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Critical insights into the pathogenesis of clinical isolates of pandemic influenza A(H1N1) 2009 virus in mouse and ferret models.

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CRITICAL INSIGHTS INTO THE PATHOGENESIS OF
CLINICAL ISOLATES OF PANDEMIC INFLUENZA A(H1N1)2009 VIRUS
IN MOUSE AND FERRET MODELS

By

Jeremy V. Camp
B.S. Auburn University, 2003
M.S. Georgia Southern University, 2006

A Dissertation
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ABSTRACT

CRITICAL INSIGHTS INTO THE PATHOGENESIS OF CLINICAL ISOLATES OF PANDEMIC INFLUENZA A(H1N1)2009 VIRUS IN MOUSE AND FERRET MODELS

Jeremy V. Camp
March 26, 2014

Influenza A virus (IAV) is a minus-sense, segmented, single-stranded RNA virus that infects the respiratory tract of humans and can cause severe illness. Novel IAV variants perpetually emerge on every continent, and the emergence of variants with increased transmissibility and/or pathogenesis in the human population is a serious concern for global public health. Infection with IAV typically causes an acute, self-limiting upper respiratory tract disease. However, severe IAV disease is characterized by infection of the lower respiratory tract which can lead to pneumonia and may result in the development of acute respiratory distress syndrome (ARDS). Viral and host contributions to the development of ARDS are poorly understood, however IAV pathogenesis has been linked mutations in the receptor binding protein and the viral polymerase. Ferrets and mice are two important laboratory animal models for studying IAV pathogenesis. In 2009, a novel H1N1 subtype IAV (H1N1pdm) emerged in the human population and displayed variable pathology in humans. Using a mouse model, we show the variability of clinical isolates of H1N1pdm is driven by viral mutations,

and that the timing of the inflammatory response is correlated with disease severity. To investigate spatiotemporal aspects of potential host and viral contributions to influenza pathogenesis, we developed a live imaging platform for ferrets infected with a human clinical isolate of H1N1pdm. We detected an early recruitment of neutrophils into ferret lungs following infection, which accumulated at foci of H1N1pdm infection within specific anatomical regions of the lung by 24 hours post-infection. The neutrophil response was biphasic, characterized by the recruitment of two populations with differing gene expression profiles, and baseline neutrophil levels were increased throughout the entire lung, including areas with no apparent viral infection. Changes in the viral microenvironment resulted in the regeneration of lung epithelium during recovery phase of infection, and this was imaged with PET-CT using a radiolabeled glucose analog. In summary, these data illustrate critical features of the immune response to IAV, and emphasize important considerations about the timing and accuracy of innate immune responses in studying viral pathogenesis.

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CHAPTER I

INTRODUCTION

1. Establishing a role for neutrophils in influenza A viral pneumonia

Influenza A virus (IAV) infection is the cause of a respiratory disease that poses a significant global public health concern (1-5). Influenza disease is commonly relatively mild and self-limiting, although highly pathogenic forms exist (6-13). The major complication from IAV infection is the formation of severe influenza pneumonia (SIP) which may develop into an acute respiratory distress syndrome (ARDS) (7-9, 11-15). The reason(s) why infection with IAV may lead to severe viral pneumonia and ARDS is poorly understood, but is thought to involve both host and viral factors. The respective and combined contributions of the host immune response and viral factors to the timing and severity of SIP are poorly understood. Neutrophils are immune cells that are well-known to be present during many types of lung diseases associated with ARDS, and may contribute to acute lung injury (16-25).

Neutrophils are present in the respiratory tract during infection with mild seasonal IAV, moderate and severe IAV infection, and highly pathogenic avian influenza viruses (HPAI) (7, 8, 14, 26-30). During SIP and HPAI infection, an increase in the number of neutrophils in the lower respiratory tract (LRT) is correlated with disease severity (7, 8, 14, 28-30).

However, neutrophils are poorly studied with respect to viral infection, respiratory viral disease, and specifically IAV infection. Herein, the contribution of neutrophils to IAV disease pathogenesis is reviewed.

IAV poses a concern for global public health due to emergence of strains with increased human transmission and/or increased pathology (2, 5, 31-34). In 2009, an H1N1 subtype IAV (H1N1pdm) emerged that was observed to have increased transmission and caused moderate to severe pathology relative to seasonal human IAV (1, 6, 28, 35-46). Clinical isolates of H1N1pdm have relatively little genetic variability yet caused variable clinical outcomes from moderate to severe pathology, including ARDS (1, 30, 38, 44, 45). Therefore they are well suited to understand host and viral contributions to IAV pathogenesis. Laboratory animal models, such as mice and ferrets, provide a way to study and test hypotheses about the pathogenesis of IAV, specifically about the contributions of viral and host determinants to severe disease (47-50). There are common occurrences in the formation of severe IAV (including SIP and ARDS) in humans, ferrets, and mice; these include increased cytokine secretions in the lung, diffuse alveolar damage (bronchointerstitial pneumonia in veterinary pathology), and neutrophilic infiltration (9, 14, 47, 50-56).

The lung has a global inflammatory response to infection, and this response includes the infiltration of neutrophils and macrophages in response to chemotactic signaling which originates in the lung (16, 18, 25, 57-64). However, the immune system requires additional information to control specific causes of infection. In other words, although similar cellular and systemic signaling systems are activated in response to infection of the lungs, in general the type I interferons (IFN) and interferon-stimulated genes (ISGs) are necessary to signal a benevolent immune response during a viral infection (65-68). During experimental IAV infection in mice, a breakdown of transmission of “viral” information causes malevolent immune responses, and a dysregulated immune response is lethal (e.g., via suppression of type I IFN, or productive infection of major IFN-producing cells, the alveolar macrophages) (69-75). Ultimately, all forms of respiratory infection require resolution of the infection and

inflammation. Interferons are essential components of initiating sterilizing immunity via the adaptive immune system (*i.e.*, resolution of infection), at which point the resolution of inflammation can effectively proceed (67, 68, 76). In regards to resolution of both infection and inflammation, neutrophils may be a keystone cell type in determining pathologic outcomes in the viral microenvironment. Although the mechanisms are poorly understood, through their direct antiviral actions and indirect actions on the lung microenvironment (*e.g.*, efferocytosis of apoptotic neutrophils by macrophages), neutrophils have the ability to influence outcomes towards successful resolution as well as towards the formation of ARDS (16, 18, 25, 26, 57, 77-84). In general, the interactions of neutrophils with IAV, the antiviral microenvironment, and resident lung cells deserve further study.

Here, the role of neutrophils in IAV disease is reviewed. First, the various presentations of disease caused by IAV infection are introduced followed by a description of the viral replication cycle. This is essential for understanding viral determinants of disease severity in humans, as well as the justification for the use of animal models in studying IAV pathogenesis. Finally, the host acute (exudative) response to IAV is reviewed, focusing on the role of neutrophils in disease and data from both human, mouse, and ferret studies. The role of neutrophils in the generation of adaptive immunity and resolution have been established in a mouse model of IAV, however, this aspect of the relationship between neutrophils and IAV infection is beyond the scope of this review (26, 83, 85-87). The intention here is to present what is known about the interaction between neutrophils and early events within the airways following IAV infection. In doing so, this review will also discuss the relationships between neutrophils and other respiratory virus infections to establish a role for neutrophils in respiratory viral disease.

2. Influenza A virus pathology

Although clinical pathology suggests that a spectrum of disease results from IAV infection, there are at least three disease “phenotypes” caused by infection with IAV, listed by increasing case fatality rate: a mild URT tract infection, a SIP which can lead to ARDS, and a LRT infection which can lead to hypercytokinemia (Figure 1.1). As discussed below, the virological basis for disease phenotype is related to IAVs adaptation to humans, for which some mechanisms have been well defined; thanks in part to the use of experimental animal models (2, 32, 33, 49, 88-91). An “ideal” viral infection (*i.e.*, one that is successful for the virus and non-lethal for the host) may be considered a balance between virus replication and an immune response necessary to promote viral shedding, typical of mild seasonal (“epidemic”) IAV. In general emergent IAV, directly or indirectly from avian enzootic cycles, have increased pathology in humans, the most fatal form of which is a syndrome of complete immune dysregulation (10, 11, 14, 92-95). IAV is genetically highly variable, and mechanisms for increased disease severity are multifactorial, involving host and viral factors, and thus are poorly understood.

2.1 Uncomplicated influenza.

The majority of yearly, seasonal IAV infections in the world cause a relatively mild, self-limiting URT disease (96). Influenza disease is characterized by an abrupt onset fever, myalgia and malaise, with symptoms similar to other URT infections, such as sneezing, coryza, and rhinorrhea (7, 97). Symptoms can last anywhere from 1-5 days, and are clinically indistinguishable from other “flu”-like illnesses, including bacterial and viral infections that cause the common cold (e.g., *Streptococcus pneumoniae*, *Haemophilus influenzae*, human rhinovirus infection, human respiratory syncytial virus infection, and

coronavirus infection) (97-100). Experimental infection of humans with IAV suggests that the virus is mainly restricted to the URT, although sampling the LRT is difficult (7, 8, 99, 101). While fever typically begins two days post-infection, virus is shed from the URT in nasal secretions as quickly as 24 hours post-infection, allowing efficient transmission prior to symptom onset, and continues until 4-5 days post-infection (98, 99, 101). Rhinorrhea is coincident with neutrophilic rhinitis and shedding of necrotic nasal epithelium (7, 102, 103). Surprisingly, the LRT seems to be involved in uncomplicated IAV infection, although this observation is frequently overlooked or unaddressed in studies (8, 104). In humans, local and systemic concentrations of IL6, CXCL8/IL8, and MCP1/CCL2 correlate with increased disease severity (*i.e.*, symptoms and increased virus shedding) (99-101, 105).

2.2 Pandemic influenza and SIP.

Histopathology. The 2009 H1N1pdm virus spread quickly throughout the globe, much like previous pandemic viruses such as the 1918 H1N1 “Spanish flu”. H1N1pdm infections also presented with typical flu-like symptoms (*e.g.*, fever, cough), however there was an increased number of cases presenting with dyspnea, respiratory distress, and pneumonia (1, 6, 28, 35-37, 39-45, 106, 107). Additionally, retrospective assessments show a proportionately greater number of adolescents and adults with severe disease compared to typical seasonal influenza, and patients with comorbidities such as obesity and asthma were at higher risk of severe infection (30, 108-110). In general, the virus causes infection of URT, as well as bronchitis and bronchiolitis, and a high proportion of cases presented with severe disease in the form of viral pneumonia (6, 30, 108). Histopathologic changes in autopsies revealed extensive cytonecrosis, desquamation, and inflammatory infiltration of the bronchus and trachea, mild to severe necrotizing bronchiolitis (6, 28, 30, 111). The primary pathologic finding of SIP

was sporadic to diffuse alveolar damage with hyaline membrane formation, edema, and occasionally hemorrhage (6, 28, 30, 111). As is typical of influenza infections, some patients experienced bacterial co-infection although this was not in a majority of patients, including those dying from ARDS (6, 28, 30, 45, 105, 111-115). This may distinguish the 2009 H1N1pdm virus from the 1918 H1N1 “Spanish” IAV, for which bacterial super-infection was determined to cause a majority of the deaths (116, 117), although this may more accurately reflect improved hygiene and standard of care. As discussed below, studies of the reconstructed 1918 H1N1 IAV using animal models suggest that this virus was highly pathogenic irrespective of secondary bacterial pneumonia (53, 118-120).

Immune response. Across many cohorts of clinical patients, serum concentration of IL-6, CCL2, and CXCL8 were significantly elevated in severe cases of H1N1pdm pneumonia when compared to patients with other confirmed illnesses including seasonal IAV, mild forms of H1N1pdm, bacterial pneumonia, or other viral respiratory infection (*Human respiratory syncytial virus* [hRSV], human rhinovirus [HRV], human adenovirus [hAdv]) (105, 112-114, 121). These cytokines and chemokines remained elevated over time (up to 6 days following hospital admission) in cases of severe H1N1pdm pneumonia, where as they decreased as patients recovered from seasonal and mild H1N1pdm infection (105, 112). In severe cases of H1N1pdm infection, decreased type I IFN and ISG production was occasionally noticed compared to adult patients with seasonal IAV infection (105). The H1N1pdm viruses have received much scrutiny and a large dataset of the genetics and pathogenic phenotypes of virus isolates exists in human and animal models. The H1N1 subtype IAV are highly important viruses due to their pandemic potential, as supported by the historical record (8, 53, 122). Some H1N1pdm viruses can infect the lower respiratory tract in humans and in the ferret animal model, which makes them excellent laboratory

viruses to investigate the involvement of the LRT in pathogenesis, specifically in the development of severe disease (6, 123-127). Their overall genetic similarity makes for excellent comparison studies between natural clinical isolates, and reverse genetics systems exist to study molecular pathogenicity (1, 107, 125, 128-138). This review will focus on the H1N1pdm viruses for these reasons.

2.3 HPAI and cytokine storm.

H5N1 HPAI. Avian IAV has sporadically entered the human population in the last 20 years, emerging first in China in 1997 (11, 93, 139, 140). It is likely that this (H5N1 avian IAV) and other emergent strains result from contact with infected domestic poultry who are infected with HPAI (93). The disease caused by H5N1 IAV is characterized by diffuse alveolar damage, alveolar necrosis, and alveolar hemorrhage (human disease, including pathology, is reviewed in (94)). There is evidence of viremia and systemic spread; IAV antigen has been detected in the trachea, bronchi and alveolar pneumocytes (10, 29), as well as infrequently in the brain and gastric epithelium (9, 29, 94). The innate cellular immune response in the lungs was characterized by an increase in inter-alveolar macrophages/histiocytes (9, 10, 12, 14, 92, 141) and only moderate infiltration of lymphocytes and neutrophils (10, 29). Systemically, patient serum had high concentrations of CXCL10, CCL2, IL6, IL8, and IL10 compared to matched control patients with either seasonal H3/H1 IAV, and these concentrations were correlated with viral load in throat (9, 10, 14). In lethal cases, the result of infection and immune dysregulation led to multiple organ failure, (e.g., kidney tubular inflammation, necrotic lesions in brain, impaired liver function) and abnormal clotting. Reactive histiocytes undergoing hemophagocytosis were

frequently found in bone marrow and lungs of patients, which is indicative of diseases involving hypercytokinemia.(9, 11, 14, 92, 94, 142).

Continuing emergence of avian IAV. Other events involving avian IAV transmission to humans are known, and are often associated with veterinary or other animal workers; for example a 2004 case of H7N7 infection in a veterinarian in Europe showed severe fatal pneumonia and DAD (143). It was reported that 1 L of serosanguineous fluid was drained from his chest upon autopsy. In 2013, another avian IAV emerged in Southeast Asia; this time an H7N9 virus (12, 13, 141, 144). The histopathology was similar to H5N1; severe pneumonia, diffuse alveolar damage, and epithelial necrosis were common features of infection with both viruses (12, 13). Patients infected with either virus had high levels of CXCL10, CCL2, IL-6, and CXCL8 in the plasma and displayed peripheral blood leukopenia and neutrophilia (10, 14, 92, 141, 142, 144). There were slightly more bacterial co-infections in cases of H7N9 compared to H5N1 avian IAV (12, 13, 92). In a direct comparison, serum from patients with infected with H5N1 avian IAV had higher concentrations of IFN α and IFN γ in the blood and lower levels of IL8, whereas the opposite was true for patients with H7N9 (145). Similarly, CXCL9 and CXCL10 were higher in patients with H5N1 avian IAV, whereas CCL4 concentrations were higher in patients with H7N9 avian IAV (145). Infection with either virus resulted in higher blood C-reactive protein (CRP) (141).

3. Influenza A virus components and replicative cycle

IAV was first isolated in 1933 as a “filterable agent” that was capable of infecting ferrets and satisfied Koch’s postulates in ferrets (146-148). It caused a respiratory tract disease in ferrets that was very similar to influenza disease in humans (147-149). Currently, many aspects of

IAV replication cycle are understood through the use of cell culture techniques. Modern molecular genetic techniques have developed a reverse genetics system, which allows direct manipulation of the viral genome to study biochemical properties of viral proteins (128, 150). Today, efforts by the WHO and agencies in collaborating countries have standardized techniques for the isolation and description of clinical IAV to provide constant, world-wide IAV monitoring with the goal of preventing or predicting future pandemics .

3.1 Virion structure and components.

IAV are enveloped, minus sense, RNA viruses in the family Orthomyxoviridae (151). Each virion carries one copy of the genome, which is composed of eight segments of viral RNA (151, 152) (Figure 1.2). In the virion, each genomic segment is bound with a polymeric helical viral nucleoprotein and a single molecule of the trimeric viral RNA-dependent RNA polymerase (RDRP) (153-156). This ribonucleoprotein (RNP) complex is encased in a shell of matrix (M1) protein, which is enveloped by a phospholipid bilayer membrane derived from host plasma membrane (157, 158). The IAV viral envelope contains three transmembrane proteins: the HA, the NA, and the M2 ion channel protein (158-162). The pleiomorphic virions are approximately 100 nanometers in diameter, and filamentous virus particles are produced which are typically greater than 1 micrometer in length (163-165). The complexities of virion assembly are not fully understood, particularly with respect to the formation of these filamentous particles and the production of virions lacking a complete genome (158, 163, 165-167).

3.2 Receptor binding, fusion, and entry.

Influenza viruses bind to glycosylated host proteins via the viral HA, which has a specificity for glycans with terminal SA residues (159, 168, 169) (Figure 1.3, step 1). Currently, it is thought that this receptor binding maintains the virus in close proximity of the plasma membrane until the virus can be taken up by clathrin-mediated endocytosis, although other forms of uptake are possible (170, 171). Clathrin-mediated endocytosis results in a gradually acidified endosome, which allows a two-step conformational change in the HA protein (172, 173). The HA is classified as a class II membrane fusion protein, and the conformational change results (1) in the insertion of an alpha helical domain near the cleavage site into the host's endosomal membrane followed by (2) a "pinching" movement at approximately pH 5.5 which fuses the host endosomal membrane with the viral envelope (159, 172, 174-176) (Figure 1.3, step 2). For this high energy fusion reaction, the HA must undergo a maturation step prior to endocytosis in which a host protease cleaves a residue on the pre-fusion conformation of the HA protein to prepare the protein for membrane fusion (177-181). During endocytosis, the M2 ion channel allows for the acidification of the interior of the virion simultaneously during the acidification of the endosome by host processes (182, 183). This prepares the matrix (M1) protein to release the viral RNPs into the cytoplasm once the membrane fusion event has occurred (157, 184, 185).

3.3 Viral replication and gene expression.

Nuclear localization signals designate transport of the RNP and other viral proteins to the host cell's nucleus where replication and transcription can occur (184, 186-189) (Figure 1.3, step 3). Once in the nucleus, the viral RDRP initiates these processes by creating complementary plus-sense copies of the gene segments (cRNA), which can be then replicated

back into vRNA (190, 191). The RDRP has a “cap-snatching” function, which removes a 5’-terminal methylated structures from host mRNA and attaches it to viral cRNA to make viral mRNA (192, 193). IAV lack methyltransferases, so this is an essential step for mRNA translation initiation on ribosomes (192, 193). This is also necessary to evade intracellular immune systems which detect 2’-*O* and 5’-penultimate unmethylated RNA structures in the cytoplasm (194, 195). Viral mRNA is exported and translated on ribosomes in the cytoplasm, while transmembrane glycoproteins are co-translated on the endoplasmic reticulum (Figure 1.3, step 4).

3.4 Non-structural gene products.

There are several non-structural gene products encoded by IAV. All seem to have the primary function of modulating the host’s immune response (151, 196-198) (Figure 1.3, step 5). As discussed below, NS1A protein reduces anti-viral type I IFN responses in infected cells through a variety of hypothesized mechanisms (reviewed in (199-201)). The IAV PB1-F2 is thought to modulate host cell apoptosis by targeting the mitochondrial activator MAVS and interfering with mitochondrial membrane potential (196, 197, 202, 203). Recently, several other gene products have been discovered (e.g., PB1-N40, PA-X) by identifying start codons in alternate reading frames (204). The approach to understanding these has been to use reverse genetics systems to create viruses with mutations in the polypeptide’s ORF or insert a stop codon to disrupt translation of the putative polypeptide in a way that does not affect the full-length protein and test the effects compared to the wild type virus *in vitro* or *in vivo* (205-207). With this approach, it is difficult to control the effects on other gene products; therefore the relative importance of these lesser-known non-structural proteins pathogenesis is currently unknown.

3.5 Virion assembly and budding.

As IAV proteins are being made, the RNP complex escapes the nucleus via the interaction of NS2 (nuclear export protein, NEP) with nuclear pore proteins (208). Transmembrane proteins, such as the HA, NA, and M2, are translated on rough ER and transported through the Golgi body to the plasma membrane (151). Currently, it is thought that the cytoplasmic domains of glycoproteins, the M1, and the M2 proteins coordinate the assembly of all eight RNPs at cytosolic locations near plasma membrane rafts of viral membrane proteins (166, 209, 210) (Figure 1.3, step 6). New virions deform the membrane outwards, eventually budding off from the host membrane (158, 166) (Figure 1.3, step 7). Release from the host cell is performed by the NA protein, which is a viral receptor destroying enzyme. Destroying SA receptors on the infected host cell may be beneficial by preventing re-infection of that cell. There is strong evidence in both natural and experimental systems for coevolution of the HA and NA proteins to enable an efficient balance between receptor binding and receptor destruction (211-215).

4. Influenza A virus ecology and epidemiology

4.1 Sources of IAV variation and subtypes.

There are two important rules governing the transmission cycle of IAV in nature: (1) the error-prone viral RDRP creates mutations which allow viral progeny to quickly adapt to new selective pressures (*e.g.*, “drift” over time within a host taxon), (2) optimized virus “solutions” to replication become genetically fixed across host taxa due to host-adapting selective pressures such as receptor binding (2, 32, 216). It is thought that host herd

immunity is the driving factor behind drift, and this makes influenza unique amongst RNA viruses ((217), reviewed in (218)). However, variation of the glycoproteins does not exist in a spectrum and certain “solutions” have been fixed in the IAV gene pool (219, 220). “Fixed” subtypes of IAV have been classified according to antigenic characteristics of the envelope glycoproteins, HA or NA (*i.e.*, antibody cross-reactivity) (2, 32, 221). This system is well-supported genetically, and viruses are identified using an “H” number and “N” number to specify the antigenic subtype of each. There are 16 subtypes of HA and 9 subtypes of NA which have been described from influenza viruses found in mammals and birds (219, 220, 222, 223). Recently two additional influenza-like viruses have been described from bats which have H17N10 and H18N11 glycoprotein subtypes (224-227). Biologically, the two membrane glycoproteins, HA and NA, have important roles in the viral life cycle, and are important features for the generation of host adaptive immunity via neutralizing antibodies.

4.2 IAV zoonoses.

Host restriction is intrinsically linked to the initial receptor binding step; thus HA subtypes remain antigenically stable within reservoir species with some drift (reviewed in (216)).

Subtypes of HA can be classified by their ability to bind specific SA terminal linkages ((223), reviewed in (159)). For example, avian IAV have undergone selection to bind preferentially to SA forming a terminal linkage to the carbon-3 of a penultimate galactose (*i.e.*, alpha 2,3-linked SA), which is the most common type of linkage in the avian gastro-intestinal tract (95, 169, 228, 229). In mammalian hosts, the isomeric linkage of SA to the carbon-6 of galactose is more common, resulting in alpha 2,6-linked SA which are found throughout the mammalian respiratory tract (95, 169, 228-230). The first step in determining whether transmission between taxa can occur is if receptor binding can occur in the new taxon. Once

in a new host species, gradual mutations in the HA and other viral proteins (*i.e.*, drift) may allow for increased efficiency of replication and transmission and establish these viruses within a new enzootic cycle (216, 231). For example, equine H7N7 and H3N8 IAV circulates in horses, canine H3N8 IAV circulates in dogs and originated from the horse strain, and swine H1N1, H1N2, H3N2, and the “variant H3N2” IAV circulates in domestic pigs (2, 38, 232-234). Throughout the last century there is evidence that novel H1N1, H2N2, and H3N2 subtypes of IAV have entered an endemic human cycle of transmission from an epizootic event and are now responsible for “seasonal” influenza (122). As the viruses drift and adapt with their host, the subtype proteins retain their original antigenicity; thus, for example, the canine, equine, human, and swine “H3” are genetically distinct, yet they are more similar to one another than any are to an “H1” (reviewed in (2, 216)).

4.3 Avian IAV.

Birds are natural reservoirs for all HA and NA subtypes of IAV (with the recent exceptions noted above), and as such all known influenza A viruses are avian influenza viruses (235-238). Avian viruses may infect domestic poultry, *i.e.*, chickens or turkeys (order Galliformes), and can be divided into highly pathogenic avian influenza viruses (HPAI) or low pathogenicity avian influenza viruses (LPAI) according to their pathogenicity in domestic birds (236-238). HPAI causes significant mortality in humans but transmits poorly between humans, and thus the 2003 H5N1 and 2013 H7N9 IAV outbreaks in China were contained (10, 11, 145, 239, 240). The reason(s) for the poor transmissibility of avian IAV between mammals is not known, although recently two laboratories have attempted to experimentally answer this question using ferrets (89, 91).

4.4 Epizootic transmission of IAV.

The hypothesis explaining the emergence novel IAV in the human population is that swine act as intermediate hosts between human population and the avian reservoirs (2, 31-34). Pigs have the ability to become infected with an avian virus and may foster adaption of avian viruses to mammalian SA linkages (241, 242). Additionally, coinfection allows for the reassortment of gene segments into novel viruses with altered virulence (241-244). Combined with the population size of modern pig farming (60 million in the U.S. alone) and the conditions in which the pigs are kept (i.e., overcrowding), this creates conditions for the emergence of novel IAV variants. An excellent recent example is the March 2009 emergence of a triple reassortant H1N1 IAV in a single pig farm in Mexico (H1N1pdm), which spread globally within three months and eliciting a WHO worldwide pandemic alert by June 2009 (1, 132). There is evidence that this H1 is the ancestor of the 1918 H1N1 virus that has remained in swine reservoirs, and in the years following the 2009 H1N1pdm, a reassortment event with H3N2 viruses have been associated with swine at agricultural fairs in the U.S. (1, 234, 245)

4.5 Human IAV.

Influenza viruses are present year-round in the human population, and the seasonality may be related to cold weather influencing human behavior (*e.g.*, spending winters indoors and increasing transmission rates) and favoring the persistence of the virus on fomites (246). Since the beginning of the 20th century, three main subtype combinations are readily transmitted within the human population: H1N1, H2N2, and H3N2 (122). For most of the last century, a novel antigenic form has displaced the previously circulating forms, *e.g.*, the

“Asian flu” H2N2 IAV from 1957 no longer circulates in the human population (122). The 1957 H2N2 IAV was instead replaced by a reassortment event with circulating H3N2 viruses, which were subsequently related to the cause of the 1968 H3N2 “Hong Kong” IAV pandemic (122). However the latest 2009 H1N1 failed to displace the seasonal human H3N2, and has even led to the formation of variant H3N2 IAV, which is a reassortant of the two as a direct result of mixing in the pig “vessel” (1, 132, 234, 247). Since 2005 other subtypes, particularly highly pathogenic subtypes, have been transmitted to humans from avian species, although so far they have resulted in dead ends for the virus (13, 141, 144). In summary, viral reassortment (antigenic shift) and random mutations under selection the reservoir host (antigenic drift) account for genetic complexity and adaptability of IAV (238). In the last 100 years, several emergent IAV have been recorded with varying levels of pathogenicity and transmissibility in humans.

5. Studying pathogenesis of IAV.

5.1 Mouse model of IAV.

Mouse IAV disease. Mouse IAV disease is characterized by increased infection of the respiratory epithelium (55, 248, 249) (Figure 1.4). This is often dose-dependent, and viral replication is typically in both the URT and the LRT (reviewed in (248, 249)).

Histopathologic findings after mouse intranasal IAV infection include pneumonia, alveolar edema, and mixed inflammatory infiltrates (248, 249). Infection of the LRT is not always fatal in mice, although the severity of disease in mice is difficult to assess. Signs of severe disease in mice include: hypothermia (as opposed to fever in humans), lethargy, hunched posture, ruffed fur, dehydration, and weight loss, the latter of which is typically used as a

humane endpoint (248, 249). Mouse respiratory epithelial cells display mainly alpha 2,3-linked SAs, and viruses with increased binding affinity to this receptor are known to replicate to a higher titer in mice which may result in a lethal disease (50, 230, 247). Human isolates (e.g., seasonal IAV) do not replicate well in mouse lungs and cause minimal disease, however 1918 H1N1 IAV (“Spanish” flu) and avian IAV both infect mouse lungs to high titer and systemic spread of viral infection is frequently noted (50, 55, 118, 250-254). This may be due to receptor binding affinity of the HA, as well as the presence of a multi-basic HA cleavage site in avian IAV (253-255). As discussed below, the innate immune system of mice has many similarities to humans.

Benefits and limitations of mice in IAV research. Mice are the most frequently used experimental animal model in modern biological laboratories, and in IAV research. (49, 50, 248, 249, 256, 257)(Figure 1.4) Genetically, laboratory mice are well-defined, homogeneous, and capable of experimental manipulation (*i.e.*, transgenic mice). Housing and husbandry costs are relatively low which allows for a comfortable level of statistical replication in experiments. Reagents and tools are commercially readily available for mice, which are important for studying the immune system. However mice are not natural hosts of IAV infection, and their lung anatomy, including the frequency and distribution of IAV receptors within the respiratory tract, is different compared to humans (49, 50, 248, 249, 256, 257). Mice are capable of becoming infected with certain IAV and may develop lower respiratory disease that has similarities to human influenza disease in a general sense (*i.e.*, a viral inflammatory immune response and the development of specific adaptive immunity) (49, 50, 248, 249, 256, 257). The pathogenicity of a given IAV isolate (e.g., human or avian) is variable between mouse strains, and does not always match human disease susceptibility (250, 258). The most well-studied host factor that contributes to increased pathology in mice

is the lack of a functional Mx1 gene (an ISG) in certain laboratory mouse strains (259-262). To overcome these limitations to studying IAV pathogenesis, strains of IAV may evolved to increased pathogenicity upon experimental passage through mice (49, 50, 248, 249, 256, 257).

5.2 Ferret model of IAV.

Ferret IAV disease. Ferrets are considered the best laboratory animal model for human IAV pathogenesis and transmission (47, 49, 50, 249, 256, 257, 263-266). They have a similar anatomy and physiology of the respiratory tract as compared to humans, including SA receptor frequency and distribution (267, 268) (Figure 1.4). They can be infected with human isolates of IAV without adaptation, and they are, themselves, natural hosts of IAV with human-like SA linkages (267, 268). Ferrets display signs of disease following infection that are very similar to humans, including fever, malaise, rhinorrhea, sneezing, dyspnea, and inappetence (47, 49, 50, 249, 256, 257, 263-266). Pathology from experimental IAV infection of ferrets may match human IAV pathology depending on IAV strain, specifically URT disease (71, 102, 103, 269) and bronchointerstitial pneumonia (related to human diffuse alveolar damage), although the latter is not as common as expected, possibly due to the practice of inoculating with an intranasal bolus (48, 270-272). Similar to human cases, infection with avian IAV causes infection of the LRT, and pathology shows severe pneumonia, inflammatory infiltrates of the lung, blood neutrophilia and leukopenia (56, 266). However ferrets display distinct neurological symptoms after infection with avian H5N1 IAV, a feature that was not common during human outbreaks (56, 273, 274). Importantly, viral traits that are associated with increased pathogenicity in humans are thought to be similar in ferrets: for example, viral receptor affinity that affects infection of the LRT verses

URT, increased polymerase activity, and to viral mechanisms of immune modulation (e.g., type I IFN suppression by viral NS1A) (56, 69, 89, 91, 275, 276). The ferret model is an enormous benefit for evaluating novel IAV for pandemic potential, measuring aspects of both transmission and pathogenicity, as well as testing vaccines and therapeutics for human use.

Benefits and limitations of ferrets in IAV research. Although ferrets are an excellent model for studying transmission and pathogenicity of IAV, and despite their use in IAV research for 80 years, the ferret model is poorly characterized compared to the mouse (47, 48, 148, 249, 264). The complete genome and transcriptome of the domestic ferret have been completed and published only very recently (277, 278). Laboratory ferrets are outbred and are therefore genetically diverse. In addition, there are very few ferret-specific tools and reagents commercially available, and these are poorly validated (279, 280). As a result, aspects of the ferret immune system remain unknown with respect to specific similarities/differences to the human immune (compared to the mouse, about which much more is known). Ferrets require special handling, housing and care, they are expensive and they are considerably larger than mice (47, 48, 264). This has the effect of reducing experimental sample size and, ultimately, reducing the statistical power achieved with many ferret studies. Perhaps for these reasons there is still variation in the experimental methods employed in IAV studies using ferrets (e.g., inoculation volume, sex, and age are not standardized), and clinical endpoints continue to become better defined (263, 265, 266, 272). Finally, many commercial vendors do not house ferrets in barrier facilities and it is possible, and very likely, that many research ferrets are not naïve to influenza infection.

6. Viral determinants of increased pathogenicity.

6.1 HA tropism and cleavage.

Sialic acid receptor binding. As discussed above, variation in viral HA leads to changes in amino acid constellations that affect receptor binding preference. This leads to increased efficiency of infection within a given host taxon. Just as different species have different frequencies of SA linkages, epithelial cell types within a given host various have different frequencies of certain SA linkages. The central dogma of the increased pathogenicity of avian IAV in humans has been that a preference of avian IAV for alpha 2,3-linked SAs enables the virus to replicate in the lower respiratory tract (52, 88, 231, 281, 282). IAV that cause severe pneumonia, such as H1N1pdm and 1918 H1N1 have an increased ability to bind to alpha 2,3-linked SAs, whereas mild seasonal IAV and H1N1pdm viruses with low pathogenicity have binding affinities to alpha 2,6-linked SA (88, 116, 282-284) (Figure 1.1). Further support of the link between receptor specificity and disease in a natural system came in 2013, where emergent avian H7N9 IAV had dual receptor specificity and caused viral pneumonia and ARDS, and not hypercytokinemia (145). The fact that H5 does not bind alpha 2,6-linked SA may be tied to the unique pathogenicity of avian H5N1 IAV, a disease marked by hypercytokinemia (88, 94, 145, 282) (Figure 1.1).

HA determines site of infection. The human URT and most of the LRT contain alpha 2,6-linked SAs, whereas the alveolar epithelium, submucosal epithelium, and alveolar macrophages contain mostly alpha 2,3-linked SAs (228, 229, 282). It is not known how avian H5N1 IAV is able to bypass the anatomical and chemical defenses of the URT to begin infection of the LRT, however ferret aerosol inoculation suggests IAV can be deposited in

the LRT (285). On the contrary, it has been shown that if H1N1pdm viruses are manipulated to have altered tropism to alpha 2,6- or alpha 2,3-linked SAs, this has no major effect in pathogenicity in ferrets (116). Therefore, properties of these viruses other than HA binding affinity (*e.g.*, binding avidity of HA or contributions of other gene segments) cannot be ignored. In summary, replication in the URT may progress to pneumonia and ARDS if viral HA allows LRT replication; but replication in the LRT, alone and without URT involvement, is linked to a disease characterized by hypercytokinemia. Likewise, it is clear that HA receptor affinity is important for transmission of avian IAV to mammals and between mammals, and is therefore an important not only as a virulence but also for pandemic potential.

HA maturation and cleavage. Other features of the HA that are related to increased pathogenicity are related to viral entry and membrane fusion. The immature HA requires cleavage by host proteases so that fusion can occur during the reduced pH of the endosome (177, 179, 181). A feature of the HA of HPAI, compared to LPAI, is the presence of a polybasic cleavage site (177-180, 286). The polybasic cleavage site allows for cleavage by the ubiquitous protease furin, which makes it possible for the virus to replication in many mammalian tissues (178, 181). Additionally, it was recently observed that some H1N1pdm isolates carried a mutation that altered their specific pH of fusion (287). HA typically fuses within the late endosome at pH 5.5, and specific amino acid mutations that stabilize the pre-fusion complex may reduce the pH of fusion (172, 174, 287-290). This may change the endosomal compartment where IAV undergoes fusion, although the ultimate effects of this are unknown.

6.2 Replication in mammalian cells.

The rate of viral replication in the host cell is dependent upon the RDRP. Host adaptation has shaped a key residue in the polymerase subunit, PB2, which allows increased RDRP activity in either mammalian or avian cells (275, 291-296). Avian IAV adapted to human cells (E627K) have a distinct increase in RDRP activity at 33°C, the temperature of the human respiratory tract, whereas a glutamate at the site has a maximum RDRP activity at 42°C (296, 297). Similar to the viral properties bestowed by HA, this alteration is important for pathogenicity as well as the transmission and pandemic potential of avian IAV in mammals.

6.3 NS1A and IFN.

Many viruses have evolved strategies to counteract an efficient host response to infection, which almost unanimously includes the ability to interrupt the type I IFN system (66-68). IAV accomplishes this through NS1A, a non-structural protein that is involved in direct and indirect disruption of the type I IFN response system (198, 298). Using reverse genetics techniques, IAV lacking NS1A functionality or lacking NS1A altogether can be made and can infect cells (299). However, these viruses are very sensitive to cellular type I IFN stimulation, and cells infected with these viruses produce much more type I IFN than cells infected with wild-type IAV (299-301). Through studies with these viruses, it has been shown that NS1A masks cytoplasmic viral nucleic acids by interacting with dsRNA molecules (299-301). This effectively hides (sequesters) dsRNA from cytoplasmic PRRs such as 2'5' oligoadenylate synthase (OAS)-RNaseL, protein kinase R (PKR), and the RLR

family of helicases (such as RIG-I and MDA5). The binding of cytoplasmic viral dsRNA by RIG-I or MDA5 begins downstream transcriptional activation of type I IFN (299-301). In addition, NS1A may also bind these host cell factors, either directly or via co-binding to dsRNA, to inhibit the translation of host cell mRNA to counteract establishment of an anti-viral state (198, 302). The interactions of NS1A are an illustration of IAV as a master at effecting an acceptable and necessary level of inflammation and cellular activation (291, 303-305), and the pathogenicity of some isolates may result from an inability of IAV to manage the balance between cell activation and immune suppression (199, 306). For example, delay of type I IFN signaling via NS1 mediated increased virulence in ferrets (69). In this scenario, inflammation (*e.g.*, NFkB-mediated effectors) would result in leukocytic infiltration without the appropriate information (*i.e.*, type I IFN) to generate a “correct” anti-viral response (65, 69, 70, 300, 301, 307). Powerful effector cells like neutrophils may respond inappropriately, and begin a cascade of immune dysregulation (70, 71, 301).

7. Immune response to IAV infection

7.1 Site of infection.

IAV infects and replicates in epithelial cells of the respiratory mucosa (151). The human respiratory system is divided functionally and anatomically into URT and LRT at the larynx, by convention. The function of the respiratory system is to facilitate gas exchange between the host blood and the atmosphere. The LRT is the primary site of gas exchange, and it is highly sensitive to (and highly protected from) inflammation. The URT serves as a mechanical and chemical barrier, protecting the sensitive LRT from infection and debris.

The upper respiratory tract. The URT is made up of the nasal cavity, the sinuses, and the pharynx (often the laryngopharynx and larynx are included in the URT). The nasal cavity and nasopharynx are lined with a pseudostratified columnar epithelium which contains both ciliated and non-ciliated cells. Anatomical barriers in the URT include the vibrissae and nasal turbinates (conchae) which block large particles and vortex air to remove smaller particulate matter. During this process the air is warmed and humidified, and receptors transmit chemical information to the brain via the olfactory nerves. Particulates which become trapped in the mucous of the URT are moved up and out of the URT via beating of the ciliated epithelial cells, rhinorrhea and sneezing. The nasal turbinates are heavily vascularized, and during inflammation may be the site of extreme leukocytic infiltration. Neutrophils, mast cells, and eosinophils contribute to both allergic and infectious inflammation which leads to rhinitis and associated symptoms (especially rhinorrhea).

The lower respiratory tract. The LRT begins below the larynx, and includes the trachea and main bronchus. These structures are supported by cartilage, and the mucosal barrier consists of a thick layer of a psuedostratified, ciliated, columnar epithelium with non-ciliated columnar and basal cells (Figure 1.5). Fibroblasts and antigen presenting cells are found in the lamina propria, which is supported by a submucosa that is thick with connective tissue. Specialized non-ciliated cells, goblet and/or clara cells, are vacuolated secretory cells within the epithelial layer which contribute primary chemical defenses to the respiratory mucosae in the form of mucous containing defensins and other innate immune effector proteins (Figure 1.5). Similarly, invaginations of the bronchial epithelium which extend into the submucosae contain patches of additional specialized secretory epithelial cells, the seromucinous (or submucosal) glands (Figure 1.5). They also contribute to the mucosal lining which protects the epithelium and is the vehicle of the mucociliary elevator. These features of the URT and

the upper portion of the LRT appear to exist solely to protect the sensitive alveolar spaces below.

Beginning with the branching of the trachea into the main bronchi, the LRT (the lung, proper) is divided into lobes fed by branches of the bronchi which split into smaller bronchioles and even smaller alveoli. This arrangement creates an enormous surface area in a minimal volume. The alveoli are arranged in acini and are composed of two types “alveolar” epithelial cells: the thin type I pneumocytes at the air/water interface and the cuboidal type II pneumocytes that secrete surfactant lipids and proteins (“I” and “II” in Figure 1.5). Associated with the bronchioles are pulmonary blood vessels which branch into smaller capillaries that cover the alveolar clusters as the lung microvasculature. The individual capillaries are typically composed of a single cell of endothelium creating a tube that is thinner than a single red blood cell. The thin capillaries and thin type I pneumocytes facilitate the efficient transport of gases between the blood and air. The barriers to infection and debris in the alveoli consist of surfactant proteins and patrolling alveolar macrophages. Surfactant proteins help buffer the surface tension of the mucosal lipid layer to prevent the collapse of the alveoli. Surfactant protein-A and surfactant protein-D are lectins (specifically they are C-type lectins, collectins, that resemble C1q complement protein) that opsonize certain viruses for phagocytosis by alveolar macrophages, including IAV (reviewed in (308)).

Alveolar macrophages. Alveolar macrophages are resident phagocytic myeloid cells within the lungs, and are the most abundant immune cells in the lung, comprising over 90% of cells in lung lavage fluid (reviewed in (309, 310)). Alveolar macrophages perform anti-inflammatory functions during homeostasis, and their capacity as phagocytic cells constantly

clears debris from the sensitive alveoli . Despite extensive anatomical and chemical barriers, the alveoli inevitably receive both harmful and harmless antigens (311). Alveolar macrophages rely on immune “ignorance” to maintain homeostasis, and suppress dendritic cell and T cell activation via secretion of IL-10, TGFb, and/or NO *in vitro* (311, 312). Phenotypically alveolar macrophages are similar to dendritic cells, and they perform similar functions by constantly sampling the alveolar microenvironment for antigens and PAMPs (313). For the most part they are self-renewing cells, but they can be replaced by bone marrow-derived monocytes upon depletion (314, 315). They are in constant contact with the pneumocytes, and this provides essential feedback for both cells in the form of CD200/CD200R which maintains an anti-inflammatory state (316-318). As discussed below, they are the “keystone” cells in the lung, and they have important functions in modulating inflammation, cell death, and regeneration in the sensitive LRT (313, 319-323).

7.2 Cellular response to IAV infection.

The initial universal response to the detection of viral PAMPs is the secretion of type I IFN (reviewed in (66, 68)). The release of type I IFN triggers interferon receptor pathways which activate transcriptional regulators to initiate production of ISGs via JAK-STAT signaling (reviewed in (324)). This operates in an autocrine and paracrine manner in an effort to stop the infection process and prevent neighboring cells from becoming infected. The ISGs include pro-inflammatory cytokines and chemokines such as type I IFN, CXCL1 and CXCL10, as well as cytoplasmic anti-viral proteins such as Mx1, OAS, RNaseL, and PKR (reviewed in (325)). Similarly, these initiate well-studied inflammatory systems, such as activation of the transcriptional regulator NF-kappaB, to release other cytokines and chemokines, such as IL-6, CCL2, and CXCL8. The net effect on cells is an increased ability

to detect viral PAMPs, a blockade on cytoplasmic translation, the initiation of apoptosis, the display of viral epitopes on major histocompatibility complexes, and pro-inflammatory signaling to alert neighboring cells and recruit inflammatory cells. IAV has mechanisms to buffer the activation of these cascades via NS1A-mediated suppression of type I IFN translation or anti-apoptosis via PB1-F2 (196-198, 300, 301).

7.3 Epithelial cell response to infection.

In the URT, IAV infects epithelial cells. Non-immune cells (non-myeloid) have cellular sensors of viruses that rely on detection of viral nucleic acids, and mammalian immune systems rely chiefly on the presence of dsRNA in the cytoplasm to recognize these viruses (326, 327). Double-stranded RNA is a common intermediate to the replicative cycles of both RNA and DNA viruses. RLRs are cytosolic helicases, such as RIG-I or MDA-5, which recognize dsRNA (327, 328). These activate pathways leading to transcription factors that initiate type I IFN secretion from the cell (Figure 1.6). Similarly, PKR is capable of recognizing and responding to viral dsRNA after activation via type I IFN signaling. TLRs (for detecting PAMPs in the extracellular and endosomal spaces) are typically not as abundant in epithelial cells as they are in myeloid cells (326, 327, 329).

In vitro infection of well-differentiated human primary respiratory epithelial cell culture systems and primary lung explants allows the study of the interaction between IAV and epithelial cells without confounding comorbidities in human patients (75, 330-334). In addition, this allows side-by-side comparison of various IAV isolates in the same human genetic background. Infection of these cultures with many IAV isolates causes the

production of type I IFN, IL-6, CCL2, and CXCL8, indicating that the epithelial cells contribute to the immune response, specifically by recruiting leukocytes to the site of infection (331, 334-338). There are differences between the response to infection with seasonal, H1N1pdm, and HPAI viruses; for example, seasonal IAV infection causes increase in the cytokines mentioned above, indicating that H1N1pdm viruses may be suppressing the immune response of these cells (viral titers were similar) (331, 334) and there is indication that this is strain-specific (331, 333, 335, 336). Interestingly, this was only true for apical secretion of cytokines and chemokines from these cells – we have shown that H1N1pdm viruses have increased basal secretion of cytokines and chemokines, such as IL6, CXCL8, CCL2, CCL4, and CCL5, from IAV-infected well-differentiated normal human bronchial epithelial cells grown in collagen-coated inserts (331). Moreover, infection of human epithelial cells with H5N1 viruses causes a comparatively higher level of IL6, CXCL8, CXCL10, TNF α , and CCL5 secretion in side-by-side studies (332, 334). Heat-inactivated virions (i.e., fusion-competent but non-replicating) were sufficient to activate human neutrophil chemoattractant gene signatures, such as CXCL1 and CXCL8 (333). Finally, necrotic epithelial cells are typical of severe influenza infection, and these cells provide a source of immune signals, as they release CXCL8 upon death (338).

7.4 Alveolar macrophages shape the viral microenvironment.

Alveolar macrophages play an important role in contributing to and limiting inflammation from epithelial cells and infiltrating macrophages during infection with IAV. In mice, depletion of alveolar macrophages prior to IAV infection results increased viral replication in the lungs, increased cytokine and chemokine production in the lungs, increased neutrophil infiltration, and increased lung pathology (322, 323, 339). Unlike epithelial cells, *in vitro*

experiments using human alveolar macrophages show that they release a wide variety of cytokine, chemokines, lipid mediators, and ROS into the environment upon sensing virus or via cytokine/chemokine receptors (75, 340-342). While pneumocytes are capable of immune signaling and release of cytokines following IAV infection, alveolar macrophages are the cells that shape the early lung microenvironment following IAV infection, and determine the pattern infiltration of leukocytes to the lung (61, 320-322, 337, 343-345).

Disrupting homeostasis. Upon introduction of IAV into the alveolar space, the alveolar space contains virus, pulmonary surfactant, and alveolar macrophages (Figure 1.5). Upon infection of either the epithelium or the alveolar macrophage, homeostatic cross-talk between alveolar macrophages and epithelial cells (e.g., CD200/CD200R) may become temporarily disrupted as type I IFN is released into the alveolus (316, 346). An important consequence of type I IFN in the alveoli is the triggered apoptosis of infected epithelial cells by activated macrophages via death receptors (65, 343, 344, 347, 348). Additionally, infection with IAV causes increased apoptosis and necrosis in epithelial cells and in monocytes without addition aid of death receptor ligation (202, 251, 334, 349-352). Desquamation of alveolar epithelium is characteristic of ARDS; on histological preparations from severe human cases the alveolar epithelium is replaced by an eosinophilic hyaline membrane (6-8).

Infection of alveolar macrophages and sensing IAV. *In vitro* studies using mouse (73, 353) and human alveolar macrophages (75, 341, 342) show that infection is abortive, suggesting that alveolar macrophages are not contributing to increased virus in the lung during infection. In mice, infection with a pathogenic IAV causes a necrotic depletion of alveolar

macrophages, which can be rescued by administering GM-CSF (313, 354). However, abortive infection is critical for sensing infection by activating virus-specific PRR and initiating adaptive immunity via MHC-I presentation on antigen presenting cells (355, 356). Detection of pathogens via TLR signaling relies heavily on myeloid cells, such as plasmacytoid DCs and alveolar macrophages (326, 357, 358), and detection of IAV relies heavily on RIG-I signaling (327). Both of which lead to induction of the type I IFN system and provides a crucial link with the innate and adaptive immune systems (67, 329, 348). Although there is an established trend that IAV with increased pathogenicity stimulate a decreased inflammatory response from alveolar macrophages *in vitro* (74, 340, 341, 359), to my knowledge no studies have investigated the effects on the normal anti-inflammatory functions of alveolar macrophages during IAV infection (*e.g.*, TGF β secretion).

7.5 Systemic inflammation.

As the local inflammatory environment grows, macrophages and endothelial cells amplify and transmit the inflammation systemically in the form of chemokines, pyrogenic cytokines, and growth-stimulating factors (360) (Figure 1.6). Chemokines recruit innate immune cells to the site of infection (reviewed in (361)). Following infection, cells such as natural killer cells (NK), inflammatory monocytes and macrophages, as well as neutrophils infiltrate the airways from peripheral blood circulation (70, 253, 360, 362, 363). As discussed below, these cells become activated while in the peripheral blood circulation, continue to activate during migration to the site of inflammation, and then perform effector functions to at the site of inflammation in response to the microenvironment. The activation of the innate immune response is immediate and acute, whereas the adaptive immune system requires time but is more specific. In the following, the role of specific early innate immune cells are discussed.

NK cells. In the context of viral infection, NK cells recognize “missing self” and the presentation of death receptors to destroy infected epithelial cells. Interestingly, mammalian NK cells are known to contain cell surface proteins, NKp46 and NKp44 in humans (NCR1 in mice and ferrets), which activates the cell upon recognition of IAV HA (364-367). IAV seems to have NK evasion strategies that include direct infection of NK cells as well as increasing the display of NK cell-inhibiting ligands on infected cells (368). Even so, NK cells seem to be dispensable for the response to influenza, and patients lacking NK cells recover fully from IAV infection (369, 370). In animal models, NK cells are essential for clearing viral infection, and they accomplish this by contributing to the generation of a robust adaptive immune response (*i.e.*, Th1), potentially through the release of massive amounts of IFN γ (367, 371, 372).

Inflammatory Monocytes and Macrophages. Macrophages are terminally differentiated cells present in tissues (315). During inflammation their precursors (monocytes) are recruited from the peripheral blood circulation and the bone marrow to the site of infection to differentiate in response to *in situ* signals (reviewed in (309)). These signals may polarize these cells to differentiate towards various phenotypes, some of which have pro-inflammatory features and some have anti-inflammatory functions to various degrees (373). Infiltrating inflammatory monocytes and macrophages have a different phenotype than alveolar macrophages, and have different susceptibilities to infection by IAV (359, 374, 375). Depending on the *in vitro* preparation of macrophages and the viral strains used, their importance in IAV disease is still uncertain. However, in general it seems that infiltrating monocytes and macrophages have a clear pro-inflammatory response to IAV infection (72,

351, 376-378). In a CCR2^{-/-} mouse model of IAV, even a minor delay in infiltrating macrophages drastically increases the influx of neutrophils into the lung by exacerbating the CXCL1/CXCL2 response (379). Macrophages are the main producers of CXCL9 and IFN β (380) following mouse IAV infection. An interesting experiment used intraperitoneal injection of IAV to show that infection of inflammatory macrophages by IAV stimulates an immune response *in vivo* without contribution of epithelial cells (375).

Neutrophils. Neutrophils are another subset of phagocytic myeloid cells, and exist in steady-state as mature, terminally differentiated cells. They are well known to be associated with acute lung injury and inflammation in the lungs, and are typically among the first immune cells to arrive during many types of infection and inflammation (16, 18, 381). They are well-studied with respect to bacterial infection, although their role in viral infection is not clear. Neutrophils are present during acute phases of IAV infection of both the URT and LRT, as well as during infection with other respiratory viruses (343). A common feature of the immune response to pathogenic influenza viruses in human and animal models is an excessive infiltration of neutrophils into the LRT (70, 85, 86, 253, 323). However, experimental depletion of neutrophils prior to infection with IAV leads to increased lung pathology, increased inflammatory cytokines and chemokines in the lung, and increased virus replication (84, 86). Neutrophil activation and their migration to the lungs are steps that are critical to recovery from IAV infection (26, 78-80, 83, 84, 86, 323). Neutrophils are short-lived during inflammation, and contribute significantly to the cellular debris that accumulates in the lung during infection, although they are capable of phagocytosis of IAV-infected epithelial cells (79). There is very little known about neutrophils in the context of viral

infection. In the next section, the possible roles for neutrophils in IAV disease will be discussed in detail.

8. Neutrophils in IAV infection

Neutrophils are increased in the lungs and blood after infection with pathogenic IAV in mice, humans, and ferrets (294, 363, 382). Neutrophils are necessary for recovery from severe, but not mild, IAV infection (86, 381). Studies in mice show that neutrophils have effects during both early and late stages of disease (85). Signals from both alveolar macrophages and macrophages initiate neutrophil chemotaxis and activation to the lungs. For example, TLR7 recognition of IAV dsRNA and Myd88-mediated release of TNF α and CCL3 by mononuclear cells is important for neutrophil recruitment to the site of infection (383, 384). Interestingly, TLR3^{-/-} mice have increased neutrophilia and fewer macrophages in the lungs, yet have increased survival after infection with IAV (385, 386). In cell co-culture, human neutrophils were seen to interact specifically with IAV-infected cells (387), although the nature of this interaction in the infected lung is unknown. Transgenic mice have been used to study the contribution of specific cytokines and chemokines to inflammation following IAV infection, particularly as this relates to “hypercytokinemia”, and are summarized in Tables 1.1-1.4. The signals from the infected lung are propagated systemically by endothelial cells, which recruit and tether neutrophils. The complex interactions governing neutrophil extravasation, migration through the interstitium, and crossing the alveolar epithelium are well known in relation to many forms of ALI and the development of ARDS with the exception of conditions surrounding viral infection (16-18, 20, 388, 389). Due to the mechanical

stimulation of passing through the small diameter of the microvasculature of the lung, neutrophils in the lung are more activated than peripheral neutrophils (17).

8.1 Neutrophil migration.

Neutrophil chemotaxis in humans is thought to be mediated by many factors, such as the chemokine CXCL8, cytokines IL-1 and TNF α , and complement C5a (388, 390, 391). During both mild and severe IAV disease, patients show increased blood CRP and activation of C5a (392), as well as increased secretion of CXCL8, TNF α , and IL-1 in nasal washes which correlate with disease severity (99, 101, 105, 393-395). Studies using mice have offered insight into the relationships between inflammation and neutrophil chemoattraction during influenza infection. For example, chemical reduction of C5a during IAV infection reduced lung neutrophilia (396). Similarly, cytokines such as IL-1b and IL-6 are important for neutrophil activation and migration to the lungs during mouse IAV infection (Table 1.1) (397-399). Mice do not possess CXCL8, but CXCL1 and CXCL2 have equivalent functions. Neutrophils contribute CXCL2 to the IAV-infected mouse lung to further stimulate neutrophil recruitment (380). More recently it was shown that removing a CXCL1 repressor (*Setdb2*) does not increase recruitment of neutrophils to the lungs of mice infected with IAV PR8, rather it reduces the ability to respond to bacterial superinfection (400). In addition, it was shown that another ISG, CXCL10, operates on a unique subset of CXCR3⁺ neutrophils present during mouse IAV infection in an autocrine manner, increasing chemotaxis, oxidative burst and enhancing inflammation (380) (Tables 1.3 and 1.4). Finally, aryl hydrocarbon receptor is somehow linked to increases in NO and neutrophilia in the lungs of IAV-infected mice independently of known neutrophil chemoattractants or mechanisms of neutrophil extravasation (401-404).

8.2 Neutrophil extracellular PRRs and phagocytosis.

Neutrophils are phagocytic cells, and their methods for sensing extracellular pathogens relies on TLRs (390). Stimulation of neutrophils through TLRs has been recorded to promote cytokine secretion (CXCL8 and TNF α via NF κ B and AP-1), ROS formation, phagocytosis, granule secretion, NET formation, and migration (388, 390, 391). Human neutrophils highly express nucleic acid-detecting TLRs, specifically endosomal TLR8 (405, 406), but do not express nor respond to activators of TLR3 or TLR7 (82, 405, 406). TLR4 is required for LPS-induced neutrophil migration to the lung (407); however, TLR4-stimulation does not lead to the production of type I IFN in neutrophils (82, 408). Surfactant protein D, a lung collectin, is an innate immune defense against a variety of viruses, which marks them for phagocytosis by neutrophils which cause the production of ROS (409-415). Human neutrophil defensins are short basic peptides released from neutrophil granules during inflammation (388, 415, 416). They have been shown to interact with IAV, reducing infectivity, and promote neutrophil phagocytosis and clearance of IAV (415-419). Defensins may also buffer the oxidative burst from neutrophils that follows from phagocytosis of viruses that have been opsonized by surfactant protein D (415-417). One study indicated that neutrophils do not interact with immunoglobulin-bound IAV (420), however another showed that protective anti-IAV antibody therapy only protected mice in the presence of neutrophils (78).

8.3 Neutrophil intracellular PRRs.

Neutrophils express sialic acid receptors and may become infected with IAV (26, 421, 422). Neutrophils infected with IAV have increased apoptosis, but infection does not result in the

production of virus (422). Infection of human neutrophils with IAV treated at 56°C to denature the viral replicase but not HA suggested that infection alone, but not replication, is sufficient to stimulate the release of CXCL8 and CCL4 in human neutrophils (423). Neutrophils infected with IAV have rapid up-regulation (<9 hpi) of type I IFN pathways, including cytoplasmic PRRs, IFN β , and ISGs (422), which is counter to the long-held dogma that neutrophils were incapable of gene expression. This is may be due to RLR sensing of viral dsRNA, as neutrophils transfected with poly(I:C) (a viral RNA mimic) have a similar response (82). Additionally, neutrophils express nod-like receptors (NLRs) but it is unclear how these interact with IAV infection (424, 425). In general, inflammasome and pro-IL1 activation following IAV infection is poorly understood (307, 397, 398, 426). However, studies of IAV infection using caspase-1, IL-1 β , or IL-1R transgenic mice show modulation of neutrophil infiltration and pathology, and suggest that it is an IAV subtype-dependent effect (397, 426-430) (Tables 1.1 and 1.2). Furthermore, it has been demonstrated that the HA of some IAV isolates suppresses neutrophil activation, providing further evidence for IAV subtype-dependent effects on neutrophils (421, 422, 431).

8.4 Neutrophil activation and degranulation.

Activation of TLRs and RLRs trigger degranulation and the upregulation of CD11b (82), which pairs with CD18 to form the “Mac-1” integrin dimer that binds collagen (432). This facilitates migration through tissues, and release of gelatinase or collagenase (MMP-2 and MMP-9) from neutrophils assist in clearing connective tissue from the path. At the site of infection, neutrophils release microbial effectors (reviewed in (81, 388)). Neutrophils develop granules sequentially (azurophilic, specific, gelatinase, secretory) and secrete granules in the reverse order (388). Secretory and gelatinase granules are released shortly

after endothelial transmigration and contain membrane proteins essential for movement (extracellular matrix [ECM]-binding integrins) and pathogen recognition (immunoglobulin [FcR] and complement receptors) (388). Specific and azurophilic granules contain tissue-destroying enzymes and anti-microbial proteins. For example, neutrophil MPO may contribute to lung injury during IAV infection (77), however it may have direct antiviral effects on IAV (433). An investigation found no difference between IAV infection of a wild-type and neutrophil elastase knockout mouse, measuring lung function, chemokine secretion, and neutrophil recruitment (434) (Table 1.5). The contents of the granules can be secreted to destroy ECM (such as MMP-9) or directed towards phagosomes to destroy engulfed microbes. The production of hypochlorous acid (HOCl) is the main oxidant used in phagosomal killing, and its production is dependent on the generation of reactive oxygen by the neutrophils. Interestingly, infection by IAV was related to the inhibition of phagosomal killing of bacteria (431).

8.6 Oxidative burst.

Neutrophil degranulation primes neutrophils for ROS generation by mobilizing NADPH oxidase components to the plasma membrane (388) and exocytosis of MPO. IAV infection causes the generation of reactive oxygen species in neutrophils (435). Oxidative burst is thought to have direct microbial effects, however the direct effect on IAV has not been published (436). IAV infection benefits from the presence of ROS in the environment (437, 438), yet IAV also suppresses NADPH oxidase activity within infected phagocytes (439, 440). Many have investigated the effects of ROS and NO on lung inflammation during IAV infection and found that reduction of oxidative stress in the form of both ROS and NO alleviates IAV-dependent lung injury (439, 441-446) (Table 1.5). For example, oxidized lipids in the

lung environment may trigger TLR4, activating immune cells and contributing to increased lung injury (447-449).

8.7 Neutrophil netosis.

Neutrophils undergo a form of programmed cell death called netosis, in which NETs are formed (450). NETs have the effect of killing many pathogens, including bacteria (389), fungi (451), and protozoans (452). NETs are becoming the focus of study in autoimmune disease atherosclerosis, since they damage endothelium (453-455). Recently, it was shown that hantavirus stimulates NET production during infection which leads to the generation of autoantibodies and may provide a mechanism for the hemorrhagic fever caused by Old World hantaviruses (456). NETs are typically found with histones, MPO, and neutrophil elastase, and the effect is to isolate the effects of these molecules directly onto the pathogen surface with a “sticky” NET of nucleic acid. NETs contribute to acute lung injury and alveolar capillary damage during IAV infection (381). Yet very little is known about the relationship between NETs and viral infection in viral disease pathogenesis (457).

8.8 Contribution to the viral microenvironment.

Resolving inflammation. At some point, neutrophil infiltration into the lung must stop, and increased neutrophils are present in lethal cases of ARDS. This may be regulated by secretion of IL-1RA and chemokine-destroying factors by recruited macrophages (373, 458). Additionally, it has been proposed that efferocytosis of apoptotic neutrophils is a key step in resolution of inflammation (459-461), and occurs in the lung during bacterial pneumonia (462). It is unclear if this happens during IAV infection. Finally neutrophils act as antigen

presenting cells during IAV infection of mice and enhance cytotoxic T cell responses (26, 83), contributing to resolution via the production of an adaptive immune response.

Prolonging inflammation. Factors that prolong the life span of neutrophils in the lungs increase the probability that they may contribute to immunopathology. IL-6 and G-CSF are immune mediators present in the lung during infection and are known to prolong survival of neutrophils in mouse lungs following IAV infection (463). Both neutrophils and macrophages are known to phagocytose apoptotic epithelial cells in mouse lungs during IAV infection (79). The cells may be recruited via chemokines or damage receptors. For example, necrotic IAV-infected epithelial cells are a source of CXCL8 (338), which attract neutrophils to dying cells. In addition, neutrophils can detect DAMPs such as S100A9 (464). It has been shown that extracellular S100A9 is abundant during IAV infection in mice (465). Antibody-mediated neutralization of S100A9 decreased lung inflammation in mice and improved disease outcome (465). Apart from potential tissue-destroying effects of neutrophil proteases, the presence of NETs may induce even more inflammation in the lungs (80, 381). Thus, there are limited data supporting directly malevolent actions of neutrophils (Table 1.5), yet factors that increase their presence and prolong their survival in the lung are correlated with increased disease severity.

9. Neutrophils in viral respiratory disease

There appears to be a correlation between IAV infection and neutrophils, but this may not be a causal relationship. In bacterial pneumonia, there is direct evidence of the importance of neutrophils in disease: bacterial PAMPs upregulate neutrophil activating and chemoattractant chemokines, bacteria have defined anti-neutrophil functions, and some bacteria, *e.g.*,

Mycobacterium tuberculosis, rely on neutrophils to establish their granulomatous niche (19, 466, 467). Similarly, in ARDS there are data that directly support the role of neutrophils as both beneficial and detrimental (16, 57, 58). To substantiate a link between neutrophils and IAV, evidence for the role of neutrophils in viral respiratory disease is briefly reviewed below. Although scarce, evidence presented here relies heavily on prospective clinical data as well as pathological reports from human cases of respiratory infection of confirmed viral and bacterial etiologies. Specific animal models are not considered here due to differences in the types of cells called neutrophils between species, as well as limitations in animal models to directly recapitulate human disease from these specific pathogens.

For both viral and bacterial etiologies, the most severe clinical complications result from infection of the LRT. Infection of the LRT by viruses such as human parainfluenza viruses, H1N1pdm, HPAI, New World hantavirus infections (causing hantavirus pulmonary syndrome), *Severe acute respiratory syndrome-related coronavirus* (SARS-Cov), and Middle East respiratory syndrome coronavirus (MERS-CoV) may develop into ARDS (22, 23, 51, 63, 143, 468-470). Clinically defined, ARDS has three phases; and most patients die within the first phase, the acute or “exudative” phase (reviewed in (25, 57, 58)). This phase is characterized by an increased immune response with high production of pro-inflammatory cytokines and chemokines, increased neutrophil infiltration and accumulation in the alveoli, and disruption of the alveolar epithelial-capillary barrier, which leads to increased vascular permeability and edema (57). The distinctive role for lung neutrophil infiltration in viral infection is summarized as follows: some, but not all, viruses that infect the LRT result in clinically-defined ARDS, and lung neutrophil infiltration is associated with viruses that do and do not lead to ARDS (16, 18, 22, 23, 51, 77, 143, 468-472). Therefore, perhaps there are

specific viral factors or virus-induced host factors that indicate a common mechanism for neutrophil infiltration into the respiratory tract (Table 1.6).

9.1 Viruses that cause neutrophilic infiltration.

Human rhinovirus. Infection with HRV is a well-studied virus for which there are several studies on the link between neutrophils and disease (87, 473-475) (Table 1.7). HRV are plus-sense single-stranded RNA viruses with an icosahedral nucleocapsid in the *Picornaviridae* family, genus *Enterovirus* (476). HRV virions enter nasal epithelial cells via endocytosis, yet, unlike influenza, infection does not cause major damage to the nasal epithelium (476-478). Neutrophilic rhinitis, increased vascular permeability, and mucus hypersecretion are the key pathological features of HRV infection (87, 473, 474, 477), and infected epithelium seems to be the source of large amounts of neutrophil chemotactic molecules, particularly CXCL8 and kinins (473, 475, 479, 480). Interestingly, *in vitro* studies have shown that TLR3 and MDA5, but not RIG-I, are important for detecting dsRNA, and HRV capsid is recognized by TLR2 on the epithelial surface (473, 481). Thus, viral recognition shares features with hRSV, but is somewhat different than with IAV (300, 327, 482). Even so, *in vitro* infection of nasal epithelial cells with HRV indicates similar secretion of inflammatory mediators is the result of these respiratory diseases: HRV causes upregulation of IL-1, IL-6, CXCL8, GM-CSF, CCL5, CCL11, and type I IFN (483). It has been established that there are virus-specific and cell-specific differences in sensing RNA viruses via primarily TLR- and/or RLR- pathways (and even in a preference for RIG-I versus MDA5), yet these pathways may have similar general endpoints, such as chemokine and cytokine signaling (326, 327, 329).

Paramyxoviruses. Numerous viruses in the family *Paramyxoviridae* infect the human respiratory tract and may cause significant pathology (Table 1.6). Paramyxoviruses are minus-sense single-stranded RNA viruses that include hRSV, which is the leading cause of childhood bronchiolitis and pneumonia during interpandemic years of IAV transmission (484). Infection with hRSV causes neutrophilic infiltration in the LRT, where thick luminal mucous plugs filled with neutrophils may cause bronchiolar obstruction (485, 486). Nasal washes and BAL from patients with hRSV contain neutrophil chemoattractant chemokines (21, 487-489) and there is evidence that neutrophils and other respiratory cells may recognize hRSV virions directly through TLR2/6 (482, 490). Other viruses within this family include *Measles virus*, *Human metapneumovirus* (MPV), and the highly pathogenic emerging viruses, *Hendra virus* and *Nipah virus*; all of which have reports of neutrophilic infiltration at sites of infection to various degrees (491-494). Interestingly, there are no reports of neutrophils associated with infection from any of the human parainfluenza viruses (PIV) (495). Human PIV infects ciliated epithelium of the respiratory tract and may cause bronchiolitis and alveolitis (470). However, infection does not cause a cytopathic effect, which is thought to be due to viral proteins which block PRR signaling and apoptosis (470, 496). Even so, cases of severe PIV infection have elevated chemokines in nasal washes (CCL3, CCL4, CCL5, CXCL8, CXCL9, and CXCL10), some of which are neutrophil chemoattractants indicating that neutrophils may be present at some point in the disease progression (470, 480, 495-497).

Coronaviruses. Members of the family *Coronaviridae* are plus-sense single-stranded RNA viruses and infect a wide variety of mammalian and avian species (498) (Table 1.7). Two genera of coronaviruses are important human pathogens: members of the genus *Alphacoronavirus* include *Human coronavirus NL63* (HCoV-NL63); and the genus

Betacoronavirus includes HCoV-HKU1 and the highly pathogenic SARS-CoV and MERS-CoV (498). Human coronaviruses, such as HCoV-HKU1, HCoV-NL63, HCoV-OC43, and HCoV-229E, have recently been identified and are thought to cause both URT and LRT infections (499, 500). Very little is known about specific pathology associated with HCoV infection. However, much more is known about infection with SARS-CoV and MERS-CoV. These emergent viruses infect pneumocytes in the LRT and may cause a severe disease that can lead to ARDS and even have pathophysiological symptoms of hypercytokinemia (22-24). Infection with either of these viruses is correlated with increased IL-6, CCL2, CXCL8, and CXCL10 in the blood and respiratory tract, and neutrophils are noted in respiratory tissues from lethal cases of infection with either of these viruses (23, 24, 501).

Other respiratory viral infections. Human adenovirus (hAdV, members of the family *Adenoviridae*, genus *Mastadenovirus*) infections have been documented to cause an initial neutrophilic alveolitis that may progress to a viral pneumonia (specifically serotype hAdv-7), which may be related to the release of CXCL8 and CXCL10 from infected epithelial cells (502) (Table 1.7). Human cytomegalovirus (family *Herpesviridae*) may cause a viral pneumonia that includes hyaline membrane formation and neutrophilic alveolar exudates (503). Autopsies from patients who died from hantavirus pulmonary syndrome, caused by infection with New World hantaviruses, were seen to have neutrophilic infiltration surrounding antigen positive lung lesions (504) (Table 1.7). These cases are particularly remarkable, since hantaviruses infect and cause inflammation of the endothelium, often with no apparent damage to the epithelium (504). However, hantavirus pulmonary syndrome (HPS) is a severe pulmonary infection characterized by interstitial pneumonitis, focal hyaline membrane formation, and left-shifted neutrophilic leukocytosis (504).

9.2 Comparisons of respiratory viral infections.

Cytopathic effect. A key question is whether virus-induced cytopathy drives neutrophilia or whether it is the result of host response to viral infection. Infections with H1N1pdm, HPAI, SARS-CoV, and MERS-CoV are thought to cause acute lung injury which results in ARDS; characterized by excessive damage to the alveolar epithelium and involving the infiltration of neutrophils (7, 8) (Tables 1.6 and 1.7). However, less pathogenic strains such as HRV infections do not cause significant damage to the respiratory mucosa, yet neutrophils are present (477, 478). Conversely, viruses that cause moderate, focal cytopathy in the lungs, for example seasonal IAV and hRSV, are known to cause neutrophilic infiltration (478-480). Therefore, neutrophils are not necessarily associated with direct cytopathic effect, nor are they exclusively associated with severe disease.

Host response to viral versus bacterial infection. Specific host responses to infection may act as a driving force behind neutrophil infiltration. As evidenced above, many viral infections associated with neutrophil infiltration have RNA genomes. Host cells detect RNA viruses primarily through RLR as well as TLR, whereas bacteria rely on a different group of PRRs to detect extracellular PAMPs (327, 328, 357, 505). Because of the importance in diagnostics, many studies have focused on biomarkers that identify bacterial versus viral infection (*e.g.*, (506, 507)). Although it is debated, these studies routinely point to CRP as a correlate of bacterial infection (506-511). However, cases of IAV and hRSV infection are known to activate complement and often show increased CRP (392, 396, 512, 513). In short a single reliable biomarker of bacterial versus viral infection has yet to be found. In a recent study, it was among a group of early conserved differentially expressed genes across species (mouse and macaque) and pathogens (IAV and bacterial), all of which were the direct result

of an upregulation of ISGs (505). For example, IFN β , IL-1 α , IL-6, IL-23 α , TIMP1, M-CSF, ISG15, CCL2, CCL5, CXCL10, and CD40 were all common to the early host immune response to lung infection (505). The control of signaling through TLR versus cytoplasmic is controlled by complex intracellular adaptor proteins (*e.g.*, allowing the characteristically bacteria-specific TLR4 to signal the upregulation of viral-specific type I interferons (514)), the implications of this require much more research (reviewed in (515)).

Specific immune response signatures to different viruses. The relative number of neutrophils during viral infection may reflect the different strategies used by viruses to manipulate the host defenses, and viral infections such as HRV, hRSV, and IAV may create unique or “signature” immune responses that are involved in neutrophil attraction relative to MPV, PIV, or HCoV (the latter group have fewer neutrophils during infection). In other words, where there are often redundancies in a typical “viral” immune response, investigating differences between these patterned immune responses *en masse* between viruses may be informative (*e.g.*, (516)). Unfortunately, there are very few studies that approach this topic. In general, clinical studies have shown that viruses causing increased concentrations of CXCL8, TNF α and IL-1 are associated with increased neutrophilia, confirmed in patients with HRV, IAV, and hRSV infection (480, 517), yet CXCL8 is only correlated with symptom scores for HRV and IAV (480). Interestingly, the lung microenvironment to hRSV has been shown to be different from IAV, specifically in the presence of IL-4 (508, 518). It is thought that this is driven by the presence of alternatively activated macrophages during RSV infection (519). It is not known if this directs differences in neutrophil chemoattraction, yet IL-4 is known to drive a Th2 (“bacterial” or antibody-biased) immune response (520).

CXCL10. Patients with ARDS resulting from infection with SARS-CoV (501), H1N1pdm (105), or H5N1 HPAI (10, 54, 330, 521) had a common marker of inflammation: high levels of CXCL10. A prospective study of patients with severe respiratory disease found BAL concentrations of CXCL10 (but not CXCL8) and eotaxin/CCL11 were significantly upregulated in confirmed cases of viral infection (HRV, IAV, hRSV, MPV, HCoV-NL63, PIV, hAdv, or polyomavirus) compared to non-viral respiratory disease (522). CXCL10 is an ISG that binds the CXCR3 receptor on NK cells and plasmacytoid DCs and induces chemotaxis, apoptosis, cell growth, and angiostasis (523-527). CXCL10 is released during many viral infections, not just respiratory viral infections. Although it is not a classical neutrophil chemottractant, it is also known to operate in an autocrine manner with a unique subset of CXCR3+ neutrophils during influenza infection (380).

9.3 Neutrophils in the viral microenvironment.

In general, immune activation pathways that involve the activation of NFkappaB lead to the secretion of neutrophil chemotactic chemokines (reviewed in (528)). This is heavily driven by PAMPs, and during a viral infection type I IFNs and ISGs are the unique elements in the virus-inflamed lung environment (reviewed in (66-68, 324, 325)). This single difference between viral and bacterial infections could have drastic effects on the actions of neutrophils once in the lung. Moreover, without this information (*e.g.*, pathogenic viruses that suppress type I IFN) neutrophils may respond to inflammation in an inefficient way, potentially with pathologic effects (65, 69-71, 298, 300, 301, 422, 529, 530). Neutrophils are known to respond to IAV and type I IFN by upregulation of ISGs (422). In systemic lupus erythematosus, neutrophils may be a large contributor of type I IFN (531, 532). During increased inflammation, left-shifted or immature neutrophils emerge from the bone marrow –

a classic sign for sepsis, but also known to be present during some severe viral respiratory diseases (*e.g.*, HPS) (504). It is known that immature neutrophils do not express IFN α/β receptor, nor many other cytokine receptors (530). It is unknown what affect this would have during increased inflammation during pathogenic influenza infection, although their role in other inflammatory conditions suggests it may affect their functions (529, 530, 533, 534).

Concluding remarks.

Are neutrophils associated with viral infection? Are they associated with respiratory viral infection? When, where, and how are they associated? In the context of viral infection, what is a productive response of neutrophils? Is a productive response determined by type I IFN? There is substantial data that suggest neutrophils are a part of a viral response to infection. Neutrophils are among the first responders to IAV infection in the lung, and they remain in great numbers throughout the development of ARDS. However, many questions remain before it is determined what part they play in mild and severe disease.

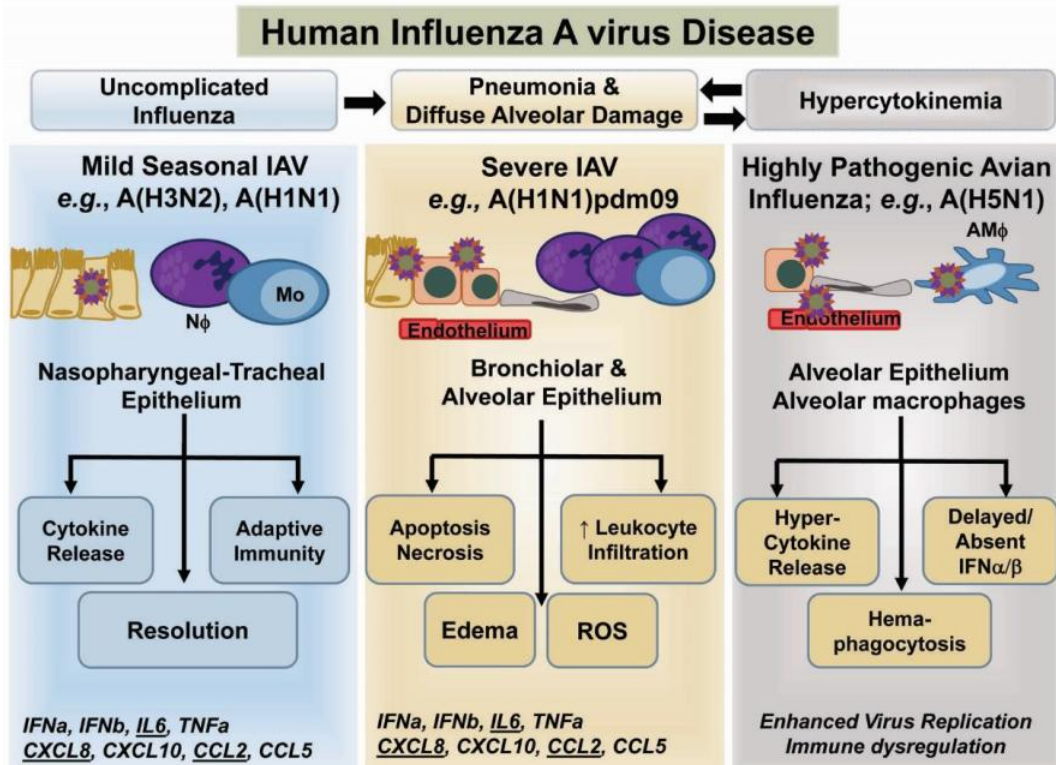


Figure 1.1 Three disease states caused by human infection with influenza A virus.

Human infection with influenza A virus (IAV) produces three main types of disease.

Seasonal human IAV (left) causes upper respiratory tract disease that is characterized as an acute, self-limiting flu-like illness. Seasonal IAV and some emergent IAV can cause a severe influenza pneumonia (center), which involves infection and inflammation of the lower respiratory tract. Some highly pathogenic avian IAV (right) cause a lethal infection characterized by a dysregulated immune response; this can also lead to severe pneumonia. Beneath each heading are the viral strains that typically cause each disease, following by a cartoon diagram showing primary site of viral replication. Arrows below point to pathological outcomes, followed by typical immune responses measured in humans (increased pathology is associated with increases in the underlined immune proteins).

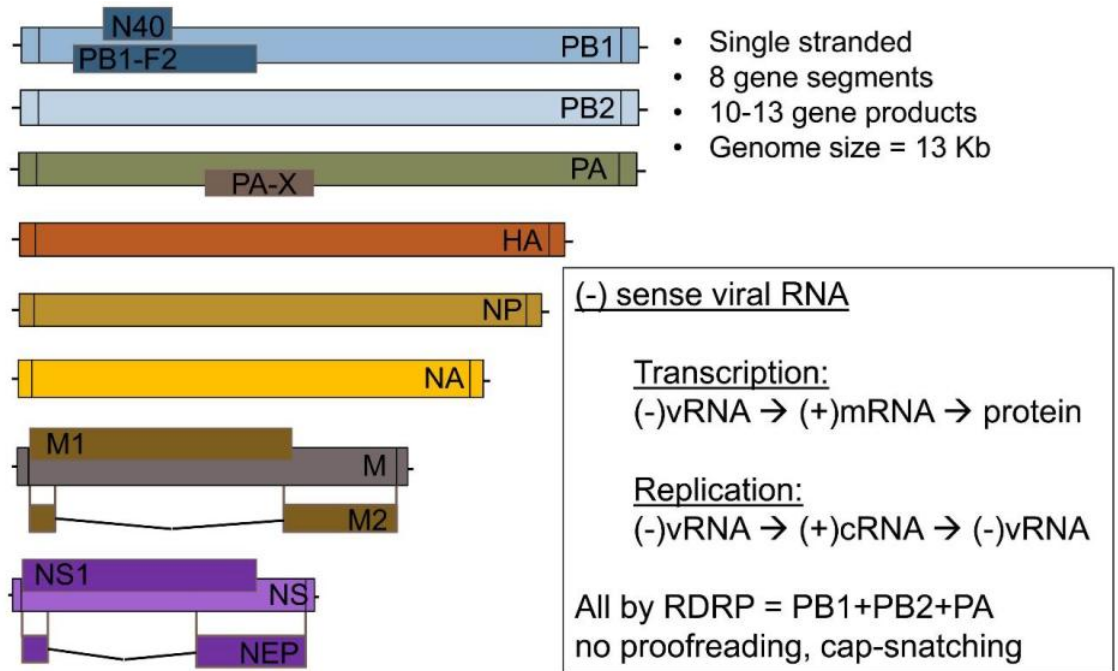


Figure 1.2 Influenza A virus genome.

Influenza A viruses have an 8-segmented, single-stranded, negative sense RNA genome that codes for 10-13 gene products. The cartoon shows the arrangement of those gene products along each gene segment. Gene segments are sized relative to each other, and the total genome is over 13 kb in length. The basic formulas for making protein and replicating the genome are shown in the box; most steps are performed by the viral RNA-dependent RNA polymerase (RDRP), which consists of three viral proteins.

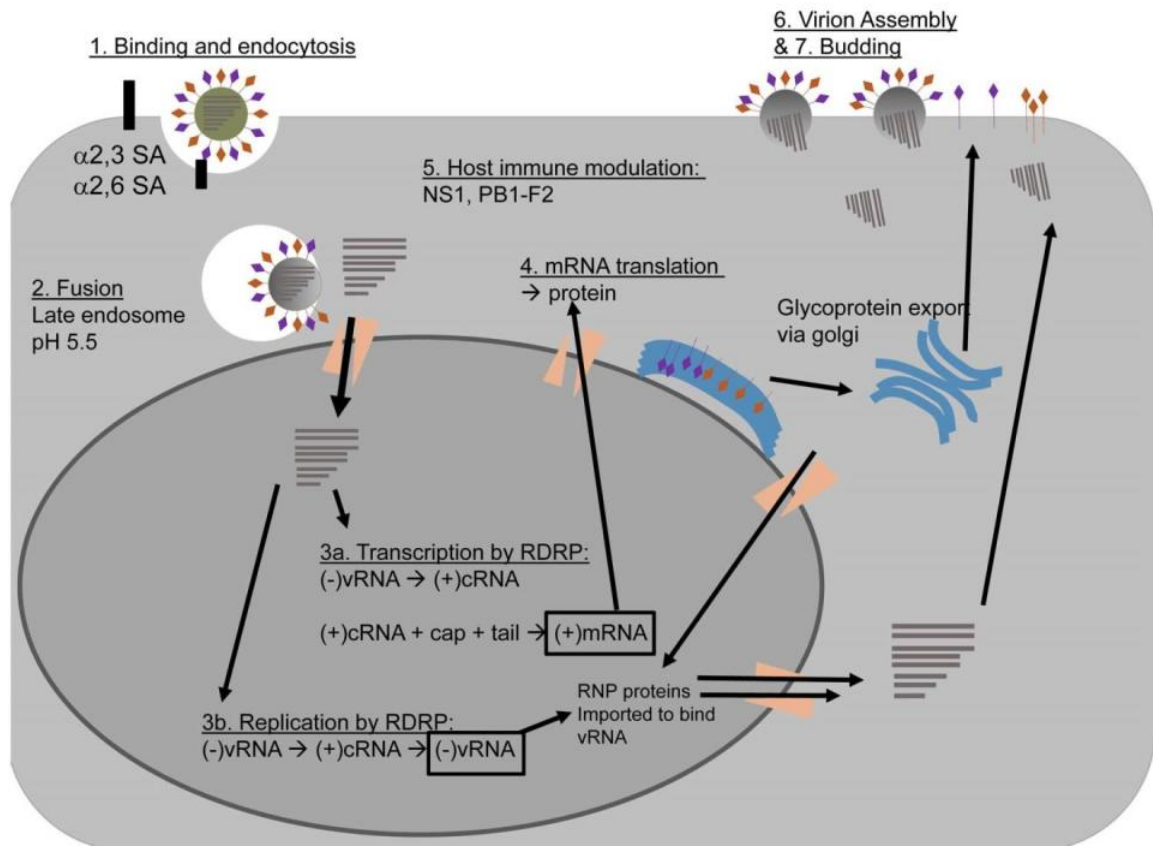


Figure 1.3 Influenza A virus life cycle diagram.

Seven major steps in the influenza A virus life cycle include: (1) binding to host cells via sialic acid residues (SA) and entry into cells via clathrin mediated endocytosis; (2) fusion to host cell membrane allows release of viral genome into cytoplasm and import into nucleus; (3) viral genome transcription (3a) and replication (3b) occur in the nucleus by the viral RNA-dependent RNA polymerase (RDRP); (4) mRNA is exported for translation by host ribosomes; (5) some proteins modify the host immune response, glycoproteins are exported to the cell membrane through the Golgi body and proteins that form the ribonucleoprotein (RNP) are imported back to the nucleus to bind to new vRNA, which is exported from the nucleus (6) to the plasma membrane where virions are assembled and (7) bud out from the host plasma membrane.

Animal Models of Influenza A Virus Infection

	<u>Mouse</u>	<u>Ferret</u>
Signs/Symptoms	Weight Loss Viral replication in respiratory tract Lethality (LD ₅₀) Seroconversion	
	Ruffled fur, Hunched posture, Hypothermia	Sneezing, Coughing Rhinorrhea, Lethargy Fever
Advantages	Inexpensive Well-characterized genetics Abundance of reagents Inbred strains	Similar to humans in: receptor distribution clinical symptoms pathology in resp. tract
Disadvantages	Not a natural host Lack clinical symptoms No virus transmission Mouse strain-dependent	Expensive USDA/special housing No standardized methods No immunological reagents Outbred

Figure 1.4 Animal models of influenza A virus infection

Mice and ferrets are commonly used animal models to study influenza A virus pathogenesis. Advantages and disadvantages are listed for each species.

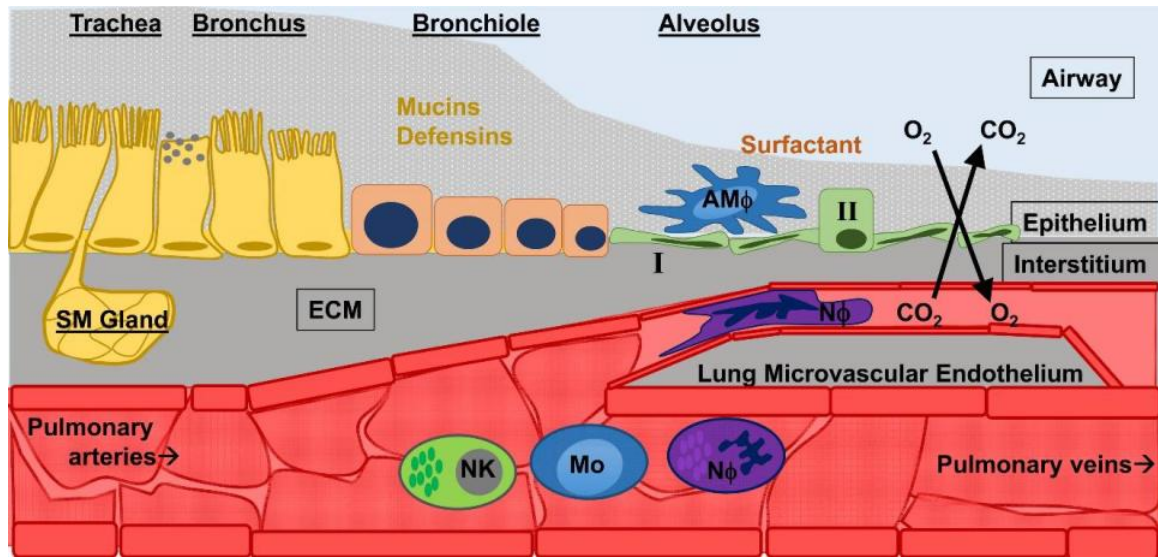


Figure 1.5 Cartoon diagram of normal human airways

The upper airways are on the left, consisting of ciliated pseudostratified epithelium with occasional secretory cells and submucosal (SM) secretory glands embedded within the extracellular matrix (ECM). In the lung, the airways branch into bronchioles and end in alveoli, which are made of type I (I) and type II (II) pneumocytes which are in near-constant contact with alveolar macrophages (AM ϕ). The alveoli are the site of gas exchange, working in close proximity to the lung microvascular endothelium to expel carbon dioxide (CO₂) from the pulmonary arteries and carry oxygen (O₂) through the pulmonary veins back through the heart and into circulation. The airway epithelium is lined with mucus containing mucins, defensins; and the alveoli contain surfactant proteins that aid in surface tension and innate immune defenses. Because the entire blood volume passes through the lungs, innate immune cells (such as natural killer cells, NK; monocytes, Mo; and neutrophils, N ϕ) are transient through the lungs and must navigate the capillary beds near the alveoli.

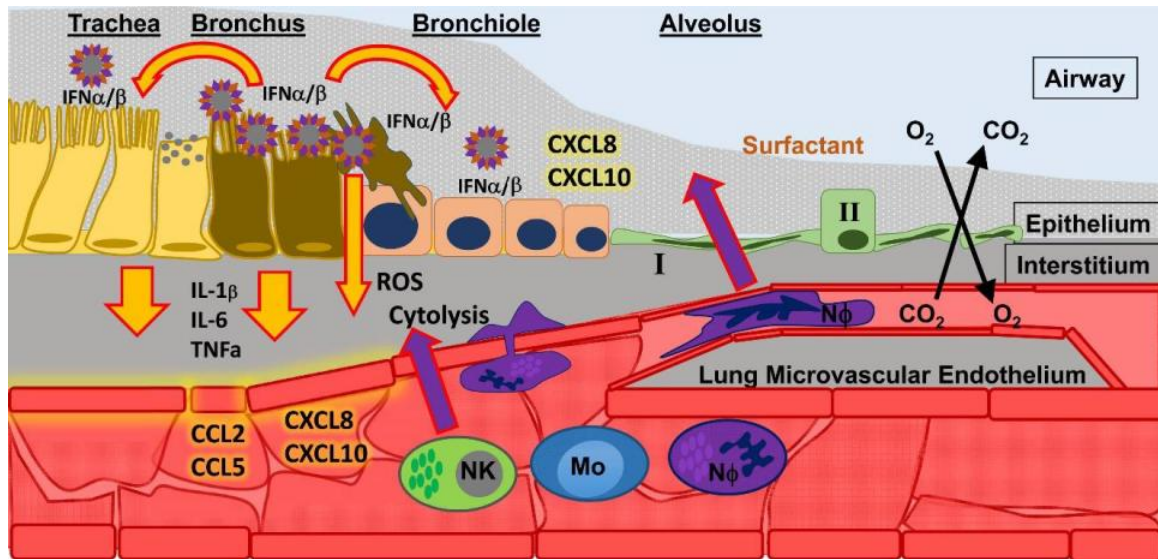


Figure 1.6 A cartoon diagram of IAV infection in the human respiratory tract.

Infection of epithelial cells in the bronchus results in the release of type I interferons (IFN α/β) which signal to nearby cells. The result of IAV α/β signaling is the release of proinflammatory cytokines (e.g., IL-1 β , IL-6, TNF α) that signal to endothelial cells, which help spread inflammatory signals (chemokines such as CCL2, CCL5, CXCL8, CXCL10) throughout the blood to recruit innate immune cells to the site of infection. Recruited of innate immune cells (such as natural killer cells, NK; monocytes, Mo; and neutrophils, N ϕ) must interact with activated endothelium to leave the blood stream and crawl towards the site of infection. There they can perform effector functions to control infection, such as releasing reactive oxygen species (ROS) and directly killing infected cells (cytolysis).

Table 1.1 Effect of cytokine knockout mice infected with IAV on neutrophils

<u>Mouse Model</u>	<u>Virus</u>	<u>Leth?</u>	<u>Dose</u>	<u>Effect on Lung Neutrophils</u>	<u>Effect on Disease Phenotype compared to wt</u>	<u>Ref</u>
IL-1 β ^{-/-}	H3N2 A/Taiwan/3446/2002	No	5x10 ⁵ PFU	Same as wt	Same as wt	(428)
IL-1 β ^{-/-}	H3N2 A/TW/3446/02 with PA gene from A/VN/1194/04	No	5x10 ⁵ PFU	Same as wt	Delayed pathology	(428)
IL-1 β (Casp 1 ^{-/-})	H3N2 A/Taiwan/3446/2002	No	5x10 ⁵ PFU	Same as wt	Same as wt	(428)
IL-1 β (Casp 1 ^{-/-})	H3N2 A/TW/3446/02 with PA gene from A/VN/1194/04	No	5x10 ⁵ PFU	Increased	Weight loss, delayed pathology	(428)
IL-1 β (Casp 1 ^{-/-})	H1N1 A/PR/8/1934	70% Survival	6x10 ⁴ PFU with 0.1% alum	Reduced	Increased Lethality	(426)
IL-1 β (Casp 1 ^{-/-})	H1N1 A/PR/8/1934	70-80% Survival	8000 EID ₅₀	Reduced	Increased Lethality	(535)
IL-6 ^{-/-}	H5N1 A/Hong Kong/483/1997	Yes	10 ³ MID ₅₀	n/c	Same as wt	(430)
IL-6 ^{-/-}	H5N1 A/Hong Kong/486/1997	No	10 ³ MID ₅₀	n/c	Same as wt	(430)
IL-6 ^{-/-}	H5N1 A/Vietnam/1203/04	Yes	10 ³ EID ₅₀	n/c	Same as wt	(536)
IL-6 ^{-/-}	H1N1 A/PR/8/1934	No	3000 EIU	Decreased	Features of chronic infection, delayed viral clearance	(463)
TNF ^{-/-}	H5N1 A/Vietnam/1203/04	Yes	10 ³ EID ₅₀	n/c	Same as wt	(536)
IL-18 ^{-/-}	H3N2 X31 (A/PR/8/1934)	No	10 ⁴ PFU	Increased	Increased virus in lungs early, cleared virus same as wt	(362)

Leth? = “yes” means it is a lethal model of disease; wt refers to wild-type mice; Casp 1 = caspase 1; PFU = plaque forming units; EID₅₀ = 50% egg infectious dose; MID₅₀ = 50% mouse infectious dose; EIU = egg infectious units, n/c = no change.

Table 1.2 Effect of cytokine receptor knockout mice infected with IAV on neutrophils

<u>Mouse Model</u>	<u>Virus</u>	<u>Leth ?</u>	<u>Dose</u>	<u>Effect on Lung Neutrophils</u>	<u>Effect on Disease Phenotype compared to wt</u>	<u>Ref</u>
IFN α BR ^{-/-}	H1N1 A/PR/8/1934*	Yes	6.7 LD ₅₀	Same as wt	Same as wt	(380)
IFN α BR ^{-/-}	H3N2 X31 (A/PR/8/1934)	Yes	800 TCID ₅₀	Reduced	Protection	(65)
IFN α BR ^{-/-}	H1N1 A/California/04/2009	Yes	330 TCID ₅₀	Reduced	Protection	(65)
IFN α BR ^{-/-}	H1N1 A/PR/8/1934	LD ₅₀	10000 PFU	Increased	Increased lethality	(70)
IL-1R1 ^{-/-}	H5N1 A/HK/483/1997**	Yes	10 ³ MID ₅₀	Increased	Delayed Death	(429)
IL-1R1 ^{-/-}	H5N1 A/HK/483/1997	Yes	10 ³ MID ₅₀	Increased	Delayed Death	(429)
IL-1R1 ^{-/-}	H5N1 A/HK/483/1997	Yes	10 ³ MID ₅₀	Same as wt	Same as wt	(430)
IL-1R1 ^{-/-}	H5N1 A/HK/486/1997	No	10 ³ MID ₅₀	Slightly less (n.s.)	Reduced Weight loss	(430)
IL1-R1 ^{-/-}	H1N1 A/PR/8/1934	No	100 PFU	Reduced	Increased Lethality	(397)
IL1-R1 ^{-/-}	H1N1 A/PR/8/1934	No	100 PFU	Reduced	Increased Lethality	(397)
IL1-R1 ^{-/-}	H1N1 r1918	Yes	10 ⁶ PFU	Same as wt	Increased Complement Gene Expression	(427)
IL-6R ^{-/-}	H1N1 A/PR/8/1934	No	3000 EUI	n/c	Increased Lethality	(463)
IL-17RA ^{-/-}	H1N1 A/PR/8/1934	Yes	1 LD ₉₀	Reduced	Protection	(399)
TNF-R1 ^{-/-}	H5N1 A/Hong Kong/483/1997	Yes	10 ³ MID ₅₀	Reduced	Delayed Death	(429)
TNF-R1 ^{-/-}	H5N1 A/Hong Kong/483/1997	Yes	10 ³ MID ₅₀	n/c	Reduced leukopenia	(430)
TNF-R1 ^{-/-}	H5N1 A/Vietnam/1203/04	Yes	10 ³ EID ₅₀	n/c	Same as wt	(536)
TNF-R1 ^{-/-} TNF-R2 ^{-/-}	H5N1 A/Vietnam/1203/04	Yes	10 ³ EID ₅₀	n/c	Increased survival (n.s.)	(536)
TNF-R1 ^{-/-} TNF-R2 ^{-/-}	H1N1 r1918	Yes	10 ⁶ PFU	Reduced	Delayed death	(427)
TNF-R1 ^{-/-} TNF-R2 ^{-/-} IL1R ^{-/-}	H5N1 A/Hong Kong/483/1997	Yes	10 ³ MID ₅₀	Reduced	Delayed Death	(429)

Leth? = “yes” means it is a lethal model of disease; wt refers to wild-type mice; Casp 1 = caspase 1; PFU = plaque forming units; EID₅₀ = 50% egg infectious dose; MID₅₀ = 50% mouse infectious dose; EIU = egg infectious units, n/c = no change. *PR = Puerto Rico, ** HK = Hong Kong.

Table 1.3 Effect of chemokine receptor knockout mice infected with IAV on neutrophils

<u>Mouse Model</u>	<u>Virus</u>	<u>Leth?</u>	<u>Dose</u>	<u>Effect on Lung Neutrophils</u>	<u>Effect on Disease Phenotype compared to wt</u>	<u>Ref</u>
CCR2 ^{-/-}	H1N1 A/PR/8/1934	No	3.3 PFU	Increased	Increased lung CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL10	(379)
CCR2 ^{-/-}	H1N1 A/PR/8/1934	70-80% Survival	5 HAU	Increased, delayed	Reduced lethality, increased virus in lung, increased CCL2, CXCL10 in lung	(537)
CCR5 ^{-/-}	H1N1 A/PR/8/1934	70-80% Survival	5 HAU	Increased	Increased lethality, increased virus in lung, , increased CCL2, CCL5, CXCL10 in lung	(537)
CCR2 ^{-/-} CCL3 ^{-/-}	H1N1 A/PR/8/1934	70-80% Survival	5 HAU	n/c	Reduced lethality, increased virus in lungs	(537)
CXCR2 ^{-/-}	H1N1 A/PR/8/1934	No	10-100 PFU	Reduced	Increased lung macrophages Decreased T cell recruitment	(538)
CXCR3 ^{-/-}	H1N1 A/PR/8/1934	No	3.3 PFU	Reduced	Same as wt	(539)
CXCR3 ^{-/-}	H1N1 A/PR/8/1934	Yes	6.7 LD ₅₀	Reduced	Increased Survival	(380)

Leth? = “yes” means it is a lethal model of disease; wt refers to wild-type mice; Casp 1 = caspase 1; PFU = plaque forming units; EID₅₀ = 50% egg infectious dose; MID₅₀ = 50% mouse infectious dose; EIU = egg infectious units, n/c = no change.

Table 1.4 Effect of chemokine knockout mice infected with IAV on neutrophils

<u>Mouse Model</u>	<u>Virus</u>	<u>Leth?</u>	<u>Dose</u>	<u>Effect on Lung Neutrophils</u>	<u>Effect on Disease Phenotype compared to wt</u>	<u>Ref</u>
CCL2 ^{-/-}	H5N1 A/Vietnam/1203/04	Yes	10 ³ EID ₅₀	n/c	Same as wt	(536)
CCL3 ^{-/-}	H5N1 A/Hong Kong/483/1997	Yes	10 ³ MID ₅₀	n/c	Same as wt	(430)
CCL3 ^{-/-}	H5N1 A/Hong Kong/486/1997	No	10 ³ MID ₅₀	n/c	Same as wt	(430)
CCL5 ^{-/-}	H1N1 A/PR/8/1934	No	3.3 PFU	Same as wt	Same as wt	(539)
CXCL10 ^{-/-}	H1N1 A/PR/8/1934	Yes	6.7 LD ₅₀	Reduced	Increased Survival	(380)

Leth? = “yes” means it is a lethal model of disease; wt refers to wild-type mice; Casp 1 = caspase 1; PFU = plaque forming units; EID₅₀ = 50% egg infectious dose; MID₅₀ = 50% mouse infectious dose; LD₅₀ = 50% mouse lethal dose, n/c = no change. PR = Puerto Rico.

Table 1.5 Effect of neutrophil effector knockout mice on IAV infection

Mouse Model	Virus	Leth?	Dose	Effect on Lung Neutrophils	Effect on Disease Phenotype compared to wt	Ref
Cybb ^{tm1} Mice (gp91 ^{phox} deficient)	H3N2 X31 (A/PR/8/1934)	No	50 HAU	Increased	Increased lung cellularity, Decreased lung consolidation, Increased TNF α , IL6, CCL2, Decreased virus in lung	(439)
Elane ^{-/-}	H3N2* A/Memphis/1/1971 Written as H3N1 in pub.	No	10 ^{4.5} PFU	Same as wt	Same as wt	(434)
NETs (PAD4 ^{-/-})	H1N1 A/WSN/33	50% Survive	1 LD ₅₀	Same as wt	Same as wt	(457)

Leth? = “yes” means it is a lethal model of disease; wt refers to wild-type mice; Casp 1 = caspase 1; HAU = hemagglutinating units; PFU = plaque forming units; LD₅₀= mouse 50% lethal dose, n/c = no change.

Table 1.6 (-) sense RNA respiratory viruses that cause increased neutrophil infiltration during infection

Virus Family	Virus species	Host/ model	Primary cell target	Pathology	N	Ref
Orthomyxoviridae (-)	Influenza A virus	Hu,NHP, Mo,Fe,Sw	epithelial cells (URT, LRT)	Mild: necrotic rhinitis and tracheitis Moderate: necrotic bronchiolitis and alveolitis; Severe: diffuse alveolar damage, hypercytokinemia (HPAD)	(+++)	(87, 473, 474)
Paramyxoviridae (-)	Human respiratory syncytial virus	Hu	epithelial cells (URT,LRT)	Severe: necrotic bronchiolitis and alveolitis, obstructed bronchioles and giant cell formation	(+++)	(485, 486)
Paramyxoviridae (-)	Human metapneumovirus	Hu			(+)	(493, 494)
Paramyxoviridae (-)	Hendra/Nipah virus	Hu			(++)	-491
Paramyxoviridae (-)	Measles virus	Hu, Mo	Resident myeloid cells of the lung	Severe: bronchiolitis obliterans	(++)	-492
Paramyxoviridae (-)	Human parainfluenza virus	Hu	Ciliated epithelium	Bronchiolitis and alveolitis	No?	-495
Bunyaviridae (-)	New World hantavirus	Hu	Lung microvascular endothelium	Hantavirus Pulmonary Syndrome, endothelial inflammation and focal antigen-positive sites in lung	(+)	-504

Families and species of (-)-sense RNA respiratory viruses for which the presence or absence of neutrophils in the respiratory tract has been documented. Also listed are: the host species (Human, Non-Human Primate, Mouse, Ferret, Swine) in which neutrophils have been known to be increased following infection; the primary site of infection (URT = upper and LRT = lower respiratory tract); the typical pathology associated with increased neutrophils; the relative amount of neutrophil infiltration; and references.

Table 1.7 (+) sense RNA and DNA respiratory viruses that cause increased neutrophil infiltration during infection.

<u>Virus Family</u>	<u>Virus species</u>	<u>Host/ model</u>	<u>Primary cell target</u>	<u>Pathology</u>	<u>N</u>	<u>Ref</u>
Picornaviridae (+)	Human rhinovirus	Hu	Epithelial cells	Mild to moderate: neutrophilic rhinitis; Severe: acute LRT, bronchiolitis and alveolitis	(+++)	-87
Adenoviridae (DNA)	Human adenovirus (HAdv3, HAdv7)	Hu		Bronchitis and alveolitis		-502
Coronaviridae (+)	Human coronavirus (NL-63 or OC43)	Hu			(+/-)?	
Coronaviridae (+)	Severe acute respiratory syndrome coronavirus	Hu, Fe	Epithelial cells	Alveolitis, ARDS, hypercytokinemia	(++)	-23
Coronaviridae (+)	Middle east respiratory syndrome coronavirus	Hu, Fe	Epithelial cells	Alveolitis, ARDS, hypercytokinemia	(++)	-24
Filoviridae (+)		NHP		Experimental aerosolized infection; hemorrhagic fever, associated with antigen and necrosis in lungs, neutrophilia	(+)	(540, 541)

Families and species of (-)-sense RNA respiratory viruses for which the presence or absence of neutrophils in the respiratory tract has been documented. Also listed are: the host species (Human, Non-Human Primate, Ferret) in which neutrophils have been known to be increased following infection; the primary site of infection (URT = upper and LRT = lower respiratory tract); the typical pathology associated with increased neutrophils, including Acute Respiratory Distress Syndrome and notes on the circumstances in which neutrophils were increased; the relative amount of neutrophil infiltration; and references.

CHAPTER II

PHENOTYPIC DIFFERENCES IN VIRULENCE AND IMMUNE RESPONSE IN CLOSELY RELATED CLINICAL ISOLATES OF INFLUENZA A 2009 H1N1 PANDEMIC VIRUSES IN MICE

Overview

To capture the possible genotypic and phenotypic differences of the 2009 influenza A virus H1N1 pandemic (H1N1pdm) strains circulating in adult hospitalized patients, we isolated and sequenced nine H1N1pdm viruses from patients hospitalized during 2009-2010 with severe influenza pneumonia in Kentucky. Each viral isolate was characterized in mice along with two additional H1N1 pandemic strains and one seasonal strain to assess replication and virulence. All isolates showed similar levels of replication in nasal turbinates and lung, but varied in their ability to cause morbidity. Further differences were identified in cytokine and chemokine responses. IL-6 and KC were expressed early in mice infected with strains associated with higher virulence. Strains that showed lower pathogenicity in mice had greater IFN γ , MIG, and IL-10 responses. A principal component analysis (PCA) of the cytokine and chemokine profiles revealed 4 immune response phenotypes that correlated with the severity of disease. A/KY/180/10, which showed the greatest virulence with a rapid onset of disease progression, was compared in additional studies with A/KY/136/09, which showed low virulence in mice. Analyses comparing a low (KY/136) versus a high (KY/180) virulent strain showed a significant

difference in the kinetics of infection within the lower respiratory tract and immune responses. Notably by 4 DPI, virus titers within the lung, bronchoalveolar lavage fluid (BALf), and cells within the BAL (BALc) revealed that the KY/136 replicated in BALc, while KY/180 replication persisted in lungs and BALc. In summary, our studies suggest four phenotypic groups based on immune response that result in different virulence outcomes in H1N1pdm strains with a high degree of genetic similarity. *In vitro* studies with two of these isolates suggested that the more virulent isolate, KY/180, replicates productively in macrophages and this may be a key determinant in tipping the response toward a more severe disease progression.

Introduction

The 2009 pandemic H1N1 influenza virus (H1N1pdm) arose through reassortment of two preexisting swine influenza viruses, a Eurasian avian-like virus and a North American triple reassortant virus (44, 127, 542). The risk factors associated with human cases of H1N1pdm mirrored those of seasonal influenza (543). As observed with seasonal influenza, the most common underlying chronic conditions among hospitalized patients were respiratory disease, asthma, cardiac disease, and diabetes (6, 30, 543-546).

However, in contrast to seasonal influenza, a greater proportion of severe and fatal cases had a pre-existing chronic illness. A second notable difference was the age distribution of hospitalized and severe cases. Children less than 17 years old had the greatest rates of hospitalization per capita and adults over 64 had the greatest rates of mortality per capita. Retrospective clinical studies focused on surveillance H1N1pdm sequences present in ICU admissions suggest that pre-existing medical conditions may be a more important

factor in severity rather than particular viral variants (547). However, this does not explain why a greater proportion of persons with pre-existing medical conditions had more severe disease than typically observed for seasonal influenza. Further, previously unreported comorbidities such as morbid obesity have been widely suggested for H1N1pdm for increased risk for admission to the ICU and death (548). At this time, it is difficult to rule out the contribution of viral variants to the resulting illness observed with the various comorbidities. Nonetheless, the course of illness and the progression to more severe disease are most likely due to the combined interplay of the individual's health, the intrinsic phenotype of the infecting viral variant and the treatment regime.

In contrast to seasonal influenza viruses, the H1N1pdm viruses replicate well and show greater pathogenicity with viral antigen in the bronchiolar epithelia and the alveolus by day 3 post-infection (DPI) (127, 247, 542). In the BALB/c mouse, A/California/04/09 and other H1N1pdm viruses show lethality, but only at the highest dose of $10^{6.5}$ plaque forming units (PFU) (247, 542). Studies of infection of BALB/c with several different 2009 H1N1pdm virus isolates show high virus titers in nasal turbinates (NT) and lung tissues by 3 DPI. Virus titers show a slight decrease by 6 DPI in lung and NT although the decrease varies among strains, too. Proinflammatory cytokines and chemokines were elevated for most mice in whole lung specimens at 3DPI for KC, IL-6, IL-12(p40), G-CSF, M-CSF, MCP-1 MIG, MIP-1 β , and LIF. By 6 DPI, IL-10 was present, albeit low, whereas some of the cytokines were reduced (e.g., MIP-2, IL-6), but most remained elevated (247, 542). The levels of cytokines and chemokines in humans or mice do not suggest hypercytokinemia common to H5N1 and 1918 viruses (46, 142, 222).

The overall genetic distance among H1N1pdm isolates remained low with 7 distinct clades in the first wave of the pandemic, while in the second wave a single viral lineage dominated (107). Molecular surveillance, while important, will not confirm potential phenotypic differences *in vivo* resulting from amino acid changes associated with viral variants in newly emerging strains or the potential for mixed infections and the genetic diversity of the intrahost viral populations (549). Animal studies can complement molecular surveillance in monitoring the potential pathogenicity and immunogenicity of circulating strains. Further, such studies have potential to reveal the efficacy of treatment regimes. Herein we report the isolation, sequence, and characterization of nine H1N1pdm influenza A viruses from adult patients hospitalized in Kentucky during the second pandemic wave, September 2009 and April 2010. Four of the nine patients died and all of the patients for whom data was available had an underlying chronic condition. This group of clinical isolates, with high genetic similarity, was characterized for virus load, virulence, and host immune response in mice. Immune responses in the lungs suggest four distinct immunological phenotypes that correlate with the observed mortality in mice. This study underscores the potential variability in the virulence of 2009 H1N1 influenza A strains circulating in Kentucky during the pandemic. These data suggest the hypothesis that the high severity of disease seen in certain hospitalized patients may be related to infection with H1N1pdm viral variants that, due to cell tropism and replication levels, may exacerbate certain types of disease associated with comorbidity.

Results

Isolation and sequence analyses of H1N1pdm isolates from hospitalized patients

H1N1pdm strains were isolated from nasopharyngeal swab samples obtained from nine de-identified patients enrolled in the Severe Influenza Pneumonia Surveillance (SIPS) project, a clinical study of hospitalized patients with severe community-acquired pneumonia in Kentucky from December 2008 – December 2011 (Table 2.1). Hospitals included in the SIPS project included two rural areas of Kentucky, one in the east and one in the midwest, and one in an urban area (Louisville). The midwest is active in agriculture and one of the highest livestock producers of hogs and pigs. The age of the patients ranged from 31 to 58 and included five females and 4 males. Many of those patients had underlying comorbidities commonly associated with severe influenza disease such as obesity, diabetes and chronic obstructive pulmonary disorder (COPD) (Table 2.S1) Four of the nine patients died.

Viruses were isolated by passage through both Madin-Darby canine kidney (MDCK) cells and eggs and virus isolates were designated according to a tracking number that contains no patient identifier information. Herein, isolates will be referred to by the tracking number and virus isolation method (*e.g.*, A/Kentucky/180/2010 egg-passaged isolate will be referred to as “KY/180E”). Viruses were amplified and the virus titers were determined by 50% tissue culture infectious dose (TCID₅₀), plaque forming units (PFU) and hemagglutination assays in Madin-Darby canine kidney (MDCK) cells (data not shown). Viral titers measured in TCID₅₀ and PFU assays were similar for MDCK-

adapted viruses and egg-adapted isolates (e.g., median titer $\sim 10^7$). Some viruses (A/KY/96/09, A/KY/99/09, A/KY/108/09, A/KY/180/10) showed a 10-100 fold higher titers in MDCK cell culture than in egg, but some (A/KY/80/09, KY/110/09, KY/136/09) did not. Egg-adapted viruses that were not recovered included A/KY/104/09 and A/KY/190/10. Hemagglutination titers were generally lower from egg-adapted viruses and showed variability among isolates (data not shown).

Universal and gene-specific primers were designed and used to amplify and sequence full-length cDNAs of all viral segments from each isolate. The amino acid sequences encoded by each gene were deduced and compared. All of the isolates have stop codons in PB1-F2 at positions 12, 58, and 88 (data not shown) (550). Amino acids that showed polymorphisms are listed for each viral protein for the majority of genes (Table 2.S2). The most amino acid changes were observed in HA1 and acidic polymerase (PA) with 18 and 9 mutations collectively across the 7-8 the KY isolates (not all isolates were completely sequenced). Neuraminidase and the two basic polymerase subunits (NA, PB1 and PB2) each had 4-5 mutations while the nonstructural protein 1 (NS1) and HA2 showed 3 mutations collectively. In contrast, matrix (M1), nucleoprotein (NP) had only 1 mutation and there were none in M2, NS2 or PA-X. Of the mutations only a few have been previously studied functionally to our knowledge. For example, in the HA1/HA2 of KY/180E we noted changes in D222G, S83P, S183P, and E374K. E374K was identified as a vaccine escape mutation (551). The S183P and the Q293H mutations in HA1 (found in KY/180E and KY/96E, respectively) have been reported by others (552-555), and have

been associated with alterations in receptor binding and increases in disease severity in humans (106, 134).

Differential progression of virus replication and disease in mice infected with H1N1pdm strains

To make an initial assessment of the levels of replication and virulence potential of each of the clinical H1N1pdm isolates, groups of six DBA2 mice were intranasally-infected and monitored daily for clinical signs and body weight. In addition to the nine clinical isolates, we included two pandemic strains (A/CA/07/09 and A/NY/18/09) and a seasonal H1N1 strain (A/BN/59/07). On days three and six, three mice each were euthanized and analyzed for viral load and antibody titers (Table 2.2). All mice infected with influenza strains showed weight loss (Table 2.2). Mice infected with KY/96M, KY/80M, KY/180E, KY/80E, and NY/18E showed the greatest weight loss in this short study (Table 2.2). Mice infected with the seasonal influenza strain BN/59E showed the least weight loss (about 9%). All other mice infected with H1N1pdm influenza isolates (KY/136M, KY/99M, KY/136E, KY/110E, CA/07E, KY/108M, KY/110M, KY/104M, and KY/108E) showed weight loss averages ranging from 13% to 26%, respectively.

The mortality rates for mock- and virus-infected groups were expressed as a percentage of lethal infections (Table 2.2). BN/59, CA/07, KY/110 and KY/136 showed no lethality and low morbidity; whereas KY/190 and KY/180 showed the greatest lethality and substantial weight loss. Both KY/80 and NY/18 showed lethality and weight loss. On 3

and 6 days post-infection (DPI), three animals were sacrificed and the lung tissues were collected for virus titration analysis (TCID₅₀ assay of lung tissue homogenate using MDCK cells). Virus titers of lungs from each mouse-infected group showed relatively little difference between the various isolates on 3 and 6 DPI ($p > 0.05$ using pairwise Wilcoxon Rank-Sum test without correction for multiple comparisons, Table 2.2). Mice infected with egg-adapted viruses showed a higher mortality than several of the corresponding MDCK isolates (*e.g.*, see KY/96, KY/99, KY/108). However, both egg and MDCK-adapted KY/80 and KY/180 showed lethality in mice.

The influenza-specific serum IgG antibody responses in mice were measured at 6 DPI by ELISA using antigen prepared from inactivated NY/18 virus (Table 2.2) and reciprocal endpoints were determined. Strains KY/80E, KY/180, and KY/190 had no detectable IgG antibody by 6 DPI. The seasonal strain and all other pandemic strains produced detectable IgG antibody; the highest endpoint titers were collected from BN/59, KY/108E, NY/18E, KY99M, KY/136, and KY/110M. In some cases, these numbers reflect a small numbers of animal remaining at 6 DPI.

Cytokine and chemokines responses in H1N1pdm isolates show four distinct phenotypic profiles

Mouse cytokine and chemokine panels were employed to gain a broad overview of the immune response at 3 and 6 DPI in the lungs and sera of mice infected with each of the viral isolates (Figures 2.1 and 2.2). No significant difference was seen between egg- and

MDCK-passaged isolates; therefore, the combined data are presented in these two figures. There was an overall increase in proinflammatory cytokines (TNF α , IL-1 β , IL-6 and KC) albeit different levels were noted. The levels of TNF α and IL-1 β were greater in the lungs on day 3 in viral isolates with greater lethality (*e.g.*, KY/180, KY/190) as compared to those with no lethality (*e.g.*, KY/136, $p < 0.05$). These same cytokines were higher in the mice with no lethality on day 6 as compared to those showing lethality. A similar temporal pattern was noted with some chemokines (*e.g.*, RANTES, Figure 2.2). Interferon-gamma (IFN γ) and IL-10, an anti-inflammatory cytokine, were present on day 6 in mice infected with all but the most lethal strains, KY/180, KY/190 (Figure 2.1, $p < 0.05$).

With the exception of isolate KY/110 and KY/104, the chemokines CCL2 (MCP-1), CCL3 (MIP1 α), CXCL9 (MIG), and CXCL10 (IP-10) displayed similar levels in all isolates on 3 DPI followed by a relative increase in animals infected with nonlethal versus a decrease in animals infected with lethal isolates at 6 DPI (Figure 2.2 and Table 2.2). IFN γ stimulates both IP-10 and MIG, and as expected the IFN γ levels were low in lethal isolates as compared to nonlethal isolates at 6 DPI. Cytokines and chemokines that showed limited response in mice with any viral infection included Eotaxin, GM-CSF, IL-1a, M-CSF, IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-12p40, IL-13, IL-15, IL-17, MIP2, LIX, MIP1 β , RANTES, IL-12p70, and VEGF (data not shown).

A principal components analysis (PCA) was performed using 11 cytokine/chemokine measurements from the lungs of mice infected with each virus (Figure 2.S1). The

cytokines and chemokines included in the analysis were selected because they were determined to be the most significantly different between isolates on 3 and 6 DPI by generalized linear model fitness testing (data not shown). The first two components explained 72% of the variance (Figure 2.S1) and the third component explained an additional 10% (data not shown). CCL3, TNF α , IL-10, IL1 β , and IFN γ were all highly correlated with the first component dimension (>80%), meaning these cytokines were principally important in differentiating the isolates. CXCL10, KC and G-CSF were similarly highly correlated with the second component (>70%), and CXCL9 was highly correlated with the third component dimension (83%, data not shown). The remaining variables, IL-6 and CCL2, were not highly correlated with the components used in this model, although they were significantly different between isolates on 3 and 6 DPI. The mean for each isolate on 3 and 6 DPI are plotted according to the coordinates of the first two components in Figure 2.3 (and Figure 2.S1).

The method used for computing this PCA is similar to k-means cluster analysis, and therefore we expected to observe the isolates to group according to their cytokine/chemokine signatures. To better visualize these clusters, we connected each isolates' 3 DPI coordinate to its 6 DPI coordinate with an arrow (Figure 2.3). According to this method of using a "time-resolved" PCA, the isolates were observed to cluster clearly into four groups representing various trajectories of disease.

The first three groups of isolates could be differentiated by their immunogenicity and low to moderate lethality. The first group includes isolates with a low lethality in mice, and

was exemplified by infection with the seasonal strain BN/59, and 2009 pandemic strains CA/07, KY/80 and KY/136. The second group (KY/96, KY/99, and KY/104) included those strains with moderate lethality in the mouse model, and was similar to the first group in course of disease, but was characterized by increased inflammatory cytokines (e.g., IL-6, KC-like, and G-CSF) and chemokines (e.g., CXCL9). In contrast to the first group, these had a higher level of the anti-inflammatory cytokine IL-10 later in disease, as well as increased IFN γ at 6 DPI. The third group of influenza strains (KY/108 and KY/110) was the most immunogenic compared to the other isolates, and differed from the two groups above in that they show the highest levels of inflammatory cytokines late in infection (e.g., IL-6, TNF- α , IL-1 β) but were similar to Group 2 in terms of lethality. They showed the highest level of chemoattractant chemokines, particularly CCL2, CCL3, and CXCL10, and produced the highest levels of IL-10 and IFN γ detected in this study.

Finally, the fourth group of isolates, consisting only of KY/180, KY/190, and NY/18, were the most lethal of the viruses screened in mice. Their proinflammatory cytokines were elevated throughout the course, but not different from 3 DPI in Group 1 isolates. Most notably, these isolates failed to produce any IFN γ or IL-10 by 6 DPI. In Figure 3, the trajectory of these isolates point in an opposite direction from all other isolates, perhaps indicating a different course of disease. In summary (see Table 2.S5), the immune responses of the virus isolates clustered similar to other phenotypic markers of virulence and lethality (Table 2.2).

In-depth comparison of the temporal progression of survival, viral load and immune responses of two clinical isolates, KY/136E and KY/180E, with low and high virulence, respectively.

KY/136E and KY/180E were selected from the nine H1N1pdm clinical isolates for further characterization as representatives of lower and higher virulence based on the apparent disease in mice. These isolates had similar concentrations of lung cytokines and chemokines on 3 and 6 DPI compared to other isolates and clustered together in the PCA. However, the time-resolved PCA revealed that the isolates differed in the progression of immune responses in the course of infection. To gain additional insight, we first assessed dose response to infection in DBA/2 mice. Mice were infected with 10^0 , 10^2 or 10^5 TCID₅₀ of KY/180E or KY/136E and examined daily for clinical signs. Each day, mice were weighed and data from each dose group are presented in Figure 4A (KY/136E) and Figure 4B (KY/180E). The Kaplan-Meier survival curves of mice infected with KY/180E or KY/136E (Figure 2.4C and 2.4D, respectively) confirmed the observations reported above in terms of the general lethality of each virus, although KY/136E did show lethality in 1-2 mice on 10 DPI (Figure 2.4C). Mice infected with KY/180E succumbed to infection starting at 3 DPI at the high dose and on 6 DPI for the middle dose (Figure 2.4D). Animals were humanely euthanized upon showing a moribund state or upon a 25% loss in body weight as described in the materials and methods.

The levels of infectious virus present in the upper and lower respiratory tracts, nasal turbinates (NT) and lung, respectively, were measured at 1, 3 and 5 DPI at the three

different doses (10^0 , 10^2 or 10^5 pfu per mouse) of KY/180E or KY/136E (Table 2.S3). As expected, levels of virus were greatest in mice with higher doses of infection. Mice infected with KY/180E persisted at higher levels of virus over the 5 day period as compared to KY/136E which had approximately 2-3 logs lower virus in the lungs and had virus levels that decreased from 3 to 5 DPI. The infectious dose (ID_{50}) and lethal dose (LD_{50}) were $<10^0$ TCID₅₀/mouse and $10^{1.2}$ TCID₅₀ per mouse, respectively, for KY/180E (data not shown).

To further dissect the immune responses of these two viruses, multiplex cytokine/chemokine bead arrays were employed to insight into the temporal patterns for the key cytokine and chemokine profiles responses noted in the broad survey of all the isolate. Mice (5 per group/per day) were infected with each of the isolates and humanely euthanized on 1, 3, and 5 DPI (Figure 2.5 and 2.6). There was an overall increase in proinflammatory cytokines (TNF α , IL-1 β , IL-6 and KC) in the lungs of all mice, however, there were much higher levels of all cytokines in lungs of mice infected with KY/180E. Further the responses were much earlier and correlated with the high levels of infection noted in the lung. In contrast with KY/180E, KY/136E-infected mice showed a gradual progression in immune response, however, the overall responses on 6 DPI of KY/136E remained low. Interferon-alpha (IFN α) and IL-12p70 levels were higher in the lungs of KY/180E-infected mice as compared to KY/136E-infected mice (Figure 2.S2). Virus-infected mice that recovered from KY/136E infection showed a high IL-10 response by 6 DPI whereas KY/180E-infected mice showed no response (Figures 1 and 5, $p < 0.05$). As expected, and also shown in Figure 1, the IFN γ levels were low in the

lethal KY/180E isolate as compared to the robust response observed in the nonlethal KY/136E isolates at 6 DPI (no statistically significant difference).

Mice infected with the middle dose of KY/180E that survived after 6 DPI, and produced high concentrations of IFN γ , eventually became moribund with all but one euthanized by Day 10. These data suggest that the immune response differences between the two isolates are due to the high levels of infection in the lung by KY/180E. The levels of cytokines and chemokines in the lung support this observation (Figures 2.S3-2.S6), showing a dose response for both isolates on 1, 3, and 5 DPI in mouse lung homogenate. Notably, a dose of 10^2 pfu/mouse of KY/180E showed a progressive increase in chemotactic chemokines to 5DPI whereas a higher dose (10^5 pfu/mouse) began declining in concentration of these analytes after 3DPI (Figure 2.S3). Similar patterns were seen for proinflammatory cytokines in the lung (Figure 2.S5) and cytokines involved in adaptive immunity initiation, IFN γ and IL12p70 (Figure 2.S6). The endpoint titer IgG responses of the mice for the low and middle doses were similar for both viruses (Figure 2.7; all significantly different from control using Student's *t*-test, $p < 0.05$). Further, the HI titers were also very similar except for the lower dose, which was about 2-fold higher in mice infected with KY/180E (Figure 2.7).

The leukocyte chemoattractant chemokines CCL2, CXCL10, KC (CXCL8-like), and G-CSF were greater and earlier in the lungs of mice infected with high doses of KY/180E than with a similar dose of KY/136E (Figure 2.6, $p < 0.05$). As expected, cytopins of BAL revealed that KY/180E contained more neutrophils and monocytes on Day 4

compared to KY/136E (Figure 2.8A,B, $p < 0.05$). Additionally, we observed a higher proportion of macrophages in the lungs in the low dose group of KY/136E compared to the higher dose group ($p < 0.05$, Figure 2.8C). Despite there being a greater number of infiltrating leukocytes in the lungs of KY/180E-infected mice, these mice had a delayed clearance of virus in the lung.

Given the apparent clearance of virus from the lungs of KY/136E (Table 2.S4), we were interested in whether the apparent differences in lethality of the two viruses could be due to the site of virus replication. We tested virus titers from bronchoalveolar lavage fluid (BALf), from the cellular pellet of the BALc (BALc) of the mice, and from the homogenized lung after lavage using TCID₅₀ assay. Samples were collected on 3 and 4 DPI because we began to see decreases in viral titer in the lungs of KY/136E-infected mice after 3DPI. In our earlier studies (Table 2.S4), the virus titer in lung reflects lung, BALf, and BAL cells combined and we observed a nearly 3 log difference between KY/136E and KY/180E. When BAL is collected before isolation of lung tissue, we found the majority of infectious KY/136E virus present in lungs of mice was localized in the cellular fraction of the BAL (Figure 2.9). In contrast to KY/180E, no infectious KY/136E virus was present in the BALf by 4 DPI. Flow cytometric analysis revealed that the influenza NP-positive cells in the BAL from both isolates were also Gr1-positive (clone RB6-8C5), a marker of mouse phagocytic leukocytes, *i.e.*, Ly6C/Ly6G positive (Figure 2.8D).

Infection and replication of H1N1pdm viruses in macrophage cell lines

Given the differences noted within the levels of virus in BALf and BALc, we used a BALB/c mouse macrophage cell line (RAW264.7) and a C57BL/6 mouse macrophage cell line (NR-9465) to test for the ability of KY/136E and KY/180E to infect and replicate in macrophage cells. Confocal microscopy at 24 hours post-infection showed that both viruses were able to infect macrophage cell lines (Figure 2.10). However, KY/180E was more efficient at replication, reaching 10-fold higher virus titers that persisted longer in both cell culture systems (Figure 2.11 and Figure 2.S7). Although the cell culture supernatant was positive at 24 hours post-infection for both viruses *in vitro*, KY/180E was the only isolate detected from the cellular component in BAL (Figure 2.8; $p < 0.05$ at 24 and 48 hours post-infection). These observations agree with our findings in the DBA/2 mouse model and suggest that KY/180 is more successful *in vivo* in replication and production of infectious virus in macrophages (Figure 2.8).

Discussion

Herein we present data characterizing several isolates of H1N1pdm influenza virus taken from human patients with severe pneumonia in Kentucky, USA. Clinical surveillance from foci around the world showed that the majority of human cases of H1N1pdm were mild (36, 556). Severe disease from H1N1pdm infection was more likely to be seen in patients with pre-existing chronic illness (557). It was initially observed that antigenic and genetic variation in circulating pdmH1N1 2009 influenza viruses was less than what is seen during seasonal human influenza A (H1N1) (38). Similarly, the isolates listed here show relatively low variation, differing at only 43 amino acid positions. Each

isolate contained from 2 to 12 unique non-synonymous mutations from the consensus sequence.

Three of the 4 lethal cases occurred in western Kentucky, and 1 of these lethal cases (KY/180) contained a virus isolate with the avian-like HA RBS (G222). The D222 is most commonly associated with human H1 strains while the G222 is common in avian strains. The HA 222 position, D222G/N/S/E/Y, was a common polymorphism in severe cases of influenza in human patients, which is within the HA receptor binding site, RBS (547, 558-561). Additionally, the D222G has been associated with increased pathogenesis in some animal models possibly due to increased replication of the virus (125, 129, 131, 558, 559, 562, 563). The aspartic acid functions in binding of the receptor and as a calcium antigenic site. Each of these amino acids will give rise to a specific binding affinity to the α 2-6-sialic acid receptor with the D222G increasing α 2-3-sialic acid receptor binding specificity (125, 224, 563). However, it is clear that other amino acids constellations at positions 183, 186, 187, 216 and 224 will also influence these interactions (127), and may result in different overall outcomes. Additional amino acid signatures in closely related H1N1pdm isolates have also been implicated in differences in virulence in animal models. For example, in a study in a cynomolgus macaque model of two highly similar strains of H1N1pdm, A/Mexico/4487 and A/Mexico/4108, showed notable heterogeneity in virulence (137). The amino acid variations responsible for the differences noted in these studies have not yet been reported. The HA1 of KY/180E also has a P183 (noted as 186 in (552)) rather than a S183 at the Sb antigenic site, which also has been noted to affect receptor specificity ((553-555)). This site, independently or

together with the D222G mutation, is thought to allow binding to α -2,3 linked sialic acid residues. This substitution may allow the virus to more efficiently enter and replicate in the lower airway epithelium (276, 555, 564, 565).

Other mutations observed in the HA gene of the Kentucky isolates have also been reported by others. Belser, *et al.* (2010) published data that showed A/CA/04/09 also has the S83P mutation in HA (as in KY/180E), although this mutation is not a known pathogenicity determinant (247). Ilyushina, *et al.* (2010) found the S183P (as seen in KY/180E) mutation arose from serial passage of A/CA/04/09 in mice and resulted in increased pathogenicity (553). Xu, *et al.* (2011) report isolates from China show similar variability and share some of our mutations in HA (S83P, T203S, and V321I; seen in KY/180E, KY/96E, and KY/80E, respectively), as well as mutations in NS1 (V123I, seen in KY/99E) and PA (V14I, seen in KY/180E) (566). Melidou *et al.* have data that suggest V321I mutation in HA may be associated with increased disease severity (134). Finally, HA1 amino acid mutation Q293H, identified in KY/96E, has been associated with increased severity of disease observed in human cases during the pandemic (106, 134). We include a summary of those mutations that correlate with virulence in humans and mice (Table 2.S6). An understanding of these and other signatures associated with more virulent phenotypes will benefit insight into the biology of the virus, patient management and public health responses.

In total, KY/180E contains mutations that differ from KY/136E in seven amino acid sites in the HA protein, four sites in PA, two sites in NA, PB1 and PB2, and one site in each of M1, NS1, and NP. Comparison of the CA/07/09, NY/18, NL/602 isolates with the KY isolates showed additional variation among all isolates (Table 2.S2). Specifically, CA/07/09, NY/18, NL/602 were similar to the KY consensus in M, HA2, PA, PB1 and PB2. In HA1, a Q223R occurred in NY/18 only. In NA, a D248N change occurred in CA/07 and NL/602. In NS1, a D247N was noted for KY/110, CA/07, NY/18, and NL/602. Most additional changes were conserved hydrophobic changes such as Val to Iso or Met to Leu. No differences were observed among the KY, CA/07/09, NY/18, NL/602 isolates in M2 or NS2 (Table 2.S2). Despite the small apparent genomic variation between H1N1pdm isolates, we and others have shown that there exists substantial variation in the course of disease, pathogenicity, and immune response in human patients, in animal models, and in primary cell culture (106, 135, 137, 566, 567). As virulence determinants of influenza viruses typically involve the genes encoding the HA, NA, and polymerase proteins, we would expect changes in these genes to be of future interest for biological function (568). Additional genome diversity that might give rise to unique phenotypes during infection may be revealed by deep sequencing or isolation of clones that sample the population of the virus (549).

To gain insight into the potential phenotypic variability inferred from the genotypic variability each virus was screened in DBA2 mice. All IAV tested so far, H1N1pdm, 1918 H1N1, and seasonal influenza A H1N1 virus, infect DB2 with varying levels of lethality without adaptation (569), and express comparatively high levels of

proinflammatory cytokines and chemokines (570, 571). Herein all DBA2 mice showed high levels of infection with all isolates similar to infection of BALB/c mouse with H1N1pdm viruses (247, 542) with relatively little difference between the various isolates on 3 and 6 DPI ($p > 0.05$ using pairwise Wilcoxon Rank-Sum test without correction for multiple comparisons, Table 2.2). However, the isolates did show three groupings in lethality (lethal, moderately lethal and not lethal) in DBA2 mice with KY/180, KY190 and KY/96 showing the highest lethality. Using a statistical method, PCA, we asked if based on these differences in lethality we might be able to cluster cytokine and chemokine responses across the H1N1pdm isolates. To our knowledge, this is the first time this method (a “time-resolved” PCA) has been used to discriminate phenotypic characteristics of viruses. Classically PCA is used as a method to reduce multivariate data so that they are better suited for a predictive statistical model. The most common contemporary usage of PCA in microbiology is during the analysis of gene expression arrays to check reproducibility of replicates. We chose to use the PCA method because of its similarity to common clustering algorithms (specifically, k-means clustering), and therefore its potential to elucidate clusters of the immune response of mice after infection with genetically similar influenza isolates. Our approach relied on two defined time points to detect differences between virus isolates using expression of multiple immune markers. Although some of the markers used in this analysis may be redundant in a virus model (*e.g.*, pro-inflammatory IL-6 and CXCL10), subtle differences that may go unnoticed are seen using this multivariate approach, particularly in the timing of these responses (and not necessarily the magnitude). Therefore, using indicators of adaptive immune priming (IFN γ) and reduction of inflammation (IL-10) were critical to the

successful implementation of this strategy. More data of this kind from other influenza isolates and other respiratory virus infections may assist in the development of this method, and may reduce the number of soluble immune markers needed for analysis in a clinical setting.

Analyses of our data suggest that there are at least two main trajectories of H1N1 influenza infection in these mice: one that successfully resolves infection, and one that does not. The majority of the isolates followed a trajectory that led to resolution and clearance of the virus from the lungs. These isolates could be further clustered into three subgroups, and these subgroups differ according to the overall immunogenicity of the isolates (*i.e.*, the levels of cytokines/chemokines in the lung). In general, the isolates showing the least mortality in DBA2 mice share in common a trajectory of disease that is characterized by a gradual increase, then decrease, in inflammatory cytokines and leukocyte chemoattractant chemokines, and concomitant increase in anti-inflammatory cytokines and IFN γ . The isolates causing the highest lethality in the DBA2 mouse (KY/180E, KY/180M, and KY/190M) showed an early rise in the proinflammatory cytokines and chemokines, and a delayed or absent rise in IL-10 and IFN γ . The lethal isolates were clearly differentiated using the time-resolved PCA in that they showed a trajectory that was opposite of all other isolates. The small sample size of human patient data and their confounding comorbidities limit the inferences that can be made connecting locale, age, sex, and length of hospital stay with genotype and pathogenicity of the isolates in the human patients. Recent data suggest that human polymorphisms in

genes that restrict virus infection (e.g., *Ifitm3* gene) may also play an important role in outcome (572). A final challenge is there are no relevant scoring systems for influenza pneumonia. A pneumonia severity index (PSI) is commonly used in hospitals by clinicians for community acquired pneumonia, but the comorbidities (e.g. high BMI, diabetes) complicate interpretation.

Selecting two isolates, KY/180E and KY/136E we sought to better define two of these trajectories in DBA2 mice. We initially observed KY/136E had a low level of immunogenicity in mice, which included an increase in IL-10 and IFN γ by day 6.

Although mice infected with KY/136E are similar to KY/180E in concentration of IFN α , G-CSF, CCL2, KC, IL-6, CXCL10, and other inflammatory cytokines and chemokines in the lung, the isolates differ in the timing of these responses. Increases in these cytokines and chemokines are typically seen during infections with H1N1pdm influenza isolates (112, 395, 542). For example, some studies show that IL-6 is released in response to influenza infection and that levels of IL-6 in the upper respiratory tract and in blood correlate with symptoms (101, 393, 573).

Type-I interferons are known to initiate the anti-viral response to influenza virus infection. One hypothesis to explain a more severe course of illness is that the virus is capable inhibiting the effect of type-I interferon signals via NS1 protein (298, 306). The NS1 protein from KY/136E differs from KY/180E in a single amino acid at site 112 (112M versus 112I, respectively). By day 1 post-infection, mice infected with KY/180E

had a higher level of IFN α in the lung. Despite an increased IFN α and IL12-p70 response, mice infected with KY/180 failed to produce IFN γ , IL-10, and serum antibodies by 6 DPI. This most closely resembles the type of aberrant immune response seen during infections with the pandemic 1918 Influenza A (H1N1) (574, 575) and with severe seasonal influenza isolates (71). Others have shown similar patterns of inflammation with isolates of pandemic 2009 H1N1 influenza (135, 560, 576).

Cytokines and chemokines such as CCL2 (MCP-1), CCL3 (MIP1 α), CCL5 (RANTES), and CXCL10 (IP10) are responsible for activating leukocytes and attracting them to the lung compartment to clear infection (337). Indeed, there were a higher number of leukocytes, including elevated numbers of neutrophils and monocytes, seen in the lungs of KY/180E-infected mice on days 3 and 4 post-infection. It is known that certain pathogenic H1N1 strains, such as the 1918 H1N1 strain, causes the increased neutrophil recruitment to the lung due to increased chemokine responses (577, 578), which was seen with KY/180E. Neutrophils are important for assisting in clearing influenza-infected cells from the lungs directly and indirectly (579, 580), but the precise mechanisms remain to be discovered (78, 86, 577). Although important to the resolution of influenza virus infection, neutrophils contain cytotoxic granules that may cause severe pathology, and may contribute to morbidity and mortality (84). An increase in neutrophils late in disease confers the pathogenicity seen in highly pathogenic strains of influenza viruses (86).

Natural Killer (NK) cells are known to be potent innate immune cells that recognize influenza hemagglutinin (581). Classically, NK cell effector functions include release of IFN γ and direct cytotoxicity to influenza-infected cells by granule exocytosis (*e.g.*, granzyme B and perforin). Mice infected with KY/180E have higher numbers of peripheral NK cells at 3 DPI compared to KY/136E using flow cytometry (data not shown). Consistent with this finding, CCL5 (RANTES) was increased in the lungs and serum of mice infected with KY/180E compared to KY/136E (Figure 2.2). NK cell deficiency in mouse models of influenza infection leads to increased pathogenicity (582, 583). Despite the increase in NK cells in KY/180E-infected mice, it has been shown that some influenza isolates may counteract this effector response, causing lysosomal degradation of the ζ chain, which is a critical component of the NK-activating receptor (584). Additionally, DBA2 mice lack the NKG2A activating receptor that is responsible for recognizing the down-regulation of MHC-I that typically occurs with a viral infection (585). Therefore, KY/180E may be able to exacerbate this deficiency by decreasing NK cell effector function. It has been shown that NK cells also contribute to immune pathology in response to influenza and other respiratory virus infections (586).

Finally, this study and others have shown that some strains of influenza virus are capable of infecting macrophages (587-590), and may alter their functionality in response to infection (591, 592). During influenza infection, alveolar macrophages are critical in clearing virus from the lungs and are key producers of IFN γ to stimulate the adaptive immunity (321, 353, 378, 577, 578, 586, 593-595). Upon infection, influenza viruses

may interfere with their normal function (341, 595, 596). Of the two isolates tested here, mice infected with KY/136E showed clearance of virus from the lung by day 4. (341, 595, 596). Our *in vitro* data using mouse macrophage cell lines indicates that KY/180E was able to produce infectious virus better than KY/136E. Additionally, mice infected with KY/180E had a higher number of Gr-1 positive infiltrating cells into the lungs by day 4, a delayed IL-10 production, and very low levels of IFN γ resulting in a deficient virus clearance. Both our *in vivo* and *in vitro* data show that KY/180E can infect macrophages, which may affect the cell's ability to release IFN γ and explain the increase pathogenicity observed with this virus compared to KY/136E. Future studies will focus on understanding the mechanism(s) involved in the virus-induced deregulation of macrophage function *in vitro* and *in vivo*. Understanding the genetic basis for the differences in the interaction between the virus and macrophages as well as other pulmonary and immune cells will contribute to understanding the course of influenza infection in patients, and assist in predicting potential virulence during future outbreaks.

Materials and Methods

Detection of influenza in clinical nasopharyngeal swab specimens

Nasal swab samples were provided by the SIPS project. A nasopharyngeal swab was taken from all patients meeting the case definition, which was defined as a patient admitted to an intensive care unit with the physician diagnosis of community-acquired pneumonia. The University of Louisville Institutional Review Board Human Subject Protection Program Office (HSPPO) approved this study prior to any data collection (#08.0399). Informed consent was waived because it was a retrospective chart review and

the data were analyzed anonymously. The nasopharyngeal swabs were taken as part of 'standard of care' diagnosis. Detection of influenza virus was accomplished using the Luminex Respiratory Viral Panel (RVP) detection kit, recently approved for *in vitro* diagnostics by the FDA. The RVP is a reverse-transcriptase, real-time PCR assay which is multiplexed to detect 12 viral targets in a single reaction well. Those targets include Influenza A, Influenza A-subtype H1, Influenza A-subtype H3, Influenza B, RSV-A, RSV-B, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, human metapneumovirus, Rhinovirus and Adenovirus. Nasal swabs were collected from each patient and placed in Universal Transport Media (Copan Diagnostics, Inc, Murietta, CA). A 5.0 µl aliquot was combined with 20 µl of RVP mastermix and processed through several rounds of amplification, including a single-tube multiplex reverse-transcriptase PCR, followed by a multiplex target-specific primer extension protocol, a bead-hybridization step, and finally a data acquisition step in the Luminex reader. Results are qualitative and read-outs are Positive, Negative or a No Call (equivalent).

Viruses and virus isolation

The viruses CA/07, NY/18, and BN/59 were kindly provided by the Centers for Disease Control and Prevention, Virus Surveillance and Diagnosis Branch, Influenza Division. Viruses from clinical cases from KY present in nasopharyngeal swab specimens were isolated and propagated in the chorioallantoic cavity of 10-day-old embryonated chicken eggs and MDCK cell line purchased from ATCC (CCL-34). The allantoic fluid containing infectious particles or MDCK supernate was harvested 72 h after inoculation. MDCK cells were infected in DMEM virus culture medium (DMEM containing 2%

BSA, 1% PEN/STREP, and 2 µg/mL of Trypsin-TPCK). The infectious virus titer of the resulting seed stock was determined by TCID₅₀ and the titer calculated by Reed and Muench (597), and confirmed by plaque assay on MDCK cells. Egg passage E2 was used for the studies involving KY/180E and KY/136E reported herein.

RNA isolation and sequencing

To sequence each of the gene segments of each H1N1pdm isolate, we followed the method described by Inoue et al (598) with minor modifications. In brief, total RNA was isolated with MagMAX AI/ND Viral RNA isolation kit (Ambion) from each virus seed stock solutions. Five µL of RNA was used to synthesize cDNA with SuperScriptase III (Invitrogen) using the FWuni12 and RVuni13 primers based on those reported by Inoue et al (598) and the cDNAs were PCR-amplified based with Accuprime (Invitrogen) The amplified products were separated by agarose gel electrophoresis and the DNA bands corresponding to the size of each segment were purified using the Wizard SV gel Clean-Up System (Promega). The purified DNAs were used as templates for automated dideoxy sequencing with BigDye 3.1 cycle sequencing kit (Applied Biosystems). To sequence the entire gene we used approximately 500 bp overlapping gene-specific primers in both directions. For the sequencing of HA gene, we used FWuni12, RVuni13, HA462_f (5'-GACTCGAACAAGGTGTAACGG-3') and HA1202_r (5'-GTCAATGGCATTCTGTGTGCTC-3') as sequencing primers. For the sequencing of NA gene, we used FWuni12, RVuni13, NA_521r (5'-TGACCAAGCGACTGACTCAA-3'), NA376_f (5'-CCCTTGGAATGCAGAACCTT-3') and NA905_f (5'-CGTGGGTGTCTTTCAACCAGAA-3'). The sequences were assembled with the

SeqScape™ program (Applied Biosystems). Alignments of nucleic and amino acid sequences were completed to identify polymorphisms with other H1N1pdm isolates and sequences for all isolates were submitted through the Influenza Research Database and uploaded into the GenBank (accession numbers provided in Table S4).

Ethics statement for animal studies

Mice studies were approved by the University of Louisville Institutional Animal Care and Use Committee with Veterinary Medicine tasked to monitor and support all animal experiments. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facilities where this research was conducted in a fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Mice studies

Six to nine-week-old female DBA/2 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the vivarium managed by UofL at the Research Resource Center or at the Regional Biocontainment Laboratory. The mice received food and water *ad libitum*, and all experiments were conducted in accordance with rules of the Institutional Animal Control and Use Committee of UofL. Mice were anaesthetized by isoflurane inhalation and infected intranasally with in a total volume of 30 µl at the dose as outlined in the figure legends and text. Viruses were adjusted to the dose required in

PBS (pH 7.2). Animals were observed daily for morbidity and measured for weight loss. All mice showing more than 25% body weight loss were considered to have reached the experimental end point and were euthanized humanely.

Mice were euthanized on the days noted in the results and on figure legends and blood, nasal turbinates and lung tissues were collected and stored appropriately until analyses. For collection of BAL after euthanasia, the neck and thoracic area were disinfected by soaking with 70% ethanol, and the trachea exposed by dissection. An 18G catheter needle was inserted into upper trachea, the needle removed, and the outer catheter sheath moved into the lower trachea. A 3 mL syringe filled with 1 mL of DPBS was used to carefully inject DPBS into the lungs, and aspirate BAL fluid. Collected fluids were transferred to an ice-cold, sterile 15 mL conical tube on ice and repeated once more as above. BAL fluids were immediately centrifuged at 500g for 10 minutes to separate fluids and cells. After centrifugation, supernatants are separated and kept separately at -80°C freezer until used for TCID₅₀ assay. Cells pellets were washed once by adding 2 mL ice-cold DPBS and centrifuged again at 500g for 10 min. Cells were resuspended in 1 mL DPBS and kept at -80°C until used.

TCID₅₀ assay for viral load in tissues and bronchial lavage fluid

Tissues were homogenized in ice-cold virus culture medium (DMEM containing 2% BSA, 1% PEN/STREP) within a biosafety cabinet. Tissue homogenates were clarified by centrifugation (4000g for 20 min at 4°C) prior to storage at -80°C, and later analyzed by TCID₅₀ or Luminex cytometric bead array (next section). For measurement of the level of infectious virus present in tissue samples, the titers of the virus were determined by

TCID₅₀ assay by titration of the clarified tissue homogenates or BALf on MDCK cells. The limit of virus detection was typically 10^{1.5} TCID₅₀/ml or as indicated in each figure. Virus titers were calculated by the method of Reed and Muench (597), and are expressed as the mean log₁₀ TCID₅₀ per milliliter. Tissues in which no virus was detected were given a value of 10^{1.0} TCID₅₀/ml for calculation of the mean titer.

Quantification of cytokine and chemokine levels in lungs

Sera and lungs from mice euthanized on days as noted in figure legends and text were analyzed by using Luminex® xMAP® technology-based assay kit (Millipore) according to the manufacturer's protocol. The final reaction plate was read with a Luminex 100 or FlexMAP 3D machine and specific concentrations were calculated from a standard using Luminex xPONENT software.

Statistics and PCA

R (version 2.13.0) base statistical package and GraphPad software package were used in the analysis of data and generation of figures. Generalized linear models were constructed using each analyte included in the initial screen of Kentucky isolates in DBA/2 mice as a response variable (cytokine/chemokine concentrations) and isolate, DPI, and egg vs. MDCK stock, were used as predictors. The stock designation of the virus was removed from the model after it was seen that it did not contribute to explain significant proportion of the variance. Log likelihood ratios were used to compare the relative fit of each model, and the top 12 models showing the highest parameter estimates for the isolates were chosen for inclusion into the PCA (*i.e.*, the cytokines/chemokines

that were the most different between isolates, controlling for DPI, selected from the best-fit models). The R package “FactoMineR” (version 1.14) was used to perform the PCA. Statistically significant differences between multiple groups was assessed using Kruskal-Wallis tests followed by *post hoc* tests using pairwise Wilcoxon Rank Sum tests unless otherwise noted. Multiple comparisons were adjusted according to Holm’s method and a p -value < 0.05 is considered significant.

Cytospin and flow cytometry

BAL cells were centrifuged at 300g for 5 min and washed twice in PBS. Cells were resuspended in 0.4 mL PBS and loaded into Shandon Cytospin™ centrifuge funnels. The cytocentrifuge chambers were spun at 1000 rpm for 5 min. Slides were air dried in a BSC and then were fixed and stained with Eosin and Methylene Blue (Kwik-Diff, Thermo Scientific). Slides were inspected under a light microscope.

For flow cytometry analysis, BAL cells were washed in PBS as above and stained with antibodies specific to mouse CD3 (500A2 Pacific Blue, BD Biosciences), Gr-1 (RB6-8C5 phycoerythrin, eBioscience), and CD49b (DX5 phycoerythrin-Cy7, eBioscience). Cells were then washed and fixed in 4% paraformaldehyde for 15 minutes at room temperature, followed by permeabilization with 0.2% saponin in 2% BSA for 20 minutes. Cells were then stained intracellularly for influenza nucleoprotein using a fluorescein isothiocyanate conjugated antibody (NP-FITC, AbCam #ab20921) for 30 minutes. Cells were washed and analyzed on a FACSAria II flow cytometer (BD).

Mouse macrophages experiments

RAW264.7 cells (ATCC #TIB-71) were seeded onto glass chamber slides (LabTek) and allowed to rest overnight. The cells were gently washed in PBS and virus was added at 1.0 MOI. The cells were washed and fixed at 24 hours post-infection and permeabilized with 0.2% saponin in 10% FBS. Cells were stained for intracellular influenza nucleoprotein as above, and a nuclear counter stain was included for the final 10 minutes (TO-PRO3, Molecular Probes). Cells were washed, mounted under a coverslip with ProLong Antifade media (Invitrogen), and visualized using a Zeiss LSM/710 confocal microscope. Kinetics of infection were determined by infecting mouse macrophage cell lines, RAW264.7 or BEI Resources #NR9456 (NIAID, NIH), in triplicate wells of a 24-well plate in DMEM supplemented with 0.2% BSA, 1% Pen/Strep, 2.5% L-glutamine, and 25 mM HEPES. Supernatants were taken and centrifuged prior to performing a TCID50 assay using MDCK cells. Virus titers were confirmed at 24 hours using a plaque assay.

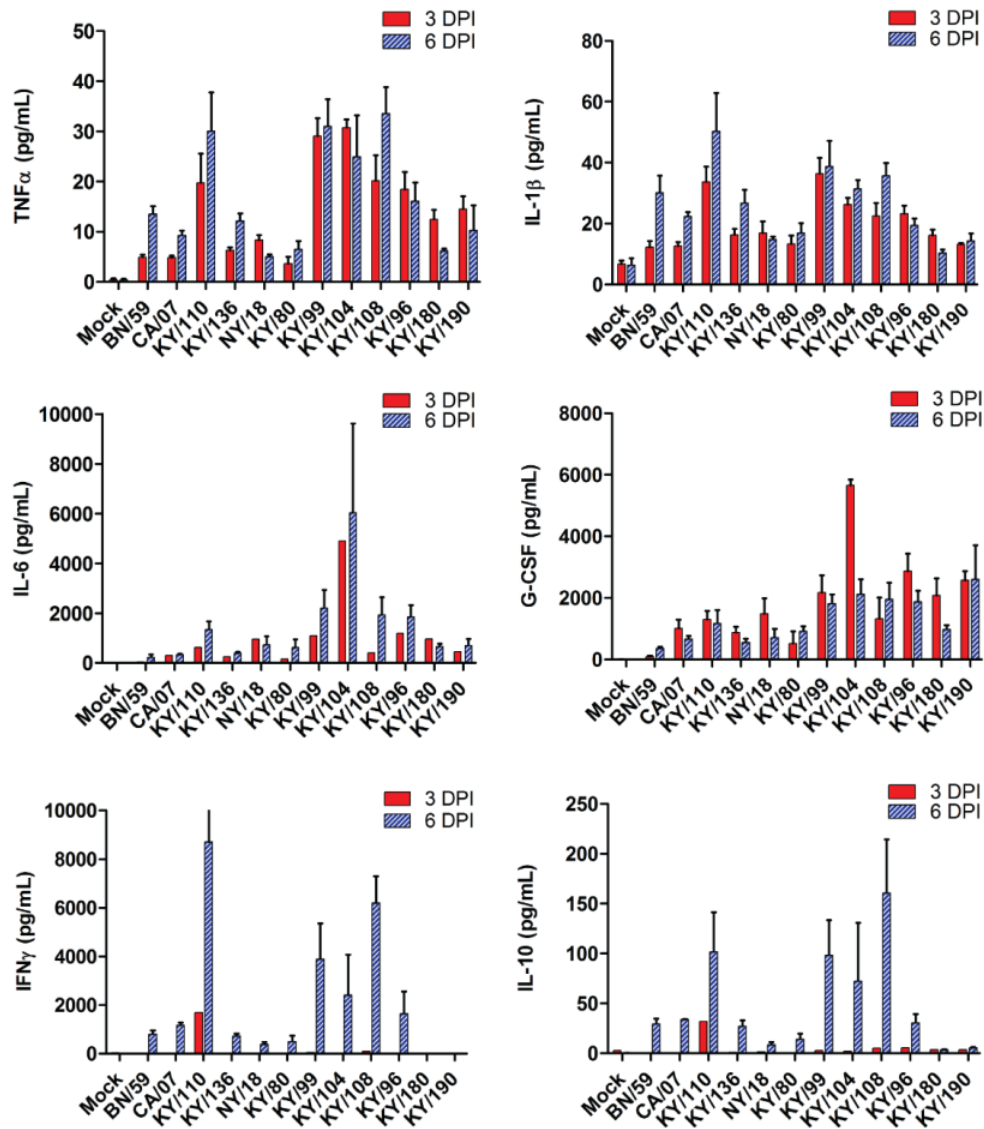


Figure 2.1 Cytokine levels in mice infected with pandemic and seasonal H1N1 influenza viruses.

Six to eight week old DBA/2 mice were infected intranasally with 10^5 TCID₅₀ with a seasonal virus isolate (BN/59), Kentucky (KY/80, KY/136, KY/96, KY/99, KY/104, KY/108, KY/108, KY/110, KY/180, KY/190) or other H1N1 pandemic isolates (CA/07, NY/18) from 2009. Cytokine levels were measured at 3 and 6 DPI as described in the materials and methods and presented as mean +/- SEM (n=6 per group, although fewer animals were available for lethal isolates).

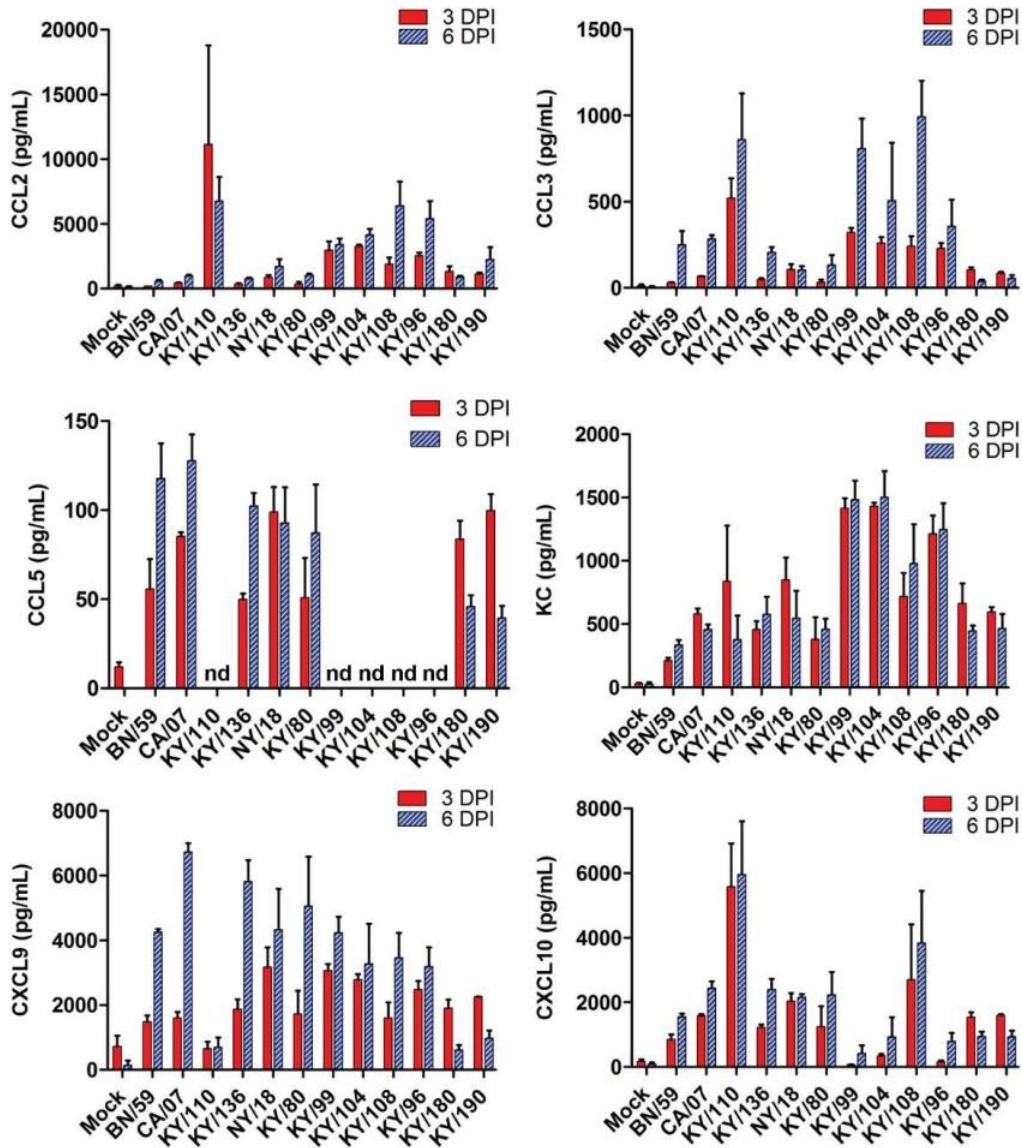


Figure 2.2 Chemokine levels in mice infected with pandemic and seasonal H1N1 influenza viruses.

Six to eight week old DBA/2 mice were infected intranasally with 10^5 TCID₅₀ with a seasonal virus isolate (BN/59), Kentucky (KY/80, KY/136, KY/96, KY/99, KY/104, KY/108, KY/108, KY/110, KY/180, KY/190) or other H1N1 pandemic isolates (CA/07, NY/18) from 2009. Chemokine levels were measured at 3 and 6 DPI as described in the materials and methods and presented as mean +/- SEM (n=6 per group when possible).

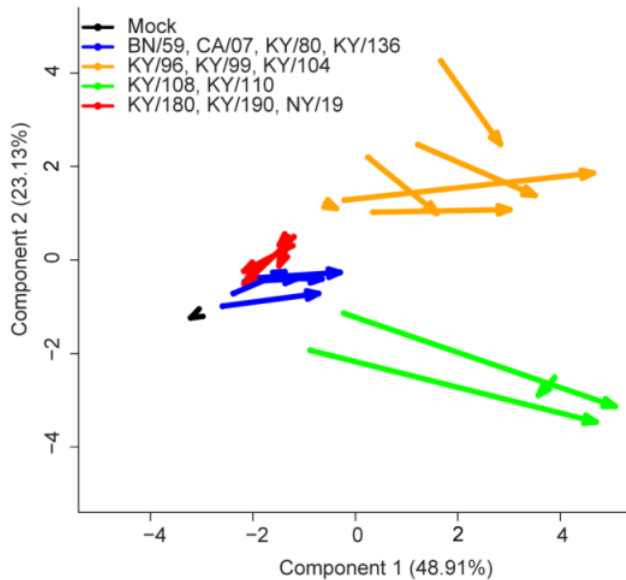


Figure 2.3 Principal components analysis of immune responses in lungs of mice to infection with pandemic and seasonal influenza viruses.

A principal components analysis was performed using the 14 cytokines/chemokines analytes shown to be the most significantly different across all isolates from days 3 and 6 (determined by generalized linear model fitness testing, data not shown). The data were normalized and scaled (zero mean-centered) cytokine responses after influenza infection at 3 and 6 DPI for all viruses. The ordinate and abscissa represent the first and second components from the PCA, which explain approximately 72% of the variance. Each arrow represents the mean of a virus isolate tested in mice. Arrow tails represent day 3 components and arrow heads represent day 6 components. Using this tool to visualize the immune response, the arrows depict the trajectory of disease of the various influenza isolates as tested in DBA/2 mice. Additionally, the 12 virus isolates clustered into four distinct patterns: Group 1, BN/59, CA/07, KY/80, KY/136; Group 2, KY/96, KY/99, KY/104, KY/108; Group 3, KY/108, KY/110; Group 4, KY/180, KY/190, NY/18 (See Table 2.S5).

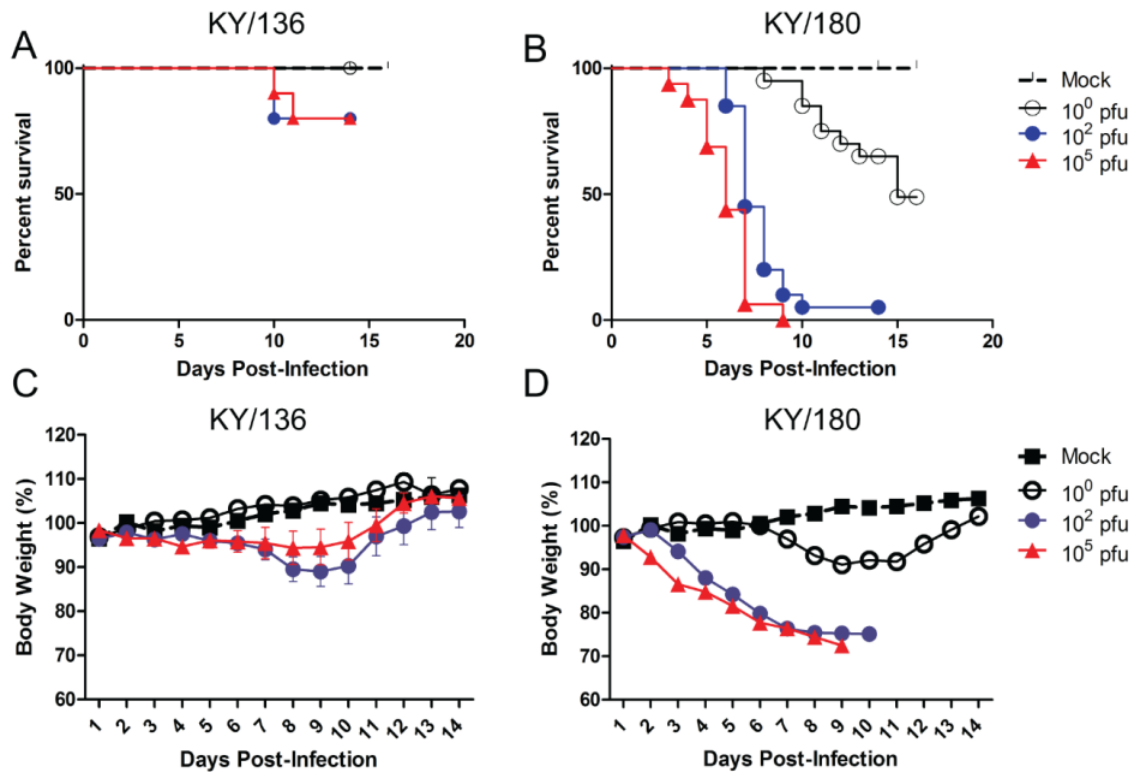


Figure 2.4 Weight loss and Kaplan-Meier curve of mice infected with KY/136 and KY/180.

Mean weight change (+/- SEM) after six to eight week old DBA/2 mice were infected intranasally with 10^0 , 10^2 and 10^5 TCID₅₀ with KY/136E (A) or KY/180E (B). Mice were examined daily for clinical signs and weighed (n=10 mice per virus group). Kaplan-Meier Survival curves for these mice show KY/136E to have low lethality (C) compared to KY/180E (D).

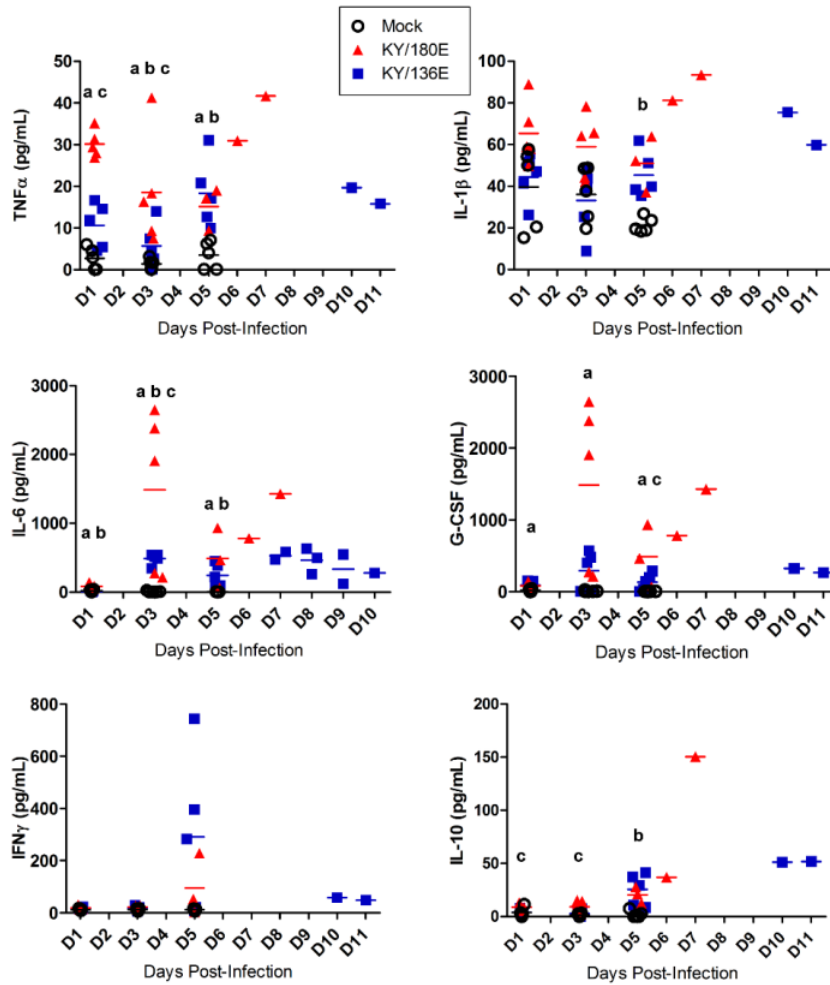


Figure 2.5 Cytokine levels in mice infected with KY/136 or KY/180.

The levels of notable cytokine responses are shown for 1, 3 and 5 DPI in six to eight week old DBA/2 mice that were infected intranasally with 10^5 TCID₅₀ of KY/180E or KY/136E (n=10 mice per virus group per time point). Statistical significance was determined by day using Kruskal-Wallis test followed by pairwise Wilcoxon Rank Sum post hoc test with Holm's adjustment for multiple comparisons. P-values < 0.05 are indicated by the following method: "a" = KY/180 is significantly different from mock; "b" = KY/136 is significantly different from mock; "c" = KY/180 is significantly different from KY/136.

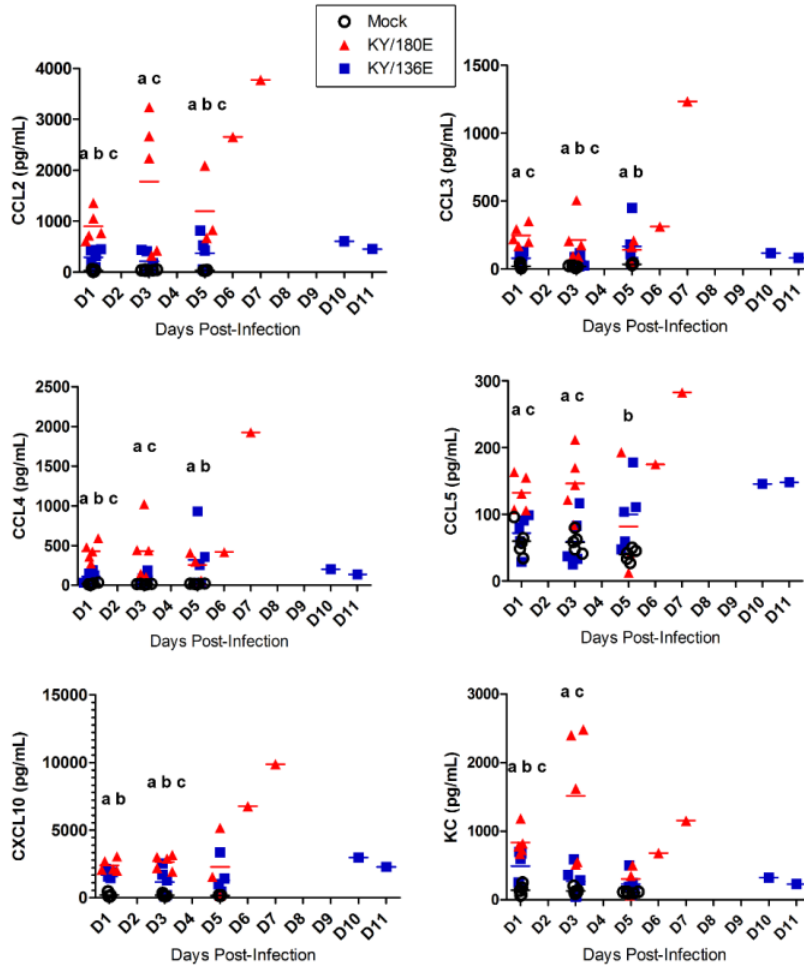


Figure 2.6 Chemokine levels in mice infected with KY/136 or KY/180.

The levels of notable chemokine responses are shown for 1, 3 and 5 DPI in six to eight week old DBA/2 mice that were infected intranasally with 10^5 TCID₅₀ of KY/180E or KY/136E (n=10 mice per virus group per time point). Statistical significance was determined by day using Kruskal-Wallis test followed by pairwise Wilcoxon Rank Sum post hoc test with Holm's adjustment for multiple comparisons. P-values < 0.05 are indicated by the following method: "a" = KY/180 is significantly different from mock; "b" = KY/136 is significantly different from mock; "c" = KY/180 is significantly different from KY/136.

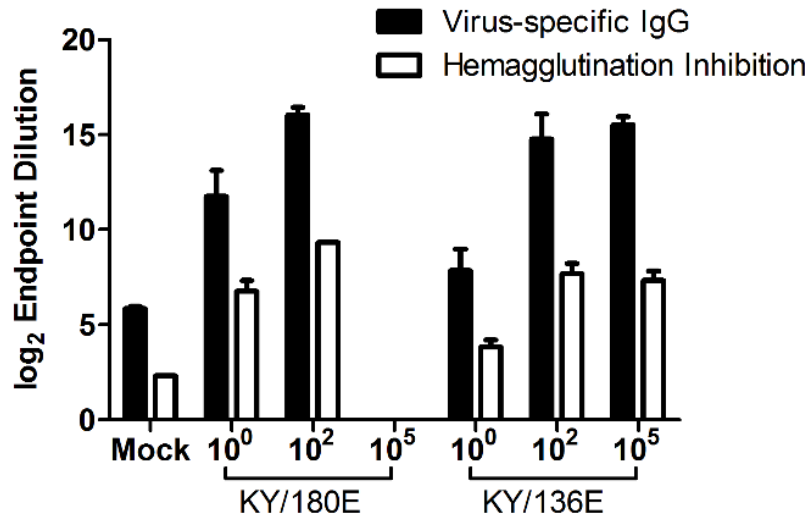


Figure 2.7 IgG responses of mice infected with different doses of KY/136 or KY/180.

Serum from day 20 post-infection was taken from six to eight week old DBA/2 mice that were infected intranasally with 10⁰, 10² and 10⁵ TCID₅₀ of KY/180E or KY/136E (n=10 mice per virus group per time point). Influenza-specific (A/NY/18/09 BPL-inactivated whole viral antigen) IgG titers were measured by ELISA and presented as average log₂ endpoint titers (+/-SEM). No mice survived to day 20 at the high dose of KY/180. All endpoint titers for animals infected with influenza virus isolates were significantly different from mock (p < 0.05). Endpoint titers for both IgG and HI from animals infected with KY/180 were significantly different from the same dosage amount of KY/136 (p < 0.05).

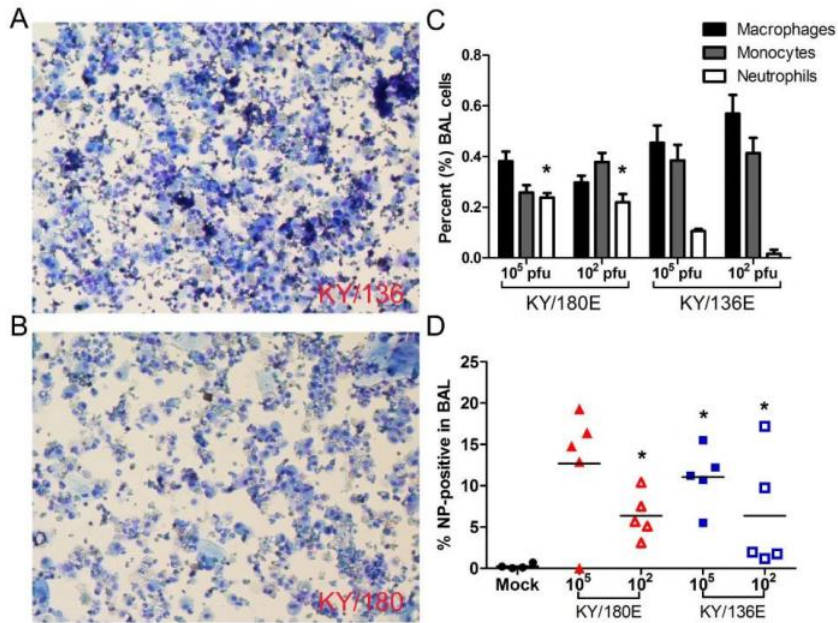


Figure 2.8 Cells within bronchial lavage fluid of mice infected with KY/136 or KY/180.

The cells within the bronchoalveolar lavage fluid (BALf) at 4 DPI from DBA/2 mice infected with 10⁵ TCID₅₀ of KY/136E (A) or KY/180E (B) (n=5 mice per virus group per time point) were affixed to slides by cytopspin centrifuge and stained (Kwik-Diff). Microscopic images from three fields per slide were counted by three blinded, independent observers. (C) The average number of macrophages, non-specific monocytes, and neutrophils are presented as a percent of the total cell count (+/- SEM). There were significantly more neutrophils in the BALf of mice infected with KY/180E virus (p < 0.05, indicated by asterisks). (D) Cells in the BALf were fixed, permeabilized, and stained with anti-influenza nucleoprotein (NP)-FITC antibody conjugate and analyzed by flow cytometry. There were significantly more NP-positive cells in mice infected with KY/180 at a dose of 10² TCID₅₀, and in mice infected with KY/136 compared to mock-infected controls (p < 0.05, indicated by asterisks). 80% of NP-positive cells were Gr-1 positive (macrophage or neutrophil).

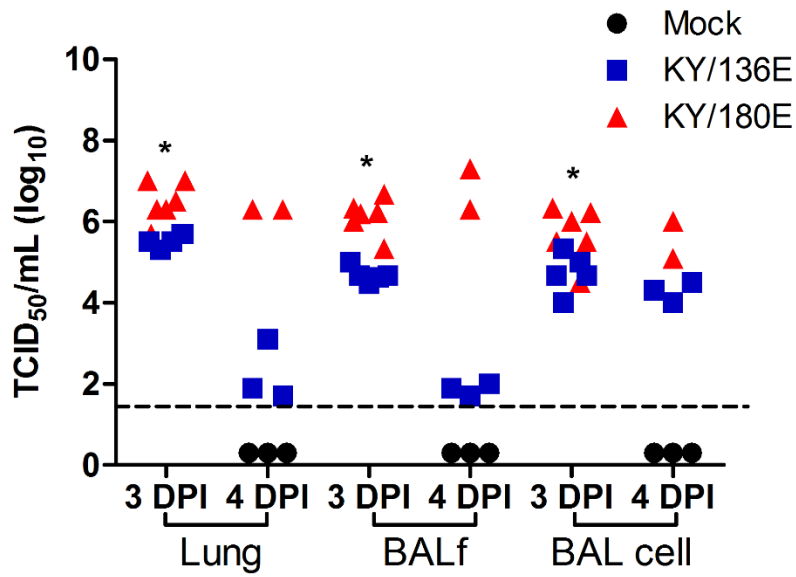


Figure 2.9 Virus titer the in lung compartments of mice infected with KY/136 or KY/180.

Bronchoalveolar lavage was taken from six to nine week old DBA/2 mice that were infected with 10^5 TCID₅₀ KY/136E or KY/180E on days 3 and 4 post-infection. Cells were separated from the lavage fluid by centrifugation and the fluid (BALf), cellular (BAL cell), and whole lung homogenate were tested separately for virus by TCID₅₀ assay on MDCK cells. Lung compartments taken from mice infected with KY/180 had statistically higher virus titers at 3 DPI than from mice infected with KY/136 ($p < 0.05$, indicated by asterisks on the figure). It is not possible to compute statistical differences from 4 DPI, as there were only two mice infected with KY/180 in that experiment that survived to 4 DPI.

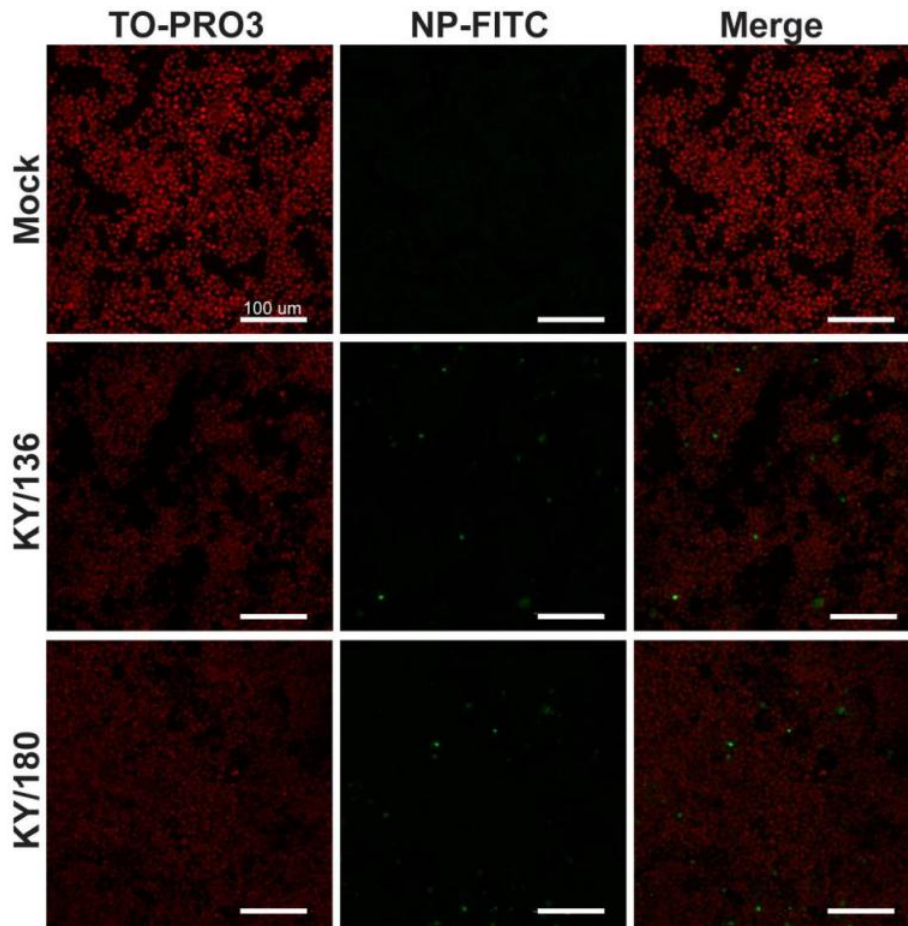


Figure 2.10 Detection of influenza in mouse macrophage cell lines.

Mouse macrophage cell line, RAW264.7, was infected in vitro with MOI=1 of each influenza isolate (high pathogenic isolate, KY/180, and low pathogenic isolate, KY/136) on chambered microscopy slides. At 24 hours post-infection cells were fixed, permeabilized, and stained using a FITC antibody conjugate specific for influenza A (H1N1) nucleoprotein (green). Cells were counterstained with a nuclear dye (TO-PRO3, Molecular Probes, red), and visualized on a Zeiss LSM710 confocal microscope. Both isolates tested were observed to infect macrophages. Scale bars indicate 100 microns.

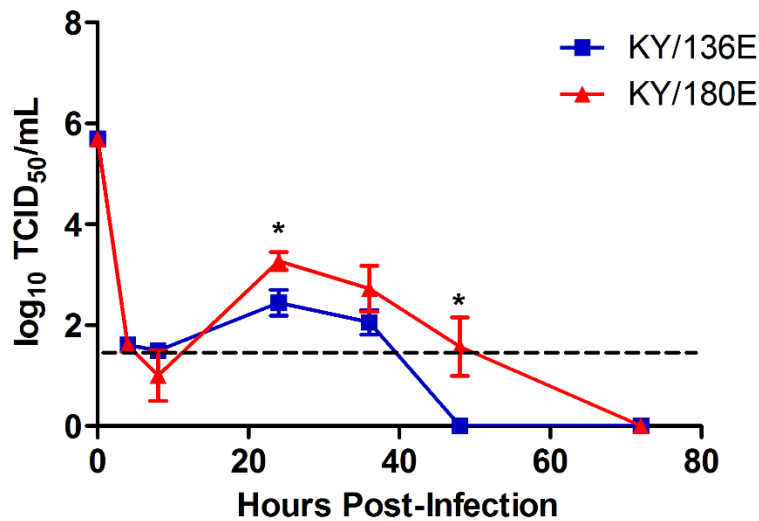


Figure 2.11 Replication of KY/180E and KY/136E in mouse macrophage cell lines.

Mouse macrophage cell line, RAW264.7, was infected in vitro with the influenza isolates on a 24-well plate at an MOI = 1.0. After one hour of incubation, the cells were washed and returned to the incubator. Cell culture supernatants were collected over time and virus titer was determined by TCID₅₀ assay. Both KY/180E and KY/136E replicate in mouse macrophage cell lines. KY/180E had significantly higher titers of virus detected at 24 and 48 hours post-infection ($p < 0.05$, indicated by asterisks on the figure). Pathogenic isolate KY/180E was able to replicate better in mouse macrophages compared to the low pathogenic isolate, KY/136E.

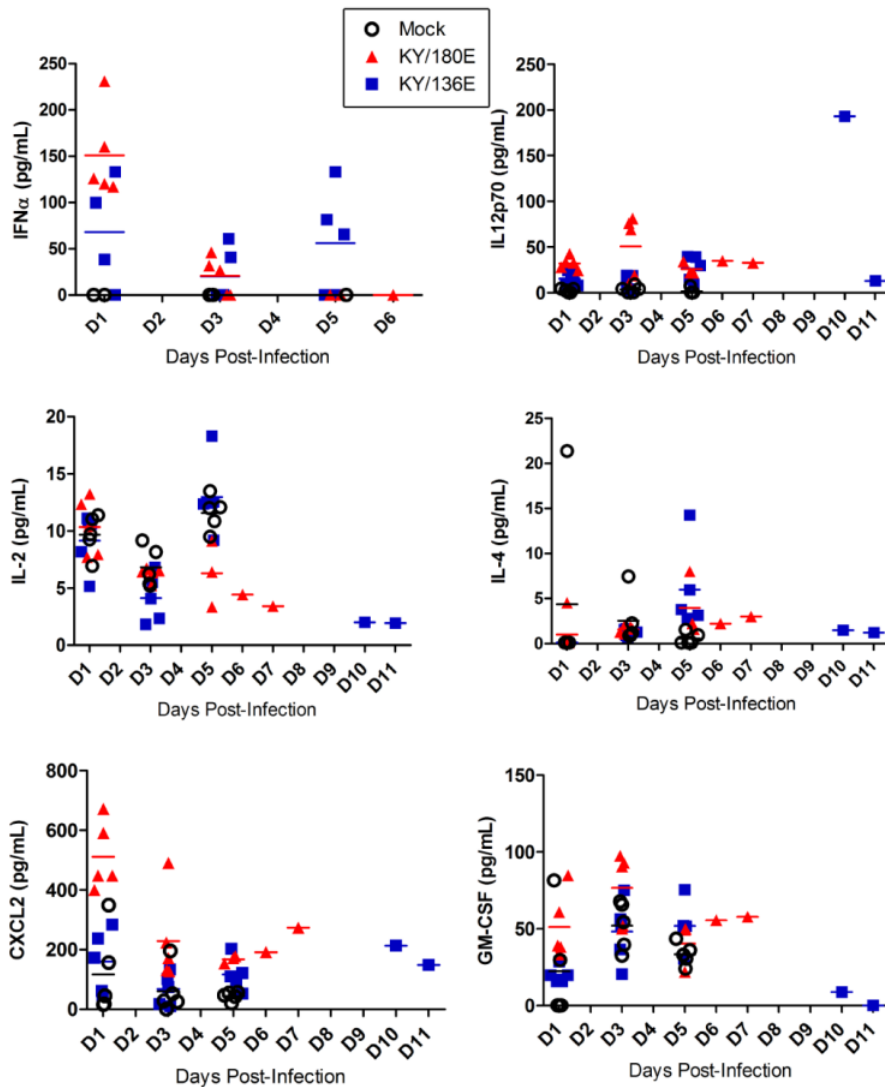


Figure 2.S2 Cytokine and chemokine profiles from mouse lung homogenate.

DBA/2 mice were infected with KY/180E, KY/136E (10^5 pfu), or mock-infected with PBS. The mice were sacrificed 1, 3, and 5 days post-infection (n=5 mice per group-day). Samples from moribund mice taken after Day 5 post-infection were also analyzed when available. Bars indicate mean concentration.

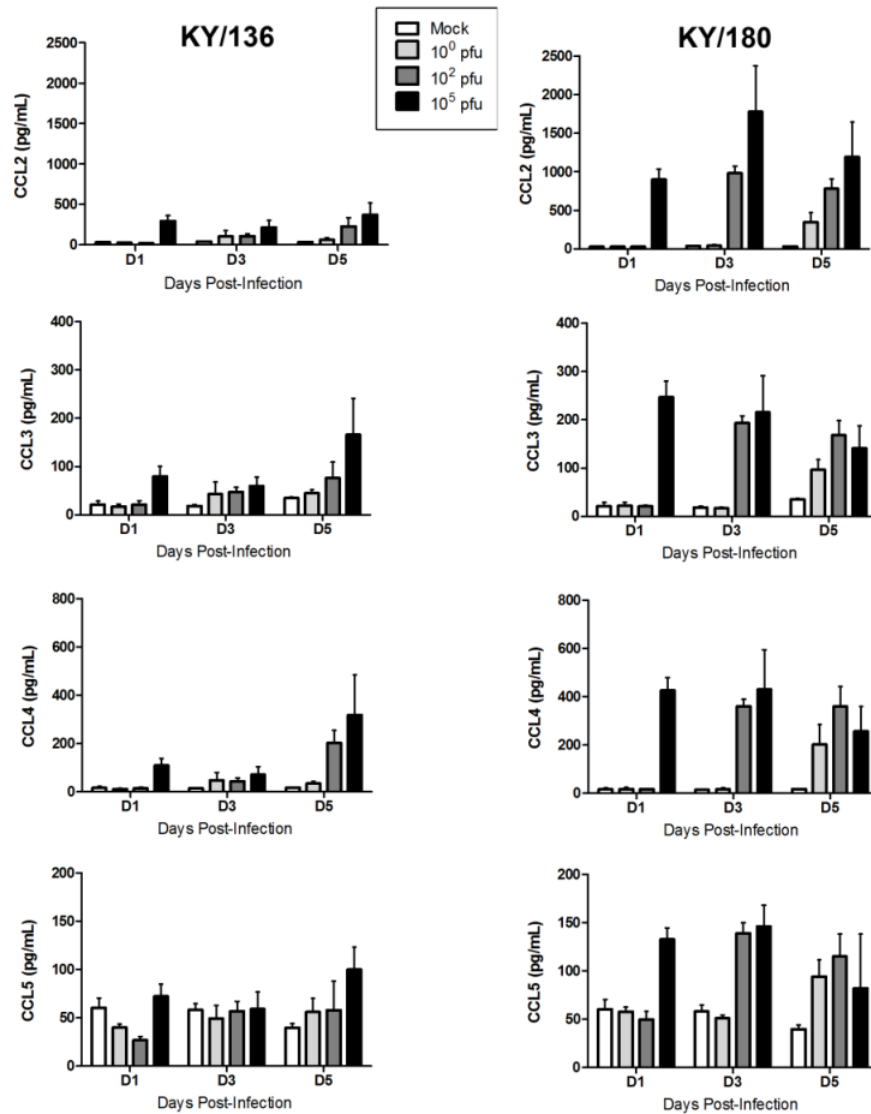


Figure 2.S3 Dose response of chemokines in the lungs of mice infected with KY/136 or KY/180 influenza A (H1N1) virus isolates.

Mice were infected with 10^0 , 10^2 , or 10^5 pfu of virus and samples were collected upon euthanasia on days 1, 3, or 5 post-challenge (D1, D3, and D5, respectively). $n = 5$ mice per dose-day for all groups except for mice infected with 10^5 pfu of KY/180 where only 2/5 mice survived to D5.

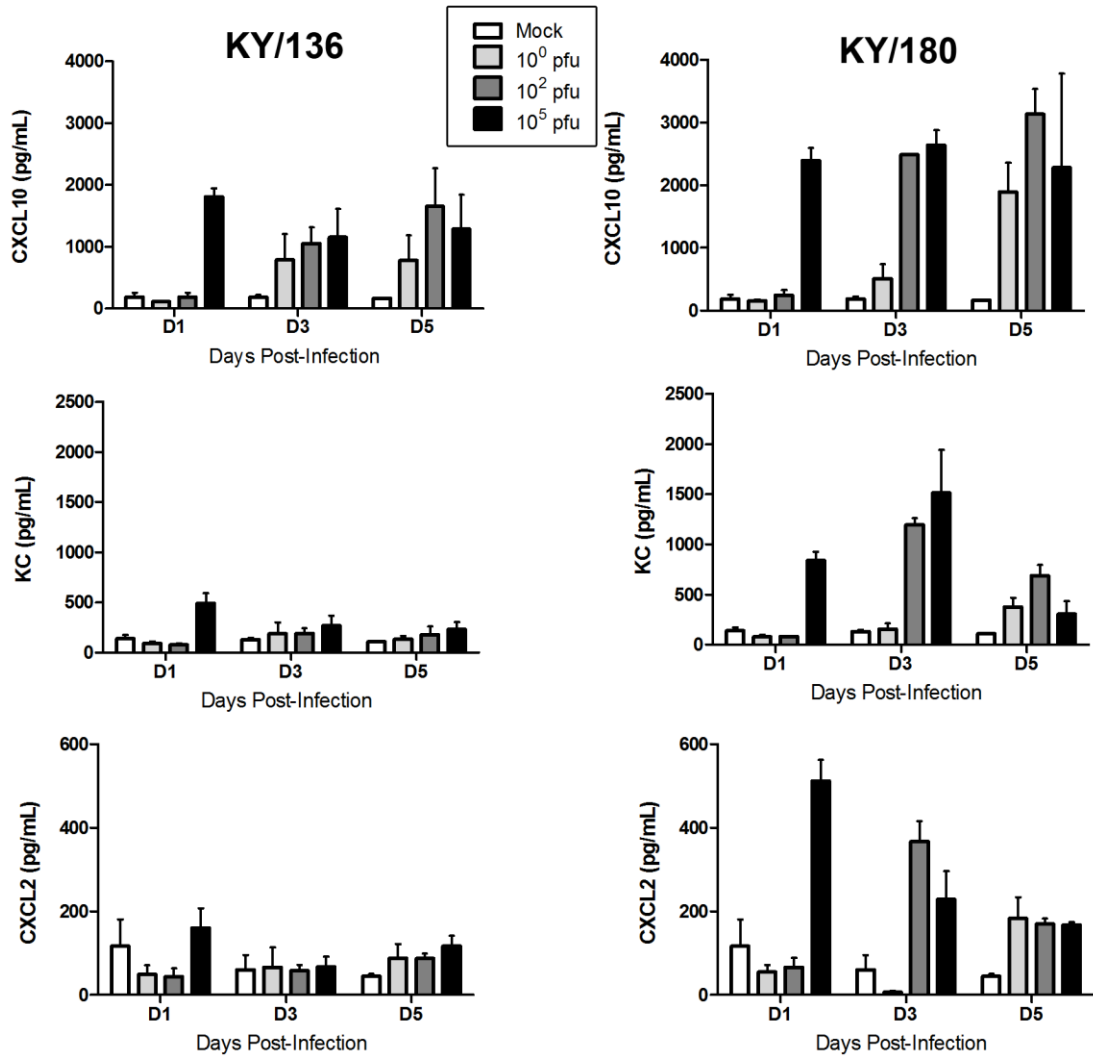


Figure 2.S4 Dose response of chemokines in the lungs of mice infected with KY/136 or KY/180 influenza A (H1N1) virus isolates.

Mice were infected with 10⁰, 10², or 10⁵ pfu of virus and samples were collected upon euthanasia on days 1, 3, or 5 post-challenge (D1, D3, and D5, respectively). *n* = 5 mice per dose-day for all groups except for mice infected with 10⁵ pfu of KY/180 where only 2/5 mice survived to D5.

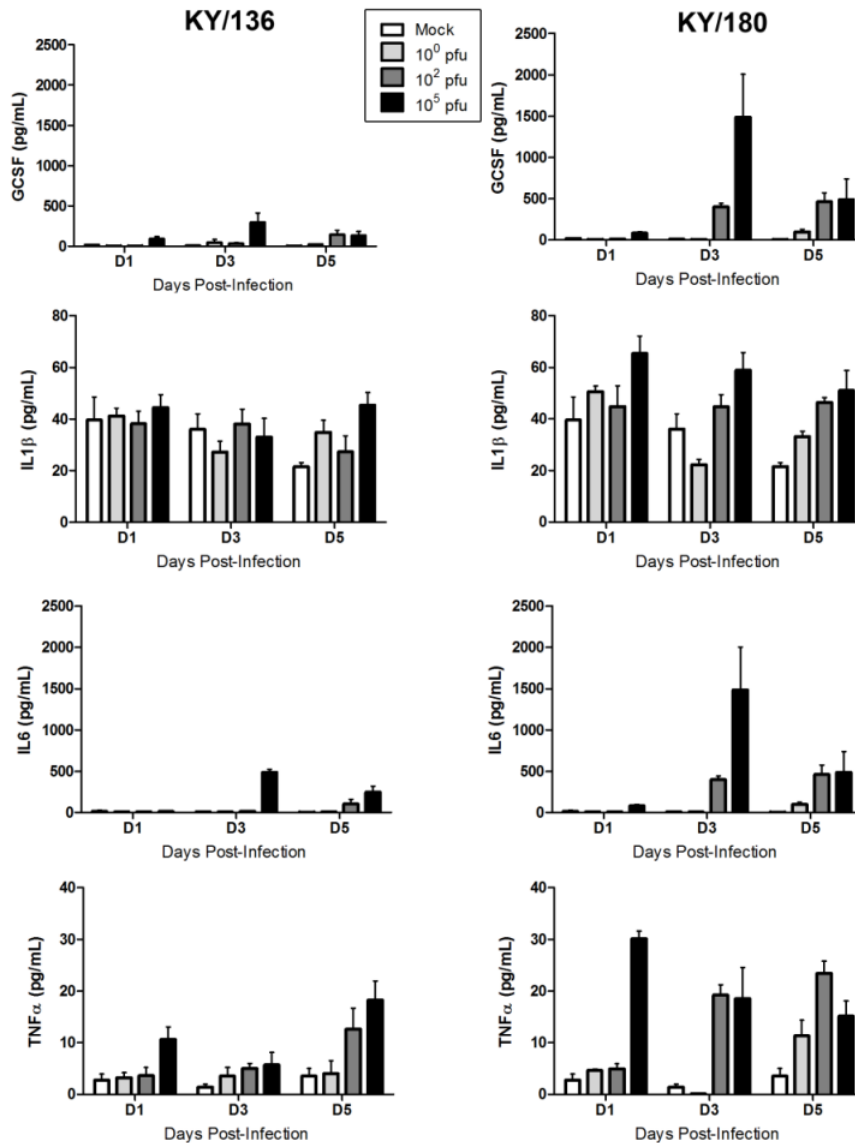


Figure 2.S5 Dose response of cytokines in the lungs of mice infected with KY/136 or KY/180 influenza A (H1N1) virus isolates.

Mice were infected with 10^0 , 10^2 , or 10^5 pfu of virus and samples were collected upon euthanasia on days 1, 3, or 5 post-challenge (D1, D3, and D5, respectively). $n = 5$ mice per dose-day for all groups except for mice infected with 105 pfu of KY/180 where only 2/5 mice survived to D5.

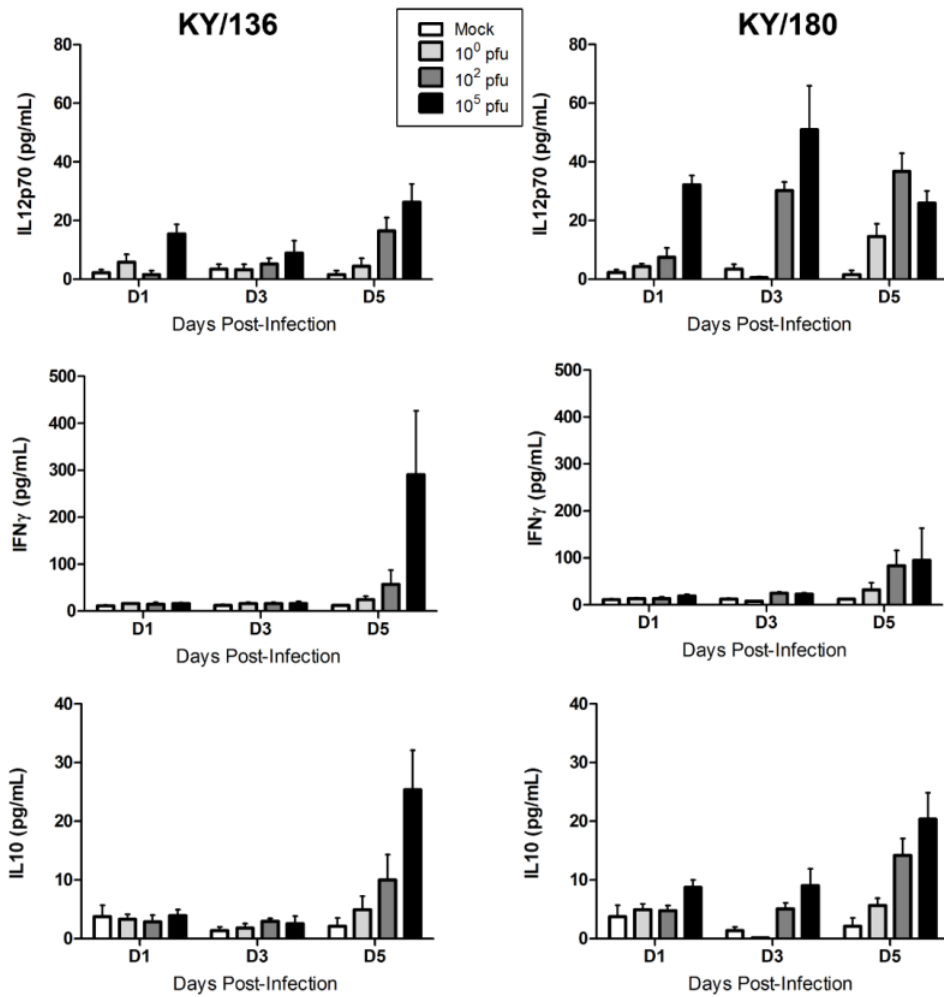


Figure 2.S6 Dose response of cytokines in the lungs of mice infected with KY/136 or KY/180 influenza A (H1N1) virus isolates.

Mice were infected with 10⁰, 10², or 10⁵ pfu of virus and samples were collected upon euthanasia on days 1, 3, or 5 post-challenge (D1, D3, and D5, respectively). n = 5 mice per dose-day for all groups except for mice infected with 10⁵ pfu of KY/180 where only 2/5 mice survived to D5.

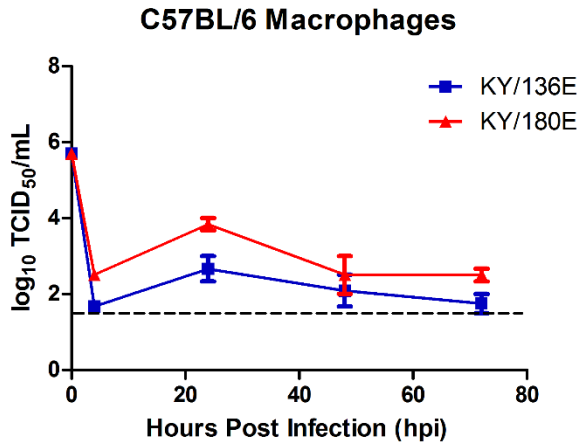


Figure 2.S7 Replication kinetics of KY/180E and KY/180E isolates in C57BL/6 mouse macrophages.

The macrophage cell line, BEIR #NR-9465, was infected at 1.0 MOI (two independent experiments at n=3 per experiment) and clarified supernatants were taken at 4, 24, 48, and 72 hour post-infection. Virus titers were measured by TCID50 assay on MDCK cells.

Table 2.1 General patient data for nasal swabs used for virus isolation.

Sample ID/ Locality Code*	Patient Age/Sex	Hospital Admission Date	Nasal Swab Sample Date	LOS [‡] (Days)	Comorbidity	Mortality
80/3	31/F	9/30/2009	9/30/2009	10	COPD, MRSA, BMI=123.6	Died
96/2	51/M	10/24/2009	10/26/2009	4	ND	Survived
99/3	35/F	10/28/2009	10/29/2009	3	ND	Survived
104/2	58/M	10/30/2009	11/4/2009	2	ND	Died
108/6	57/F	11/2/2009	11/3/2009	19	COPD, Diabetes Renal Disease, BMI= 79.7	Survived
110/6	54/F	11/3/2009	11/4/2009	3	Diabetes	Survived
136/3	46/M	12/10/2009	12/11/2009	8	Diabetes, MRSA	Survived
180/2	53/M	3/24/2010	4/1/2010	19	COPD, Renal Disease	Died
190/2	55/F	4/10/2010	4/15/2010	12	ND	Died

*locality, 2=western Kentucky (KY); 3=eastern KY; 6=Louisville metro; [‡]LOS, Length of stay in hospital; MRSA, methicillin-resistant *Staphylococcus aureus*; COPD, chronic obstructive pulmonary disease; BMI- body mass index; ND, none determined.

Table 2.2 Summary of viral titers, lethality of H1N1pdm isolates in DBA2 mice.

Inoculum	DP I	Virus titer (Lung)	Lethality
Mock	3	<1.5 *	0.0
	6	<1.5 *	0.0
KY/80M	3	7.2 ± 0.5	0.0
	6	6.4 ± 0.9	33.3
KY/80E	3	7.6 ± 0.1	0.0
	6	7.0 ± 0.3	33.3
KY/96M	3	7.0 ± 0.5	0.0
	6	7.2 ± 0.5	0.0
KY/96E	3	7.3 ± 0.4	0.0
	6	7.4 ± 0.4	100.0
KY/99M	3	6.4 ± 0.2	0.0
	6	5.8 ± 0.7	0.0
KY/99E	3	7.4 ± 0.9	0.0
	6	7.3 ± 0.3	33.3
KY/104M	3	6.6 ± 0.9	0.0
	6	6.9 ± 0.5	33.3
KY/108M	3	7.5 ± 0.6	0.0
	6	6.6 ± 0.4	0.0
KY/108E	3	6.5 ± 0.5	0.0
	6	6.4 ± 0.8	33.3
KY/110M	3	5.9 ± 0.4	0.0
	6	6.2 ± 0.9	0.0
KY/110E	3	5.9 ± 0.2	0.0
	6	6.8 ± 0.7	0.0
KY/136M	3	5.3 ± 0.3	0.0
	6	5.8 ± 0.9	0.0
KY/136E	3	5.8 ± 0.4	0.0
	6	5.2 ± 0.5	0.0
KY/180M	3	6.8 ± 0.3	0.0
	6	5.7 ± 0.7	100.0
KY/180E	3	6.9 ± 0.4	33.3
	6	6.4 ± 0.1	66.7
KY/190M	3	6.8 ± 0.5	0.0
	6	6.3 ± 0.3	100.0
NY/18E	3	6.9 ± 0.6	0.0
	6	6.8 ± 1.0	33.3
CA/07E	3	7.2 ± 0.6	0.0
	6	6.9 ± 0.8	0.0
BN/59E	3	6.7 ± 0.0	0.0
	6	5.9 ± 0.1	0.0

Legend: Virus titer (log₁₀ TCID₅₀/mL at a limit of detection = 10^{1.5} TCID₅₀ / mL)

Table 2.S1 Comorbidities associated with severe, hospitalized influenza pneumonia patients.

ID	Gender	BMI	Tamiflu Therapy	PSI	PSI Class	COPD	Diab	Cancer	Other Infect	Invasive Vent Fail
80	Female	123.6	Tamiflu 75 mg Q12H x 7 days	61	II	yes	no	no	MRSA	yes
96	Male	21.9	No	91	IV	no	no	no	None	yes
99	Female	76.90	No-Flu vaccine only	54	II	no	no	no	None	0
104	Male	26.63	Tamiflu 75mg x2 doses	123	IV	no	yes	no	<i>Proteu spp.</i>	yes
108	Female	79.71	Tamiflu 75 mg daily x 5 days	106	IV	yes	yes	no	None	no
110	Female	21.40	Tamiflu 75 mg daily x 5 days	73	III	no	yes	no	None	no
136	Male	24.4	Tamiflu 150mg daily x 5 days	76	III	no	yes	yes	MRSA	yes
180	Male	49.4	Tamiflu	93	IV	yes	no	no	None	yes
190	Female	56.4	Tamiflu	75	III	yes	yes	no	none	no

Legend: BMI, body mass index; PSI, pneumonia severity index; COPD, Chronic obstructive pulmonary syndrome; MRSA, Methicillin-resistant Staphylococcus aureus; Inf, infection; vent, ventilation failure

Table 2.S2 Variants noted in amino acid sequence alignments of H1N1pdm clinical isolates.

	M1		HA1													
A.A.	30	39	83	83	84	97	116	121	137	147	183	186	199	200	203	
A.A.	S	G	S	S	S	D	I	S	P	N	S	A	V	F	T	
Change	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	
	G	E	P	F	N	N	M	G	T	S	P	T	A	L	S	
CA07	A	G	C	C	G	G	A	A	C	A	T	G	T	T	T	
NY/18	A	G	T	C	G	G	A	A	C	A	T	G	T	T	A	
NL/602	A	G	T	C	G	G	A	A	C	A	T	G	T	T	T	
KY/80	A	G	T	C	G	G	A	G	C	A	T	G	T	T	A	
KY/96	A	G	T	C	G	G	A	A	C	A	T	G	T	T	T	
KY/99	A	G	T	C	G	G	G	A	C	A	T	G	C	T	A	
KY/104	A	G	T	C	G	A	A	A	C	A	T	G	T	T	A	
KY/110	A	A	T	C	G	G	A	A	A	A	T	G	T	T	A	
KY/136E	A	G	T	T	G	G	A	A	C	A	T	G	T	T	A	
KY/180E	G	G	C	C	A	G	A	A	C	A	C	A	T	T	A	
KY/180M	NA	G	C	C	A	G	A	A	C	G	C	A	T	T	A	
KY/190	NA	G	C	C	A	G	A	A	C	G	T	G	T	C	A	
	HA		HA2						PA							
A.A.	222	223	234	293	321	374	440	499	14	387	465	471	538	581	610	
A.A.	D	Q	V	Q	V	E	S	E	V	V	I	N	E	M	E	
Change	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	
	G	R	I	H	I	K	L	K	I	I	T	S	K	L	D	
CA07	A	A	G	G	A	G	C	G	G	G	T	A	G	C	A	
NY/18	A	R	G	G	G	G	C	G	G	G	T	A	G	A	A	
NL/602	A	A	G	G	G	G	C	G	G	G	T	A	G	C	A	
KY/80	A	A	G	G	A	G	C	G	G	G	T	A	G	A	A	
KY/96	A	A	G	T	G	G	C	A	G	A	T	A	G	A	A	
KY/99	A	A	G	G	G	G	C	G	G	G	T	G	G	A	A	
KY/104	A	A	G	G	G	A	C	G	G	G	T	A	A	A	A	
KY/110	A	A	G	G	G	G	C	G	NA	NA	NA	NA	NA	A	NA	
KY/136E	A	A	A	G	G	G	C	G	G	G	C	A	G	A	A	
KY/180E	G	A	G	G	G	A	C	G	A	G	T	A	G	A	T	
KY/180M	G	A	G	G	G	A	C	G	NA	NA	NA	NA	NA	NA	NA	
KY/190	A	A	G	G	G	A	T	G	NA	NA	NA	NA	NA	NA	NA	
	PA			PB1					PB2							
A.A.	647	654	716	14	196	215	83	563	662	736	33	176	183	340	584	
A.A.	N	Q	K	V	E	P	A	R	T	K	K	I	L	K	V	
Change	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→ I	
	D	E	Q	I	G	Q	G	K	N	G	R	T	M	N		
CA07	A	C	A	G	G	C	C	G	C	A	A	T	C	A	G	
NY/18	A	C	A	G	A	C	C	G	C	A	A	T	C	A	G	
NL/602	A	C	A	G	A	C	C	G	C	A	A	T	C	A	G	
KY/80	A	C	A	G	A	C	C	G	C	A	A	T	C	A	G	
KY/96	A	C	A	G	A	A	C	G	C	A	G	T	A	A	G	
KY/99	G	S	A	G	A	C	C	G	C	A	A	T	C	A	G	
KY/104	A	C	A	G	A	C	C	A	C	A	A	T	C	A	G	
KY/110	NA	NA	NA	G	A	C	C	G	A	A	A	C	C	A	G	
KY/136E	A	C	A	G	A	C	G	G	C	A	A	T	C	T	G	
KY/180E	A	C	C	A	A	C	C	G	C	G	A	T	C	A	A	

Table.s.S2 cont'd

	NA								NP			NS1		
A.A.	79	106	108	150	220	248	396	407	100	181	373	112	123	154
A.A.	S	I	I	K	R	D	I	V	I	A	T	I	V	G
Change	→	→	→	→	→	→	→	→	→	→	→	→	→	→
	P	V	V	R	K	N	K	I	V	D	I	M	I	R
CA07	T	G	A	A	G	A	T	G	G	C	C	A	G	G
NY/18	T	A	A	A	G	G	T	G	A	C	C	A	G	G
NL/602	T	A	G	A	G	A	T	A	G	C	T	A	G	G
KY/80	T	A	A	A	G	G	T	G	A	C	C	A	G	G
KY/96	C	A	A	A	G	G	A	G	A	C	C	A	A	G
KY/99	T	A	A	A	G	G	T	G	A	C	C	A	G	G
KY/104	T	A	A	A	G	G	T	G	A	C	C	A	G	G
KY/110	T	A	A	A	G	G	T	G	A	C	C	A	G	A
KY/136E	T	A	A	G	G	G	T	G	A	A	C	G	G	G
KY/180E	T	A	A	A	A	G	T	G	A	C	C	A	G	G

*NA = not yet sequenced; for KY/190 only the HA gene has been sequenced.

Table 2.S3 GenBank accession numbers of H1N1pdm isolates.

10Virus ID	HA	M	NA	NP	NS	PA	PB1	PB2
A/Kentucky/190/10/E	JX875013	*	*	*	*	*	*	*
A/Kentucky/180/10/E	CY099332	JX875027	CY099333	JX875032	JX875031	JX875030	JX875028	JX875029
A/Kentucky/180/10/M	JX875056	*	*	*	*	*	*	*
A/Kentucky/136/10/E	CY099330	JX87521	CY099331	JX875026	JX875025	JX875024	JX875022	JX875023
A/Kentucky/110/09/E	JX875054	JX875049	JX875052	JX875055	JX875053	*	JX875050	JX875051
A/Kentucky/104/09/E	JX875047	JX875041	JX875044	JX875048	JX875046	JX875045	JX875042	JX875043
A/Kentucky/99/09/E	JX875019	JX875014	JX875016	JX875020	JX875018	JX875017	JX875015	*
A/Kentucky/96/09/E	JX875039	JX875033	JX875036	JX875040	JX875038	JX875037	JX875034	JX875035
A/Kentucky/80/09/E	JX875011	JX875005	JX875008	JX875012	JX875010	JX875009	JX875006	JX875007
A/Netherlands/602/09	CY039527	CY046944	CY039528	CY046943	CY046945	CY046942	CY046941	CY046940
A/NewYork/18/09	GQ232064	FJ984348	FJ984350	FJ984352	GQ232063	FJ984354	FJ984353	FJ984351
A/California/07/09	FJ969540	FJ966975	FJ984386	FJ969536	FJ969528	FJ966977	FJ969531	FJ984387

* not yet sequenced

Table 2.S4 Virus titers (TCID₅₀/ml*) on 1, 3, and 5 days post-infection in lungs and nasal turbinates of DBA/2 mice infected with KY/180E and KY/136E.

	Dose	Lung			NT		
		1 DPI	3 DPI	5 DPI	1 DPI	3 DPI	5 DPI
KY/136E	10⁰	< 1.5*	2.86 ± 2.15	0.68 ± 1.52	< 1.5	< 1.5	< 1.5
	10²	1.42 ± 1.95	3.78 ± 2.23	2.50 ± 0.00	< 1.5	< 1.5	1.94 ± 1.89
	10⁵	5.04 ± 0.86	3.64 ± 2.17	< 1.5	< 1.5	3.64 ± 0.76	2.92 ± 0.29
KY/180E	10⁰	0.50 ± 1.12	2.76 ± 2.61	4.40 ± 2.47	< 1.5	< 1.5	< 1.5
	10²	4.86 ± 1.77	6.54 ± 0.36	6.42 ± 0.44	0.50 ± 1.12	5.22 ± 0.54	5.32 ± 0.75
	10⁵	7.54 ± 0.34	6.18 ± 0.46	6.38 ± 0.30	5.40 ± 0.26	4.80 ± 0.45	4.96 ± 0.36

Legend: Dose = pfu/mouse. Values in mean log₁₀ TCID₅₀/mL ± S.D. (*n* = 5 mice per dose-day); * Limit of detection = 10^{1.5} TCID₅₀ / mL.

Table 2.S5 Relative magnitude of immune responses of H1N1pdm isolates in mice in groups revealed by principal component analysis clustering.

Cytokine/ Chemokine	Function	DPI	Group 1	Group 2	Group 3	Group 4
IL-6	Inflammation	3	+	+++	+	+
		6	+	++++	+++	+
TNF α	Inflammation	3	+	+++	+++	++
		6	+	+++	++++	+
KC	PMN attraction	3	+	+++	++	++
		6	+	+++	++	+
G-CSF	PMN proliferation	3	+	+++	++	+++
		6	+	++	++	++
CXCL9 (MIG)	T-cell chemoattractant	3	+++	++++	++	+++
		6	++++	+++	++	+
CCL2 (MCP-1)	Monocyte chemoattractant	3	+	+++	++++	++
		6	++	+++	++++	++
IL-1 β	Inflammation	3	+	++	++	+
		6	++	++	+++	+
CXCL3 (MIP1 α)	PMN attraction	3	+	++	+++	+
		6	++	+++	++++	+
CXCL10 (IP10)	Chemoattractant	3	++	-	++++	++
		6	+++	+	++++	+
IFN γ	Adaptive immunity	3	-	-	+	-
		6	+	++	+++	-
IL-10	Anti- inflammation	3	-	-	+	-
		6	+	++	+++	-

Legend: Group 1, BN/59, CA/07, KY/80, KY/136; Group 2, KY/96, KY/99, KY/104, KY/108; Group 3, KY/108, KY/110; Group 4, KY/180, KY/190.

Table 2.S6 Summary of references to mutations in influenza A (H1N1) isolates with observed virulence.

Gene product	Mutation	Source	Citation
HA1	S83P	mouse, <i>in vitro</i>	3, 12
HA1	S183P	mouse	21, 22, 93, 94
HA1	T203S	mouse	44
HA1	D222G	human, mouse, macaque	10, 30, 31, 32, 34, 36, 38, 37, 43, 89
HA1	Q293H	mouse	44
HA1	V321I	mouse	3, 24, 44
HA2	E374K	human	95
NA	I106A	mouse	44
NS1	V123I	mouse	44
PA	V14I	mouse	44
PB2	K340N	human	95

CHAPTER III

MOLECULAR IMAGING REVEALS A PROGRESSIVE PULMONARY INFLAMMATION IN LOWER AIRWAYS IN FERRETS INFECTED WITH 2009 H1N1 PANDEMIC INFLUENZA VIRUS

Overview

Molecular imaging has gained attention as a possible approach for the study of the progression of inflammation and disease dynamics. Herein we used [^{18}F]-2-deoxy-2-fluoro-D-glucose ([^{18}F]-FDG) as a radiotracer for PET imaging coupled with CT (FDG-PET/CT) to gain insight into the spatiotemporal progression of the inflammatory response of ferrets infected with a clinical isolate of a pandemic influenza virus, H1N1 (H1N1pdm). The thoracic regions of mock- and H1N1pdm-infected ferrets were imaged prior to infection and at 1, 2, 3 and 6 days post-infection (DPI). On 1 DPI, FDG-PET/CT imaging revealed areas of consolidation in the right caudal lobe which corresponded with elevated [^{18}F]-FDG uptake (maximum standardized uptake values (SUVMax), 4.7-7.0). By days 2 and 3, consolidation (CT) and inflammation ([^{18}F]-FDG) appeared in the left caudal lobe. By 6 DPI, CT images showed extensive areas of patchy ground-glass opacities (GGO) and consolidations with the largest lesions having high SUVMax (6.0-7.6). Viral shedding and replication were detected in most nasal, throat and rectal swabs and nasal turbinates and lungs on 1, 2 and 3 DPI, but not on day 7, respectively.

In conclusion, molecular imaging of infected ferrets revealed a progressive consolidation on CT with corresponding [^{18}F]-FDG uptake. Strong positive correlations were measured between SUVMax and bronchiolitis-related pathologic scoring (Spearman's $\rho = 0.75$). Importantly, the extensive areas of patchy GGO and consolidation seen on CT in the ferret model at 6 DPI are similar to that reported for human H1N1pdm infections. In summary, these first molecular imaging studies of lower respiratory infection with H1N1pdm show that FDG-PET can give insight into the spatiotemporal progression of the inflammation in real-time.

Introduction

In March of 2009, an outbreak of a novel variant of H1N1 influenza A virus was reported in cases of influenza illness in Mexico (35). By June 11, the World Health Organization raised the pandemic alert level to its highest level, declaring the first influenza pandemic in over 40 years (35). Unlike seasonal influenza viruses, this novel H1N1 pandemic strain (H1N1pdm) tended to affect younger healthier populations and had an increased risk of morbidity and mortality (37, 39, 40) with 12-30% of the population developing clinical influenza, 4% of those requiring hospital admission, and 1 in 5 requiring critical care (599). In general, however, infection of the H1N1pdm was relatively mild in most persons, although a fatal viral pneumonia with acute respiratory distress syndrome occurred in approximately 18,000 cases.

In contrast to seasonal influenza in human cases, H1N1pdm infections showed a tropism for the lung similar to H5N1 (30). The ability of H1N1pdm viruses to infect the lower

respiratory track has been attributed to a broader specificity in the binding of the viral hemagglutinin (HA) to α 2-3- in addition to α 2-6-linked sialic acid (SA) receptors (284, 336). It is reasonable that the lung tropism of the H1N1pdm contributed to the severity of disease in those individuals with preexisting complications such as asthma and chronic obstructive pulmonary disease (COPD) (6, 30, 544-546). Data from limited human autopsies and animal studies of various pandemic strains also suggest contribution of the host innate immune response and the virus in the progression of disease (46, 394, 600, 601).

Molecular imaging can potentially play a strong role in basic infectious disease research and clinical response by providing a noninvasive, spatiotemporal measurement of viral infection and host inflammation (602, 603). To explore the potential utility of molecular imaging in influenza infection, we chose the ferret (*Mustela putorius furo*) model. Ferrets have been used as an animal model of influenza infection and pathogenesis since 1934, when they were reported to develop an acute respiratory tract illness when exposed to influenza viruses from humans and swine (149). In contrast to mice, the ferret can be infected by human isolates without adaptation and display signs and symptoms of infection such as sneezing and nasal secretions that are similar to what is seen in humans (43, 47, 49, 256). The ferret is an attractive model for imaging influenza pulmonary infections given the ferret's long trachea, large lung capacity, and bronchiolar branching. These anatomical features can potentially bridge imaging with histopathologic evaluation. Finally, the ferret more closely mimics humans in distribution of sialic acid

(SA) receptors in the respiratory tract with higher α -2-6 SA in the upper respiratory tract and α 2-3 SAs- in the lower (228).

Historically, plain film (x-ray) radiography and computed tomography (CT) have been useful for clinical assessments of influenza disease severity in clinical cases (41, 604). These imaging modalities are limited by characterizing only anatomic changes in the lung parenchyma, such as ground-glass opacity (GGO) and consolidations, which represent different degrees of interstitial and alveolar filling by cells, edema, and inflammatory exudate (605). In contrast, positron emission tomography (PET) imaging with the radiotracer [^{18}F]-2-fluoro-2-deoxy-D-glucose ([^{18}F]-FDG) can provide data on metabolic activity of cells by measuring sites of increased glycolysis from leukocyte chemotaxis and accumulation, and provide increased sensitivity in detection of cells during inflammation. [^{18}F]-FDG, an analog of glucose that is moved into cells via facilitated transport, is commonly used in PET imaging as a radiotracer in clinical and basic science research. Recent studies have demonstrated the utility of [^{18}F]-FDG in assessment of infectious disease burden in animal models of schistosomiasis and tuberculosis (606-608). PET/CT has been used in the assessment of one hospitalized H1N1pdm patient and revealed an intense inflammatory response (609). The uptake of [^{18}F]-FDG in humans and animals suggests the predominant presence of activated neutrophils (610-613). In studies of mice infected with influenza A virus, neutrophils play a critical role in protection and recovery from infection, and participate in the process of adaptive immunity to the virus (78). Coupled with molecular virology and pathology, molecular imaging with important probes of disease has enormous potential to reveal early critical

factors that contribute to the clinical progression of illness as well as accelerate screening, the efficacy and mechanistic studies of vaccines and antiviral therapies (602, 603).

To test the hypothesis that FDG-PET/CT imaging could reveal the spatiotemporal nature of H1N1pdm inflammation and disease progression, we chose the ferret model of H1N1pdm influenza infection. Further, for these studies we chose a low passage clinical isolate, A/Kentucky/180/2010 or KY/180, herein, which has a change in the HA1 gene, D222G, which correlates with increased severity of disease in patient cases from several countries (558, 559, 562, 563). In mice, infection with H1N1pdm engineered to include this change show increased viral titers and pathology, however, in ferrets there do not seem to be any major differences in clinical signs, transmissibility or pathogenicity (125, 614). Our results show for the first time, the spatiotemporal progression of inflammation with CT and PET using [¹⁸F]-FDG in ferrets infected with H1N1pdm in conjunction with histopathology and viral titers over a seven day period. Importantly, the extensive areas of patchy GGO and consolidation seen in the ferret model at 6 days post-infection (DPI) are similar to that reported for human H1N1pdm infections (609). In vivo imaging with these modalities for anatomic (CT) and molecular (PET) data suggests increased pulmonary inflammation as the amount of circulating virus becomes undetectable. These results suggest that molecular imaging will be a great asset in gaining insight into the temporal and spatial progression of the inflammatory process caused by influenza virus infection.

Results

Characterization of KY/180 in female ferrets

Due to the size of the Siemens Trimodal gantry for PET/CT imaging we chose four month old females rather than male ferrets. The pandemic H1N1 isolate KY/180 employed in these studies was isolated from nasal swab sample provided by the Severe Influenza Pneumonia Surveillance project, a clinical study of hospitalized patients with influenza pneumonia in Kentucky. The patient had a severe course of influenza disease and died after 19 days. Sequencing of the HA1 gene from this isolate revealed the D222G mutation, which has been associated with severe disease in human cases (558, 559, 562, 563). The second passage of the KY/180 seed was employed in the characterization of infection in the female ferrets. The 50% infectious dose in female ferrets was determined to be $10^{0.07}$ TCID₅₀ (data not shown). In group 1, six ferrets were mock infected with PBS. In group 2, six ferrets were infected intranasally (i.n.) with KY/180 with a 0.5 ml dose of $0.5 \times 10^{5.7}$ TCID₅₀ per naris. Ferrets were monitored for temperature for 10 DPI, and for body weight and clinical symptoms for 28 DPI. Two animals in each group were euthanized on days 2, 14 and 18 to determine virus and HI titers in blood, lung and several additional organs at 2 DPI.

In figure 3.1A, the body weight changes are shown for mock- and KY/180-infected ferrets. Body weight showed a drop on day 2 where the weight remained for the remainder of the study. Figure 3.1B shows the average temperatures of the mock- and KY/180-infected ferrets for the first 10 DPI. Temperature peaked on days 1 and 5 for KY/180 infected animals with a mean temperature of 103.4°F (SD=1.71°F) and 103.2°F

(SD=0.52 °F), respectively. The average hemagglutination-inhibiting (HI) serum antibody titer in the blood on day 14 was 540 reciprocal dilution and the average total serum influenza-specific IgG by ELISA was 7610 reciprocal dilution (Fig. 3.1C). In studies to determine the infectious dose, additional tissues were taken from animals infected at with KY/180 with a 0.5 ml dose of $0.5 \times 10^{4.7}$ TCID₅₀ per naris. On day 2, viral titers were the highest in the nasal turbinates ($10^{6.25}$ TCID₅₀/g) followed by the caudal lung ($10^{6.0}$ TCID₅₀/g) and trachea ($10^{3.75}$ TCID₅₀/g). The lowest levels of virus were observed in the cranial lobe of the lung ($10^{3.2}$ TCID₅₀/g). Viral titers were measured by TCID₅₀ in nasal turbinates, trachea, right cranial lobe of the lung, right caudal lobe, brain, liver, spleen, kidney, duodenum, jejunum, ileum, colon and rectum. KY/180 was detected in jejunum in one animal ($10^{2.0}$ TCID₅₀/g), but was not detected in any other tissues (data not shown).

FDG-PET/CT imaging of the H1N1pdm-infected ferrets show progressive inflammation

Female Fitch ferrets were divided into five groups, with two per group of animals that were mock-infected with PBS (group 1) or intranasally infected with KY/180 (groups 2-5), in a 0.5 ml dose of $0.5 \times 10^{6.0}$ TCID₅₀ per naris, on day 0 (Table 1). Group 5 was the only group that was imaged each day; while groups 2-4 were imaged and sacrificed on days 1, 2 and 3 post-infection. This study design permitted evaluation of the progression of imaging with infection and pathology in two animals each day as well as continuous imaging of the lungs in one cohort over the seven-day time-period.

FDG-PET and CT images of the H1N1pdm-infected and mock-infected ferrets were successfully obtained and fused for two ferrets on days 1, 2, 3 and 6 (Fig. 3.2 and 3.3). Volumes of interest (VOI) and corresponding maximum standardized uptake values (SUVMax) were generated for any metabolically active lesions in the lung as well as background activity in the lungs, liver, heart, thymus, and thoracic lymph nodes. Baseline imaging prior to infection showed no focal areas of lung consolidation on CT and background standard uptake values (SUVMax) of the [¹⁸F]-FDG levels ranged from 0.7-1.0 for PET (Fig. 3.2A and Fig. 3.3A). Each figure shows the one two-dimensional coronal plane that were standardized across days to provide a similar orientation and do not necessarily represent the SUVMax as that plane may be out of view.

By 1 DPI, an area of consolidation was identified on CT in the right caudal lobe with corresponding radiotracer uptake on PET (Fig. 3.2B, SUVMax of 4.7). Consolidative areas in the right caudal lobe increased by day 2, with a persistently elevated SUVMax of 3.1 (data not shown). By day 3, the consolidation increased in the right caudal lobe (Fig. 3.2C and Fig. 3.3C, SUVMax of 3.7 and 4.4, respectively) and also appeared in the left caudal lobe (SUVMax of 3.2) of ferret 2214 (Fig. 3.3C). By 6 DPI, there were widespread areas of patchy consolidation on CT with multiple areas of increased radiotracer uptake in both ferrets in caudal and cranial lobes (Fig. 3.2D and Fig. 3.3D, SUVMax of 6.0 and 7.6 on the right, 4.2 and 4.6 on the left, respectively). These results suggest that inflammation progresses into the lower respiratory airways after infection into the upper part of the lower respiratory system. A ferret from the uninfected cohort was also imaged on day 6, with no focal appearance of consolidation on CT and no

evidence of increased [¹⁸F]-FDG uptake on PET (image not shown, background SUV of 0.6).

Viral shedding and replication of the nasal turbinates and lung tissues

To measure viral shedding, each day each ferret was swabbed in the nasal, throat and fecal passages and the viral titer was measured by TCID₅₀ (Table 2). The highest levels of viral shedding were measured in the throat swabs. Nasal swabs also showed viral shedding for most animals, while the presence of virus in rectal swabs was low although detectable in a few animals.

Replication of H1N1pdm in nasal turbinates and lungs were determined post-mortem from the right caudal lobe of the lung taken on euthanasia (Table 3.3). Four sections were taken per lobe to provide greater insight into the spread of the virus in the tissue (Fig. 3.4). High levels of virus were detected in all nasal turbinate samples at 1, 2, and 3 DPI (95% C.I. = 5.43 +/- 1.00 TCID₅₀/mL). Virus was also detected in a majority of lung sections from 1, 2, and 3 DPI. It was absent in the lung sections from one animal on 2 DPI, although it was present in the ferret's nasal turbinates, suggesting that the timing of infection in this animal was slower than the others. Of note, this same animal had a focus of consolidation on CT and radiotracer uptake on PET. This observation also suggests that, while the virus may have been undetectable by TCID₅₀, low levels of virus had entered the lower respiratory system. No animals on day 7 post-infection had detectable virus in the nasal turbinates or the sampled lung tissue. Virus was not detected in nasal, throat and fecal passages or the sampled lung tissue from ferrets in any of the controls.

Histopathology of H1N1pdm-infected and mock-infected ferrets

Upon necropsy, all but the right caudal lobe of the ferret lung was fixed with paraformaldehyde. Following fixation, sections were taken for histopathology from the right and left cranial lobes, left caudal lobe and the middle accessory lobe. Representative photographs from slides of the left caudal lobe are shown in figure 3.5. The ferrets in the control group had intact bronchiolar walls with very minimal infiltration by neutrophils with the exception of the left caudal lobe from control animal 2206 sacrificed on Day 1. Possible causes of this pattern of change may be an underlying systemic vasculopathy which is typically confirmed by evaluation of other organs that were not collected (e.g., kidney, spleen, liver).

In general pulmonary lesions associated with influenza infection were roughly comparable at Days 1 and 2 and consisted of variable suppurative or necrosuppurative bronchiolitis and mixed cell alveolitis at minimal to moderate severity levels. By 1 DPI, there were some small foci of inflammation without much infiltration of the bronchi or bronchioles. There was an increased severity of inflammatory findings in lung lobes from infected ferrets on day 3. Specifically, more extensive infiltration of neutrophils can be seen within the bronchiolar lumen, along with necrosuppurative bronchiolitis and mixed cell alveolitis. At Day 7, lesions observed in the lung lobes continued to exhibit an increased severity compared to the majority of lung lesions seen at 1 and 2 DPI. Bronchiolar epithelial hyperplasia and cytokaryomegaly were noted in addition to bronchiolitis.

FDG-PET/CT and histopathology show positive correlation during infection

To evaluate potential correlations between PET/CT with histopathology, the SUVMax of lesions in the right and left lung of each ferret were compared with the cumulative histopathology scores assigned by the veterinary pathologist (Fig. 3.6). On average, the SUVMax was higher in the right lung than the left lung but the slopes and Spearman's correlation coefficients (ρ) were similar between the two sides. The highest correlation was seen between the cumulative bronchiolitis score and SUVMax (ρ of 0.71 and 0.75 on the right and left, respectively). The next highest was between the cumulative bronchitis score and SUVMax (ρ of 0.69 on the right and 0.67 on the left). A weaker positive correlation was seen between the cumulative alveolitis score and SUVMax (ρ of 0.47 on the right and 0.57 on the left).

Discussion

Herein, we show for the first time the feasibility of utilizing [^{18}F]-FDG PET coupled with CT imaging of H1N1pdm in ferret to track the progression of pulmonary disease in real-time. We chose a low passage clinical isolate, KY/180, which has a change in the HA1 gene, D222G. The D222G change in H1N1pdm correlates with increased severity of disease in patient cases from several countries (558, 559, 562, 563). The patient from which we obtained the KY/180 isolate also had a severe course of influenza illness over a period of 19 days that resulted in death. Recently, studies in mice and ferrets infected with pandemic influenza viruses A/California/04/2009 and A/Netherlands/602/2009 engineered with the D222G mutation have shown that the D222G mutation are lethal in

mice, but not ferret (125, 614). The lethality in mice, but not ferrets, has been attributed to the greater abundance of α 2-3-SA in the mouse model (125, 230). All of these viruses have an affinity for α 2,6-SAs associated with attachment to and replication in cells of the upper respiratory tract as shown by the high levels of viral replication in the nasal turbinates. Thus, infection of ferrets with these H1N1pdm isolates engineered with D222G and our clinical isolate have not correlated with clinical findings in patients. These results in ferrets are not surprising given that 80% of the fatal cases of H1N1pdm had underlying medical conditions and bacterial infections (30). Discovery of the molecular components of the host response that may promote pathogenesis will be critical for defining new treatments.

Noninvasive imaging can provide real-time in vivo monitoring of the progression of infection, inflammation and disease that may give insight into the mechanisms that modulate disease progression. Recently, Veldhuis Kroeze *et al.*, presented data on the monitoring of pulmonary lesions of H1N1pdm influenza virus-infected ferrets with CT scanning which correlated with disease progression and severity (615). As those studies demonstrate, CT is a powerful tool, but it will not give the molecular details that can be provided by PET or SPECT imaging of probes that target critical host responses such as neutrophil invasion. In our study we coupled CT scanning with the [18 F]-FDG radiotracer and show infection and inflammation of influenza infection in the lower respiratory system with foci of increased [18 F]-FDG uptake corresponding to areas of lung opacity on CT, with underlying inflammation on necropsy. In comparison to human CT imaging studies of influenza, the molecular images in the ferret show strong similarity. CT

findings in patients with confirmed influenza infection show patchy ground-glass opacities in segmental multifocal distributions, mixed with areas of consolidation in the lung (6, 41, 616). Moreover, the few case reports of human influenza in which lungs were imaged by [¹⁸F]-FDG PET demonstrate areas of high uptake in these ground-glass opacities and consolidation (609). Our study similarly demonstrates this pattern in the ferret model, also showing patchy opacities on CT with high uptake of radiotracer on PET, with necroscopy-based confirmation of inflammation in the left caudal lobe. Specifically, we show the ferret lung demonstrated progressive consolidation on CT and FDG uptake on PET predominantly in the right caudal lobe, which progressed to the left caudal lobe by day 3 p.i.. By day 6, the diffuse metabolically active lesions seen on PET/CT were similar to what has been reported in the human literature during the 2009 H1N1 pandemic (609).

Histopathologic evaluation of the lungs confirmed the progressive nature of the pulmonary lesions and corroborated the radiologic data. Suppurative and necrosuppurative bronchiolitis seen on days 1 and 2 became progressively worse by days 3 and 7 post-infection. The inflammation tended to be patchy or multifocal and an entire lung lobe was never uniformly affected, corresponding to the multiple patchy lesions seen on PET/CT by the end of the study. This also agreed with the analyses of the viral titers in various sections of the right caudal lobe and the PET/CT imaging. Our analyses of viral titers in the four representative sections suggest different levels of infiltration of the lobe. The histopathologic scoring for bronchiolitis correlated the best with the SUVMax of the lesions seen in the right and left lungs on PET. In general, the severity of

infection and inflammation on imaging can be represented by (1) the volume of affected lung (i.e. the percentage of diseased lung relative to total lung capacity), and (2) the extent of parenchymal destruction (disruption of pulmonary architecture) and inflammatory cell migration. Our study first aims to correlate FDG uptake measurements with histology, thereby analyzing the extent of parenchymal destruction and cellular infiltrates.

It should be considered that there can be variation in matching FDG uptake with histologic severity because more severe architectural distortion can lead to necrosis with more dead cells, therefore showing less uptake of radiotracer among metabolically inactive dead cells and nonviable tissue. Our study, however, shows that progressing inflammatory infiltrates on histology in the studied time period after acute influenza infection corresponds to radiologic trends. Additionally, our study demonstrates spatial progression with increased size and number of abnormal foci in the lung parenchyma during acute infection.

Ultimately, utilizing these new imaging tools, we envision a number of future experiments to delineate potential differences in the course of H1N1pdm and H5N1 isolates infection in ferrets. We also plan to explore additional radiotracers that might reveal potential differences in host responses in the immune system and the process of acute injury in the lung. Future studies will assess differences in presentation of those who recover from infection versus those who eventually succumb to infection such as with more lethal isolates such as H5N1. This model should be valuable in rapid

assessment of the effect of various treatments on pulmonary inflammation and damage. Finally, these first PET/CT imaging approaches could be extended to a number of other important pulmonary infections caused by pathogens such as hantaviruses, respiratory syncytial virus, and SARS CoV, to gain further insight into the spatiotemporal *in vivo* dynamics of disease progression (603).

Materials and Methods

Virus and cells

The influenza H1N1pdm virus, A/Kentucky/180/2010, (KY/180; GenBank CY99332 and CY99333) was isolated from the nasal swab of a severe hospitalized case (hospitalized in March 2010) provided by the Severe Influenza Pneumonia Surveillance project, an ongoing clinical study of hospitalized patients with influenza pneumonia in Kentucky (courtesy of Dr. Julio Ramirez). The virus was isolated and passaged in the allantoic cavity of ten-day-old embryonated hens' eggs at 37°C. The allantoic fluid was harvested 72 h after inoculation, pooled and stored in aliquots at -80C until use. The infectious virus titer of the resulting seed stock was determined by TCID₅₀ (50% tissue culture infectious dose) and the titer calculated by Reed and Muench (597) and confirmed by plaque assay on MDCK cells. Passage E2 was used for the studies reported herein.

Ferrets

Ferret studies were approved by the University of Louisville Institutional Animal Care and Use Committee. University of Louisville has Veterinary Medicine tasked to monitor and support all animal experiments. Research was conducted in compliance with the

Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research will be conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

All female Fitch ferrets were obtained from Triple F Farms (Sayre, PA). Ferrets were selected after screening blood samples for the presence of influenza antibodies using a hemagglutination inhibition assay (HI). Ferrets that were seronegative for seasonal and pandemic viruses were shipped directly to the University of Louisville Regional Biocontainment Laboratory and acclimated for seven days prior to initiation of the studies. Animals were fed Teklad Laboratory Diet #2072 (Harlan/Teklad, Madison, WI) and water *ad libitum*.

For the characterization of the progression of infection of the KY/180 clinical isolate we utilized four month old, female ferrets. Prior to infection with virus, ferrets were anesthetized with 0.05 mg/kg atropine, 5.0 mg/kg ketamine, and 0.08 mg/kg dexmedetomidine intramuscularly. Subsequently, six animals were inoculated intranasally (i.n.) with 0.5 mL of infectious virus per naris as a bolus, which was diluted to $10^{5.7}$ TCID₅₀/mL in phosphate buffered saline (PBS). Six additional animals were inoculated by i.n. with 0.5 mL of infectious virus per naris as a bolus with PBS (mock). Anesthesia was reversed with 0.4 mg/kg atipamezole. Ferrets were monitored daily for temperature and clinical symptoms. On day 2 post-infection, two ferrets were taken to measure viral

titers in blood, lung, brain, trachea, nasal turbinates, spleen, kidney, thymus, liver, duodenum, jejunum-ileum, large intestine, and rectum. Gross pathology was defined during necropsy for the lung. At 14 and 28 days post-infection two additional ferrets from each group were analyzed for viral titers and pathology in the lung.

For molecular imaging studies, twelve, four-month-old female Fitch ferrets were utilized. Animals were fed food and water *ad libitum* except 4 h prior to and during CT/PET imaging. On day 0, ferrets were anesthetized prior to inoculation with virus or PBS with ketamine, dexmedetomidine and atropine. Eight animals were inoculated i.n. with 0.5 mL of infectious virus per naris as a bolus, which was diluted to $10^{6.0}$ TCID₅₀/mL in phosphate buffered saline (PBS). Four ferrets were inoculated i.n. with 0.5 mL PBS per naris as a mock-infected control. For imaging, on days 0, 1, 2, 3 and 6, anesthetic induction and maintenance were achieved with 1-3% isoflurane. Blood glucose levels were checked prior to administration of the radiolabeled tracer to ensure that they were within normal limits, which typically range from 62-134 mg/dL in ferrets (617). Glucose was provided to animals to compensate for body fluids lost during imaging. Typically 30 mls of Lactated Ringer's solution (Hospira) was administered subcutaneously (s.q.) following completion of the imaging. Animals were monitored for body temperature and vital signs during imaging.

CT and PET imaging

Imaging was performed on 0, 1, 2, 3, and 6 days post-infection (DPI). Each day, four ferrets were imaged with CT and PET on hardware designed for preclinical animal

studies, including microCT and microPET, respectively. Two ferrets were euthanized the each day and necropsied to obtain tissue samples for virologic and histopathologic analyses (please see study design Table 1). Image acquisition was conducted with a Siemens Inveon Trimodal Scanner (Siemens Preclinical, Knoxville, TN), which is a small animal imaging platform that combines microPET, microCT, and microSPECT modalities within one unit. This combination facilitated co-registration of PET and CT images as the study subject was kept in a uniform position on the scanner bed, minimizing potentially large motion artifacts as a result of repositioning the animal between each scan. The Inveon microCT scanner features a variable-focus tungsten X-ray source with an achievable resolution of 20 μm and a detector with a maximum field of view (FOV) of 8.4 cm x 5.5 cm. The source-to-object distance was 263.24 mm and the source-to-detector distance was 335.67 mm. The Inveon PET detector provided an axial field of view (FOV) of 12.7 cm with a spatial resolution of 1.44 mm. PET images were reconstructed using a 2D-filtered backprojection algorithm with attenuation correction provided by microCT imaging. For the microCT scan, the following imaging settings were used: two bed positions, 80 kVp, 500 μA , 500 ms exposure time, and 4 x 4 binning. After each ferret underwent microCT imaging, the bed position was reset and microPET imaging with ^{18}F -FDG (PETNET, Louisville, KY) began immediately. For each ferret, 2 mCi of ^{18}F -FDG was administered (i.p.) with a 60-90 min uptake period. Radioactive dose was confirmed with an Atomlab 500 Dose Calibrator (Biodex Medical Systems Inc., NY).

Image processing and analyses

All imaging data were processed with PMOD software (v3.1; PMOD Technologies Ltd., Zurich, Switzerland). MicroCT data were received from the Inveon platform as DICOM files and PET data as microPET files. Scans were imported into the program's local SQL database with the units for the PET radiotracer in kBq/cc. PET images were co-registered with the CT images with re-slicing done as necessary to facilitate later calculations. For analysis of 18F-FDG levels, the standardized uptake value (SUV) was used. SUV is a widely used semi-quantitative measure that normalizes radiotracer uptake in a given region of interest based on body weight, and calculated for this study as follows:

$$SUV = \frac{\text{Tissue Concentration} \frac{9kBq}{mL}}{\text{Injected}} \text{Dose (kBq)} * \text{Body Weight (g)} \quad \text{Eq. 1}$$

For all calculations, animal weights were expressed in kilograms and FDG activity in megabecquerels. For each image series, SUVs for each voxel were calculated using an external filter in PMOD, with the radionuclide half-life set at 6586.2 sec for 18F-FDG. For each pulmonary lesion, an ellipsoid volume of interest (VOI) was generated that encompassed the structure. Then, automatic isocontour detection was used to refit the VOI by setting a threshold of 50-60% of the difference between the maximum and minimum intensity SUVs in the ellipsoid VOI such that $0.5 * (SUV_{\max} - SUV_{\min})$. In cases where the automated threshold included contiguous structures in the VOI, manual refitting in conjunction with the co-registered CT scan was used to exclude those surrounding structures. For all VOIs, maximum SUV (SUV_{\max}) and average and standard deviation of all pixels in the volume ($SUV_{\text{Mean}} \pm SD$) were calculated. For CT analysis, image interpretation was performed by a radiologist (in consultation with the scientific team) having more than ten years of diagnostic experience along with

formal certifications by the American Board of Radiology (ABR) and the American Board of Nuclear Medicine (ABNM). Lesions on CT were identified using conventional criteria and terminology; Ground-glass opacity (GGO) is defined in this study as hazy increased lung opacity, with discernible underlying lung architecture such as visible bronchial and vascular structures, representing partial displacement of air in interstitial and alveolar airspaces; Consolidation is defined in this study as high density lung lesions (more dense than GGO) in which vascular and bronchial margins are obscured, representing complete displacement of alveolar air (605).

Viral titers in swabs and tissues

On days 1, 2, 3 and 6 and prior to euthanasia, swabs were taken from each ferret from nasal, throat and rectal regions. Following scheduled euthanasia, the nasal turbinates and the right caudal lobe of the lung from each ferret, which was divided laterally into four segments, were isolated. All swab and tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until analyzed for virus titer by TCID₅₀. Frozen tissues were weighed and diluted 10% weight per volume into cold DMEM with 1% penicillin/streptomycin and 0.2% BSA before being homogenized and centrifuged to remove debris. Tissue homogenate and swabs were serially diluted 10-fold in DMEM with 2 µg/mL TPCK-Trypsin, 0.2% BSA, 4.5 g/L glucose, 1% penicillin/streptomycin, 2 mM L-glutamine, and 25 mM HEPES. Each sample was analyzed in quadruplicate following incubation in 96-well plates with Madin-Darby canine kidney (MDCK) cell monolayers at 37°C in 5% CO₂ for three days. Supernates were collected from each well were assayed for hemagglutination activity using 0.5% turkey red blood cells as an indicator of infection.

Viral titers were expressed as \log_{10} TCID₅₀ / mL and were calculated using the Reed-Muench method (618).

Hemagglutination inhibition assay (HI)

The HI test quantitates serum antibody to influenza virus which can prevent agglutination of turkey RBCs (Fitzgerald Industries International Inc., MA). Heat-inactivated serum samples were treated with receptor-destroying enzyme (Sigma-Aldrich) for removing nonspecific inhibitors (followed by RBC adsorption) and were diluted 2- fold serially from initial dilution of 1:10. HA antigen (8 HA units in 25 μ L) were added onto each well and incubated for 1 h at RT. Following antigen-antibody reaction, 50 μ L of 0.5% turkey RBC were added to each well and incubated for 1 h at RT. HI negative wells were scored based upon a diffuse sheet of agglutinated RBCs covering the bottom. HI positive wells were scored if they showed a well circumscribed button of nonagglutinated RBCs.

Histopathology

Lungs were inflated and stored in 10% neutral-buffered formalin. Three lung sections were placed into cassettes per lung section (right cranial, left cranial, left caudal, and right middle lobe) until they were trimmed, paraffin-embedded, and sectioned. Sections were mounted on glass slides and stained with hematoxylin and eosin for microscopic evaluation at Experimental Pathology Laboratories, Inc. by a veterinary pathologist.

Sections were examined for the presence of abnormal findings including suppurative and necrosuppurative inflammation; epithelial hyperplasia and cytokaryomegaly; and fibrinous and exudative changes. Changes were graded with a standardized scale of 0-5, with 0

classified as “not present”, 1 as “minimal”, 2 as “slight / mild”, 3 as “moderate”, 4 as “moderately severe”, and 5 as “severe / high.” For each ferret, a composite score for pathological changes was generated based on the locations in the respiratory tract (alveoli, bronchioli, bronchi) for statistical evaluation.

Statistical analyses

All statistics were performed using R version 2.13.0 and GraphPad Prism 5. For each image, mean SUVMax and standard deviations were obtained. For each ferret, SUVMax values were correlated with histopathologic scoring using Spearman's Rho (ρ).

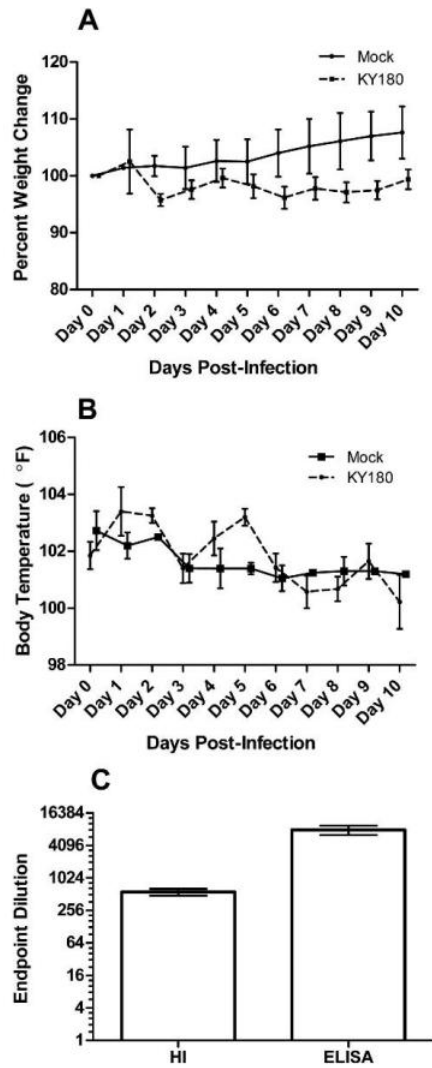


Figure 3.1 Characterization of KY/180 in female ferrets.

A cohort of six control and six KY/180-infected ferrets were examined for A) body weight and B) temperature over a period of 10 days. C) Blood was examined for the presence of HI and antibody titers at 14 days.

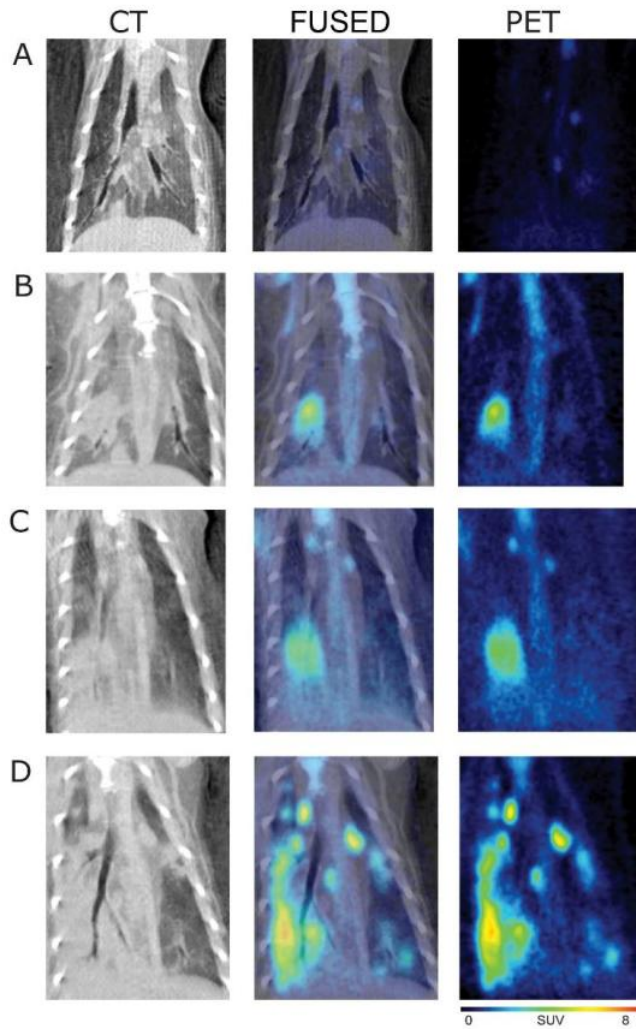


Figure 3.2 18F-FDG PET, CT, and PET/CT fusion images of the thorax in H1N1pdm-infected ferret 2213.

A) Day 0 shows background activity in the lung with minimal uptake in the mediastinal and subcarinal lymph nodes. B) Day 1 post-infection demonstrates an area of developing consolidation in the right caudal lobe corresponding to increased radiotracer uptake. C) By day 3, the consolidation appears in the right caudal lobe and into the left caudal lobe. D) By day 6, there are multiple lesions in the lung parenchyma bilaterally with intense radiotracer uptake.

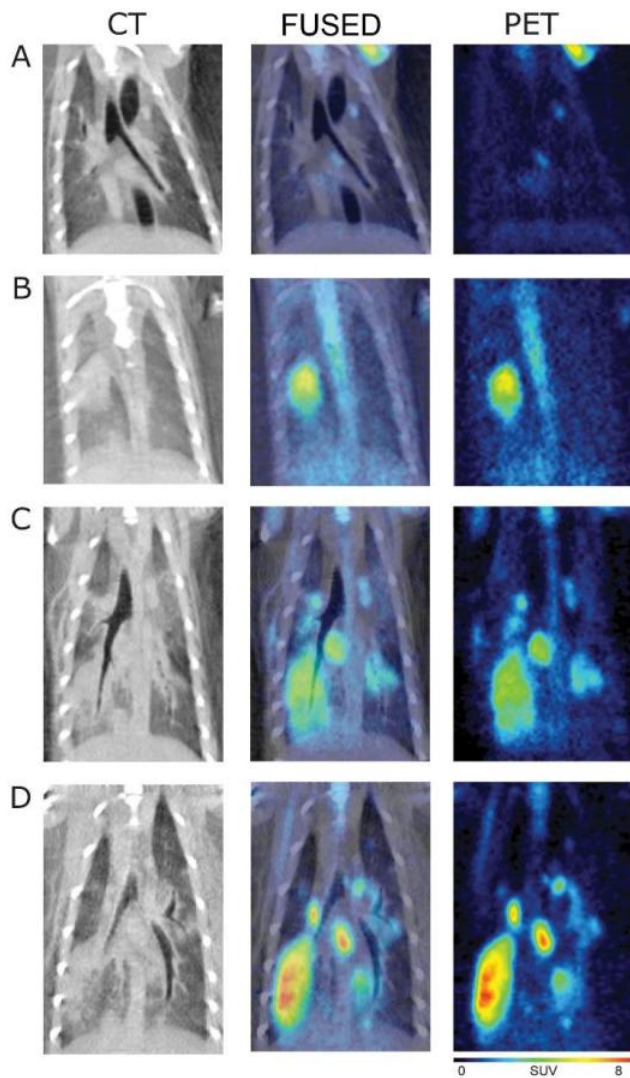


Figure 3.3 18F-FDG PET, CT, and PET/CT fusion images of the thorax in H1N1pdm-infected ferret 2214.

A) Day 0 shows background activity in the lung with minimal uptake in the mediastinal and subcarinal lymph nodes. B) Day 1 post-infection demonstrates an area of developing consolidation in the right caudal lobe corresponding to increased radiotracer uptake. C) By day 3, the consolidation has spread in the right caudal lobe and into the left caudal lobe. D) By day 6, there are multiple lesions in the lung parenchyma bilaterally with intense radiotracer uptake.

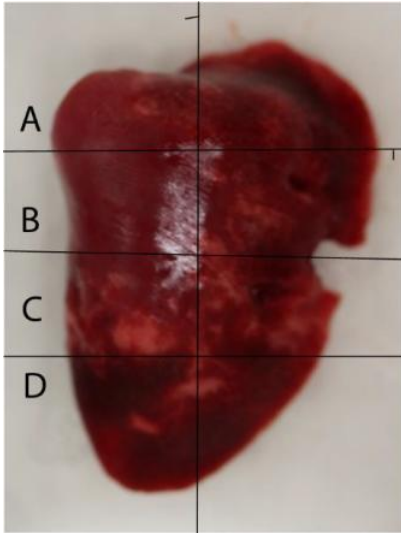


Figure 3.4 Right caudal lobe of ferret.

The right caudal lobe of each ferret was divided into 4 sections, A (top), B (middle), C (middle-lower) and D (bottom) for measurement of viral replication. Please refer to table 3.3 for viral replication titers measured from each tissue section.

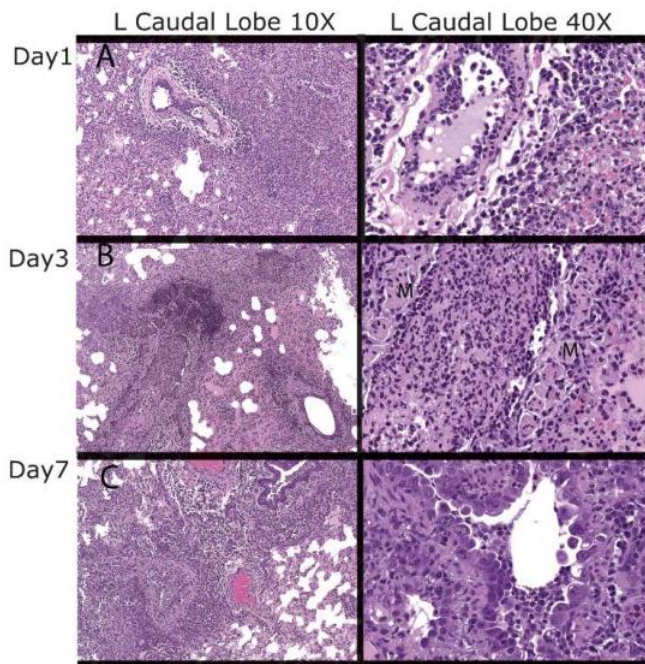


Figure 3.5 Histopathologic evaluation of the lung caudal lobe in H1N1 infected ferrets.

A) On day 1, there are small foci of inflammation (10X) in animal 2208. A higher 40X magnification of the bronchiole (shown in the 10X magnification with a box) exhibits neutrophils within the lumen with loss of part of the epithelial layer. Mixed cell alveolitis surrounds the bronchiole. B) On day 3, a bronchiole with necrosuppurative exudate is highlighted at 10X for animal 2212. In the 40X magnification (from the region in the 10X magnification with a box), muscle (M) defines the bronchiole from which the epithelium has been sloughed and the lumen is filled with neutrophils (necrosuppurative bronchiolitis). C) On day 7, a bronchiole with necrosuppurative exudate is surrounded by an extensive area of mixed cell alveolitis. Cytokaryomegaly is depicted by the variable large size and shape of the epithelial cells with piling up of the bronchiolar epithelium (hyperplasia) as shown in 10X image, and also visible in this bronchiole from another section of the left caudal lung lobe from animal 2214 (40X image).

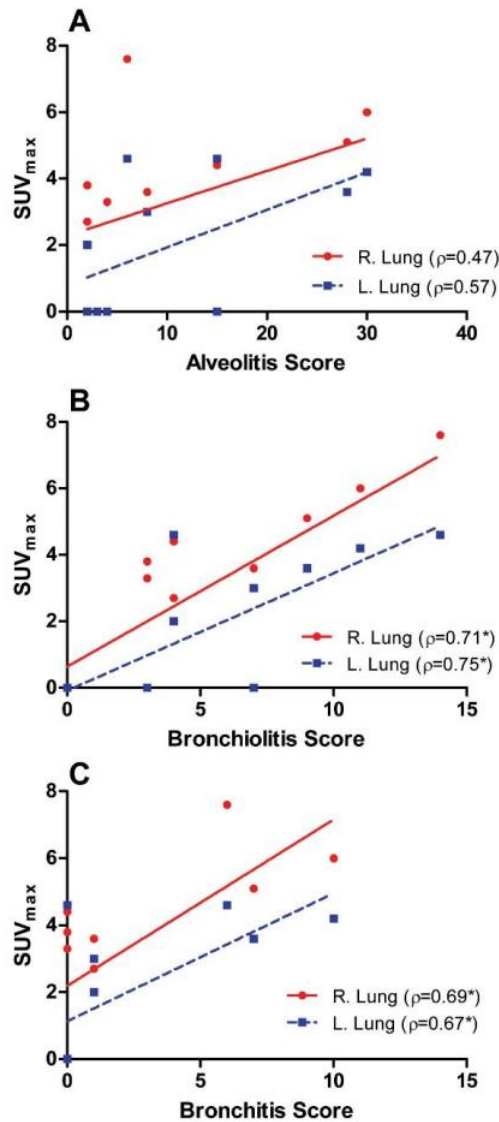


Figure 3.6 Correlations between SUVMax of lung lesions on FDG-PET versus histopathologic severity scores.

A) SUVMax versus cumulative alveolitis severity shows minimal positive correlations in the right (red) and left (blue) lung. B) SUVMax versus cumulative bronchiolitis severity shows high positive correlation in both lungs. C) SUVMax versus cumulative bronchitis severity demonstrates moderate correlation.

Table 3.1 Study design for ferret imaging and sample collection*

Group	Animal ID	Day 0	Day 1	Day 2	Day 3	Day 6	Day 7
1	2206/2207	£	†	†			
	2203/2210				†		†
2	2208		£†				
	2209		£†				
3	2202			£†			
	2204			£†			
4	2211				£†		
	2212				£†		
5	2213	£	£	£	£	£	†
	2214	£	£	£	£	£	†

†Date for necropsy; £ Date for imaging; *Swab samples were collected for viral shedding daily and upon necropsy

Table 3.2 Viral shedding.

Group	Animal ID	Area	Day 1	Day 2	Day 3	Day 7	
1	2206	Nasal	0*				
		Throat	0				
		Rectal	0				
	2207	Nasal			0		
		Throat			0		
		Rectal			0		
	2210	Nasal				0	
		Throat				0	
		Rectal				0	
	2203	Nasal					0
		Throat					0
		Rectal					0
2	2208	Nasal	0				
		Throat	4.7				
		Rectal	2.2				
	2209	Nasal	4.7				
		Throat	5.3				
		Rectal	0				
3	2202	Nasal	3.0	3.5			
		Throat	3.5	3.5			
		Rectal	1.2	0			
	2204	Nasal	0	3.0			
		Throat	3.5	3.5			
		Fecal	0	0			
4	2211	Nasal	0	0	0		
		Throat	3.8	4.7	4.3		
		Rectal	1.0	0	0		
	2212	Nasal	0	3.5	0		
		Throat	4.5	4.3	3.5		
		Rectal	1.0	0	0		
5	2213	Nasal	3.8	4.3	3.5	1.5	
		Throat	5.5	2.7	5.0	2.0	
		Rectal	0	0	0	0	
	2214	Nasal	4.0	3.7	3.3	0	
		Throat	6.7	3.7	5.3	0	
		Rectal	2.7	0	2.3	0	

*Numerical values are log base 10 TCID₅₀/g

Table 3.3 Distribution of H1N1pdm in right caudal lung and nasal turbinates

Day	Animal ID	RC A	RC B	RC C	RC D	NT
1	2208	6.7*	6.5	5.0	5.7	5.7
	2209	0	6.5	0	1	6.8
2	2202	0	4.4	3.3	4.5	5.0
	2204	0	0	0	0	4.5
3	2211	4.2	0	0	2.8	6.0
	2212	5.8	6.5	3.5	6.3	4.7
7	2213	0	0	0	0	0
	2214	0	0	0	0	0

*Numerical values represent log base 10 TCID₅₀/g; Abbreviation: right caudal (RC) lung lobe divided into sections A through D as illustrated in figure 3.4, nasal turbinate (NT)

CHAPTER IV

LOWER RESPIRATORY TRACT INFECTION OF THE FERRET BY PANDEMIC INFLUENZA A(H1N1)2009 VIRUS TRIGGERS BIPHASIC, SYSTEMIC AND LOCAL NEUTROPHIL RECRUITMENT

Overview

Infection of the lower respiratory tract by influenza A viruses results in an increase in inflammation and immune cell infiltration in the lung. The dynamic relationships among the lung microenvironments, the lung and systemic host responses during infection remain poorly understood. Herein, we used an extensive systematic histologic analysis coupled with live imaging to gain access to these relationships in ferrets infected with the pandemic A(H1N1)2009 virus [H1N1pdm]. Neutrophil levels rose in lungs of H1N1pdm-infected ferrets 6 hours post-infection and became concentrated at areas of H1N1pdm-infected bronchiolar epithelium by 1 dpi (days post-infection). In addition, neutrophils were increased throughout the alveolar spaces during the first 3 dpi, and returned to baseline density by 6 dpi. Histochemical staining revealed neutrophil infiltration in the lungs occurred in two waves, at 1 and 3 dpi, and gene expression within microenvironments suggested two types of neutrophils. Specifically, CCL3, but not CXCL8/IL-8, levels were greater within discrete lung microenvironments, and coincided with increased infiltration of neutrophils in the lung. We used live imaging of ferrets to monitor host responses within the lung over time with ¹⁸fluorodeoxyglucose (FDG).

Sites within the H1N1pdm-infected ferret lung with high FDG had high levels of proliferative epithelium. In summary, neutrophils invaded the H1N1pdm-infected ferret lung, globally, and focally, at sites of infection. The microenvironments with increased neutrophils, did not correlate with FDG, and hence, FDG-uptake may reflect prior infection and inflammation that has experienced damage as reflected by bronchial regeneration of tissues in the lungs at sites of high FDG.

Importance

Severe influenza disease is characterized by an acute infection of the lower airways that may rapidly progress to organ failure and death. Well-developed animal models that mimic human disease are essential to understanding the complex relationships of the microenvironment, organ and system in controlling virus replication, inflammation, and disease progression. Employing the ferret model of H1N1pdm infection, we used live imaging and comprehensive histological analyses to address specific hypothesis regarding spatial and temporal relationships that occur over the progression of infection and inflammation. We show the general invasion of neutrophils at the organ level (lung), but a distinct pattern of localized accumulation within the local microenvironment at the site of infection. Moreover, we show that these responses were biphasic within the lung. Finally, live imaging revealed an early and sustained host metabolic response at sites of infection that may reflect damage and repair of tissues in the lungs.

Introduction

The annual impact of seasonal influenza A viruses (IAV) on public health is fairly predictable and is mostly preventable in normal, healthy populations with currently available vaccine strategies (619-621). However, novel IAV variants arising from antigenic shift in zoonotic reservoirs are not predictable and may not be immediately preventable with contemporary vaccines (622-624). In the last 100 years, IAV have emerged with high transmissibility and virulence (1918 H1N1), high transmissibility and moderate virulence (2009 H1N1 pandemic), and low transmissibility and high virulence (H5N1, H7N9) (122, 625, 626). Virulence factors of IAV have been attributed to specific mutations in the hemagglutinin (HA) and polymerase (PB2) viral proteins (627, 628). Moreover, the specific sialic acid-binding affinity of the HA determines the cell types infected in the respiratory tract and hence the location of infection (i.e., upper versus lower) (116, 281). In general, IAV strains that are able to infect the lower respiratory tract cause more inflammation and have greater lethality (52, 281, 294, 629).

Controlled inflammation is required for resolution of IAV infection, yet increased inflammation is correlated with more severe influenza disease, and a dysregulated immune response to infection with highly pathogenic avian IAV (HPAI, *e.g.*, H5N1) may be fatal (9, 253). Seasonal H3N2 and H1N1 IAV typically infect only the upper respiratory tract and result in mild inflammation of the nasopharynx and trachea. Infection and increased inflammation in the lung was observed in cases of the 2009 pandemic H1N1 IAV (H1N1pdm), which caused increased mortality compared to seasonal strains (6, 30, 42, 127, 132, 630). Studies in humans and ferrets show that

highly pathogenic avian influenza (HPAI) viruses (*e.g.*, H5N1) and the H1N1pdm viruses infect and replicate well in the lower respiratory tract (95, 629), yet cases of H1N1pdm virus had much lower lethality compared to H5N1 and did not cause severe immune dysregulation. Thus, the precise relationship between location of infection and location of inflammation is complex, and pathogenesis may depend on other specific immune responses to certain IAV isolates.

We know very little about how most respiratory viral infections, and the resulting immune responses, progress in real-time within the local microenvironments of the upper and/or lower respiratory tract in humans or in animal models. Live, whole body imaging of pathogens or host responses may offer a bridge to gain insight into these dynamics (603). During the 2009 H1N1pdm outbreak, molecular imaging of patients revealed that FDG (fluorodeoxyglucose) increases and becomes concentrated in sites of consolidation and ground-glass opacities within the lung (6, 42, 602, 609). This motivated our exploration of live, FDG-imaging in the ferret model of IAV infection (631). The ferret serves as a critical animal model to ask questions regarding IAV disease progression given the similarity in its clinical signs and disease with those in humans (266, 272, 631, 632). Similar to those observations in human cases of H1N1pdm disease, H1N1pdm-infected ferrets have a focal pattern of FDG uptake in the lungs that correlated with bronchiolitis and bronchitis histopathology scores (631).

We and others have shown that IAV infection initiates in discrete foci of the respiratory tract (285, 631, 633). Viral replication triggers inflammation in the form of cytokines,

chemokines, and the infiltration of innate immune cells in the lungs (30, 135, 360).

Cellular uptake of FDG in acute lung injury correlates with inflammatory cell infiltration (612, 634). We and others have hypothesized that cellular uptake of FDG in the lung following infection may be due to inflammatory cell infiltration, *e.g.*, neutrophils and monocytes (631, 635, 636). Neutrophils are among the first to respond to many types of inflammation, particularly bacterial infection (16, 18, 60). Neutrophils perform many functions during bacterial infection (*e.g.*, phagocytosis, release of anti-bacterial proteases, reactive oxygen species) yet very little is known about their contributions to viral infections (472). In mild and severe human cases of influenza, neutrophils and neutrophil chemoattractant signals are elevated in blood and nasal lavage and are independent of bacterial co-infection (6-8, 30, 99, 105). In mouse models of influenza, depletion of neutrophils results in increased virus replication and increased disease severity (84, 577, 637, 638). However, it is known that neutrophils are increased in the lungs of mice during severe IAV infection (85, 253, 294, 363, 639).

Currently, we know very little about the timing and spatial distribution of neutrophils as they infiltrate the lungs following viral infection, and importantly, how they function in disease resolution or exacerbation. Herein, we used an extensive systematic histologic analysis coupled with live imaging to probe the temporal dynamics of virus distribution and inflammation within microenvironments of lungs of ferrets infected with a clinical H1N1pdm isolate. Given their importance early in infection, we focused on neutrophils and hypothesized that neutrophils would migrate to sites of IAV infection preferentially, creating an inflammatory microenvironment (16, 80, 472, 636). IAV distribution,

neutrophil accumulation and selected immune gene expression profiles were measured within discrete sections of a lung lobe. We show that recruitment of neutrophils to the lung following infection occurs in two waves, coincides with chemokine gene expression, and may reflect two different states of neutrophil activation. Using extensive histological sampling, we show that neutrophilic inflammation correlates with the temporal and spatial distribution of IAV in the lungs of infected ferrets. Importantly, the neutrophil recruitment and accumulation occurs at the tissue level (*i.e.*, the whole lung) and at sites of IAV infection. Finally, we show that FDG uptake in the infected lungs is not due to neutrophilic infiltrates, but correlates with epithelial cell proliferation and regeneration following infection.

Materials and Methods

Virus and viral titers.

Ferrets were infected with a clinical isolate of IAV H1N1pdm (A/Kentucky/180/2010) taken from a fatal case of H1N1pdm (130) that has been previously described in ferret (631), DBA/2 mouse (130) and human primary cell culture (331) models. Virus was grown in 10-day old embryonated hen eggs and diluted in phosphate buffered saline (PBS). The concentration of virus stock was determined using 50% tissue culture infectious dose assay (TCID₅₀) in Madin-Darby canine kidney cells as described previously (631).

Ferrets, ferret virus challenge, and ethics statement.

Four month old (16-20 weeks) female Fitch ferrets were obtained from Triple F Farms (Sayre, PA, USA). Ferrets were determined to be seronegative for A/Uruguay/716/2007 (H3N2), seasonal A/Brisbane/59/2007 (H1N1), and A/Kentucky/180/2010 (H1N1pdm) subtypes of IAV by hemagglutination inhibition assay (631). Animals were housed for an acclimation period of at least one week at the University of Louisville Regional Biocontainment Laboratory prior to virus challenge (this research facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International). Animals were maintained on a 12-hour light/dark cycle and provided Teklad Laboratory Diet #2072 (Harlan/Teklad, Madison, WI) and water *ad libitum*. The research described herein complied with federal statutes and regulations relating to animal experimentation, including the Animal Welfare Act, and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, published by the National Research Council, 1996. Experiments involving ferrets were approved by the University of Louisville Institutional Animal Care and Use Committee.

Ferrets were inoculated with 10^6 TCID₅₀ influenza A/Kentucky/180/2010 (H1N1pdm) as a 1 mL bolus. To prepare animals for infection with virus, ferrets were sedated with intramuscular 0.05 mg/kg atropine, 5.0 mg/kg ketamine, and 0.08 mg/kg dexmedetomidine. Sedated ferrets were scruffed and held with their nose pointing upwards and neck extended while 0.5 mL of virus or PBS was given per naris. Following intranasal (i.n.) inoculation, animals were held in place for 1 min before anesthesia was reversed with 0.4 mg/kg atipamezole. Following infection, ferrets were measured daily

for temperature and weight and monitored twice daily for clinical signs (dyspnea, nasal and ocular discharge, loss of appetite, neurological signs, sneezing, lethargy, anorexia, diarrhea, and other abnormalities) through study completion. Body temperatures were measured via subcutaneous implanted microchip IPTT transponder (BioMedic Data Systems, Inc.). Right caudal lung tissues were snap frozen on liquid nitrogen and stored -80°C until homogenized and stored in TRIzol® reagent (Invitrogen). Blood was taken in sodium-ethylenediaminetetraacetic acid tubes and stored in TRIzol®. Other lung tissues were taken for immunohistochemistry (IHC) as described below.

Analysis of gene expression levels by RT-PCR.

Previously, the right caudal lung lobe of each ferret was divided into four sections and each section was homogenized for quantification of virus by determining the TCID₅₀ (631). Following homogenization, a portion of the lung tissue was placed in TRIzol® reagent and stored at -80°C. Herein, total RNA was extracted from these samples and 1 µg RNA was used to make cDNA using Superscriptase III. Ferret mRNA gene-specific primers were generated using published ferret sequences (Table 1). Primers were designed to span exons when possible by comparing to known human and canine splice sites. Primers were chosen to identify cell type-specific targets (*e.g.*, granulocyte colony stimulating factor receptor [G-CSFR, *Csf3r* gene], myeloperoxidase [MPO, *Mpo* gene], and neutrophil elastase [ELANE, *Ela2* gene] transcripts which are specific to neutrophils; monocyte colony stimulating factor receptor [M-CSFR, *Csf1r* gene] which is used as a macrophage/monocyte marker; NCR1 [*Ncr1* gene] which is a natural killer cell receptor; and CD11b [*Itgam* gene] which is an integrin expressed on inflammatory leukocytes), as

well as soluble inflammatory signals (interleukin-6 [IL-6, *Il6* gene], CXCL8 [also known as interleukin-8, *Il8* gene], interferon-beta [IFN- β , *Ifnb1* gene], CCL2 [also known as monocyte chemotactic protein 1, *Ccl2* gene], CCL3 [also known as monocyte inducible protein 1 alpha, *Ccl3* gene], and tissue necrosis factor-alpha [*Tnf*]). Real time-polymerase chain reaction was performed using SYBR Green dye on a ViiA™ 7 real time PCR machine (Applied Biosystems). Primers were validated using mitogen-stimulated whole blood from donor ferrets (Marshall Farms; data not shown). Transcript fold-increase measurements were compared to housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase [*Gapdh*]) and average mock-infected animals using the delta-delta-cycle threshold method ($\Delta\Delta Ct$); the fold change is expressed here as the $2^{-\Delta\Delta Ct}$ value.

Histology.

Lungs were inflated with 10% neutral-buffered formalin and fixed for 48 h. Lungs were trimmed, placed into cassettes, dehydrated through ethanol to Limonene-D, paraffin-embedded, and sectioned at 5 μ m. For systematic sampling of the lungs, the right and left caudal lung lobes (RCa and LCa, respectively) were trimmed into four consecutive transverse slices approximately 0.5-cm thick moving from the lung hilum to the posterior of the lobe, as illustrated in Fig. 1. Sections (1, 2, 3) followed the main bronchus to approximately the lower-middle of the lung, as shown on Fig. 1 (left panels). The sections were paraffin-embedded and two serial 5 μ m sections were mounted onto each glass slide (Fig. 1, middle panel). Slides were dewaxed in 3 xylene washes, and rehydrated in decreasing ethanol gradients to water for IHC, and regions of the slides were photographed and analyzed (*e.g.*, square regions on Fig. 1, right panel).

IHC and tissue staining.

For detection of IAV nucleoprotein (NP), antigen was retrieved with 1% Pronase in CaCl₂ solution and was detected with a primary monoclonal antibody (HB65 clone EVS238 from East Coast Bio, Inc.)(640) and avidin-biotin complex amplification (Vector Labs) was used to visualize the antibody with diaminobenzidine (DAB) peroxidase substrate. The slides were dehydrated through increasing ethanol gradients, cleared in xylenes, and mounted under coverglass with toluene solution. Neutrophils were stained pink with a 30 min incubation in naphthol AS-D chloroacetate esterase (NACE) at 37°C according to manufacturer's instructions (Sigma-Aldrich) and one serial section was counterstained with hematoxylin to identify anatomical features. Slides were mounted with coverglass under an aqueous mounting medium (Vectamount AQ, Vector Labs). For each NACE-stained slide, the next consecutive slide was similarly stained for IAV antigen. Rehydrated tissue slides were incubated in 3% hydrogen peroxide and avidin/biotin blocking buffer (Vector Labs) for 15 min each at room temperature before blocking in 5% fetal calf serum for 1 h at room temperature. For both NACE and NP staining, one section per slide was counterstained with hematoxylin for anatomical reference, and a second unstained section was used for image processing as described below. Antigen retrieval for Ki-67 and histone-3 was performed by heating rehydrated slides in a 72°C water bath overnight in target retrieval buffer (Dako). A monoclonal antibody cross-reactive to ferret Ki-67 (Dako, clone MIB-1) was used as above to stain with DAB peroxidase substrate, and rabbit polyclonal anti-human histone-3 antibody (Abcam) was stained red with alkaline phosphatase substrate Vector Red (Vector Labs).

Sirius red staining was performed by incubating rehydrated tissue slides for 30 min in saturated picric acid containing 0.1% Sirius Red and 0.1% Fast Green, then dehydrating and mounting under Permount (Fisher Scientific).

IHC analyses.

Images from IHC slides were analyzed with the “Threshold Colour” plug-in of the FIJI package of ImageJ software (641). Briefly, pixels of a certain hue and saturation (bright pink for NACE-stained neutrophils, red for Sirius Red, and brown for DAB-stained NP-positive or Ki-67-positive cells) were selected and the percentage of total pixels was calculated for each image. The percent consolidation was calculated for each image by converting each image to high contrast 8-bit (black-and-white) and calculating the percentage of black pixels (*i.e.*, the number of pixels containing tissue, eliminating airspace). A Java computer language script (available upon request) was generated to analyze all images in the same way using validated settings. Neutrophil density is reported here as the percent NACE-positive pixels divided by percent consolidation in a single image. To validate this measurement, two independent observers counted the number of NACE-positive neutrophils in 50 images and there was a 95% correlation between the automated method and visual inspection (data not shown).

Comprehensive, systematic sampling of the caudal lung lobes was performed by a blinded observer choosing four regions per NACE-stained tissue section and photographing three images per region at 20X magnification (squares in right panels of Fig 1). To correlate the amount of neutrophils with foci of IAV infection, NP antigen-

positive areas were photographed and their anatomical locations were recorded (i.e., bronchial epithelium, bronchiolar epithelium, submucosal epithelium, alveolar region). The same site was located on the NACE-stained slides and photographed (i.e., a serial section of tissue no greater than 15 μm away from the NP-positive site). IAV NP antigen-negative regions were anatomically site-matched to NP-positive focus and located in the same general regions (e.g., a region of bronchiolar epithelium that was antigen-negative was selected on a slide where bronchiolar epithelium was antigen-positive, and therefore was always < 0.5 cm from the original antigen-positive focus). NACE-positive neutrophils, IAV NP-positive cells, and Sirius Red-stained collagen were measured using automated image analysis as described above. The number of Ki-67-positive cells per slice was estimated by two observers using a scoring index (642, 643).

Ferret molecular imaging.

Ferrets were anesthetized by isoflurane induction and 2 mCi of FDG was administered intraperitoneally as previously described (631, 644). The dose was confirmed before and after injection with an Atomlab 500 Dose Calibrator; Biodex Medical Systems Inc., NY. Ferrets were kept warm under sedation with isoflurane for 1 h prior to intubation and during imaging using the Siemens Trimodal as described previously (631, 644). Following imaging, ferrets were removed from isoflurane, given intramuscular ketamine (5 mg/kg) and dexmedetomidine (0.08mg/kg), and immediately euthanized by cardiac exsanguination.

PET-CT Image Analysis.

After PET and CT images were acquired from the Trimodal scanner, PET images are aligned to CT anatomical reference space in order to provide one-to-one voxel correspondence between anatomical and functional images. Since pathology on CT (*e.g.*, consolidation) can confound true lung volume and boundary estimation, the lungs were segmented from CT images using a robust algorithm (*i.e.*, interactive region growing) proposed in (645) by which users had chance to correct any mis-segmented lung portion interactively if necessary. Then, significant uptake regions were segmented using affinity propagation PET delineation algorithm (646). Once all the metabolically active lesions were identified and delineated, maximum standard uptake values (SUV_{max}) and maximum intensity values were calculated as an evaluation metric for the FDG uptake. These measurements were taken from regions coinciding with histopathology slices, locating the slices on the registered and segmented PET-CT scans manually. The alignment between histology and PET-CT was ensured by processing 9-parameter affine registration and using prior knowledge about the anatomy, particularly the airway structures and the lung lobes. For airway structure extraction, we used the hybrid algorithm for delineation of the airway tree as proposed in (647). Due to the 2D nature of the histopathology slices, registration operations were all done in 2D. After alignment of the histopathology slices into PET-CT scans were satisfied, *in vivo* imaging correlation with histopathology slices was explored.

Statistical analyses.

All statistics were performed using R version 2.13.0 and GraphPad Prism 5. In general, nonparametric statistical tests were used as stated in the results, even when sample size was large non-independent measurements within the same ferret made parametric analyses undesirable. Pairwise *post hoc* tests were adjusted for multiple comparisons using Bonferroni's method. For generalized linear modeling of neutrophil density, variables such as time post-infection, individual ferret, lung lobe within ferret, and sample region within lung lobe approximated a nested block design with repeated measures within ferrets, and within lobes within ferrets. The fully factorial model was reduced using stepwise regression to find the best fitting model as determined by significant reduction in Akaike information criterion. For all statistical tests, the Type-I error rate was set to $\alpha = 0.05$; although values $p < 0.1$ are given consideration here due to sample size limitation and pseudoreplication as discussed below.

Results

Previously we have shown that infection of ferrets with H1N1pdm (A/Kentucky/180/2009) causes moderate disease, evidenced by the brief febrile period, low weight loss, and clinical signs recorded during infection (*i.e.*, lethargy, rhinorrhea) (631). Virus was detected from nasal swabs, throat swabs, nasal turbinates, and right caudal lung lobes on 1, 2, and 3 dpi, but was undetectable by 7 dpi (one animal had low amounts of virus in nasal and throat swabs on 7 dpi) (631). Histopathology revealed a focal bronchitis and bronchiolitis characterized by infiltration of leukocytes (neutrophils and exudate macrophages) during H1N1pdm infection (631). Cellular uptake of FDG

correlates with leukocytic infiltration in certain model systems (612, 635, 636). In this ferret model, we have observed progressive increase of FDG uptake in distinct foci of the H1N1pdm-infected ferret lung (631). Therefore, herein, we tested the overarching hypothesis that FDG uptake during ferret IAV infection was due to infiltrating neutrophils and macrophages. We first determined which leukocytes were likely to be increased in these regions by measurement of immune gene expression profiles within discrete lung microenvironments.

Lung microenvironments of IAV infection have increased CCL2 and CCL3 gene expression

First, the expression of signature immune genes was analyzed in the right caudal lobe of one mock and two H1N1pdm-infected ferrets on 1, 2, 3, and 7 dpi. As stated above, the right caudal lobe had the greatest levels of FDG uptake following infection (631).

Primers for IAV HA and inflammatory cytokines and chemokines were used to confirm the presence of virus and the host immune response to infection. Genes were chosen to detect cytokines and chemokines that are known to be increased during the acute phase of influenza infection and are important for leukocyte activation and chemoattraction (*e.g.*, IFN β , TNF α , IL-6, CCL2, CCL3, and CXCL8). First, IAV HA mRNA was detected at 1, 2 and 3 dpi, but not 7 dpi, and was not uniformly distributed within the right caudal lobe (data not shown). This matched the pattern of IAV infection as measured by TCID₅₀, previously reported from the same ferret lung sections (631). The fold-change in gene expression of inflammatory cytokines (IFN β , TNF α , IL-6) and chemokines (CCL2, CCL3, CXCL8) in sections were compared to mock-infected controls. No significant

change in inflammatory expression levels was detected in the blood (data not shown). In some lung sections, the cytokines IL-6 and TNF α (not shown) were elevated at 2 dpi, but not in all sections from infected ferrets. Other inflammatory signals (*e.g.*, IFN- β , CXCL8) were not elevated above mock-infected animals (data not shown). Gene expression levels of CCL2 and CCL3 were both slightly increased at 1 dpi and significantly increased on 3 dpi ($p < 0.05$, Fig. 2). Both CCL2 ($p = 0.01$) and CCL3 ($p = 0.04$) were significantly increased in all lung sections expressing viral HA gene mRNA (compared to all HA-negative sections, including those within the same ferret; Mann-Whitney $U = 105$ & 73 , respectively for CCL2 and CCL3).

Neutrophil-specific gene expression is detected in lung microenvironments

Primers for leukocyte-specific genes which encode cell surface receptors and other cell-specific proteins were used to determine the presence of neutrophils (G-CSFR, MPO, ELANE, CD11b), macrophages (M-CSFR, CD11b), and natural killer cells (NCR1, Granzyme A, CD11b) in the lung. The monocyte/macrophage specific gene, *Csf1r* (M-CSFR), was increased slightly at 2 and 3 dpi, and increased expression of the natural killer cell receptor, *Ncr1* (NCR1), was detected in some sections at 2 and 3 dpi (data not shown). Some lung sections had increased expression of the leukocyte cell surface integrin, CD11b, and the median expression value of CD11b in HA-positive sections was significantly increased above mock (median = 2.6, $p = 0.01$, Wilcoxon test, data not shown). Expression of the neutrophil growth factor receptor gene, *Csf3r* (G-CSFR), was increased in lung sections at 3 dpi ($p < 0.05$) (Fig. 3A). Expression of *Csf3r* was not significantly increased in tissue sections that were positive for HA gene expression

compared to HA-negative sections (Mann-Whitney U test). Neutrophil specific granule genes (*e.g.*, *Mpo*, *Elane*) were not significantly upregulated compared to the mock-infected animals (data not shown).

Neutrophils have biphasic infiltration into the lungs of IAV-infected ferrets

The major inflammatory signals in the right caudal lung sections were the neutrophil-specific gene *Csf3r* and the leukocyte chemoattractants CCL2 and CCL3. To confirm the temporal pattern of neutrophils detected by gene expression in the lungs of these ferrets, NACE staining was used to label neutrophil specific granules. One histological preparation per lung lobe (except right caudal lobe which was used for gene expression, above) from H1N1pdm-infected and mock-ferrets were stained and inspected for neutrophils. To estimate the number of neutrophils within the stained histological sections, image analysis software was used to determine the amount of NACE-positive pixels per unit of tissue in an image (20X objective) of the NACE stained lung, as described in the methods (expressed as the estimated “neutrophil density”). Image analysis of sites chosen haphazardly within each tissue slice revealed that the overall density of neutrophils in the lung was biphasic, showing a maximum at 1 dpi ($p < 0.05$) with another peak at 3 dpi (n.s.) following a decrease at 2 dpi (Fig. 3B). Thus, the major peak of neutrophils did not correspond to the major peak of neutrophil-specific gene expression in the lung. The other lung lobes showed a wide range of neutrophil density, similar to the gene expression data, suggesting there were foci of neutrophil infiltration throughout the lungs.

Neutrophils may hone to specific sites of IAV infection in the lungs

To determine the precise distribution of IAV within the lungs, IHC was used to detect NP antigen in paraffin-embedded sections of the remaining lung lobes (except of right caudal lobe). Infection was detected in bronchial, submucosal, and bronchiolar epithelial cells (Fig. 4A-D), but not alveolar epithelium (not shown). IAV NP was detected from all infected ferrets at 1 and 3 dpi, and from one animal at 2 dpi; but was not detected from infected animals 7 dpi. H1N1pdm infection was highly focal in the lung, and was primarily restricted to sites in bronchioles and bronchi (Fig. 4A-D). Similarly, the pattern of antigen positivity matched the IAV mRNA expression, the pattern of chemokine and neutrophil-specific gene expression and the pattern of NACE staining. Counterstaining NP-labeled sections with hematoxylin allowed the identification of increased numbers of inflammatory macrophages and neutrophils, some of which were antigen-positive (Fig. 4D), at foci of infection in the bronchioles. Therefore, neutrophils appeared to hone to the sites of infection in specific anatomic regions.

Comprehensive sampling of the lung lobes for neutrophil infiltration

The preliminary data suggested that neutrophils infiltrate the lungs and hone to sites of IAV infection, driven by chemokine gradients (*e.g.*, CCL3). However, the data were correlative because the sampling was not comprehensive and data were obtained from different lobes (*i.e.*, gene expression from the right caudal lobe, neutrophil and NP staining from the four remaining lung lobes). Therefore, we performed a second ferret study to confirm these observations using a systematic histological sampling of the lung. Six female ferrets were infected with 10^6 TCID₅₀ of H1N1pdm (plus 2 mock-infected)

and 2 animals were euthanized per time-point at times that were established to have peak neutrophil infiltration (1 dpi and 3 dpi) as well as an early time post-infection (0.25 dpi). Left and right caudal lung lobes were formalin-fixed, then sectioned into four 0.5 cm cross-sections from the hilum moving posteriorly, embedded in paraffin, and prepared for histology (three cross-sections shown in Fig. 1). Tissue slices were NACE-stained and photographed to quantify neutrophils. To establish factors important neutrophil distribution in the lung, a methodical sampling was performed to provide data for a generalized linear model. Four regions from each slice were chosen blindly, and three non-overlapping images were taken from each region to quantify the number of NACE-positive pixels as a function of total tissue (excluding airways) per image using ImageJ (Fig. 1). This established a 3-D grid of sampling locations within the caudal lung lobes, wherein three images are taken from locations within each caudal lung lobe ($n = 48$ images per lung lobe).

Neutrophils infiltrate the right caudal lobe at early times after infection

Step-wise model testing of the generalized linear model revealed that the best-fitting model included time post-infection, lung lobe (left versus right), and the distance from the lung hilum. *Post hoc* analyses confirmed that neutrophils were significantly increased at 1 dpi and at 3 dpi as in the previous study (Fig. 5A, $p < 0.001$ in the left and right caudal lobes), however increased neutrophil density was measured in the lungs of infected animals above mock as early as 0.25 dpi (Fig. 5A, $p < 0.05$ in the left caudal lobe, n.s. in the right caudal lobe). The distribution of neutrophils was highest in the right caudal lobes at 0.25 and 1 dpi (Fig. 5A, $p < 0.001$) compared to the left, but not at other

time points. Additionally, it is important to note that a range of neutrophil density was observed, yet, in general, infected animals had higher baseline levels of neutrophils in either lobe at 0.25 dpi, 1 dpi, and 3 dpi. Thus, neutrophils appeared to be focally distributed throughout the lung, and these observations (*e.g.*, increased right caudal lung) suggest that they are driven by IAV infection.

Neutrophils are increased at sites of IAV infection.

To test the hypothesis that neutrophils were increased at foci of IAV-infected epithelial cells, IHC staining for IAV NP was performed in serial sections of the NACE-stained tissues taken from right and left caudal lobes as described above and Fig. 4. Using NP-stained serial sections as a reference, images were taken from three areas of NACE-stained tissue slices defined by their distance from NP-positive. First, three non-overlapping images were taken from each antigen-positive site. For each antigen-positive site, two other sites were chosen and three non-overlapping images were analyzed: sites < 1 cm from antigen-positive sites (within the same histology slice), and sites > 1 cm from antigen-positive sites (other histology slices within the same lobe). These three groups of images were also compared to images taken of NACE-stained mock-infected controls, and image analysis software was used to calculate the estimated neutrophil density as described above. There was no linear relationship between distance from focus of infected cells and density of neutrophils, *i.e.*, neutrophils were increased in all sites relative to mock-infected control animals (Fig. 5B, $p < 0.01$). However, a pattern was revealed by resolving the spatial distribution over time: neutrophil density was significantly greater at NP-positive sites and at sites <1 cm from known NP-positive sites

at 1 dpi (Fig. 4, 5B, $p < 0.01$). There was no difference in spatial distribution at 3 dpi, and neutrophil density remained increased relative to mock-infected controls (Fig. 5B, $p < 0.01$).

Neutrophils are increased at specific locations of IAV infection.

To test the hypothesis that anatomical location explained the distribution of neutrophils after infection, sites of IAV-positive bronchi (*e.g.*, Fig. 4A), seromucinous glands (*e.g.*, Fig. 4B), and bronchioles (*e.g.*, Fig. 4C, 4D) were identified (no antigen-positive alveolar epithelial cells were detected). These sites and antigen-negative sites within the same ferret (matched to a similar anatomic region) were located on NACE-stained serial sections, imaged (Fig. 4E-G), and analyzed using image analysis software to calculate the estimated neutrophil density as described above. There was a significantly higher density of neutrophils in IAV-positive bronchioles compared to controls (Figs. 4C, 4D, 4G, 4H, and 5C, $p < 0.01$). Neutrophils were not increased in bronchi or seromucinous glands above antigen-negative regions (Figs. 4E, 4F, and 5C). Furthermore, neutrophils were increased in bronchioles and alveoli where there was no IAV-positive antigen present by IHC (Fig. 5C). Interestingly, certain areas containing neutrophils in the lumen of bronchioles near IAV-positive epithelial cells had extracellular Histone H3 staining (data not shown), an indicator of neutrophil extracellular trap formation (NETosis). However, there were insufficient sites of NETosis and a lack of available reagents for the ferret model to pursue investigation of this phenomenon further at this time.

Approach to analyze PET- FDG images to achieve direct spatial correspondence with histology

We have previously reported that molecular imaging of ferrets using FDG-PET showed progressive increase in inflammation following infection with H1N1pdm (correlated with bronchiolitis and bronchitis pathology scores) that was greatest in the right caudal lobe but also focally distributed throughout the other lung lobes (631). We hypothesized that this may be due to increased inflammatory cell infiltration at sites after infection (612, 631, 635, 636). Therefore, we injected infected ferrets with FDG and imaged them with PET-CT prior to euthanasia on 0.25, 1, and 3 dpi (Fig. 6). Using the same ferrets in the second study, we analyzed PET-CT data similar to histological data (*i.e.*, a 3D grid) and achieve direct spatial correspondence between the histology and imaging. Automated airway segmentation of CT images was first performed to extract the lungs and airways from the images (Fig. 6). Then, stacked 3D image constructions were used to locate the exact positions of tissues taken for IHC using anatomical features as references (such as branching of bronchi, location of the heart, and bone structure) as well as gross anatomical photographs taken during necropsy to ensure accurate correspondence between CT and IHC. Next automated registration of PET-CT images was performed to ensure PET-CT were aligned, which then allowed us to measure the uptake of FDG at sites taken for histology (Fig. 6).

FDG uptake did not match the pattern of neutrophil infiltration

We tested whether FDG uptake was associated with sites of increased neutrophil infiltration. The stacked CT images were used to locate the regions from which NACE

image analysis had been performed, and FDG uptake (SUVmax) was determined on PET images from these same regions. There was an early increase in FDG SUVmax at 0.25 dpi, which immediately decreased below mock levels before gradually increasing to 3 dpi (Fig. 6). For both left and right caudal lobes, uptake in mock-infected animals was significantly greater than uptake at 1 dpi ($p < 0.05$, Fig. 7A). Temporally, this did not align with the pattern of neutrophil infiltration (Fig. 5A). Due to differences in resolution between the two modalities (PET-CT and IHC) we averaged the three non-overlapping images from each region measured by IHC and this compared to a single SUVmax from that region. Over all time-points, there was no association between FDG uptake and the neutrophil density (data not shown). However there was a significant linear association between SUVmax and neutrophil density at 1 dpi, although the association was comparatively weak ($R^2 = 0.26$) and was likely due to outliers (non-zero slope, $p < 0.001$, data not shown). This finding was very surprising, therefore we attempted to identify what was responsible for the FDG uptake using this data set for which we had confidence in our correspondence between PET-CT and histology.

FDG uptake did not match sites of IAV infection

We tested the hypothesis that FDG uptake occurs in sites of H1N1pdm infection in ferret lungs. Using a similar approach to the one above for NACE-stained sections, we measured FDG-PET uptake (SUVmax) at sites of IAV-positive cells determined by IHC. We also measured SUVmax at two other sites based on distance from IAV-positive cells: < 1 cm from any known IAV-positive area, and < 1 cm from any known IAV-positive area, both were measured from regions within the same ferret lobe. There was

significantly less FDG uptake at sites of IAV-positive cells compared to mock-infected animals ($p < 0.01$ for all regions compared to mock, Fig. 7B). Because this approach was also potentially confounded by the disparity in resolution between PET-CT and microscopic imaging modalities, we tested whether IAV infection may increase the uptake of glucose *in vitro* by infecting immortalized cell lines with several IAV isolates. We experimentally infected several human cells and cell lines (e.g., A549 and well-differentiated human bronchial epithelial cells line, 16HBEo-), and we observed slightly increased uptake of FDG in some but not all treatments (data not shown). Thus, we may infer that increased FDG uptake in infected ferrets was not associated with IAV infection directly.

FDG uptake measured proliferation in the lungs following IAV infection

We then tested the hypothesis that proliferating cells were responsible for the increased FDG uptake in lung tissues by detecting Ki-67 protein with IHC. Ki-67 is a nuclear protein that is present in high amounts during cellular proliferation. In order to estimate the amount of proliferating cells, we used a Ki-67 proliferation index (Fig. 8, images show examples of each score, 0-3) which is an established method for grading tumors clinically (642). No significant regions of proliferating epithelium were detected in the ferrets imaged at 0.25 dpi, 1 dpi, and 3 dpi; therefore we included histological samples from the previous study described above (*i.e.*, two infected ferrets and one mock ferret were taken at each of 1, 2, 3, and 7 dpi, the right caudal lobe is excluded from these sections) (631). Three non-overlapping images were taken haphazardly from each of three anatomical regions per tissue slice and were scored by two independent observers in

a blinded fashion. Both alveolar and bronchiolar regions had baseline levels of proliferating cells and there was no difference between lung tissues taken from mock and infected animals in these anatomical regions (not shown). However, there was an increase over time in the amount of proliferating cells in the bronchi, which peaked at 7 dpi ($p < 0.01$; Fig. 8).

Discussion

The pathology of human infection with IAV is often only known from lethal endpoints, taken at autopsy (6-9). In humans, severe IAV infection affects the LRT, and results in the massive infiltration of neutrophils (6, 42). Similarly, severe IAV disease in ferrets is characterized by early infiltration of neutrophils (123, 253). With respect to neutrophils in the lungs during IAV infection, pathology reports from lethal, human influenza pneumonia typically state the presence of suppurative necrosis, mixed cellular infiltrates, or neutrophilic infiltration (6-8, 30, 272, 630). However, few studies have directly addressed simple questions about the relationship between neutrophils and IAV infection. For example: when do neutrophils arrive in the lungs following IAV infection? What is the pattern of neutrophil infiltration, *i.e.*, is it specific to anatomical locations? Is it specific to sites of IAV? Or is it specific to sites of damage and necrosis? How important are the various inflammatory signals released in viral microenvironments to the activation and chemoattraction of neutrophils during IAV infection? Answers to these questions will provide a basis for answering more important questions, such as how does the severity of disease affect neutrophil infiltration (or vice versa)? Severe IAV infection often results in a form of acute lung injury and the formation of ARDS, and neutrophils

are implicated as both contributing to and recovering from this syndrome (16, 18, 60). Based on mouse studies, the role of neutrophils during IAV infection show importance in protection from lethal disease, however much remains unknown about their relationship in the ferret model (84, 85, 577, 637, 639).

Despite the importance of the ferret model in the study of IAV pathogenesis, and despite it being used for nearly 80 years for this, the ferret model is challenging. For example, there are no ferret-specific commercially available reagents, and the cross-reactive antibodies are unverified (279, 280). To overcome the limitations in availability of immunological reagents, we have explored the use of molecular imaging as a tool to study the pathogenesis of IAV in the ferret lung (631). Molecular imaging modalities, such as PET, rely on the *in vivo* accumulation of injectable non-species-specific radioisotope-labeled probes that can be imaged in 3-dimensions, and images can be fused with CT images to provide anatomical references (645, 648). Their use in infectious disease imaging is quickly developing (635, 645), and preliminary data provides the foundation for the use of FDG-PET imaging in diagnosis or prognosis of clinical cases of IAV (42, 609, 631). To further develop this tool, and to expand the tools available for the use of ferret model, we established a model of FDG-PET during ferret infection with H1N1pdm (631, 644). To complement the comprehensive 3-D nature of the images provided by PET-CT fusion, we chose a systematic sampling of the lung to measure infiltrating neutrophils in ferrets. Our sampling approach differed from methods previously used to determine the spatial and temporal dynamics of IAV infection in the ferret (123, 132, 270), which typically focus measurements to the site of infection and/or

lung lesions. Additionally we relied on gene expression in discrete sections of tissue to test changes in the viral microenvironment because antibody-based staining of tissues (e.g., IHC) or cells (e.g., flow cytometry) is currently unavailable. In doing so, we were able to test many hypotheses about neutrophil infiltration following H1N1pdm infection.

Neutrophils migrate into the lungs early and accumulate in sites of infected bronchioles. The increase of neutrophil density in the lungs until 1 dpi may be due to the increase in chemokine transcription within specific lung microenvironments (649, 650). Leukocyte chemoattractant chemokines such as CXCL8, CCL2, and TNF α are known to be present at sites of infection following experimental human IAV infection (99), and levels are increased in patients with H1N1pdm compared to seasonal IAV (105, 112). Previously we and others have shown increased secretion of leukocyte chemoattractant chemokines (TNF α , CCL2, CCL3, and CXCL8) following *in vitro* infection of well-differentiated normal human bronchial epithelial cell culture with H1N1pdm compared to seasonal IAV infection (331, 334). In IAV-infected ferrets, we and others have shown increased gene expression of other leukocyte chemoattractants (CCL2, CCL3, and CXCL10) as well as leukocyte-specific genes (G-CSFR, M-CSFR, CD11b, and CTSB) in the blood (126, 516, 600). Therefore, it is likely that neutrophils infiltrate the lungs following H1N1pdm infection following chemokine gradients to the site of infection. Moreover, we observed that the distribution of neutrophils was higher in the right caudal lobes at 1 dpi, which matched our observations and others' that i.n. and aerosol challenge of ferrets with IAV shows a preference for infection of the right caudal lobe (272, 285, 631).

It remains unknown what specific signal drives neutrophil chemoattraction in ferrets during IAV infection. Herein, we demonstrate that CCL3 was upregulated in discrete locations within the lung that were correlated with the presence IAV HA mRNA, neutrophils and a neutrophil-specific gene (G-CSFR) (Figs. 2 and 3). The significant increase in gene expression for CCL3 occurred at 3 dpi, which indicates another chemoattractant may be more important for early recruitment at 1 dpi (Fig. 2). Similarly, depletion of neutrophils from a mouse model of influenza infection decreased the amount of CCL3, suggesting that neutrophils may also be a source of this chemokine (577). In this study, we found that ferrets did not upregulate CXCL8 following infection with H1N1pdm. CXCL8 is the major chemoattractant chemokine for neutrophils during many human infections (651). It is known that neutrophil chemoattraction is somewhat species specific, and some mammals do not possess CXCL8. For example, in mice the chief neutrophil chemoattractant chemokines are CXCL1 and CXCL2 (652). In humans, neutrophils display the chemokine receptors CCR1 and CCR5, which are important for their chemotaxis and oxidative burst in response to CCL3. The ferret genome contains orthologs for these gene sequences (NCBI Gene ID: 101693329 and 101692743, respectively) (649, 653), however it is not known if these are similarly expressed on ferret neutrophils. Additionally, neutrophil chemoattractants not commonly associated with viral infection, such as eicosanoid molecules (leukotriene) and complement, were not measured (472). In general, sectioning the lung lobe revealed the highly focal nature of inflammatory gene expression in the lungs, which matched the focal nature of viral distribution in this ferret model. Although not shown, the increased expression of IL6

and TNF in certain sections but not others indicates a complex relationship between infection and immune response.

We measured the focal nature of infection in two ways: sampling discrete sections of a single lung lobe for gene expression and virus titration, as well as an unbiased semi-comprehensive histological analysis of two lung lobes. Using NACE-stained tissues, the latter showed a range of neutrophil densities that was greater in infected animals compared to mock-infected. By resolving the distance from NP-positive cells over time, we show that neutrophils become greatly enhanced at specific viral foci by 1 dpi (Fig. 5B). We observed occasional neutrophil NETosis at sites of IAV-infected cells, but we were unable to make any further statistical correlation to spatial or temporal aspects of disease without further sampling. Together, these observations indicate that neutrophils migrate to sites of IAV infection, and perform functions there. Thus, we conclude that inflammation caused by IAV was focal and driven by the presence of IAV-infected cells, yet the entire lung experiences increased infiltration as a result of infection. This may be due to the specific migratory patterns of neutrophils through the lung to sites of inflammation (654). The increased “background” neutrophil infiltration in infected animals (*i.e.*, before and after 1 dpi and at 1 dpi away from foci) was not obvious by standard hematoxylin and eosin IHC - neutrophils were uniformly distributed throughout the lung at a density that was quantifiably greater than mock-infected controls.

We detected two waves of neutrophils following H1N1pdm infection using the histochemical stain, NACE. As discussed above the peak of the first wave occurred at 1

dpi, and was concentrated at sites of influenza infection. A second wave of neutrophils was detected at 3 dpi, by both NACE staining and gene expression signatures. This biphasic pattern of neutrophils following H1N1pdm infection has been reported elsewhere (123). Neutrophil-specific transcriptional markers were only detected in lung microenvironments at 3 dpi (*e.g.*, G-CSF receptor [*Csf3r*], and *Itgam*, the alpha-m subunit of the Mac-1 integrin leukocyte activation marker, CD11b) (Fig. 3). Thus, there appears to be two different pools of neutrophils invading the lung following infection which differ in their gene expression status, and the second may be responding to or producing CCL3. Currently, it is thought that steady-state neutrophils are mature, terminally differentiated cells with limited cell-specific gene regulation, *e.g.*, mature neutrophils have pre-formed granules, but neutrophils themselves are capable of gene transcription in response to certain stimuli (655, 656). This may explain why gene expression of neutrophil granule contents were not detected (MPO, ELANE, CTSB). We hypothesize that the first wave at 1 dpi consists of mature neutrophils, and the second at 3 dpi represents a more immature population, perhaps as a result of emergency granulopoiesis, that have been recruited to the lung from the bone marrow as a direct result of IAV infection and systemic inflammation, and thus contain mRNA for the neutrophil growth factor receptor, G-CSFR (472, 657). Additionally, these gene signatures provide support for the biphasic infiltration of neutrophils observed in other lung lobes, showing a relationship between systemic activation and recruitment of neutrophils and their subsequent concentration at specific viral microenvironments. These observations should be studied further, as this may indicate distinct functions of these two pools of neutrophils.

The lung is a known reservoir of steady-state neutrophils, perhaps due to the large blood volume of the lung capillaries (62, 472, 658). An early increase in airway and circulating blood neutrophils is a feature of IAV infection in humans (7, 8), and is common in experimental IAV infection in mice (130, 253, 363) and ferrets (123, 266, 659). We show this happens as early as 6 hours post-infection (Fig. 5A,B). However, increased numbers of neutrophils in the lower airways has been correlated with increased influenza disease severity (85, 123, 132, 253, 600), and fewer neutrophils are found in the lungs of ferrets infected with virus isolates causing less severe influenza disease (123, 659). Excessive neutrophil infiltration may contribute to acute lung injury by causing alveolar-capillary damage (577, 578, 639). Conversely, neutrophils are essential to the recovery from IAV infection as part of the innate immune response as well as the adaptive immune response (84, 319, 637, 638). For example, depleting mice of neutrophils prior to IAV infection leads to increased viral replication and increased clinical disease (85). This suggests that the balance between damage from excessive neutrophil infiltration and the generation of controlled immune responses during IAV-infection is a critical determinant of disease outcome. This study shows a unique pattern of neutrophils and suggests they are honing to specific sites of infection and inflammation during H1N1pdm-infection. It is not known how these patterns of infiltration will change in response to a more severe IAV infection or to infection with a HPAI which causes immune dysregulation.

As it pertains to our findings, a majority of neutrophil research has been centered on their relationship to acute injury (particularly acute lung injury) and bacterial infection

(reviewed in (16),(18), and (60)). Indeed, pulmonary bacterial co-infections are clinically common during and following IAV infection and are thought to represent the major risk factor for mortality from an IAV infection. Bacterial co-infection was not evident in any of the ferrets examined by histopathology. No further effort was made to detect bacteria (*e.g.*, PCR, IHC, or direct culture) since commensal bacteria are known to exist throughout the respiratory tract, and any pathogenic bacterial infection would be distinctive on histopathology. Further, our methodology for ferret husbandry and *i.n.* infection is in line with contemporary standards, and we would not expect bacterial co-infection to occur in this controlled laboratory model. Previous studies have shown that neutrophils are capable of recognizing IAV and IAV-infected cells (660-663), although disagreements in data suggest that neutrophil responses to IAV are dependent on the viral strain (661-664). The role of neutrophils in viral disease remains a topic that is quickly growing (80, 456, 471, 665) and deserves further research.

We have hypothesized that increases in FDG uptake following infection is due to increases in inflammatory cell infiltration in the lungs (612, 631, 635, 636). Large FDG lesions (SUV_{max} > 3) begin to be detected in the lungs of IAV-infected ferrets at 3 dpi and increase (SUV_{max} ≥ 5) until 7 dpi (631). However, the density of neutrophils in the lung did not match the temporal or spatial pattern of FDG uptake in the lung. We previously reported greater uptake of FDG in the right caudal lung where we and others have measured increased viral replication and neutrophilic infiltration (272, 285, 631). Another hypothesis to explain increased FDG uptake is that IAV increases cellular glucose metabolism (666). We found that infection with IAV isolates caused an increase

in FDG uptake by immortalized human epithelial cell lines (A549) *in vitro*, but not by differentiated human bronchial epithelial cells (16HBEo-) (data not shown) (331, 631). Increased glucose promotes the acidification of cellular endosomal compartments, a step required for IAV infection of cells (666, 667). In hospital clinics, FDG is used to detect metabolically active tumors throughout the body, including the lungs, as it is taken into cells through glucose transporters and accumulates (648, 668, 669). In this study, we did not detect an increase in gene expression of GLUT1 (*Slc2a1*) or GLUT4 (*Slc2a4*) in lung tissues (data not shown). Thus, we could not detect IAV-dependent increased glycolysis *in vivo* via uptake of FDG at the times tested in this study (Fig. 7). We have reported gradually increasing FDG uptake until 7 dpi (SUVmax scores >5) (631) which is after IAV is cleared from the lungs. Therefore we cannot support the hypotheses that FDG uptake is related to neutrophil infiltration nor inflammation directly within the IAV-infected microenvironment.

We tested the hypothesis that FDG uptake was related to lung regeneration and repair after IAV-induced injury and inflammation. IHC staining for the nuclear antigen, Ki-67, detects cells undergoing proliferation (*i.e.*, cells in S, G2, and M phase of the cell cycle) (670) and is used on biopsy tissue as a diagnostic tool to confirm tumor diagnosis or the presence of neoplastic tissue (671). In lung cancer, Ki-67 staining is known to be correlated with FDG uptake measured by PET imaging (672, 673). We found a low background of cell proliferation in the lungs, especially in bronchioles, but higher in alveolar spaces. There was marked increase in the proliferation of cells within the bronchial epithelium up to seven dpi with IAV (Fig. 8). This matches the timing of FDG

uptake in ferrets seen previously (631), and corresponds to the increase in bronchiolitis and the presence of bronchiolar cytonecrosis, which was replaced by metaplastic bronchiolar epithelium beginning as early as 3 dpi and detected fully at 7 dpi. It is still unknown if proliferating cells contribute to the early FDG uptake (< 3 dpi) in infected ferrets. Small foci of FDG are often seen in locations within the thorax soon after infection. This may indicate the initiation of an adaptive immune response (*e.g.*, within lymph nodes by migratory dendritic cells, stimulated with IFN signaling from NK cells).

The often extensive cytonecrosis of the airway epithelium, followed by metaplasia of pneumocytes and large airway epithelium (depending on the severity of disease), is a common feature of infection by many IAV subtypes, including H1N1pdm (6, 8).

Normally, lung cells are quiescent and only enter the cell cycle after infection and injury as part of the regeneration process (reviewed in (674)). We have reported a significant enrichment of genes associated with cellular proliferation in primary human bronchial epithelial cell culture after IAV infection and found reduced virus replication in undifferentiated versus well-differentiated bronchial epithelial cells (331). Others have shown that ferrets infected with mild seasonal viruses, but not HPAI, also show significant enrichment of genes involved in cellular growth and proliferation (675).

Recently, IAV has been used to elucidate the method by which the distal airways rebuild from basal-like stem cells, a process which may continue for up to one month post-infection (676). Our studies suggest FDG could be used to probe the recovery of the

lungs after infection. Such data could be valuable for screening compounds that assist repair and regeneration of the lungs during this sensitive time.

Conclusions

The microenvironmental dynamics of IAV infection are important for controlling virus replication, inflammation and disease progression (52, 95, 126, 281, 660), however processes of inflammation extend beyond the site of infection (9, 85, 253, 360). Whereas *in vitro* experimental approaches are important for defining cellular and subcellular effects of virus infection, well-defined relevant animal models are essential for understanding how these cellular interactions define organ and organismal responses to infection (52, 600, 629, 632). These data support the importance of the whole organ response to infection, as well as immunological changes that occur at viral foci.

Neutrophils migrate into the lungs early and accumulate in sites of infected bronchioles, potentially responding to chemokines released by IAV-infected cells. A second wave of neutrophils is detected a 3 dpi, which have altered gene expression profiles suggesting differences in maturation/phenotype. Live imaging of infected ferrets suggested increased uptake of FDG at sites of cellular proliferation, and not necessarily at sites of leukocytic infiltration. Future studies should address several questions raised by the data herein, such as the site-specific actions of neutrophils; the mechanism behind the biphasic temporal distributions; and the phenomenon of epithelial regeneration in the context of the entire lung or entire organism during infection.

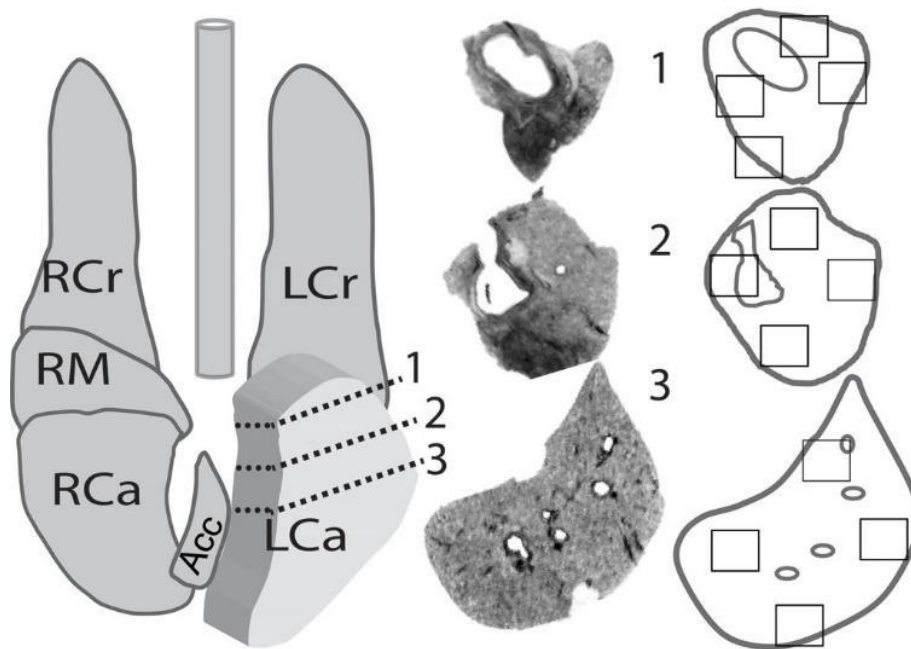


Figure 4.1. Sampling scheme for experiments on the temporal and spatial distribution of inflammation and infection in ferrets after H1N1pdm challenge.

A comprehensive histological sampling of ferret lungs was performed at time points following intranasal infection of ferrets with 10^6 TCID₅₀/ferret H1N1pdm virus (A/Kentucky/180/10). Samples were taken at 0.25, 1, and 3 dpi from 2 infected ferrets per time point, and 2 mock-infected ferrets at 3 dpi, immediately following PET-CT imaging. The diagram of a ferret lung (left) shows the method by which the left caudal lobe was divided (dashed lines on expanded lobe) for histology. The right caudal lobe (cuts not shown) similarly was divided into at least 4 cross-sections along the main branching bronchus. The resulting cross-sections of tissues are shown in the center column as black-and-white photos of histological slides, numbered according to locations on the lung diagram. To the right, outlines of the tissues were given to a blinded observer to select four regions for IHC analysis (black squares on the right). This sampling scheme for IHC was used to analyze the pattern of neutrophil infiltration, as well as

computed tomography and positron emission tomography images. Ferret lung lobes are labeled as follows: RCr, right cranial lobe; LCr, left cranial lobe; RCa, right caudal lobe; RM, right middle lobe; Acc, accessory lobe.

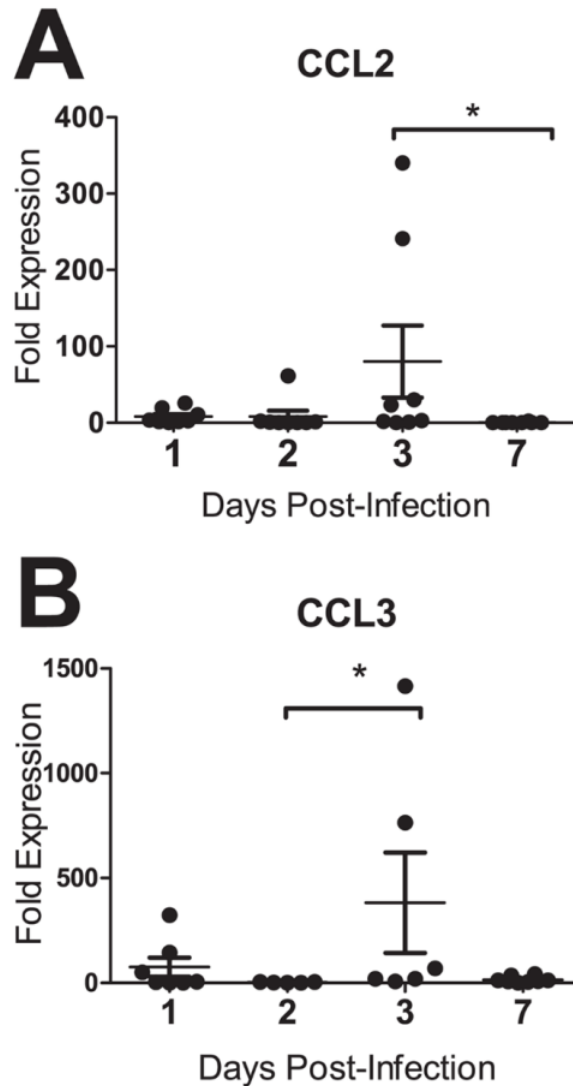


Figure 4.2. Inflammatory cytokine and chemokine gene expression in the ferret lung after infection with influenza A virus (H1N1pdm).

Female ferrets were infected with 10^6 TCID₅₀ per ferret of H1N1pdm virus, A/Kentucky/180/2010 ($n = 2$ ferret per timepoint). The right caudal lung lobe from each H1N1pdm-infected and mock-infected ferrets ($n = 1$ ferret per timepoint) was taken upon euthanasia on days 1, 2, 3, and 7 post-infection and divided into four sections. Each section was homogenized separately and cDNA synthesis was prepared for gene expression analysis. Ferret gene-specific primers were developed to cross exons and RT-

PCR was performed to identify gene expression of leukocyte chemoattractant chemokines (A) CCL2 and (B) CCL3. Fold expression is compared to expression of housekeeping controls (GAPDH) and mock-infected animals using the delta-delta Ct method. Asterisks indicate significant differences between days by nonparametric statistical tests, corrected for multiple comparisons..

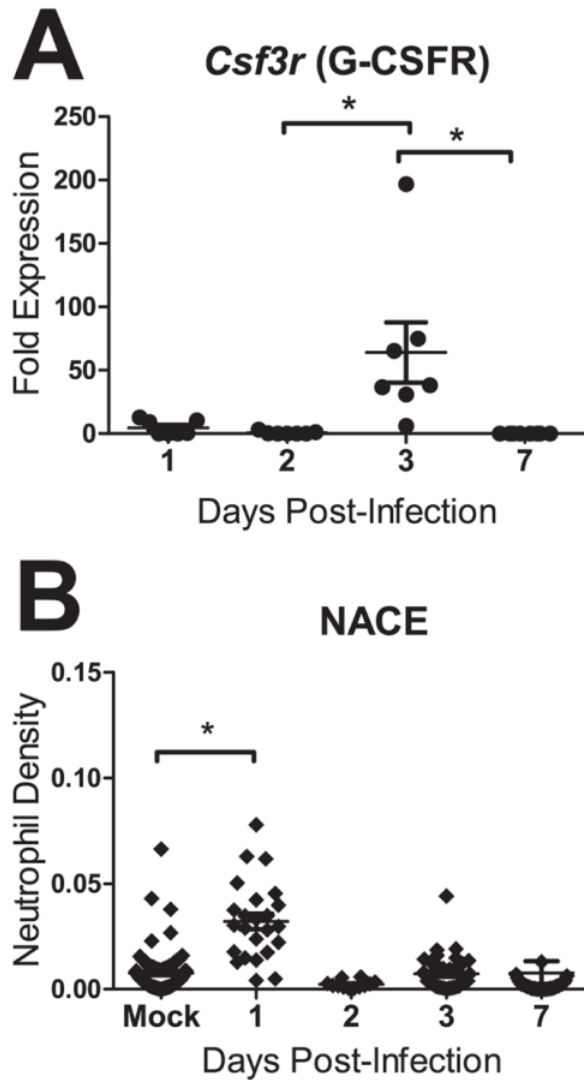


Figure 4.3. Neutrophils were detected in ferret lung sections after infection with influenza A virus (H1N1pdm).

Female ferrets were infected with 10^6 TCID₅₀ per ferret of H1N1pdm virus, A/Kentucky/180/2010 ($n = 2$ ferret per timepoint). (A) The right caudal lung lobe from H1N1pdm-infected and mock-infected ferrets ($n = 1$ ferret per timepoint) was taken upon euthanasia on days 1, 2, 3, and 7 post-infection and divided into four sections. Each section was homogenized separately and cDNA synthesis was prepared for gene expression analysis. Ferret gene-specific primers were developed to cross exons and RT-

PCR was performed to identify gene expression of a neutrophil-expressed gene (*Csf3r/G-CSFR*) relative to housekeeping controls and mock-infected animals. Asterisks indicate significant differences between days by nonparametric statistical tests, corrected for multiple comparisons. (B) Other lung lobes (left cranial, right cranial, left caudal, and middle) taken from each ferret were prepared for IHC, and one slice from each was stained for neutrophils (NACE). Neutrophils were quantified in 20X images taken from 3 regions on each of the NACE-stained slides using ImageJ image analysis software. The number of NACE-pixels divided by the number of total tissue-containing pixels are presented as the “neutrophil density”. Asterisk indicates significant difference in distribution compared to mock-infected ferrets using non-parametric statistical tests.

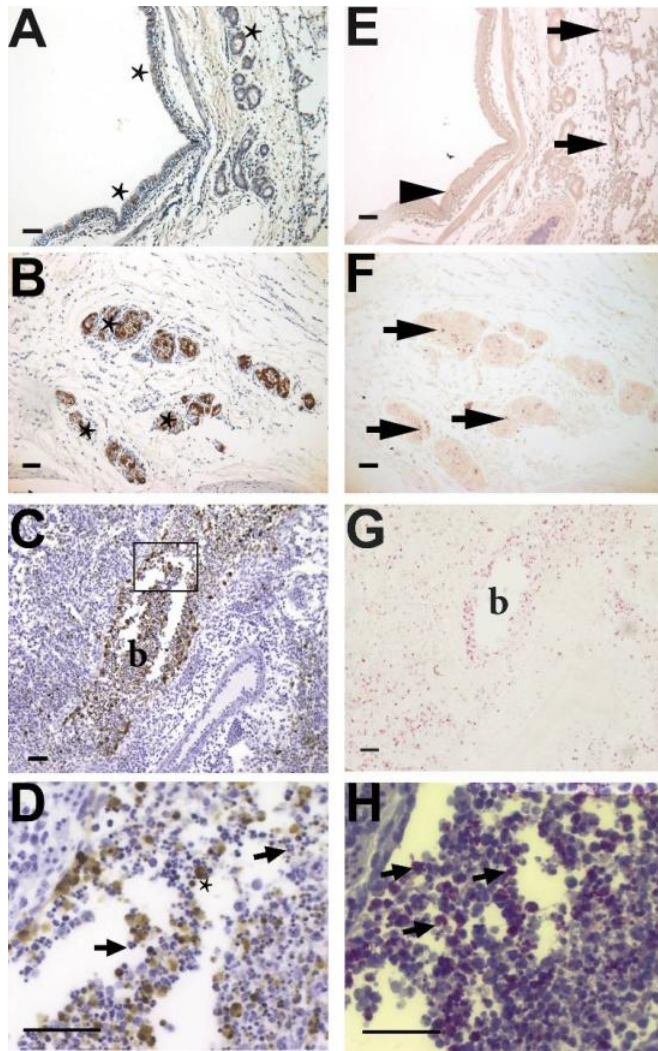


Figure 4.4. Immunohistochemical staining of influenza nucleoprotein and neutrophils in ferret lungs.

Histological preparations from the right caudal lobe of a female ferret 1 day post-infection with 10^6 TCID₅₀ of an H1N1pdm virus isolate (A/KY/180/2010). On the left panels (A-D), influenza antigen (nucleoprotein, NP) is stained brown with hematoxylin (blue) counterstain. On the right panels (E-H) neutrophils are stained pink with naphthol AS-D chloroacetate esterase (NACE) in a serial section of the same region of lung as the IHC NP stain (A-D) (without counterstain in E-F to show bright pink NACE stain, blue hematoxylin counterstain in F). (A) Influenza-infected cells (stars) in the pseudostratified

columnar epithelium of the bronchus of a ferret 2 days post-infection. (B) Infected seromucinous glands (three are marked with stars but more are visible). (C) Infection of the bronchiolar epithelium (lumen marked “b”) was more common than other sites, and antigen can be seen within luminal cells. (D) Magnification of the indicated region in (C) shows that many NP-positive cells (star) are macrophages, and the lumen of the bronchiole is filled with polymorphonuclear neutrophils (arrows show two but many more are visible). (E) Few neutrophils are present within the epithelial layer of the bronchus at sites of influenza-infected epithelial cells (arrowhead), but more are present within the alveolar spaces adjacent to this site (arrows). (F) Inflammatory cells within the infected submucosal glands are neutrophils (three areas are indicated with arrows but more are present in each gland). (G) Extensive neutrophil infiltration in the bronchiolar lumen (“b”). (H) Magnification of a serial section of (G) that is stained for NACE and counterstained with hematoxylin to show the morphological features defining neutrophils (arrows show three but many more are visible). Panels A-C and E-G were taken with 10X objective, D and H were taken with 40X objective. Horizontal black bars in all panels are 50 μm long.

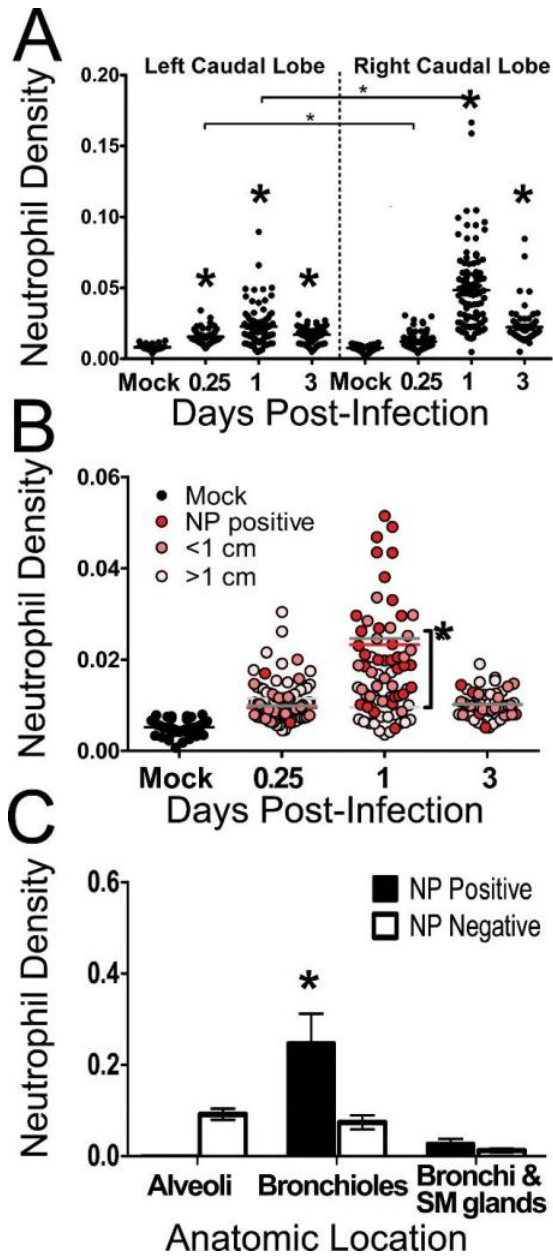


Figure 4.5. Neutrophils in the lungs of ferrets after infection with influenza A virus (H1N1pdm).

Ferrets were infected with 10^6 TCID₅₀ of an H1N1pdm virus isolate (A/KY/180/2010), and lung tissues were taken upon euthanasia and processed for histology. Caudal lung lobes, taken from two infected ferrets per day (0.25, 1, and 3 days post-infection, dpi), were systemically divided and prepared for histology. Neutrophils were quantified from

histochemical staining with naphthol AS-D choroacetate esterase (NACE) using image analysis software, and data are presented as neutrophil density - the number neutrophil-positive pixels per total tissue pixels on each image. (A) Neutrophil density is quantified over time, dividing data by lobe. Asterisks indicate significant differences from mock-infected animals, and significant comparisons between lobes are shown as bars with asterisks above (nonparametric statistical tests, $p < 0.05$). (B) Influenza nucleoprotein (NP) was identified on histological sections with IHC. The neutrophil density was calculated from the serial NACE-stained section, grouped according to distance from known NP-positive cells and matching sites of IAV-positive cells to distant sites within the same ferret (red, pink, and white dots to indicate NP-positive, < 1 cm from NP-positive, and > 1 cm from NP-positive sites, respectively) to mock-infected animals (black dots). Data are plotted over time, and means within a given timepoint are shown colored according to distance (red, pink, and grey lines for NP-positive, < 1 cm from NP-positive, and > 1 cm from NP-positive sites, respectively). Asterisk indicates significant difference between sites within a given timepoint. (C) Neutrophil density was calculated from pictures taken at sites of known influenza infection (NP-positive) and grouped by anatomic region (alveoli, bronchioles, and bronchi with submucosal [SM] glands). These images were site-matched within the same ferret (< 1 cm from known IAV-positive sites) to anatomic regions that had no IAV-positive cells (NP-negative). *N.B.*, NP-positive cells at alveolar sites were leukocytes; and mock-infected animals are not shown on this panel, but are shown above. Asterisks indicate significant difference between NP-positive and NP-negative sites.

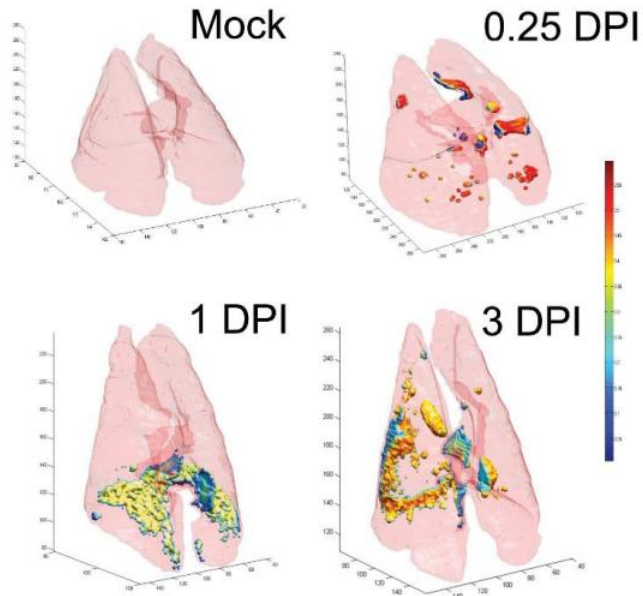


Figure 4.6. Rendering of PET-CT images of ferret lungs at different time points post-infection with H1N1pdm.

Stacked whole lung images give a 3-dimensional view of ferrets at three timepoints following infection with 10^6 TCID₅₀ H1N1pdm virus (A/KY/180/2010); only one ferret shown per time point, including a mock-infected shown at 3 days post-infection (DPI). Ferrets were injected with an intraperitoneal dose of radio-labeled [¹⁸F]-fluorodeoxyglucose (FDG) and imaged using PET-CT one hour later. For each image, the light pink surface corresponds to the border of the lung which was segmented from CT images by an automated CT analysis. PET-CT fusion images were used to define the uptake of FDG within the segmented lung space, and the intensity of FDG uptake is illustrated as small colored volumes within the lung borders. Regions of FDG uptake are colored according to voxel intensity within the FDG-PET image as shown on the color scale on the right (from <0.05 in blue to >0.5 in dark red).

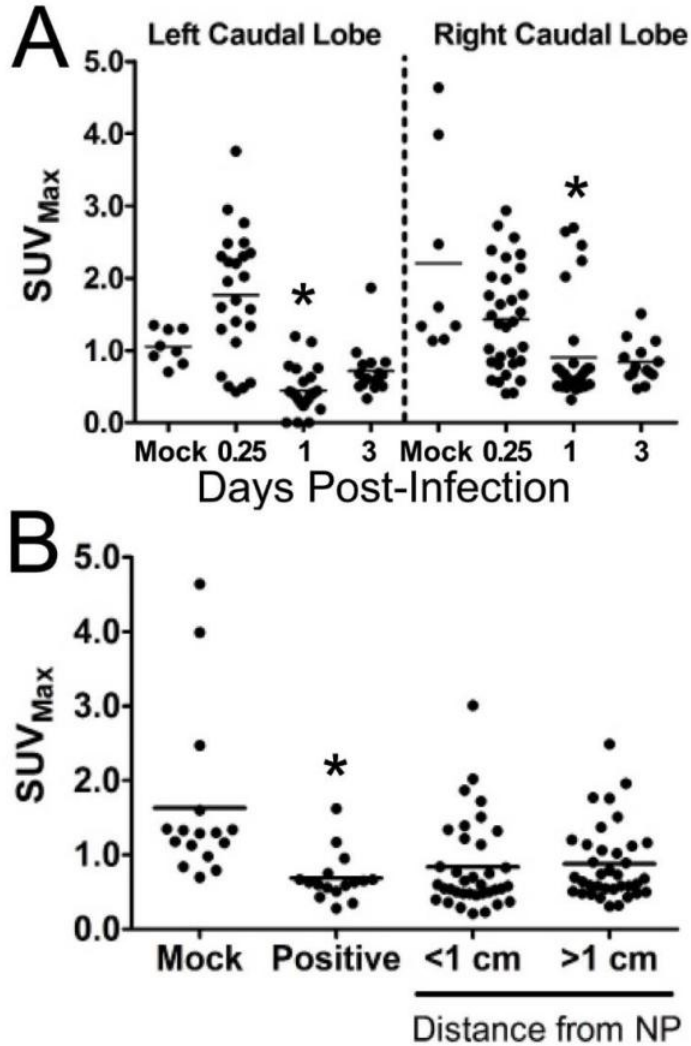


Figure 4.7. Distribution of cellular uptake of radiolabeled FDG during influenza infection in ferrets.

Female ferrets were infected with 10^6 TCID₅₀ of H1N1pdm virus (A/KY/180/2010). A radiolabeled glucose analog ([¹⁸F]-fluorodeoxyglucose, FDG) was injected at 0.25, 1, and 3 days post-infection and ferrets were imaged 1 h later ($n = 2$ per time point). Ferrets were euthanized immediately after imaging and lung tissues were prepared for histology.

(A) The temporal distribution of FDG uptake (maximum standard uptake value, SUV_{max}, within a defined volume) was measured in images taken by positron emission tomography after locating histology slices using anatomical references in registered

computed tomography images. These areas matched areas analyzed for neutrophil infiltration, and approximate a 3-D grid of each ferret caudal lung lobe. (B) The spatial distribution of FDG uptake at sites in the ferret lung grouped by distance from IAV nucleoprotein (NP)-positive cells within the lungs. The presence of H1N1pdm was measured with immunohistochemistry staining for viral nucleoprotein. Asterisks indicate statistically significant differences from mock-infected animals.

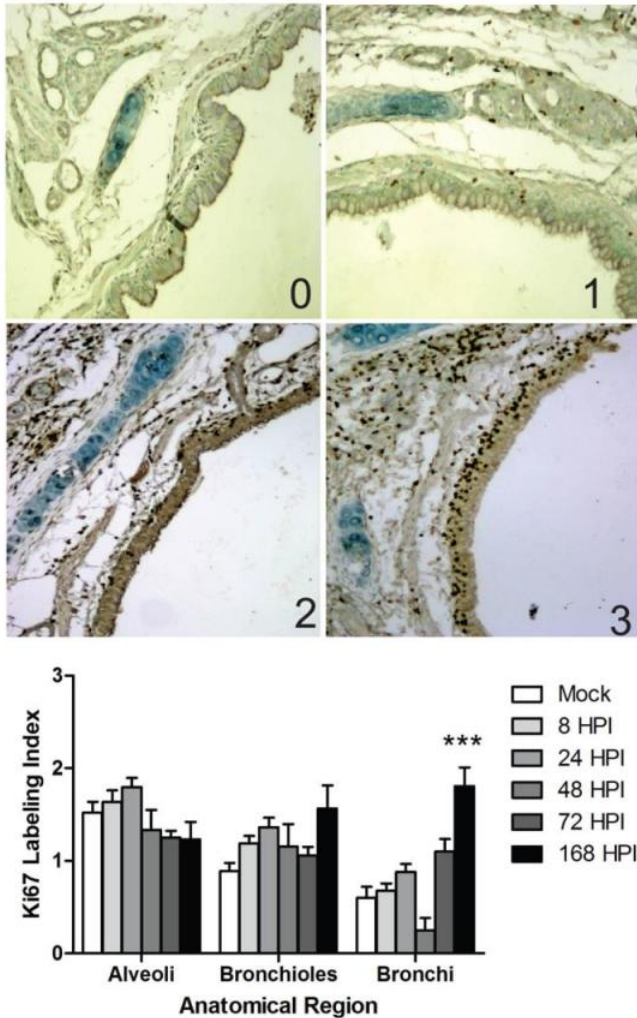


Figure 4.8. Proliferation of lung epithelial cells measured by Ki-67 labeling index.

The top four images are representative of the scoring system used to grade (labeling index values of 0-3) the extent of lung cellular proliferation (Ki-67 antigen positivity) in immunohistochemical sections taken from ferrets infected with 10^6 TCID₅₀ H1N1pdm virus (A/KY/180/2010). The left and right cranial, left caudal, and middle lobes were taken at necropsy (at 8, 24, 48, 72, and 168 hours post-infection, HPI) and prepared for histology. Various anatomical locations were analyzed in one lobe of each ferret per timepoint, including bronchi, bronchioles, and alveolar spaces. The images were grouped

anatomically and scored by two independent observers for the amount of Ki-67 positive cells using the guides above. The bottom figure shows the mean proliferation index per anatomic region over time. For reference of scale, the height of the numbers is 50 μm in the images. Asterisks indicate significant difference from mock within each anatomical region (***, $p < 0.01$) using nonparametric statistical tests.

Table 4.1. Primers used to identify ferret leukocyte-specific and inflammatory genes.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ELANE	GCTGCTGAATGACATCGTG	CACGTTCTGTTGATGGTG
CCL2	CAGAAGTGGGTCCGGGATTC	TTCATGGCTTTGCAGTTTGGG
CSF3R	AGAGCTGGAAGATGGAGCAT	TAGAGCTGAAAGGGCCTGAT
CCL3	GGTCTTCTCTGCACCATTTG	CTTGCGTGTAACTGCTTGG
MMP9	AGCGAGAGACTCTTCACCCA	CAGTGGTGCAGGTGGAGTAA
CSF1R	ATGGAGACACCAAGCTCACA	CATCTTGGAAGCCTACGTGA
CD11B	CTGGAGCTGCCTGTGAAATA	ATAATGCGGCTGGTCTTCTC
TNFA	CCAGATGGCCTCCAATAAT	CAGCTTCAGGGTTTGCTACA
IL6	ACCGGTCTTCAGGAGTTTCA	AAGAACTGGAGCAGGTGTTTG
IFNB	TCCCAGAAGTTCCAGAAGGA	TCGACAATGCTCTCATTCCA
IL8	GTGAAGCTGCAGTTCTGACG	GGGCCACTGTCAATCACTCT
SLC2A1	CTGCTGAGCGTCATCTTCAT	TTCTTCAGCACACTCTTGGC
NCR1	GCACCGAGAAGCAGAATCTT	GCTGGTACTTCACAGCCTCA
GZMA	AGCTCACTGTGTCCTGAACAA	TCAAAGCACGGATAAGGAAA
MPO	TCCCTGAGGAGGAAGAGATG	GCTGGTGTGAGCATGTCAGT
GAPDH	AGCTGAATGGGAAGCTCACT	CTGCTTCACCACCTTCTTGA
IAV HA	CACCAGTCCACGATTGCAATA	ATGGGAGGCTGGTGTATAGC

CHAPTER V

CONCLUSIONS

Influenza A viruses (IAV) may cause severe respiratory disease, and emergent strains with increased virulence have the potential to infect the world's population within a short time frame. These points have been illustrated in just in the last fifteen years with the emergence of H5N1 in 2003, the re-emergence of a swine-origin H1N1 resembling the 1918 Spanish influenza in 2009, and the emergence of avian-origin H7N9 in 2013. Fortunately, in the aftermath of each virus outbreak, none had both the virulence and transmissibility that is feared of the next IAV pandemic. Importantly, these events have resulted directly or indirectly in increased surveillance and increased funding into IAV research, and valuable data has been collected. To date, few infectious diseases have received more attention than the 2009 H1N1 "pandemic" IAV (Figure 5.1). This dissertation uses H1N1pdm infection of mouse and ferret animal models to investigate the timing and magnitude of general and specific host immune responses to IAV viruses. In conclusion, the findings from the experiments presented herein are summarized in the context of very recent publications in the field of IAV research, highlighting the benefits and limitations of animal models for understanding IAV pathogenesis in humans.

The timing of an IAV infection

The earliest responses to IAV. The studies presented here help establish a temporal-spatial course of disease for H1N1pdm and provide a model to test determinants of lethality (Figure 5.2). Shortly following H1N1pdm infection, the virus enters cells and begins to replicate its genome. The ferret model provided information that neutrophils arrive in the lungs as early as 6 hpi (Figure 5.2). This coincides with the gene expression of type I IFN and release of ISG products, such as CXCL10, from *in vitro* infection of human primary epithelial cell culture with H1N1pdm at 6 hpi (331, 332, 334, 335). Others have shown that virus detection and inflammatory response genes, such as IL-6, CXCL8, and CCL5, are upregulated as early as 3 hpi following *in vitro* infection of human primary bronchial epithelial cells with both seasonal H1N1 and H1N1pdm (330, 333). Detectable secretion of these products into cell culture media is detected by 24 hpi (330-332, 334, 335).

The expanding inflammation. Host responses within the viral microenvironment are important determinants of influenza pathogenesis; however these effects are transmitted systemically (360, 363). The systemic amplification of these signals, which influence the recruitment of inflammatory cells during IAV infection, is determinants in the outcome of IAV disease (9, 14, 94). This amplification occurs through endothelial cells, which are also important for leukocyte recruitment to the site of infection (20, 60, 64, 360, 472, 538). In mouse models, it has been suggested that increased inflammation and increased inflammatory leukocytic infiltration in the lungs contributes to the increased

pathogenicity of IAV (85, 86, 130, 136, 139, 247, 253, 323, 363, 375, 379, 430, 539).

What remains unclear is the connection between inflammation at the site of infection and how this amplification of inflammation systemically affects disease outcome, specifically with regard to innate leukocyte recruitment and activation.

The severity of disease is related to the quality of the “signal.” The fidelity of this immune signal – the timing and the context – is critical to the responses of recruited white blood cells to a localized virus infection in order to contain the infection. We have established the recruitment of neutrophils to sites of inflammation and infection is very specific in space and time during a non-lethal H1N1pdm infection of ferrets (Chapter IV). Infiltrating leukocytes respond to the IAV-infected lung viral microenvironment by releasing effectors, such as ROS or additional chemoattractants, to additionally shape the microenvironment (441, 442). An abundance of activating signals for infiltrating leukocytes may contribute to the creation of an unrestricted “macroenvironment,” one that results in unmanageable inflammation for the host (86, 253, 380, 381, 396). Increased inflammation (due to viral determinants or chronic inflammatory disease in the host) may alter critical timing of the recruitment of immune cells, which may not be able to respond appropriately (e.g., recruited immature neutrophils do not have type I IFN receptors (530)). Moreover, IAV strains which alter specific signals, such as suppression of type I IFN production from infected cells (69, 199) or disabling neutrophil responses (421, 422, 431, 440), increase disease severity in ferrets and mice, respectively. In these

scenarios, the absence of a typical “viral” lung microenvironment restricts valuable information to needed to elicit an appropriate response.

The importance of macrophages in the immune response to IAV infection

Airway macrophages are also capable of responding quickly to IAV infection, as shown by experiments which infected primary human macrophages in cell culture (74, 341, 342, 374, 377, 595, 677). Macrophages infected with H1N1pdm are able to secrete CXCL10, TNF α , IL6 as early as 8 hpi (74), and increased secretion by 24 hpi (75, 342, 359, 377, 678) (Figure 5.2). More importantly, experiments using co-culture systems have shown that these interactions may regulate epithelial apoptosis within a viral microenvironment during IAV infection via TRAIL-DR5, and CD40-CD40L, and therefore may be important mediators of mucosal barrier integrity during severe infection (61, 65, 312, 341, 343, 344, 347, 352, 353, 373). These experiments have shown that type I IFNs are extremely important signals regulating these responses and therefore establish the viral microenvironment. Thus, multiple *in vitro* systems have indicated that the response of specific cell types to IAV infection is immediate, gene expression can be detected as early as 3 hpi, and secretion of immune mediators can be detected within the first 24 hours following infection. However, more research should focus on the use of coculture systems to reveal the importance of cellular interactions at the site of infection since these are physiologically relevant to the establishment of the viral microenvironment.

While co-culture models of infection and immune response are essential to establish a role for macrophages in IAV pathogenesis, it remains to be demonstrated whether IAV modulates the *in vivo* macrophage response to infection. Thus far, demonstrating viral replication within airway or exudate macrophages provides valuable background to support mechanistic studies of the affect infection has on the functions of macrophages during IAV infection (73, 74, 359, 375). Apart from their role as pathogen sensors and inflammatory signalers, resolution of infection (and inflammation) relies in part on the phagocytic functions of macrophages to clear debris and necrotic cells within these microenvironments (61, 79, 459, 679). This causes the release of anti-inflammatory cytokines which, in the case of increased inflammation, may not be sufficient to tip the balance towards recovery (61, 79, 459, 679). Indeed, the most severe form of IAV in humans causes a hypercytokinemia, which is characterized by both pro- and anti-inflammatory signals simultaneously expressed (9, 10, 14, 94). Much remains to be discovered about these critical fine-scale interactions at IAV viral foci. As we demonstrate, the ferret is an excellent model in which to pursue these questions.

Animal models for the study of influenza A virus pathogenicity in humans

Clinically, severe respiratory disease, including IAV, progresses in one of four ways: early or late recovery, and early or late death (680). Children and young adults (<65 years of age) were at increased risk for hospitalization from H1N1pdm infection during the first two waves of the outbreak in 2009-2010 (133, 681, 682). Additionally, patients with certain comorbidities experienced a more severe disease (*e.g.*, obesity, chronic lung

disease)(133, 682). It was important to establish whether the variability in human response to H1N1pdm virus was solely host comorbidity or if viral variants contributed to differential pathogenicity. The laboratory mouse is genetically homogeneous, and therefore variability in disease outcome following H1N1pdm infection is driven by virus-specific factors. Using the mouse model, we and others have shown that the variability in human responses to H1N1pdm infection may have been due, in part, to circulating genetic variants of H1N1pdm (130, 136, 247, 683-686). Importantly, key differences in disease between mice infected with closely related IAV strains was the timing and quantity of chemokines in the lungs and the resulting recruitment of inflammatory cells such as neutrophils (130, 136, 685, 687). Moreover, genetically similar H1N1pdm viruses produced variable host responses in outbred animal models as well including ferrets and macaques (127, 130, 132, 247, 683, 686). Animal models were essential to understanding these very basic features of the 2009 H1N1 global pandemic, when only genetic information and patient reports were available (127, 132). Therefore, it is important to understand the benefits and limitations in animal models, which currently offer the best information for inferring human IAV disease severity.

Testing the limits of the mouse model: host determinants of pathogenicity

In nature, there is no such thing as “mouse influenza” (as far as we know, and there is limited interest in understanding IAV pathogenesis in mice for the benefit of mice). It is known that IAV virulence determinants are mouse strain-specific, further illustrating the need for caution in using mouse models (136, 250, 683, 684). Thus, our discovery of

three-to-four distinct mouse immune responses to H1N1pdm infection, although a useful tool to understand differences between H1N1pdm isolates (viral determinants), may not offer great insight into pathogenicity in humans (host determinants). One group has recently demonstrated that the variability among genetically similar H1N1pdm isolates is only observable in certain mouse strains, indicating that our discovery may not even extend to mice other than DBA/2J mice (130, 136). However, certain aspects of IAV infection of the respiratory tract seem to be similar for many mammalian species. In humans, increased cytokines and chemokines in the blood and respiratory samples (e.g., nasal lavage) during H1N1pdm infection were correlated with increased disease severity (105, 112). Cytokines and chemokines such as IL-6, TNF α , CCL2, CXCL8, and CXCL10 were also higher during infection with a more lethal H1N1pdm virus isolate in macaques (*e.g.*, (686)), ferrets (*e.g.*, (71)), and mice (*e.g.*, (130, 683)). It has been demonstrated that release of these chemokines are related to the increased recruitment of inflammatory leukocytes to the site of IAV infection in mice (85, 86, 130, 247, 294, 323, 363, 539, 683). However, the precise relationship between increased cytokines and chemokines and disease severity remains unclear, and this may be where the limit of a mouse model is drawn.

Testing the limits of the mouse model: viral determinants of pathogenicity

While interpretation of host determinants of increased IAV pathogenicity must require strict prudence, is the same true for the interpretation of data showing viral determinants of increased pathogenicity in the mouse? Does pathogenicity of emergent IAV hold true

for all mammals? Are there species-specific determinants of pathogenicity and how are they linked to host response? This is an interesting problem in the field.

Preliminary data linking viral traits and immune response

Using the advances in reverse genetics, it is possible to test hypotheses regarding the contribution of specific amino acid constellations in viral proteins that affect the timing and magnitude of specific immune responses. For example, in we (chapter III) and others have observed that H1N1pdm viruses possessing an HA with an alpha 2,3-linked SA binding affinity are lethal in DBA/2 mice (*e.g.*, a D222G mutation in HA (125, 129, 131, 685)), which supports the hypothesis that receptor binding drives pathogenicity in mice (50, 125, 230, 247). In preliminary studies currently underway, we have used reverse genetics to create a virus with this HA molecule on the background of another H1N1pdm virus that is lethal in mice but does not have alpha 2,3-linked SA affinity (A/Netherlands/602/2009 [= “NL602”] differs from KY180 HA at 6 amino acid positions). Materials and methods for these experiments were similar to those in chapter II (*e.g.*, 6-8 week old female DBA/2 mice were infected intranasally with 30 μ L virus). There were no differences in lethality or morbidity (weight loss) between KY180- and NL602-infected mice (10^2 TCID₅₀/mouse), indicating that NL602 has increased virulence independent of alpha 2,3-linked SA binding (Figure 5.3). Moreover, we did not observe differences in virus in the bronchoalveolar lavage (BAL) at 3 dpi, nor in cytokine and chemokine secretion in the BAL at 3 dpi between mice infected with either wildtype viruses or the reassortant virus (10^5 TCID₅₀/mouse) (Figure 5.4). However, at this

dosage, mice infected with viruses possessing an alpha 2,3-linked SA binding HA had reduced lung cellularity and an altered balance between airway macrophages and neutrophils following infection (Figure 5.5). Thus, for H1N1 viruses in mice, specific mutations in HA may be traced to surprising effects beyond receptor tropism, and these effects are related to the interaction between systemic and local inflammatory responses (254, 688, 689).

Host adaptation approach to identify virulence determinants

As mentioned previously, this specific polymorphism in HA (D222G/N/S) was also associated with increased virulence in humans (125, 547, 558, 559, 561-563, 690, 691). Thus, perhaps we can infer that increased affinity to avian-type SA linkages is a common virulence determinant for mice and humans. To attempt to identify additional viral variants that have increased virulence in humans for which mice can be used to study pathogenicity, we may try a reverse approach. Adaptation of IAV (and many other viruses) within a host species typically leads to attenuation of the virus upon coevolution over many cycles of continued transmission (682). Perhaps we can identify viral variants that have disappeared from the H1N1pdm gene pool following prolonged transmission between humans and ask: were these associated with increased pathogenicity in humans, and were these associated with increased pathogenicity in mice? Researchers in the United Kingdom divided sequenced human H1N1pdm isolates into first/second/third wave to show human adaptation (692). Elderfield, *et al.* showed that third wave H1N1pdm isolates induced less type I IFN in human cells *in vitro*, had

distinct mutations in HA which increased tropism for alpha 2,6-linked SA, and increased polymerase activity within mammalian cell culture (692). More to the point, these mutations were less lethal in mice (136).

A similar analysis is presented in Table 5.1, comparing the circulating polymorphisms of H1N1pdm in humans taken three years after the 2009 emergence of H1N1pdm in the United States to those circulating during 2009 (*i.e.*, during the first two waves of the H1N1pdm). The HA, PA, and PB2 genes are compared, since these are established virulence factors for many IAVs and mutations within these genes are known to contribute to virulence differences (177, 251, 275, 294). For simplicity, polymorphisms are compared to two Kentucky H1N1pdm sequences of known lethality in mice (130). In general, the results suggest that increased pathogenicity was associated with rare variants that were likely not adapted to mammals.

Polymerase proteins: For example, 3 of the 4 polymorphic sites in PA have mutations in 2009 that disappear from the circulating human viruses by 2012 (Table 5.1). Therefore, PA I14, PA D610, and PA Q716 are likely avian-adapted mutations, and they may contribute to altered fitness and/or virulence in mammals. This is not surprising since it is known that the PA and PB2 both came from avian-adapted ancestors (1). Therefore, reverse genetics approaches that focus on the contribution of these mutations to virulence in the mouse model may be beneficial to understanding human pathogenicity.

Hemagglutinin: Interestingly, only one of the selected sites in the HA protein shows potential adaptation to mammals (HA2 E47K). The lysine at position 47 of HA2 went from 20% of the circulating viruses to 100%. This polymorphism was shown to be involved in stability of the virion, and specifically the pH stability of the HA (287). This type of change is a known avian-to-mammalian host-adaptation, and it is important for fusion and entry of the virus into the host cell (693, 694). Using syncytium formation assays and erythrocyte lysis assays (common assays to determine pH of fusion for viral hemagglutinin proteins), we determined that there was no difference in pH of fusion for this viral polymorphism alone (HA2 E374K, unpublished data). This is further supported by the finding by others that an additional mutation is needed to alter pH of fusion for H1N1pdm viruses (287). The other polymorphisms in HA show limited evidence of human adaptation. Therefore, the isolate with decreased mouse lethality had the dominant variants (>90% of 998 isolates analyzed) in human cases of H1N1pdm isolated in the U.S. in 2012. HA is under many selective pressures, including herd immunity as well as host adaptations, and thus variants identified here may not necessarily indicate attenuation (or increased virulence). However, one of these variants was extremely well-studied (D222G/N/S/Y), and illustrates the type of evidence needed to connect viral genotype with phenotype in humans using animal models.

HA1 D222G, a case in point

Mice typically are not susceptible to infection with human isolates of IAV, and therefore it is common practice to adapt H1N1 and H3N2 IAV to mice by serial passage (249).

The immediate result is an IAV that replicates well in the respiratory tract of mice and causes increased lethality in mice (255, 695). Some of the variants isolated from Kentucky patients during the first and second waves of H1N1pdm had mutations in HA that have arisen from mouse adaptation and these mutations, alone, are known to increase the lethality in mice: D222G and S183P (255, 685). The mutation D222G, near the SA binding site in the head of the HA molecule, was also discovered from reconstructed samples of the 1918 Spanish flu, and was responsible for increased transmission of the virus between ferrets (276). This specific mutation, a glycine at position 222, is a virulence determinant in macaques (689) and in ferrets (125), and results in altered receptor tropism in human cell culture (563) and on glycan array (125). The G222 substitution is thought to allow the HA increased binding to alpha 2,3-linked SA while maintaining alpha 2,6-linked SA tropism, whereas the D222 virus has affinity to only alpha 2,6-linked SA (125, 276, 563, 689). Therefore, again, increased binding to avian-type receptors is an important virulence determinant considering the distribution of receptors in the host, particularly when there is an increase in avian-type receptors in the lower respiratory tract.

A fascinating study showed that the HA D222 mutation may function as a site for diversifying viral population (696). Memoli, *et al.* found this pattern of adaptation within a single patient with H1N1pdm over time, where the dominant D222 variant switched to a G222 dominant population. They turned to the ferret model to test the virulence of these populations, since the ferret is more similar to humans with respect to the

relationship of virus tropism and virulence. Ferrets were infected with a population of 100% D222 variants HA, a population where G222 was the major variant (8% D222), or a plaque-purified 100% G222 IAV population. They found that only the plaque-purified isolate (100% G222) had increased pathogenicity, but also led to the greatest diversity of the quasispecies group after infection (127, 132). This illustrates the implications of how viral quasispecies can affect the course of IAV disease, and how quasispecies can complicate testing hypotheses for a specific viral variant. Ultimately, it is important to frame these types of questions in the context of understanding human disease. Two additional studies ($n = 7$ and 357) showed that the D222 mutant was significantly associated with mild human cases of H1N1pdm, and the G222 mutant (or more specifically the D/G222 quasispecies) was associated with more severe cases of H1N1pdm (690, 691). In all, this well-studied mutation from H1N1pdm shows the level of evidence required to make a convincing argument for HA position 222 as a viral determinant of pathogenicity, and the utility of animal models for establishing this. Interestingly, both D222G and S183P emerged from a single passage within a BALB/c mouse model, perhaps eluding to the fact that more than one variant contributes to differential pathogenicity of quasispecies (138).

Future directions

We showed that increased H1N1pdm replication in mouse macrophage cell culture was associated with the isolate that caused increased lethality. This increased replication was not seen in human monocyte-derived macrophages (Gerlach, *et al.* unpublished).

However, viruses with increased lethality in mice may show differences in human monocyte-derived macrophage immune signaling (Gerlach, *et al.* unpublished). It is not known if this is true for mouse macrophages, or what the implications are of this in humans. Additionally, we observed increased neutrophils in the bronchoalveolar lavage (BAL) of mice infected with a lethal isolate compared to a non-lethal isolate of H1N1pdm (130). Because very little is known about neutrophils and IAV infection, we turned to the ferret model to validate this finding (Chapter IV). The ferret model showed that neutrophils are, indeed, recruited to the lungs and may become concentrated at sites of H1N1pdm infected epithelium. This establishes a foundation for further research into their role in IAV pathogenicity. Much more is known about neutrophils in mice, in general, and therefore cautious research on IAV and neutrophils may move back into the mouse model.

For both ferrets and mice, an important part of the neutrophil story is missing: the role of viral determinants of increased pathogenicity. What drives the increased lethality in mice? And is the increase in neutrophils related to the increased lethality in mice? Perhaps a proof-in-principle would be to make a virus that increases neutrophils in the lungs of mice without causing increased pathogenicity. These approaches to these questions in mouse models of H1N1pdm rely on the use of reverse genetics. For IAV, plasmid-based reverse genetics systems allow genetic manipulations of the viral genome – site directed mutagenesis, (limited) insertion of genes, or knock-out of certain gene functions – to study IAV pathogenicity (150, 697, 698). Currently, the best technique

involves simultaneously co-transfecting 8 plasmids into 293T cells (an easily transfectable cell line) and Madin-Darby canine kidney (MDCK) cells (for IAV, an easily “infect-able” cell line). The plasmids, one for each virus gene segment, contain bi-directional mammalian promoters to make both viral mRNA (for viral protein production necessary to make infectious virions) as well as minus-sense viral nucleic acid. Viruses are rescued and passaged in hen eggs or in MDCK cells to make virus stocks.

The Kentucky clinical isolates of H1N1pdm published in Camp, *et al.* (2013) provide unique tool to study IAV pathogenesis (130). There are 50 known polymorphisms among all 8 isolates, 22 between the lethal and non-lethal isolates which were focused on for publication (130). With the exception of D222G, none have been convincingly studied for their role in increased lethality. However, Camp, *et al.* (2013) show that lethality is not necessarily the only endpoint available; it may be possible to identify mutant viruses that increase lung neutrophilia, for example. (This may not be probable, as the host response is similarly complicated and inter-related.) It is fairly well established that mutations that affect receptor binding determine the pathogenicity in humans, mice, ferrets, and macaques; however, it has been shown that this is not always the case. Recently, experimental manipulation of H1N1pdm viruses to purposefully alter their receptor binding preference (*i.e.*, their affinity for only alpha 2,3-linked or only alpha 2,6-linked specificity) did not show altered replication or virulence in the ferret (688). Perhaps this binary paradigm for receptor specificity and virulence (*i.e.*, high versus low virulence is due to avian versus mammalian SA distribution in the LRT versus

URT, respectively) is more complicated, and perhaps differences in the frequency and distribution of complex sialic acid linkages within a specific host's respiratory tract is most important for understanding virulence (688), or perhaps it is just the remnants of a viral quasispecies in this specific experiment masking effects of specific viral variants (690, 691, 696). Again, this illustrates the fascinating complexities of understanding viral determinants of pathogenicity, and the importance of animal models to test hypotheses about IAV pathogenesis.

Influenza A viruses are looming and carry a potential to infect every human on the planet. The study of influenza A viruses is fascinating and complex, owing to their impressive variability. As a tool, they will continue to provide important insights into immune functions important for human disease.

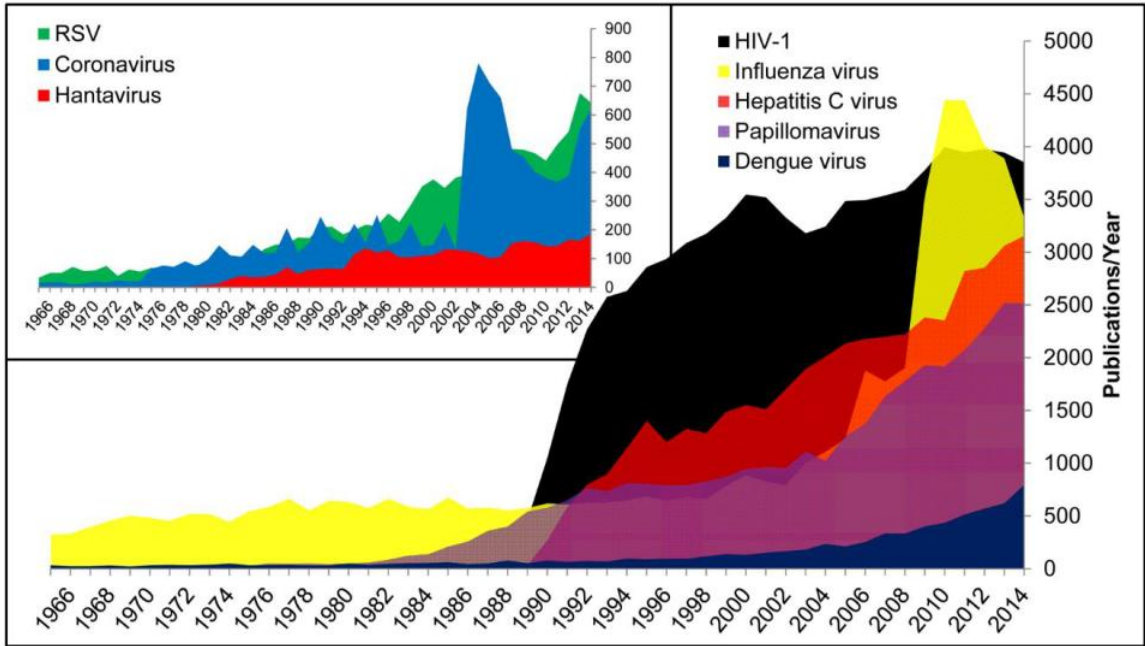


Figure 5.1 Research interest in viruses affecting humans.

The relative amount of scientific research devoted to each of 5 selected viruses is measured by the number of publications per year over the last 50 years (inset shows 3 additional viruses on a different scale over the same time frame). Source: NCBI PubMed searches for given terms; RSV = Respiratory syncytial virus; HIV-1, human immunodeficiency virus type 1.

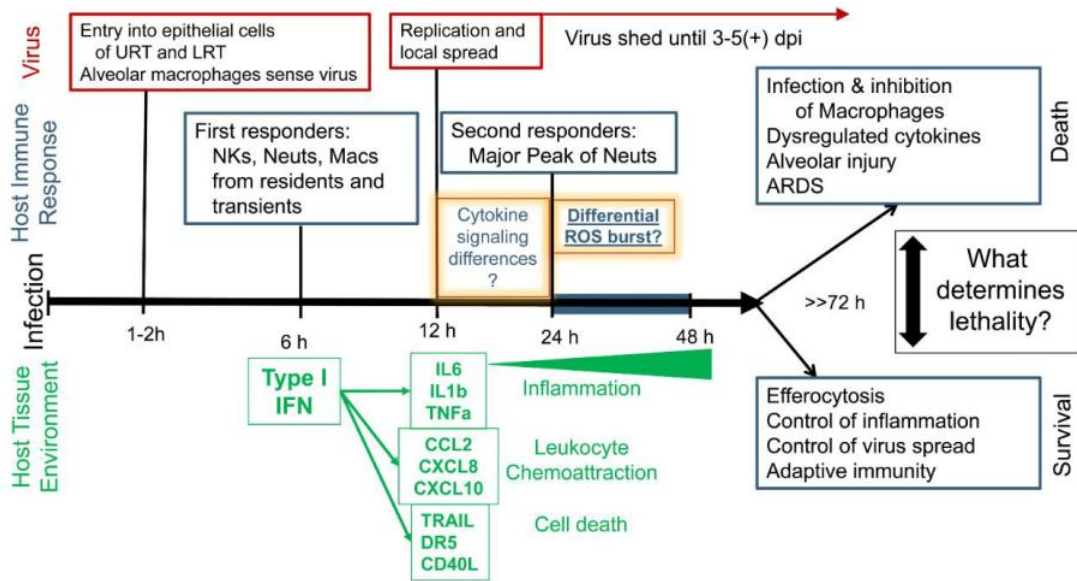


Figure 5.2 A course of disease following influenza A virus infection.

This figure is a prototype course of disease following infection with influenza A virus. Shown are major events in the viral replication cycle (red), the host immune response (blue), and the effects on the host tissue environment (green). A critical points for the

formation of severe disease is established following 48 hours.

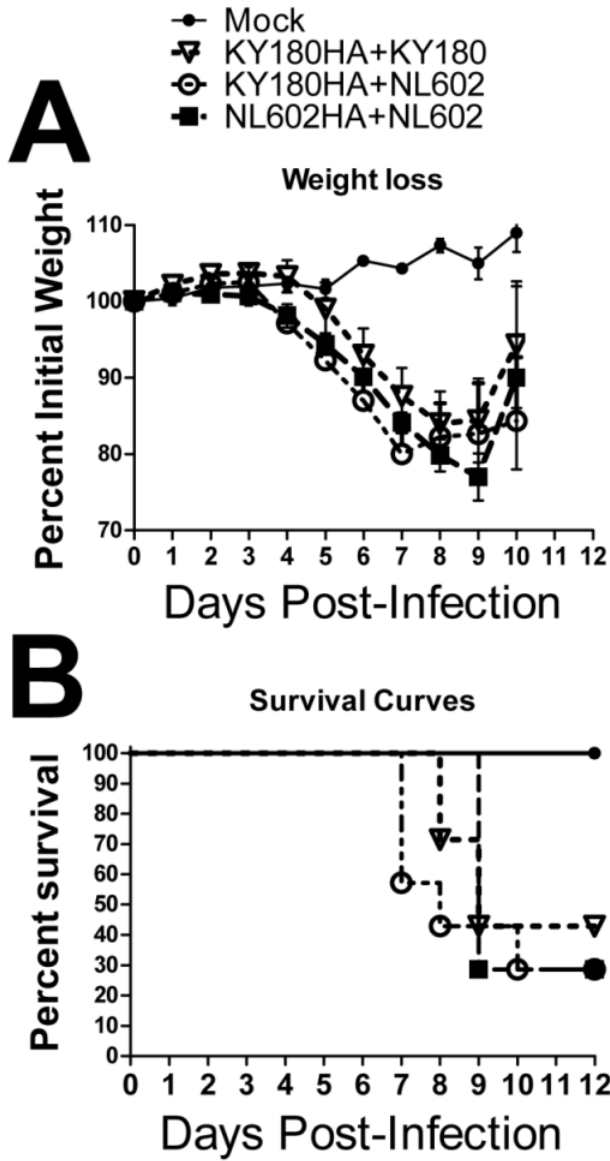


Figure 5.3 Weight loss and survival of mice infected with wild type and reassortant influenza A(H1N1)pdm09 viruses.

Mice were infected with human clinical isolates of influenza A(H1N1)pdm09 (H1N1pdm) viruses to test the hypothesis that the alpha 2,3-linked sialic acid (SA) binding is a major virulence determinant for mouse infection. It is known that

A/Kentucky/180/2010 (KY180) is lethal in DBA/2 mice, and the hemagglutinin (HA) binds alpha 2,3-linked SA. The A/Netherlands/602/2009 (NL602) isolate is also lethal in DBA/2 mice, however it does not possess an alpha 2,3-linked SA affinity. We used reverse genetics to create a reassortant virus that had the hemagglutinin (HA) of KY180 with the remaining gene segments from NL602 and infected mice intranasally with 10^2 TCID₅₀ of each virus. (A) All viruses caused weight loss following infection and (B) the infection was 70%-80% lethal for all viruses.

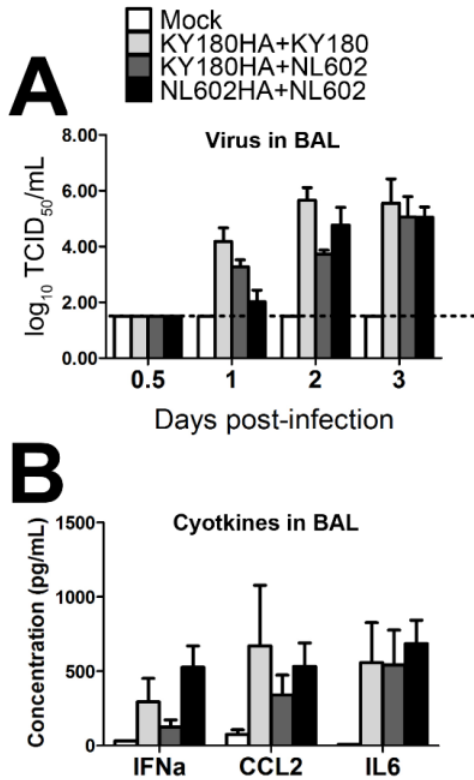


Figure 5.4 Virus replication and immune response in the lungs of mice infected with wild type and reassortant influenza A(H1N1)pdm09 viruses.

DBA/2 mice were infected with human clinical isolates of influenza A(H1N1)pdm09 (H1N1pdm) viruses: A/Kentucky/180/2010 (KY180) or A/Netherlands/602/2009 (NL602). Both isolates are lethal in DBA/2 mice, however the hemagglutinin (HA) from KY180 possesses alpha 2,3-linked SA binding affinity. We used reverse genetics to create a reassortant virus that had the HA of KY180 with the remaining gene segments from NL602 (KY180HA+NL602) and infected mice intranasally with 10^5 TCID₅₀ of each virus. (A) All viruses were detected at similar titers in the bronchoalveolar lavage (BAL) and (B) the interferon alpha (IFNα), interleukin-6 (IL6), and CCL2 were present in similar concentrations in the BAL at 3 dpi.

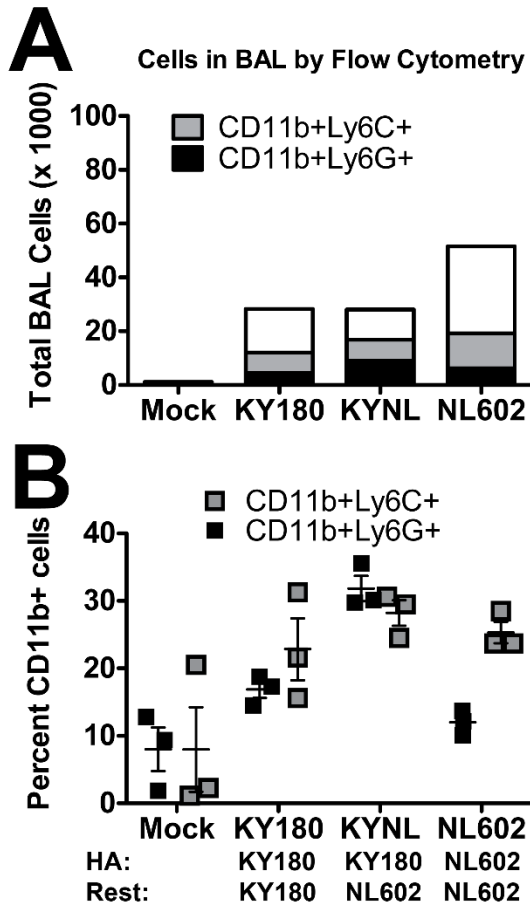


Figure 5.5 Cellular infiltration into the lungs of mice infected with wild type and reassortant influenza A(H1N1)pdm09 viruses.

DBA/2 mice were infected with human clinical isolates of influenza A(H1N1)pdm09 viruses: A/Kentucky/180/2010 (KY180) or A/Netherlands/602/2009 (NL602). Both isolates are lethal in DBA/2 mice, however the hemagglutinin (HA) from KY180 possesses alpha 2,3-linked SA binding affinity. We used reverse genetics to create a reassortant virus that had the HA of KY180 with the rest of the seven gene segments from NL602 (KY180HA+NL602) and infected mice intranasally with 10^5 TCID₅₀ of each virus. (A) Flow cytometry was used to measure the population of cells within the

bronchoalveolar lavage (BAL) at 3 dpi. The total number of CD11b+ cells was counted and the number of CD11b+Ly6G+ and CD11b+Ly6C+ cells are shown stacked within the total number of CD11b+ cells. The animals infected with viruses containing KY180HA had reduced overall lung cellularity compared to NL602-infected mice. (B) The percentage of Ly6G+ (neutrophils) cells and Ly6C+ (macrophages) cells relative to all CD11b+ cells in the BAL show that there are proportionately fewer neutrophils in the BAL of mice infected with the virus possessing the NL602HA at 3 dpi.

Table 5.1: Variants in influenza A(H1N1)pdm virus in the human population.

<u>HA</u>	<u>HA1:84</u>		<u>HA1:183</u>		<u>HA1:186</u>		<u>HA1:222</u>		<u>HA1:234</u>		<u>HA2:47</u>	
	Virul.	A.A.	Low	High	Low	High	Low	High	Low	High	Low	High
2009	100%	S	100%	0%	98%	2%	96%	1%	0%	100%	80%	20%
2012	93%	S	98%	2%	96%	3%	97%	1%	0%	90%	0%	100%
Total	97%	N	99%	1%	96%	4%	96%	1%	0%	97%	48%	51%

<u>Polym.</u>	<u>PA:14</u>		<u>PA:465</u>		<u>PA:610</u>		<u>PA:716</u>		<u>PB2:340</u>		<u>PB2:584</u>	
	Virul.	A.A.	Low	High	Low	High	Low	High	Low	High	Low	High
2009	87%	V	0%	99%	99%	1%	87%	13%	3%	97%	100%	0%
2012	99%	I	0%	100%	99%	0%	100%	0%	1%	98%	100%	0%
Total	86%	I	0.2%	99%	99%	0.6%	86%	13%	3%	97%	100%	0%

Hemagglutinin (HA, top), and two polymerase subunit proteins (PA & PB2, bottom) of H1N1pdm viruses isolated from patients in the United States in 2009 and 2012. Frequency of specific amino acid (A.A.) mutations at each site (top row) expressed as a percentage of the total known polymorphisms at each site for each year. The variants are divided into low virulence and high virulence (Virul.) based on the lethality of two viruses in DBA/2J mice.

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APPENDIX

PUBLISHED WORK

Listed below are co-authorship statements and acknowledgments for each of the previously-published chapters (chapters II and III). This information includes funding sources, author affiliations, author contributions, and complete citation information. Also included below is the co-authorship statement and acknowledgments for Chapter IV, which was undergoing peer review for publication at the time this dissertation was submitted. As stated below, previously-published chapters (*i.e.*, chapters II and III) are open-access articles distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. More information about Creative Commons licenses can be found at the following website: creativecommons.org/licenses. Reproduction of Chapter IV may be subject to publisher permission after the fact when it and if it is accepted for publication.

Chapter II – Co-authorship statements

Chapter II is composed of the following publications:

Camp JV, Chu Y-K, Chung D-H, McAllister RC, Adcock RS, Gerlach RL, Wiemken TL, Peyrani P, Ramirez JA, Summersgill JT, Jonsson CB (2013). Phenotypic Differences in

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The following analyses were performed by authors other than me:

- (1) Collection of clinical specimens was performed by TLW, PP, JAR, and JTS
- (2) Virus isolation from clinical specimens and collection of mouse samples from the screen of all isolates in DBA2 mice was performed by YKC.
- (3) Sequencing of virus isolates was performed by DHC, YKC, RCM and RSA
- (4) The final manuscript was written and edited by JVC and CBJ.

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The following analysis was performed by authors other than me:

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Chapter IV – Co-authorship statements

Chapter IV has been submitted to the Journal of Virology and is under peer review as of 4/24/2015.

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5. Camp, J.V., T.L. Svensson, A. McBrayer, C.B. Jonsson, P. Liljestrom, C.E. Bruder. 2012. "De-novo transcriptome sequencing of a normalized cDNA pool from influenza-infected ferrets." *PLoS One*. 7(5):e37104.
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7. Jonsson, C.B., J.V. Camp, A. Wu, H. Zheng, J.L. Kraenzle, A.E. Biller, C.D. Vanover, Y.K Chu, C.K. Ng, M. Proctor, L. Sherwood, M.C. Steffen, D.J. Mollura. 2012. "Molecular imaging reveals a progressive pulmonary inflammation in lower airways in ferrets infected with 2009 H1N1 pandemic Influenza virus." *PLoS One*. 7(7):e40094.
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10. Gerlach, R.L., J.V. Camp, Y.K. Chu, C.B. Jonsson. 2013. "Early host responses of seasonal and pandemic influenza A viruses in primary well-differentiated human lung epithelial cells." *PLoS One*. 8(11): e78912.
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12. Xu, Z, U. Bagci, A. Mansoor, G. Kramer-Marek, B. Luna, A. Kubler, B. Dey, B. Foster, G.Z. Papadakis, J.V. Camp, C.B. Jonsson, W.R. Bishai, S. Jain, J.K. Udupa, and D. Mollura. 2015. "Computer-Aided Pulmonary Image Analysis in Small Animal Models." *Medical Physics*. *In press.
13. *Camp, J.V., U. Bağcı, B. Squier, Y-K. Chu, M. Fraig, S. Uriarte, H. Guo, D.J. Mollura, C B. Jonsson. 2015. "Lower respiratory tract infection of the ferret by 2009 H1N1 pandemic influenza A virus triggers biphasic, systemic and local neutrophil recruitment." *Journal of Virology*. *In review.

Presentations and other scientific contributions

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“Phenotypic differences in virulence and immune response in closely related clinical isolates of influenza A 2009 H1N1 pandemic viruses in mice.” Poster at 2013 American Society for Virology Conference, State College, PA.

“Multi-focal Neutrophil Infiltration and Inflammation in Lungs of Ferrets Infected with 2009 H1N1 Influenza A Virus Clinical Isolate.” Orthomyxovirus Pathogenesis Workshop Presentation at 2014 American Society for Virology Conference, Ft. Collins, CO

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- T. L. Bissell Award, Georgia Entomological Society, 2006
- Georgia Entomological Society Scholarship, 2005
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- Member, Entomological Society of America, 2005 – 2007
- Member, American Society for Virology, 2010 – present
- American Society for Virology Student Travel Grant, 2011, 2013
- Integrated Program in Biomedical Sciences Fellowship, University of Louisville, 2011-2013

Employment History

Immunology Core Manager, August 2010 – August 2011

Center for Predictive Medicine for Biodefense and Emerging Infectious Diseases
Regional Biocontainment Laboratory
University of Louisville, Louisville, KY

Biologist, January 2009 – August 2010

Project: NIH C26 contract, Development of the ferret model of influenza
Laboratory of Colleen B. Jonsson, Ph.D.; Supervisor, Carl E. Bruder, Ph.D.
Department of Viral Biochemistry
Southern Research Institute, Birmingham, AL

Research Associate, February 2007- December 2008

Project: Mucosal immunology and HIV vaccination strategies
Laboratory of Pamela Kozlowski, Ph.D.
Gene Therapy Group
LSU Health Sciences Center, New Orleans, LA

Research Assistant, June 2005- December 2006

Exhibit curation and population genetics of the tick, *Ixodes scapularis*
Laboratory of Lorenza Beati, M.D., Ph.D.
U.S. National Tick Collection
Institute for Arthropodology and Parasitology, Statesboro, GA

Graduate Teaching Assistant, August 2004- December 2006

Department of Biology

Georgia Southern University, Statesboro, GA

Field Biologist, September 2001 – August 2004

Project: Ecology of Eastern Equine Encephalitis virus mosquito vectors

Laboratory of Eddie W. Cupp, Ph.D.

Vector Biology Laboratory

Auburn University, Auburn, AL