UNDERSTANDING FEATURES CONTRIBUTING TO SPECIES-SPECIFIC INFLUENZA VIRUS REASSORTMENT AND IDENTIFICATION OF NOVEL INFLUENZA A VIRUS PATHOGENICITY FACTORS

by

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(Under the Direction of Dr. Ralph Tripp)

ABSTRACT

The segmented genome of Influenza A viruses (IAV) allows for genetic exchange resulting in evolutionary shift. The 2009 pandemic was a result of a triple reassortment virus from avian, swine, and human origin which emerged from swine. Swine represent a mixing vessel for IAV supporting the replication of multiple lineages. Here, we investigated the role of the triple reassortment internal gene (TRIG) cassette in mediating species specific reassortment in swine with preferential reassortment of the glycoprotein genes hemagglutinin and neuraminidase. We also establish that *Petrous Alecto* kidney epithelial cells are permissive to infection with human and avian strains of IAV while supporting co-infection resulting in the generation of novel IAVs.

Immediately after the introduction of the 2009 influenza virus pandemic, reverse zoonosis occurred in swine resulting in continued circulation of the pandemic H1N1 virus in swine. Continued circulation allowed for reassortment to occur resulting

in the circulation of novel swine influenza viruses with altered pathogenic phenotypes as that observed with the pH1N1. In 2012, H3N2 variant (H3N2v) viruses were identified in Iowa state fairs in people which resulted from zoonotic transmission from swine. H3N2v infection resulted in enhanced morbidity and mortality and morbidity with the inability to transmit from person to person. Therefore, we investigated the pathogenic potential of reassortant viruses developed between the 2009 pH1N1 virus and an endemic circulating strain of swine influenza. To this end, we identified that specific genetic constellations and individual genes derived from swine could significantly enhance the pathology and immunopathology in ferrets and mice respectively. We show that the swine NA and PA can drive acute lung injury post infection through up regulation of MIP-2 resulting in increased neutrophil recruitment promoting inflammation and tissue damage.

INDEX WORDS: Influenza, reassortment, pathogenicity, acute lung injury

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DEDICATION

This work is dedicated to my fiancé, Shannon Hudkins, and my mother, Debbie Alford, without your continued support and faith none of this would be possible. You are both the most influential women in my life! Love you both

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Influenza A viruses (IAV) have caused several human pandemics in the last century, including one that emerged in 2009 resulting from a triple reassortment of viral genomic segments in swine. IAV is a significant public health concern resulting in >500,000 deaths/year worldwide a feature resulting in significant economic burden (1).

IAV is a member of the family *Orthomyxoviridae*, and is a pleomorphic, enveloped particle that contains a lipid membrane derived from the host cell. IAV has a segmented negative sense RNA genome consisting of 8 segments that encode for 10-12 proteins (2).

Due to the segmented nature of the influenza genome, co-infection of influenza viruses can occur and genetic reassortment of the 8 genome segments can result in novel reassortment viruses such as the 2009 pandemic H1N1 virus (3). This phenomenon is referred to as genetic shift. The mechanism by which influenza viruses preferentially reassort is currently unknown, but recent insights have provided some bits of information on what viral factors may be contributing to reassortment. Under unbiased circumstances, IAVs of the same strain reassort without barriers and preference allowing for significant viral mixing (4). However, when two independently unique strains of influenza virus co-infect the same cell an array of viral and host specific

barriers exist that preferentially support or restrict viral reassortment. IAV viral gene segments encode a genome packaging signal in the untranslated region of the viral RNA (5-7). This packaging signal is responsible for directing genome packaging at the cell membrane during viral assembly (8, 9). Genome packaging signals also insure that no more than 8 specific genes are packaged per virion (10). Influenza packaging signals exhibit strain and gene specific differences and that packaging signals may have a significant role in mediating the propensity for independent influenza viruses to reassort.

The polymerase complex of IAV is relatively well conserved (2), but work has shown that substitutions of polymerase genes between different strains of IAV show significantly high levels of incompatibility resulting in viruses with suboptimal fitness (11-15). Host specific determinants of IAV reassortment are extremely important, but no known mechanisms have been elucidated, but selective pressure via the immune response or host genetics drive the final checkpoint on whether a virus is optimally fit for replication and transmission.

Previous reports have shown that swine are susceptible to infections of influenza virus from avian and human lineages, and swine have been described as a mixing vessel' for influenza. For >80 years influenza evolution was relatively stagnant with classical swine H1N1 virus existing as the primary endemic strain of IAV (16). In 1998, most likely with enhanced production capacity of swine, multiple outbreaks of H3N2 and H2N2 viruses occurred in in swine populations in North Carolina and quickly spread through

the United States (17). Co-infection of swine resulted in the generation of novel reassortment viruses as double and triple reassortment viruses (18). The double reassortment virus failed to establish itself in the swine population; however, the triple reassortment viruses flourished and became the new primary strain of swine influenza. Interestingly, it appeared that reassortment continued with exchange of different glycoproteins, but the internal genes of the triple reassortment viruses remained stable and were later referred to as the Triple reassortment internal gene (TRIG) cassette. It is believed that the TRIG cassette provides selective advantage to support IAV reassortment in swine (19).

Until recently, swine were believed to be susceptible to multiple lineages of influenza virus due to sialic acid distribution in the respiratory tract, but recent published data has shown that the sialic acid linkage profiles in the lungs of humans and swine are not substantially different. Some unknown swine host factors are definitively contributing to this concept of swine as a mixing vessel for influenza.

Since the emergence of the 2009 pH1N1 virus, increased surveillance of swine influenza has been ongoing along with studies that are investigating the potential for generation of novel reassortant viruses resulting in co-infection of the pH1N1 and current circulating strains of seasonal influenza. Reverse zoonosis was observed with 2009 pandemic only months after the outbreak started (20), and in 2011, H3N2 variant viruses were identified in Iowa state fairs which resulted from reassortment of H3N2 swine TRIG viruses obtaining the pandemic M gene which resulted in zoonotic

transmission and a high incidence of mortality; however the H3N2v viruses failed to establish human-to-human transmission (11, 21-23).

The 2009 pandemic resulted in significantly high levels of transmission, but was relatively mild when compared to the other 3 pandemics as only about 12,000 deaths were observed, but the pandemic exhibited some very interesting characteristics (24). The primary population affected was young healthy adults which mirrored that which was observed with the 1918 pandemic and this was attributed to the elderly having preexisting immunity to the virus. pH1N1 infection was accompanied by noteworthy cytokine expression and those with exacerbated cases observed significant lower respiratory tract replication.

With continued circulation in swine of the 2009 pandemic it harbors the potential to continue to reassort and obtain additional virulence factors or pass on the genes associated with enhanced transmissibility to generate viruses with increased pandemic potential.

Influenza viruses harbor multiple pathogenicity factors defined by independent genetic components which results in host damage or susceptibility modulating the host-virus interaction. Two specific proteins which have garnered less attention are neuraminidase (NA) and the acidic protein (PA). NA is responsible for cleavage of sialic acids mediating viral release during budding. NA also facilitates infection via cleavage of sialic acids in the mucus in order free trapped virions (25, 26). NA acts in the manner described to release bound virions to mediate infection. PA is a major component of

the polymerase complex and exhibits endonuclease activity enabling the cap-snatching activity of the influenza RNA dependent RNA polymerase (27). PA and NA have been associated with enhancing pathogenicity and disease outcome, but the specific mechanisms on how they alter immunopathology are still unknown.

Severe cases of influenza related illness are associated with the onset of acute lung injury and in the most severe cases acute respiratory distress syndrome. Acute lung injury is acute hypoxemic respiratory failure with bilateral radiographic opacities often correlating with hydrostatic pulmonary edema (28). Neutrophils are a major cellular component of acute lung injury exacerbating tissue damage through the expression of oxidants, proteinases, matrix metalloproteinases, and cationic peptides (29). IAV infection results in the rapid recruitment of neutrophils post infection often resulting in viral clearance; however when the neutrophil response goes unchecked, neutrophil persistence can significantly contribute to enhanced immunopathology leading to ALI (30).

This work highlights multiple aspects of influenza reassortment and viral factors contributing to immunopathology associated with IAV induced ALI. Here, we show that bat epithelial cells are not only susceptible to both human and avian IAV include highly pathogenic avian influenza viruses, but also support influenza co-infection resulting in reassortment and the generation of novel isolates post infection. We also purport the potential role of the TRIG cassette in supporting species specific reassortment in swine in comparison to humans. Finally, we show that circulating swine IAV harbor genetic

potential for enhancing circulating strains of seasonal IAV through reassortment in particular NA and PA, and swine NA and PA can in cohort or independently drive enhanced acute lung injury through increased MIP-2 expression leading to a robust neutrophil response.

Specific Aims

Central Question 1: What drives species specific reassortment potential of swine epithelial cells compared to human epithelial cells, and can *Pteropus alecto* epithelial cells support influenza replication and reassortment?

Hypothesis 1: Swine epithelial cells are differentially susceptible to influenza viruses as compared to human epithelial cells, allowing for improved opportunity for co-infection, reassortment, and adaptation resulting from inclusion of the TRIG cassette.

Central Question 2: What is the pathogenic potential and compatibility of reassortants that occur between the pandemic H1N1 and endemic swine viruses?

Hypothesis 2: Reassortment between pH1N1 and swTRIGH1N1 results in novel reassortants with increased pathogenicity characterized by an altered innate immune phenotype

Rationale: Justification for this work is to provide insight into viral or host determinates that could contribute to generation of novel influenza reassortants in swine and identify novel pathogenicity factors through reassortment of pandemic H1N1 with current endemic swine IAV. A further understanding of the factors involved in

swine susceptibility could provide future insight into vaccine design for prevention of future pandemics or provide more predictive power for how current circulating strains of influenza could result in pandemics or novel viruses, while Identifying novel pathogenicity factors can provide implications for enhanced surveillance in pandemic preparedness.

Specific Aim 1: Establishment of a methodical approach for identification of naturally occurring reassortants resulting from co-infection of human (calu3) and swine (LLC-PK1) cell lines with the current circulating strains A/California/04/09 and A/Swine/Illinois/02860/2009. Establishing a high throughput screen for identification of reassortants resulting from co-infection is a necessary precursor for differentiating reassortment patterns

Specific Aim 2*:* Identification of differential reassortment patterns resulting from co-infection of either human or swine epithelial cells *in vitro* and resolution of potential viral factors contributing to reassortment in swine, and evaluating the reassortment potential in bat epithelial cells. This will provide evidence that swine epithelial cells have a higher propensity for generation of novel reassortants. Also, identification of reassortment rates in each cell line provides an efficient method for evaluation for future experiments. Identification of viral factors contributing to enhanced reassortment potential could provide significant insight into the mechanisms modulating species-specific reassortment.

Specific Aim 3. Characterization of the differential pathogenesis *in vitro* and *in vivo* observed with the isolated reassortment viruses compared to the parent viruses.

Pathogenesis studies of potential reassortants will provide us will crucial information on what viral factors are contributing to fitness of these reassortment viruses.

Specific Aim 4: Identify viral genes or gene constellations which contribute to enhanced viral pathogenesis resulting in acute lung injury, and potential mechanisms underlying ALI induction post IAV infection.

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CHAPTER 2

LITERATURE REVIEW

History

The first described record of influenza A virus (IAV) infection was documented in 1878 following an outbreak of what is now referred to as highly pathogenic avian influenza virus (HPAI) which occurred in Northern Italy. Additionally, it is speculated that the disease which led to the death of Hippocrates in 412 BC was possibly influenza virus (1) based on the description of his clinical symptoms. IAV was officially described as a novel pathogen in 1880 and was commonly referred to as fowl plaque (2).

IAV belongs to the family *Orthtomyxoviridae* which encompasses a wide array of negative-sense, single-strand, segmented RNA viruses including Influenza B and C, Thogotovirus, Isavirus, and Quaranfilvirus. The envelope of the IAV encapsulates eight negative sense RNA segments which encode for ten to twelve identified proteins. IAVs are differentiated and labeled according to genus, species, isolate location, isolate number, and the year isolated. IAVs are additionally subtyped based on the specific antigenic subtype of the virus. IAV subtypes are identified based on the antigenic similarity of the hemagglutinin (HA) and neuraminidase (NA) proteins. 17 individually

distinct HA subtypes and 10 NA subtypes exist (3) currently with newly identified subtypes added as they are discovered.

Pandemics and significance

In the past century, four IAV pandemics: 1918, (Spanish Flu), 1957 (Asian flu), 1968 (Honk Kong Flu) and 2009 (Swine Flu) have occurred resulting in significant morbidity and/or mortality worldwide. While not referenced as typical pandemics a number of pseudopandemics have also occurred. The term pseudopandemic is used to describe mild less represented pandemics resulting in global spread without significant outcomes. A number of pseudopandemics have occurred throughout the 20th century i.e. 1977 Russian influenza episode (4).

The most notable of the four pandemics is the 1918 Spanish flu as defined by the significant global burden attributed to the severity of the disease resulting in approximately 50 million deaths worldwide, and the probability that it helped end World War I (5). The 1918 influenza pandemic was unique in that it observed a triphasic outbreak curve meaning that three independent periods of robust transmission and incidence were observed (6). It is estimated that approximately a third of the US population became infected. Mortality rates attributed to the 1918 pandemic ranged from 2.5 % globally to 70% in isolated populations compared with standard seasonal influenza viruses which exhibit a mortality rate less than 0.1 % (7). Mortality associated with IAV is typically identified in young and elderly individuals and correlates with disruption of pulmonary function leading to acute lung injury (ALI), acute respiratory

distress syndrome (ARDS), and pneumonia (8, 9). Therefore, it was of significant interest that the 1918 pandemic more strongly affected young healthy adults. Approximately 99% of all individuals that succumbed to infection were under the age of 65 (10). It is believed that the origin of the 1918 pandemic resulted from a reassortment event in avian species and resulted from zoonotic transmission between humans and avian species (11, 12). Sequencing analysis of the 1918 H1 HA showed a low level of species specific adaptations compared to circulating strains of avian influenza viruses (13). Of the 47 known human-specific antigenic adaptations, 37 correlate with the avian consensus sequences, providing additional support for direct transmission from avian to humans without an intermediary host (14). The 1918 H1N1 shows high degrees of similarity to the oldest collected sequence data for classical swine H1N1 viruses. The 1918 pandemic was identified as a H1N1 subtype and has been genetically reconstructed in laboratories to identify genetic markers for disease outcome and severity (15). Two mutations in the HA receptor binding site, E190D and G225D, were identified which correlate with adaptation to bind to alpha 2,6 sialic acid receptors. Current influenza dogma suggests that sialic acid receptor expression differences in the respiratory tract of mammalian and avian provides a trans-species barrier that IAV must bridge to transmit efficiently from avian to human and vice versa. Human IAV preferentially bind alpha 2,6 terminally linked sialic acids while avian influenza viruses bind alpha 2,3 linked sialic acids. Therefore, the mutations E190D and G225D were essential for establishing the susceptibility of humans to the 1918 pandemic even though the H1 sequence exhibited high conservation with avian influenza HA sequences

(16, 17). Infection with the 1918 pandemic virus results in significant pathogenicity in laboratory animals (18-23). Mortality associated with the 1918 pandemic was most often associated with secondary bacterial infections due to the lack of non-existence of broad spectrum antibiotics in 1918 (24). The 1918 pandemic was the worst recorded influenza pandemic in the past century with approximately 50 million deaths worldwide.

The 1957 pandemic was the result of an H2N2 strain of IAV which was first identified in China and quickly emerged in the US and Europe (25-27). The H2N2 pandemic originated in the Chinese province of Guizhou and quickly spread throughout China. Eventually, it spread to Singapore as well (28). The H2N2 resulted from a reassortment event in birds resulting in zoonotic transmission to humans (29). The 1957 H2N2 was a direct descendant of the 1918 pandemic virus with the inclusion of three avian influenza genes resulting HA and NA in the H2N2 subtype along with the avian PB1 (30, 31).

Different population infection dynamics were again observed dissimilar to what was seen in 1918. The Asian flu infected primarily young healthy adults and exhibited a high rate of mortality with a case fatality rate of approximately 50% in children (32). Biphasic characteristics with a U-shaped mortality curve were observed with the Asian flu, but differences in severity were not observed between waves with incidences of mortality mostly associated with viral pneumonia rather than secondary bacterial infections which is likely due in part to the emergence and proliferation of broad spectrum antibiotics administered early in the course of the infection limiting secondary bacterial growth (32).

The 1968 Hong Kong pandemic was caused by an H3N2 virus which was derived from a reassortment between a human and Eurasian avian strain of IAV and originated in southeast Asia (33). The HA gene segment of the human strain was replaced by the avian HA along with PB1 (33). The disease manifestations mirrored those observed in 1957, and like the 1957 pandemic, the 1968 event yielded increased mortality in the young and elderly (34).

The most recent pandemic occurred in 2009, and surprisingly the pandemic virus was an H1N1 virus. As H1N1 viruses have been endemic in humans, the emergence of an H1N1 pandemic was unanticipated as many experts predicted the next pandemic would involve a novel/emerging subtype for humans i.e. H5 or H7.

In March 2009, two American students were hospitalized with diagnoses of influenza-like illness (35, 36). After the initial diagnosis, a number of additional cases were rapidly identified in the Mexico City area (37,38). Within two months of the initial event human-to-human transmission was identified in multiple countries on different continents confirming the existence of a global pandemic (39). In the first year, the 2009 pandemic had caused approximately 12,000 deaths with a case fatality rate ranging from 0.2 - 1.23% with most cases and deaths in victims ranging from 5-59 years of age which was unusual as most seasonal influenza viruses affect primarily the elderly and young (40-42). Interestingly, it is now believed that the elderly most likely had some levels of preexisting immunity to the 2009 pandemic which provides some support for the low attack rate observed in the elderly.

The clinical manifestations of the 2009 pandemic were relatively mild. Clinical symptoms ranged from fever, cough, sore throat, shortness of breath (dyspnea), headache, and rhinorrhea (42). Gastrointestinal signs were observed in some cases, which differs significantly from seasonal IAV clinical symptoms. Pathological studies on patients which succumbed to infection showed penetration of the virus to the lower respiratory tract which is also different than that which is observed with seasonal influenza virus (43-45). The 2009 pandemic quickly became the predominant circulating strain of influenza in humans excluding the previous seasonal strains from circulation in the general population.

The 2009 pandemic resulted from zoonotic transmission from swine. The 2009 pandemic was a triple reassortment virus derived from swine, avian, and human origins. The NA and M genes were derived from Eurasian avian-like swine viruses, and the remaining 6 genes were derived from endemic triple reassortment swine viruses with avian PB2 and PA, human PB1, and swine HA, NP, and NS genes (46).

Influenza ecology

IAV have a vast host range including many wild and domestic avian species, and an eclectic range of mammalian species including humans, bats, and seals. The primordial reservoir for IAVs are aquatic birds specifically those in the orders Anseriformes (ducks geese and swans) and Charadriformes (shorebirds and gulls) (47). The most common aquatic avian reservoir in particular are dabbling ducks (48), but IAV has been isolated from 100 different aquatic bird species with very little information

regarding the overall endemicity of the virus or overall ecological role of the avian species. Influenza viruses in aquatic birds preferentially replicate in the gastrointestinal tract resulting in fecal-oral transmission, which is essential in maintaining efficient bird to bird transmission (49).

Interspecies transmission is most common between wild and domesticated waterfowl through co-mingling in ponds and rice paddies in Asia (50). Continued transmission occurs between both domesticated and wild waterfowl and domesticated terrestrial poultry such as chickens, quail, and turkeys through interactions and backyard poultry farms and live bird markets, which coincidently act as a mediation point for zoonotic transmission of avian influenza isolates to a variety of other mammalian hosts including humans (51).

Due to the high propensity for exposure of domesticated poultry to wild waterfowl, it is expected that domesticated poultry represent one of the many hosts unique to influenza ecology. Most of the HA and NA subtypes which replicate efficiently in wild ducks replicate quite efficiently in domesticated ducks with the exception of typical gull isolates derived from HA subtypes H14 and H15 (52). Quail however support the replication of all HA subtypes with no apparent signs of morbidity (53). Quail have also been proposed as a potential intermediate host for bridging the mammalian interface for IAV as they possess a unique distribution of sialic acids in their upper and lower respiratory tract (54). IAV are thought to bind to host cells through an interaction between the hemagglutinin receptor binding site and terminal sialic acids on the host cell. Avian influenza viruses preferentially bind sugars with a specific glycolinkage,

particularly 2,3 linked terminal sialic acids, while mammalian adapted virus preferentially bind alpha 2,6 linked sugars. This phenomenon has been propagated as dogma for some time, but sugar specificity is not the sole driver of species specificity in IAV as a number of factors have significant roles in mediating interspecies transmission. As quail express both 2,3 and 2,6 sialic acids it is believed that quail present a direct intermediary for adaptation of avian influenzas to humans (54).

Turkeys and chickens are the remaining terrestrial domestic poultry species susceptible to IAV infection. Turkeys are exceptionally permissive to IAV infection and support the replication of many HA and NA subtypes of IAV(55). However, chickens represent a problematic host for IAV. Even though chickens are extremely susceptible to a wide range of IAV subtypes, most IAV fail to establish continued circulation in them (56). Two distinct Subtypes H6N1 and H9N2 virus are the only known influenza viruses which have established lineages in chickens (56).

Relative to avian species most influenza variants are regarded as low pathogenic isolates relative to the overall pathogenicity of said virus in a given avian species. Highly pathogenic isolates are traditionally defined as H5 or H7 viruses which harbor a multi basic cleavage site. This results in systemic dissemination of the virus and high rates of morbidity and mortality in avian species, especially domesticated chickens (57).

As quail were discussed as a possible bridge species for adaptation of avian like virus to become transmissible to mammalian species, swine have been implicated as a mixing vessel for IAV (58-60). Swine are a well described host for IAV. Currently swine only support the replication of a limited number for influenza subtypes including H1, H2

and H3 viruses with a N1, or N2 neuraminidase (59). However, additional evidence exists showing that swine can support a wide range of influenza subtypes including highly pathogenic H5 and H7 viruses (61, 62). Interestingly, the level of pathogenicity associated with highly pathogenic strains in poultry is not observed in swine (62). Highly pathogenic H5N1 infection of swine results in a relatively asymptomatic outcome with low rates of transmissibility. Influenza endemicity in swine exhibits strong geographical limitations. Geographical distribution of influenza virus lacks significant regional diversity as evidenced by 80 years of swine influenza virus stability from 1918 to 1998 in the United States (59, 63). It has been recently identified that swine most likely act as a true intermediary for IAV between avian and humans. The majority of transmission events occur by reverse zoonosis from humans (64, 65). Avian viruses typically lack the ability to sustain replication in the swine host. Until present, the only avian virus with sustained endemicity in swine was the Eurasian avian lineage of viruses (66). Uniquely, swine present a host with limited restrictions on influenza reassortment. Influenza seasonality is no longer observed in swine (due to changes in production practices) as it is observed in humans allowing continued circulation and genetic shift and drift throughout the year. In association with the observed year-round circulation, high rates of reassortment are identified in swine. In our recent paper, we highlight the propensity for swine specific reassortment and the probable role of the triple reassortment internal gene cassette (TRIG) identified in endemic US swine influenza viruses (67). Continued circulation of IAV in swine continues to elicit species specific adaptations which generate

novel strains for which humans lack pre-existing immunity as evidenced by the 2009 pandemic (52).

Humans have represented an interesting host in the dynamics of influenza ecology. H1, H2, and H3 influenza viruses predominant in humans with the historical majority of circulating strains stemming from previous pandemic events (68). In humans, viral displacement is often observed with introduction of novel subtypes with antigenic differences compared with the seasonal strains of human influenza virus (68). Human influenza virus ecology lacks the geographical diversity observed in swine, most likely due to global travel and the transportation of imports and exports worldwide. As discussed, influenza viruses in swine observed minimal to no seasonality. However, in humans, influenza virus seasonality is biphasic and based on geographical location. Temperate regions correlate influenza seasonality with late fall/winter corresponding to enhanced virus survival during winter months, higher population densities indoors due to weather, etc. On the other hand, seasonality is less identifiable in tropical locales, but spikes in influenza prevalence can be observed during the rainy season for many of the same reasons seasonality exists in temperate locales (69). Influenza reassortment in humans is less prominent than that observed in swine, but it does still occur. Human susceptibility to newly emerging strains and a wide range of influenza subtypes is multifaceted with heavy dependence on population immunity and population genetics, particularly those associated with individual polymorphisms associated with immunity (70, 71).

Influenza viruses also persist in a wide array of other mammalian species including dogs, horses, cows, and aquatic mammals (47). Recently bats have been identified as a potential reservoir for influenza viruses (72,73). Two distinct influenza viruses (H17N10 and H18N11) were identified using next generations sequencing techniques in indigenous bats of Guatemala (72, 73). Interesting, the protein function of the glycoproteins of the H17N10 and H18N11 viruses function differently than that observed in current strains of IAV (74). The bat-derived HAs do not specifically bind sialic acids and harbor unique receptor binding domains (75). With the absence of interactions with sugars the NA is no longer required to catalyze the cleavage of sialic acids (76, 77). The information provided on these proteins is in respect to reconstructed protein sequences from the sequencing results. Efforts to rescue and grow these unique viruses have thus far been unsuccessful. While this information is still unique, and with evidence that these subtypes do not follow canonical replication pathways for influenza, there are still many questions left to answer. However the most applicable portion of these findings is that bats may have an interesting role in not only the history, but perhaps the present ecology of IAV.

IAV Protein function and pathogenicity factors

IAV is in the family *Orthomyxoviridae* which are characterized by a segmented negative sense RNA genome. IAV is a pleomorphic virus with an envelope derived from the cell membrane. The IAV genome consist of 8 negative sense RNA gene segments encoding 10-12 known proteins dependent on viral isolate. IAVs encode 4 structural

proteins matrix proteins 1 and 2 (M1, M2), Hemagglutinin (HA), and neuraminidase (NA), a viral ribonucleotide protein complex (vRNP), consisting of the RNA dependent RNA polymerase (encoded by acidic protein (PA), basic protein 1 (PB1), and basic protein 2 (PB2) and the influenza nucleoprotein (NP). Finally, IAVs translate 4 additional non-structural proteins which facilitate viral replication (non-structural protein 2 (NS2) or nuclear export protein (NEP) or mediate the host immune response (non-structural protein 1 (NS1), basic protein 1-frame 2 (PB1-F2), and the newly described PA-X protein (136). Details highlighting all the described proteins will be described in the following section with emphasis placed particularly on the NA and PA proteins.

The two major immunogenic proteins for IAVs are the surface glycoproteins HA and NA. The influenza HA is the most well described influenza protein. It is also the most well defined pathogenicity factor as the cleavage site for avian influenza virus isolates can define the systemic spread of infection and level of pathogenicity. HA is a multifunctional protein, which mediates binding, internalization, membrane fusion, and budding (3). HA is a homotrimeric protein with its nascent form regarded as HA0 (78). The HA protein is composed of three distinct domains. First, the globular head which is the viral determinant for antigenicity. The globular head is the highly variable domain of HA (78). Each HA subtype (1-18) has significant genetic variability, and antibodies typically do not provide cross protection across different HA subtypes. Located in the globular head of HA is the receptor binding domain (RBD) (79). The HA RBD specifically binds sialic acids, in particular terminal linked sialic acids with terminal linkages with either alpha 2,3 or alpha 2,6 specific linkages. The transmembrane domain of HA is

relatively stable and stalk specific antibodies provide heterologous protection (80). The HA0 protein is cleaved by host proteases exposing the fusion domain of HA which is responsible for viral envelop fusion with the endosomal membrane during infection mediating vRNP release into the cytoplasm of the cell (81).

The second glycoprotein translated by IAV is the neuraminidase protein. NA is a glycoside hydrolase enzyme that is responsible for the cleavage of glycosidic linkages of neuraminic acids (3, 82). In the case of the influenza, the influenza NA specifically cleaves terminal 2,3 and 2,6 linked sialic acids mediating viral release from the cell. NA is a homotetramer in its quaternary form (82). NA is one of the key antigenic proteins expressed by IAVs. However antibodies directed against the NA protein have very limited neutralizing capacity during viral infection and release, but provide some level of neutralizing capacity protecting animals during vaccine/challenge studies (83). On the other hand, NA is the predominant target for current influenza therapeutics. Drugs such as oseltamivir specifically block the sialic acid cleavage activity of the influenza neuraminidase inhibiting viral release (84, 85).

The NA protein is not nearly as well described as the HA protein and functional and mechanistic studies on its role in pathogenicity are just now being recognized. Previous work has shown that NA may have a significant role in mediating cleavage of sialic acids in the mucus allowing for efficient penetration through a dense, (50um) thick mucus layer in the lungs (86-88). Initial observations were elucidated using normal human bronchial epithelial cells. These cells produce a mucus layer *in vitro* and provide a more accurate translatable model to *in vivo* studies. Primary cells treated with

oseltamivir, the neuraminidase inhibitor, showed significant reduction in viral infectivity (89). This work provided evidence that the influenza NA may be having a significant role during infection rather than just viral release. Most recently, two independent studies have shown that in fact NA does have a pivotal role in penetration of the mucus layer in order for IAV infection to occur. Existing data indicates that IAV does in fact preferentially bind to sugars contained within the mucus layer providing a sponge like mechanism for prevention of IAV attachment (88). However, current literature shows that the IAV NA actively cleaves the mucin sugar interactions in an attempt to overcome retention in the mucus layer in order to mediate/improve infectivity (87). The observed role for mediating mucus dissolution has been shown in both human and swine models.

During genome replication in frame deletions of the NA gene have been observed, particularly in the stalk domain of the protein. H5 subtype viruses appear to select for a 20 amino acid deletion in the stalk region of the NA (90). A number of studies have shown that NA truncation in the stalk domain mediates influenza pathogenicity (91, 92). Current highly pathogenic strains of avian influenza exhibit this 20 amino acid truncation. Generation of a highly pathogenic strain with the full length neuraminidase using reverse genetics resulted in a significant reduction in virulence in ducks (93). The neuraminidase also has a significant role in host adaptation particularly from wild aquatic birds to domesticated poultry (94) which most likely corresponds with the reduction in virulence observed in ducks with the full length NA. Neuraminidase has been implicated in the neuro-virulence of specific influenza strains (95).

The two remaining structural proteins for IAVs are M1 and M2 encoded by the M gene (2). IAVs can exist in spherical or filamentous forms (2). The spherical form of AV appears to be associated with lab based adaptations while filamentous derivatives appear in clinical specimens and can enhance the transmissibility of IAVs (96, 97). Influenza morphology has been dependently attributed to the M gene and in particular the M1 encoded by the matrix gene. The matrix gene encodes two independent proteins. The M1 protein is the matrix protein which lies beneath the viral envelope. The M1 protein preferentially binds viral ribonucleoprotein complexes providing a structural support for the internal components of the virion (98). The M1 protein additionally has an important role in viral assembly during replication. M1 is responsible for recruitment of viral components to the membrane for assembly and is indispensable for genome packaging and viral budding (99, 100). When mice are administered M1-specific monoclonal antibodies a substantial reduction in the prevalence of filamentous influenza viruses is observed with a corresponding increase in spherical virions (101).

M2, the second protein translated from the matrix gene is a tetrameric transmembrane protein (2). The transmembrane domain of the M2 protein forms a hydrogen ion channel which mediates endosomal release of the viral RNPs (102). The low pH of the endosome triggers the M2 protein to acidify the interior of the virion which mediates release of vRNPs from M1 through weakening of the electrostatic interaction (102). The proton channel activity of M2 is not limited to early events of viral replication as M2 mediates trans-Golgi pH homeostasis preventing conformational

changes in the HA protein (103). Finally, the M2 protein cytoplasmic domain exhibits interaction with M1 assisting in genome packaging (104-106).

Specific M gene segments have strong implications in influenza disease pathogenesis. As the M gene has been shown to modulate virion morphology, it was hypothesized that the M gene may have a significant role in mediating transmission of IAV (107). Recent evidence in guinea pig studies shows that specific M genes are required for contact transmission to occur between guinea swine (107, 108). Interestingly, the M gene has also been shown to have a substantial role in modulating morbidity and mortality (109). Following the recent 2009 pandemic, reverse zoonosis occurred with the pandemic H1N1 virus allowing for continued exchange of viral genes with swine influenza viruses. To this end, H3N2 variant viruses have emerged that are endemic swine viruses with the pandemic H1N1 M gene resulting in enhanced virulence. Importantly however, the described H3N2 variant viruses fail to transmit from person to person (109-111).

IAVs express between 6 and 8 non-structural proteins. Three proteins in particular form a heterotrimer representing the viral encoded RNA dependent RNA polymerase. PA, PB1, and PB2 trimerize to form the RdRp and interact with the nucleoprotein NP in order form intact vRNPs required for replication. The influenza RNP complex transcribes viral RNA into positive sense viral mRNA segments with host cell derived cap structures at the 5’ end and a polyadenylated 3’ tail. The RdRp is also responsible for transcription of complementary vRNA segments (112). The PB2 protein promotes cap snatching from mature host mRNAs. PB2 specifically recognizes type I

mRNA cap structures and enzymatically cleaves host mRNAs with its endonuclease domain (113). The PB2 protein is often associated with host tropism, as a number of essential amino acid have been identified in order for transmission to occur between humans including E627K, D701N, and T271A (114, 115).

The second component of the polymerase complex is PB1. PB1 establishes the backbone of the viral RNA polymerase. PB1 is the workhorse component of the vRNP complex. PB1 exhibits nucleotide polymerization activity (116). PB1 directly interacts with PA and PB2 via the N terminus and C terminus of the protein respectively (117). Very little information regarding the role of PB1 in pathogenicity is published, but the PB1 gene does encode a second protein PB1-F2. PB1-F2 is transcribed due to a shift in the open reading frame of PB1 resulting in a short 90 amino acid protein that is not encoded by all IAVs (118). It appears that the PB1-F2 protein when expressed, is relatively well conserved (119). One well known strain, the 1918 pandemic, does in fact encode PB1-F2. From these studies it has been well established that PB1-F2 has a significant role in viral pathogenicity. PB1-F2 exhibits pro-apoptotic activity and can also directly regulate the cellular innate immune response (120-123).

The final component of the influenza RdRp is the PA protein. PA is a multifunctional protein that exhibits endonuclease activity and vRNA binding (124). It was initially described that PB2 exhibits cap-snatching activity, but it appears that perhaps both PB2 and PA may facilitate cap snatching as the endonuclease domain is inhibited by influenza specific endonuclease inhibitors (125). Therefore there is some evidence that both PB2, and/or PA may havesignificant roles in cap-snatching.

IAVs lack proof reading activity (2), and it has been observed that when a single amino acid mutation in introduced into the IAV PA, significant increases in the production of defective interfering genome segments were observed (126). The increase in short defective RNA segments triggers enhanced RIG-1 activation via the triphosphate caps still applied to the vRNA segments (127, 128). This interaction triggers up-regulation of the IFN pathways resulting in enhanced expression of Type I and III interferons which have a significant role in not only mediating infection and viral clearance, but can also increase immunopathology during infection (129, 130). The PA protein has been shown to drive significant differences in pathogenicity of IAVs. In a recent study, the authors show that two strains of highly pathogenic avian influenza exhibit differing levels of pathogenicity in mice. The authors demonstrated that a single amino acid change in the PA gene at position 353 contributed to the variable levels of pathogenicity observed therefore establishing an overall role in how PA can affect pathogenicity particularly in a mammalian model for influenza infection (131, 132). The PA from highly pathogenic strains of avian influenza have also been shown to activate influenza replicon activity enhancing apoptosis and interferon expression. An additional study shows that a specific amino acid changes in the PA protein inhibits viral clearance from ferrets as well enhancing replication *in vitro* (85I) and pathogenicity in mice (133). As such, there appears to exist sufficient evidence that differences in PA can modulate pathogenicity, and interestingly, PA also has a significant role in host adaptation. Avian influenza viruses with the introduction of human PAs or specific mutations in PA (T552S) can facilitate quicker replication kinetics with enhanced pathogenicity in mice (134).

PA-X is a newly described protein encoded by some IAVs. The PA-X protein is translated from the second open reading frame of the PA gene generated by ribosomal frame-shifting (135). The PA-X protein is comprised of the N terminal endonuclease domain and a T terminal domain derived from the ribosomal frameshift (136). The PA-X appears to be evolutionarily conserved in a species dependent manner (137). Initial observations claimed that PA-X expression led to decreased pathogenicity. However more recent studies have shown that that PA-X expression correlates with host protein synthesis suppression assisting repression of the antiviral response to infection (138-140).

The nucleoprotein NP is the final critical component of the polymerase complex and forms the structural backbone for vRNP complexes. The NP protein is essential for nuclear import of vRNP complexes as is necessary for replication of vRNA. The NP protein has an important role in host specificity, and is involved in the maintenance of host specific gene pools (141). The NP protein is relatively stable with low rates of genetic drift due to the innate lack of selective pressure, but differences are observed between isolates of avian and mammalian origin. It is also believed that this is due in part to the tissue tropism for replication in the respiratory epithelium versus the gut in avian species (142). It has been determined that NP may facilitate other roles during viral replication, but the exact mechanism is not known. Research has shown that NP does interact with importin alpha (most likely mediating vRNP transport), filamentous actin, nuclear export receptor, and a DEAD box helicase (143).

The final two proteins encoded by IAV are translated by the NS gene via an alternative splicing mechanism resulting in two independent proteins: nonstructural protein 1 (NS1) and nuclear export protein (NEP) (144). NS1 is a multifunctional protein involved primarily in mediating cellular type I interferon responses in order to facilitate viral replication (145). NS1 has been shown to regulate RIG-I activity through blocking RIG-s interaction with double-stranded RNA preventing IFN expression (145). NS1 also directly inhibits PKR signaling and disrupts the cellular processing and nuclear export of host mRNA (146). As described, NS1 disrupts RIG-I and PKR signaling resulting in IFN antagonism highlighting the major functional role of NS1. NS1 specifically interacts with PKR through its N-terminal RNA binding domain acting to block signaling (147). NS1 also exhibits some alternative functions for modulation of the cellular response to infection. NS1 interacts directly with TNFa inhibiting its expression (148). Finally, NS1 also inhibits viral replication through mediation of vRNA synthesis through interaction with cellular translation initiation factors and poly-A binding proteins which results in enhanced vRNA processing and replication (149). NS1 has also been implicating in having contradictory roles during apoptosis. NS1 can act either as a pro- or anti-apoptotic factor, but the mechanisms are still unclear. However, the most likely mechanism is through interaction with the phosphoinositol 3 kinase pathway (150).

NEP was formerly named non-structural protein 2, and has been renamed due to the protein activity involved in transport of vRNP complexes from the nucleus to the cytoplasm. Influenza NEP interacts with CRM1 much like the human immunodeficiency virus protein REV, and nuclear export of influenza vRNPs is mediated by

Crm1/exportinin 1(151, 152). Nuclear export is initiated by identification of a leucine rich nuclear export signal found in NEP (152).

Genetic shift and reassortment

The IAV genome is segmented, a structural feature which permits genetic shift/reassortment to occur between two independent influenza strains. When co-infection of a single cell occurs, a maximum of 256 possible genetic variants can be produced as viral progeny. This extremely high propensity for genetic variance allows for the virus to quickly undergo an evolutionary genetic shift resulting from selective pressure or pure random mixing. Intricate evolutionary relationships exist between the segmented nature of the influenza genome and the promiscuity of its RdRp which results in introduction of point mutations and a slower evolutionary path. Therefore the dynamics between IAV genetic shift and drift present a functional relationship that results in a high degree of genetic variance of IAVs globally and also potentiates the possibility of influenza pandemics to occur. The three most recent pandemics appear to have occurred solely through genetic reassortment (4). The Hong Kong and Asian flu pandemics resulted from reassortment events which occurred in avian species while the 2009 pandemic reassortment event occurred in swine.

There are a myriad of factors which govern influenza reassortment, and without bias influenza reassortment can occur at a high frequency. Thus, experiments were conducted using silent mutations in two identical viruses to identify the frequency of reassortment without host or viral constituents. *In vitro* co-infection experiments using

these matched viruses allowed the investigators to ascertain an 88% reassortment rate for the viruses in question (153). Additionally, the authors show that the only factor contributing to reassortment is the propensity for co-infection to occur, and similar results were identified in guinea swine (153, 154).

As described co-infection has a important role in the prevalence of reassortment. However, when sequential infection occurs , for example with one strain 24 hours prior to re-infection with an independent strain, this phenonem begs the question, what is the likelihood of reassortment occurring? This is most likely related to the role of the immune response preventing co-infection from occurring.

In a recent study in guinea swine, it was demonstrated that 24 hours appears to be the upper time limit during which sequential infection can occur and a high number of reassortants be identified (154). One of the major factors driving influenza reassortment is viral gene incompatibility host and cellular tropism. Different viral strains, especially from different species, show signs of incompatibility which results in failure to mediate the host immune response and defects in replication. Most genetic incompatibilities result from two major factors: Influenza genome segments contain RNA packaging signals which mediate packaging of all 8 genome segments into the virion (155, 156). Genetic variations in packaging signals can lead to viruses which exhibit genetic incompatibility and fail to generate progeny virus. Influenza genome packaging appears to be mechanistically regulated via RNA-RNA interactions between genome segments (157). The RNA packaging signal is localized in the 3' to 5' untranslated region of each gene with variable lengths and differences between each

segment (158, 159). Of particular importance is that the packaging signals differ between strains of IAV and can facilitate preferential reassortment (160). The exact mechanism of genome packaging and genetic compatibility still needs to be elucidated and many unanswered questions still exist.

Strain specific tissue tropism may also have a significant role in mediating viral reassortment. It was recently shown that different swine influenza strains exhibits unique tissue tropisms in swine. Specific strains of swine influenza closely related to the pandemic H1N1 virus replicated efficiently in the nasopharyngeal, bronchial, and alveolar epithelium; while swine TRIG viruses and Eurasian swine viruses preferentially replicate din the lower respiratory tract (315). Therefore, it is likely to report that tissue tropism driven by strain specific differences has a significant role in the ability of different viruses to reassort.

Functional protein dynamics and interactions also drive influenza virus compatibility. Factors which drive particular functions during infection and replication are required to balance to ensure optimal viral fitness through maintenance of equilibrium between life cycle determinants. The best example of functional agreement occurs with the glycoproteins HA and NA. A balance in the binding activity of the NA and sugar cleavage activity of NA needs to be compatible (161). As described previously. the IAV RdRp is a trimeric protein consisting of PA, PB1, and PB2. Polymerase compatibility is essential for viral fitness. If physical interactions are disrupted between the 3 proteins, suboptimal replication can be observed resulting in reduced viral replication (162). IAVs must also maintain an intricate balance between

mediation of the cellular immune response and viral replication (163). In the end, reassortants which emerge as fit viruses have undergone strong selective pressure during infection. Selection pressure is the final, and often the most important, feature driving influenza reassortment as the viruses must maintain transmission either enzoonotically or zoonotically.

Swine Influenza

The first serological evidence of emergence into swine occurred around 1918 with the emergence of the classical swine H1N1 viruses. The 1918 pandemic and the elemental classical swine strain have strong similarities, and mostly likely branched off the evolutionary tree at similar time points. There is no direct evidence that the 1918 pandemic virus resulted as a zoonotic event from swine. On the other hand using full genome analysis it appears that both the 1918 pandemic and classical swine viruses resulted from direct transmission from avian species with a period of adaptation to each individual host (63). The primary seasonal influenza A viruses continuously change particularly with the introduction of IAV pandemics. Unlike what has been shown by continuous primary strain inclusion and exclusion in humans, influenza remained relatively stable in swine for 80 years with limitations in genetic shift (165). Although surveillance efforts were limited to that which is observed now, viruses of the H1N1 and in particular classical swine H1N1 were essentially the only influenza variants observed in swine (4). The clinical signs in humans infected with swine flu included fever, loss of appetite, lethargy, difficulties breathing, cough, and low mortality.

In 1931, Shope was the first individual to show Koch's postulates for swine influenza by validating swine influenza virus as the etiological cause of the disease (166), although it was noted by others that disease was exacerbated by co-infection with *Haemophilus influenza suis* (167). Shope established both mouse and ferret models for influenza replication, and showed using antigenic techniques that influenza could infect humans and result in seroconversion confirmed by either swine influenza specific or cross-reactive antibodies (168, 169).

A surveillance study established in 1978 showed that 25% of all swine tested exhibited serological evidence for classical H1N1 which was influenced by vaccination in some herds.. This data suggested that IAVs had a appreciably high prevalence in the swine population. This is not to say that H3N2 viruses were not circulating in swine herds, but simply at lower (1.1%) rates (170-172).

Swine influenza had approximately 80 years of relative stability, but major shifts occurred in the late 1990's coinciding with major changes in swine production practices. The average swine operation increased significantly from 1992 to 1998 from 945 head to 4,646 head respectively (316). In 1998, the seroprevalence of H1N1 dropped 18.6% to 7.6% while H3N2 prevalence increased from 1.2% to 8% (173). The H3N2 viruses being identified were H3N2 human viruses.

In August 1998, multiple outbreaks of H3N2 were identified in North Carolina and quickly spread to Minnesota, Iowa, and Texas often in conjunction with swine that were transported to Midwestern states for finishing (174). The etiologic agents of the outbreaks were of H3N2 origin, but not solely of human H3N2 origin. The outbreak

spread quickly and established endemicity across the US (175). The outbreaks were of two distinct viruses resulting from multiple reassortment events. One virus resulted from a double reassortment event resulting from the classical swine H1N1 with human H3N2 HA, NA, and PB1, while the second virus resulted from a triple reassortment event with avian origin PB2, PA, human PB1, HA, and NA, and classical swine NP, M , and NS (175). The double reassortment virus failed to establish itself in the swine population; however, the triple reassortment virus flourished and rapidly became endemic in the swine population and became the primary circulating strain of swine influenza (176). Researchers have hypothesized that the establishment of the triple reassortment virus into the swine population is directly related to the triple reassortment internal gene cassette (TRIG) human PB1, avian PA and PB2, and swine NP, M, and NS which provides a stable interface for rapid and efficient antigenic switching through reassortment of the HA and NA glycoproteins resulting in evasion of herd immunity (67, 109).

After the emergence of the TRIG viruses in 1998, surveillance studies ranging from 1998-2001 showed that influenza prevalence in swine boomed to 31.7% with 2/3 testing positive based on seroprevalence as H1 and 1/3 testing positive for H3 viruses (177). The primary viruses observed were the H1N1 and H3N2 with the described TRIG cassette. The influenza genetic diversity was no longer limited to just H3N2 and H1N1 strains as a number of unique swine influenza viruses featuring a TRIG cassette were isolated post-1998 including H1N2 and H3N1 (178, 179)

The history of swine influenza is significantly different based on geographical differences, and the role in production practices efficiently transporting virus from one

region to another. In 1979, zoonotic transmission occurred with an avian H1N1 strain which entered the European swine population resulting in the avian-like swine lineage which excluded the previous classical swine H1N1 strain (66). Continued evolution occurred in Europe, with reassortment of the Eurasian avian like swine virus with human seasonal H3N2 resulting in human like swine H3N2 lineage viruses in the 80s and H1N2s in the 1990s (180, 181). These viruses still exist as the primary circulating strains of influenza in swine in Europe.

Swine have been regarded as an optimal mixing vessel for the development of zoonotic viruses that can be introduced into the avian or human populations. The mixing vessel hypothesis is a three-part hypothesis proposed in the 1980s of which all three parts have been validated, but with differing mechanisms than originally postulated (182-184). The first part of the mixing vessel hypothesis states that swine are susceptible to both human and avian strains of IAV. The first evidence for this occurred in 1970 when a human H3N2 isolate was isolated from swine in 1970 (185), and in 1979, the first direct evidence for an avian H1N1 isolate was identified in swine (185). Currently, there are surveillance and phylogenetic data which supports this claim going back to the early 1900's. Initial reports state that swine are more susceptible to both avian and human strains of influenza due to the sialic acid distribution profile of the swine respiratory tracts. This dogma has been refuted with recent evidence proving that the sugar distribution profile of humans and swine is not considerably different. Therefore purporting that swine are a more appropriate mixing

vessel than humans and avian species is not fully supported (186), consequently, elucidating the mechanism for reassortment in swine is of utmost importance.

The second part of the mixing vessel hypothesis states that reassortment of human, avian, and swine influenza viruses can occur in swine, and this was first identified with the viruses which emerged in the US swine populations as described previously. Finally, the last part of the mixing vessel hypothesis states that swine can transmit novel reassortant viruses to humans with pandemic potential. This was supported by the emergence of the 2009 pandemic H1N1 virus, which resulted from zoonotic transmission of a triple reassortant virus from swine (46).

Acute Lung Injury (ALI)

Acute lung injury (ALI) is a syndrome often associated with extensive disease mediated by a wide array of etiologies from bacterial to viral infections. All diagnosed patients with acute respiratory distress syndromes (ARDS) also can be interchangeably characterized as exhibiting ALI. However, not all ALI patients are clinically diagnosed with ARDS as ALI is defined as a mild form of ARDs. ARDS is clinically characterized as acute hypoxemic respiratory failure with bilateral radiographic opacities often associated with hydrostatic pulmonary edema (187). ALI is defined as a mild form of ARDS characterized by less severe hypoxemia (188). Specifically, ALI is defined as ARDS with a partial pressure of oxygen PaO_2/fractions of inspired oxygen ratio <300 (189). ALI diagnosis is relatively abstract and devoid of a gold standard for evaluation. Accurate diagnosis is attributed to the expertise and overall evaluation of a group of

experts. Since ALI is a milder version of ARDS, a large percentage of cases remain undiagnosed. On a cellular level, ALI is attributed to enhanced neutrophil recruitment with enhanced expression of systemic and tissue specific cytokines and chemokines, acute phase reactants, and matrix remodeling enzymes (190, 191).

Influenza-Induced ALI and Chemokine Expression.

Acute lung injury (ALI) can be mediated through the recruitment of inflammatory leukocytes to sites of infection or damage mediated in part by the establishment of pro-inflammatory chemokine gradients in the periphery and lung. Leukocyte recruitment is mediated by cascade beginning with release of leukocytes from the bone marrow, accumulation in the periphery, and trans-endothelial migration. Chemokines are a large family of small molecular weight proteins which bind the superfamily of G-protein coupled serpentine receptors (192). Chemokines mediate the intracellular communication between infiltrates and resident lung cells. Chemokines are subdivided into 4 families C, CC, CXC and CX3C subfamilies. Chemokine families are differentiated based on their cysteine residues and the amino acids separating them (192). For instance, CC chemokines are standard chemokines with two adjacent cysteine residues, and C chemokines have one less cysteine residue compared with the others lacking the 1st and third conserved cysteine residue (192) . CXC chemokines are represented by chemokines lacking one cysteine residue at the N terminus of the protein, and Cx3C chemokines are type II transmembrane proteins with a chemokine head attached to a mucin-like stalk domain (192). Perhaps the most important subfamily in relation to

recruitment of pro-inflammatory immune cell mediators is the ELR+ subfamily of the CXC motif group of chemokines (192). CXC ELR+ chemokines exhibit potent chemoattractant properties for infiltrating neutrophils (193). Neutrophils and macrophages have been implicated as the key infiltrating leukocytes contributing to ALI. However, neutrophils and macrophages are not the only direct mediators of ALI as NK cells and other inflammatory leukocytes have been shown to have a contributory role in mediating ALI (194). Therefore the CXC ELR-chemokines also have an important role in ALI as they are responsible for trafficking of NK cells and T cells to the lung. Also novel neutrophil subsets have been identified that exhibit CXCR3 expression which binds the CXCL10 ligand which is an ELR (-) chemokine potentially implicating ELR(-) chemokines as recruitment factors for neutrophils along with NK and T cells (195). CXCL1 and CXCL2 have been implicated as the predominant chemokines involved in mediating ALI , and treatment with chemokine neutralization antibodies and CXCR2 antagonists which interrupts the interaction of CXCL1 with CXCR2 protects mice during ALI onset reducing the inflammatory response (196-200). During tissue damage or infection, expression of CXCR2 enhances acute lung injury while blocking experiments result in neutrophil retention in the bone marrow and abrogation of ALI (201-205). In humans, a direct correlation between the expression of ELR+ chemokines and ALI has been observed (206). CXCR2 knockout mice showed significant reduction in a hypoxia induced ALI model, a ventilator induced model, and an LPS-induced model leading to reduced morbidity and neutrophil retention in the bone marrow (207-209).

Influenza viruses induce a potent chemokine and cytokine response post-infection. It has been shown that influenza viruses mediate a robust CXCL2 (macrophage inflammatory protein-2;MIP-2) response post-infection that is linked to a drives the pathological outcome following infection in mice and ferrets (212). i It has also been shown in mice that infection with A/Puerto Rico/8/1934 H1N1 elicited up regulation of RANTES, MIP-2, and IP-10 5-15 days pi (213). In a post-mortem study of patients with fatal cases of 2009 pandemic H1N1 significant expression of IL-8 (CXCL8), a ligand for CXCR1 and 2, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 1-B (MIP-1B) was observed (214). Using CXCR2-/- mice, it has been established that CXCR2 is the primordial chemokine receptor mediating neutrophil recruitment during IAV infection. This study showed that when using CXCR2-/- mice infection no longer results in neutrophil recruitment to the lung; however differences in viral titer and clearance are not observed (215). This evidence shows the overall brevity of chemokines in the onset of ALI and IAV infection and more importantly the establishment of the chemoattractant potential for rapid influx of neutrophils and ALI onset.

CXCL1 and 2 and CXCR2 are a small subset of the overall mediators modulating the onset of ALI. One major chemokine that has been elucidated to have a significant role is IL-8 in humans or KC in mice. Il-8 like MIP-2 is a potent neutrophil chemoattractant. Initial observations showed that in patients with ALI the serum levels of IL-8 were substantially upregulated (191). However, subsequent studies could not corroborate this association, but established that IL-8 expression alone did not mediate

tissue damage and enhanced disease. It was able to demonstrate that the Il-8 auto antibody complexed with IL-8 was the major prognostic indicator of disease onset and that differential levels of this complex correlated directly with mortality (216-220). In mice, the induction of anti-KC:KC complexes resulted in the development of severe pulmonary inflammation and tissue damage (221-223). Auto IL-8 antibodies are unique in that they consist of a single IgG3 or 4 molecule and bind with high affinity to IL-8 inhibiting the interaction of IL-8 with target cells such as neutrophils (216, 224). AutoIL-8-IL-8 complexes are known to have a role in mediating neutrophil apoptosis (216, 224, 225). Anti-IL-8- complexes somehow mediate enhanced expression of BCL-X and repression of pro-apoptotic proteins BAX and BAK mediating neutrophil survival (226, 227). In the same paper, the authors further establish that the FcgammaRIIa (FcγRII)portion of the Anti-IL-8 molecule is directly responsible for anti-apoptotic signaling cascade during this distinct interaction (228, 229). IL-8 targets the complex to the neutrophil where the anti-IL-8 initiates a signaling cascade mediating neutrophil survival and enhanced disease (226). Overall in mice, the contribution of both MIP-2 and KC have been evaluated and the level of association of MIP-2 and KC with enhanced inflammation has been extensively studied. Using siRNA suppression of both KC and MIP-2 in mice, investigators showed significant reduction in the numbers of neutrophils being recruited to the lung along with a significant reduction in the expression of local IL-6 expression. However, the effect of knocking down MIP-2 significantly mediated the systemic pro-inflammatory response and a significant inhibition in the recruitment of total neutrophils post-traumatic hemorrhage and shock (222).

KC has a significant role in mediating early neutrophil recruitment early post infection with IAVs resulting in fulminant acute lung injury (230). In early studies with human bronchial epithelial cells, IAV infection resulted in enhanced expression of IL-8 post infection (231). In a recent study in 2014, it was shown that treatment with human cathelicidin (LL-37) significantly reduced the expression of IL-8 post infection which resulted in modulation of the neutrophil response to infection mitigating the disease outcome of infection (232). In the past few years, H7N9 avian influenza viruses have emerged in the human population resulting from zoonotic transmission. Infection with H7N9 viruses results in significant lung inflammation often resulting in ARDs. From clinical serum samples collected from patients with ARDs subsequent to an infection with H7N9, an elevated number of pro-inflammatory cytokines and chemokines were identified including IL-8 (233). A number of other chemokines have been implicated in mediating ALI during influenza infection including IP-10 and RNATES but this review will focus primarily on those chemokines known to have a role in ALI and which are defined potent neutrophil chemoattractants (234, 235). As evidenced here a number of papers support the notion that significant upregulation of MIP-2 and IL-8 complexes post-infection mediate significant neutrophilia. Neutrophils have been implicated as the primary cellular factor responsible for mediating inflammation and tissue damage resulting in acute lung injury. In the following section, we describe alternative regulators of neutrophil recruitment which act in multiple different mechanisms through alteration of chemokines and cytokines, or modulation of the extracellular matrix.

Contribution of matrix metalloproteinase.

Extracellular matrix proteins such as matrix metalloproteinase 9 (MMP-9), have a substantial role in regulating leukocyte migration and tissue repair. MMP-9 participates in recruitment of neutrophils to the lung post infection, and as neutrophils migrate to the lung they become the predominant producer of MMP-9 resulting in dissolution of the extracellular matrix facilitating enhanced migration (249). MMPs compose a family of zinc-dependent endopeptidases that catalytically cleave collagen and additional components of the extracellular matrix. MMPs are separated into five distinct subsets based on substrate specificity and structural properties: 1) gelatinases, 2) stromelysins, 3) collagenases, 4) matrilyssins, 5) and membrane type MMP (236,237). The major physiological role of matrix metalloproteinases is tissue remolding and wound repair (236, 237). However, matrix metalloproteinases can specifically and non-specifically cleave non-extracellular matrix related proteins including cytokines, growth factors, and cell surface receptors (238, 239). In relation to ALI, MMPs are most involved in cytokine and chemokine regulation and extracellular remodeling as it relates to neutrophil recruitment from the periphery to sites of injury. Levels of MMP activity have been directly linked with enhanced acute respiratory distress syndrome resulting in one or more of the following sequelae: multiple organ failure, neutrophilia, and/or acute mortality. MMP levels are used diagnostically as markers of acute lung injury and neutrophilia (240). many cytokines and chemokines can be cleaved by MMPs resulting in activation or inactivation. One example as it relates to inflammation is that the membrane-bound form of tumor necrosis factor alpha is proteolytically cleaved by

MMP-7 resulting in the biologically active form of TNFα (241). In addition, TGFβ is proteolytically cleaved from its latent complex from a panel of MMPs. Interestingly, IL-1β one of the key contributors to ARDS can be cleaved by MMPs resulting in activation and inactivation dependent on the MMP involved (242). As discussed, MMPs are upregulated during neutrophilia, and may be having a role in modulation of the chemokine gradient responsible for neutrophil recruitment. Recent evidence has shown that MMP-7 can cleave the inactive KC/syndecan-1 complex allowing penetration of KC into the alveolar space resulting in establishment of the chemotactic gradient for neutrophil recruitment (243). Although the direct effect MMPs have on the induction and severity of ALI and ARDS is not well defined, it has been established that MMP-3 and MMP-9 knockout mice are resistant to severe lung injury development using an immune model of ALI. MMP-3 and MMP-1 deficient mice also showed reduced neutrophilia resulting in faster disease resolution and a reduced onset of ALI (244). Therefore, MMPs may have a significant role in mediation of ALI. As discussed, influenza virus infection results in significant up regulation and alteration of chemokine gradients, especially KC. Common MMP-P inhibitors include a wide array of tetracyclines including doxycyclin, COL-3, and CMT. Rats undergoing ventilator induced injury exhibited significant clinical signs associated with ALI while treatment with CMT-3 showed significant reduction in histopathological lesions and instances of ALI (245). The severity of ARDS was abrogated in swine pretreated with CMT-3 prior to LPS induced ARDS (246). These studies provide supporting evidence for the direct role of MMPs mediating ALI and ARDS.

MMPs can act in a variety of mechanisms enhancing damage through recruitment of neutrophils and increased activation of pro-inflammatory cytokines. However, MMPs may also have a significant role in inhibiting the immune response and mediating tissue repair, but as discussed, MMPs, when knocked out have been shown to have an important effect on disease outcome. Therefore, the activity, role, and the tempo/pattern of expression/secretion of MMPs is relative to the type of injury being induced. Here, we discuss how influenza infections modulate expression of MMPs ultimately driving influenza induced ALI. Mice treated with doxycycline survived a lethal H3N2 influenza challenge with no appreciable differences in viral titers. Reduced levels of MMP-2 and MMP-9 were observed post-treatment resulting in reduced protein leakage, inflammation, and lung injury (247). Influenza pathogenesis is known to be strain-dependent with a wide array of genomic contributions to virulence(2). The propensity for genetic drift makes identification of such genes important for evaluating the pathogenic and pandemic potential of influenza viruses. Therefore, a number of studies investigate strain-dependent virulence to identify viral factors enhancing viral pathogenesis. Comparative studies were completed in mice which investigated the virulence between A/WSN/33 (WSN H1N1) and A/Panama/2007/99 (Panama H3N2). The results showed that WSN induced higher levels of pathology and immune-mediated disease linked with enhanced oxidative stress and elevated MMP-9 levels (248). Neutrophil infiltration post-infection is an integral host immune response. During influenza infection, it has been shown that MMP-9 upregulation post influenza infection has been tied to MAPK signaling and TNFα expression not only in the lung, but

systemically during a lethal infection (250) . It was previously discussed that MMPs mediate cleavage; this is apparently a dichotomous role as MMPs have a role in regulation of TNFα while TNFα regulates an additional MMP mediating neutrophil recruitment and migration post infection. The exact mechanistic role of MMPs during influenza pathogenesis is still incomplete, but with the current research there exists a strong association between influenza upregulation of MMPs and acute lung injury. As discussed neutrophil migration is facilitated by MMP expression and chemokine and cytokine expression implicating a significant role of neutrophils in exacerbating or alleviating ALI.

Neutrophil Mediated Lung Injury and Influenza:

CXCR1 and CXCR2 are the major chemokines involved in neutrophil recruitment. Inhibition of CXCR2 expression through antagonist treatment or knock-out mice show reduced neutrophil recruitment to sites of infection with significant reduction in the onset of inflammation and tissue damage. As discussed previously, a hallmark characteristic of acute lung injury (ALI), and acute respiratory distress syndrome is the enhanced expression of CXCL1, CXCL2, and their cognate receptors (251). CXCL1 and CXCL2 are potent chemoattractants for neutrophils. During ALI, elevated levels of temporal and spatial CXCL1 and CXCL2 are observed (252). The localization of CXCL1 and CXCL2 mitigate the natural neutrophil trafficking cascade. Neutrophils are innate immune cells and important for pathogen control However, neutrophils that go unchecked may inflict extensive tissue damage via production of destructive enzymes

and compounds. Neutrophil mediation of pathogens is elicited through the release of reactive oxygen species (ROS) and proteases which result in pathogen killing (314), but as mentioned extensive tissue damage can occur when the neutrophil response goes uncontrolled. Suppressor of cytokine signaling (SOCs) and signal transducer and activator of transcription both differentially control neutrophils. SOCS1 and 3 expression has been shown to significantly down regulate the recruitment of neutrophils during infection; while STAT1 signaling and increased expression results in increased recruitment of neutrophils during injury (322). IAV infection results in enhanced expression of SOCS1 and SOCs3 resulted in delayed viral clearance through inhibition of neutrophil trafficking (322). Neutrophil trafficking involves a three step signaling cascade mediated by chemokine gradients established post-infection or damage induction. Neutrophils originate in the bone marrow with a small fraction remaining in the blood stream (205, 253). Neutrophil egress into the periphery is the first checkpoint in the neutrophil response to infection or inflammation. Neutrophil egress from the bone marrow into the periphery is mediated by enhanced expression of CXCL1 and CXCL2 the ligand for CXCR2 by endothelial cells and repression of CXCL12 the ligand for CXCR4 on osteoblasts (202). Regulation of this balancing act has been established and evidence has shown that expression of granulocyte colony-stimulating factor (GCSF) alters the balance of CXCR4 and CXCR2 resulting in neutrophil egress during acute inflammation (205, 254). Therefore, tissue specific chemokine gradients are responsible for trafficking of neutrophils to effector sites, but neutrophil initialization is also modulated by chemokines systemically.

The next step in neutrophil trafficking is mediated by resident leukocytes including macrophages, mast cells, and even epithelial cells. Signaling begins through recognition of pathogen associated molecular patterns (PAMPS) or damage associated molecular patterns (DAMPs) via pattern recognition receptors (PRRs). Some examples of PAMPS and DAMPS include formyl peptides, bacterial DNA, viral RNA/DNA, and mitochondrial DNA (255). Exposure of PAMPS and DAMPs to PRRs results in the release of a plethora of pro-inflammatory molecules including neutrophil chemoattractants. Along with induction of the chemokine gradient endothelial cells actively upregulate the expression of adhesion molecules important for endothelial migration and targeting of neutrophils to sites of stimulus. Neutrophils traffic to sites of damage/infection via the blood stream (256). The migration from the periphery into the endothelium of the target tissue is the second major check point in neutrophil migration. Transmigration into the endothelium occurs in post capillary venules and is regulated by the neutrophil adhesion cascade resulting in diapedesis into the endothelium. The adhesion cascade occurs in a stepwise fashion including: slow rolling, rolling, activation, adhesion strengthening, arrest, and intraluminal crawling (257, 258). The adhesion cascade is mediated initially by the presence of selectins and integrins. Rolling is mediated by selectins which are transmembrane proteins expressed on the surface of leukocytes, and the specific selectins which are upregulated are dependent on the stimulus and level of activation.

Adhesion is mediated by the presence of integrins specifically B1 and B2 integrins. Integrins mediate cell adhesion through interaction with cell adhesion

molecules such as intracellular adhesion molecules -1 (ICAM-1), ICAM-2, and vascular cell adhesion molecule-1 VCAM-1) (260). During typical bacterial and viral infections neutrophil adhesion to the endothelium is mediated by B2 integrins, but during ALI the use of B1 and B2 is observed (260). Each distinct part of the adhesion cascade includes multiple reversible checkpoints which mediate transmigration. Once transmigration is initiated this step is irreversible. Transmigration into the endothelium occurs via two distinct mechanisms. Neutrophils can transmigrate via endothelial cell junctions or directly through an endothelial cell referred to as para- and trans- cellular migration respectively (261).

Leading up to the final checkpoint, neutrophils penetrate the perivascular basement membrane in a PECAM-1 and MMP-9 dependent manner (262), but the exact mechanism is still unknown. Final migration to the site of inflammation is likely mediated by the expression of lipid mediators and chemokines. Unlike chemokines, lipid mediators such as LTB4 and FPR1 are rapidly produced, but have a very short half-life. Chemokines are transcriptionally regulated and have lower half-lives due to protease resistance most likely mediated through association with glyosaminoglycans. Chemokines act in long distance recruitment of neutrophils while lipid mediators act to recruit neutrophils via short distances (263). The radial proximity of CXCR2 mediated recruitment has a larger proximity when compared to lipid mediators, and neutrophils have a stronger affinity for lipids which can drive further migration while at the peak of the chemokine gradient (256).

IAV immunopathology is typically associated with a robust cytokine response mediating enhanced inflammation, and neutrophils have a dynamic role during influenza infection. Evidence has shown that neutrophils are critical to mounting an effective immune response to mediate viral clearance and that they also contribute to enhanced disease pathology and acute lung injury during IAV infection. Neutrophils initiate defense via two mechanisms: phagocytosis, or the mediation of pathogen destruction via a complex array of mechanisms including oxidants, proteinases and cationic peptides. Independent of phagocytosis neutrophils produce oxidants such as hydrogen peroxide and super oxide which exhibit potent microbial activity, but may also be lethal to healthy uninfected neighboring cells (264). Neutrophils also secrete the serine protease neutrophil elastase which is known to have significant antimicrobial activity, but has been shown to be significantly upregulated in the serum of patients with ALI and ARDs (265). Specifically, the level of BAL and serum elastase correlates with the level of ALI and ARDS severity (266, 267). Elevated levels of serum elastase have also been identified in patients diagnosed with IAV induced encephalopathy (317). Exogenous elastase induces ALI induction in mice directly (268-270). Recent studies show that neutrophil elastase can interact with cell surface receptors activating transduction pathways to release specific growth factors and pro-inflammatory cytokines and trigger apoptosis through PAR-1 (271-274). Stimulation of PAR-1 using the agonist TFLLR-NH2 significantly increased influenza morbidity and resulted in significant lung inflammation (318).

Granules within neutrophils also contain the proteolytic enzymes and cationic peptides. When neutrophils phagocytose an organism/cell these proteins are quickly released into the phagosome to induce proteolysis of the target without inducing harm to the environment or the neutrophil. When the compounds are no longer contained in their phagosome and released into the extracellular space the cytosolic potential of these compounds can result in significant tissue damage and pathology. Neutrophil granule proteins are comprised of several subsets including: azurophilic, specific, gelatinase, and secretory vesicles. Neutrophil granule expression is dependent on the state of myelopoeisis (275). Early stage granules typically contain membrane bound receptors while late granule expression/release contain proteolytic enzymes and antimicrobial polypeptides (276). Human neutrophil peptides interact with surfactant protein D and act in both in viral clearance and neutrophil uptake of IAV and bacteria, but also overexpression leads to increased pathology in the host (319). The neutrophil granular proteins have been shown to independently contribute to induction of ALI. In a neutrophil deficient model of *Streptoccous pyogenes* infection the incidence of ALI was completely abolished. Influenza A viruses inhibit the release of myeloperoxidase and acid phosphatase which reduced the bactericidal capacity of the host therefore enhancing the chance of bacterial secondary infections (320). When neutrophil intracellular components were supplied intranasally during infection the recurrence of ALI-like symptoms was observed (277).

Neutrophils also produce cationic polypeptides as antimicrobial agents which can also initiate cell to cell signaling through cell surface proteins known as alarmins

(278, 279). One major example of the cationionic polypetides expressed by neutrophils is lactoferrin, an iron binding protein which has potent antibacterial and antiviral activity(280). Neutrophils also express the cationic peptides LL-37 and defensin. LL-37 is the active form of hCAP18 (281). LL-37 exhibits potent anti-microbial activity, and can contribute to inflammation via regulation of monocytes and lymphocytes (282, 283). LL-37 modulates the neutrophil response to IAV infection particularly through interaction with IL-8 expression (319). In patients with ARDS, LL-37 is significantly upregulated in the BAL when compared to normal controls (284). LL-37 forms duplexes with host DNA resulting in activation of immune system and a pro-inflammatory cytokine response. Necrotic cells isolated from models of ALI show enhanced levels of DNA-LL-37 associations. LL-37 has also been shown to enhance neutrophil survival through inhibition of apoptosis in neutrophils resulting in neutrophil accumulation during ALI (285).

The final mechanism of microbial killing by neutrophils is via the expression of oxidants and reactive oxygen species. Reactive oxygen species are generated primarily in the phagosome via NADPH oxidase and nitric oxide synthase. Neutrophils in particular form superoxoide anion, hydrogen peroxide, and hydroxyl radicals. Reactive oxygen species have broad spectrum antimicrobial activity, but also supply intracellular signals (termed redox signaling) resulting in protein and lipid oxidation (286, 287).

Recently, it has been shown the inhibition of reactive oxygen species by the use of apocyanin enhances protein expression of suppressor of cytokine signaling 1 and 3 (two

key regulators of cytokine signaling) resulting in reduced inflammation during IAV infection (314).

Exogenous treatment of patients intranasally with oxidants results in induced lung injury (288). Oxidants and reactive oxygen species have a role in ALI and ARDs as elevated levels are observed in patients diagnosed with ALI and ARDS, and levels correlate with oxidant and reactive oxygen levels in the serum (289-291). Reactive oxygen species induce disruption of the endothelium through disruption of the tight junctions. This occurs through initiation of phosphorylation of focal adhesion kinases which results in disruption and enhanced permeability of the endothelium (292).

A newly described mechanism of neutrophil killing is the formation of neutrophil extracellular traps. NETS (296, 297). The formation of neutrophil nets is instigated by a stimuli such as infection triggering extrusion of DNA fibers littered with intracellular proteins including histones, elastase, MMPs, and bactericidal proteins (298-306). These proteins have been shown to modulate the tissue microenvironment and mediate infection through a net-like mechanism. The persistence of neutrophil nets has been shown to contribute to enhanced disease during infection. Influenza infection results in the formation of NETS which correlates with excessive endothelial damage during IAV infection (321). Neutrophil NET formation is mediated though expression of reactive oxygen species, IL-8, or phorbol myristrate acetate (295, 307)

Neutrophils may mediate ALI and ARDS using a myriad of mechanisms most often in a pathogen or stimuli specific manner, but ALI has also been diagnosed in children with neutropenia indicating neutrophil independent mechanisms for ALI

induction exist as well (308). Therefore neutrophil independent and dependent mechanisms of ALI have been identified.

The nature of the neutrophil response observed during influenza infection is often attributed to the overall pathogenicity of the viral strain. Some associate mild disease with protective mechanisms of neutrophils. For example, mice depleted of neutrophils and infected with A/X-31 H3N2 (X31) , typically a mild influenza infection, exhibited enhanced viral titers and pulmonary inflammation (312). Even though a strong debate exists between the beneficial and detrimental role of neutrophils, recent studies provide concrete evidence that regardless of the influenza strain present, uncontrolled neutrophil proliferation leading to neutrophilia during influenza infection leads to overt clinical signs of ALI and ARDs. In our recent study described in chapter 3 we show that through reassortment in swine, new viral gene constellations can drive enhanced pathology through enhanced expression of MIP-2 leading to excessive neutrophil trafficking.

Conclusions

The 2009 pandemic was the first pandemic of the 21st century and resulted from zoonotic transmission from swine. The 2009 pandemic was a triple reassortment virus resulting from genetic exchange of viruses from avian, swine, and human origin. It is still unclear what molecular factors and mechanisms appear to drive reassortment in swine compared to other species, but research indicates that swine influenza viruses can rapidly reassort especially in regards to the HA and NA glycoproteins purporting a high

level of genetic compatibility between the internal gene cassette of swine IAV and a myriad of different HA and NA subtypes. Therefore, it is important to elucidate any mechanism that may contribute to species specific reassortment potentials. Soon after the emergence of the 2009 pandemic in humans, reverse zoonosis back into swine was quickly observed only a few months later. As the pandemic H1N1 virus continues to circulate in the swine population it is important to identify specific genes or gene constellations from swine influenza viruses which may enhance the overall pathogenicity of the pandemic virus. Identification of as yet unknown swine influenza pathogenicity factors would allow for better pandemic preparedness and enhance surveillance efforts in swine, as well as potentially drive swine vaccination programs to minimize any reassortment potential via uncontrolled virus circulation in herds.

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CHAPTER 3

BAT CELLS (KIDNEY) FROM PTEROPUS ALECTO ARE SUSCEPTIBLE TO IFLUENZA A VIRUS INFECTION AND REASSORTMENT

Dlugolenski D, Jones L, Tompkins SM, Crameri G, Wang LF, Tripp RA.(2013). *Influenza and Other respiratory viruses*. Reprinted here with permission of the publisher

Abstract

Waterfowl are primary hosts for IAVs (IAV), however there is sporadic infection of swine and other species that pose a risk for zoonotic spread. Yellow-shouldered bats were shown to be hosts of an IAV thereby constituting a potential novel reservoir. We show that *Pteroptus alecto* kidney cells (PaKi) are susceptible to infection and sustain replication of A/WSN/33 (H1N1) and A/Vietnam/1203/04 (H5N1). Importantly, we show that co-infection of PaKi cells results in novel reassortants.

Body

Numerous species are susceptible to infection with IAVs (IAV) including wild and domesticated avian species, horses, dogs, ferrets, swine, whales, and seals (15). IAV often caus*es asymptomatic infections, particularly in wildfowl and shorebirds (15), and a*dditional species are known to serve as intermediate hosts which may provide a niche in the evolution and ecology of IAVs. Bats are a species of interest contributing to approximately 25% of all known mammalian species, and *bats* have emerged as a primary reservoir for a variety of zoonotic diseases spanning numerous virus families including the *Paramyxoviridae*, *Rhabdoviridae*, *Cornonavir*idae, and *Filovir*idae (2, 8, 14). Bats of the genus Pteropus (flying foxes) were first identified as a reservoir of Hendra virus (5, 16). Bats have also been identified as reservoirs for other zoonotic diseases such as SARS-COV and Ebola virus. Previously, in the 1970s, influenza hemagglutinin and neuraminidase proteins were isolated from the lungs and trachea of *Nyctalus noctula* bats (9). Recently, a new lineage of IAV was identified from genetic material isolated

from rectal swabs of bats indigenous to Guatemala, revealing novel HA and NA subtypes (13). To date, efforts to isolate and grow this newly identified virus have been unsuccessful. However, the identification of bats as a potential reservoir for IAV is important to understand particularly as related to host-pathogen interactions which include replication, transmission, and adaptation.

In this study, the susceptibility of *Pteropus alecto* kidney (PaKi) cells (3) to infection with IAVs was investigated. Initially, the ability of a human strain of IAV, i.e. A/WSN/33 (H1N1) was investigated for its ability to infect PaKi cells. Cells were infected with A/WSN/33 H1N1 at an MOI of 0.01 in DMEM supplemented with 5% fetal bovine serum (FBS) without the addition of exogenous trypsin for 24 hours at 37°C. Infected cells were then examined by immunoflourescence using a mouse anti-NP primary antibody and a goat anti-mouse Alexaflour 488-conjugated secondary antibody and visualized using confocal microscopy. As shown in Figure 1A, NP staining was observed in the nucleus and cytoplasm of PaKi cells indicating that bat cells are susceptible to infection with IAV.

Next, the replication kinetics of several IAV strains including A/WSN/33 (H1N1), A/California/04/09 (H1N1), A/Vietnam/1203/04 (H5N1), and A/Swine/Illinois/02860/08 (H1N1) were examined in PaKi cells infected at a MOI of 0.01 at 37°C at 24 and 48 hpi by $TCID_{50}$ titers. Attempts were made to determine the replication kinetics of A/California/04/09 and A/Swine/Illinois/02860/08, but due to exogenous trypsin sensitivity of PaKi cells resulting in loss of the cell monolayer, this was not possible. However, these viruses were able to infect PaKi cells as evidenced by NP staining at 12

hpi (data not shown). Since the HA of A/Vietnam/1203/2004 contains a polybasic cleavage site allowing for cleavage by an endogenous furin-like protease (12), and the HA of A/WSN/33 is cleaved by the serum protease plasmin, the addition of exogenous trypsin was not necessary (7), thus the replication kinetics were determined. For A/WSN/33 and A/Vietnam/1203/2004, the inoculum was supplemented with 5% FBS and 0.5% L-Gln, respectively. As shown in Figure 1B, A/WSN/33 virus replicated efficiently at 24 and 48 hpi with titers of $10^{4.1}$ and $10^{5.25}$ TCID50/ml, respectively. Similarly, the A/Vietnam/1203/04 virus also replicated efficiently, with titers of $10^{4.1}$ and $10^{5.6}$ TCID50/ml at 24 and 48 hpi, respectively. Infection with A/Vietnam/1203/04 induces substantial cytopathic effect (CPE) 48 hpi with almost full disruption of the cell monolayer. Infection with A/WSN/33 induced a minimal amount of CPE visible at 48 hpi. These findings show that PaKi cells are not only capable of supporting IAV infection, but also sustain IAV replication.

From these findings, the propensity for the generation of reassortant viruses was examined following co-infection of PaKi cells with A/California/04/09 and A/Swine/Illinois/02860/08 at an MOI of 3 per virus in DMEM supplemented with 0.5% L-glutamine. Because these viruses require the presence of trypsin for multiple rounds of replication, infections were limited to one round of replication. Supernatants were collected at 24 hpi and plaqued on MDCK cells. Individual plaques were picked and amplified in MDCK cells. Supernatants were collected at 72 hpi and RNA was isolated using the RNeasy kit (Qiagen). cDNA was synthesized from the purified RNA using the Verso cDNA synthesis kit (Thermo Scientific). The residual RNA was precipitated as

described previously (4), and cDNAs were further purified using the QiaQuick PCR Purification kit (Qiagen). cDNA from individual plaques were then screened using triplex taqman qPCR specific for each virus with primers and probes specifically targeting the HA, NA, and PB2 gene segments (Table 1). Reactions were performed using the Quantifast Multiplex qPCR kit (Qiagen).

A total of 77 plaques were screened and two reassortants identified. Of the two reassortants, positive Ct values ranging from 24-35 were observed for the A/Swine/Illinois/02860/08 NA and the A/California/04/09 HA and PB2 genes, and the other isolate exhibited positive Ct values for the A/Swine/Illinois/02860/09 HA and A/California/04/09 NA and PB2 genes (Figure 1C). The reassortants were subjected to full genome sequencing. One step RT-PCR was performed on viral RNA using virus-specific amplification primers. Amplified products were gel purified using the QIAquick gel purification kit (Qiagen) according to manufactures' protocol. Full genome sequencing using primers encompassing all 8 genes from A/California/04/09 and A/Swine/Illinois/02860/09 were used. Sequencing reactions were carried out with the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and sequenced on an ABI 3130-XL Genetic Analyzer. Sequencing verified that one isolate was a reassortant viruses resulting from co-infection of PaKi cells (figure 1c). The identification of a two reassortants prior to sequencing probably resulted from non-specific cross reactivity of the multiplex screen. Collectively, these studies provide the first demonstration that bat cells can sustain replication of IAV and support the generation of influenza virus reassortants.

As shown, bat epithelial cells like swine cells, are susceptible to infection by both avian and human IAV strains. Swine have served as a reservoir for the past three pandemics including the 2009 pandemic that resulted from a triple reassortant virus derived from human, swine, and avian lineages (6, 10). Importantly, given the results presented here, additional studies are warranted that aim to determine the role of bats in the ecology of IAV reassortment and the identification of additional mixing vessels for the generation of reassortant IAV isolates. It has been identified that bats have complex direct and indirect interactions with multiple reservoirs of IAV. Interactions have been observed in urban Hong Kong in which local fruit bats and bird species fed on flowering plants in the same locale (2). These distinct interactions present an opportunity for transmission of IAV from birds to bats. With HPAI actively circulating in avian species in China (11), the identification of a novel HA and NA subtype in bats (13), and initial evidence for support of HPAI replication in bat epithelial cells establishes the need for additional studies investigating the potential of bats as a reservoir for IAV, the host-pathogen interactions between bats and IAV, and the need for increased surveillance in bat populations.

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Table 1. Reassortant screen multiplex primers and probes

	Forward Primer	Reverse Primer	Probe
ILL HA	GGTGTTCATCATCCGCCTAACAT	GCCTCTACTCAGTGTGAAAGCATACCAT	CAACTACTACTGGACTCTGCTGGAACC
ILL NA	TGTGCATAGCATGGTCCAGCTCAA	AAAGGATATGCTGCTCCCGCTAGT	AGGGCGACCCAAAGAGAACACAAT
ILLPB2	TGGCCCAGTCCACTTCAGAAATCA	ATAAACACTGCCTGTTCCACCAGC	GACGAGGTTTCTCCCGGTGGCTGGT
CAL HA	CACTCTCCACAGCAAGCTCA	CTTCTTGCTGTATCTTGATGACC	ATCACTCTCCACAGCAAGCTCAT
CAL NA	TGATTTGGGATCCGAACGGATGGA	AAAGGATATGCTGCTCCCGCTAGT	AGGGCGACCCAAAGAGAACACAAT
CAL PB2	TGACATCAGAGTCACAGCTGGCAA	TCTGCTGACACTGCTGCTCTTCTT	AAACAAGGTTTCTCCCAGTAGCCGGCG

Table 1. Reassortant screen multiplex primers and probes

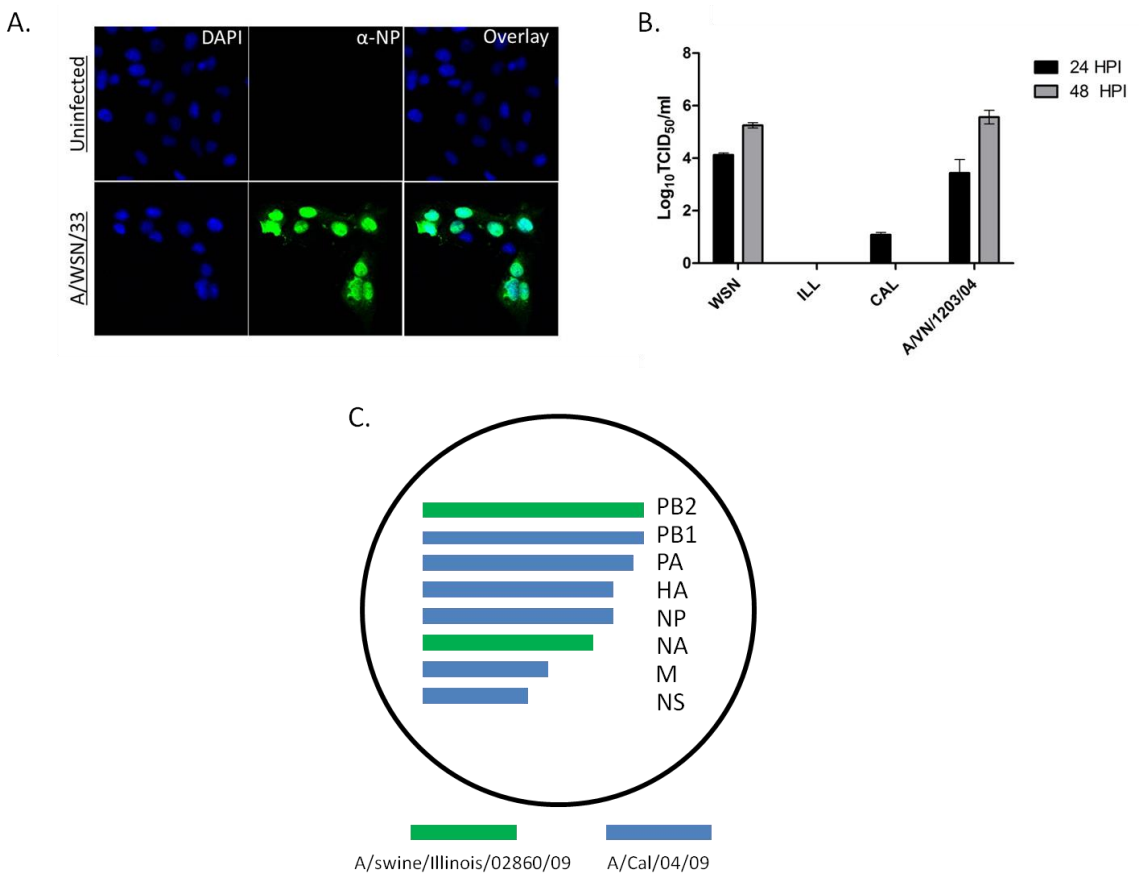

Figure 1. Susceptibility of bat epithelial cells to infection with multiple strains of IAV and the propensity for co-infection and development of a novel virus. (A). PaKi cells were infected with A/WSN/33 at an MOI 0.01 or mock infected with media. Cells were fixed 12 hpi with 2% formalin and stained using a mouse anti-NP primary antibody and goat anti-mouse Alexa flour 488 secondary antibodies. Cells were visualized using confocal microscopy. (B) PaKi cells were infected with A/WSN/33 and A/VN/1203/04 supplemented with 5% fetal bovines serum and A/swine/Illinois/02860/09 (ILL) and A/California/04/09 (Cal) supplemented with 1ug/ml TPCK-treated trypsin at a 0.01of MOI. TCID50 assays were conducted on cell supernatants isolated 24 and 48 hpi. Data is presented as the average of three independent experiments performed in duplicate.

(C) PaKi cells were co-infected with A/Swine/Illinois/02860/09 and A/California/04/09 with a MOI of 3.0. Cell supernatants were collected and evaluated for the presence of reassortant viruses. Shown is a schematic of the single reassortant isolated post co-infection of PaKi cells. The viral isolate was confirmed by Sanger sequencing.

CHAPTER 4

SWINE PA AND NA GENE REASSORTMENT INTO HUMAN H1N1 IS ASSOCIATED WITH AN ALTERED PATHOGENIC PHENOTYPE LINKED TO INCREASED MIP-2 EXPRESSION

Dlugolenski D, Jones L, Howerth E, Wentworth D, Tompkins SM, Tripp RA..(2015). *Journal of Virology*. Reprinted here with permission of the publisher

Abstract

Swine are susceptible to infection by both avian and human influenza viruses, and this feature is thought to contribute to novel reassortant influenza viruses. In this study, the influenza virus reassortment rate in swine and human cells was determined. Swine cells co-infected with 2009 pandemic H1N1 (huH1N1), and an endemic swine H1N2 (A/swine/Illinois/02860/09) virus (swH1N2), resulted in a 23% reassortment rate that was independent of alpha 2,3 or 2,6 sialic acid distribution on the cells. The reassortants had altered pathogenic phenotypes linked to introduction of the swine PA and NA into huH1N1. In mice, the huH1N1 PA and NA mediated increased MIP-2 expression early post-infection resulting in substantial pulmonary neutrophilia with enhanced lung pathology and disease. The findings support the notion that swine are a mixing vessel for influenza reassortants independent of sialic acid distribution. These results show the potential for continued reassortment of the 2009 pandemic H1N1 virus with endemic swine viruses, and the potential for reassortants to have increased pathogenicity linked to the swine NA and PA genes which are associated with increased pulmonary neutrophil trafficking that is related to MIP-2 expression.

Importance

IAVs can change rapidly via reassortment to create a novel virus, and reassortment can result in possible pandemics. Reassortments among subtypes from avian and human viruses led to the 1957 (H2N2 subtype) and 1968 (H3N2 subtype) human influenza pandemics. Recent analyses of circulating isolates have shown that multiple genes can

be recombined from human, avian and swine influenza viruses leading to triple reassortants. Understanding the factors that can affect IAV reassortment is needed for the establishment of disease intervention strategies that may reduce or preclude pandemics. The findings from this study show that swine cells provide a mixing vessel for influenza reassortment independent of differential sialic acid distribution. The findings also establish that circulating NA and PA genes could alter the pathogenic phenotype of the pandemic H1N1 virus resulting in enhanced disease. The identification of such factors provides a framework for pandemic modelling and surveillance.

Introduction

Each year IAVs (IAV) cause epidemics with high morbidity and some fatality in humans (69). In avian species, IAV infection generally is associated with mild disease and it's only when the virus crosses species barriers that pathogenic traits are attributed to infection (70). IAV is frequently associated with 'spill-over' events as numerous species are susceptible to IAV. Often reassortment of IAV leads to a subtype or variant that has not previously infected humans, and as the virus adapts and transmits more easily, a pandemic event may loom. IAV is the cause of >30,000 deaths in the United States and >500,000 worldwide annually (71). In the past century, four IAV pandemics have occurred, i.e. 1918, 1956, 1968, and 2009 (72), where the past three events resulted from reassortment events occurring between IAVs of different origins (73). The 2009 pandemic resulted from triple reassortment of human, avian, and North American and

Eurasian swine origins (74), and through competitive exclusion (4), this pandemic strain has become the dominant circulating virus replacing the previous seasonal H1N1 strains of IAV .

Since the emergence of 2009 pandemic pH1N1, reverse zoonosis to swine has been globally observed (75-82). North American classical swine viruses, European or Eurasian avian like swine viruses, and triple reassortment of internal gene swine viruses (TRIG) co-circulate in swine populations today (83). TRIG viruses emerged in the US swine population in 1998 and quickly became the predominant circulating strains in the United States (84). The TRIG cassette exhibits high genetic stability and has been shown to provide an efficient platform for glycoprotein exchange between viruses to subvert herd immunity (85). Reverse zoonosis of the pH1N1 virus into swine, the propensity to generate novel reassortant viruses with increased pathogenicity and pandemic potential is of concern (86). Recently, 320 confirmed cases and an estimated 2000 total cases of swine influenza zoonosis involving swine H3N2 variant (H3N2v) were observed in Iowa (87, 88). H3N2v viruses resulted from reassortment of H3N2 TRIG viruses with inclusion of the pH1N1 M gene. The M gene from pH1N1 is linked with human-to-human transmission through modulation of neuraminidase (NA) activity and morphology (89). Given the apparent role of the M gene it is important to understand how the individual genetic components of IAV affect the pathogenic phenotype and pandemic potential of IAV. It is important to note that additional genetic reassortments have been identified with H3N2v including inclusion of the pandemic H1N1 Acidic Protein (PA) (90)

The nonstructural proteins, particularly NS1, are regulators of the host response to infection and subsequent pathogenicity (91, 92). Recent evidence shows that protein subunits of the polymerase complex substantially contribute to the pathological outcome of IAV infection, but the overall role in immunity and disease pathogenesis is less defined compared to NS1. The polymerases from the 1957, 1968, and 2009 pandemics were derived from reassortants (73). Protein components of the RNP complex have been implicated in various aspects of influenza virulence and pathogenicity in a variety of mammalian models including mice, ferrets, and swine (93-97). For example, the well described amino acid K627 in the PB2 protein enhances polymerase activity leading to increased transmission, replication, and pathogenicity in mammals (98).

PA modulates replication kinetics, species specificity, and differential pathogenicity of IAV (99). Differences in PA proteins have been directly linked with increased and decreased levels of viral transcription and replication (93, 100). PA from highly pathogenic avian influenza (HPAI) virus isolates contributes to increased cytokine production levels, while also increasing pathogenicity in mice (101). PA has been shown to induce differential levels of interferon production and apoptosis early in infection along with also contributing to increased defecting interfering genome production leading to a more robust cellular innate response via activation of the RIG-I pathway (102, 103) . Studies investigating the pH1N1 PA have shown that a single amino acid change, at position 552, can lower the species barrier for infection in mammals (99). Resulting from a frame-shift, the PA gene encodes a second small protein called, PA-X,

which hijacks the host immune response through host protein shutdown mechanisms thereby modulating the antiviral and apoptotic pathways (104).

The pathogenic role of the neuraminidase (NA) protein is not well understood, and NA is not often associated with viral pathogenicity in mammalian hosts. NA facilitates viral release from infected cells by cleavage of sialic acids between the host cell and the HA protein(105). Recently, NA was identified to mediate dissolution of the mucus layer of the epithelium through cleavage of mucosal sugars during infection potentially implicating different NA subtypes in enhancing IAV pathogenicity (106, 107). Genetic reassortment of PA and NA could play a significant role in mediating the pathogenic outcome of emerging IAVs.

In this study, the TRIG cassette was identified to support species-specific reassortment of A/swine/Illinois/02860/09 (swH1N2) and A/California/04/09 (huH1N1) in swine through screening of the HA, NA, and PB2 genes that resulted from co-infection. Interestingly, preferential inclusion of the swH1N2 NA gene into huH1N1 was observed. IAV reassortants resulting from co-infection of swine cells showed increased replicative fitness and increased pathogenicity attributed to reassortment of the swH1N2 HA, NA and PA genes. The swine HA, NA, and PA mediate increased pathogenicity in ferrets and mice early post-infection with evidence of enhanced necrosis and lymphocytic infiltration resulting in acute lung injury. Intranasal infection of BALB/c mice with the triple reassortant (rILL346) was associated with pulmonary recruitment of neutrophils followed by NK cells early during infection and induction of a robust pro-inflammatory environment characterized by IL-1□, TNF□, and IL-6 expression.

Subsequently, using reverse genetic-derived viruses, it was independently determined that the swH1N2 NA and PA increase viral pathogenesis by enhancement of neutrophil and natural killer (NK) cell recruitment into the lungs of mice post-infection. These studies showed that viruses harboring the swH1N2 NA and PA recruit neutrophils early during infection which promotes stimulation of a robust pro-inflammatory (IL-6, IL-1β, and TNFα) cytokine response increasing overall pathology in the lungs of mice. Neutrophils exhibit extensive cross-talk with innate effector cells types modulating NK cell maintenance, survival, and activation (108). Here it's shown that swH1N2 PA contributes to NK cell recruitment and enhanced expression of IFNγ. Finally, swine PA and NA enhance MIP-2 expression resulting in the observed increases in neutrophil recruitment to the lung.

Materials and Methods

Ethics statement

All animal experiments were performed in accordance to the national guidelines provided by the "The Guide for Care and Use of Laboratory Animals" and The University of Georgia Institutional Animal Care and Use Committee (IACUC). The animal use protocol A2012 06-025-Y2-A2 was approved by the University of Georgia IACUC.

Cells and Viruses.

Madin Darby Canine Kidney cells (MDCK) (ATCC # CCL-34) were cultured in Dulbecco's Modified Eagle Medium containing high glucose (Hyclone) supplemented

with 5% heat-inactivated Fetal Bovine serum (FBS), and were maintained at 37°C with 5% CO_2. LLC-PK1 cells (ATCC#CL-101) were cultured in Dulbecco's Modified Eagle Medium containing high glucose (Hyclone) supplemented with 5% heat-inactivated Fetal Bovine serum (FBS), and were cultured at 37°C with 5% CO_2. Calu-3 cells (ATCC# HTB-55), were cultured in DMEM high glucose supplemented with 20% FBS, 1mM L-glutamine, 1mM HEPES, 1x non-essential amino acids, and were cultured at 37°C with 5% CO_2. A/California/04/09 (huH1N1) was kindly provided by the CDC and A/swine/Illinois/02860/09 (swH1N2), kindly provided by Marie Culhane. Viral stocks were cultured in MDCK cells using infection media (DMEM high glucose supplemented with 1mM L-glutamine with 1ug/ml TPCK treated trypsin). Viral stocks and samples were never introduced to embryonic chicken eggs in order to eliminate potential egg-derived mutations. "Viruses were received as single passage cell preps in MDCKs. Neither virus was introduced into eggs for amplification. All viral stocks used were used from a C3 stock which is representative of 3 passages in MDCKs including the initial prep generated from the clinical sample. Viral stocks were confirmed by sequencing with Big Dye Terminator v3.1 Cycle sequencing kit (Life Technologies). Stock viral titers were quantified using plaque assays as previously described (109). *In vitro* viral infections were performed in infection media.

In vitro Co-infections and plaque purification

Co-infections of LLC-PK1 and Calu-3 cells were performed in infection media. Each infection is performed at an MOI=3 with each independent virus; therefore,

totaling a MOI=6 during co-infection with 2 viruses. At 24h post-infection (hpi), supernatants were harvested and stored at -80°C. Co-infection supernatants from LLC-PK1 and Calu-3 cells were plaque purified. Supernatants were serially diluted from 10^{-1} to 10^{-6} on MDCK cells. The supernatants were allowed to absorb to the cell monolayer for 1h. Cells were rinsed 3x with PBS, and over-laid with a 1:1 mixture of 2% agarose and 2x overlay media: 2x MEM (Invitrogen) supplemented with, 0.3% NaHCO3, 4mM L-glutamine, and 0.4M HEPES. The plates were incubated for 72h at 37°C with 5% CO_2. After 72h pi, plaques were isolated. Agar plugs from individual plaques were applied to MDCK cells and grown for 72h in infection media. 72h pi RNA was isolated from supernatants using the RNeasy mini RNA isolation kit (Qiagen). Residual DNA was eliminated via treatment with DNAse–I (Invitrogen). cDNA was synthesized using the Verso cDNA synthesis kit (Thermo Scientific) using random hexamers for non-specific amplification and 200ng RNA. Residual RNA was removed from newly synthesized cDNA using standard RNA salt precipitation methods as previously described (110). RNA free cDNA samples were further purified using the Qiaquick PCR purification kit (Qiagen).

Multiplex qPCR.

Quantitative PCR (qPCR) reactions were first optimized in monospecific PCR reactions using previously described TAqMan primers and probes specific for the HA, NA and PB2 genes of huH1N1 and swH1N2 (111). PCR reactions were performed using the QuantifFast multiplex qPCR kit (Qiagen) and carried out on a Stratagene MX3500p Real-

time PCR system. Primer/probe pairs were optimized for specificity against the parental and non-parental virus. Two triplex qPCR reactions were used to screen for influenza recombinants between the huH1N1 and swH1N2 HA, NA, and PB2 gene segments. Triplex qPCR reactions were performed using the QuantiFast multiplex qPCR kit (Qiagen), 0.5uM forward and reverse primers, and 0.2uM probes were used for each reaction. Multiplex reactions were performed on a Stratagene MX3500p Real-time PCR system. Cycling conditions included an initial denaturation step of 95°C for 5mins and amplification was performed during 40 cycles including denaturation at 95 °C for 30 secs and annealing of 55°C for 30 secs.

Sequencing.

Stocks of validated viral reassortants derived from co-infection LLC-PK1 cells were propagated in MDCK cells in infection media. Viral reassortant titers were determined using a standard influenza plaque assay protocol as described above(109). RNA was isolated from viral stock samples using the RNeasy Kit (Qiagen) according to the manufacturer's protocol. One step RT-PCR was performed on viral RNA using viral specific amplification primers. Amplified products were gel purified using the QIAquick gel purification kit (Qiagen) according to the manufacture's protocol. Full genome sequencing using primers encompassing all 8 genes from huH1N1 and swH1N2 were used. Samples were sequenced using the Sanger method with the Big Dye Terminator v3.1 Cycle sequencing kit (Life Technologies) on an ABI 3130-XL Genetic Analyzer. Sequences of reassortants were aligned with the parent strains using Clustal W analysis.

Quantitative PCR.

Total RNA was isolated from infected Calu-3 cells using RNeasy mini kits (Qiagen). Reverse transcription was performed using a Verso cDNA synthesis kit (Thermo Scientific). Validated Human specific Taqman gene expression primer/probe sets and master mix (Life Technologies) were used to amplify and quantify IFNα, IFNβ, and IFNλ, according to manufacturer's protocols. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a housekeeping gene to normalize gene expression. All qPCR reactions were performed on a Stratagene Mx3005P.

In vitro viral Kinetics.

Calu-3 cells were infected with huH1N1, swH1N2, and reassortant viruses at an MOI=0.01. Virus was diluted in infection media. Supernatant from individual wells was collected at 6h, 12h, 18h, 24h, 48h, and 72h pi. Viral titers were determined on each sample collected using $TCID_{50}$ (112).

Mouse Studies

Specific-pathogen free, 6-8 week old female BALB/c mice (National Cancer Institute, NCI) were used in all experiments. Mice were housed in microisolator cages and were provided food and water *ad libitum*. Mice were anesthetized by intraperitoneal injection of Avertin (180-250 mg/kg, Sigma). Mice were intranasally challenged with $5x10^5$ PFU of huH1N1, swH1N2, and viral reassortants diluted in PBS.

Five mice (n=5) were used for each experiment, and three independent experiments were conducted for all mouse experiemnts

Lung Viral Titers.

IAVviral burden in lungs of mice was determined by $TCID_{50}$ on whole lung supernatants. Lungs were aseptically removed from infected and control mice at 0.5, 1, 2, 4, 6, 8, and 10 days pi. Lungs were collected and homogenized at 4°C in 1 ml of DMEM/high glucose (HyClone) using a gentleMACS Dissociator (Miltnyi Biotec). The lung homogenates were pelleted and the supernatant collected. Viral titers were determined by $TCID_{50}$ on MDCK cells as previously described (112).

Flow Cytometry.

Bronchoalveolar lavage (BAL) samples from mice were collected with 1 mL PBS at days 2, 4, and 10 pi. For flow cytometry, BAL cells were incubated in FACS staining buffer (PBS containing 1% BSA) with FcγIII/II receptor antibody (BD) and with one of the following antibody groups: Group 1: conjugated anti-Gr-1, conjugated anti-Ly6G, Group 2: conjugated anti-CD3, conjugated anti-DX5, conjugated anti-NKp46, Group 3: conjugated anti-SiglecF, conjugated anti-CD11c, conjugated anti-CD11b, conjugated anti CD45, conjugated anti-CD69, Group 4: conjugated anti-CD4, conjugated anti-CD8, conjugated anti-CD44, and conjugated antiCD69 (BD Biosciences). Cells were acquired on a LSRII flow cytometer (BD Biosciences) and data analyzed using Flow Jo software (v7.6.5).

Mouse Pathology.

Mice were euthanized and the lungs collected and inflated through the trachea with 10% neutral buffered formalin (NBF) on days 2 and 4 pi. Lungs were immersed in 10% NBF, fixed overnight, trimmed, embedded in paraffin, sectioned at 4 micrometer thickness, and stained with hematoxylin and eosin. Histopathological evaluation of the lungs was performed by a board-certified veterinary pathologist. Lesion severity was scored on 0-4 scale.

Luminex bioassay and indirect ELISA

Bronchoalveolar lavage (BAL) samples from mice were collected with 1mL PBS at days 2, 4 and 10 pi. The cellular fraction was removed by centrifugation. Cytokine expression levels including IFNγ, IL-1α, IL-1B, IL-2, IL4, IL-5, IL-6, IL-10, IL-12p40, IL-15, IL-17, and TNFα were analyzed in BAL supernatant samples using the Milliplex MAP kit (Millipore) per the manufacturer's instructions and samples were analyzed using a Luminex 200. Mouse bronchoalveolar lavage (BAL) wash was also evaluated for the presence of MIP-2 at 12h and 24h pi by ELISA following the manufacturer's instructions (eBioscience).

Ferret Studies.

Healthy, 16-18 week old, castrated outbred male Fitch ferrets weighing between 0.8 and 1.0 kg (Triple F Farms) were used for all ferret experiments. Ferrets were

housed individually in microisolator cages and provided food and water *ad libitum*. All ferrets were microchipped with subcutaneous implantable temperature transponders IPPT300 (Biomedic Datasystems). All ferrets were tested prior to infection for sero-conversion against huH1N1 and swH1N2 using a Hemagglutinin Inhibition (HI) assay (113). Ferrets were anesthetized using a ketamine, glycopyrrolate, and dexmedetomine mixture (KGD) composed of 8mg/kg ketamine, 0.01 mg/kg glycopyrrolate, and 0.04 mg/kg dexmedetomidine intramuscularly. KGD offers a deeper plane of sedation and allows for implementation of infections without the induction of sneezing during intranasal inoculation. The described method for sedatation is part of our AUP required by the University of Georgia Animal Resource and IACUC. Ferrets were intranasally challenged with 10^6 PFU of huH1N1, swH1N2, or viral reassortants in 500ul PBS. In order to facilitate recovery from sedation, ferrets were provided 0.2 mg/kg antisedan/atipamezole (Zoetis) recovery agent intramuscularly(114). Body weight and temperatures were evaluated every other day pi, and clinical signs of infection including nasal and ocular discharge, sneezing, loose stool, and trouble breathing were evaluated daily. Nasal washes were collected on days 2, 4, 6, 8, 10, and 12 pi using 2ml PBS under anesthesia with 30mg/kg ketamine. The amount of ketamine used allows for a more mild sedation that insures artificial induction of sneezing can occur to assure proper nasal washing. The described method for sedation is part of our AUP required by the University of Georgia Animal Resources and IACUC. Cloanal swabs were conducted at day 2 and 4 pi. Nasal washes and cloanal swabs were transferred to 1.5 ml tubes and centrifuged for 1 min at 10,000g. Viral titers from nasal wash samples were determined

by $TCID_{50}$. Ferrets were anesthetized at day 14 pi with KGD and bled by intracardiac (IC) puncture(115). Following blood collection, ferrets were humanely euthanized with 0.5ml Beuthaniasia-D (Merck). Ferret serum was evaluated for the presence of influenza-specific IgG antibodies using standard microneutralization and hemagglutination inhibition assays(116). Three ferrets (n=3) were used for each experiment and 2 independent experiments were conducted.

Ferret Pathology and Immunohistochemistry.

Cranial and caudal lungs, trachea, and nasal turbinates were collected at day 2 and 4 pi. Two ferrets per group were sacrificed for analysis (N=2). All tissues were preserved in 10% neutral buffered formalin. Bone was decalcified 14 days prior to nasal turbinate analysis. Tissues were routinely processed and stained with Hematoxylin and Eosin (H&E). Immunohistochemical (IHC) analysis was performed using a mouse anti-NP influenza monoclonal antibody (ATCC; H16-L10-4R5) on lung and nasal tissues infected with huH1N1, swH1N2, and reasssortant viruses. Tissues were deparaffinized and subsequently blocked with a protein blocking agent (Dako Cytomaton) and labeled using a streptavidin- biotin immunoperxoidase system. The reaction was visualized with a 3,3'-diaminobenzidine substrate (Dako) .

Reverse Genetics.

Recombinant viruses rhuH1N1-HA, NA, PA, and HNP were generated by amplification of the genomes using M-RTPCR and cloning into modified reverse genetics

plasmids (117). Sequence verified reverse-genetic plasmids representing each of the 8 gene segments were co-transfected into HEK-293T/MDCK co-cultured monolayers as described (117, 118) Briefly, 0.6 µg of plasmid for each gene segment was mixed and incubated with 15 µl of Lipofectamine 2000 (Invitrogen) at 20^{o}C for 20 min. The Lipofectamine-DNA mixture was transferred to 90% confluent 293T/MDCK cell monolayers in a 35mm tissue culture dish and incubated at 33^{o}C with 5% CO_2 for 8h. Transfection supernatant was replaced with 3 ml of Opti-MEM I medium (Invitrogen) supplemented with 0.3% BSA fraction V (Invitrogen), 3 µg/ml TPCK-trypsin (Worthington) and 1% antibiotic-antimycotic (Invitrogen). Three days post transfection, supernatant was collected and viruses were propagated in MDCK cells at 33^{o}C

Neuraminidase Activity Assay.

Total protein concentrations of viral stocks were determined using a BCA assay (Pierce). A total of 0.1 mg protein from each viral stock was used to measure the NA activity of each sample. 0.1 mg protein was treated with 10 ug MUNANA substrate and incubated at 37 degrees Celsius for 30 min. The reaction was stopped using 1M glycine pH 10.7. The cleavage of MUNANA by neuraminidase present in each sample was determined using a fluorimeter with an excitation of 365nm and 450nm emission wavelength.

Statistics.

All statistical analyses were performed using GraphPad software (San Diego, CA). Statistical significance was determined using one-way ANOVA followed by a post-hoc Bonferroni comparison test. $p<0.05$ was considered significant.

Results

The triple reassortment internal gene cassette (TRIG) influences IAV reassortment in a species-dependent manner.

Zoonotic transmission of the 2009 pandemic H1N1 virus ensued from a triple reassortment of avian, swine, and human origin (3). Swine have receptors to which swine, avian and mammalian influenza viruses bind which increases the potential for viruses to exchange genetic sequences and produce new reassortants (119-121), a feature increasing the potential for pandemic viruses. However, the sialic acid distribution profile in the swine respiratory tract does not solely support the reassortment potential (122-124). Current endemic swine IAVs in the US are composed of the classical swine H1N1 viruses and reassortant TRIG viruses (18). The genotypic components of the TRIG cassette exhibit high genetic stability resulting in a backbone that can support different glycoprotein combinations providing a potential mechanism for reassortment resulting in evasion of herd immunity (20). These findings suggest that the TRIG cassette may provide a selective advantage for influenza reassortment in a species-dependent manner.

To determine if there were differential reassortment rates between human and swine cells, a human lung epithelial (Calu-3) cell line and swine kidney epithelial (LLC-PK1) cell line were evaluated for their susceptibility to infection. Calu-3 and LLC-PK1 cells were infected with A/California/04/09 (huH1N1), and A/swine/Illinois/02860/09 (swH1N2), an endemic swine TRIG IAV. SwH1N2 and huH1N1 exhibited similar infectivity and had similar replication kinetics at 24h pi. However, Calu-3 cells supported a higher level of virus replication for both huH1N1 and swH1N2 at 24h pi (5.7- 6.1 log_{10}-PFU/ml) compared with LLC-PK1s (4.3-5.2 log_{10}-PFU/ml)(data not shown). Calu-3 and LLC-PK1s cells were co-infected with a 1:1 ratio of huH1N1 and swH1N2 at a MOI=3 with each virus and supernatants were collected 24h pi. Individual viral isolates that resulted from virus co-infection were purified and amplified in MDCKs for 72 hours. Each individual plaque isolate was screened using a previously described multiplex qPCR approach directed against the HA, NA, and PB2 genes of each respective virus (111). One-hundred individual plaques derived from co-infections of both Calu-3 and LLC-PK1 cells were screened. Influenza reassortants were not identified from co-infection of Calu-3 cells based on the HA, NA, and PB2 screen (Figure 1B). The distribution of wild-type viruses after co-infection was greatly skewed with 76% of the viruses harboring the HA, NA, and PB2 gene constellation derived from the swH1N2 virus (Figure 1B). Interestingly, co-infection of Calu-3 cells resulted in preferential replication of the swH1N2 virus independent of similar replication kinetics between huH1N1 and swH1N2. In contrast, co-infection of LLC-PK1 cells resulted in a 23% reassortment prevalence based on the genetic rearrangement of the HA, NA, and PB2 genes (Figure 1A). This data

represents reassortment as it relates to the HA, NA, and PB2 genes but this cannot preclude additional reassortants that may have occurred in human epithelial cells. All reassortants were validated by full genome sequence analysis. Additional rearrangement of internal genes was observed but limited providing evidence for stability and high genetic compatibility of the TRIG cassette. Nucleotide differences were observed through sequences analysis with the parental strains; however, amino acid substitutions were not observed when compared to the parental strains therefore all mutations were synonymous. Percentages are represented as introduction of swH1N2 genes into the huH1N1 virus as confirmed by full-genome sequencing. In this case, 91% of the reassortants isolated from co-infection of LLC-PK1 cells resulted in the introduction of swH1N2 glycoprotein genes into the huH1N1 virus, and in particular, 65% of the total reassortants resulted from inclusion of the swH1N2 NA (Figure 1C).

Co-infection in swine cells results in novel reassortants with altered phenotypes.

It has been previously shown that IAV reassortants having genetic exchange of regulatory genes such as NS, or replication machinery such as PA and PB2, can alter the viral kinetics and immune modulatory phenotype of IAVs (26-30). Thus, replication kinetics were evaluated for reassortants having genetic exchange between structural and non-structural genes among the parental viruses, swH1N2 and huH1N1. rILL346 encodes the swH1N2 HA, NA, and PA and the remaining genes are comprised of segments derived from the huH1N1 virus; while rILL18, has the swine origin PB2 and NS genes and the remaining genes were derived from the huH1N1 virus (Figure 2A). The

permissibility and susceptibility of Calu-3 cells was evaluated with the described reassortants. The supernatants from infected (MOI = 0.01) Calu-3 cells were collected 24h, 48h, and 72h pi. Viral titers were evaluated by $TCID_{50}$ and end point titers expressed as $TCID_{50}$/ml. Interestingly, rILL346 showed a significant increase in virus replication at 24h, 48h, and 72h pi compared to the wildtype viruses. Most interesting were the differences observed at 24h pi with rILL346 replicating to a ~100-fold higher mean viral titer compared to the parental viruses ($10^{7.4}$ vs. $10^{5.7}$ and $10^{5.25}$ $TCID_{50}$/ml) (Figure 2B). rILL18 replicated efficiently in Calu-3 cells, but no substantial differences were evident compared to the parental strains. However, infection with rILL346 and rILL18 resulted in differential plaque formation. Plaque morphology is often associated with cell-to-cell spread that is linked to viral fitness (125). Plaques generated in MDCK cells following infection with rILL346 were large and uniform compared to moderate sized plaques made by the parental viruses. Plaques from rILL18 exhibited an attenuated phenotype compared to the parental viruses (Figure 2C). These findings indicate that rILL18 may replicate to similar levels *in vitro*, and the addition of the swH1N2 NS or PB2 to the genome does not appear to alter viral replication. The increase in replication kinetics and morphological differences in plaque formation may suggest increased viral fitness of rILL346.

Differences in viral replication often correlate with differences in the antiviral response, in particular the type I and III IFN responses, which is dependent on many host and viral factors. It has been shown that viral components of replication such as the polymerase complex and the interferon (IFN) antagonist activity of NS gene can affect

the antiviral response (26-30). The type I and III IFN responses elicited by rILL346, rILL18, swH1N2, and huH1N1 infection of Calu-3 were investigated. Calu-3 cells were infected with a MOI=1.0, and cell lysates collected 12h and 24h pi. RNA purified from cell lysates was evaluated using one step qPCR with TaqMan primers and probes directed against IFNα, IFNβ, IFNλ (IL-29) and normalized against the housekeeping gene, HPRT. Comparison of the Type I IFN responses (IFNα /IFNβ) showed modest expression levels, but no sigficant differences were observed between viruses (data not shown). However, infection with rILL346 and swH1N2 resulted in robust expression of IFNλ at 12h and 24h pi. Infection with rILL346 significantly ($p<0.001$) increased (~30 fold) IFNλ mRNA expression 12h pi compared to huH1N1 and rILL18 (Figure 2D). At 24h pi, infection with rILL346 and swH1N2 induced significantly ($p<0.001$) higher (100-fold) IFNλ mRNA expression compared to huH1N1 and rILL18 (Figure 2D). Ultraviolet (UV)-inactivated viruses were used a controls to determine if virus replication was required for induction of the responses observed in gene expression. Although infection with rILL346 and swH1N2 induce a substantial antiviral state, the viruses appear to subvert the antiviral activity of IFNλ, a feature that potentially may contribute to inceased cell pathogenesis.

swH1N2 HA, NA, & PA is associated with increased pathogenicity in ferrets.

With evidence for increased replicative fitness (Figure 2B&C) and implications for modulation of the antiviral type III IFN response in Calu-3 cells (Figure 2D), the ferret model was used to assess potential pathogenicity of rILL346 and rILL18. Ferrets were

intranasally infected with rILL346, rILL18, swH1N2, or huH1N1, and those infected with rILL346 exhibited increased morbidity (Figure 3). Infection of ferrets with rILL346 resulted in substantial clinical signs characterized by a high degree of sneezing, lethargy, difficulty breathing, and nasal discharge throughout the course of infection. Only mild nasal discharge was observed with infection with huH1N1, and no clinical signs were observed with swH1N2 and rILL18. However, significant ($p>0.05$) changes in weight loss were not observed between groups, but infection with rILL346 and swH1N2 resulted in the most weight loss during the course of infection (Figure 3A). There were no appreciable differences in viral shedding at day 2, 4, or 6 pi with undetectable levels of virus at day 8 pi (Figure 3B). Virus replication was not detectable from cloanal samples. Since there were no detectable differences in virus replication, it is likely that changes in pathogenicity were attributed to modulation of the host immune response to infection. This is supported by the levels of influenza-specific antibody neutralization titers elicited in response to infection. In this case, rILL346 and swH1N2 induced higher influenza-specific antibody titers compared to rILL18 and huH1N1 (Figure 3C). Antibody production is dependent on $CD4^+$ T cell activation, which is partially dependent on the host innate immune response to infection (126). Increased influenza-specific antibody titers can directly correlate with robust induction of the innate immune response to infection (127).

Histopathological and immunohistochemistry (IHC) analyses were performed on lung and nasal turbinates of intranasally infected ferrets. No discernible differences were observed in the distribution of virus throughout the respiratory tract of infected

ferrets by IHC staining (data not shown). The main lesions observed in the lungs occurred at day 2 and 4 pi characterized by multifocal necrotizing bronchiolitis and alveolitis along with a level of bronchial gland necrosis. rILL346 and swH1N2 induced the highest level of pulmonary lymphocytic infiltration that occurred at day 2 and 4 pi. rILL18 and huH1N1 infection was associated with minimal pulmonary lymphocyte trafficking at days 2 and 4 pi, and at day 4 pi, pulmonary lymphocyte levels were more substantial compared to rILL346 or rILL18. Infection with rILL346 and swH1N2 also resulted in higher levels of type II hyperplasia and bronchiolar epithelial hyperplasia compared to rILL18 and huH1N1 (Figure 3E). Overall, rILL346 and swH1N2 exacerbated lung pathology at day 2 and 4 pi with no overt changes in viral shedding (Figure 3D).

Infection of the upper respiratory tract is often attributed to increase transmissibility of IAVs (128). Infection with rILL346 and swH1N2 exhibited the most severe pathology in the nasal turbinates at day 2 pi. rILL346 infection had extensive mucosa involvement of the nasal cavity, loss of the epithelium, severe congestion, hemorrhage in the lamina propria, mild edema, necrotic debris, and lymphocytic infiltration. Interestingly, at day 4 pi, huH1N1 had similar pathological outcomes as observed with rILL346 at day 2 pi while rILL346 associated disease severity decreased over time. Ferrets infected with rILL18 had less severe pathology in the nasal turbinates and olfactory type epithelium at day 2 pi compared to the other viral infections. Specifically, rILL18 infection was associated with very minor epithelial damage, mild edema, and very few lymphocytes in the lamina propria. Overall, rILL346 and swH1N2

infection induced the highest degree of pathology in the nasal turbinates of ferrets early throughout infection (Figure 3D).

rILL346 is associated with pulmonary neutrophil and NK cell recruitment.

rILL346 is a reassortant virus derived from three swH1N2 genes segments (HA, NA, and PA) with the remaining genes from huH1N1. The evidence of enhanced lung pathology in ferrets following infection with rILL346 and swH1N2 (Figure 3D) suggested that swH1N2 HA, NA, and PA genes affected viral replication (Figure 3B), and likely immune-mediated pathogenesis. To elucidate a mechanism, mice were similarly infected with rILL346, rILL18, swH1N2, and huH1N1 so that the pulmonary immune response (Figure 4A) and lung histopathology (Figure 4B&C) could be evaluated. Mice were infected with $5x10^5$ PFU virus and lung viral titers were determined at days 2, 4, 6, 8 and 10 pi. All viruses replicated to similar titers at day 2 pi, but clearance of rILL346 and swH1N2 was delayed and these viruses replicated to substantially higher lung virus titers at days 4 and 6 pi (Figure 4A), whereas rILL18 and huH1N1 were cleared by day 6 pi (Figure 4A). Lung histopathological analysis revealed that at early time points of infection, e.g. day 2 pi, rILL346 infection was associated with enhanced lung pathology compared to rILL18, swH1N2, and huH1N1 infection (Figure 4B) that was characterized by numerous focal points of necrotic bronchiolar epithelial cells either in the lining of the epithelium or desquamated in the bronchiolar lumen (Figure 4C). The pathology associated with rILL346, rILL18, and swH1N2 infection was similar at day 4 pi, and huH1N1 induced mild pathology throughout the course of infection (Figure 4B).

As there is a threshold at which the pro-inflammatory response facilitates influenza virus clearance and/or mediates disease, the cytokine levels for a panel of pro-inflammatory cytokines were evaluated in mice. Bronchoalveolar lavage (BAL) samples harvested at days 2 and 4 pi were evaluated for expression of IFNγ, IL-1α, IL-1β, IL-2, IL-4,IL-5, IL-6, IL-10, IL-12p40, IL-15, IL-17, and TNFα. Infection with rILL346 was associated with increased protein levels of IL-6 (Figure 5A), IL-1β (Figure 5B), and TNFα (Figure 5D) at day 2 pi. Infection with swH1N2 resulted in significant ($p<0.001$) but delayed expression of IL-6 (Figure 5A), IL-1β (Figure 5B), and TNFα (Figure 5D) with trends showing increased levels of expression as well at day 4 pi. At day 4 pi, IFNγ expression was significantly ($p<0.001$) upregulated following infection with rILL346 and swH1N2 compared to huH1N1 and rILL18; however only rILL346 infection caused an exuberant pro-inflammatory response in the lung early after infection.

As expected from the cytokine profiles (Figure 5), pulmonary infiltration by neutrophils and NK cells (Figure 6) correlated with increased pro-inflammatory cytokine expression levels suggesting these cells contributed to a role in disease outcome. Infection with rILL346 resulted in substantially higher recruitment of leukocytes at day 2 pi in mice (Figure 6A). BAL-derived neutrophils ($Gr\text{-}1^{hi}Ly6G^{hi}$) expressed high levels of pro-inflammatory cytokines at various times pi including IL-1β, IL-6 and TNFα. At 24h pi, significant ($p<0.01$) neutrophil (Figure 6B) recruitment following rILL346 infection correlated with elevated levels of IL-1β, IL-6, and TNFα in the BAL specimens (Figure 5). This is consistent with the finding that neutrophils are involved in the activation and maintenance of NK cells during influenza infection (129), and with neutrophils cytokines

such as IL-15 promoting survival and trafficking to sites of infection (130-132). Moreover, activated NK cells express large concentrations of IFNγ post-interaction with NKp46 and sugar residues expressed on the surface of the HA protein (133). Infection with rILL346 resulted in considerable NK cell recruitment at day 4 pi (Figure 6C) reflecting high levels of IFNγ expression pi (Figure 5C).

Swine H1N2 NA and PA affect neutrophil infiltration while PA modulates NK cell trafficking.

In order to elucidate the genetic components from swH1N2 that contribute to the increased immune pathology in the lung following rILL346 infection (Figure 4), reverse genetic viruses were constructed as single gene reassortants (rhuH1N1-swHA, NA, PA) in the huH1N1 backbone. An additional reverse genetics virus was developed to mimic the naturally derived rILL346 that contains the swine HA, NA, and PA (rhuH1N1-swHNP). Pulmonary leukocyte (CD45+) recruitment in the BAL was determined in mice infected with rhuH1N1-swHA, rhuH1N1-swNA, rhuH1N1-swPA, and the triple reassortant rhuH1N1-swHNP. At day 2 pi, CD45+ leukocyte numbers in the BAL were significantly ($p<0.05$) enhanced compared to infection with the parental huH1N1 virus (Figure 7A). Only infection with rhuH1N1-swHNP resulted in significant ($p<0.001$) recruitment of BAL-derived leukocytes (CD45+) compared to both parental viruses (Figure 7A) with results comparative to that observed with rILL346 at day 2 pi (Figure 6A). At 48h pi, infection with rhuH1N1-swNA, rhuH1N1-swPA, and rhuH1N1-swHNP resulted in significant ($p<0.05$) enhancement of GR-1^{hi}Ly6G^{hi} neutrophils in the BAL

(Figure 7B). At day 4 pi, significant ($p<0.05$) levels of NK cells (CD3-DX5+NKp46+) are observed in the lungs of mice infected with rhuH1N1-swPA and rhuH1N1-swHNP (Figure 7C).

Alteration of the innate immune response by the swH1N2 HA, NA, and PA has downstream implications on T cell activation.

T cell development and activation is in part dependent on the quality of the innate response reflected by the pro-inflammatory environment (134). The levels of CD8+ (CD3+CD8+CD44+CD69+) and CD4+ (CD3+CD8+CD44+CD69+) T cell activation were determined in the BAL at day 10 pi as a percentage of the total CD8+ (CD3+CD8+) and CD4+ (CD3+CD4+) T cells. Mice infected with rILL346 had the highest level of CD8+ (CD3+CD8+) and CD4+ (CD3+CD4+) T cell activation (CD44+CD69+) (Figure 8), compared to mice infected with rILL18 and the parental viruses. Antibody titers were evaluated and the results were correlated with the findings observed in ferrets (Figure 3C). rILL346 and swH1N2 infection was associated with a higher level of influenza-specific IgG antibodies, but there were no differences in the IgG subtypes (IgG1 and IgG2a) expressed (data not shown). These findings are relevant as NK cell activation and expression of IFNγ has been implicated in regulating CD8+ T cell priming and affecting differences in T cell activation (135).

swH1N2 NA and PA affect levels of MIP-2 expression linked to neutrophil recruitment to the lungs

Neutrophil recruitment is generally mediated by the chemoattractant MIP-2 (136), which is expressed in part by virus-infected epithelial cells. In this study, infection with rhuH1N1-swNA or rhuH1N1-swPA resulted in significant ($P<0.05$) MIP-2 expression in the BAL at 24h pi (Figure 9A). Infection with rhuH1N1-swHNP did not result in significant ($p<0.05$) MIP-2 expression above that expressed in response to the parental viruses, but did increase MIP-2 expression, and differences in MIP-2 expression were not observed at 12h pi (data not shown). These findings indicate that MIP-2 expression is associated with swH1N2 NA and PA genes, a feature that appears to recruit neutrophils early during infection. As expression of MIP-2 could be affected by the virus replication rate, the differences in virus replication kinetics were examined at 12 and 24 hpi between the reverse genetic derived and parental viruses. There were no detectable differences in replication between viruses at 12 and 24 hpi Figure 9B).

Other factors that may contribute to the differences observed were investigated. Total protein was normalized from rILL346, rILL18, huH1N1, and swH1N2 viral stocks derived from cellular supernatants and tested for cleavage activity based on the cleavage of the fluorescent substrate MUNANA. The *Clostridium perfringens* NA protein was used as a positive control. The NA protein of rILL346 and swH1N2 is shared while rILL18 and huH1N1 share NA proteins. Results were compared relative to the cleavage activity of protein isolated from huH1N1. Therefore, it was evident that both rILL346 and swH1N2 exhibited ~50% more activity based on the cleavage of the substrate MUNANA compared to the huH1N1 NA (Figure 10A). The cleavage activity observed with rILL18 mimicked that detected from huH1N1. As both rILL346 and swH1N2 share

the same NA protein it is feasible to conclude that the swH1N2 NA is substantially more enzymatically active when compared to the huH1N1 NA. These findings indicate a potential role for NA-mediated differences. The influenza PA gene encodes a second protein PA-X, which is expressed, based on a ribosomal frame shift (137). PA-X has been shown to have a role in modulation of the innate immune response through up-regulation of antiviral cytokine and chemokine expression through a host protein shutdown mechanism (88, 137, 138). CLUSTALW analysis of the amino acid sequences from the N terminal and C terminal regions of the PA from huH1N1 and swH1N2 show extensive amino acid changes (Figure 10B). Alterations in the protein sequence of PA-X could directly affect the mechanistic role of PA-X resulting in direct modulation of the antiviral state of infected cells.

Discussion

Swine are considered a mixing vessel for the development of reassortant influenza viruses with pandemic potential. Initial findings suggested that the differential distribution of α2,3 and α2,6 sialic acids along the swine respiratory tract were key to supporting the 'mixing vessel' hypothesis, but more recent studies examining sialic acid receptor distribution profiles in swine and humans indicate that such differences are not significant or observed (47-49). The swine influenza TRIG cassette has been hypothesized to have a role in supporting increased glycoprotein exchange in swine, a feature that may increase the reassortment prevalence and increasing the overall fitness of influenza in swine perhaps by evasion of herd immunity (20). Calu-3 and LLC-

PK1 cells do not have detectable differences in the levels of α2,3 and α2,6 sialic acid expression profiles (data not shown). Additionally, sialic acid binding profiles for the huH1N1 and swH1N2 viruses are not notably different and both viruses preferentially bind α2,6 terminal sialic acids (139). Thus, it remains unlikely that differences in sialic acid expression and HA specificity are primary causes of differences linked to reassortment potential in these cell types. Thus, the findings in this study support the TRIG cassette as a platform for enabling species-specific reassortment in swine compared to humans. It is shown that co-infection of swine epithelial cells with swine- and human-derived TRIG viruses resulted in 23% reassortment prevalence with preferential exchange of the NA glycoprotein while no reassortment viruses were identified in human epithelial cells based on mixing of the NA, HA, and PB2 genes.

Influenza reassortant viruses resulting from swine can emerge with pandemic potential as evidenced by the 2009 pH1N1. The pH1N1 virus has established itself as the dominant circulating seasonal H1N1 virus, and based on extensive surveillance studies in swine, reverse zoonosis has been identified worldwide (10-17). pH1N1 harbors the ability to transmit very efficiently from human-to-human based on transmission analyses from the 2009 outbreak and detailed ferret transmission studies (140). With evidence of rapid reverse zoonosis, the potential for continued reassortment in swine is probable. NS, PA, PB2, HA, and NA genes have all been implicating in modulating the pathogenicity of influenza viruses, and this study defines the contribution of swine derived NS, PA, PB2, HA, and NA to enhance pH1N1 virulence. It also shows that the introduction of swine NA and PA directly increase pathogenicity of the pH1N1 viruses in

mice and ferrets resulting in acute lung injury modulated by increased lymphocyte infiltration and destruction of the endothelial-epithelial barrier. Swine NA and PA genes are linked to enhanced expression of MIP-2, a potent neutrophil chemoattractant, which results in elevated levels of neutrophil trafficking to the lungs of infected animal's early post-infection. Neutrophils have a notable role in influenza pathogenesis and neutrophilia is consistent with acute respiratory distress syndrome (141). Neutrophils are short-lived, phagocytic granulocytes which may reduce influenza infection through phagocytosis of influenza-infected epithelial cells, or formation of neutrophil extracellular traps (NETS) that reduce infection(142). Neutrophils are directly associated with influenza viral clearance, but also when in abundance in the BAL, correlate with increased disease severity(143). In this study, evidence is provided for this association with both the swine NA and PA genes. Neutrophils cause extensive damage to the epithelial endothelial barrier via production of reactive oxygen species, secretion of proteases, and robust induction of a pro-inflammatory cytokine environment (144). Elevated levels of induced IL-6, IL-1β, and TNFα comprise the elevated pro-inflammatory environment induced by neutrophils mediated through swine NA and PA and are the correlative cause of increased epithelial damage in the lungs. The exact mechanism by which swine NA and PA mediate neutrophil-associated acute lung injury needs to be further elucidated.

Neutrophils are essential innate effector cells forming the first line of defense against several pathogens. They have a role in the engulfment of the pathogens, and the release of reactive oxygen species (ROS) and proteases that contribute to host defense.

Neutrophil recruitment to sites of infection can be mediated by an array of chemoattractants. These chemoattractants synergistically cooperate to mediate recruitment to sites of infection (145) . Neutrophils can be recruited to sites of infection via the expression of proinflammatory cytokines including IL-6, IL-1β, TNFα, KC, MIP-2, and MCP-1 which represent inflammatory signals which mediating the trafficking cascade of neutrophils (145, 146). Enhanced IL-6, IL-1β, and TNFα may also contribute to continued neutrophil recruitment at later times post-infection, an effect that seems linked to swine NA and PA genes. In this study, elevated expression of MIP-2 was observed 24h pi which appears to correlate and may be responsible for triggering downstream neutrophil migration that was observed at 48h pi. The findings of this study suggest that swine NA and PA are individually culpable for the elevated MIP-2 expression, and this may be associated with regulation of cellular pathways linked to changes in viral infectivity. NA is responsible for cleavage of sugars in the mucus layer in the lungs and can potentially lead to increased levels of infection (106). The *in vivo* viral kinetics data does not fully support this hypothesis, but the viral replication kinetics in human epithelial cells does. The swine NA also exhibits increased cleavage activity compared with the huH1N1 as evidence by the increased munana cleavage activity of the swH1N2 compared with huH1N1. It is important to note that the PA gene encodes a second protein, PA-X, through a frame-shift(137). PA-X can have a direct role in host protein synthesis shutdown modulating antiviral and apoptotic pathways (104, 137, 138). Clustal W alignments of the PA-X proteins of huH1N1 and swH1N1 show a low degree of similarity. The exact mechanism by which the swine NA and PA enhance MIP-

2 expression and how MIP-2 is mediating the downstream recruitment of neutrophils to the lung needs to be further elucidated.

Collaborative cross-talk occurs between neutrophils and NK cells at sites of inflammation mediating recruitment, activation, and maintenance of both neutrophils and NK cells (129). Neutrophil depleted mice have impaired NK cell maturation exhibiting hyper proliferation and weak survival (147). Neutrophils also express high levels of IL-15, which promotes NK cell activation (130-132). Early recruitment of neutrophils by PA likely mediates the activation and maintenance of NK cells observed at 4 DPI. NK cells also contribute significantly to influenza disease pathogenesis (148-150), but the exact mechanism is not well understood.

The predominant viral strains circulating in swine are typically represented by H1, H2, and H3 strains of IAV, but as pre-existing immunity to H1 and H3 subtype viruses is widespread in the human population, it is unlikely that the viral threat would be posed by recombinant IAV representing H1 and H3 subtypes (151). However, H2 viruses harboring an array of neuraminidase proteins circulate in swine and avian populations (152-154). H2 viruses have been absent from the human population for greater than 50 years marked by the 1957 pandemic(155); therefore, pre-existing immunity to a potential pandemic with H2 viruses exhibits elevated pandemic potential and risk. Not only do the described reassortants here pose a potential risk for elevating the virulence of current H1N1 circulating strains but could elevate the potential risk of H2 viruses emerging with pandemic potential with enhanced virulence compared with the 2009 pandemic H1N1 virus.

In summary, the swine TRIG cassette contributes to species-specific reassortment in swine and presents a foundation for establishing swine as a mixing vessel for IAV. As evidenced by extensive global surveillance studies , the pH1N1 was rapidly reintroduced into swine, resulting in continued reassortment of the pH1N1 with endemic strains of IAV resulting in variant strains of IAV that result in enhanced virulence to humans as portrayed by the H3N2v viruses (81, 87, 88). As shown here, swine influenza viruses harbor gene components that can directly modulate the pathogenic outcome of potential reassortant viruses. In particular, swH1N2 NA and PA modulate MIP-2 expression resulting in pulmonary recruitment of neutrophils which establish a pro-inflammatory cytokine environment in the lung. The pro-inflammatory cytokine environment promotes sustained recruitment of neutrophils leading to their activation in the lung. Modulation of the innate immune response by NA and PA contribute to acute lung injury in both the mouse and the ferret models of infection. These findings necessitate the need for elucidating the mechanisms that promote viral reassortment in swine, and the propensity that the described reassortants viruses described may be actively circulating in swine populations argues for increased surveillance and research on identification of swine influenza pathogenicity markers.

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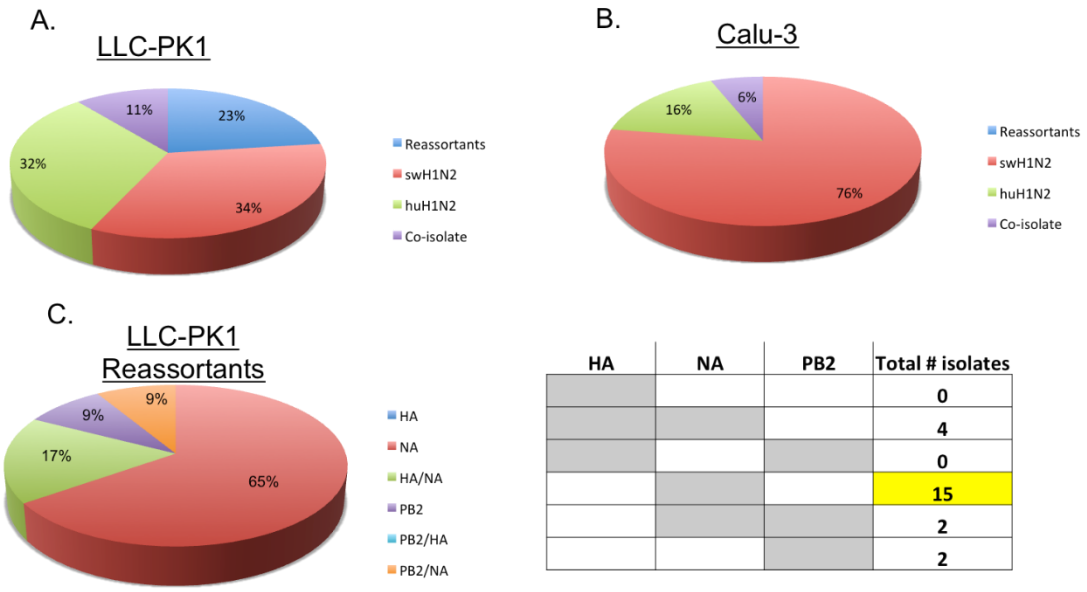


HA	NA	PB2	Total # isolates
			0
			4
			0
			15
			2
			2

Figure 1. Comparison of influenza reassortment outcomes in swine and human cells following co-infection with huH1N1 and swH1N2. Co-infections were performed in LLC-PK-1 cells (A) and Calu-3 cells (B) with huH1N1 and swH1N2. Primers and probes specific to parental strains huH1N1 and huH1N2 HA, NA and PB2 gene segments were used to screen the reassortment prevalence in both cell lines. 100 individual plaque purified isolates from each cell line were screened. Co-infection of LLC-PK1 cells resulted in a reassortment prevalence of 23% with a large percentage of reassortants resulting in glycoprotein gene exchange (C). The total number of reassortment profiles isolated is displayed in the profile matrix and the denoted genotype is profiled in grey with the prevalent reassortment genotype highlighted in yellow (C).

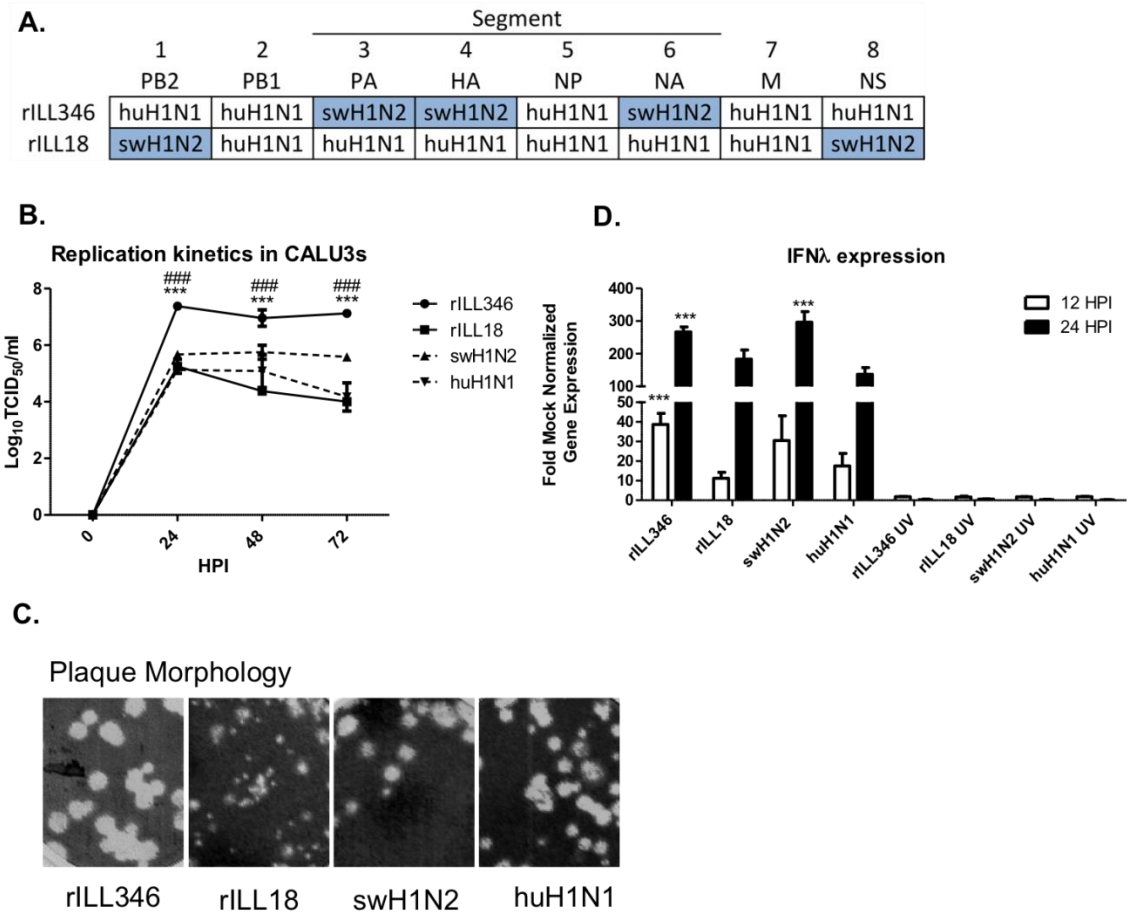
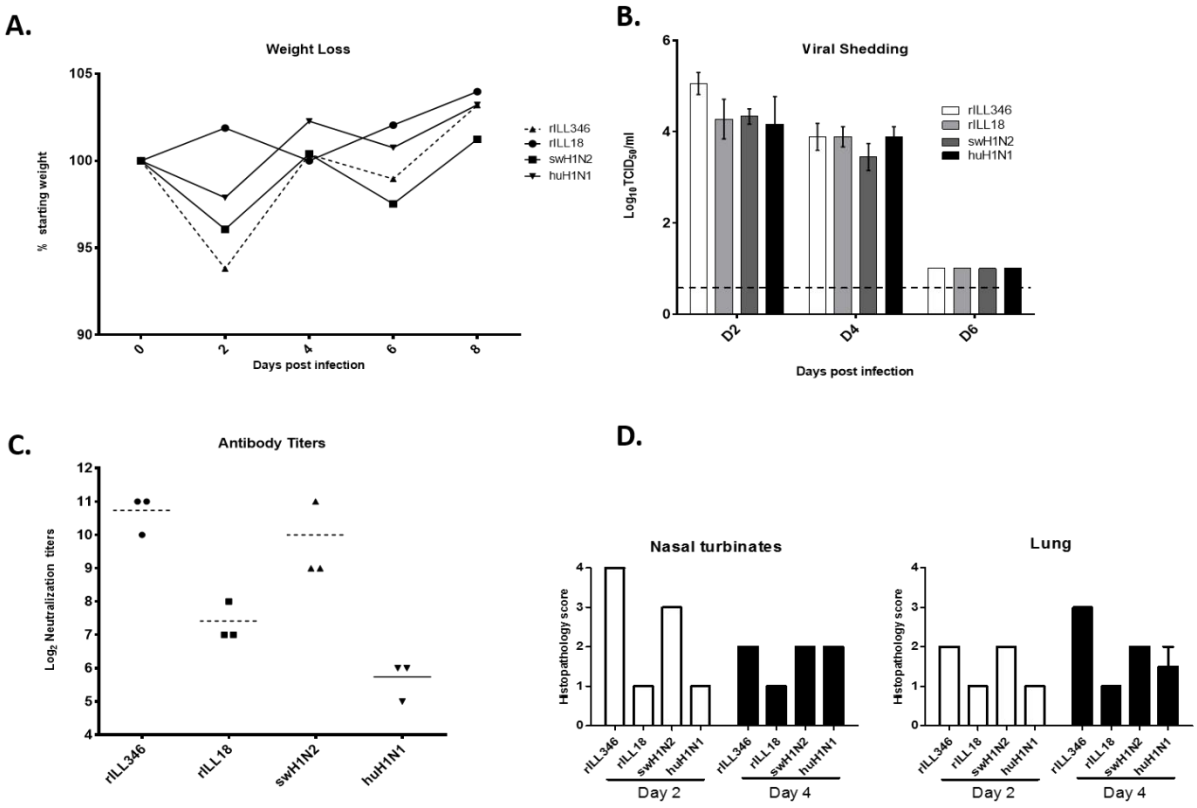


Figure 2. Influenza reassortants derived from huH1N1 and swH1N2 viruses exhibit differential phenotypes *in vitro*. Viral replication (A) in Calu-3 cells was determined. Calu-3 cells were infected (MOI=0.01) and supernatants collected at 0h, 24h, 48h, and 72h pi to determine viral titers. Endpoint titers are expressed as mean $\log_{10}$ TCID50/ml $\pm$ standard deviation. The plaque morphology (C) of each viral reassortant and parental virus were compared in MDCK cells and scanned for comparison. *In vitro* induction of the Type III IFN response elicited by infection with rILL346, rILL18, and the parental viruses were evaluated by qPCR (D). IL-29 (IFNλ) specific primers and probes were used

to evaluate the relative Type III IFN expression in Calu-3 cells at 12 and 24 HPI (MOI=1.0). UV- inactivated viral samples were used as replication controls. Fold-changes and standard deviations were calculated based on triplicate Ct values relative to mock infected cells and the housekeeping gene HPRT. All data is representative of three individual experiments. Asterisks (*) denote significance related to the pH1N1 parental virus while hash (#) represents a p-value significant compared to the swH1N2 parental virus. Results were considered signiFicant with a p-value ≤0.05 (*/#)≤0.01 (**/##), and ≤0.001 (***/###).


A.
Weight Loss
% starting weight
Days post infection
rILL346
rILL18
swH1N2
huH1N1
B.
Viral Shedding
Log10TCID50/ml
D2
D4
D6
Days post infection
C.
Antibody Titers
Log2 Neutralization titers
D.
Nasal turbinates
Lung
Histopathology score
Day 2
Day 4

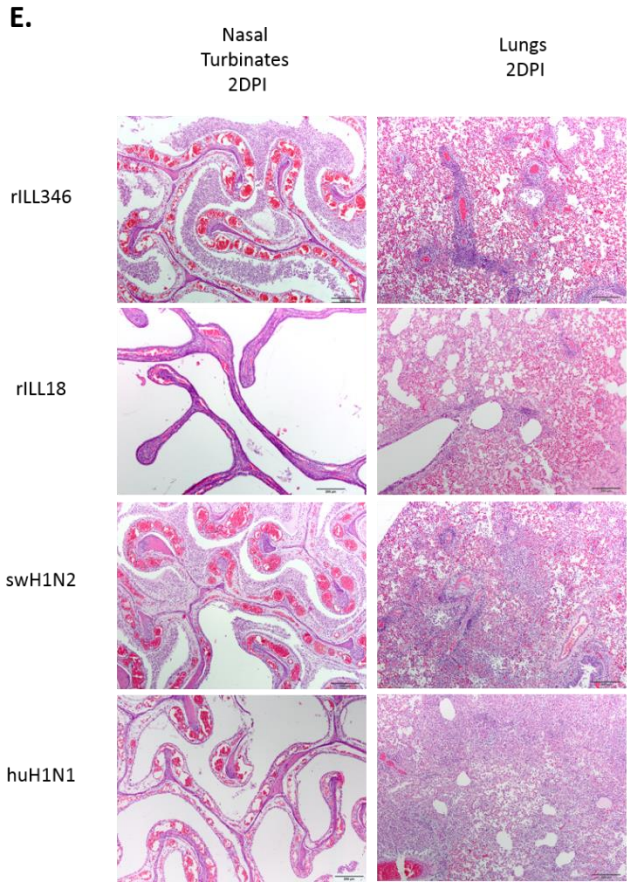

E.
Nasal Turbinates 2DPI
Lungs 2DPI
rILL346
rILL18
swH1N2
huH1N1

Figure 3. rILL346 mediates increased viral pathogenicity in ferrets – an effect associated with swine HA, NA and PA genes. Ferrets were intranasally infected with 10^6 PFU virus and weight loss (A) was evaluated on a daily basis for N=3 ferrets per group and is represented as the average percentage of the pre-infection weight of the ferret. Viral titers (B) were determined from nasal washes at days 2, 4, and 6 pi. Endpoint titers are expressed as mean $\log_{10}$ TCID50/ml $\pm$ standard deviation. The limit of virus detection was 10 TCID50/ml. Influenza-specific antibody responses (C) were measured based on microneutralization assays of serum collected from infected ferrets at day 14 pi. Neutralization titers are presented as the $\log_{10}$ serum dilution at which virus could no longer be detected. Histopathology (D) was performed on lungs and nasal turbinates of infected ferrets. Nasal turbinates and lung tissues were collected at days 2 and 4 post infection from N=2 ferrets per time point per virus and lesion severity was scored on a 4 point scale relative to the overall pathology induced during infection. Representative H&E stained sections (E) of ferret nasal turbinates and lung tissues 2 dpi. All data is representative of two independent experiments.

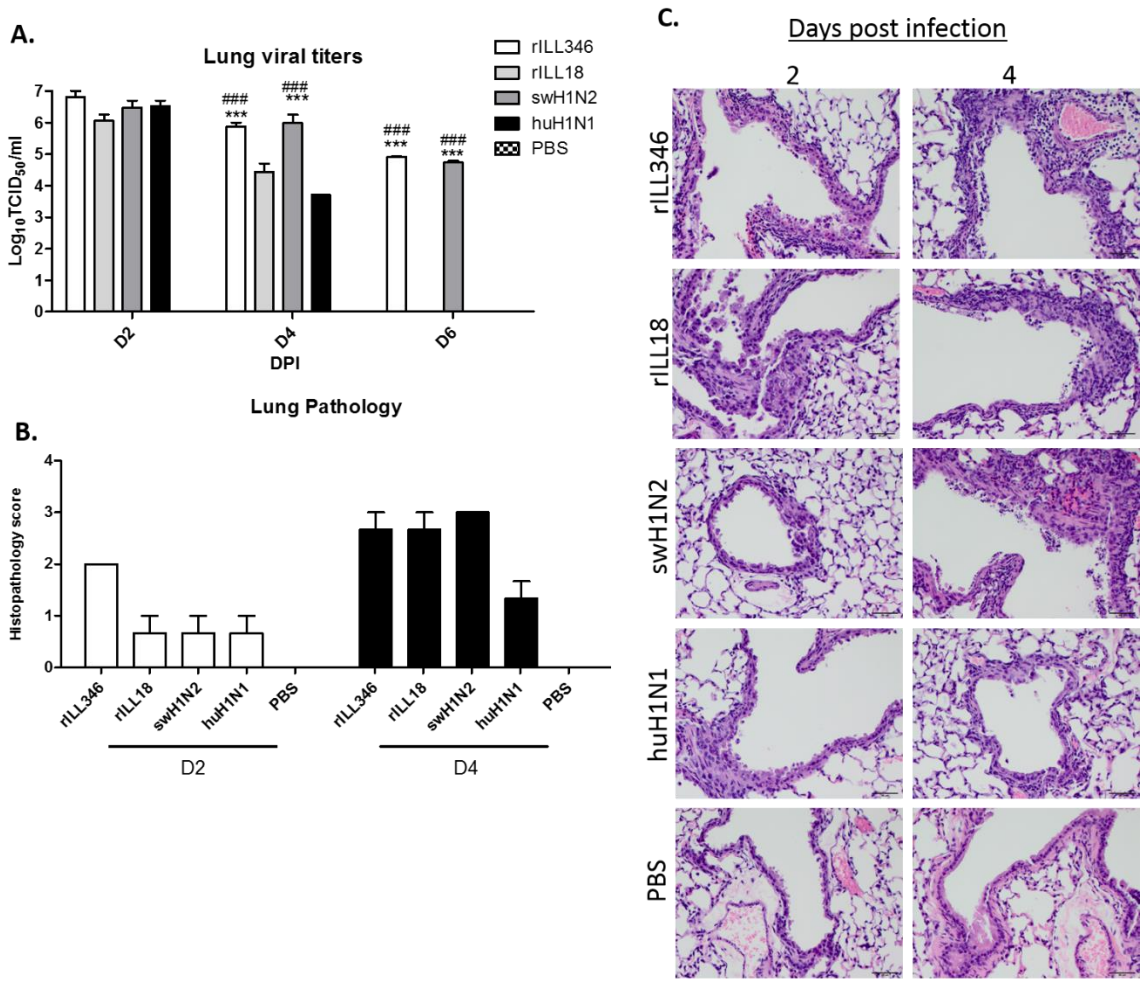


Figure 4. rILL346 infection is associated with exacerbates pathogenicity in BALB/C mice compared to the parental viruses. BALB/c mice were intranasally infected with $5x10^5$ PFU of rILL346, rILL18, huH1N1, or swH1N2. Lungs were collected and homogenized at days 2 and 4 pi and lung virus titers (A) determined. Endpoint titers are expressed as the mean log_{10} TCID50/ml $\pm$ standard deviation. The limit of virus detection is 10 TCID50/ml. Histopathological analysis (B) was performed on lungs of infected mice. Arrows presented in representative H&E stained sections (C) of lung tissue isolated from mice at 2 and 4 DPI show sites of leukocytes infiltration and alveolar damage. All data is representative of three individual experiments. Asterisks (*) denote significance

related to the pH1N1 parental virus while Hash (#) represents a p-value significant compared to the rILL18 reassortant virus. Results were considered significant with a P value ≤0.05 (*/#) ≤0.01 (**/##), and ≤0.001 (***/###).

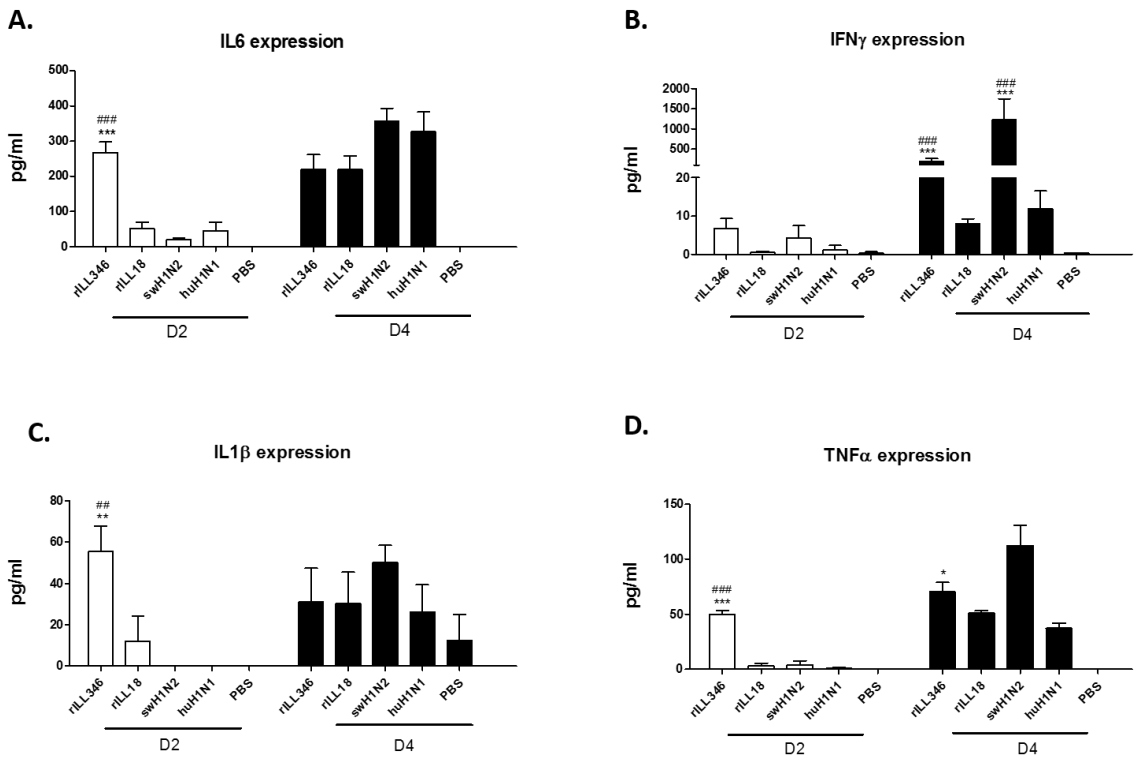


Figure 5. Early expression of pro-inflammatory cytokines linked to rILL346 infection. Proinflammatory cytokine expression levels in the BAL of mice infected with rILL346, rILL18, huH1N1, and swH1N2 were evaluated at day 2 and 4 pi. BALB/c mice were intranasally infected with $5x10^5$ PFU virus. At days 2 and 4 pi, brochoalveolar lavage (BAL) was collected from infected mice. The BAL fluid was for expression of IL-6 (A), IFNγ (B), IL-1β(C), and TNFα (D) by Luminex. All data is representative of three individual experiments. Asterisks (*) denote significance related to the pH1N1 parental virus while Hash (#) represents a p-value significant compared to the rILL18 reassortant virus Results were considered significant with a P value ≤0.05 (*/#)≤0.01 (**/##), and ≤0.001 (***/###).

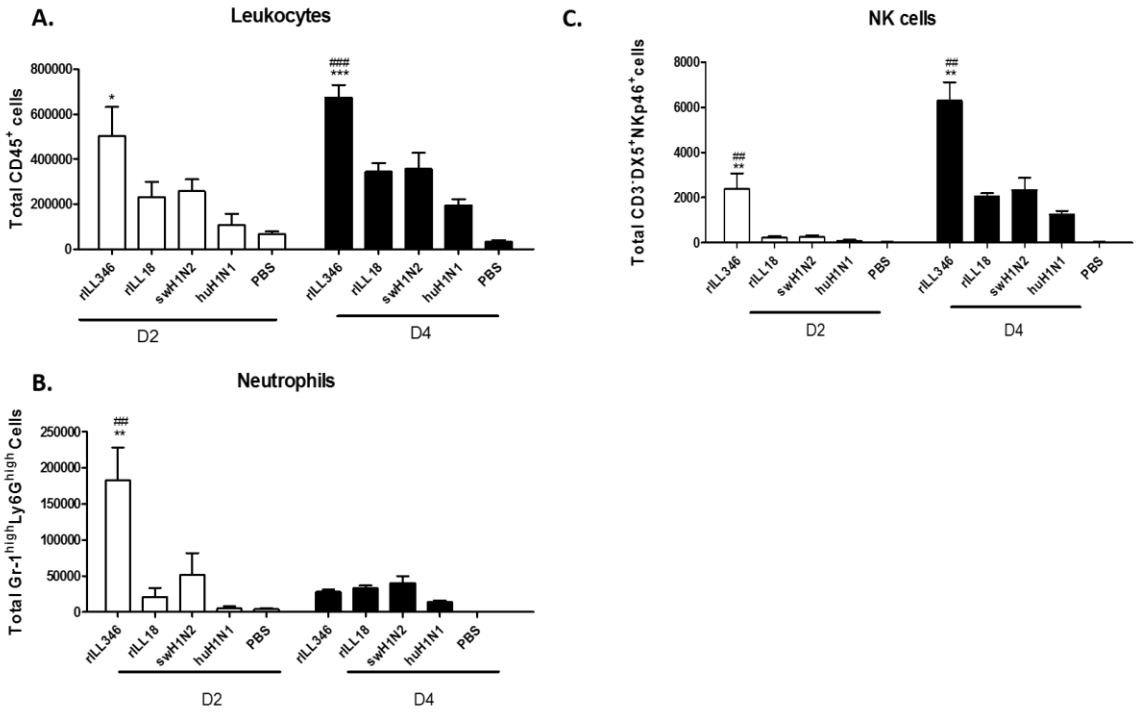


Figure 6. Infection with rILL346 and rILL18 affects pulmonary innate cell trafficking. Pulmonary cellular infiltration in mice infected with rILL346, rILL18, huH1N1, and swH1N2 at day 2 and 4 pi. BALB/c were intranasally infected with $5x10^5$ PFU virus and BAL fluid was collected on days 2 and 4 pi. A) Pan leukocyte (CD45+), B) NK cell (CD3-(DX5+NK46p+), and C) Neutrophil (Gr-1^{1hi}, LY6G^{hi}) cell infiltration was determined by flow cytometry. All data is representative of three individual experiments. Asterisks (*) denote significance related to the pH1N1 parental virus while hash (#) represents a p-value significant compared to the swH1N2 parental virus. Results were considered significant where p-value ≤0.05 (*/#)≤0.01 (**/##), and ≤0.001 (***/###).

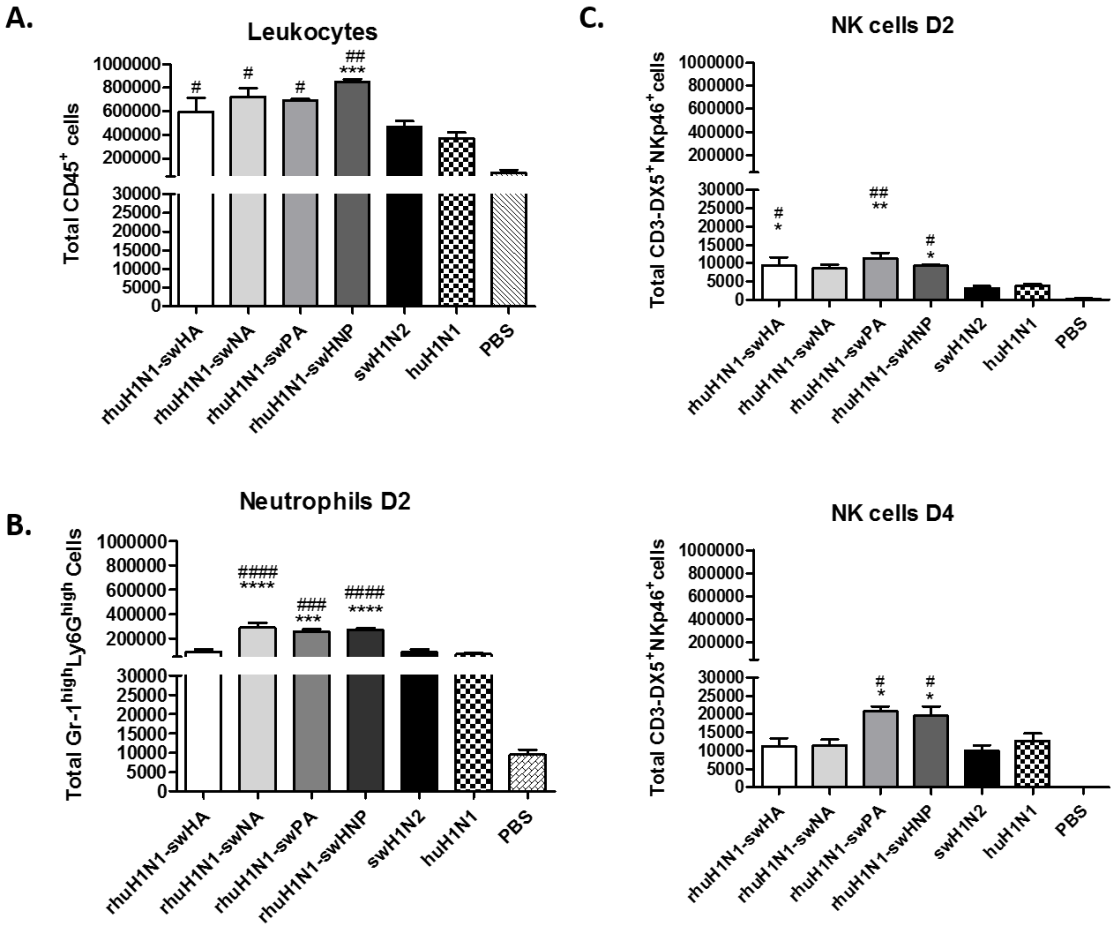


Figure 7. swH1N2 PA and NA contribute to increased levels of neutrophil and NK cell recruitment. To elucidate the genetic components that may be contributing to neutrophil and NK cell pulmonary recruitment the reassortant viruses d were rescued to assess the contribution of the swine HA, NA, and PA genes. Swine PA, HA, NA genes were introduced into the huH1N1 backbone individually (HA, NA, AND PA) or together (HNP). Mice were intranasal infected with $5x10^5$ PFU vbirus, and BAL was collected at days 2 and 4 pi to assess the recruitment of A) leukocytes (CD45+) B) neutrophils (Gr-1^{hi}, $LY6G^{hi}$), and C) NK cells (CD3-(DX5+NK46p+) into lung. All data is representative of

three individual experiments. Asterisks (*) denote significance related to the pH1N1 parental virus while hash (#) represents a p-value significant compared to the swH1N2 parental virus. Results were considered significant with a P value ≤0.05 (*/#)≤0.01 (**/##), and ≤0.001 (***/###).

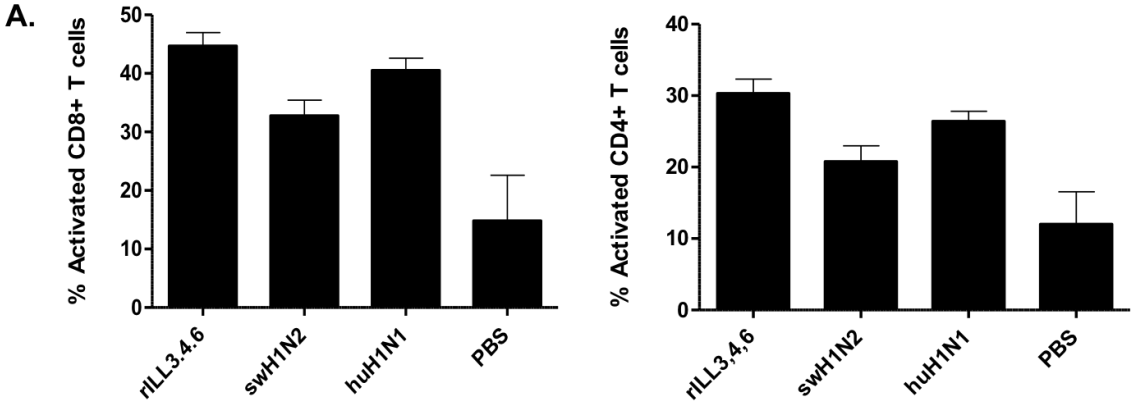


Figure 8. Modulation of the CD4 and CD8 T cell response following rILL346 infection. T cell recruitment to the lungs of infected mice was determined following infection with rILL346, rILL18, huH1N1, and swH1N2 at day 10 pi. BALB/C were intranasally infected with $5x10^5$ PFU virus. BAL fluid was collected at day 10 pi and the percentage of activated CD4+ (CD3+CD4+CD44+CCD69+) and CD8+(CD3+,CD8+,CD44+,CD69+) T cells from the total CD4+ (CD3+CD4+) and CD8+ (CD3+CD8+) population determined. All data is representative of three individual experiments.

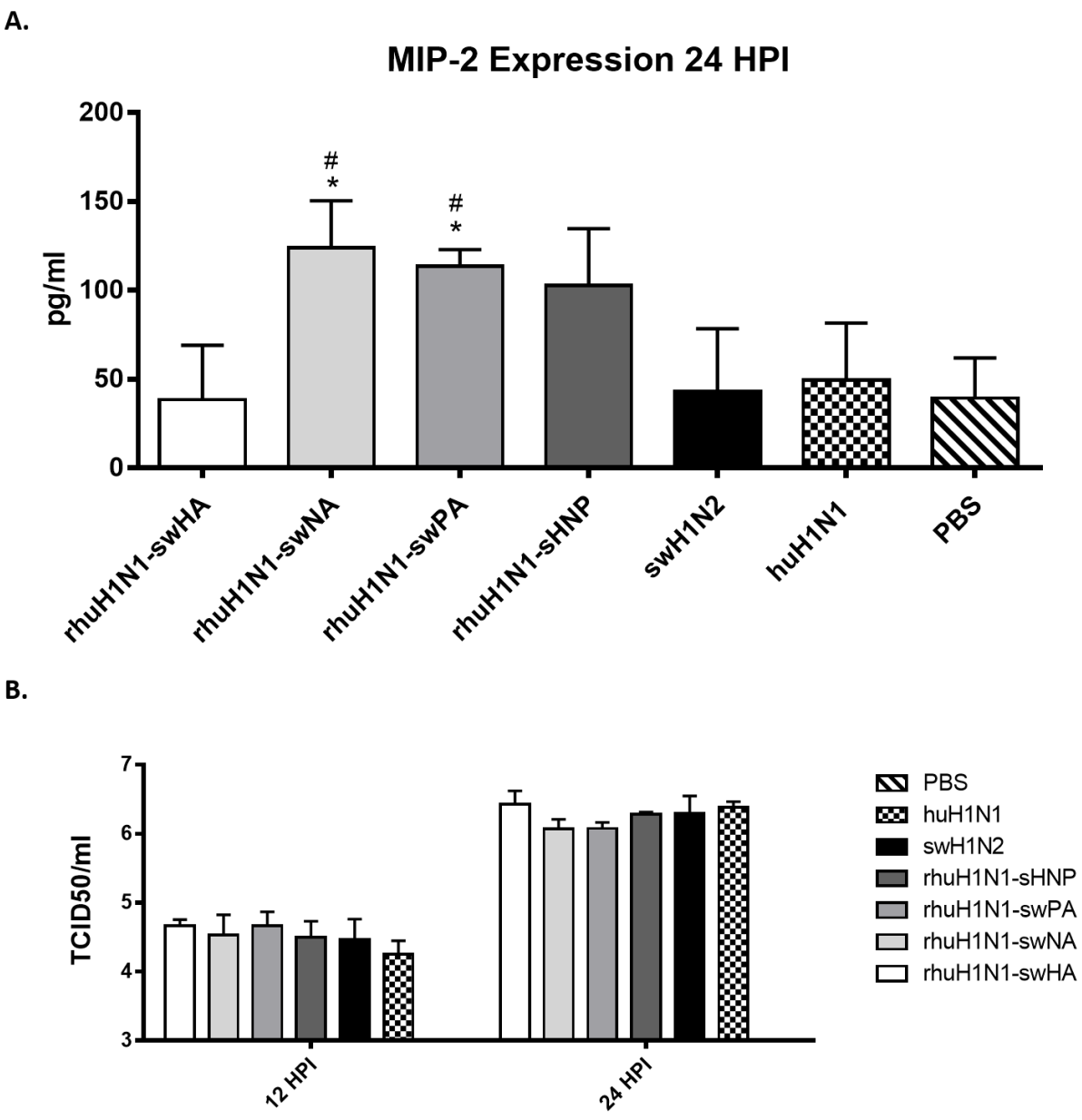


Figure 9. swH1N2 NA and PA genes affect early MIP-2 expression. MIP-2 expression in the BAL was measured by ELISA at 24 hpi (A). Mice were intranasally infected with $5x10^5$ PFU of reverse genetic-derived viruses where the swH1N2 HA, NA, PA, HNP were introduced into the huH1N1 virus along with the parental viruses. BAL fluid was collected at 24h pi. Lung viral load was measured by TCID50 on BALB/c mice infected

with reverse genetic derived viruses or the parental swH1N2 and huH1N1 viruses. Viral titers were determined at 12 and 24 hours post infection (B). All data is representative of three individual experiments. Asterisks (*) denote significance related to the pH1N1 parental virus while hash (#) represents a p-value significant compared to the swH1N2 parental virus. Results were considered significant with a P value ≤0.05 (*/#) ≤0.01 (**/##), and ≤0.001 (***/###).

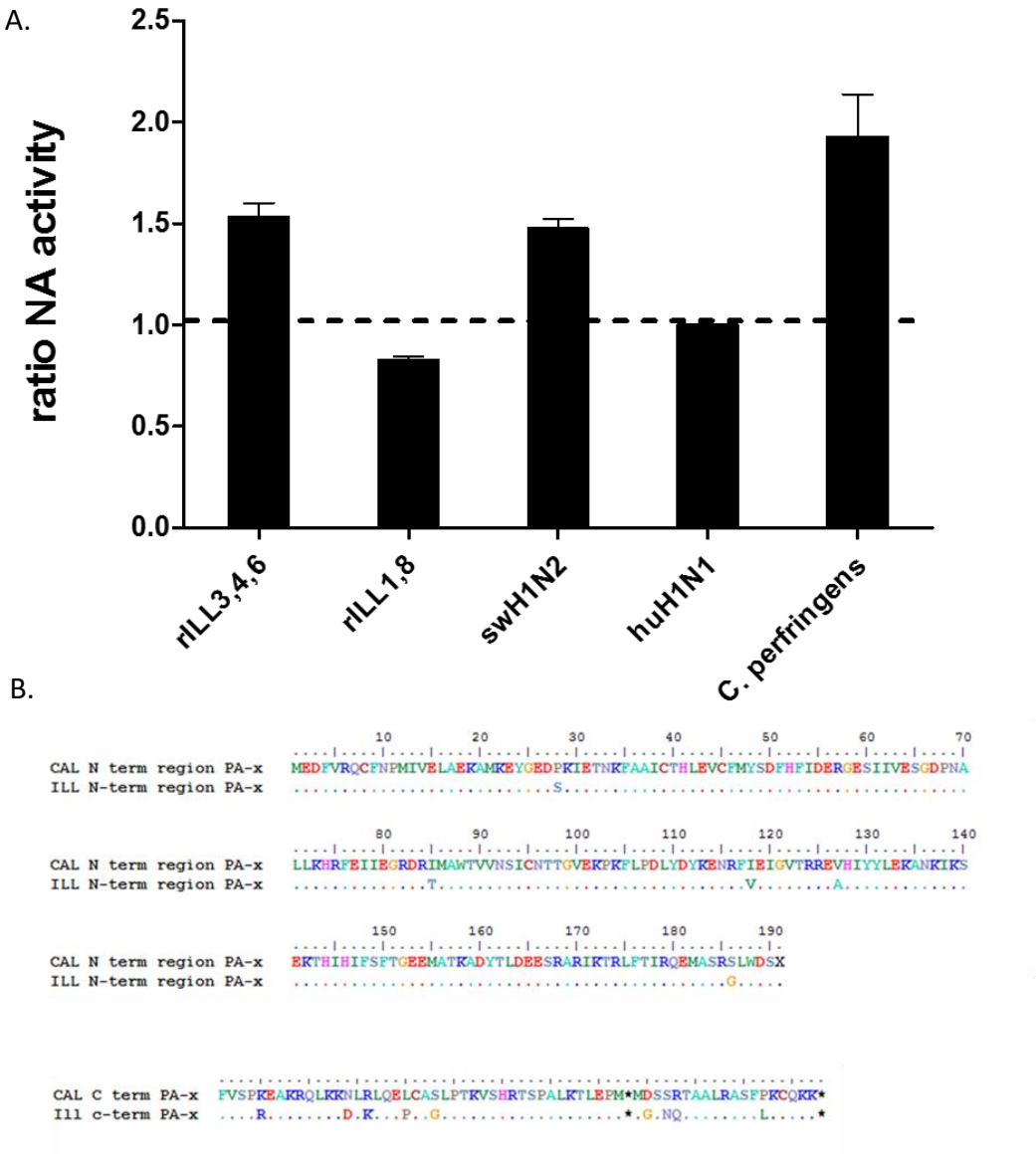


Figure 10. Potential viral associated factors contributing to NA and PA mediated pathogenicity. NA activity was compared between rILL346, rILL18, swH1N2, and huH1N1. 1.0 mg of protein was used to normalize for variation between viral samples. 1.0mg of viral protein was mixed with MUNANA for 30mins at 37 degrees Celsius in the absence of light. Viral samples were compared relative to huH1N1 cleavage activity. Data is representative of three independent experiments

CHAPTER 5

CONCLUSIONS

Influenza A virus (IAV) is the only virus to cause 4 different pandemics in the past century. The 1957 and 1968 pandemic viruses resulted from reassortment of two independent strains in avian species; while the 2009 influenza pandemic resulted from a triple reassortment of avian, human, and swine lineages and originated in swine (ref). Swine occupy an important niche in influenza ecology, and represent a mixing vessel for influenza viruses as defined by the ability of swine to support IAVs from avian and human lineages. The mechanisms which govern influenza reassortment are still unknown, but some insights have been garnered on viral constituents which drive influenza reassortment including gene specific packaging signals, but still much needs to be elucidated. Reverse zoonosis of the 2009 pandemic virus was identified quickly after outbreak of the pandemic allowing continued evolution of the pandemic virus and circulating swine IAVs. Viruses with enhanced pathogenicity have been identified due to continued reassortment in swine. Therefore, the emergences of a virus with pandemic potential is of utmost concern. Identification of the constituents of pathogenicity circulating in swine and mechanisms driving enhanced virulence need to be determined. The central questions addressed by this study were: What drives the species specific reassortment potential of swine epithelial cells compared to human epithelial cells, and

can *Pteropus alecto* epithelial cells support influenza replication and reassortment? What is the pathogenic potential and compatibility of reassortants that occur between the pandemic H1N1 and endemic swine viruses? Our hypotheses were: *Hypothesis 1:* **S**wine epithelial cells are differentially susceptible to influenza viruses as compared to human epithelial cells, allowing for improved opportunity for co-infection, reassortment, and adaptation resulting from inclusion of the TRIG cassette. *Hypothesis 2:* Reassortment between pH1N1 and swTRIGH1N1 results in novel reassortants with increased pathogenicity characterized by an altered innate immune phenotype. The *specific aims* addressed were:

Specific Aim 1: Establishment of a methodical approach for identification of naturally occurring reassortants resulting from co-infection of human (calu3) and swine (LLC-PK1) cell lines with the current circulating strains A/California/04/09 & A/Swine/Illinois/02860/2009. In Chapters 3 and 4, we required a method to investigate the generation of IAV reassortants with a high level of accuracy and high throughput in order to process a large array of samples in a timely manner. To this end, we developed a multiplex qPCR approach described in Chapters 3 and 4, using a triplex array of primers and probes. As we were focused primarily on investigating the role of the TRIG cassette during reassortment we developed gene specific primers and probes against the glycoproteins genes in order to identify glycoprotein gene exchange. We included an internal gene control to identify stability of the TRIG cassette using PB2 specific primers and probes. We developed a highly efficient method for identifying IAV

reassortants between A/California/04/09 and A/swine/Illinois/02860/09 with a 100% accuracy.

Specific Aim 2: Identification of differential reassortment patterns resulting from co-infection of either human or swine epithelial cells *in vitro* and resolution of potential viral factors contributing to reassortment in swine, and evaluating the reassortment potential in bat epithelial cells. Using the described method in Aim 1, we were able to comparatively investigate the reassortment potential of swine versus human epithelial cells. We identified, that swine support a reassortment rate of approximately 20% with preferential reassortment of the glycoprotein genes. On the other hand, we were unable to identify influenza reassortants resulting from co-infection of human epithelial cells. Therefore, it was evident that some viral or host specific mechanism was driving species specific reassortment in swine. As both viruses harbor the TRIG cassette and as we observed preferential glycoprotein exchange and increased stability of the TRIG cassette, we conclude that the TRIG cassette is a contributory factor mediating species specific reassortment in swine. Using the described methods in Aim 1, we were also able to establish the susceptibility of *Pteropus alecto* cells to infection and the propensity for co-infection and development of novel reassortants viruses. Using both human and avian strains of IAV we establish that bat cells can support replication of influenza, and may represent a novel mixing vessel for continued evolution of IAVs.

Specific Aim 3. Characterization of the differential pathogenesis *in vitro* and *in vivo* observed with the isolated reassortment viruses compared to the parent viruses. In Chapter 4, we identify a reassortant virus from *in vitro* co-infection of LLC-PK1 cells

which replicated to higher titers and drove a more robust type III cytokine response *in vitro* compared to the parental strains. rILL346 has the swine NA, PA, and HA with remaining genes derived from the pandemic H1N1 virus. Therefore, we established pathogenesis of this virus in ferrets. rILL346 significantly enhanced the immunopathology of the lungs of ferrets as represented by histopathological analysis of the lung and nasal turbinates. No appreciable differences were observed in viral titers therefore implicating the host specific response in mediating the observed pathology. We then establish the immunophenotype of the virus in mice compared to the parental strains. From these studies, we established that rILL346 drives enhanced pathology through recruitment of neutrophils mediating a robust pro-inflammatory cytokine response.

Specific Aim 4: Identify viral genes or gene constellations which contribute to enhanced viral pathogenesis resulting in acute lung injury, and potential mechanisms underlying ALI induction post IAV infection. From specific Aim 3, we identified that rILL346 enhanced the immunopathology of infection via neutrophil recruitment. We therefore needed to establish the specific genetic component or constellation responsible for mediating this enhanced response. To this end we developed single gene reassortants using standard reverse genetic techniques and further investigated the contributing genotype driving the specific pathological response. To this end we establish, that the NA and PA independently and synergistically drive the enhanced recruitment of neutrophils post infection resulting in a more robust pro-inflammatory response. We further established the mechanism by which these two genes recruit

neutrophils to the lung. Here, we show that NA and PA upregulate MIP-2, a potent neutrophil chemoattractant , expression in the lung at 24 hours post infection mediating the recruitment of neutrophils 24 hours later.

Taken together, this work establishes that swine influenza viruses harbor specific genes which can modulate the host response to infection resulting in increased immunopathology in acute lung injury mediated by neutrophilia in the lung resulting from genetic exchange of the swine NA and PA genes. This is important as this research establishes that swine epithelial cells support a high reassortment rate for continued evolution of human and swine IAVs therefore providing a species specific platform for the development of IAVs with enhanced pandemic potential. This work calls for continued surveillance of swine influenza viruses therefore identifying potential pathogenicity markers and establishing the pandemic potential of current circulating viruses.