune response resembles that of humans more closely than mice, cotton rats are considered a better model. However, limited animal availability and the aggressiveness of this species have hampered the acceptance of this model resulting in a paucity of published studies utilizing cotton rats (62).

Due to their low cost and small size, guinea pigs are more often used in influenza research recently (61). Like ferrets, guinea pigs are susceptible to unadapted human isolates and can transmit the virus from infected to uninfected animals over a maximum distance of 91 cm (78).

Since domestic poultry and pigs are natural/intermediate hosts for influenza viruses, utilizing these species has resulted in insights into reassortment and host adaptation (62;79).

Dogs and cats have been used as model species as well, especially after naturally occurring H5N1 infection and transmission was reported in cats (80). Most recently, clinical influenza infection and cross-species transmission has been documented in dogs in Florida with equine H3N8 influenza virus (81). The exposure of humans to cats and dogs makes transmission studies relevant.
However, there is currently no evidence that dogs transmit infections to other animal species (61).

1.2 Acute Respiratory Distress Syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) was first described as adult respiratory distress syndrome in humans in 1967 (82). This clinical condition requires hospitalization and can be brought forth by a plethora of causes, including near-drowning, aspiration of gastric content, pancreatitis, intravenous drug use, abdominal trauma as well as bacterial and viral pneumonia (82-84).

ARDS is very well defined by a list of clinical parameters: acute onset, radiological evidence of bilateral pulmonary infiltrates, a ratio of partial pressure of arterial oxygen to the fraction of inspired oxygen (PaO₂/FiO₂) of less than 300, and no clinical evidence for elevated pulmonary arterial pressure (85).

This definition has been challenged and re-defined several times. Using the Delphi method, additional positive end-expiratory pressure (PEEP) restrictions in defining hypoxemia, specific radiographic evaluation criteria and quantification of pulmonary compliance were introduced (86). Most recently, a panel of experts endorsed by the American Thoracic Society modified the definition of ARDS again. Specific cut-offs for PaO₂/FiO₂ ratios were established to classify patients as having mild, moderate and marked ARDS. In addition, this so-called “Berlin definition” recommended the abolishment of the term acute lung injury (ALI) and stated that any ancillary variables besides PaO₂/FiO₂ ratios did not contribute to predicting mortality and therefore removed these from the definition (87).
The cited criteria of ARDS pose some practical challenges in diagnosing the condition, for example because of dynamic changes in blood oxygenation, which are also greatly influenced by PEEP and as well as the subjectivity of chest radiograph evaluation (88-90).

Recent retrospective studies documented that the incidence rate of ARDS within the United States (US) fell from 81 to 38 cases per 100,000 people per year (91). The authors concluded that this trend was due to improved treatment protocols including low tidal volume ventilation, restrictive transfusion policy and increased intensivist staffing. However, there has been a long controversy in the literature regarding the accuracy of mortality rates (reviewed in 83).

1.2.1 ARDS and influenza

One of the known risk factors of developing ARDS is viral infections. The two main virus types involved are Herpesviridae causing nosocomial viral pneumonia and community-acquired viral pneumonia with the latter group including influenza viruses (92). In addition, humans infected with various different strains of influenza A viruses, including the recent 2009 H1N1 pandemic have been reported to develop pneumonia progressing to ARDS (93-95). It has been shown that even in the severe 1918 pandemic, the histopathological changes observed in affected lung tissues are not significantly different from those occurring during seasonal outbreaks. Therefore it has been concluded that victims of the 1918 Spanish flu also developed ARDS (18). Besides antiviral drugs and avoidance of
corticosteroids, therapeutic management of influenza-induced ARDS does not differ from ARDS caused by other factors (92).

Bacterial coinfections are common amongst critically ill influenza A patients, with *Staphylococcus aureus* being the most common agent. Coinfections diagnosed within 72 hours of admission have been associated with significantly higher morbidity and mortality (94).

Studies have shown that survivors of H1N1-induced ARDS with an intensive care unit (ICU) stay displayed long-term effects, including reduced diffusion capacities, psychological impairment and poor health-related quality of life (96).

### 1.2.2 Mouse models of ARDS

Directly translating the clinical criteria defining ARDS in the human to animals has been challenging (97). In 2011, the American Thoracic Society published a workshop report proposing that the main features of ALI in animals include histological evidence of tissue injury, alteration of the alveolar capillary barrier, presence of an inflammatory response, and evidence of physiological dysfunction (97). To diagnose ALI, three of these four criteria must be present. In addition, the committee classified findings as “very relevant” and “somewhat relevant” for each criterion and proposed that at least one “very relevant” point is checked off in three of the four overarching criteria to confirm diagnosis of ALI in research animals. For example for the feature “histological evidence of tissue injury”, accumulation of neutrophils in the alveolar or interstitial space as well as
thickening of alveolar walls are classified as “very relevant”, whereas atelectasis and hemorrhage are considered to be “somewhat relevant” (97).

There are a number of differences in the gross and anatomic anatomy in mice compared to humans. These include the absence of bronchial arteries in mice, the decreased thickness of the blood-gas barrier and alveolar diameter, fewer branches of conducting airways, increased numbers of Clara cells in the distal airways, and extensive bronchial-associated tissue (98;99).

When diagnosing ALI in mice via histology, one has to remember that the common feature in humans of hyaline membranes is rarely present in mice, despite clear evidence of increased alveolocapillary permeability (97;100). The cause of this is currently elusive; however one publication described hyaline membrane formation in the post-mortem histologic evaluation of animals that had succumbed to influenza infection (101). It is therefore possible that simple euthanasia of research mice prevents the formation of hyaline membrane in mice with ARDS.

There are also significant differences in the inflammatory response. Mice have fewer circulating neutrophils than humans and do not express defensins (99).

The three most commonly used mouse models of ALI over the last five years are mechanical ventilation with high tidal volumes or high peak inspiratory pressures, pulmonary or systemic administration of endotoxin, and inhalation or instillation of live bacteria (97).
The majority of animal models or ARDS focus on reproducing known risk factors such as sepsis, lipid embolism secondary to bone fracture, acid aspiration and ischemia-reperfusion of pulmonary or distal vascular beds (reviewed in 100). While not one of the most commonly used model of ARDS, induction of pulmonary injury using influenza virus instillation has been well established (30;102-104).

1.3 Adenosine signaling in the lung

Adenosine is an endogenous purine nucleoside with important signaling function and a very short half-life (105). It is released constitutively from respiratory epithelial cells in the lung in the nonamolar range (106). Adenosine is generated from Adenosine-5'-triphosphate (ATP) metabolism by cell surface enzymes called ectonucleotidases (107). While ATP release in health is very low in the lung (300-500 fmol•min⁻¹•cm⁻²) (106;108), respiratory epithelial cells release up to threefold more ATP under stress such as mechanical forces (109) or viral infection (110). The exact mechanism leading to the release of ATP from non-mucous respiratory epithelial cells remains unknown, mainly due to the lack of pharmacologic agents that promote ATP release (107). Recent data suggests that intracellular calcium ion mobilization is required to induce hypotonic ATP release; however, the reported ATP concentrations are still rather low, suggesting that additional and/or alternative upstream signals are required (108;111-113). Other findings have shown that
thrombin induces rapid ATP release from A549 cells in a RhoA-dependent fashion. While the exact mechanism is not known at this point, it has been speculated that cytoskeletal rearrangements facilitated by Rho enables hemichannels such as connexin and pannexin to facilitate ATP release (107;114;115).

The ecto-apyrase (CD39, NTPDase 1), which is expressed on the apical surface of respiratory epithelial cells (116), hydrolyzes ATP and ADP in the bronchoalveolar lining fluid to AMP (117). AMP is then hydrolyzed to adenosine by the ecto-5'-nucleotidase (CD73). CD73 is abundantly expressed in lung (118), and is rate-limiting for extracellular adenosine formation (119). CD73 expression increases in ALI in mice (120) and in influenza-infected cells in vitro (121). Adenosine plays a key role in regulation of pulmonary fluid dynamics (122) and lung inflammation (123). Steady-state extracellular adenosine levels (124) are comparable between C57BL/6 mice and man (125), but local adenosine levels can increase up to 100-fold in response to oxidative stress (126). However, adenosine bioavailability is limited by reuptake into cells (127), degradation by adenosine deaminase to inosine (128), or rephosphorylation by adenosine kinase (117).

Adenosine signals through four specific adenosine receptor (AdoR) subtypes (A₁, A₂a, A₂b, and A₃) (127), all of which are expressed on alveolar type I and type II cells (125), macrophages (129) and neutrophils (123) in mice. A₁-AdoR, which predominates in the mouse lung (130), has the highest affinity for adenosine of
any AdoR subtype (131). While A₁ and A₃ are Gᵢ-coupled and result in an adenylyl cyclase inhibition, A₂A and A₂B result in adenylyl cyclase activation (132-134). Usually, studies describing the effects of adenosine on respiratory epithelial surfaces investigated the A₂B receptor only (135).

1.3.1 In lung disease
All inflammatory cells are regulated by purinergic receptors and ectonucleotidases. It is widely accepted that, for example, bacterial products can cause ATP release which terminates in conversion to adenosine and receptor signaling (136). In addition, adenosine can be released due to hypoxia, lung injury, or chronic inflammation (137;138). However, the exact role of adenosine remains unclear and it has been documented that it can be either proinflammatory or protective in the lung (137-139). On the one hand adenosine/A₁-AdoR interactions have been shown to have a proinflammatory role in acute lung injury (131), and to contribute to the pathogenesis of ARDS (140). A₁-AdoR signaling can promote neutrophil activation (141), adhesion (142), and chemotaxis (143), all of which are important to the pathogenesis of acute lung injury (144) and possibly influenza pathogenesis (28;145). Similar evidence exists for chronic inflammatory lung conditions, where studies in mice have shown that lowering adenosine levels can reverse pulmonary phenotypes (137;146). On the other hand there is a significant amount of literature available documenting that adenosine signaling protects various tissues from hypoxic or ischemic damage by preconditioning via increased mitochondrial K-ATP channel
activity as well as induction of manganese superoxide dismutase (reviewed in 139). In addition, a reduced inflammatory response during reperfusion injury has also been attributed to adenosine signaling (147).

With regards to the process of alveolar fluid clearance, adenosine has been shown to increase chloride secretion via cystic fibrosis transmembrane regulator (CFTR) by signaling through the A1-AdoR. This results in reduced rates of alveolar fluid clearance, impaired pulmonary gas exchange and development of pulmonary edema (110).

Adenosine levels are also abnormally high in chronic lung conditions, such as asthma, chronic pulmonary obstructive disease, cystic fibrosis and idiopathic pulmonary fibrosis. This has been shown to result in a multitude of changes including altered mucociliary clearance response as well as destruction of parenchyma secondary to inflammation (148). In addition it has been shown that the A1-AdoR itself is upregulated in asthma causing airway hyperresponsiveness via contraction of bronchiolar smooth muscle cells (149;150).

A potential explanation for the discrepancies within the literature regarding positive and negative effects of adenosine is that the different outcomes reflect adenosine signaling through its different receptors. There is a gap of knowledge as to which outcome and effect can be attributed specifically to which adenosine receptor subtype, and oftentimes studies utilize non-specific adenosine inhibitors that alter all receptor subtypes at the same time versus employing selective ligands for each receptor type.
1.4 Figures

Figure 1.1: Influenza A virion displaying major virus features.
### Table 1.1: Summary of influenza proteins and their function (61).

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Proteins encoded</th>
<th>Functional unit</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB2</td>
<td>Polymerase subunit</td>
<td>Binds caps of host cell mRNAs</td>
</tr>
<tr>
<td>2</td>
<td>PB1</td>
<td>Polymerase subunit</td>
<td>Polymerase catalytic subunit</td>
</tr>
<tr>
<td></td>
<td>PB1-F2</td>
<td>Non-structural protein</td>
<td>Proapoptotic factor, possible immune evasion function</td>
</tr>
<tr>
<td>3</td>
<td>PA</td>
<td>Polymerase subunit</td>
<td>Polymerase subunit</td>
</tr>
<tr>
<td>4</td>
<td>Hemagglutinin (HA)</td>
<td>Surface glycoprotein</td>
<td>Viral attachment and fusion</td>
</tr>
<tr>
<td>5</td>
<td>Nucleoprotein (NP)</td>
<td>Internal protein</td>
<td>Viral RNA synthesis</td>
</tr>
<tr>
<td>6</td>
<td>Neuraminidase (NA)</td>
<td>Surface glycoprotein</td>
<td>Virion release</td>
</tr>
<tr>
<td>7</td>
<td>Matrix protein 1 (M1)</td>
<td>Internal protein</td>
<td>Regulates RNP nuclear import</td>
</tr>
<tr>
<td></td>
<td>Matrix protein 2 (M2)</td>
<td>Surface protein</td>
<td>Ion channel that facilitates viral RNP uncoating</td>
</tr>
<tr>
<td>8</td>
<td>NS1</td>
<td>Non-structural protein</td>
<td>Evasion of host immune response</td>
</tr>
<tr>
<td></td>
<td>Nuclear export protein (NEP or NS2)</td>
<td>Nuclear export protein</td>
<td>Viral RNA nuclear export</td>
</tr>
</tbody>
</table>

RNP: ribonucleoprotein
1.6 References


definitions, mechanisms, relevant outcomes, and clinical trial coordination.


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Chapter 2: **H1N1 influenza A virus infection results in ARDS-like disease in mice.**

2.1 Abstract

Many patients with severe influenza A infection develop acute respiratory distress syndrome (ARDS). Despite the amount of research that is being conducted in mice in the field of influenza studies, the mouse model of ARDS is poorly characterized. It was our aim to determine whether influenza A H1N1-infected mice exhibit lung dysfunction consistent with consensus criteria for acute lung injury and ARDS in humans. We therefore infected 8-12 week-old BALB/cAnNCr mice intranasally with influenza A/WSN/33. Carotid \( P_aO_2 \) was measured following mechanical ventilation on 100% \( O_2 \) with 1.2 kPa PEEP. Lung water was detected by magnetic resonance imaging. Lung function was analyzed by the forced-oscillation technique. Bronchoalveolar lavage inflammatory mediators were assayed by ELISAs. These experiments showed that \( P_aO_2:F_iO_2 \) ratios (≥ 600) in uninfected mice were normal, but declined to ≤ 300 at day 2 and ≤ 200 by day 6 following influenza infection. Influenza induced a progressive increase in lung water content and lung volume over days 2-6, together with decreased static and dynamic lung compliance and increased airway resistance. Bronchoalveolar lavage fluid keratinocyte cytokine and receptor for advanced
glycation end-product levels were elevated in influenza-infected mice. In conclusion and based on human American/European Consensus Conference (AECC) criteria, mice infected with influenza A H1N1 virus develop acute lung injury as early as day 2 and show progression to ARDS by day 6. Functional endpoints based on AECC criteria are clinically-relevant and may be more predictive of disease outcome in murine influenza studies than histopathology alone.

2.2 Introduction
Influenza A virus causes highly-contagious acute respiratory illness in humans (1). Despite vaccination and the use of antiviral drugs, the incidence of seasonal influenza-related disease in the United States has increased during the last two decades (2), and now accounts for 200,000 hospitalizations and more than 36,000 excess deaths per year (3). Moreover, the recent pandemic of influenza A/California/09 virus H1N1 (“swine flu”) was estimated to have infected around 61 million people in the United States between April 2009 and April 2010, resulting in approximately 275,000 hospitalizations and 12,500 excess deaths (4).
Severe primary influenza pneumonia can progress to acute lung injury and even the acute respiratory distress syndrome (ARDS) (5). The American/European Consensus Conference (AECC) defines ARDS as a clinical syndrome characterized by the acute onset of significant hypoxemia (PaO₂:FiO₂ [P:F] ratio
≤ 200) in the presence of bilateral chest X-ray infiltrates and the absence of left atrial hypertension (6). Less severe hypoxemia (P:F ≤ 300) is defined as evidence of acute lung injury (6). Subsequently, various refinements to the original AECC criteria have been suggested (7). In particular, Ferguson et al. (8) included requirements for the use of positive end-expiratory pressure (PEEP) when determining P:F ratios, presence of bilateral airspace disease on chest X-ray, and either predisposing lung disease or decreased lung compliance. Experimentally, additional predictors of poor ARDS outcomes have been identified, including impaired alveolar fluid clearance (AFC) (9), onset of multi-organ dysfunction syndrome (10), and elevated plasma or bronchoalveolar lavage (BAL) fluid levels of the inflammatory biomarkers IL-8, surfactant protein-D, and the receptor for advanced glycation end-products (RAGE; a marker of alveolar type I epithelial cell injury) (11).

A significant fraction of patients hospitalized during the recent H1N1 swine flu pandemic developed ARDS (12), and lung histopathology consistent with ARDS was reported in a retrospective study of the 1918 pandemic (13). Although ARDS is less commonly reported as an outcome in interpandemic “seasonal” influenza outbreaks, it has been associated with poor prognosis (3), and may be under-diagnosed as a proximate cause of ARDS (14). However, to date, development of ARDS has not been conclusively demonstrated in influenza A-infected mice, rendering studies of its pathogenesis more challenging. We have previously shown that mice infected with a lethal dose of a mouse-adapted influenza H1N1
viral strain (A/WSN/33) develop decreased peripheral oxygenation, increased lung water content, and decreased alveolar fluid clearance, all of which are suggestive, but not diagnostic, of progression to ARDS (15). We hypothesized that we could develop new methods that would allow us to demonstrate that influenza A H1N1-infected mice exhibit changes in lung function consistent with AECC criteria for the diagnosis of ARDS in human patients. Our studies are the first to demonstrate that AECC criteria can successfully be adapted to definitively establish the presence of ARDS in murine models, and also the first to demonstrate that lung injury consistent with current criteria for the diagnosis of ARDS develops in mice infected with H1N1 influenza A.

2.3 Materials and methods

Infection of mice. Pathogen-free, 8 to 12 week-old BALB/cAnNCr mice of either sex (maintained in sterile caging) were infected intranasally with 10,000 plaque-forming units of mouse-adapted H1N1 influenza virus strain A/WSN/33 in 50 μl PBS with 0.1% BSA, as previously described (16), except using light isoflurane anesthesia. For all studies, data for each group were derived from a minimum of two independent infections. All mouse procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. Care and handling of all animals was in accordance with the NRC/NIH Guide for the Care and Use of Laboratory Animals.

Measurement of arterial blood gases. Mice were anesthetized by
intraperitoneal injection of valium/ketamine, as in previous studies (15). Anesthetized animals were tracheotomized, cannulated, and connected to a Model 687 volume-controlled mouse ventilator (Harvard Apparatus, Holliston, MA, USA). Tracheotomized mice were ventilated at 160 breaths/minute on 100% O₂ (F₁O₂ = 1.0). Tidal volume was set at 8 ml/kg and 9 torr (1.2 kPa) PEEP was applied. After 15 minutes, the left carotid artery was ligated cranially and a 200 μl blood sample was collected using a 0.5 ml Monovette® heparinized blood gas syringe (Sarstedt Inc., Newton, NC, USA). PₐO₂, PₐCO₂, arterial O₂ saturation (SₐO₂), and hematocrit, together with plasma pH, and sodium (Na⁺), potassium (K⁺), and bicarbonate (HCO₃⁻) ion concentrations were measured using an EG6+ cartridge in an iSTAT® point-of-care analyzer (both Abbott Laboratories, Abbott Park, IL, USA).

**Magnetic resonance imaging of the lung.** Mice were anesthetized with 2.5% isoflurane mixed with 1 liter/minute carbogen and were maintained on 1-1.5% isoflurane during imaging. For imaging, each animal was placed prone on a temperature-controlled mouse bed and inserted into a 35 mm diameter quadrature volume coil prior to positioning in the magnetic resonance imaging (MRI) scanner. MRI was performed using an 11.7T BioSpec® high-field small animal MRI system with a 94/30 magnet (Bruker BioSpin Corp., Karlsruhe, Germany), as in prior studies (17). During the experiment, physiologic parameters (ECG, respiratory rate and body temperature) were continuously monitored using a small animal monitoring and gating system (Model 1025,
Small Animal Instruments, Inc. Stony Brook, NY, USA). Heart rate was maintained between 350-450 breaths per minute by adjusting the level of anesthesia. Core temperature was maintained at 37°C using a warm air blower. Each animal was imaged for approximately 30-60 minutes (10-30 minutes for initial calibrations followed by 30 minutes of data acquisition). Respiratory and cardiac-gated axial T1-weighted FLASH (TR = 150 ms, TE = 2.3 ms, Flip Angle = 45°, navgs = 4) and T2-weighted RARE images (TR = 3500 ms, TE = 12 ms, Rare Factor = 8, navgs = 4) were acquired contiguously over the entire lung, from 1 mm below the diaphragm moving cranially as far as the thoracic inlet. The acquisition parameters for both the T1- and T2-weighted multi-slice scans were as follows: FOV = 25 mm x 25 mm, slice thickness = 1 mm, matrix size = 256 x 256. A region-of-interest that included the lung was manually outlined in T1-weighted images by a blinded expert observer. Lung volumes were calculated by summing the outlined regions-of-interest from each contiguous slice. Fluid volumes were calculated by setting a global threshold in the T2-weighted images just above the background intensity value and summing the segmented voxels within the lung region-of-interest. For image display purposes (Figs. 2A and 2B), but not for data analysis (Figs. 2C and 2D), T1- and T2-weighted images were overlaid and identified by false color (red for T1-weighted images, green for T2-weighted images). Image contrast for Figs. 2A and 2B was also adjusted to improve visualization.

**Measurement of lung mechanics.** Mechanical properties of the mouse lung
were assessed using the forced-oscillation technique (18;19). Mice were anesthetized with valium/ketamine and tracheotomized, as above, then given pancuronium intraperitoneally (0.08 μg/kg). Animals 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 1.5-2.2 torr (200-300 Pa) PEEP. Following two total lung capacity maneuvers to standardize volume history, pressure and flow data (reflective of airway and tissue dynamics) were collected during a series of standardized volume perturbation maneuvers. Volume-stepped discontinuous pressure-volume loops were generated for each mouse and used to determine static lung compliance. Total lung resistance and dynamic compliance were calculated using the single-compartment model, as in our previous studies (20).

**Measurement of bronchoalveolar lavage fluid proinflammatory mediators.** Levels of keratinocyte cytokine (KC, the murine homolog of CXCL-8) and murine RAGE were measured by ELISA (R & D Systems, Minneapolis, MN, USA). Both assays were performed in accordance with manufacturer’s instructions. For BAL fluid cell counts, cell viability was determined via trypan blue exclusion. Cell types were differentiated on cytospin preparations using Wright-Giemsa stain and standard hematological criteria.

**Other methods.** All other methods were performed as previously described (15;16;21).

**Statistical analyses.** Descriptive statistics were calculated using Instat 3.05
Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. Differences between group means were analyzed by 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

**Influenza A virus infection of BALB/c mice results in severe hypoxemia, hypercapnia, and acute respiratory acidosis.** We hypothesized that severe primary influenza A H1N1 infection might serve as a form of direct lung injury and therefore act as a predisposing factor for development of ARDS. Using our previously-established protocol, mice were infected intranasally with 10,000 plaque-forming units of influenza A/WSN/33 (a mouse-adapted H1N1 influenza A strain, which is pneumotropic following intranasal inoculation (22)), under light isoflurane anesthesia. In our hands, this infection protocol results in severe disease by 6 days post-infection (d.p.i.), with 100% mortality by 8 d.p.i. (a median of 7 days to death). Based on our previous studies, we evaluated lung function at 2, 4, and 6 d.p.i. These timepoints were selected as being reflective of maximal impairment of alveolar fluid clearance (51% at 2 d.p.i. (15)), peak viral replication in the lungs (\( 2.8 \times 10^6 \) plaque-forming units/g lung tissue at 4 d.p.i. in the current study), and the onset of severe morbidity and mortality (6 d.p.i.). To ensure that any disease present was the result of primary, uncomplicated influenza infection,
we performed histopathologic evaluation of Gram-stained lung tissue sections. No evidence of secondary bacterial pneumonia or sepsis was found in any influenza-infected mice (not shown).

All uninfected mice that were mechanically-ventilated on 100% O\textsubscript{2} for 15 minutes (with 9 torr [1.2 kPa] PEEP) had normal P:F ratios (≥ 600; Fig. 2.1A) and S\textsubscript{a}O\textsubscript{2} values of 100% (Fig. 2.1B). These animals were also normocapnic, or only mildly hypercapnic (Fig. 2.1C), and had normal plasma pH values (7.4; Fig. 2.1D). In contrast, influenza A virus-infected mice exhibited very significant impairment of pulmonary gas exchange (Fig. 2.1A). At 2 d.p.i., hypoxemia in influenza-infected mice was of a severity that in humans would be consistent with diagnosis of acute lung injury (P:F ≤ 300) (6). P:F ratios in influenza A virus-infected mice declined slightly further at 4 d.p.i., and by 6 d.p.i. were consistent with development of frank ARDS (P:F ≤ 200) (6). Moreover, S\textsubscript{a}O\textsubscript{2} values in many animals were significantly below 100% by day 6, despite ventilation on 100% O\textsubscript{2} (Fig. 2.1B). Influenza-infected mice were also hypercapnic from 2-6 d.p.i. (Fig. 2.1C), and developed acute, uncompensated respiratory acidosis (reduced plasma pH without compensatory elevation of plasma HCO\textsubscript{3}\textsuperscript{-} levels) as early as 2 d.p.i. (Fig. 2.1D). Hypercapnia and acidosis were less severe at 6 d.p.i., suggesting the onset of metabolic compensation by this timepoint. Nevertheless, mean alveolar to arterial (A-a) gradients increased from 64 torr (8.5 kPa) at day 0 to 470 torr (62.7 kPa) at 6 d.p.i. (http://www.mdcalc.com/a-a-o2-gradient).

Influenza A virus infection of BALB/c mice results in hyperkalemia and
**elevated hematocrit.** Although influenza A virus infection had no effect on either plasma osmolarity or Na\(^+\) levels, plasma K\(^+\) content increased significantly at 6 d.p.i. (Table 2.1). Moreover, HCO\(_3^-\) levels were significantly reduced at 2-4 d.p.i., when acidosis was most severe. At 6 d.p.i., when plasma pH had recovered somewhat, some increase in plasma HCO\(_3^-\) was detected. Finally, the hematocrit progressively increased over the course of influenza infection. This change, which has been reported previously (23), may reflect increased erythropoiesis (secondary polycythemia) in the face of prolonged hypoxemia.

**Influenza A virus infection of BALB/c mice results in radiologic evidence of bilateral airspace disease consistent with ARDS.** In human patients, the presence of bilateral infiltrates on chest X-ray are considered to be an essential criterion for a diagnosis of ARDS, as it is currently defined by both the AECC (6) and Ferguson *et al.* (8). Because of the poor resolution of both radiography and computed tomographic scanning in mice, we elected to visualize chest infiltrates by MRI. For these studies, we modified the method of Serkova *et al.* (17). This technique allowed us to both identify areas of pulmonary edema (water signal in T2 images) and to quantitate total lung volume (using T1 images of thoracic structures) and thereby calculate the percentage of lung volume occupied by edema fluid. As expected, water signal was virtually absent from MRI scans of uninfected mice (Figs. 2.2A and 2.2C). Influenza A virus infection resulted in a significant increase in both pulmonary edema (Figs. 2.2B and 2.2C) and total lung volume (Fig. 2.2D) from 2-6 d.p.i. At 2 d.p.i., heterogeneous foci of fluid-
filled tissue were present in both lungs in a perihilar distribution. By 6 d.p.i., these regions had coalesced and expanded to the point where large sections of the lung and, in some mice, entire lung lobes became completely fluid-filled. Moreover, the volume of aerated lung decreased significantly as disease progressed (from $520 \pm 14 \text{ mm}^3$ at day 0 to $417 \pm 18 \text{ mm}^3$ at 6 d.p.i.), despite the overall increase in total lung volume.

**Influenza A virus infection of BALB/c mice induces alterations in lung mechanics consistent with ARDS.** Evidence of decreased lung compliance is a component of the diagnostic criteria for human ARDS outlined by Ferguson *et al.* (8). Others have reported that increased airway resistance may also be present in ARDS (24). We therefore analyzed lung mechanics in influenza A virus-infected mice, mechanically ventilated on a flexiVent computer-controlled piston ventilator. Using this technique to generate volume-stepped discontinuous pressure-volume loops, we found that static lung compliance declined as early as 2 d.p.i., and was significantly reduced at 4-6 d.p.i. (Fig. 2.3A). Mean pressure-volume loops shifted to the right and decreased in area from 2-6 d.p.i., indicating both a decline in lung compliance and a reduction in recruitable lung volume as infection progressed (Fig. 2.3B). Likewise, dynamic lung compliance decreased significantly throughout the course of infection (Fig. 2.3C). Finally, total lung resistance significantly increased from 2 d.p.i. (Fig. 2.3D).

**Influenza A virus infection of BALB/c mice results in elevated levels of clinically-relevant inflammatory biomarkers in bronchoalveolar lavage fluid.**
In human patients with ARDS, BAL fluid levels of the proinflammatory cytokine IL-8 and RAGE have been shown to be predictive of outcome (11). Similarly, BAL RAGE levels have been shown to be an indicator of lung injury in mice (25), while influenza-associated mortality is reduced in RAGE-knockout mice (26). We found that infection with influenza A virus resulted in elevated BAL levels of KC (the murine homolog of IL-8; Fig. 2.4A) and RAGE (Fig. 2.4B) at 2-6 d.p.i. The degree of increase in levels of both mediators remained relatively constant throughout infection.

Neutrophil recruitment is also a prominent feature of the early phase of acute lung injury (27;28), and neutrophil-mediated damage has been implicated in the pathogenesis of ARDS (29). As in previous studies (30), we found that influenza A virus infection of BALB/c mice resulted in progressive leukocyte infiltration into the lung, together with peribronchiolar and interstitial neutrophil accumulation (not shown). This was accompanied by a very significant elevation in BAL fluid neutrophil counts by 6 d.p.i., together with a smaller increase in alveolar macrophage and lymphocyte counts (Fig. 2.4C). No eosinophils were detectable in BAL fluid at any timepoint.

2.5 Discussion

In the current study, we developed novel methodologies and modified existing techniques in order to determine whether the severe lung injury present in influenza-infected mice could be shown to conform to criteria used for diagnosis
of ARDS in human subjects in the ICU (6;8). This was necessary because no existing murine ARDS model has been shown to fully conform to these diagnostic criteria (31). Using this battery of techniques, we demonstrated that, like human patients (12), mice infected with a lethal dose of a mouse-adapted strain of H1N1 influenza (A/WSN/33) exhibited progressive deterioration in lung function that ultimately resulted in development of ARDS, as it is currently defined for human subjects by both AECC criteria (6) and those of Ferguson et al. (8): influenza-infected mice developed acute and severe hypoxemia (mean P:F ratio ≤ 200 at 6 d.p.i.), reduced static and dynamic lung compliance, and progressive bilateral pulmonary infiltrates, all as a consequence of direct lung injury (influenza pneumonitis). These changes were accompanied by the onset of hypercapnia, respiratory acidosis, hyperkalemia, and increasing pulmonary inflammation. They were also temporally correlated with severe cachexia, impaired alveolar fluid clearance, increased alveolar permeability, and increased lung water content, which we have shown previously to be present with progressively increasing severity from 2-6 d.p.i. in mice infected with this strain of influenza virus (15).

ARDS is a common sequel of primary infection with both pandemic and seasonal influenza A strains in humans (3;12;13). In fatal human influenza cases, multiple ARDS-like histopathologic changes are evident: interstitial edema and neutrophilic inflammatory infiltrates, formation of hyaline membranes in alveoli, bronchioles, and bronchi, varying degrees of acute intra-alveolar edema and/or
hemorrhage, and diffuse alveolar damage, together with necrotizing bronchitis and bronchiolitis and capillary thromboses (13;31). With the exception of hyaline membrane formation, similar pulmonary histopathology was observed in the current study. It has been proposed that mice may not develop hyaline membranes because these are an end-result of prolonged mechanical ventilation at high tidal volume (31). For a variety of reasons, however, the majority of both influenza and ARDS pathogenesis studies in the mouse model have not used human-relevant functional end-points to define ARDS, but instead rely on such readouts as mortality rate, histopathology, and wet:dry weight ratios, which have little predictive value clinically (32). We felt that, in order to assert a diagnosis of ARDS in influenza-infected mice, it would be necessary to develop new methods that would be of relevance to currently-accepted human criteria for diagnosis of ARDS, such as those proposed by the AECC (6).

Alterations in arterial blood gases and, in particular, alterations in gas exchange in ventilated patients receiving 100% O$_2$ are clinically-important diagnostic criteria for ARDS (6;8). Since P$_a$O$_2$ is merely a function of O$_2$ concentration and solubility (Henry’s Law), there is no physical reason why it should vary by species. It ought therefore be possible to derive comparable P:F ratio values in normal humans and normal mice under similar ventilation conditions. However, in the majority of studies in which P$_a$O$_2$ has been measured in mice with lung injury, even normal controls have P:F values around 400 (33-35), which in humans would be considered indicative of moderate hypoxemia. Moreover, previous investigators
have not measured $P_aO_2$ in mice under conditions that would conform to AECC standards: these measurements have generally been performed in spontaneously-breathing animals, in the absence of PEEP, often using anesthetics with known respiratory suppressant effects (33;34). Given the limitations of current methodologies to measure arterial blood gases in spontaneously-breathing mice, we developed a novel technique that would allow us to measure P:F ratios in mechanically-ventilated animals, with appropriate PEEP. Using this new method, we found that uninfected mice exhibit mean P:F ratios that are comparable to those of healthy human subjects ($\geq 600$). We also found that influenza A H1N1 infection resulted in an acute reduction in P:F ratios, which was evident as early as 2 d.p.i.. Moreover, P:F values were consistent with ARDS ($\leq 200$) in virtually all influenza-infected animals by 6 d.p.i. In addition, influenza-infected mice exhibited a progressive decrease in their ability to maintain $S_aO_2$ values of 100% when ventilated on 100% $O_2$, together with significant hypercapnia. Taken together, these findings are consistent with development of an increasingly larger V-Q mismatch and shunt fraction over the course of infection (approximately 25% by 6 d.p.i., based on standard iso-shunt diagrams). Our MRI and flexiVent findings support this possibility, since we found that an increasing percentage of total lung volume becomes filled with fluid as disease progresses, and that both static and dynamic lung compliance are reduced by more than 50% by 6 d.p.i. Thus, progressive lung injury develops early in the course of influenza A infection in mice, which progresses to full-blown
ARDS by day 6. To our knowledge, we are the first group to formally demonstrate that mice infected with influenza A H1N1, or indeed any strain of influenza, develop alterations in lung function consistent with the current AECC definition of ARDS. These findings are of significance to our understanding of the pathogenesis of H1N1 influenza, which has recently become the dominant circulating seasonal and pandemic influenza A serotype worldwide (36). Moreover, ours is the first study in which P:F ratios comparable to those in normal humans have been achieved in normal mice.

In our previous studies, we used pulse oximetry to measure the degree of hypoxemia (reduced peripheral O₂ saturation) in conscious influenza-infected mice breathing room air (15). While we found that influenza infection resulted in a statistically-significant decline in peripheral O₂ saturation from as early as 2 d.p.i., we did not see a clinically-significant drop in this parameter until 6 d.p.i. This contrasts with the current study, where we found that pulmonary gas exchange was severely impaired as early as 2 d.p.i. This discrepancy may result from the relative insensitivity of pulse oximetry in mice. We hypothesize that this insensitivity is a consequence of the 10-fold higher respiratory rate in conscious mice than in humans, which results in a far higher alveolar ventilation rate (~580 ml/kg/minute for a 25g mouse vs. ~60 ml/kg/minute for a 70 kg male human). A higher alveolar ventilation rate in spontaneously-breathing mice would tend to promote effective hemoglobin saturation even in the presence of impaired alveolar gas exchange, since it would result in a higher steady-state PₐO₂. Taken
together, these findings suggest that, while pulse oximetry may be simple to perform and repeatable from day-to-day on the same subject, it is a relatively insensitive readout of the capacity for pulmonary gas exchange in mice. This is in contrast to human subjects, where a strong predictive correlation has been demonstrated between $S_aO_2:F_iO_2$ and $P:F$ ratios (7).

MRI has not previously been used to evaluate progression of pulmonary edema in influenza-infected mice, but our findings suggest that this technique may be of great value for this application. The use of separate T1- and T2-weighted scans to identify body structures and lung water, respectively, allowed us to accurately and unambiguously quantify lung water content by analysis of false-color images. Moreover, because mice were imaged under moderate isoflurane anesthesia via nose cone, this approach allowed us to recover individual mice after imaging and hence follow the progression of edema formation and changes in its spatial distribution over the entire course of infection on an individual animal basis (which of course, cannot be done using wet:dry weight ratio as a readout of pulmonary edema). The MRI changes which we identified in influenza-infected mice may have resulted from nucleotide-mediated impairment of alveolar fluid clearance, which we demonstrated previously in this model (15). Moreover, they are entirely consistent with those reported in human subjects with ARDS: computed tomographic scanning of ARDS patients has shown the presence of heterogeneously-distributed collapsed areas in the lungs, accompanied by areas of overdistension (37). In addition, pigs with experimental ARDS were shown to
exhibit a similar increase in total lung volume and decrease in aerated lung volume to that seen in the current study (38).

To our knowledge, no previous studies have systematically evaluated changes in baseline lung mechanics over the course of influenza infection in mice. We found that influenza A virus infection resulted in both a progressive decrease in lung compliance and an increase in total lung resistance. These changes in lung mechanics, which were qualitatively and quantitatively comparable to those observed in human ARDS (39), are consistent with the progressive development of interstitial and intra-alveolar edema which we identified by MRI: edema fluid in the airspaces will both dilute pulmonary surfactant (lowering compliance) and reduce the effective diameter of small airways (increasing resistance), while fluid-filled alveoli and interstitial tissues will compress small airways (also increasing airway resistance). Moreover, in addition to the rightward shift in P-V loops (which clearly demonstrates the decrease in static lung compliance), the mean area enclosed by the P-V loops was reduced after influenza infection. This suggests that the volume of recruitable lung tissue progressively decreased in influenza-infected mice, despite the increase in total lung volume observed in our MRI studies. Together, these alterations in lung fluid volume may have resulted in development of increased dead space, V-Q mismatch, and shunt formation, all of which may have contributed to development of progressive hypoxemia and hypercapnia in influenza-infected mice.

We found that alterations in lung function were evident in influenza-infected mice
before both peak viral replication at 4 d.p.i. and peak epithelial injury at 6 d.p.i. (as assessed in our previous studies by BAL protein and lactate dehydrogenase content, and epithelial permeability to FITC-albumin (15)). This suggests that lung injury in influenza infection may not be a direct consequence of viral replication, but instead may result from an excessive innate immune response to the virus. Previous investigators have also correlated the pathology induced by fatal influenza infection with an excessive immune response (40). In particular, the rapid accumulation of proinflammatory cytokines after infection (the so-called “cytokine storm”) is thought to play a prominent role in mediating the high morbidity and mortality associated with infection by highly-pathogenic avian H5N1 and human pandemic influenza strains (41;42). However, as in ARDS (43), cytokine blockade has had only limited therapeutic success in severe influenza infection (44), and cytokine-deficient mice infected with highly-pathogenic avian H5N1 influenza strains are not protected from disease (45). Neutrophils also appear to be important in limiting influenza replication in vivo (46-48), but, as in ARDS (29), excessive pulmonary neutrophil infiltration may play a role in pathogenesis of severe disease induced by pandemic influenza strains (30;48;49). We found that development of ARDS in influenza-infected mice was temporally associated with elevated intrapulmonary levels of the neutrophil chemoattractant KC, and a progressive increase in neutrophil infiltration of the lungs. While this by no means implies causation, it is consistent with previous influenza pathogenesis studies (50).
In conclusion, we have demonstrated that infection of BALB/c mice with a lethal dose of mouse-adapted influenza A H1N1 virus results in development of ARDS, as currently defined by AECC criteria. These studies are the first to demonstrate that AECC criteria can successfully be adapted to murine ARDS models, and also the first to demonstrate development of ARDS consistent with AECC criteria in mice infected with H1N1 influenza A virus. The functional assays that we have developed to identify and characterize ARDS in mice, such as P:F ratio measurements in mechanically-ventilated animals with PEEP, more directly reflect the clinical situation than histopathologic studies do, and we propose that their use will provide improved, more predictive outcome measures for murine studies of the pathogenesis and treatment of both influenza infection and ARDS (and also, perhaps, for studies of influenza pathogenesis in other animal models). Indeed, it is quite possible that some current ARDS models may actually be found to be inconsistent with AECC criteria if these methods are applied to them. Finally, our studies indicate that some aspects of influenza pathogenesis (particularly those related to development of lung injury and its treatment) can be safely modeled using mouse-adapted low-pathogenicity influenza strains such as A/WSN/33 or A/PR/8/34, thereby avoiding the need to work with highly biohazardous highly-pathogenic strains under stringent biocontainment conditions.
2.6 Figures

Figure 2.1: Influenza A virus infection of BALB/c mice results in severe hypoxemia, hypercapnia, and acute respiratory acidosis.
Effect of influenza A virus infection for 2-6 days on: (A) $P_aO_2$: $F_iO_2$ ratios, with individual datapoints, mean values, and quartiles displayed; (B) Arterial $O_2$ saturation (% $S_aO_2$); (C) $P_aCO_2$; and (D) plasma pH. All mice were anesthetized with valium/ketamine and mechanically-ventilated on 100% $O_2$ ($F_iO_2 = 1.0$) with 9 torr (1.2 kPa) PEEP. $n=8-12$ per group. *$P<0.05$, ***$P<0.0005$, compared with uninfected mice (day 0). †$P<0.05$, compared with influenza-infected mice at 2 d.p.i.
Figure 2.2: Influenza A virus infection of BALB/c mice results in radiographic evidence of bilateral airspace disease. (A) Representative mid-thoracic magnetic resonance image from an uninfected mouse. LV: left ventricular lumen. (B) Representative mid-thoracic magnetic resonance image from an influenza-infected mouse at 6 d.p.i. T1-weighted images (body tissues) are colored red. T2-weighted images (edema) are colored green. (C) Effect of influenza A virus infection for 2-6 days on lung water content (% of total lung volume). <0.1% T2-weighted (edema) signal was detected in normal mice. (D) Effect of influenza A virus infection for 2-6 days on total lung volume. All mice were anesthetized with isoflurane and imaged using an 11.7T BioSpec® high field small animal magnetic resonance imaging system with respiratory and cardiac gating. n=6-8 per group. *P<0.05, **P<0.005, ***P<0.0005, compared with uninfected mice (day 0).
Figure 2.3: Influenza A virus infection of BALB/c mice induces alterations in lung mechanics consistent with ARDS.
Effect of influenza A virus infection for 2-6 days on: (A) Static lung compliance ($C_{st}$); (B) Mean P-V loop profile in uninfected mice and influenza-infected mice at 6 d.p.i.; (C) Dynamic lung compliance ($C_{dyn}$); and (D) Total lung resistance (R). All mice were anesthetized with valium/ketamine and mechanically ventilated on a flexiVent computer-controlled piston ventilator, with 8 ml/kg tidal volume, at a frequency of 150 breaths/minute, against 1.5-2.2 torr (200-300 Pa) PEEP. n=5-7 per group. **$P<0.005$, ***$P<0.0005$, compared with uninfected mice (day 0).
Figure 2.4: Influenza A virus infection of BALB/c mice results in elevated levels of clinically-relevant inflammatory biomarkers in bronchoalveolar lavage fluid.

Effect of influenza A virus infection for 2-6 days on bronchoalveolar lavage fluid levels of: (A) Keratinocyte cytokine (KC); (B) Receptor for advanced glycation end-products (RAGE); and (C) Alveolar macrophages (AMs), neutrophils (PMNs), and lymphocytes (SLs). n=6-8 per group. *P<0.05, **P<0.005, ***P<0.0005, compared with uninfected mice (day 0).
# 2.7 Tables

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<td>Na⁺ (mmol/L)</td>
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<td>K⁺ (mmol/L)</td>
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<td>HCO₃⁻ (mmol/L)</td>
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<td>15.4 ± 0.9</td>
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<tr>
<td>Hematocrit (%)</td>
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<td>48.7 ± 1.1*</td>
<td>51.2 ± 2.2**</td>
<td>53.6 ± 0.8***</td>
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*±: days post-infection

*: P<0.05, **: P<0.01, ***: P<0.001, compared to uninfected mice

Table 2.1: Effect of influenza A/WSN/33 infection on plasma electrolytes and hematocrit.
2.8 References


30. Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive


Chapter 3: Post-infection A77-1726 treatment improves cardiopulmonary function in H1N1 influenza-infected mice

3.1 Abstract

Acute respiratory disease is associated with significant morbidity and mortality in influenza. Because antiviral drugs are only effective early in infection, new agents are needed to treat non-vaccinated patients presenting in late-stage disease, particularly those who develop acute respiratory distress syndrome. We found previously that the de novo pyrimidine synthesis inhibitor A77-1726 reversed influenza-induced alveolar fluid clearance impairment. Patients with acute respiratory distress syndrome and intact alveolar fluid clearance have lower mortality than those with compromised fluid clearance. We therefore investigated the effects of treatment with nebulized A77-1726 (67.5 mg/kg) on indices of cardiopulmonary function relevant to the diagnosis of acute respiratory distress syndrome in patients. Eight to 12 week-old BALB/cAnNCr mice were inoculated intranasally with 10,000 pfu/mouse influenza A/WSN/33 (H1N1). Pulse oximetry was performed daily. Alveolar fluid clearance, lung water, and lung mechanics were measured at 2 and 6 days post-inoculation in live, ventilated mice by BSA instillation, magnetic resonance imaging, and forced-oscillation techniques, respectively. A77-1726 treatment at 1 day post-inoculation delayed mortality.
Treatment at days 1 or 5 reduced viral replication at day 6, and improved alveolar fluid clearance, peripheral oxygenation, and cardiac function. Nebulized A77-1726 also reversed influenza-induced increases in lung water content and volume, improved pulmonary mechanics, reduced bronchoalveolar lavage fluid ATP and neutrophil content, and increased IL-6 levels. The ability of A77-1726 to improve cardiopulmonary function in influenza-infected mice and to reduce the severity of ongoing acute respiratory distress syndrome late in infection suggests that pyrimidine synthesis inhibitors are promising therapeutic candidates for management of severe influenza.

3.2 Introduction

Influenza A viruses cause a highly-contagious acute respiratory illness in humans (1). Currently, seasonal influenza accounts for 200,000 hospitalizations and more than 36,000 excess deaths per year in the United States alone, and is the 8th leading cause of attributable annual mortality in this country (2). Moreover, the recent pandemic H1N1 “swine influenza” strain (A/California/09) was estimated to have infected around 61 million people in the United States alone between April 2009 and April 2010, resulting in approximately 275,000 hospitalizations and 12,500 excess deaths (3).

At the present time, annual vaccination is the mainstay of influenza prophylaxis (1). However, rapid influenza virus mutation necessitates annual vaccine reformulation, and current limitations in the speed of vaccine production can
prove problematic when novel influenza strains arise (4). Moreover, attitudes to
vaccination in the general population are by no means uniformly positive (5), and
vaccination rates in the United States are consequently inadequate to fully
control influenza transmission, particularly in high-risk populations (6).
Furthermore, it has been estimated that around 85% of developing countries may
have no access to vaccines during influenza pandemics (7).
Given the current limitations of influenza vaccination, it is highly likely that a
substantial percentage of the population will become infected and require
treatment during the next pandemic. Neuraminidase inhibitors such as
oseltamivir are highly effective at reducing viral replication, disease severity, and
transmission from person-to-person in influenza. However, development of viral
mutants that are resistant to oseltamivir is increasingly common (8). Moreover,
these antivirals are not always widely-available, can be costly, and may be of
only limited use, even in the early stages of infection (9;10). Many patients
present later in disease, and those with severe influenza may go on to develop
acute respiratory distress syndrome (ARDS), which is associated with poor
prognosis (11). Once ARDS has developed, non-specific (and often costly) in-
patient supportive care is the only available treatment option (12;13). Hence,
there is a “therapeutic gap” in influenza, with no virus-specific therapies currently
available to prevent, retard, or manage progression of severe disease to ARDS
in those non-vaccinated subjects who present later in infection when antivirals
are no longer effective. This gap may be of particular significance during
pandemics, in which large numbers of patients could develop disease severe enough to require hospitalization and care in already over-burdened intensive care units (ICUs) (12;14). We found previously that mice infected with a lethal dose of H1N1 influenza A/WSN/33 exhibited nucleotide-mediated bronchoalveolar epithelial dysfunction (impaired alveolar fluid clearance [AFC]) (15). Effective AFC is crucial to efficient gas exchange in the lung (16), and ARDS patients with intact AFC have lower morbidity and mortality than those with compromised AFC (17). We therefore hypothesized that, by reducing intrapulmonary nucleotide levels, early or late post-infection treatment with the de novo pyrimidine synthesis inhibitor A77-1726 (A77) might improve cardiopulmonary function, delay progression to ARDS, and reduce ARDS severity, thereby help to fill the existing “therapeutic gap” in influenza.

3.3 Materials and methods

Mouse inoculation. Eight to 12 week-old BALB/cAnNCr mice were inoculated intranasally with 10,000 plaque-forming units of H1N1 influenza A/WSN/33 in 50 μl PBS with 0.1% BSA under light isoflurane anesthesia. Mice were individually marked and weighed daily. Data for each experimental group were derived from a minimum of two independent infections. All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee.
Administration of nebulized A77. A77 (EMD Biosciences, La Jolla, CA) was reconstituted in DMSO and aliquots of stock solution frozen. Conscious, influenza-infected mice were restrained (SoftRestraints; SciReq, Montreal, Canada) then exposed to 67.5 mg/kg nebulized A77 (from the stock solution diluted 1:1000 in sterile normal saline) using a computer-controlled nose-only aerosol system (inExpose; SciReq). The full A77 dose was delivered to each mouse over a 5-minute period, admixed to room air at a bias flow rate of 2 l/min.

Magnetic resonance imaging of the lung. Isoflurane-anesthetized mice were imaged using a 9.4T BioSpec® high-field small animal magnetic resonance imaging (MRI) system (Bruker BioSpin Corp., Karlsruhe, Germany). Respiratory and cardiac-gated axial T1- and T2-weighted images were acquired over the entire lung. The lung region-of-interest in T1-weighted images was manually outlined by a blinded observer. Lung volumes were calculated by summing outlined regions-of-interest from each contiguous slice. Fluid volumes were measured by setting a global threshold in T2-weighted images and summing the segmented voxels within the lung region-of-interest. For image display purposes (but not data analysis) in Figure 3.4, T1- and T2-weighted images were overlaid and identified by false color, with image contrast adjusted to improve visualization.

Other methods. Preparation of viral inocula and measurements of AFC rates, viral titers, cardiopulmonary function, lung water content, lung mechanics, and bronchoalveolar lavage fluid (BALF) cells and mediators were performed as
previously described (15). Additional details of all methods are provided in an online supplement.

**Statistical analyses.** Descriptive statistics were calculated using Instat 3.05 (GraphPad, San Diego, CA). Weight loss curves were compared by a Log-Rank (Mantel-Cox) test, using Prism software (GraphPad). \( P < 0.05 \) was considered statistically significant. All data are presented as mean ± SEM.

### 3.4 Results

**A77 ameliorates nucleotide-mediated inhibition of alveolar fluid clearance in influenza-infected mice.** We found previously that influenza A/WSN/33 induced AFC impairment at 2-6 days post-inoculation (d.p.i.) and development of lethal primary influenza pneumonia at 6-8 d.p.i. (15). In initial proof-of-concept studies we therefore determined the effects of early (1 d.p.i.) or late (5 d.p.i.) exposure to a single dose of nebulized A77 on AFC at 2 and 6 d.p.i. AFC rates in untreated, influenza-infected mice were comparable to our earlier studies (Fig. 3.1A). Treatment with vehicle (sterile saline + 0.01% DMSO) at 1 d.p.i. had no effect on AFC at 2 d.p.i. (not shown). A77 nebulization at 1 d.p.i. almost completely restored normal AFC at 2 but not 6 d.p.i. A77 treatment at 5 d.p.i. significantly improved AFC at 6 d.p.i.

AFC inhibition in mice infected with replication-competent influenza virus results from increased BALF nucleotide levels (15). In agreement with these findings,
BALF ATP content significantly increased at 2 and 6 d.p.i. (Fig. 3.1B). These effects were reversed by A77 treatment at 1 or 5 d.p.i.

Addition of the epithelial Na\(^+\) channel (ENaC) inhibitor amiloride (1.5 mM) to the AFC instillate reversed the increase in AFC observed in A77-treated mice at 2 d.p.i. (Fig. 3.1C). This indicates that A77 increases ENaC-mediated AFC in influenza-infected mice.

The cystic fibrosis transmembrane regulator (CFTR) Cl\(^-\) channel inhibitor CF\(_{inh}\)-172 (100 μM) reduces AFC in uninfected mice, but improves AFC in untreated animals at 2 d.p.i. by blocking increased Cl\(^-\) secretion (15). As in uninfected mice, CF\(_{inh}\)-172 reduced AFC in A77-treated animals at 2 d.p.i. (Fig. 3.1C). Under Cl\(^-\)-free conditions, which maximize the electrochemical gradient for epithelial Cl\(^-\) secretion, A77 treatment restored normal AFC at 2 d.p.i. (Fig. 3.1D), indicating that A77 restores normal CFTR function in influenza-infected mice.

Because of the significant effects of early and late post-infection A77 therapy on AFC, we investigated its impact on other aspects of murine lung function, particularly those relevant to diagnosis and assessment of ARDS in human subjects.

**A77 prolongs survival, despite increasing post-infection weight loss and having only limited effects on influenza replication.** Treatment with nebulized A77 at 1 d.p.i. increased median time-to-death from 6 to 7 days, although it did not prevent mortality (Fig. 3.2A). Despite this protective effect, A77 treatment at 1 d.p.i. increased the rate of weight loss in infected mice (Fig. 3.2B). At 6 d.p.i.,
body weight was 3% lower in A77-treated animals, although this difference may not be of major clinical importance. Weight loss at 6 d.p.i. was not significantly altered by treatment with nebulized A77 at 5 d.p.i.

Lung homogenate influenza titers did not differ between untreated and A77-treated mice at 2 d.p.i. (Fig. 3.2C), but were reduced at 6 d.p.i. in mice treated with A77 at 1 or 5 d.p.i. However, this effect was modest (< 0.5 log).

**A77 improves cardiopulmonary function.** Carotid $S_aO_2$ declined very significantly by day 6 d.p.i. (Fig. 3.3A), without a compensatory increase in respiratory rate (Fig. 3.3B). Heart rate (Fig. 3.3C) and cardiac contractility (pulse distension; Fig. 3.3D) declined progressively over the course of infection. Similar myocardial dysfunction has been reported previously in patients hospitalized for both seasonal and "swine flu" influenza (18;19).

Treatment with nebulized A77 (67.5 mg/kg) at 1 d.p.i. moderately improved $S_aO_2$ and significantly increased heart rate and cardiac contractility at 2 d.p.i. A77 treatment at 1 or 5 d.p.i. significantly improved $S_aO_2$, heart rate, and pulse distension at 6 d.p.i.

**A77 ameliorates ARDS-like bilateral airspace disease.** In human patients, the presence of bilateral infiltrates on chest X-ray is considered to be an essential criterion for a diagnosis of ARDS (20;21). However, neither radiographic nor computed tomographic imaging provides sufficient resolution and contrast for assessment of pulmonary edema in mice (22). We therefore employed a modified MRI method to accurately and unambiguously quantify lung volume and
water content throughout both lungs over the course of infection (23). Because MRI imaging was performed under moderate isoflurane anesthesia, animals could be recovered, allowing us to follow the progression of edema formation and changes in its spatial distribution over the course of infection on an individual animal basis. As expected, water signal (from T2-weighted scans) was virtually absent in uninfected mice (mean signal < 0.1% of total lung volume, as determined from T1-weighted scans of thoracic structures). Influenza infection resulted in significant pulmonary edema at 2-6 d.p.i. (Figs. 3.4A and 3.4C). At 2 d.p.i., heterogeneous foci of fluid-filled tissue were present in both lungs in a perihilar distribution (not shown). By 6 d.p.i. these regions had coalesced and expanded to the point where large sections of the lung and, in some mice, entire lung lobes became completely fluid-filled (Fig. 3.4C). Comparable heterogeneously-distributed areas of collapsed and over-distended lung tissue have been reported in computed tomographic scans of ARDS patient lungs (24). In contrast, following A77 treatment at 1 d.p.i., T2-weighted (water) signals were almost completely absent from the lungs at both 2 and 6 d.p.i. (Figs. 3.4A and 3.4D).

Influenza induced a significant increase in total lung volume at 6 d.p.i. (Fig. 3.4B), most likely as a result of increased alveolar recruitment (25). However, the mean volume of aerated lung did not significantly increase (from 520 ± 14 mm$^3$ in uninfected mice to 577 ± 30 mm$^3$ at 6 d.p.i.). Lung volumes at 2 and 6 d.p.i. were normal in mice treated with A77 at 1 d.p.i. (Fig. 3.4B).
**A77 attenuates development of pulmonary edema.** Influenza infection resulted in a significant increase in lung wet:dry weight at 2 and 6 d.p.i. (Fig. 3.5A), which correlated very significantly ($P<0.005$) with the percentage of fluid-filled lung volume measured by MRI. A77 treatment at 1 but not 5 d.p.i. blocked the influenza-induced increase in lung water content.

Development of pulmonary edema in untreated mice was accompanied by a significant increase in BALF protein and LDH at 6 d.p.i. (Figs. 3.5B and 3.5C). Treatment with nebulized A77 at 1 or 5 d.p.i. had no effect on these markers of bronchoalveolar epithelial integrity.

**A77 improves lung mechanics.** Total lung resistance increased significantly at 2 and 6 d.p.i. (Fig. 3.6A). In addition, airway hyperresponsiveness to the bronchoconstrictor methacholine was present at 2 d.p.i. (Fig. 3.6B). Both dynamic and static lung compliance declined significantly at 2 and 6 d.p.i. (Figs. 3.6C and 3.6D, respectively). These alterations in the mechanical properties of small airways and lung parenchyma in influenza-infected mice are qualitatively and quantitatively comparable to those observed in human ARDS (21).

Treatment with A77 at 1 d.p.i. normalized total lung resistance and reversed airway hyperresponsiveness at 2 d.p.i. A77 administration at either timepoint also reduced airway resistance at 6 d.p.i. A77 treatment at 1 d.p.i. improved dynamic compliance at 2 but not 6 d.p.i., and mice treated with A77 at 5 d.p.i. had normal dynamic compliance at 6 d.p.i. However, A77 treatment had no effect on static
lung compliance. These findings suggest that A77 primarily improves lung compliance by reducing resistance to airflow rather than parenchymal stiffness.

**A77 decreases pulmonary histopathology.** Influenza A virus infection resulted in mild bronchiolitis at 2 d.p.i., which was not significantly impacted by A77 treatment at 1 d.p.i. (Figs. 3.7A and 3.7B). Marked necrosuppurative bronchopneumonia, alveolar histiocytosis, and perivascular lymphocyte cuffing were evident by 6 d.p.i. in untreated mice (Fig. 3.7C). Severity of histopathologic lesions at this timepoint was significantly ameliorated by A77 treatment, particularly when administered at 1 d.p.i. (Figs. 3.7D and 3.7E).

**A77 reduces bronchoalveolar lavage fluid neutrophil levels.** Tissue pathology in untreated mice was accompanied by a significant increase in BALF total cell counts at 2 and 6 d.p.i. (Fig. 3.8A). By 6 d.p.i., neutrophils accounted for approximately 70% of all cells in BALF (Fig. 3.8B). BALF alveolar macrophage and lymphocyte counts also increased significantly by 6 d.p.i. (Figs. 3.8C and 3.8D, respectively). Treatment with nebulized A77 at 1 d.p.i. reduced total cell counts at 6 d.p.i. by attenuating the neutrophilic response. Neutrophils constituted only 36% of total BALF cells in this group. A77 administration at 5 d.p.i. had a significantly greater effect on BALF neutrophil counts at 6 d.p.i. than that of treatment at 1 d.p.i. In mice treated with A77 at 5 d.p.i., neutrophils constituted only 12% of total BALF cells at 6 d.p.i. In contrast, A77 had no effects on the magnitude or kinetics of mononuclear leukocyte responses.
**A77 alters bronchoalveolar lavage fluid cytokine and chemokine responses.** In untreated mice, influenza induced profound increases in BALF IFN-γ, IL-6, KC, MCP-1 (CCL2), RANTES (CCL5), and TNF-α at 2 d.p.i. (Table 3.1). Moderate increases in other cytokines (IL-1β, IFN-γ, IL-10, and IL-12) were also detected. A77 treatment resulted in an approximately 40% increase in mean IFN-γ, KC, and TNF-α levels at 2 d.p.i., together with a 3-fold rise in IL-6. These changes were accompanied by statistically-significant increases in BALF IL-1β, IL-10, and IL-12 and a reduction in IFN-γ. However, levels of these latter cytokines remained low.

At 6 d.p.i., BALF IFN-γ, KC, RANTES, and TNF-α declined significantly relative to 2 d.p.i. in untreated mice, while IFN-γ and IL-10 content markedly increased. A77 treatment at either 1 or 5 d.p.i. increased mean BALF IL-6 and IL-10 levels at 6 d.p.i. by more than 2- and 3-fold, respectively. Both treatments also increased MCP-1, RANTES, and TNF-α, although TNF-α levels were very low at this timepoint. Finally, A77 treatment at 5 d.p.i. reduced IFN-γ content at 6 d.p.i. A77 treatment at 1 or 5 d.p.i. did not alter BALF levels of other cytokines at 6 d.p.i.

### 3.5 Discussion

Annual vaccination and antiviral drugs are the mainstays of influenza prophylaxis and therapy (1). However, antiviral drugs are only effective early in infection. Patients who present with severe influenza later in infection often progress to ARDS, which is associated with poor prognosis (11). A significant fraction of
patients hospitalized for severe primary influenza pneumonia during the “swine flu” pandemic developed ARDS (26), and histopathologic lesions consistent with this syndrome have been described retrospectively in patients infected with highly-pathogenic H5N1 (“bird flu”) and 1918 “Spanish flu” influenza A strains (27;28). Currently, the only treatment option available for ARDS is non-specific, supportive ICU care (12;13). An important “therapeutic gap” therefore exists, and novel drugs are needed to either delay the onset of ARDS or reduce its severity in this cohort of influenza-infected patients. Such drugs could reduce the potential for ICU overload in pandemic situations (29;30), maintaining the ability of the healthcare system to provide adequate care to all who require it (12;14).

Previously, we demonstrated that influenza induces rapid, nucleotide-mediated AFC impairment (15). As proof-of-concept experiments for the current study, we confirmed this effect, and demonstrated that early or late post-infection treatment with the *de novo* pyrimidine synthesis inhibitor A77 could improve AFC and reduce BALF nucleotide levels at both 2 and 6 d.p.i. Since ARDS patients with intact AFC have lower morbidity and mortality than those with compromised AFC (17), we investigated whether A77 might have additional beneficial effects in influenza-infected mice. We found that, despite having only a limited antiviral effect, A77 exposure at 1 d.p.i delayed mortality and resulted in prolonged improvements in $S_aO_2$, cardiac function, pulmonary edema, lung mechanics, and histopathology, thereby retarding progression to ARDS. Moreover, these beneficial effects of A77 treatment persisted for up to 5 days, possibly because
this drug can bind with high affinity to proteins in the alveolar lumen or within respiratory cells (http://products.sanofi.us/arava/arava.html). Most importantly, however, A77 treatment late in infection (at 5 d.p.i.) also resulted in significant improvements in $S_aO_2$, pulmonary edema, airway mechanics, and neutrophil infiltration at 6 d.p.i., by which time severe lung injury and ARDS are already present. We know of no currently-available drugs with similar efficacy – ICU mainstays such as corticosteroids and β-adrenergic agonists generally have very limited beneficial effects on lung function following the onset of severe viral acute lung injury (12). A77 is therefore a promising candidate agent to bridge the current “therapeutic gap” in influenza, and may be of particular value in management of late-stage disease.

Treatment with A77 at 1 d.p.i. significantly delayed mortality, although it did not prevent the ultimate demise of influenza-infected mice and actually increased the rate of body weight loss. Based on these criteria, which are commonly used as outcome measures in murine influenza studies, our findings may be interpreted as being indicative of limited A77 efficacy. However, infection of human subjects does not induce the extremely rapid, severe, and probably intrinsically lethal weight loss observed in influenza-infected mice (>30% in 6 days). Moreover, pre-admission changes in body weight are not considered to be of clinical significance in management of human patients with ARDS (12). In addition, weight loss and mortality in mice have been shown to be poorly-correlated with human ARDS outcomes and are often not of predictive value in determining
clinical efficacy of novel therapeutics (31). The rapid onset of severe anorexia and cachexia following influenza infection by no means negates the utility of the mouse as a model for most aspects of influenza pathogenesis. However, it may be an intrinsic limitation of this species as a model for predicting effects of novel therapeutics on ARDS severity and survival in human patients with severe disease. We therefore focused our studies on the effects of A77 treatment on indices of murine cardiopulmonary function that reflect those used to diagnose and determine the severity of ARDS in patients.

For a diagnosis of ARDS to be made in the ICU, a patient must exhibit a specific set of clinical signs which are indicative of severe lung dysfunction and injury: acute onset of significant hypoxemia (decreased $P_aO_2$ or $S_aO_2$, which are strongly correlated in humans (32)) in the presence of bilateral chest X-ray infiltrates and the absence of left atrial hypertension, together with either predisposing lung disease or decreased lung compliance (20;21). Experimentally, additional predictors of poor ARDS outcomes in patients have been identified, including impaired AFC (17). Readouts of lung function analogous to those used for diagnosis of ARDS in man are now considered to be highly-relevant and appropriate indices of acute lung injury in murine models, primarily because of their translational relevance (31). Using these readouts, we found that cardiopulmonary dysfunction and lung injury severity in influenza-infected mice were ameliorated by both early and late post-inoculation single-dose A77 treatment. Since both caloric and fluid intake can be easily controlled in
an ICU setting, the improvements in cardiopulmonary function imparted by A77 treatment may be sufficient to significantly improve survival rates in patients with severe influenza, irrespective of any effects of A77 on the rate of weight loss in influenza-infected mice.

Development of severe disease in mice infected with highly-pathogenic influenza strains has been associated with increased viral replication rates (33), induction of a “cytokine storm” (34-36), and/or excessive neutrophil infiltration (37-40). A77 improved cardiopulmonary function without significant antiviral effects, which implies that the severity of lung dysfunction in influenza may be independent of viral replication. Nevertheless, it is possible that early post-infection treatment with a combination of oseltamivir and A77, or use of a pyrimidine synthesis inhibitor with intrinsic antiviral activity such as A3 (41) might prove to have enhanced beneficial effects in our model.

The contribution of a “cytokine storm” to the pathogenesis of ARDS associated with severe influenza remains unclear. Hypercytokinemia has been associated with fatal outcome in human H5N1 influenza (42), yet TNF-α or IL-6-knockout mice are not protected against H5N1 disease in mice (35;43). We found little to no quantitative or temporal correlation between most BALF cytokines and chemokines and improved cardiopulmonary function in A77-treated mice. For example, A77 administration at 1 or 5 d.p.i. resulted in comparable improvements in cardiopulmonary function at 6 d.p.i., despite opposing effects of these treatments on BALF IFN-γ and IL-10. Relative to untreated controls, A77
nebulization at 1 d.p.i. increased IL-10 but not IFN-γ at 6 d.p.i. In contrast, exposure to A77 at 5 d.p.i. increased IFN-γ but not IL-10 at day 6. However, BALF IL-6 levels increased at both 2 and 6 d.p.i. following A77 treatment at 1 or 5 d.p.i. Hence, A77-induced improvements in cardiopulmonary function were consistently associated with elevated IL-6 levels - this was not the case for any other cytokine analyzed. Taken together, however, our data indicate that cardiopulmonary dysfunction is not the result of a generalized “cytokine storm”, although it may be associated with changes in the relative levels and/or kinetics of individual cytokine responses to influenza infection.

The functional consequences of altered cytokine profiles in A77-treated mice remain unclear. IL-6 is predominantly pro-inflammatory, and is often correlated with poor ARDS outcomes in humans (44). However its effects are pleiotropic and IL-6 can even be lung-protective in some lung injury models (45-47). Therefore, without further research, we cannot determine whether the relationship between elevated IL-6 and improved cardiopulmonary function in A77-treated mice is correlative or causative. Likewise, it is possible that the approximately 40% increase in mean BALF TNF-α levels at 2 d.p.i. in A77-treated mice may be of concern, particularly given the potential of this cytokine to exacerbate epithelial injury and capillary leak (48). However, in our previous studies, we did not find any increase in BALF protein levels or alveolar barrier permeability to instilled FITC-albumin tracers at this timepoint (15).
Improved cardiopulmonary function in A77-treated mice was associated with reduced BALF neutrophil counts at both 2 and 6 d.p.i. While only correlative, these findings suggest that beneficial effects of A77 on lung function might in part result from attenuation of the neutrophil response. Previous studies have shown that neutrophils and neutrophil-derived reactive oxygen species are involved in the pathogenesis of influenza-induced lung injury, although it is currently unclear whether they are detrimental or protective in influenza (37;49;50). Importantly, reactive species can alter both endothelial permeability and respiratory epithelial cell ion transport. Abrogation of the neutrophil response may therefore contribute to beneficial effects of A77 on lung function. However, in our earlier studies, we did not find evidence of elevated reactive species generation in our model (15).

Nucleotide-mediated AFC inhibition occurred before the onset of severe cardiopulmonary dysfunction, suggesting that AFC inhibition and consequent pulmonary edema are the primary insults induced by influenza. Alveolar edema reduces the available surface area for gas exchange and increases diffusion distance, resulting in peripheral hypoxemia and subsequent cardiac dysfunction. Likewise, edema fluid will dilute surfactant (lowering dynamic compliance) and reduce the effective diameter of small airways (increasing airway resistance). Compression of small airways by adjacent fluid-filled alveoli and interstitial tissues will further increase resistance. The fact that A77 is able to reverse almost all the detrimental effects of influenza infection supports this hypothesis, since such multiple beneficial effects would be unlikely unless this drug impacts a
single upstream event in a cascade of cardiopulmonary dysfunction. However, A77 could not prevent bronchoalveolar epithelial damage, suggesting that loss of epithelial integrity may instead be a consequence of viral or immune-mediated cytopathologic effects.

In conclusion, we have shown that early or late post-infection aerosol treatment of influenza-infected mice with the de novo pyrimidine synthesis inhibitor A77 delays mortality and significantly improves a variety of indices of cardiopulmonary function pertinent to diagnosis of ARDS in human subjects. These include $S_aO_2$, myocardial contractility, lung water content, and lung mechanics. In addition, the beneficial effects of this drug are associated with reduced neutrophil infiltration and elevated BALF IL-6 levels. Our findings suggest that A77 has the potential to retard progression to ARDS in influenza-infected human patients over a prolonged period (5 days or more). More importantly, A77 or related agents (41) may be of particular value in reducing the severity of lung dysfunction in late-stage influenza, when treatment is currently limited to supportive measures. Hence, nucleotide synthesis inhibitors such as A77 are promising candidates to fill the current "therapeutic gap" in influenza.
3.6 Figures

Figure 3.1: A77 ameliorates nucleotide-mediated inhibition of alveolar fluid clearance.

(A) Effect of treatment with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) on baseline alveolar fluid clearance rate (AFC) at 2 d.p.i. (n = 7) and 6 d.p.i. (n = 15), or A77 treatment at 5 d.p.i. (A77-Tx DAY 5) on AFC rate at 6 d.p.i. (n = 6), compared with untreated animals (n = 12 per timepoint). (B) Effect of treatment with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) on bronchoalveolar lavage fluid (BALF) ATP content at 2 d.p.i. (n = 5) and 6 d.p.i. (n = 9), or A77 treatment at 5 d.p.i. (A77-Tx DAY 5) on AFC rate at 6 d.p.i. (n = 10), compared with untreated animals (n = 5 per timepoint). (C) Effect of treatment with nebulized A77 at 1 d.p.i. (A77-Tx DAY 1) on day 2 AFC in the presence of 1.5 mM amiloride (AMIL) or 100 μM CFTR inhibitor-172 (CF-inh), compared with untreated animals (n = 7-9 per group). (D) Effect of treatment with nebulized A77 at 1 d.p.i. (A77-Tx DAY 1) on day 2 AFC in Cl⁻-free (Na⁺ gluconate) BSA (n = 7 per group). Dotted lines indicate mean values for each parameter in uninfected mice. *P < 0.05, ***P < 0.0005, vs. uninfected mice. (†)P < 0.1, †P < 0.05, #P < 0.0005, vs. infected, untreated mice (UNTx) at the same post-infection timepoint.
Figure 3.2: A77 prolongs survival, despite increasing post-infection weight loss and having only limited antiviral effects.

Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) or 5 d.p.i. (A77-Tx DAY 5) on: (A) Mortality \((n \geq 10\) per group); (B) Body weight (BWT; \% change from baseline; \(n > 15\) per group, including data from mice that die prior to 6 d.p.i.); and (C) Influenza virus replication (log titer in whole lung homogenates; \(n \geq 5\) per group). ***\(P < 0.0005\), vs. untreated mice (UNTx). † \(P < 0.05\), ‡ \(P < 0.005\), vs. untreated mice at the same post-infection timepoint.
Figure 3.3: A77 improves cardiopulmonary function in conscious mice. Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) or 5 d.p.i. (A77-Tx DAY 5) on: (A) Carotid arterial $O_2$ saturation ($S_aO_2$; %); (B) Respiratory rate (RR; breaths/minute); (C) Heart rate (HR; beats/minute); and (D) Pulse distension (PD; $\mu$m). $n > 10$ per group. Dotted lines indicate mean values for each parameter in uninfected mice. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, vs. uninfected mice. $^\dagger P < 0.05$, $^\# P < 0.0005$, vs. untreated mice (UNTx) at the same post-infection timepoint.
**Figure 3.4: A77 ameliorates bilateral airspace disease.**

Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) on: (A) Lung water content (% of total lung volume) at 2 d.p.i. ($n = 7$) and 6 d.p.i. ($n = 4$), compared with untreated animals ($n = 6$ per timepoint). $< 0.1\%$ T2-weighted (edema) signal was detected in uninfected mice ($n = 8$); and (B) Total lung volume (cm$^3$) in the same experimental groups. Dotted line indicates mean lung volume in uninfected mice. (C) Representative day 6 mid-thoracic magnetic resonance image from an untreated, influenza-infected mouse. T1-weighted images (body tissues) are pseudo-colored red. T2-weighted images (edema) are pseudo-colored green. LV: left ventricular lumen. (D) Representative day 6 mid-thoracic magnetic resonance image from an influenza-infected mouse treated with nebulized A77 at 1 d.p.i. All mice were anesthetized with isoflurane and imaged using an 11.7T BioSpec® high field small animal magnetic resonance imaging system with respiratory and cardiac gating. Images were adjusted in brightness, contrast, and color balance for a more uniform appearance. These adjustments do not obscure, eliminate, or misrepresent any information presented in the original scans. $***P < 0.0005$, compared with uninfected mice. $†P < 0.05$, vs. untreated mice (UNTx) at the same post-infection timepoint.
Figure 3.5: A77 attenuates development of pulmonary edema.
Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) or 5 d.p.i. (A77-Tx DAY 5) on: (A) Lung water content (wet:dry weight ratio; n = 6-9 per group); (B) BALF protein content (µg/ml; n = 6-10 per group); and (C) BALF lactate dehydrogenase (LDH) content (mU/ml; n = 5-7 per group). Dotted lines indicate mean values for each parameter in uninfected mice. *P < 0.05, **P < 0.005, ***P < 0.0005, vs. uninfected mice. †P < 0.05, #P < 0.0005, vs. untreated mice (UNTx) at the same post-infection timepoint.
Figure 3.6: A77 improves lung mechanics.
Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) or 5 d.p.i. (A77-Tx DAY 5) on: (A) Baseline total lung resistance ($R_{\text{BASAL}}$; cmH$_2$O.s/ml); (B) Maximal airway resistance following nebulization of 50 mg/ml methacholine ($R_{\text{MAX}}$; cmH$_2$O.s/ml); (C) Dynamic lung compliance ($C_{\text{DYN}}$; ml/cmH$_2$O x 10); and (D) Static lung compliance ($C_{\text{ST}}$; ml/cmH$_2$O x 10). All mice were anesthetized with valium/ketamine and mechanically ventilated on a flexiVent computer-controlled piston ventilator, with 8 ml/kg tidal volume, at a frequency of 150 breaths/minute, against 3 cmH$_2$O PEEP. $n = 5$-7 per group. Dotted lines indicate mean values for each parameter in uninfected mice. **$P < 0.005$, ***$P < 0.0005$, compared with uninfected mice. ‡$P < 0.005$, $#P < 0.0005$, vs. untreated mice (UNTx) at the same post-infection timepoint.
Figure 3.7: A77-1726 decreases pulmonary histopathology. Representative parenchymal and airway histopathology in hematoxylin/eosin-stained lung tissue sections from influenza-infected untreated and A77-1726-treated mice: (A) 2 d.p.i., untreated mice; (B) 2 d.p.i., mice treated with A77-1726 at 1 d.p.i.; (C) 6 d.p.i., untreated mice; (D) 6 d.p.i., mice treated with A77-1726 at 1 d.p.i.; (E) 6 d.p.i., mice treated with A77-1726 at 5 d.p.i. Scale bar = 100 μm. Original objectivelens magnification: 10x.
Figure 3.8: A77 reduces bronchoalveolar lavage fluid neutrophil levels in influenza-infected mice.

Effect of treatment of influenza-infected mice with nebulized A77 (67.5 mg/kg) at 1 d.p.i. (A77-Tx DAY 1) or 5 d.p.i. (A77-Tx DAY 5) on BALF numbers of: (A) Total cells; (B) Neutrophils (PMNs); (C) Alveolar macrophages (AMs); and (D) Lymphocytes (Lymphs). n ≥ 10 per group. All cell counts are expressed as x 10^6/ml. Dotted lines indicate mean values for total cells and alveolar macrophage counts in uninfected mice (not shown for neutrophils and lymphocytes as counts for both cell types are less than 10^4/ml in uninfected mice). *P < 0.05, **P < 0.005, ***P < 0.0005, vs. uninfected mice. †P < 0.005, ‡P < 0.0005, vs. untreated mice (UNTx) at the same post-infection timepoint.
3.7 References


tactics for use of the U.S. antiviral strategic national stockpile for pandemic

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Chapter 4: **Heterozygosity for the F508del mutation in the cystic fibrosis transmembrane conductance regulator anion channel attenuates influenza severity**

4.1 Abstract

Seasonal and pandemic influenza are significant public health concerns. Influenza stimulates respiratory epithelial Cl⁻ secretion via the cystic fibrosis transmembrane conductance regulator (CFTR). The purpose of this study was to determine the contribution of this effect to influenza pathogenesis in mice with reduced CFTR activity. C57BL/6-congenic mice heterozygous for the F508del CFTR mutation (HET) and wild-type (WT) controls were infected intranasally with 10,000 FFU/mouse influenza A/WSN/33 (H1N1). Body weight, arterial O₂ saturation, and heart rate were monitored daily. Pulmonary edema and lung function parameters were derived from wet:dry weight ratios and the forced-oscillation technique, respectively. Bronchoalveolar lavage fluid cytokines and chemokines were measured by ELISA. Relative to WT mice, influenza-infected HETs showed significantly delayed mortality, which was accompanied by attenuated hypoxemia, cardiopulmonary dysfunction, and pulmonary edema. However, viral replication and weight loss did not differ. The protective HET phenotype was correlated with exaggerated
alveolar macrophage and IL-6 response to infection and was abrogated by alveolar macrophage depletion using clodronate liposomes. Reduced CFTR expression modulates the innate immune response to influenza and alters disease pathogenesis. CFTR-mediated Cl⁻ secretion is therefore an important host determinant of disease and CFTR inhibition may be of therapeutic benefit in influenza.

4.2 Introduction
Seasonal influenza is the 8th leading cause of attributable annual mortality in the United States of America (USA), accounting for ~200,000 hospitalizations and >36,000 excess deaths per year (1). Moreover, the recent pandemic of H1N1 “swine influenza” was estimated to have infected ~61 million people in the USA between April 2009 and April 2010, resulting in approximately 12,500 excess deaths (2). Future emergence of novel and more highly-virulent influenza strains may result in pandemics with devastating loss of life. For example, the 1918 “Spanish influenza” pandemic was estimated to have caused ~50 million deaths worldwide (3). Influenza is therefore a significant public health concern. Respiratory epithelial cells are the main infection target and site of replication for influenza viruses (4;5). A primary function of these cells is to regulate the depth of the thin (8 to 10 μm) layer of fluid lining the airspaces, thereby facilitating normal gas exchange and effective mucociliary clearance. Apical amiloride-sensitive epithelial sodium (Na⁺) channels (ENaC) and cystic fibrosis
transmembrane conductance regulator (CFTR) chloride (Cl\(^-\)) channels play a central role in this process (6). ENaC-mediated active transport of Na\(^+\) ions from the alveolar lining fluid to the subepithelial interstitial space creates a transepithelial gradient for Cl\(^-\) absorption via CFTR. CFTR also regulates ENaC activity – reduced CFTR function induces ENaC hyperactivity (7). Transepithelial NaCl transport creates an osmotic gradient, causing water to move passively from the airspace to the interstitium.

ENaC and CFTR activity are primary determinants of normal lung function. ENaC inhibition and/or stimulation of CFTR-mediated Cl\(^-\) secretion leads to accumulation of fluid in the alveolar space, impaired gas exchange, and hypoxemia (8;9). Those patients with acute lung injury that have intact transepithelial ion transport have lower morbidity and mortality (10). In contrast, the absence of functional CFTR and/or ENaC-mediated Na\(^+\) hyperabsorption result in cystic fibrosis (CF), which is characterized by decreased airspace lining fluid depth, mucus dehydration, and impaired mucociliary clearance (11;12). CF currently afflicts an estimated 30,000 people in the United States and its most common cause is homozygosity for the F508del CFTR mutation (a phenylalanine deletion at position 508) (13). Although CF affects multiple organ systems, respiratory complications account for nearly 85% of CF mortality (13).

We have shown that infection of mice with respiratory syncytial virus resulted in ENaC inhibition and moderate pulmonary edema (14;15). Although infection with H1N1 influenza A/WSN/33 led to a comparable reduction in ENaC-mediated Na\(^+\)
transport, influenza also stimulated CFTR-mediated Cl\(^-\) secretion by the respiratory epithelium and induced severe pulmonary edema and hypoxemia (16-18). We therefore hypothesized that increased disease severity in influenza-infected mice (relative to those infected with respiratory syncytial virus) is a consequence of CFTR stimulation. Further, we speculated that presence of the F508del mutation would reduce CFTR-mediated Cl\(^-\) secretion, thereby attenuating pulmonary edema and disease severity. In the current study, we found that influenza-infected F508del CFTR-heterozygous (HET) C57BL/6-congenic mice had improved survival and less severe lung dysfunction than wild-type (WT) littermate controls, despite comparable levels of viral replication and weight loss. The protective phenotype in HET mice was associated with very significant increases in BALF alveolar macrophage and IL-6 content. Moreover, alveolar macrophage depletion with clodronate liposomes (CL-LIP) attenuated the IL-6 response and increased influenza severity to WT levels. Our findings suggest that CFTR-mediated Cl\(^-\) secretion is an important determinant of disease severity and that CFTR inhibition may be of therapeutic benefit in influenza.

**4.3 Materials and methods**

**Breeding and Genotyping of F508del mice.** C57BL/6-congenic F508del CFTR-heterozygous and –homozygous mutants and WT controls were generated by breeding B6.129S7-Cfr\(^{tm1Kth}\) mice (19). 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 (17;18). Mice were individually marked and weighed daily. Data for each experimental group were derived from a minimum of 2 independent infections.

**Depletion of alveolar macrophages.** Mice were treated intranasally with 100 μl of CL-LIP under light isoflurane anesthesia 48 hours prior to infection and every 72 hours thereafter (20). Clodronate was encapsulated in phosphatidylcholine/cholesterol liposomes at ~5 mg/ml liposome suspension (21). CL-LIP were a gift from Dr. Nico van Rooijen, Vrije Universiteit of Amsterdam, Amsterdam, Netherlands. Pilot studies indicated that undiluted CL-LIP and those diluted 1:1 in PBS caused comparable macrophage depletion, but undiluted CL-LIP induced more respiratory compromise. As in prior studies (20), CL-LIP were therefore administered in diluted form. The degree of alveolar macrophage depletion was determined from BALF total and differential cell counts.

**Measurement of lung mechanics.** Mechanical properties of the mouse lung were assessed in valium/ketamine-anesthetized, tracheotomized mice using the forced-oscillation technique (22) as in our previous studies (23). 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, static lung compliance, and dynamic lung compliance at baseline were calculated using the single-compartment model (22). Maximal airway responsiveness to bronchoconstrictors was measured following exposure to increasing doses of nebulized methacholine (0.1 to 50 mg/ml).

**Measurement of bronchoalveolar lavage fluid inflammatory mediators.** BALF protein was determined by a BCA assay. Murine IFN-γ, IL-6, IL-10, and CXCL-1/KC were quantified by an ultra-sensitive mouse proinflammatory multiplex electro-chemiluminescence assay (Meso Scale Discovery, Gaithersburg, MD). Murine IFN-α, CXCL-10/IP-10, CCL-2/MCP-1, and CCL-5/RANTES levels were measured using Quantikine ELISA kits (all R & D Systems, Minneapolis, MN). All assays were performed in accordance with manufacturer's instructions.

**Other methods.** Preparation of histopathologic images, bronchoalveolar lavage, and measurements of carotid arterial O₂ saturation, heart rate, lung homogenate viral titers, and lung wet:dry weight ratios were performed as in our previous studies (15;16).

**Statistical Analysis.** Survival data were analyzed by a log-rank (Mantel-Cox) test using GraphPad Prism 5.04 (GraphPad Software, San Diego, CA). 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.

4.4 Results

**Heterozygosity for the F508del CFTR mutation delays mortality following influenza infection.** Compared to WT littermate control mice, HETs showed significantly delayed mortality following infection with a lethal dose of H1N1 influenza virus (A/WSN/33). Median time-to-death increased from 7 days in WT mice to 8 days in HETs (Figure 4.1A). All WT controls died by 8 days post-infection (d.p.i.), while 25% of HETs survived. In contrast, median time-to-death did not differ between WT controls and congenic F508del CFTR-homozygous mice, and all homozygotes died (Figure 4.1B). Given the lack of impact of homozygosity on influenza mortality, subsequent studies focused solely upon differences between WT and HET animals.

**Pulmonary histopathology is attenuated in HET mice.** At 2 d.p.i., moderate interstitial pneumonitis was present in both WT mice (Fig. 4.2A) and HETs (Fig. 4.2D). At 6 d.p.i., widespread, severe interstitial bronchoalveolar pneumonitis was present in WT animals, and alveoli contained large amounts of protein-rich edema fluid with fibrin (Figs. 4.2B and 4.2C). In contrast, only moderate interstitial alveolitis was evident in HET lungs at 6 d.p.i. and alveolar spaces contained large numbers of alveolar macrophages but no edema fluid (Figs. 4.2E and 4.2F).
Infection with influenza A virus induces an exaggerated, protective alveolar macrophage response in the lungs of HET mice. Over 95% of cells in BALF from uninfected WT mice and HETs were alveolar macrophages and total cell counts did not differ between the two strains. However, BALF alveolar macrophage counts were almost 3-fold higher in HETS than WT mice at 6 d.p.i. (Figure 4.3A). BALF lymphocyte and neutrophil counts also increased in both WT and HET mice at 2 and 6 d.p.i., but did the magnitude of these increases did not differ between strains at either timepoint (Figures 4.3B and 4.3C, respectively).

To determine whether an exaggerated alveolar macrophage response contributed to delayed mortality in influenza-infected HET mice, we depleted alveolar macrophages using CL-LIP (21). As in prior studies (20), intranasal CL-LIP administration (250 µg/mouse in 100 µl) resulted in depletion of approximately 70% of BALF alveolar macrophages in uninfected WT mice at 48 hours, without inducing any loss of body weight or other toxicity. BALF macrophage counts declined from a mean of $7.3 \pm 0.5 \times 10^4$ to $2.4 \pm 0.8 \times 10^4$ ($P<0.005; n=4$).

Intranasal CL-LIP administration reduced BALF alveolar macrophage counts (and hence total cell numbers) at 6 d.p.i. by 50% in WT mice and 75% in HETs (Figure 4.3A). Final BALF alveolar macrophage numbers did not differ between CL-LIP-treated WT and HET animals. Likewise, following CL-LIP treatment, BALF neutrophil and lymphocyte numbers at 6 d.p.i. did not differ between WT and HET mice (Figs. 4.3B and 4.3C, respectively).
Treatment of WT mice with CL-LIP did not alter median time-to-death after influenza infection (n=5; not shown). In contrast alveolar macrophage depletion reduced median time-to-death to 5.5 days in influenza-infected HETs (Fig. 4.3D). Because CL-LIP treatment did not alter BALF neutrophil counts in HETs, but normalized macrophage counts in a fashion that also resulted in increased lethality, these data suggest that the heterozygous advantage in influenza-infected mice results from an exaggerated influx of alveolar macrophages into the lungs. Moreover, the lack of effect of CL-LIP on mortality in WT mice indicates that the detrimental effects of this drug on survival in HETs were not due to nonspecific toxic effects of clodronate.

**Influenza-infected HETs exhibit macrophage-dependent, replication-independent amelioration of cardiopulmonary dysfunction.** Despite the protective effect of heterozygosity for the F508del CFTR mutation on influenza mortality, and reversal of this effect by alveolar macrophage depletion, the rate of post-infection weight loss did not differ between WT, untreated HET, and macrophage-depleted HET mice (Fig. 4.4A). Carotid arterial oxygen saturation (S\textsubscript{a}O\textsubscript{2}) and heart rate did not differ between WT mice, untreated HETs, and CL-LIP-treated HETs prior to influenza infection. However, while S\textsubscript{a}O\textsubscript{2} initially declined at the same rate in all three groups following infection, untreated HETs experienced less severe hypoxemia than WT and macrophage-depleted F508del mice at 6 d.p.i. (Fig. 4.4B). Likewise, moderate tachycardia was observed in all
groups at 2 d.p.i., but only WT controls and macrophage-depleted HETs exhibited physiologically-significant bradycardia at 6 d.p.i. (Fig. 4.4C). Comparable influenza titers were detected in lung homogenates from WT and HET mice at 2-6 d.p.i. (Fig. 4.4D). As in previous studies (20;24), day 6 lung homogenate influenza titers increased by ≥ 1.5 logs in both WT mice and HETs following CL-LIP treatment. However, titers did not differ between CL-LIP-treated WT controls and HETs at 6 d.p.i.

**Heterozygosity for the F508del CFTR mutation prevents influenza-induced pulmonary edema in a macrophage-dependent fashion.** Influenza infection of WT mice for 2 or 6 days resulted in a progressive increase in lung water content (wet:dry weight ratio), indicating development of severe pulmonary edema (Fig. 4.5A). By 6 d.p.i., mean lung water content in both untreated and CL-LIP-treated WT controls had increased by 160% from baseline values. In contrast, no pulmonary edema developed in influenza-infected HETs, even at 6 d.p.i. However, day 6 lung wet:dry weight ratios increased to WT levels in alveolar macrophage-depleted HETs.

Development of pulmonary edema in both WT mice and HETs was accompanied by an increase in BALF protein content at 6 d.p.i., which is indicative of increasing damage to the bronchoalveolar epithelial barrier (Fig. 4.5B). However, BALF protein levels remained lower in HETs than WT controls at day 6. Finally, BALF protein levels at 6 d.p.i. were not significantly affected by alveolar macrophage depletion in either strain.
Detrimental effects of influenza infection on airway resistance and lung compliance are attenuated in HETs. Static and dynamic lung compliance are indices of lung tissue stiffness and resistance to inflation on inspiration, which are measured at a fixed lung volume and during normal ventilation, respectively. As in previous studies (25), static and dynamic lung compliance were higher in uninfected HET mice than in WT controls, but this effect was not statistically-significant (Figs. 4.6A and 4.6B, respectively). Following infection, both static and dynamic compliance progressively declined in WT mice over the course of infection, but remained normal in untreated, influenza-infected HETs. CL-LIP treatment of influenza-infected HETs reduced both static and dynamic compliance to day 6 WT levels.

Baseline total lung resistance to airflow did not differ between uninfected WT mice and HETs. Infection induced a progressive and significant increase in total lung resistance from 2-6 d.p.i. in both groups, but this effect was attenuated in HETs (Fig. 4.6C). Alveolar macrophage depletion had no effect on baseline total lung resistance in WT mice at 6 d.p.i., but induced a significant increase in HETs. Finally, airway hyperresponsiveness to methacholine was present at 2 d.p.i. in WT controls, but not HETs (Fig. 4.6D), indicating that influenza does not induce exaggerated bronchoconstrictive responses in HETs.

Bronchoalveolar lavage fluid cytokine and chemokine responses to influenza infection differ significantly between wild-type mice and HETs. Minimal levels of inflammatory cytokines and chemokines were detectable in
BALF from uninfected WT or HET mice (not shown). BALF IFN-α, IL-6, CCL-2/MCP-1, CXCL-1/KC, and CXCL-10/IP-10 increased significantly in WT mice at 2 d.p.i. (Table 4.1). However, with the exception of CCL-2, these responses were either absent or attenuated in HETs. In both genotypes, BALF contained minimal amounts of IFN-γ, IL-10, and CCL-5/RANTES at this timepoint.

At 6 d.p.i., IFN-α, IL-6, and CXCL-1/KC levels declined in WT mice, but all increased significantly in HETs. The difference in BALF IL-6 levels between WT and HET mice at 6 d.p.i. was particularly dramatic. IFN-γ, IL-10, CCL-2/MCP-1, CCL-5/RANTES, and CXCL-10/IP-10 increased to a comparable degree in both strains at 6 d.p.i. Finally, alveolar macrophage depletion attenuated day 6 cytokine responses in both WT and HET mice, but further increased BALF CXCL-10/IP-10 and levels.

4.5 Discussion

Despite vaccination and use of antiviral drugs, seasonal influenza is a significant threat to public health (26). Moreover, while the 2009 H1N1 “swine flu” pandemic did not cause significant excess mortality, the 1918 pandemic may have killed over 50 million people worldwide, and there is no reason to believe that future outbreaks may be any less severe (27-29). However, our understanding of basic mechanisms underlying the pathogenesis of severe cardiopulmonary dysfunction and lung injury in influenza remains limited (30). In the current study, we found that influenza-infected HET mice had improved survival and less severe lung
dysfunction than wild-type WT controls, despite comparable levels of viral replication and weight loss. This unprecedented protective phenotype in HET mice was associated with exaggerated alveolar macrophage and IL-6 responses to infection and was lost following alveolar macrophage depletion with CL-LIP. Our findings show for the first time that altered ion channel activity can modulate the pathogenesis of any pulmonary viral infection of the lung. Moreover, they suggest that CFTR-mediated Cl− secretion is an important host determinant of influenza severity and that CFTR inhibition may be of therapeutic benefit in influenza.

CFTR expressed on the apical surface of respiratory epithelial cells plays a central role in regulation of alveolar lining fluid depth. Consequently, altered CFTR activity can significantly impact normal lung function. We have shown previously that influenza stimulates CFTR-mediated Cl− secretion, which contributes to reduced clearance of excess intra-alveolar fluid (16). However, CFTR is also expressed on monocytes, alveolar macrophages, and lymphocytes (31). Multiple studies have shown that, as well as its detrimental effects on mucociliary clearance, CF results in significant alterations in respiratory epithelial cell and alveolar macrophage innate immune responses to bacterial pathogens (32). These include attenuation of type I IFN production by respiratory epithelial cells and increased production of IL-6 and CXCL-1/KC by alveolar macrophages (33;34). We and others have shown that lung function is not adversely affected by F508del CFTR heterozygosity in either mice or humans (25). However, there
is currently very little information regarding the impact of heterozygosity for F508del CFTR on immune responses to either bacterial or viral lung pathogens. We found that baseline BALF cell, cytokine, and chemokine levels did not differ between uninfected WT mice and HETs, which suggests that immune function of these cells is not intrinsically abnormal. However, our data indicate that both respiratory epithelial (type I IFN) and alveolar macrophage (IL-6) cytokine responses to influenza are altered in HETs. The cellular mechanisms underlying these alterations have not yet been defined, and it is not clear whether they are influenza-specific or extend to other viral and bacterial lung pathogens: since epithelial and alveolar macrophage expression of toll-like receptors 2 and 4 is altered in CF, it is possible that HETs might also respond differently to bacterial pathogens (35;36). Finally, the impact of homozygosity for F508del CFTR on influenza pathogenesis is unknown – although homozygote mortality did not differ from WT, this does not necessarily imply that both genotypes responded similarly to infection.

Increased mortality rates following infection with highly-pathogenic influenza strains have been correlated with several factors, including a higher rate and extent of viral replication (37), a greater neutrophil response to infection (24;38;39), development of a “cytokine storm” (40-42), and an attenuated alveolar macrophage response (20;24;37). Our findings suggest that the protective effect of heterozygosity for F508del CFTR in influenza is associated with the last of these factors. Attenuation of post-infection cardiopulmonary
dysfunction in HETs was not a consequence of differences in viral replication kinetics or BALF neutrophil counts between WT mice and HETs. Likewise, the IFN-γ response to infection did not differ between WT controls and HETs at 6 d.p.i., suggesting that reduced disease severity in HETs was not associated with a reduced “cytokine storm” per se. In contrast, exaggerated alveolar macrophage and IL-6 responses to infection were only present in HETs. Moreover, alveolar macrophage depletion with CL-LIP both reduced IL-6 production and increased disease severity at 6 d.p.i. in HETs, but had no such effects in WT controls. Furthermore, as in previous studies, CL-LIP treatment increased viral titers and lung edema and reduced BALF neutrophils in both WT mice and HETs, but only altered disease progression in the latter group (20;24).

Increased BALF IL-6 is a common component of the “cytokine storm”, but its role in influenza-pathogenesis is not fully understood: some investigators have reported an association between high BALF IL-6 and increased mortality (43), while others have found that disease severity is not reduced in IL-6-knockout mice (44;45), or even that IL-6 is protective (46). We found a profound increase in BALF IL-6 at 6 d.p.i in HET but not WT mice, which was ablated by alveolar macrophage depletion. Importantly, IL-6 can stimulate ENaC activity (47). Hence, we hypothesize that attenuation of cardiopulmonary dysfunction in influenza-infected HETs results from a combination of the effects of this mutation on both bronchoalveolar epithelial and alveolar macrophage function: following infection, mutant CFTR expressed in the epithelium has reduced capacity for Cl− secretion.
and ENaC inhibition, while expression of this mutation on alveolar macrophages results in increased IL-6 production and a subsequent increase in epithelial ENaC activity. Together, these improve transepithelial ion transport, reduce the depth of the alveolar lining fluid, attenuate hypoxemia, and improve outcome. By extension, we propose that increased CFTR-mediated Cl⁻ secretion and inadequate alveolar macrophage and/or IL-6 responses may contribute to the greater disease severity associated with infection by highly-pathogenic influenza strains.

Homozygosity for the recessive F508del CFTR mutation is the predominant cause of CF in the Western world (48). Interestingly, the F508del CFTR mutant allele is estimated to have a carrier frequency of 2-4% in populations of European descent (49). The carrier incidence is significantly lower in the Middle East and India, while this allele is almost completely absent from Southeast Asia and sub-Saharan Africa. Maintenance of this allele at such high frequency in Northern Europeans suggests that heterozygosity imparts a survival advantage (49). However, the specific selection pressure underlying the continued persistence of this mutation has not been identified to date (50). Our data suggest the intriguing possibility that a survival advantage against influenza imparted by heterozygosity for the F508del CFTR mutation may account for persistence of this allele at high-levels in persons of European descent. Clearly, however, this possibility cannot be confirmed without studies in the human population.
In conclusion, our results indicate that heterozygosity for the F508del mutation in CFTR attenuates influenza-induced cardiopulmonary dysfunction in mice. Moreover, they demonstrate that this effect is not associated with altered viral replication, an attenuated proinflammatory cytokine response, or a reduction in pulmonary neutrophil infiltrates, but is correlated with a ~3-fold increase in BALF alveolar macrophages and a 10-fold greater IL-6 response to infection. Taken together, these entirely unprecedented observations show for the first time that, as in pulmonary bacterial infections, altered CFTR function can modulate the innate immune response to, and alter the pathogenesis of, viral infections of the lung. Furthermore, they support our hypothesis that CFTR-mediated Cl⁻ secretion is an important determinant of influenza severity and that CFTR inhibition may be of therapeutic benefit in this disease. Finally, they suggest that the high carrier frequency of the F508del CFTR mutation might result from a heterozygous survival advantage against influenza.
4.6 Figures

Figure 4.1: Heterozygosity for the F508del CFTR mutation delays mortality following influenza infection.
Effect of intranasal infection with H1N1 influenza A/WSN/33 (10,000 FFU/mouse) on mortality in: (A) F508del CFTR heterozygotes (HET; n=16); and (B) F508del CFTR homozygotes (HOM; n=5). #P<0.0005, relative to wild-type C57BL/6 littermate controls (WT; n=18).
Figure 4.2: Pulmonary histopathology is attenuated in HET mice.
Representative parenchymal and airway histology in hematoxylin/eosin-stained lung tissues, showing pathologic effects of influenza infection for: (A) 2 days (original objective lens magnification 10x); (B) 6 days (magnification 10x); and (C) 6 days (magnification 20x) in WT mice; and effects of influenza infection for: (D) 2 days (magnification 10x); (E) 6 days (magnification 10x); and (F) 6 days (magnification 20x) in HETs.
Figure 4.3: Infection with influenza A virus induces an exaggerated, protective alveolar macrophage response in the lungs of HET mice.

Effects of influenza infection for 2-6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on: (A) Bronchoalveolar lavage fluid alveolar macrophages (AMs; \(n=10\)-20 per group); (B) BALF lymphocytes (\(n=10\)-20 per group); (C) BALF neutrophils (\(n=10\)-20 per group); and (D) Mortality in untreated HET mice (\(n=16\)) and CL-LIP-treated HET mice (\(n=5\)). WT: wild-type C57BL/6 mice. HET: C57BL/6 congenic mice heterozygous for the F508del CFTR mutation. *\(P<0.05\), **\(P<0.005\), #\(P<0.0005\), vs. uninfected WT mice. ‖\(P<0.0005\), vs. WT mice at the same timepoint. ¶\(P<0.0005\), vs. non-CL-LIP-treated HET mice at 6 d.p.i. Data are presented as mean ± SEM.
Figure 4.4: Influenza-infected HETs exhibit macrophage-dependent, replication-independent amelioration of cardiopulmonary dysfunction.

Effects of influenza infection for 2-6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on: (A) Body weight (BWT; % change from day 0; n=20 per group); (B) Carotid arterial oxygen saturation (S_aO_2; n=20 per group); (C) Heart rate (beats per minute; n=20 per group); and (D) Log viral titers in lung homogenates (log FFU/g; n=8-10 per group). WT: wild-type C57BL/6 mice. HET: C57BL/6 congenic mice heterozygous for the F508del CFTR mutation. **P<0.005, ‖P<0.0005, vs. WT mice at the same timepoint. #P<0.0005, vs. non-CL-LIP-treated mice of the same genotype at 6 d.p.i. Data are presented as mean ± SEM.
Figure 4.5: Heterozygosity for the F508del CFTR mutation prevents influenza-induced pulmonary edema in a macrophage-dependent fashion.

Effects of influenza infection for 2-6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on: (A) Lung water content (wet:dry weight ratio; n=6-9 per group) and (B) Bronchoalveolar lavage fluid (BALF) protein content (μg/ml; n=6-10 per group). WT: wild-type C57BL/6 mice. HET: C57BL/6 congenic mice heterozygous for the F508del CFTR mutation. **P<0.005, #P<0.0005, vs. uninfected WT mice. ‡P<0.005, ‖P<0.0005, vs. WT mice at the same timepoint. ¶P<0.0005, vs. non-CL-LIP-treated HET mice at 6 d.p.i. Data are presented as mean ± SEM.
Figure 4.6: Detrimental effects of influenza infection on airway resistance and lung compliance are attenuated in HETs.

Effects of influenza infection for 2-6 days and treatment with clodronate liposomes for 6 days (6 CL-LIP) on: (A) Static lung compliance (C_{ST}; ml/cmH\_2O, x 10); (B) Dynamic lung compliance (C_{DYN}; ml/cmH\_2O, x 10); (C) Baseline total lung resistance (R_{BASAL}, cmH\_2O.s/ml); and (D) Maximal lung resistance following nebulization of 50 mg/ml methacholine (R_{MAX}, cmH\_2O.s/ml). WT: wild-type C57BL/6 mice. HET: C57BL/6 congenic mice heterozygous for the F508del CFTR mutation. n=7-10 per timepoint for both mouse strains. **p<0.005, #p<0.0005, vs. uninfected WT mice. ‡p<0.005, ‖p<0.0005, vs. WT mice at the same timepoint. ¶p<0.0005, vs. non-CL-LIP-treated HET mice at 6 d.p.i. Data are presented as mean ± SEM.
4.7 References


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Chapter 5: **A₁-adenosine receptor gene deletion or pharmacologic blockade attenuates acute lung injury in mice infected with influenza A (H1N1)**

5.1 Abstract

Influenza causes significant morbidity and mortality. A₁-subtype adenosine receptors (A₁-AdoR) are activated in influenza-infected mice. We hypothesized that, by promoting neutrophil activation and chemotaxis, increased A₁-AdoR activation plays a significant role in induction of acute lung injury in influenza-infected mice.

Wild-type (WT) C57BL/6 and congenic A₁-AdoR-knockout (A₁-KO) mice were infected intranasally with 10,000 FFU/mouse influenza A/WSN/33 (H1N1). Alternatively, WT mice were treated daily with the A₁-AdoR antagonist DPCPX from 24 hours prior to infection. Body weight, carotid arterial O₂ saturation, and heart rate were monitored daily. Pulmonary edema was evaluated by lung weight:dry weight ratio. Lung function parameters were measured by the forced oscillation technique. BALF inflammatory mediators were quantified by ELISA.

Post-infection hypoxemia, bradycardia, pulmonary edema, and lung pathology were attenuated in A₁-KO and DPCPX-treated WT mice, but viral replication was unaffected. Influenza did not increase airway resistance or decrease lung compliance in A₁-KO mice. BALF alveolar macrophages, neutrophils, IFN-γ, and
IL-10 were significantly reduced in A<sub>1</sub>-KO and DPCPX-treated mice. DPCPX treatment also attenuated post-infection weight loss. Adenosine-mediated activation of neutrophil A<sub>1</sub>-AdoR may play a significant role in influenza pathogenesis. A<sub>1</sub>-AdoR inhibitor therapy may therefore be beneficial in patients with influenza-induced lung dysfunction and injury.

5.2 Introduction

Infection with influenza A viruses case a seasonal and contagious acute respiratory disease in humans which accounts for 200,000 hospitalizations and more than 36,000 excess deaths per year in the United States alone (1). In addition, reoccurring pandemics result in devastating losses worldwide, most notably in 1918, when at least 50 million people are believed to have died (2). The 2009-2010 H1N1 “swine influenza” pandemic is estimated to have infected around 61 million people in the United States alone within 12 months, resulting in approximately 275,000 hospitalizations and 12,500 excess deaths (3). Nucleotides are normally present at extremely low concentrations in the bronchoalveolar lining fluid (BALF), but large amounts of ATP are released apically by respiratory epithelial cells in response to cell stress. We have previously demonstrated that infection with influenza A virus results in an increase in BALF ATP levels (4). This effect is reversed by treatment with the de novo pyrimidine synthesis inhibitor A77-1726, suggesting that released ATP is derived from increased de novo nucleotide synthesis in response to infection
The nucleoside adenosine plays a key role in regulation of pulmonary fluid dynamics (6) and lung inflammation (7). Adenosine can be generated in the airspace lining fluid by sequential hydrolysis of ATP. The ecto-apyrase NTPDase 1 (CD39), which is expressed on the apical surface of respiratory epithelial cells, hydrolyzes ATP and ADP to AMP (8). AMP is then further hydrolyzed to adenosine by the ecto-5'-nucleotidase (CD73), which is also abundantly expressed in the lung (9). Adenosine signals through four specific adenosine receptor (AdoR) subtypes (A₁, A₂a, A₂b, and A₃) (10). All four are expressed on alveolar type I and type II cells (11), macrophages (12) and neutrophils (7). A₁-AdoRs, which have very high affinity for adenosine, predominate in the mouse lung (13). Adenosine/A₁-AdoR interactions have been shown to have a proinflammatory role in acute lung injury (ALI), and to contribute to its pathogenesis (10;14). A₁-AdoR signaling can promote neutrophil activation, adhesion, and chemotaxis (15), all of which may be important to the pathogenesis of both ALI (16) and influenza (17;18). We have previously shown that activation of bronchoalveolar epithelial A₁-AdoR by adenosine contributes to impairment of alveolar fluid clearance in influenza-infected mice (4).

The aim of the current study was to determine the contribution of A₁-AdoR activation by adenosine to the development of ALI in a mouse influenza model. We found that, when compared to wild-type C57BL/6 controls (WT), hypoxemia and cardiopulmonary dysfunction were attenuated in influenza A/WSN/33-
infected C57BL/6-congenic A₁-AdoR-deficient (A₁-KO) mice and in WT mice treated with the A₁-AdoR antagonist DPCPX (8-Cyclopentyl-1,3-dipropylxanthine). These effects were associated with reduced leukocyte infiltration into the lungs and lower IFN-γ responses to infection. Our findings indicate that activation of respiratory epithelial A₁-AdoR by adenosine may play a prominent role in the pathogenesis of influenza-induced ALI and that A₁-AdoR antagonists may be of therapeutic value.

5.3 Materials and methods

Animals. C57BL/6AnNCr WT mice were purchased from the National Cancer Institute (Frederick, MD). C57BL/6-congenic A₁-KO mice were kindly supplied by Dr. Jurgen Schnermann (NIDDK, Bethesda, MD). Offspring were genotyped as previously described (19). All experimental animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. Ethical considerations precluded performance of survival studies.

Mouse inoculation. Eight to 12 week-old mice of either genotype were individually marked then inoculated intranasally with 10,000 plaque-forming units (pfu) of H1N1 influenza A/WSN/33 in 50 μl PBS/0.1% BSA under ketamine/xylazine anaesthesia, as in our previous studies (4). This inoculum induces severe hypoxemia and ALI in WT mice by 2 days post-infection (d.p.i.), and results in 100% mortality by 8 d.p.i. (median time to death: 7 days) (4;5). Body weights and carotid arterial O₂ saturations were recorded every other day.
Data for each experimental group were derived from a minimum of three independent infections.

**Measurement of lung mechanics.** Mechanical properties of the mouse lung and responses to increasing doses of the nebulized bronchoconstrictor methacholine (0.1 to 50 mg/ml) were assessed by the forced-oscillation technique (20) in valium/ketamine-anesthetized, tracheotomized mice that were mechanically ventilated using a flexiVent computer-controlled piston ventilator (SciReq, Montreal, Canada), as in our previous studies (5;21).

**DPCPX treatment regimen.** Beginning one day prior to infection, WT mice were treated daily with 1 mg/kg of the specific A<sub>1</sub>-AdoR antagonist DPCPX (Tocris Bioscience, Ellisville, MO) by i.p. injection in 100 µl saline (22). Controls received an equivalent volume of vehicle.

**Other methods:** Preparation of viral inocula, measurement of carotid arterial O<sub>2</sub> saturation, heart rate, and viral titers, histopathologic evaluation, bronchoalveolar lavage, and measurement of bronchoalveolar lavage inflammatory mediators and lung water content were performed as previously described (4;5).

**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. Between-group comparisons 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.
5.4 Results

Cardiopulmonary dysfunction, but not viral replication, is attenuated in A₁-KO mice. Uninfected adult A₁-KO mice were phenotypically normal. Following infection with influenza A/WSN/33 (10,000 pfu/mouse), both WT and A₁-KO mice lost weight at a similar rate (Fig. 5.1A). However, hypoxemia and bradycardia were attenuated in A₁-KO mice at 6 d.p.i. (Figs. 5.1B and 5.1C, respectively). Lung homogenate viral titers were approximately 0.5 logs lower at 2 d.p.i. and 0.5 logs higher at 4 d.p.i. in A₁-KO mice (Fig. 5.1D). Titers did not differ between mouse strains at 6 d.p.i.

A₁-KO mice are partially protected from pulmonary edema and loss of bronchoalveolar epithelial integrity. Lung water content (wet:dry weight ratio) was significantly elevated in WT but not A₁-KO mice at 2 d.p.i. (Fig. 5.2A). At 6 d.p.i., a further increase in wet:dry weight ratios was observed in both strains, although this was attenuated in A₁-KO mice. In both strains, influenza infection was accompanied by an increase in BALF protein content at 6 d.p.i., which is indicative of increasing damage to the bronchoalveolar epithelial barrier (Fig. 5.2B). However, BALF protein levels remained lower in A₁-KO mice than WT controls at day 6.

Lung compliance and airway resistance do not change in A₁-KO mice following influenza infection. Static and dynamic lung compliance are indices of lung tissue stiffness and resistance to inflation on inspiration, which are measured at a fixed lung volume and during normal ventilation, respectively.
Static and dynamic compliance did not differ between uninfected WT and A1-KO mice (Figs. 5.3A and 5.3B, respectively). However, while infection resulted in a progressive decline in both static and dynamic compliance in WT controls, it had no effect on these parameters in A1-KO mice.

Total airway resistance did not differ between uninfected WT and A1-KO mice (Fig. 5.3C). Infection induced a significant increase in total lung resistance at 6 d.p.i. in WT animals, but had no such effect in A1-KO mice. Finally, airway hyperresponsiveness to escalating doses of methacholine was present at 2 d.p.i. in WT controls, but not A1-KO mice (Fig. 5.3D).

**Severity of lung pathology is reduced in influenza-infected A1-KO mice.**

When compared to uninfected WT controls (Fig. 5.4A), lungs from uninfected A1-KO mice were histologically normal (Fig. 5.4E). At 2 d.p.i., moderate interstitial pneumonitis was present in both WT controls (Fig. 5.4B) and A1-KO mice (Fig. 5.4F). By 6 d.p.i., WT mice had developed severe bronchiolar and peribronchiolar neutrophilic inflammation, together marked with bronchiolar epithelial necrosis (Fig. 5.4C). Marked alveolar septal inflammation was also present in WT mice at this timepoint, and large numbers of neutrophils and alveolar macrophages were visible within alveoli, which were often filled with protein-rich edema fluid (Fig. 5.4D). Interstitial pneumonitis was significantly attenuated in lungs from A1-KO mice at 6 d.p.i. and fewer infiltrating leukocytes were visible in both the peribronchiolar and alveolar spaces (Figs. 5.4G and 5.4H).

**Leukocyte infiltration of the lung in response to influenza infection is**
decreased in A₁-KO mice. BALF total cell numbers did not differ between uninfected WT and A₁-KO mice (not shown) and over 95% of cells in BALF from both strains were alveolar macrophages (Fig. 5.5A). BALF lymphocyte counts increased modestly at 2 d.p.i. in WT mice, and significant increases in neutrophil numbers were found in WT BALF at this timepoint (Figs. 5.5B and 5.5C, respectively). However, BALF lymphocyte and neutrophil levels did not increase in A₁-KO mice at 2 d.p.i., and both remained significantly lower than WT. At 6 d.p.i., there were very significant differences in BALF leukocyte numbers between strains. As in previous studies, BALF alveolar macrophage, lymphocyte, and neutrophil and counts increased dramatically from day 2 levels in WT mice. In contrast, there was no change in BALF alveolar macrophages and only a modest increase in BALF lymphocyte numbers relative to day 0 in A₁-KO mice at 6 d.p.i. Although some neutrophils were present in BALF from A₁-KO mice at this timepoint, the neutrophil response was highly-attenuated: neutrophil counts were almost 12-fold lower than in WT controls. The difference in neutrophil response between the two genotypes was reflected in measurements of whole lung myeloperoxidase activity, which increased in WT controls at 2 d.p.i. and almost doubled by 6 d.p.i. (Fig. 5.5D). However, lung myeloperoxidase activity did not increase in A₁-KO mice at 2 d.p.i. and modestly decreased at 6 d.p.i., although this effect was not statistically-significant. Finally, in contrast to BALF, we found no difference in peripheral blood total cell, monocyte, lymphocyte, or neutrophil counts between WT and A₁-KO mice at 6 d.p.i. (Table 5.1).
Bronchoalveolar lavage fluid cytokine and chemokine responses to influenza are significantly altered in A1-KO mice. Minimal levels of inflammatory cytokines and chemokines were detectable in BALF from uninfected WT or A1-KO mice (not shown). BALF IFN-γ and IL-10 levels remained very low in both WT and A1-KO mice at 2 d.p.i., while TGF-β increased to a comparable degree in both strains (Table 5.2). In contrast, IL-6 and KC (CXCL-1) increased in WT but not A1-KO animals at 2 d.p.i., and an IFN-α response to infection was only detectable in WT controls. Interestingly, however, MCP-1 (CCL-2) levels were markedly increased in A1-KO animals at this timepoint.

At 6 d.p.i., BALF IFN-α content was low in both genotypes. BALF IL-6 did not change significantly from day 2 in WT controls, but increased to WT levels in A1-KO mice at this timepoint. IFN-γ and IL-10 increased from day 2 levels in both genotypes, but these increases were less pronounced in A1-KO mice. In contrast, BALF IP-10 (CXCL-10), KC, MCP-1 (CCL-2), and TGF-β concentrations were significantly higher in A1-KO mice than WT animals at 6 d.p.i. BALF RANTES (CCL-5) increased at 2 d.p.i. and rose further at 6 d.p.i., but RANTES levels did not differ between genotypes at either timepoint (not shown). Finally, BALF from both genotypes contained very low concentrations of IL-1β, IL-12, MIP-1α (CCL-3), and TNF-α at both post-infection timepoints (not shown).

Treatment with the A1-AdoR antagonist DPCPX ameliorates hypoxemia, pulmonary edema, and inflammatory leukocyte infiltration in influenza-
infected WT mice. To determine whether A$_1$-AdoR blockade might have therapeutic potential, mice were treated daily with the specific A$_1$-AdoR antagonist DPCPX (1 mg/kg) from one day prior to infection. DPCPX significantly reduced the rate of post-infection weight loss in WT mice (Fig. 5.6A). Treatment with saline vehicle had no such effect. Likewise, DPCPX- but not saline-treated mice developed less severe hypoxemia and bradycardia than untreated animals (Fig. 5.6B and not shown, respectively). Influenza-induced pulmonary edema (wet:dry weight) was modestly attenuated by DPCPX treatment at 2 d.p.i. and more significantly so at 6 d.p.i., but was unaffected by saline administration at either timepoint (Fig. 5.6C). In addition, BALF alveolar macrophage and neutrophil counts were lower in DPCPX-treated mice at both 2 and 6 d.p.i., although this effect was only statistically-significant at day 6 (Fig. 5.6D). Finally, BALF IFN-γ, IL-10, and KC levels were significantly lower in DPCPX-treated mice than controls at 6 d.p.i. (Table 5.3). However, DPCPX treatment did not alter BALF IL-6 or TGF-β content.

5.5 Discussion
Extracellular nucleotides are important signaling molecules in the lung (10). We previously reported that influenza infection results in increased de novo nucleotide synthesis and channel-mediated release of ATP and UTP into the BALF (4;5). In addition, we showed that activation of A$_1$-AdoR by adenosine plays a key role in induction of nucleotide-mediated alveolar fluid clearance
impairment in influenza A virus-infected mice (4). Activation of A1-AdoR has been shown to have both pro- and anti-inflammatory effects in different experimental models (23). In the current study, we found that influenza-induced hypoxemia, lung dysfunction, inflammation, and ALI were attenuated in A1-KO mice and WT mice treated with the A1-AdoR antagonist DPCPX. These results indicate that the A1-AdoR has a previously-unrecognized pro-inflammatory role in the pathogenesis of influenza-induced hypoxemia and ALI.

Despite significant attenuation of disease severity in H1N1-infected A1-KO mice relative to WT controls, we found no differences in either the rate or magnitude of body weight loss between the two genotypes. In contrast, weight loss was reduced in DPCPX-treated WT mice. Development of hypoxemia in WT mice was rapid and comparable to our previous findings (4;5). Hypoxemia was reduced in both A1-KO and DPCPX-treated mice at 6 d.p.i. However, DPCPX treatment had a larger effect on hypoxemia, and this was associated with a greater reduction in lung water content than that observed in A1-KO mice at 6 d.p.i. This suggests that the degree of impairment of gas exchange following influenza infection may depend upon the severity of pulmonary edema, and the downstream detrimental effects of increased lung fluid on lung compliance and airway resistance.

Heart rates were higher in both uninfected A1-KO and DPCPX-treated WT mice than untreated WT controls. However, given that activation of A1-AdoRs on cardiac pacemaker cells in the atrioventricular node on the left side of the heart
induces bradycardia in normal mice (24), this finding is not surprising. Only infection of WT mice resulted in bradycardia, suggesting that this effect of influenza may be secondary to activation of cardiac pacemaker A₁-AdoR by adenosine. It is possible that increased epithelial adenosine production and progressive breakdown of the lung epithelial barrier following influenza infection in WT mice result in “spillover” of adenosine into the pulmonary veins (and hence the left side of the heart). Although adenosine has a relatively short plasma half-life (<10 seconds (25)), this is considerably longer than the transit time of blood through the entire cardiac vasculature of the mouse (0.1 seconds at a resting heart rate of 600 bpm) and also far longer than the time required for blood to transit from the lungs to the heart via the pulmonary veins. Moreover, pulmonary venous adenosine levels are higher than systemic concentrations even in normal subjects, suggesting baseline production by the lungs (26). However, this mechanism is yet to be confirmed in our model.

Neutrophil recruitment is a prominent feature of the early phase of ALI, and has been implicated in its pathogenesis (16). However, the role of neutrophils in influenza is less clear. In some studies neutrophil depletion resulted in enhanced viral replication and more severe disease, suggesting that these cells play an antiviral and protective role (27-29). In contrast, others have shown that influenza ALI was attenuated by neutrophil depletion or ablation of neutrophil recruitment to the lungs (30;31). Likewise, severe disease induced by pandemic influenza strains has been associated with excessive neutrophil infiltration of the lungs.
A₁-AdoR signaling is known to promote neutrophil chemotaxis, adhesion, phagocytosis and superoxide production (15). We found that pulmonary neutrophil responses to infection were highly-attenuated in A₁-KO or DPCPX-treated mice, resulting in reduced whole lung myeloperoxidase activity at 6 d.p.i. However, peripheral blood neutrophil counts did not differ between WT and A₁-KO mice at 6 d.p.i. This indicates that the severity of influenza-induced ALI is dependent upon the extent of neutrophil recruitment into the lung, rather than the systemic neutrophil response. Moreover, neither A₁-AdoR deletion nor antagonism significantly altered viral replication. Together, our data indicate that neutrophils play a pro-inflammatory role in influenza. Our findings also indicate for the first time that adenosine is an important chemotactic factor for neutrophil and mononuclear cell recruitment following influenza infection. Indeed, its role may be as or more important than that of chemokines such as KC and MCP-1: despite significant attenuation of neutrophil and mononuclear cell recruitment in A₁-KO mice at 6 d.p.i., levels of KC and MCP-1 remained higher in A₁-KO mice than WT controls.

In addition to an exaggerated neutrophil response, increased mortality rates following infection with highly-pathogenic influenza strains have been correlated with a higher rate and extent of viral replication (34), reduced alveolar macrophage responses (32;35), and development of a “cytokine storm” (36-38). We found that amelioration of influenza-induced ALI in A₁-KO or DPCPX-treated mice was not associated with significant reductions in viral replication. Moreover,
the protective effects of A1-AdoR deletion or antagonism in influenza correlated with reduced (rather than increased) BALF alveolar macrophages, suggesting that an alveolar macrophage response was not necessary for protection from ALI in our model. Reduced ALI severity in both A1-KO and DPCPX-treated mice was associated with reductions in BALF IFN-γ and IL-10, but not IL-6, at 6 d.p.i. However, while BALF KC and TGF-β increased at 6 d.p.i. in A1-KO mice, this was not the case in DPCPX-treated animals. Moreover, chemokine responses to influenza were higher in A1-KO mice than WT controls at 6 d.p.i. This suggests that only a specific subset of cytokines and chemokines contributes directly to ALI development in influenza. Further studies are needed to determine the contribution (if any) of individual soluble mediators to protection from ALI in influenza-infected A1-KO or DPCPX-treated mice.

We have shown previously that influenza induces de novo nucleotide synthesis and channel-mediated release of ATP into the BALF where it is metabolized to adenosine (4;5). In WT mice, this newly-generated adenosine activates the high-affinity A1-AdoR, resulting in increased release of pro-inflammatory mediators, recruitment of neutrophils, monocytes, and lymphocytes to the lung, and damage to the lung parenchyma. Because neutrophils and macrophages can also release ATP (39), adenosine/A1-AdoR-mediated recruitment of these cells to the lung may perpetuate a vicious cycle of adenosine generation and A1-AdoR-mediated inflammation which results in severe ALI. In contrast, despite a similar increase in adenosine generation in A1-KO mice, ALI does not develop due to the absence
of an adenosine/A₁-AdoR-mediated pro-inflammatory response to infection. Likewise, blockade of A₁-AdoR with DPCPX attenuates development of ALI in WT mice. The latter findings are consistent with earlier studies showing that pretreatment with DPCPX prevented the development of ALI in feline models of both lipopolysaccharide-induced ARDS and ischemia-reperfusion injury (40). However, we should note that other investigators found that treatment of A₁-KO mice with aerosolized lipopolysaccharide resulted in exaggerated neutrophil recruitment to the lung and increased pulmonary vascular leakage, suggesting an anti-inflammatory role for the A₁-AdoR (41).

Vaccines and neuraminidase inhibitors are the current mainstays of influenza prophylaxis and treatment, respectively (1). However, the 2009 “swine flu” pandemic demonstrated that the effectiveness of annual vaccination programs is limited by difficulties with timely vaccine production and distribution (42), uptake by the public (43), and efficacy (44). Likewise, antiviral drugs are expensive (45), often induce resistant viral strains (46), and are generally less effective late in infection (47;48). Corticosteroids and β-adrenergic agonists generally have very limited beneficial effects on lung function in patients with severe viral ALI (49). Hence, their treatment is generally limited to non-specific supportive care in the ICU. We are therefore faced with an urgent need for novel therapeutics that delay the onset of influenza-induced ALI or reduce its severity. Such therapeutics would be particularly useful during pandemics, when critical care facilities are likely to be overloaded (49). Our data suggest that A₁-AdoR antagonists may be
of value in this regard. Importantly, A₁-AdoR antagonists are in development for other indications and they appear to be safe and well-tolerated in humans (50).

In conclusion, our results indicate that influenza-induced hypoxemia, cardiopulmonary dysfunction, and ALI are either attenuated or absent in A₁-KO or A₁-AdoR antagonist-treated mice. Moreover, they demonstrate that these effects are not associated with altered viral replication but are correlated with a dramatic reduction in pulmonary neutrophils. These observations show for the first time that activation of respiratory epithelial and leukocyte A₁-AdoR by adenosine plays a significant role in neutrophil recruitment and development of ALI following influenza infection. Moreover, our findings using DPCPX indicate that A₁-AdoR antagonists have potential as new therapeutics to retard or ameliorate development of respiratory failure and ALI in influenza-infected patients.
5.6 Figures

Figure 5.13: Cardiopulmonary dysfunction, but not viral replication, is significantly attenuated in A1-adenosine receptor-knockout (A1-KO) mice. Effect of intranasal infection of wild-type C57BL/6 (WT) and A1-KO mice with H1N1 influenza A/WSN/33 (10,000 FFU/mouse) on: (A) Body weight (BWT; % change from day 0; n>25 per group); (B) Carotid arterial oxygen saturation (% \( S_aO_2 \); n=13-31 per group); (C) Heart rate (HR; beats per minute; n=11-28 per group); and (D) Lung homogenate viral titers (log pfu/g; n=5-10 per group). #P<0.001, vs. uninfected WT mice. †P<0.05, §P<0.001, vs. WT mice at the same timepoint. Data are presented as mean ± S.E.M.
Figure 5.2: A$_1$-KO mice are partially protected from pulmonary edema and loss of bronchoalveolar epithelial integrity. Effects of influenza infection for 2-6 days on: (A) Lung water content (wet:dry weight ratio; $n=7$-$12$ per group); and (B) Bronchoalveolar lavage fluid (BALF) protein content. #$P<0.001$, vs. uninfected WT mice. †$P<0.05$, ‡$P<0.005$, vs. WT mice at the same timepoint. Data are presented as mean ± S.E.M.
Figure 5.3: Lung compliance and airway resistance do not change in A1-KO mice following influenza infection.

Effects of influenza infection on: (A) Static lung compliance ($C_{ST}$; ml/cmH$_2$O, x 10; n=6-15 per group); (B) Dynamic lung compliance ($C_{DYN}$; ml/cmH$_2$O, x 10; n=6-14 per group); (C) Baseline total lung resistance ($R_{BASAL}$, cmH$_2$O.s/ml; n=6-13 per group); and (D) Maximal lung resistance following nebulization of 50 mg/ml methacholine ($R_{MAX}$, cmH$_2$O.s/ml; n=5-9 per group). #P<0.001, vs. uninfected WT mice. §P<0.001, vs. WT mice at the same timepoint. Data are presented as mean ± S.E.M.
Figure 5.4: Severity of lung pathology is reduced in influenza-infected A1-KO mice.

(A) C57BL/6 mouse, uninfected, 10x magnification; (B) C57BL/6 mouse, 2 d.p.i. with H1N1 influenza virus, 10x magnification; (C) C57BL/6 mouse, 6 d.p.i. with H1N1 influenza virus, 10x magnification; (D) C57BL/6 mouse (same as in C), 6 d.p.i. with H1N1 influenza virus, 40x magnification; (E) A1-KO mouse, uninfected, 10x magnification; (F) A1-KO mouse, 2 d.p.i. with H1N1 influenza virus, 10x magnification; (G) A1-KO mouse, 6 d.p.i. with H1N1 influenza virus, 10x magnification; (H) A1-KO mouse (same as in C), 6 d.p.i. with H1N1 influenza virus, 40x magnification. Bar in images A-C and E-G = 100μm; bar in images D and H = 50μm.
Figure 5.5: Leukocyte infiltration of the lung in response to influenza infection is decreased in A1-KO mice.

Effects of influenza infection for 2-6 days on: (A) Bronchoalveolar lavage fluid (BALF) alveolar macrophages (AMs); (B) BALF lymphocytes (Lymphs); (C) BALF neutrophils (PMNs); and (D) lung homogenate myeloperoxidase (MPO) activity. 

$n=6$-$10$ per group. *$P<0.05$, **$P<0.005$, #$P<0.001$, vs. uninfected WT mice. †$P<0.05$, ‡$P<0.001$, vs. WT mice at the same timepoint. Data are presented as mean ± S.E.M.
Figure 5.6: Treatment with the A1-AdoR antagonist DPCPX ameliorates hypoxemia, pulmonary edema, and inflammatory leukocyte infiltration in influenza-infected WT mice.

Effect of daily i.p. administration of 100 µl saline vehicle or 1 mg/kg DPCPX in 100 µl saline on: (A) Body weight (BWT; % change from day 0; n>10 per treatment group); (B) Carotid arterial oxygen saturation (% SaO2; n>10 per treatment group); (C) Lung water content (wet: dry weight ratio; n=5 per treatment group); and (D) BALF alveolar macrophages (AMs; n=5 per treatment group) and neutrophils (PMNs; n=5 per treatment group). #P<0.001, vs. uninfected WT mice in the same treatment group. †P<0.05, §P<0.001, vs. untreated WT mice at the same timepoint. Data are presented as mean ± S.E.M.
Table 5.1: Peripheral blood leukocyte responses to influenza do not differ between wild-type controls and A₁-adenosine receptor-knockout mice at 6 d.p.i.\textsuperscript{a}

\textsuperscript{a}: days post-infection.
\textsuperscript{b}: Wild-type C57BL/6 mice.
\textsuperscript{c}: C57BL/6-congenic A₁-adenosine receptor-knockout mice.
\textsuperscript{d}: $x 10^3$/μl

Data are presented as mean cells/μl ± S.E.M. (to the nearest significant figure).

\textsuperscript{*}P<0.05, vs. WT mice (by Student’s $t$-test).
Table 5.2: Bronchoalveolar lavage fluid cytokine and chemokine responses to influenza are significantly altered in A₁-adenosine receptor-knockout mice.

<table>
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<tr>
<th>d.p.i.</th>
<th>n</th>
<th>IFN-α</th>
<th>IFN-γ</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IP-10</th>
<th>KC</th>
<th>MCP-1</th>
<th>TGF-β</th>
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<tr>
<td>2</td>
<td>WT</td>
<td>8</td>
<td>505</td>
<td>18</td>
<td>560</td>
<td>55</td>
<td>N.M.</td>
<td>309</td>
<td>824</td>
</tr>
<tr>
<td></td>
<td>± 61</td>
<td>± 3</td>
<td>± 61</td>
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<td>± 85</td>
<td>± 76</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A₁-KO</td>
<td>6</td>
<td>10</td>
<td>1</td>
<td>14</td>
<td>20</td>
<td>N.M.</td>
<td>15</td>
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<td>± 3²</td>
<td>± 1</td>
<td>± 2</td>
<td>± 4</td>
<td>± 2²</td>
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<td>± 155</td>
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<tr>
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<td>4268</td>
<td>476</td>
<td>824</td>
<td>2921</td>
<td>167</td>
<td>2323</td>
</tr>
<tr>
<td></td>
<td>± 5</td>
<td>± 1148</td>
<td>± 40</td>
<td>± 72</td>
<td>± 241</td>
<td>± 16</td>
<td>± 136</td>
<td>± 77</td>
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<td></td>
<td>A₁-KO</td>
<td>9</td>
<td>28</td>
<td>538</td>
<td>550</td>
<td>209</td>
<td>6047</td>
<td>524</td>
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<tr>
<td></td>
<td>± 16</td>
<td>± 83²</td>
<td>± 121</td>
<td>± 26²</td>
<td>± 385²</td>
<td>± 138²</td>
<td>± 121²</td>
<td>± 192²</td>
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*P<0.05, **P<0.005, #P<0.001, vs. WT mice at the same timepoint (by ANOVA).
Table 5.3: Treatment of C57BL/6 mice with the $A_{1}$-AdoR antagonist DPCPX significantly alters bronchoalveolar lavage fluid cytokine responses to influenza at 6 d.p.i.$^a$

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>IFN-γ</th>
<th>IL-6</th>
<th>IL-10</th>
<th>KC</th>
<th>TGF-β</th>
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<td>476 ± 40</td>
<td>936 ± 16</td>
<td>167 ± 16</td>
<td>621 ± 77</td>
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<tr>
<td>DPCPX$^c$</td>
<td>4</td>
<td>1487 ± 648$^*$</td>
<td>450 ± 122</td>
<td>139 ± 26$^#$</td>
<td>127 ± 28</td>
<td>531 ± 67</td>
</tr>
</tbody>
</table>

$^a$: days post-infection.

$^b$: Untreated C57BL/6 mice.

$^c$: DPCPX-treated C57BL/6 mice.

Data are presented as mean pg/ml ± S.E.M. (to the nearest significant figure).

$^*P<0.05$, $^#P<0.001$, vs. UNTx mice (by ANOVA).
5.8 References


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Chapter 6: Future directions

6.1 Challenges in prevention and treatment of influenza infections

Influenza vaccination has been shown to protect a significant amount of the population against the disease (1). However, vaccine uptake in the population has been low. Even during the 2009 “Swine flu” pandemic, vaccination rates by the US population did not significantly improve. Even more importantly, the overall vaccination rate was not high enough to induced so-called “herd immunity” which would also protect unvaccinated individuals (2;3). Challenges in strain matching, adapting to the rapidly mutating virus, timely production of sufficient quantities of vaccine, distribution around the globe and limited shelf life further increase the number of problems with the commercially available vaccine (4). In addition, third world countries might not have access to vaccines due to the costs involved (5). All of these factors increase the chance of large parts of the population potentially contracting influenza and requiring adequate medical care. Neuraminidase inhibitors (e.g., oseltamivir) have been shown to significantly decrease viral load early during the course of disease. However, drug-resistant virus strains have developed after the introduction of this compound to the market (6). Even more troubling, the drug’s general effectiveness has recently been questioned (7;8).
Even if neuraminidase inhibitors are effective during the first few days after infection, many patients do not show severe symptoms and exacerbation of disease until several days later when peak viral replication rates have been surpassed. A multitude of these patients are at risk for developing acute respiratory distress syndrome (ARDS) and subsequent mortality (9). The current standard of care for patients with respiratory failure is non-specific and results in expensive in-patient supportive care (10;11).

This means that doctors today are lacking a specific treatment for patients approaching influenza-induced ARDS that is effective at a later stage of disease and has the potential of preventing acute lung injury (ALI) and the need for mechanical ventilation. This is especially important due to the fact that hospitals do not have enough ventilators to cope with needs during a pandemic or even a localized severe outbreak. Studies in the US have shown that at any given day outside of the influenza season, 75% of all mechanical ventilators are in use. During a regular flu season this number increases to 95% (12). The amount of remaining ventilators not in use is insufficient to cope with markedly increased demands during a potential pandemic (10;12). Ergo, patients would die simply because they could not be ventilated.

6.2 Re-evaluating the cytokine storm

Targeting the immune system during acute infections has been largely unsuccessful (13), pointing to the option that the cytokine storm and exuberant
inflammatory reaction are not the cause of influenza-induced mortality. For example, early corticosteroid treatment of H1N1 patients with ARDS even results in worsened outcome (14). Specific therapy against TNF signaling has never been tested in humans with influenza based on inconclusive results in mice studies as well as lack of positive effect in humans with other causes of sepsis (15). While cyclooxygenase-2 enzyme (COX-2) inhibitors have been promising in vitro (16;17), in vivo studies in mice have been equivocal (18-20) and studies in human patients are forthcoming. The list of failed immunomodulatory therapeutics is long and includes statins, high mobility group box 1 protein antagonists, angiopoietin-1 therapy and others (reviewed in 21). Many of these potential therapies showed promising results in vitro or in animal models, yet failed to be protective in human trials, further emphasizing the need for better defined and improved animal models.

6.3 Nucleotide synthesis inhibitors

Both purine and pyrimidine synthesis inhibitors have been widely used for decades to suppress the immune system and inflammatory reaction of patients with organ transplants or chronic inflammatory conditions like psoriatic arthritis (22;23).

Our studies in the mouse influenza model have shown that when administered in aerosolized form, the de novo nucleotide synthesis inhibitor A77-1726 prevents respiratory failure (24).
The systemic immunosuppressive effects of A77-1726 may be problematic when fighting a (pulmonary) infection that predisposes to secondary opportunistic bacterial infections (25). Targeted delivery to the lung via aerosol is more likely to minimize adverse systemic effects. In addition, common pyrimidine synthesis inhibitors like leflunomide are characterized by poor bioavailability and patients have to be subjected to slowly increasing doses of the drug over weeks to experience a therapeutic effect (26). This is obviously not feasible in an acute process such as caused by influenza virus infections and creates the need to further investigate active metabolites of these compounds to result in more rapidly effective drugs.

A77-1726 is not protected under patent laws anymore and to our knowledge no pharmaceutical company is developing it as a drug candidate.

6.4 A₁ adenosine-receptor antagonists

After the asthmatic Henry Hyde Salter first described the effects of strong coffee on his own airway symptoms in 1859, methylxanthines have been the focus of investigation as potential therapeutics (reviewed in 27). Early drugs however caused cardiovascular and gastric side effects (28).

Thus far, selective A₁-adenosine receptor antagonists have been suggested in the treatment of chronic respiratory conditions, resulting in decreased bronchiolar hyperresponsiveness, mucin hypersecretion and pulmonary inflammation (28). In addition it has been shown that systemic treatment is of benefit by reducing the
migration of dendritic cells to the lymph nodes and preventing leukocyte adhesion to endothelial cells (29).

Investigation of more selective A_1-adenosine receptor antagonists ultimately resulted in the development of bamiphylline as treatment for COPD patients (28). Placebo-controlled studies proved efficacy of the substance, with later double-blind clinical trials documenting efficacy even in smokers with chronic bronchitis. Oral bamiphylline has been approved for the treatment of COPD in Europe with a documented low rate of side-effects (30). Based on this success, a selective antagonist for the A_1-adenosine receptor was developed and named L-97-1 (31). This small water-soluble molecule is under development as oral therapeutic for patients with asthma by the company Endacea Inc.

In contrast, respirable antisense oligonucleotide developed to silence RNA-transcription of the A_1-adenosine receptor have been deemed unsuccessful due to disappointing results in phase 2 clinical trials (28).

Our most recent research has shown that preventing signaling through the A_1-adenosine receptor is a potential and promising therapeutic approach to reduced cardiopulmonary function during influenza infection (see Chapter 5). Since these results were obtained by using genetically engineered mice deficient for the A_1-adenosine receptor in all tissues, future studies will focus on utilizing L-97-1, a known receptor antagonist (31;32), to treat influenza-infected C57BL/6 mice to investigate the therapeutic effect. We plan on utilizing similar readouts as documented in this thesis to assess treatment effects and different treatments
regimes of nebulized drug administration. We anticipate that post-infection treatment will improve pulmonary functional readouts like blood oxygenation, pulmonary resistance, compliance and edema, while viral loads should not differ between treated and untreated mice. In addition, special interest will be paid to cytokine and chemokine levels and the pulmonary inflammatory cell population in the bronchoalveolar lavage fluid to assess L-97-1’s effect on the local inflammatory environment. Due to targeted drug delivery to the lung, we anticipate little to no systemic effects of drug treatment, however it may be of interest to relate the local pulmonary inflammatory population to the circulating white blood cell response. This might be especially interesting in light of the aforementioned positive effects of systemic treatment with other A₁-adenosine receptor antagonists.

6.5 Chloride channel inhibitors

Our work with mice heterozygous for the dF508 mutation in chloride anion channel CFTR has shown that decreased function of this chloride channel has an effect on disease severity and outcome. To our knowledge, there are currently no chloride channel antagonists being develop for treatment of ARDS or influenza-induced lung injury.

However, there is an ongoing effort of developing CFTR inhibitors as treatments for secretory bowl diseases and polycystic kidney disease (PKD). CFTR chloride anion conductance is the rate-limiting step in transepithelial fluid secretion in
epithelial cells lining the normal intestine as well as renal cysts in PKD (33). High-throughput screening has aided in the discovery of CFTR inhibitors of the thiazolidinone, glycine hydrazide and quinoxalinedione chemical classes. These small molecules are potent in the nanomolar range. While the glycine hydrazides target the extracellular CFTR pore, thiazolidinones and quinozalinediones interact with CFTR on in the cytoplasmic domain (reviewed in 33). Most recently, it has also been shown that the influenza protein M2 alters CFTR function and expression in *Xenopus* oocytes and human bronchiolar epithelial cells (34). However, investigators generally focus on the role of either normal CFTR (expressed in normal levels) or defective CFTR in cystic fibrosis during influenza infections. To our knowledge there are no studies besides our own investigating reduced chloride channel expression through heterozygosity.

We have shown that H1N1 influenza A virus impairs AFC by inhibiting ENaC-mediated Na\(^+\) absorption and increased anion secretion via CFTR (35). Concomitant inhibition of Na\(^+\) absorption and stimulation of Cl\(^-\) secretion result in severe pulmonary edema, hypoxemia, and impaired cardiac function (24). We hypothesize that CFTR is an essential, non-redundant “master regulator” of alveolar fluid clearance and blockade will be more beneficial to influenza patients than cytokine neutralization.
6.6 Hypoxia-inducible factor 1

Another interesting aspect worth investigating is the possible disease mechanism of influenza infection pathogenesis and pulmonary injury in the context of tissue hypoxia. In fatal cases, influenza patients die of multiple organ dysfunction syndrome due to tissue hypoxia (9). This disease mechanism is still poorly understood (36).

Hypoxia is a damaging condition for organisms caused by a limited supply of O$_2$ that is fundamental for efficient energy production (37). In order to survive hypoxia, organisms need to adapt their physiological functions. Under hypoxic conditions, cells change their physiologic state to "hypoxic mode", which alters metabolism to foster more efficient uptake of O$_2$ and reduce energy expenditure (37). Although risky to not generate enough ATP to maintain essential cellular functions during hypoxia, tight regulation of O$_2$ homeostasis is essential (38). In vivo as well as in vitro studies have shown that lung epithelial cells are very tolerant to severe and prolonged hypoxia with no change in ultrastructural characteristics, cell viability or ATP content (39). In the lung, hypoxia is a common feature of many respiratory diseases that result in inadequate alveolar ventilation or in pulmonary edema (40). Pulmonary edema is a feature of acute lung injury and acute respiratory distress syndrome (41;42), which can be caused by seasonal as well as pandemic influenza infection (9;43).

In areas of inflammation, local hypoxia is caused by an imbalance between O$_2$ supply and demand. Decreased oxygenation during inflammation can be caused
by vasculopathies or increased interstitial pressure. Also, inflamed tissue exhibits increased \( \text{O}_2 \) consumption due to increased metabolic activity which is compounded by the metabolic demands of infiltrating inflammatory cells (44). Adaptive responses to hypoxia utilize many different molecular pathways (45), but one single transcription factor appears to play a critical role in cellular and systemic \( \text{O}_2 \) homeostasis: Hypoxia-inducible factor 1 (HIF-1) (38).

HIF-1 has been coined “the master regulator of \( \text{O}_2 \) homeostasis” and has been shown to play a key role in cellular and systemic physiology, development, and pathophysiology. It is a heterodimeric basic helix-loop-helix-PAS transcription factor (46) consisting of two subunits, HIF-1\( \alpha \) and HIF-1\( \beta \) (47). Comparison of cDNAs encoding human and mouse HIF-1\( \alpha \) reveal a striking degree of evolutionary conservation (>90% amino acid sequence identity) (48-51). When cellular \( \text{O}_2 \) levels decrease, HIF-1\( \alpha \) expression and HIF-1 transcriptional activity increase exponentially (38). HIF-1 can mediate cell autonomous, as well as tissue-restricted, and systemic homeostatic responses to hypoxia (52).

While HIF-1\( \beta \) is constitutively expressed and permanently present in the nucleus (53), HIF-1\( \alpha \) expression is \( \text{O}_2 \)-regulated (46). Under normoxic conditions, the HIF-1\( \alpha \) subunit is synthesized and rapidly hydroxylated by prolyl hydroxylase domain (PHD) proteins which use \( \text{O}_2 \) and \( \alpha \)-ketoglutarate as substrates to catalyze the reaction (52). Because this process requires molecular \( \text{O}_2 \), HIF-1\( \alpha \) escapes degradation under hypoxic conditions, enters the nucleus and heterodimerizes with HIF-1\( \beta \) to form HIF-1 (53). This dimerization induces a
conformational change in HIF-1α that is required for high-affinity DNA binding. HIF-1 acts as a sequence-specific transcriptional activator and under hypoxic conditions it interacts with an essential binding site within the hypoxia response element (HRE). This element has been identified in numerous genes that encode proteins which mediate a variety of essential adaptive responses to hypoxia (38), including those genes encoding EPO (54), glucose transporters (55), glycolytic enzymes (56), and VEGF (57). HIF thus mediates increased O₂ delivery to the cells as well as adaption to decreased O₂ availability (38).

The three major HIF-1 mediated adaptive responses to hypoxia are: erythropoiesis, angiogenesis and changes in glucose and energy metabolism (52). The first two play key roles in tumor biology, making HIF a target for anticancer drugs (37). However, VEGF does not only stimulate angiogenesis, but also increases permeability of blood vessels, and together with other HIF gene targets like eNOS and HOX-1 (which generate potential vasodilators such as NO and carbon monoxide) increase perfusion in hypoxic and inflamed tissue (53). It has been shown that hypoxia induces the expression of virtually all glycolytic enzymes (48;58) and increases the expression of membranous glucose transporters (59). Under normoxia, glucose transport is the rate limiting step in cellular glucose metabolism. This is the reason why under hypoxic conditions, transport needs to be increased. Through its control of gene transcription, HIF-1 establishes the optimal balance between glycolytic and oxidative metabolism that maximizes ATP production without increasing toxic ROS levels (52;60).
It has been shown that HIF-1 also plays a central role in stress responses beyond hypoxia, e.g. inflammation. The inflammatory microenvironment is usually hypoxic, due to increased O₂ consumption and decreased O₂ delivery due to interrupted blood flow (61). Therefore successful elimination of pathogens requires adaptation to reduced O₂ availability (62). When uninflamed tissues are analyzed for HIF-1α, very little protein is detected, although the O₂ tension in the tissue might be as low as 3 - 5%. In inflamed tissues however, O₂ tension can drop below these levels (61) and HIF-1α accumulates (62). The inflammatory microenvironment contains a number of small molecules such as cytokines (IL-1β, TNF-α), ROS (43) and NO (63) that have been shown to activate HIF under normoxia. However, under hypoxic conditions, induction of HIF is greatly enhanced. Mechanisms range from inhibition of HIF-1α protein degradation to transcriptional and translational control of HIF-1α mRNA (44). This facilitates adaptation to hypoxia by increased glycolytic ATP production (64) but also participates in pathogen defense by up-regulation of e.g. iNOS (65). In addition, VEGF receptor-1 has been demonstrated to be present on monocytes. The activation of this receptor can lead to monocyte activation and chemotaxis; therefore, it is hypothesized that secretion of its ligand VEGF (induced by HIF) into the airspace lining fluid may play a role in the recruitment of immune cells into the alveolar space.

This is a novel approach in the investigation of influenza pathogenesis. While HIF was discovered over 20 years ago (66) and its role in response to hypoxia is well
investigated (37;38;45;52), there is a lack of knowledge concerning the importance and significance of HIF in influenza infection. It is becoming more and more evident that HIF is not just involved in responses to hypoxia, but also plays a role in infection (67;68) and inflammatory responses (53;69).

It is therefore important to define HIF’s role in influenza pathogenesis. This raises the question of whether HIF-1α stabilization is a helpful and protective host response or an effect the pathogen anticipates in order to create a more favorable cellular environment for its replication. It has been shown that in cynomolgus macaques infected with either recombinant 1918 flu strains or with H5N1, HIF-1α is markedly up-regulated in the lung within 24h p.i. (70). This was done by immunohistochemical labeling of HIF-1α and semi-quantitative scoring related to nuclear expression/translocation. Since no pulsoximetry was performed on the animals, it was not determined to which degree the primates were systemically hypoxic. The authors cite a study in humans in which treatment for hypoxia improved survival rates (71) and suggest the measurement of HIF-1 expression, e.g. in peripheral blood mononuclear cells as a possible prognostic biomarker (70). However, no further investigation of an underlying mechanism leading to in HIF up-regulation was performed.

Compounds developed as cancer treatments targeting hypoxic cell signaling may also be potential therapeutics in influenza infection. A number of small molecules have been discovered that are capable of interfering with HIF signaling on different levels. Other treatment options include hypoxia-activated prodrugs,
monoclonal antibodies and drugs carried into hypoxic tumor areas by recombinant anaerobic bacteria. Some of these strategies are currently being tested or are already FDA approved (72). Werth et. al. suggest that altering HIF signaling pathways is a promising strategy for curing life threatening infections in humans (73).

We hypothesize that influenza infection results in increased pulmonary HIF-1α levels and altered expression of downstream targets which play significant proinflammatory roles in the pathogenesis of influenza-induced pneumonia. We have previously shown that Influenza A (H1N1)-infected mice become systemically hypoxic as detected by peripheral arterial O₂ saturation measurements (24;35;74). In addition, these mice have significantly impaired alveolar fluid clearance, resulting not only in systemic, but also local tissue hypoxia by creating a thickened layer of airspace lining fluid (75). Preliminary proof-of-concept studies show that there is increased detectable HIF-1α in the lungs of infected mice over the course of disease.

6.7 Final thoughts

Taken together, the data in this thesis provides a significant contribution to the field of ARDS as well as influenza research. The newly defined mouse model of ARDS contains more meaningful readouts that will be useful in testing novel therapeutics and aid in making more informed decisions about the fate of drug candidates. New therapeutic approaches such as pyrimidine synthesis inhibitors
and A₁-adenosine receptor antagonists have shown promising results in the animal model. Furthermore, additional investigations into chloride channel inhibitors as well as the role of HIF-1α in the pathogenesis of influenza-induced pulmonary injury may yield promising targets for future studies. Taken together, these strategies aim at closing the therapeutic gap that currently exists in the treatment of patients with ARDS and influenza virus infection.

6.8 References


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