# SPECIFIC GENE CONSTELLATIONS OF SWINE INFLUENZA A VIRUSES AND DIFFERENCES IN THE HOST ANTIVIRAL RESPONSE INFLUENCES DISEASE PATHOGENESIS IN RESISTANT AND SUSCEPTIBLE MURINE STRAINS

By

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(Under the Direction of Mark Tompkins)

## ABSTRACT

The 2009 H1N1 influenza pandemic caused millions of infections and thousands of deaths in the United States alone. The spread of this virus worldwide, as well as the propensity for it to cause disease in children and young adults previously considered healthy, was a major cause for concern. The pandemic H1N1 influenza strain was the result of a quadruple reassortment event with other circulating influenza strains and entered back into the swine population via multiple reverse zoonotic events. Although this strain never became endemic in North American swine, some of its gene segments, including the matrix gene, has become predominant completely replacing the classical swine matrix gene in current circulating strains in North America. Previous studies have linked a more filamentous morphology, greater neuraminidase activity, and higher transmission efficiency with the pandemic origin matrix gene. The objective of this research was to evaluate the pandemic origin matrix gene to increase disease severity. A second objective of this research was to elucidate additional host factors that could contribute to increased disease susceptibility. Herein, we demonstrate that infection with swine influenza strains containing the pandemic origin matrix gene induces more severe histopathologic changes in the lungs resulting in greater morbidity and mortality relative to infection with swine influenza strains containing the swine origin matrix gene in the murine model. Furthermore, the increase in severity of disease can in part be attributed to the dysregulation of the host innate immune response in the form of disproportionate recruitment of

specific host innate immune cells as well as greater activation of neutrophils and NK cells during infection with swine influenza strains containing the pandemic origin matrix gene. To focus on additional host factors that contribute to influenza susceptibility, we compared the host innate immune response to influenza infection in phenotypically resistant (BALB/c) and susceptible (DBA/2) murine strains in an infection model that retained the previously established difference in morbidity, while minimizing the characteristic differences in replication. We demonstrate that the characteristic hyperinflammatory response in DBA/2 mice is attributed in part due to the dysregulation of the innate immune response distinguished by limited production of anti-inflammatory cytokines and a limited antiviral response despite high interferon production. Taken together, the research presented here demonstrates the importance of understanding the effects both viral and host factors can have on the development of disease, in order to mitigate the potential disease burden of seasonal or pandemic influenza.

Index Words: Influenza virus, swine influenza, pathogenesis, matrix gene, mouse model

pandemic influenza, interferon lambda

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# DEDICATION

To my family who helped me start this journey, to my high school biology teacher Mrs. Vogt who believed in me, and to Nicholas Curran without whom I never would have made it to this point.

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# CHAPTER 1

## INTRODUCTION

Influenza A virus along with Influenza B, Influenza C, Thogoto, Isavirus and the recently discovered Influenza D make up the Orthomyxoviridae family (1). Influenza A was first isolated from a human clinical sample in 1933. Despite its discovery more than 80 years ago and the first approved commercial use of an influenza vaccine in the United States 12 years later, influenza remains a major public health concern. Seasonal influenza is estimated to cause 3-5 million cases of severe illness and anywhere from 250 to 500 thousand deaths annually worldwide (2-4). Complications from influenza, are often due to secondary infections including: primary viral pneumonia, combined viral-bacterial pneumonia, influenza encephalopathy, and in children croup and myositis (3). These are a few of the complications that can arise and add to the direct cost of influenza infection through hospitalizations, medical fees, drugs, and testing exceeding $87 billion annually in the US alone. Aside from the significant direct cost, there is also substantial indirect cost mostly associated with loss of productivity, consequently increasing the economic impact (5). Therapeutics include: four antiviral compounds available in the US as well as several generally effective vaccines against seasonal influenza.

Current vaccines are either live attenuated virus or recombinant hemagglutinin (HA) protein. Both types are trivalent or quadrivalent and contain components of influenza A H1N1, H3N2 and components from influenza B. Vaccines against influenza A H5, H7, and other subtypes have been produced or are being developed as a precautionary measure against potential pandemics. Every year surveillance data is accumulated and the WHO releases recommendations for the vaccine strains for northern and southern hemispheres about eight months prior to the influenza season. Efficacy of the vaccine is affected by how antigenically similar the actual circulating strains are to the vaccines produced. There many other factors

that can affect the efficacy of the vaccine on an individual basis including age, immunosuppression, and genetics (6, 7).

The zoonotic potential of swine influenza viruses has been evaluated globally using surveillance data (8, 9). Although influenza infection in pigs generally causes low mortality, including infection with the pandemic H1N1 strain, it does cause significant morbidity, and symptoms can last from two to six days (10-14). This results in reduced daily weight gain and consequently a delay of animals going to market; therefore, the prevention of influenza infection of swine is economically significant (15-17). However, the ability of avian, human, and swine origin influenza strains to not only infect but also replicate well in swine with the potential for generation of recombinant viruses, has earned swine the status of mixing vessel, as well as resulted in a demand for research into the potential for new emerging strains and their pandemic potential. This is, despite the fact that the global transmission of pandemic H1N1 in swine was due to multiple introductions of the virus from humans to swine, and not the reverse (18-20). Studies have shown more than 70% of the swine influenza strains characterized since 2011 contain genes of pandemic H1N1 origin, specifically, the polymerase acidic, nuclear protein, and matrix genes (21). Other studies have linked the pandemic origin genes with an increase in efficiency of replication and transmissibility in various animal models (22). With the propensity of influenza strains to reassort in swine and an increase in viral diversity of swine influenza strains due to the constant introduction of human origin influenza strains, the efficacy of influenza vaccines in swine is of great concern (23).

In 1930, Shope demonstrated that swine influenza can infect mice, and over the years mice have become a common model to study influenza infection, specifically influenza disease pathogenesis, and are particularly useful in studying the efficacy of potential vaccines and therapeutics (24). Recent studies demonstrated that murine strains can be classified on a spectrum from susceptible to resistant based on the resulting morbidity and mortality to influenza infection (25). In light of this, our research efforts have focused on using two murine strains, one categorized as susceptible and one resistant, to study disease pathogenesis of a panel of swine influenza virus isolates from the last ten years. The overall objective of the research project presented here is to demonstrate the potential of enhanced disease in the murine model

from swine influenza strains of specific gene constellations. This project was developed around the central hypothesis that infection of mice with swine influenza strains containing a pandemic origin matrix would result in enhanced disease compared to infection with strains containing a swine origin matrix gene. The following aims were developed to address this hypothesis:

1. Investigate differences in morbidity and mortality between mice infected with swine influenza strains containing either the pandemic origin or swine origin matrix gene.
2. Investigate differences in disease pathogenesis, virus replication, and immunopathology induced by swine influenza viruses containing either the pandemic origin or swine origin matrix gene.
3. Determine differences in cytokine and chemokine profiles in mice post-infection with swine influenza strains containing either the pandemic origin or swine origin matrix gene.
4. Determine the role of interferon signaling in the host innate immune response relative to disease susceptibility in the murine model.

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# CHAPTER 2

# LITERATURE REVIEW

## Overview of Influenza A Virus

Influenza A is a negative sense single strand RNA virus with a segmented genome comprised of eight segments which encode 10-11 proteins. The eight segments in order of largest to smallest are polymerase basic 2 (PB2), polymerase basic 1 (PB1), which encodes for PB1 and PB1-F2 proteins, polymerase acidic (PA), hemagglutinin (HA), nuclear protein (NP), neuraminidase (NA), matrix (M), which codes for both M1 and M2 proteins, and non-structural (NS), which codes for both NS1 and NS2, also known as nuclear export protein (NEP) (1). This segmented genome is in part what makes genetic reassortment a possibility and is one of the mechanisms that leads to strains of influenza causing pandemics such as in 2009. Most viruses go through genetic drift which are single point mutations allowing for gradual accumulation of differences between strains; however, reassortment allows for entire gene segments to mix from different strains of influenza resulting in a new strain (2).

The virus shape is pleomorphic, appearing as either round or more filamentous. The outside structure is formed from the lipid membrane of the host cell with the virus M2 protein embedded in it and the HA and NA proteins sticking out like spikes (1). The M1 protein lies just beneath the lipid membrane with the ribonucleoprotein complex (RNP) at the core. The RNP is made up of RNA wrapped around NP with PB1, PB2 and PA attached in a double helical arrangement as first described by Duesberg in 1972 (3). Viral replication begins with the HA protein attaching to neuraminic acids (sialic acids) expressed on the host cells. In avian species the predominant receptor has an α 2,3 conformation where as in the human upper respiratory tract the predominant conformation is α 2,6 which allows for some virus-host specificity. There are hosts such as swine that possess both α 2,3 and α 2,6 receptors in their upper respiratory tract and therefore may be infected by both avian and human specific influenza strains (4). Recent research has

demonstrated that a single amino acid change can alter specificity from α 2,3 to α 2,6; however, there are other factors that also play into influenza virus-host specificity (5). After attachment, the virus enters the cell via one of the following mechanisms: clathrin coated vesicles, which is the most common method, caveolin, non-clathrin non-caveolin dependent endocytosis, or through micropinocytosis. The virus is then trafficked to a late endosome which requires several host proteins including protein kinase C (PKC), Rab5, and Rab7 (6, 7). The HA molecule is a trimer that is synthesized as a precursor, HA0, which is susceptible to protease digestion by host proteases (trypsin-like) resulting in cleavage of HA0 into two subunits, HA1 and HA2. Virus membrane fusion is mediated by the HA protein and is pH dependent. The low pH (~ pH 5.0) triggers the conformational change of HA1 and HA2 that results in exposure of the fusion peptide (8-10). The fusion peptide is inserted into the endosome membrane and the HA1 becomes aligned antiparallel to the membrane anchor of the HA2 allowing for fusion with the endosomal membrane (8, 11). This conformational change and fusion of the HA molecule to the membrane of the endosome occurs in several HA simultaneously, thereby forming a pore in the endosome membrane which releases the RNP complex into the cytoplasm of the cell (10). This process of uncoating also requires H+ ions in the endosome to be transported into the virus particle itself. The specificity of the H+ ions is dependent on a histidine residue 37 within the 97 amino acid M2 protein (12). As an ion channel, the M2 protein, is responsible for the lowering of the pH inside the virus which allows for disruption of M1 protein-protein interactions and thus releasing the RNP complex (13-18). The proteins that make up the RNP complex all have nuclear localization signals that allow for active transport into the nucleus where transcription and translation take place (19-21). Some of the proteins transport as a complex, for example PB1 was found to require co-expression of PA for transport into the nucleus (22). There is still some debate as to the specific mechanism for transport. What is known, is that the RNP proteins are recognized by Karyopherin α (importin α) (23, 24). Upon binding karyopherin α, complexed RNP proteins recruit Karyopherin β at the nuclear port allowing for transport into the nucleus (25).

The viral RNA polymerase of influenza is made up of three proteins, PB1, PB2 and PA, which together catalyze both transcription and replication of the viral genome (26-28). mRNA is synthesized from

the viral RNA (vRNA) and requires a capped primer which the PB2 protein snatches from the host RNA pol II by cleavage after purine residues within 10 – 13 nucleotides from the cap structure (29, 30). Cleavage and initiation activity requires binding of vRNA (31). Initiation of transcription occurs by the addition of a G residue and will continue by PB1 until a stretch of U where it will stutter creating the poly adenylation or the poly A tail (32-35). For transcripts such as the NS1 or M1 segments, the alternate splice transcript is controlled to approximately 10% of the unspliced transcript. For NS1, this mechanism is controlled mostly by nuclear export regulated by a negative feedback loop, although it is likely that an alternative mechanism exists (36). For M1, there are several mechanisms for control, including nuclear export and activation control of cellular splice factors SF2/ASF (37). It is well understood that influenza transcription of complementary RNA (cRNA) is primer independent; however, the mechanism of switching between mRNA and cRNA is still unclear (38-41). There is some research suggesting that the polymerase complex is structurally different between replication and transcription competent replication. There has also been data suggesting the availability of soluble NP as a factor for control based on increased stabilization with NP for cRNA. This would prevent degradation as well as assist with direct interaction with the viral polymerase thereby possible aiding in switching from capped-primer translation to vRNA replication (42-44).

The HA, NA, and M1 genes are expressed later in infection than PB1, PB2, and PA. Since M1 has replication stopping capabilities, it would require delayed expression to allow for sufficient replication for new virions (38). This delayed expression can be connected to a single mutation in the 4$^{th}$ nucleotide from the 3'end in the vRNA promoter, an otherwise very conserved region. PB1, PB2, and PA have a C in that position, which is associated with down regulation of transcription and up regulation of replication, whereas the other segments all have a U (45). The virus can also regulate viral gene expression through a variety of mechanisms including degrading host pre-mRNA post cap snatching, inhibiting host mRNA processing, degrading cellular RNA polymerase II, and preferential translation of viral mRNA transcript. New research has demonstrated a possibility that preferential translation of viral mRNA may be possible by the viral polymerase complex remaining associated with viral transcript making the cellular factor eIF4e

unnecessary (46). This is supported by the demonstration that eIF4E is inactivated in influenza infected cells (47).

The last steps of virus replication are assembly and release. The RNP complex is assembled in the nucleus and therefore needs to be exported to the cytoplasm where with other proteins packaging and release can occur. M1 is known to associate with the RNP complex while in the nucleus as well as with vRNA, NP, and nucleosomes. The association of RNP with M1 allows for RNP dissociation from the nuclear matrix. A nuclear export signal (NES) has not been found on the M1 protein; however, M1 is known to interact with NEP which does have an NES and has been shown to be critical for RNP export (48, 49). NEP is known to interact with nucleoporins as well as the cell export receptor CRM1 (50). Experiments using Leptomycin B, a CRM1 inhibitor, have demonstrated that RNP export into the nucleus is CRM1 dependent (51). One hurdle that the virus must overcome is preventing RNP reentry into the nucleus after export. This mechanism is regulated by the binding of NEP to M1, which hides the NLS of M1 thereby preventing nuclear re-entry (48). NP also assists in this mechanism by binding actin filaments in the cytoplasm thereby acting as an anchor once in the cytoplasm (52). In polarized cells, the virus buds from the apical plasma membrane. This is thought to generally restrict replication to the mucosal surface unlike other viruses, which are not restricted to budding from the apical plasma membrane and therefore can more easily produce a systemic infection (53-55). This is further validated by the localization of the HA, NA, and M2 proteins to the apical surface (56-58). The HA, NA, and M2 proteins are folded and assembled in the ER and then are exported to the Golgi where the HA and NA undergo glycosylation. Cysteine residues on the HA and M2 are also palmitolylated in the Golgi network (59). These molecules then traffic to the apical surface where they associate with lipid rafts. This association is required for efficient replication since it allows for a minimum required virion concentration of HA and NA to occur in a localized area or “bud zone” (60, 61). M2 has been shown to be excluded from these lipid rafts, which may explain its minimal content in the virion (62, 63). M2 can however, bind to cholesterol and therefore may attach at the periphery of the lipid rafts and thereby bring several lipid rafts together (64).

There are several models for how the virion is packaged; one of the current models is selective incorporation. This model requires that each protein or RNP complex has packaging signals in coding and non-coding regions; therefore, upon packaging each virion has only eight segments (65). For packaging to occur, all of the proteins must converge into one relatively small area. The mechanism for this is believed to be a function of the M1 protein; however, the M2 protein is also required (66). M1 not only is able to bind to the RNP complex during nuclear export but can also bind to the lipid membrane afterwards. M1 has also been shown to bind to the cytoplasmic tails of HA, NA, and M2, thereby associating the inner core to the plasma membrane (67-69). Initiation of budding begins with the curvature of the plasma membrane (68, 69). The M gene is linked to shape characteristics, either rounded or more filamentous, although other factors such as polarization and the intact actin cytoskeleton also contribute (66, 70-73). The cytoplasmic tail of the M2 protein has scission capability although other research has shown that Rab11 may also play a part in the process (74). Truncation of the M2 cytoplasmic tail specifically the last 16 residues but not the last 8 results in a decrease in release of infectious virions (75). Lastly the enzymatic activity of the NA protein is required for release by cleaving the sialic acid residues on the cell membrane from the HA, thereby also preventing aggregation of the virions (76-78). Therefore, the virus must contain a balance between HA and NA proteins such that attachment and entry is allowed but also release of the new virions can occur.

Several of the viral proteins can directly affect replication and transmission efficiency, such that specific mutations in those proteins can drastically alter the outcome of infection. As mentioned previously, the M1 protein along with the cytoplasmic tail of the M2 protein effect virion morphology, varying from more spherical to filamentous (73, 79-81). A single mutation such as at residue 86 of the M2 protein can decrease virus replication in a temperature dependent manner while replacing an alanine with a proline at residue 41 of the M1 protein results in a reduction of transmission efficiency (82, 83). Multiple studies have demonstrated that the NA and M genes from the pandemic H1N1 influenza strain can also confer filamentous morphology and increased neuraminidase activity (84, 85). Several recent studies have now linked a higher percentage of filamentous shaped virions and higher neuraminidase activity to subsequently

greater transmission efficiency (85-87). A study back in 2009 demonstrated that the mutation rate of the matrix gene is higher in humans and swine than in avian influenza strains giving good reason to further delve into the potential links between differences in the M gene of various influenza strains and host tropism and disease (88).

## Overview of Swine Influenza Virus Infection in North America

Koen, following the 1918 "Spanish flu" pandemic, was the first to describe influenza infection in swine (89). This H1N1 virus was later isolated from swine in 1930 by Shope, remained the dominant strain in North America through the 1990s, and is referred to as classical swine influenza virus (classical swine) (90, 91). In 1998, a human-origin H3N2 triple reassortment virus was successful in becoming established in the swine population (92). This virus contained the NP, M, and NS genes from classical swine virus, the PB1, HA, and NA genes from the human seasonal H3N2, and the PB2 and PA genes of avian origin (93, 94). The six internal genes: NP, M, NS, PB1, PB2, and PA, became known as the triple reassortment internal gene cassette (TRIG) (95). Reassortment events continued to occur, specifically between the H3N2 and classical swine H1N1 and H1N2 strains, resulting in the TRIG cassette becoming predominant; such that, most fully characterized swine influenza strains since 2000 contain these internal gene segment combinations (93, 96-99). Although the North American swine influenza lineage developed distinctly from the European and Asian lineages, the TRIG cassette has been found outside of the United States, including countries such as Korea, Vietnam, and China (100-103). It has been demonstrated that viruses with the TRIG cassette found their way to Asia through extensive animal trade (104). In 2009, a novel H1N1 virus emerged from pigs in Mexico and infected humans first in North America then spreading worldwide, becoming the first influenza pandemic of the 21$^{st}$ century (105-108). The virus also spread into pigs from humans in a series of reverse zoonotic events (109-112). This virus, later called "swine flu" and renamed pandemic H1N1 (pdmH1N1), was determined to have occurred through recombination resulting in six gene segments from the triple-reassortment North American swine lineage and two gene segments, M and NA, from the previously established Eurasian avian-like H1N1 swine influenza lineage, a phylogenetically distinct strain from any previously characterized in the U.S.(113, 114). The introduction and circulation of

pdmH1N1 in swine has resulted in another generation of reassortment viruses (115, 116). Currently, there are six main clades that are co-circulating in the U.S. including one H3-IV, three H1 from classical swine lineage (α, β, and γ), one from pandemic lineage (H1pdm), and one from human seasonal lineage (δ). The δ cluster is subdivided into δ1 and δ2 based off two separate introductions of human seasonal strains into swine, an H1N2 and H1N1 respectively, each forming a distinct and separate but closely related lineage (97, 117). Reassortment between the pdmH1N1 and co-circulating H3N2 swine viruses have resulted in influenza strains with diverse genetic constellations as well as antigenically distinct strains causing concern for the efficacy of vaccines in both swine and humans due to potentially low cross-reactivity of antibodies (118-121).

Recent research into the outcome of reassortment between circulating H3N2 swine influenza strains and the pdmH1N1 strain has demonstrated that the PA, NP, and M genes from pdmH1N1 most commonly replaced the genes from the TRIG cassette origin. Furthermore, by 2011 70% of the strains characterized showed replacement of the TRIG origin with the pandemic origin M gene (121, 122). This is a cause for concern as recent data suggests that the pandemic origin M gene confers increased neuraminidase activity as well as modulating the pleomorphic shape of the virion, together resulting in an increase in transmissibility (83, 85-87). This increase in transmissibility has been demonstrated in guinea pigs, as well as in swine (84, 123). Although increased transmissibility has not been proven in human infection, in 2010 an H3N2 variant containing a pandemic origin matrix gene did become zoonotic, infecting 364 people (120, 124-126). Furthermore, one study demonstrated that myeloid-derived dendritic cells produce a lower interferon response while secreting greater proinflammatory cytokines resulting in inflammasome activation in response to this same H3N2 variant (127). Therefore, the importance of understanding the host innate immune response to influenza infection, and further defining influenza genes that can affect disease pathogenesis and the host immune response, cannot be overstated.

## The Innate Immune Response to Influenza A Virus

The immune response first begins with recognition of a pathogen by pattern recognition receptors (PRRs). There are several types of PRRs including toll-like receptors (TLRs), nucleotide-oligomerization

domain (NOD)-like receptors (NLRs), and retinoic acid inducible gene I (RIG I)-like receptors (RLRs). There are 10 human TLR proteins and they are expressed on a variety of cell types both on the surface of the cell as well as on intracellular membranes. TLRs recognize a large variety of non-self-molecules including everything from bacterial peptides such as LPS and flagellin, fungal mannans, viral envelope proteins, DNA, and RNA (128). TLR3 and TLR7 are found on the endosomal membrane and are responsible for recognizing viral double stranded RNA and single stranded RNA respectively, including from influenza virus. TLRs are differentially expressed depending on cell and tissue type and recent research has demonstrated that plasmacytoid dendritic cells (pDCs) tend to express mostly TLR7 (129, 130). In some cases, active virus replication is needed for TLR stimulation; however, several different groups have shown that pDCs can be stimulated by inactivated virus regardless of the method of virus inactivation. Therefore, stimulation of pDCs only requires the presence of viral genomic RNA (131). TLRs 2 and 4 are the most studied of the TLRs and are known to be expressed by cells at various mucosal surfaces including the upper and lower respiratory tract; therefore, their role in influenza pathogenesis has been examined (129). Although neither one is traditionally triggered by influenza infection, stimulation of these TLRs followed by viral challenge can increase resistance to influenza infection (132). Additional research has proven a more successful immunization response using nanoparticles with HA peptides along with ligands meant to stimulate both TLRs 4 and 7 compared to immunization with any one of these by themselves (133). It is widely known that TLR stimulation is important for both the innate and adaptive immune response and is mediated by the TLR signaling proteins.

TLRs are a type I integral membrane glycoprotein and contain leucine-rich repeats, cysteine rich repeats in the extracellular domain, and a Toll/IL-1 receptor (TIR) homology domain in the cytoplasmic region (134). A ligand, such as viral RNA, binds to the leucine rich repeat domain and triggers homo- or hetero dimerization of the receptor allowing for a conformational change leading to TIR domain interaction with each other and various adapter proteins. The TLR3 signaling pathway begins with binding of the adaptor protein TIR domain-containing adaptor protein inducing interferon β (TRIF) followed by activation of the TRAF family member associated NF-κB activator (TANK) binding kinase 1 (TBK1). This leads to

the activation of both transcription factors interferon regulatory factor 3 (IRF3) and NF-κB (129). TLR7 signaling requires the adapter protein MY-D88 followed by recruitment of IL-1 receptor-associated kinase 4 (IRAK4). IRAK4 is activated by phosphorylation and can then interact with and phosphorylate IRAK1. Together they associate with TNF receptor-associated factor 6 (TRAF6) forming a complex that disassociates from the receptor allowing it to associate with transforming growth factor-β-activated protein kinase 1 (TAK1). Similar to TLR3 signaling, this leads to the activation of the transcription factor NF-κB via the IκB kinase cascade as well as induction of interferon regulatory factor 7 (IRF7) and the mitogen-activated protein kinase (MAPK) pathway (135). Transcription factors IRF3 and IRF7 can induce hundreds of interferon stimulated genes (ISGs) including Viperin. Viperin interacts with IRAK 1 and TRAF6, which helps induce the translocation of IRF7 to the nucleus feeding back into the antiviral response (136).

NOD-like receptors form an inflammasome complex consisting of nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing (NLRP), the adapter apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [CARD] (ASC), and procaspase. There are 14 NLRP family members; however, during influenza infection NLRP3, also known as NALP3, is specifically activated (137). Assembly of the NLRP3 inflammasome complex via its canonical pathway leads to the activation of caspase 1 via cleavage of procaspase-1 which results in processing and subsequent secretion of the proinflammatory cytokines IL-1β and IL-18 (138). NLRP3 activation requires two signals, the first of which is dependent on signaling via other receptors to induce expression of IL-1β, IL-18 and NLRP3. The second signal is in response to host damage, thereby causing the activation of caspase 1 (139).

Inflammasome activation in response to influenza infection has been demonstrated in a variety of cells, both human and murine derived, including macrophages, dendritic cells, bronchial epithelial cells, lung fibroblasts and other epithelial cell lines (140-144) . The first signal during influenza infection can be via TLRs or another cytoplasmic receptor, RIG I, leading to type I interferon production (144). Recent studies have demonstrated that the M2 and PB1-F2 proteins are capable of inducing inflammasome activation independently (145, 146). Other recent studies have demonstrated inflammasome activation in

response to influenza infection regulates cellular recruitment, including neutrophils and monocytic dendritic cells via IL-1β, IL-18, MIP1, and KC. These pathways have been shown to be both NLRP3 dependent and independent (142, 143, 147). Furthermore, antibody class switching, specifically to IgA and IgG can be induced by inflammasome activation (143). A recent study demonstrated that inhibition of NLRP3 early during influenza infection reduces survival; however, inhibition of NLRP3 several days into infection can increase survivability by the decrease in recruitment of inflammatory cells which otherwise are known to exacerbate disease (148). Another recent attributed increased lethality of the 1918 influenza in macaques compared to other highly pathogenic strains to dysregulation of inflammatory related genes, including IL-1β and NLRP3 (149). Therefore, while inflammasome activation during influenza infection can be beneficial, it can also be detrimental, particularly when dysregulated.

There are three cytoplasmic receptors that belong to the RLR group: RIG I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RLRs contain a DExD/H-box helicase domain, a carboxy-terminal repressor domain, and except for LGPD, two amino terminal CARDs (150). Recently it was discovered that not only can RIG I recognize viral dsRNA but also ssRNA by its 5’ triphosphate moiety and is thought to recognize influenza RNA via this moiety as well as through its 3’ untranslated region (151-153). This moiety is generally lost on cellular RNA during maturation allowing for differentiation of self and non-self by this receptor. Like RIG I, MDA5 also recognized dsRNA and its synthetic analog polyinosinic-polycytidylic acid, or Poly (I:C), in a length dependent fashion, presumably a reflection of substrate preference (150). It is widely agreed that there are other factors that contribute to the differential binding of RIG I and MDA5 thus allowing for a broad spectrum of virus recognition. Upon stimulation, RIG I undergoes a conformational change that allows the CARD-containing adapter molecule mitochondrial antiviral signaling (MAVS) to bind and localize to the outer mitochondrial membrane via its transmembrane domain. MAVS is also known as CARD adaptor-inducing interferon β (Cardif), IFN-β promoter stimulator-1 (IPS-1), and virus-induced signaling adaptor (VISA) (150). TRIM 25 is the E3 ubiquitin ligase required for the interaction of RIG I and MAVS and allows for association of lysine 63-linked ubiquitin chains and resulting activation. More recent research

has determined a second E3 ubiquitin ligase that can function instead of TRIM 25, called REUL/RNF135/RIPLET (154, 155). There are several different molecules that can exert negative control and thereby regulate interferon activation such as the deubiquinating enzyme CYLD, allowing for specific control over the pathway and its downstream effects (156). The formation of the RIGI-MAVS complex stimulates the recruitment of a cascade of proteins that leads to downstream activation of ISGs and various transcription factors as part of the antiviral response. Furthermore, it is also capable of inducing a proinflammatory response. The first to be recruited to the complex are adapter proteins from the TRAF family, either TRAF3 that mediates the antiviral response or TRAF2 and 6, which mediate the inflammatory response (157). Following the antiviral pathway, NF-κB Essential Modulator (NEMO) interacts with a K-63 poly-ubiquinated TBK1 creating a complex which is also recruited to MAVS (158). While NEMO along with IKKα and IKKβ activate the transcription factor NF-κB; TANK, recruited by TRAF3, along with TBK1 form a complex that is able to modulate the phosphorylation of IRF3 and IRF7, which are transcription factors for both, type I and type III interferons (159, 160).

## Overview of Type I and Type III Interferon Signaling

Interferons were first described in 1957 by Isaac and Lindermann as an antiviral factor in chick choriallantoic membrane. Since then interferons have been divided into three groups: type I, which include IFNα, β, ε, κ, and ω; type II also known as IFNγ; and type III, which include IFNλ1, λ2, λ3, and λ4. Type I interferons, specifically IFNα and β (IFNα/β) are the archetype for the antiviral response and have been the most studied. There are 13 IFNα homologous genes all clustered on human chromosome 9. These 13 genes result in 12 distinct gene products, IFNα1 and IFNα13 are identical, all of which share 35% amino acid sequence identity with IFNβ (161, 162).

Type I interferons are produced by almost any cell type that has been appropriately stimulated including macrophages, pDCs, fibroblasts, and epithelial cells (163). Historically, the most potent cell type for interferon production is pDCs, which may be explained by constitutive expression of high levels of IRF7 (164). IFNα/β is produced in response to a variety of stimuli including dsRNA, ssRNA, CpG DNA, and LPS; although, recent research has demonstrated that LPS signals the production of only IFNβ along

with IFNλ1 (165). In most cell types, induction of IFNα requires the activation of a positive feedback loop. This has been shown to be based on differential regulation of IRF7 (164). Unlike most subtypes of IFNα, IFNβ does not require IRF7 for activation, although IRF7 is able bind to its promotor and enhance activation (166). Instead, IFNβ is considered an early responder and requires IRF3 for induction (167). Both IRF3 and IRF7 require phosphorylation of multiple serine clusters at their C-terminus by kinases; however, while IRF7 is MyD88 dependent requiring the adaptor protein TRAF6, IRF3 is MyD88 independent and instead requires the adaptor protein TRIF. This variation in adaptor proteins may account for the differences in early and late induction (168). Induction of interferons requires coordination of multiple proteins all interacting together and for optimal induction of IFNβ, assembly of the full enhanceosome is necessary. The enhanceosome assembles on the four positive regulatory domains (PDVI – IV) of the promotor and includes transcription factors IRF3, NF-κB, c-Jun/ATF-2 (169-172). HMGI(Y) is included in the enhanceosome as well; although IRF1 and IRF7 are also capable of binding the enhanceosome, they modify the response and are therefore not required like HMGI(Y) (166, 173). Once the enhanceosome is assembled, the creb-binding protein (CRB / p300) is recruited and assembles the transcript machinery. Acetylation of HMGI(Y) by PCAF/GCN5 stabilizes the enhanceosome while acetylation by CPB destabilizes the enhanceosome as one means of regulating IFNβ induction (174).

Differential expression of the varying IFNα subtypes has long been demonstrated. Therefore, a considerable amount of effort into studying the differences in the immune response among the subtypes has arisen. While most IFNα subtypes rely on autocrine and paracrine feedback loops, IFNα4 is induced early on during infection and therefore does not require IRF7 for induction. Instead, IFNα4 relies on IRF3 for induction, similar to IFNβ (175-177). Unlike the rest of the subtypes, IFNα13 has been shown to be constitutively expressed; however, in humans it is only a pseudogene (178). Along with IFNβ, IFNα11 and α12 have been shown to be the most potent antivirals and most antiproliferative (179). Other research has demonstrated that the IFNα response varies based on viral stimulation and cell type and is able to modify the antiviral state based on the stimulation (180-182). The discovery that IFNα subtypes as well as IFNβ respond differentially to IRF3 and IRF7 depending on the method of activation, either single or by co-

activation, substantiates the theory that the subtypes have varying functions (183). The different subtypes are believed to have developed varying functions due to functional gain (184). Despite all the differences between the varying IFNα subtypes and IFNβ, they all signal through a common receptor belonging to the type II cytokine receptor family.

The type I interferon receptor is a ubiquitously expressed heterodimer composed of IFNαR1 and IFNαR2 which associate with tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) respectively (185). STAT2 is also weakly bound to IFNαR2, and upon stimulation TYK2 phosphorylates IFNαR1 strengthening the bond with STAT2 resulting in its phosphorylation along with STAT1 phosphorylation by JAK1 (167). This phosphorylation event exposes the nuclear localization signal (NLS) as well as allows for recruitment of CBP to IFNαR2 resulting in acetylation of IFNαR2 forming the docking site for the DNA binding protein IRF9 (186). The STAT1, STAT2, and IRF9 complex is known as the interferon stimulated gene factor 3 (ISGF3) transactivation complex, which can bind to various interferon-stimulated response elements (ISREs). ISREs are part of the promotors for ISGs, including IRF7 (185). This is not the only signaling pathway for type I interferons. For example, instead of formation of a STAT1-STAT2 heterodimer complex, a STAT1 homodimer complex can form which results in binding and activation of IFNγ activated sites (GAS). Type I interferons can also signal through either the G-dependent mitogen activated protein (MAP) kinase pathway or through the PI3K pathway (187, 188).

Negative regulation of interferon signaling is essential for providing a controlled and appropriate cellular response; consequently, there are several different pathways by which type I interferon induction is negatively regulated. Suppressor of cytokine signaling 1 and 3 (SOCS1, 3) downregulate signaling via inhibition of the tyrosine 701 phosphorylation of STAT1 and thereby translocation of STAT1 (189). The Src homology 2 domain-containing PTP1 (SHP1) was found to associate with the IFNαR complex and inhibit signaling via preventing tyrosine phosphorylation of Jak1 and STAT1 specifically. UBP 43 directly inhibits type I interferon signaling by binding to the receptor subunit IFNαR2, thereby preventing association with Jak1 (190). More recent research has demonstrated IRF2 as a negative regulator of IFNβ, thus allowing for modulation of the concentration of constitutively secreted IFNβ (191). This has been

demonstrated to be important for a variety of functions including maintenance of hematopoietic stem cell niche, immune cell function, and bone remodeling (reviewed in depth by Gough) (169).

Aside from the antiviral response, which will be discussed in depth subsequently, type I interferons are capable of a variety of functions including stimulating cell cycle arrest through a variety of mechanisms, and tumorigenesis via heterodimer formation between the C10 regulator of a tyrosine kinase-like (CRKL) and STAT5 (192, 193). IFNβ has also specifically been shown to induce miRNAs that subsequently affect the antiviral response (194). Type I interferons can induce a proinflammatory response not only by recruitment of neutrophils and macrophages, but also through neutrophil activation. Cytokines and chemokines including interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and CXCL1/KC are all chemoattractants for monocytes including macrophages and neutrophils and are produced in response to type I IFN signaling (195-198). Furthermore, tumor necrosis factor (TNF) induced lethal shock is reduced in IFNαR knockout mice demonstrating type I IFNs ability to mediate activation of neutrophils and as well as monocyte recruitment and consequently the proinflammatory response (199, 200). Type I interferons can bridge the innate and adaptive immune response indirectly such as via neutrophil myeloperoxidase (MPO) activity mediating DC function; however, there are more direct mechanisms by which type I IFNs carry out this function (201).

More than a decade ago, research demonstrated IFNαβ enhanced production and cross-priming of $CD8^+$ T cells via DCs (202). Further research demonstrated IFNα enhances $CD8^+$ T cell proliferation, expansion, and long-term survival in vivo as well as adjuvant activity by CpG stimulation (203-206). This enhanced activation of T cells is mediated via DC secretion of IFNα/β (207). Dendritic cells themselves are also affected by type I interferons and undergo differentiation and phenotypic maturation more rapidly in the presence of IFNα/β. This effect in turn results in enhancement of DCs function in T cell activation, antigen presentation, and cross priming (205). In addition to enhancing DC and T cell functions, type I interferons also effect B cells in several different ways. In the bone marrow, IFNα/β effects B cell generation and selection as well as inducing sensitivity to IgM ligation activity (208). Recent research has shown additional effects on B cells including increased survival and resistance to Fas-mediated apoptosis,

induction of activation markers and co-stimulatory markers, as well as enhanced differentiation and proliferation (209, 210). Furthermore, the antibody response is enhanced by IFNα post soluble antigen, chicken gamma globulin (CGG), stimulation via the IFNαR on T and B cells (206). Many roles for type I interferon in immunomodulation have been described; however, much less in known about type III interferons and their role for bridging the innate and adaptive immune response despite similar induction and signaling pathways.

The type III interferon family was discovered by two independent groups in 2003 (211, 212). The family includes IFNλ1, IFNλ2, and IFNλ3, also known as IL-29, IL-28A, and IL-28B respectively, as well as a fourth member, IFNλ4. IFNλ4 was discovered in the last few years as a frameshift mutation of IFNλ3; however, it is only present in a fraction of the human population (213). The genes coding for IFNλ, including 5 exon and 4 intron regions, are clustered on chromosome 19 (211, 214). Although functionally an interferon, the IFNλ family are structurally related to the IL-10 cytokine family (211, 215). Of the 4 proteins, IFNλ2 and IFNλ3 have the highest amino acid similarity, 96%, while IFNλ1 has only 15 - 20% similarity and IFNλ4 shows 30% amino acid similarity with IFNλ3 (211-213, 216). The majority of the differences are in the disulfide bridges, five cysteines within the three disulfide bonds versus seven cysteines, is one of the structural differences between IFNλ1 and IFNλ2, 3 respectively (217). The murine genome contains a similar region encoded on chromosome 7A3; furthermore, this region encodes two functional proteins, IFNλ2 and IFNλ3, while IFNλ1 is non-functional in mice (218). In addition, IFNλ2 and IFNλ3 are glycosylated in mice in contrast to humans where only IFNλ1 is glycosylated (211, 212, 218). Similar to type I interferons, type III interferons are mostly produced by mDCs and pDCs although they can be produced by most cell types including epithelial cells, neuronal cells, and specific populations of T cells (219-223). Interferon λ expression can be induced by a multitude of stimuli including LPS, poly I:C, bacterial infections, and viral infections (165, 224, 225). The antiviral effects of IFNλ has been noted against a wide selection of viruses including sindbis, dengue, rotavirus, norovirus, reovirus, vesicular stomatitis (VSV), respiratory syncytial (RSV), influenza, Sendai, hepatitis B (HBV), hepatitis C (HCV), human immunodeficiency virus (HIV), encephalomyocarditis, West Nile, vaccinia, foot and mouth, and herpes

simplex (HSV) (211, 226-238). As with type I interferons, type III interferons respond to viral infection via the RIG I pathway signaling through MAVS followed by TBK1 leading to IRF3 and IRF7 activation (239, 240). The promotor regions of IFNλ are comparable to type I interferons; including binding sites for AP-1, IRF3, IRF7 and NF-κB (239-241). Although very similar, only type III interferons are induced by the recently discovered cytosolic DNA sensor Ku70 (242, 243). Moreover, differences between type I and type III interferon induction have also been noted such as enhanced induction of type III but not type I interferon by mediator complex 23 (Med23) during HSV infection (244). Kinetics between varying IFNλ subtypes are also disparate, such that in response to influenza infection, IFNλ1 is expressed within eight hours, whereas IFNλ2,3 is not present until 16 hours post infection (221). In response to poly I:C stimulation, IFNλ4 is expressed within 2 – 4 hours and is down regulated by 8 hours whereas IFNλ3 remains upregulated at 24 hours (213). Not only are type I and type III interferons structurally different, but the IFNλ family signals through a distinct receptor.

Interferon λ binds to a heterodimeric receptor made up of the ubiquitous IL-10Rβ and the unique IL-28Rα (211, 212). Unlike IFNαR, which is ubiquitous, IL-28Rα is restricted to mostly epithelial cells of the respiratory and gastrointestinal tract as well as epithelial cells of the brain, kidney, large and small intestine, lung, and vaginal cavity (238, 245-248). However, mRNA expression of the IL-28R has been shown in a variety of immune cells including neutrophils, B cells, T cells, and NK cells, although whether the receptor is fully functional on all these cell types is a contentious topic (246, 249, 250). Expression of IFNλRα is regulated by a variety of mechanisms, which can affect its function. The receptor has been shown to be inducible, for example during the maturation of monocytes to macrophages, possibly by transcription factors including STAT1, AP-2, c-Jun, and p53 for which binding sites have been identified in its promotor region (251, 252). These transcription factors are responsible for a variety of functions including early differentiation of B and T cells, apoptosis, proliferation, cell cycle arrest, and immune functions which can illuminate some of the functions of IFNλ outside of its well-studied antiviral properties (252). Like IFNα/β, IFNλ is a monomer in solution and interacts with its receptor in a 1:1 ratio (211, 215, 216). The ligand interface of the receptor includes helix A, loop AB, and helix F with the most important residues being

lysine 49 and arginine 51 for IFNλ3 and arginine 49 and histidine 51 for IFNλ2 (216, 253). Although similar in sequence, the murine IFNλRα, coded on chromosome 4D3, contains only two of the three tyrosine residues in the intracellular domain while gaining three tyrosine residues elsewhere. Despite these differences, additional research demonstrated that the murine ligand-receptor interaction of IFNλ results in similar signal transduction as its human counterpart (218, 254, 255). IFNλ signals via the same JAK-STAT signaling pathway that IFNα/β uses, as described previously (211, 212, 254). However, except for the conserved STAT2 docking site, the cytoplasmic domains of IFNαR1 and IFNλRα, specifically the tyrosine residues, differ and therefore may allow for separate signaling (254). Furthermore, while IFNα induces phosphorylation of STAT1, STAT2, and STAT3, it is relatively brief compared to the prolonged phosphorylation induced by IFNλ (256). This may be one mechanism by which type I and type III interferons are able to serve different functions while signaling via the same pathway.

When IFNλ was first discovered, it was postulated that type III interferons were a limited but redundant pathway to the type I interferons. Since then, several studies have demonstrated greater induction and production of type III interferons compared to type I interferons, in response to a variety of viral infection including RSV and influenza (257-259), and the redundancy of type I and III interferons has come into question. Other studies have shown intestinal epithelial cells differentially respond to type I and type III interferons (227, 230, 260). Moreover, more recent studies have established greater efficacy by type III interferons for ISG induction and viral clearance than type I interferons against specific viruses including porcine epidemic diarrhea virus (PEDV) and rotavirus (227, 261). In one study, type I interferons were shown to prevent systemic norovirus infection in a mouse model while only type III interferons cleared persistent infection (229), further disproving the theory that type III interferons are functionally redundant to type I interferons.

Since the upregulation of ISGs is the main mechanism by which interferons induce an antiviral state, there has been continued interest into the potential differences between type I and type III induction of ISGs. Induction of the JAK-STAT signaling pathways by IFNλ results in up regulation of a variety of ISGs all of which are also induced by type I interferons, although the reverse may not be true (262). There

is more recent research that has demonstrated induction of genes specific to IFNλ and not type I interferons; however, since these papers are rare, it has yet to be widely accepted (263). On the other hand, differences in the kinetics of ISG induction by type I and type III interferons have been demonstrated repeatedly. Several studies in hepatocytes, in relation to hepatitis C virus, show that while IFNα induces an early rapid ISG response, it is also short lived relative to IFNλ, which induces a far more sustained ISG response (263-266). Furthermore, the kinetics of the ISG response to IFNλ is similar to IFNβ (267). This sustained strong ISG response by IFNλ may be in part due to the increased expression of IFNλRα, as shown during HCV infection. This substantiates earlier research that proposed a regulatory feedback loop mechanism between receptor and protein based on the ISG STAT1 (252, 264). Although the majority of this research has been done in hepatocytes and used to study the interferon response to HCV, Zhou's earlier study included a variety of cell types; therefore, the difference in ISG kinetics between type I and type III interferons is accepted as more than a phenomenon of hepatocytes (262).

There are over 300 ISGs that are induced by interferons in response to viral infection (268). The first ISG discovered to restrict influenza replication is orthomyxovirus resistance gene (Mx), in humans known as MxA and in mice as Mx1 (269-271). MxA inhibits influenza infection via the nucleoprotein, although this interaction is influenza strain dependent (272-278). Furthermore, amino acid changes to the nucleoprotein confers resistance to MxA inhibition (279, 280). Interferon induced transmembrane proteins (IFITMs) are a family of proteins which aside from cell cycling, cell adhesion and other biological functions also have been shown to have antiviral activities. IFITM 1, 2, and 3 have been shown to restrict influenza replication (281). Specifically, IFITM3 reduces morbidity and mortality associated with influenza infection, at least in part by limiting viral replication by the restriction of virus entry via the fusion pore (282, 283). However, the pH of the HA viral fusion activity affects its sensitivity to IFITM, such that the lower the pH required for activity the greater sensitivity to inhibition by IFITM2 and IFITM3 (284). Protein kinase R (PKR) is activated through the binding of dsRNA upon which it phosphorylates eukaryotic translation initiation factor 2α (eIF-2α) resulting in the cessation of all host protein synthesis including the machinery the virus hijacks for virus replication and production (285). Upon interferon stimulation, the ISG 2'5'-oligo

(A) synthetase (OAS) is activated. and thereby stimulates RNaseL activity resulting in the degradation of cellular and viral RNA. Similar to type I interferons, type III interferons have a greater effect on the immune response than just the upregulation of ISGs.

Type III interferons induce a variety of immunomodulatory effects including many of which cross between the innate and adaptive branches of the immune response. Although many immune cell types express the IL-28R as described above, only B cells were found to respond to stimulation with IFNλ (249). This refutes previous data that demonstrated neither B cells nor T cells are responsive to IFNλ stimulation (286). However, the discrepancy between these two papers may in part be because the former data was collected from human cells while the latter was collected from murine cells. Both monocytes and macrophages produce IL-6, IL-8, and IL-10 in response to IFNλ, while monocyte derived macrophages can also produce chemokines including MIP1α, MIP1β, and RANTES (287, 288). Furthermore, in monocyte-derived macrophages, IFNλ1 increases TLR induced IL-12p40 which results in increased production of IFNγ by natural killer (NK) cells due to crosstalk between the macrophages and the NK cells (289). Whether IFNλ can directly influence NK cell function in either humans or mice is under contention, current data is contradictory demonstrating both stimulation of IFNγ production by NK cells and the lack thereof (290, 291). IFNλ function seems to be complicated and situation specific as it can also suppress the pro-inflammatory response via IL-1β, in contrast to other reports of its pro-inflammatory function (250). In another example, monocyte derived dendritic cells treated with IFNλ expressed high levels of MHC I and MHC II, which in turn specifically can induce proliferation of $CD4^+CD25^+FOXP3^+$ T cells in an IL-2 dependent manner (292). The modulation of the TH1/TH2 bias by IFNλ can in part be mediated through its down regulation of IL-13 (293). Contemporary research has demonstrated an overall reduced capacity for IFNλ treated dendritic cells to stimulate T cells; while confirming previous findings of preferential proliferation of $FOXP3^+$ T cells, albeit by expansion of already existing cells not by de novo generation (294). Moreover, IFNλ along with IFNα was shown to suppress $CD4^+$ T cell proliferation in response to RSV infection (295). An explanation of the variation in T cell response to IFNλ may be a result of a difference in acute infection versus persistent infection as demonstrated by Misumi and Whitmire. In their

lymphocytic choriomeningitis (LCMV) mouse infection model, the lack of IFNλR and therefore signaling was correlated with an increased T cell response during acute infection but a decreased T cell response during chronic infection (296). In an allergic airway disease model *in vivo*, IFNλ was shown to modulate lung dendritic cell function resulting in down regulated OX40L and up regulated IL-12p70 as well all promotion of TH1 differentiation (297). Furthermore, stimulation of human PBMCs with IL-28B demonstrated a decrease in proliferation of H1N1-stimulated B cells and IgG production (298). These immunomodulatory functions along with the upregulation of ISGs create the antiviral state by which the host can control viral replication, and which must be subverted for the virus to be able to replicate and spread.

## Virus-Host Interaction

In order for influenza virus to be able to avoid or subvert the host immune response and replicate effectively, it produces two proteins that are able to interact with various cells and proteins of the immune system. As previously mentioned, the influenza NS1 protein is the main interferon antagonist and uses a variety of mechanisms to mediate its function. Influenza viral RNA is recognized by the cytosolic pattern recognition receptor RIG I resulting in stimulation of the interferon pathway. NS1 can inhibit the interferon response in a pre-transcriptional manner by directly inhibiting RIG I signaling (299). One mechanism for RIG I signaling inhibition is by NS1 binding into a complex with RIG I requiring ssRNA, thereby preventing complex formation of RIG I with its adapter protein MAVS resulting in inhibition of downstream signaling including IRF3 (151, 300). A second mechanism for inhibition is through binding to TRIM25, the ubiquitin ligase required for RIG I activation and formation of a complex with the adapter MAVS (301). Furthermore, host and virus strain dependent NS1 may also inhibit a secondary ubiquitin ligase, Riplet, which also results in prevention of continued signaling through the RIG I pathway (302). Post-transcriptionally, NS1 can inhibit interferon signaling by inhibiting the nucleo-cytoplasmic transport of signaling molecules (303-307). NS1 can also limit the effects of interferon by interacting with ISGs including PKR and OAS (1). NS1 can inhibit the activation of PKR either by binding dsRNA preventing its interaction with PKR, or via the PACT protein which activates PKR and therefore prevents the

conformational change that would normally allow for activation and phosphorylation of eIF2-α (308). Influenza further regulates the function of PKR by recruiting an inhibitor of PKR, $p58^{IPK}$ and preventing phosphorylation of eiF-2α (285). By binding to dsRNA, NS1 can prevent the activation and resulting antiviral activity of another ISG, OAS (309). Another mechanism by which NS1limits the hosts antiviral response is by inhibiting transcription factors including NF-κB, IRF3, AP-1, and Jun N Terminal kinase (JNK) although which proteins and the degree of inhibition is directly affected by the strain of influenza (300, 310-313).

The ability for influenza virus to regulate the host adaptive immune response has also been linked to the NS1 protein. A strong TH1 response balancing IFN-γ producing CD4+ and cytotoxic CD8+ is crucial for viral clearance (314). Dendritic cells are the major antigen presenting cells for naïve T cells. DC maturation enabling antigen processing and presentation is critical to initiating the adaptive immune response (315). NS1 was shown to directly affect several cytokines and chemokines including IL-12 p35, IL-23 p19, RANTES, IL-8, CCR7 and others related to dendritic cell maturation and migration as well as T cell stimulation (315, 316). The reduction of pro-inflammatory cytokines such as TNFα and IFNγ by NS1 was also demonstrated *in vivo* along with a reduction in the bone marrow lymphocyte depletion, thereby influencing disease pathogenesis (317).

The regulation of apoptosis can be important for virus replication and there are several pathways to induce or inhibit apoptosis including by PKR, OAS/RNase L, JNK, as well as the PI3K/Akt pathway. The downregulation of apoptosis was demonstrated to be interferon independent and regulated by the NS1 protein during influenza infection (318). Moreover, the activation of the PI3K/Akt pathway by NS1 early in infection has been repeatedly demonstrated, possibly with the function to prevent stress-induced cellular apoptosis, thereby allowing efficient replication (319). This activation was also demonstrated to be biphasic, and is therefore not only induced early in infection, but also late in infection (320). This interaction was found to be specific to binding of NS1 to the p85β subunit of PI3K resulting in the regulation of the PI3K/Akt pathway; however, the resulting effects of this regulation have not been fully elucidated (321, 322). The NS1 protein is not the only protein that interacts with the host immune response.

The M2 protein has also been shown to be able to modulate the host immune response. NLRP3 inflammasome activation via TLR7 is induced by the M2 protein; however, M2 is also capable of blocking autophagosome fusion with lysosomes, thereby potentially regulating cell death (146, 323). Aside from its immunomodulatory properties, the M1 protein has several regions associated with B and T cell epitopes, while an asparagine located at amino acid 31 on the M2 protein confers resistance to adamantanes (324-327). Many of these functions are dependent on the influenza strain from which the various proteins are derived, such as a mutation that confers resistance; however, the degree of disease and pathology in the host is also dependent on the host itself.

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# CHAPTER 3

# INFLUENZA PATHOGENESIS IN GENETICALLY DEFINED REISISTANT AND SUSCEPTIBLE MURINE STRAINS[1]

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[1] Samet SJ, Tompkins SM. Influenza Pathogenesis in Genetically Defined Resistant and Susceptible Murine Strains. Yale J Biol Med. 2017;90(3):471-9.

Abstract

The murine infection model is a cornerstone for influenza virus research and includes aspects such as disease pathogenesis, immunobiology, and vaccine and antiviral drug development. One compelling feature of the murine model is the availability of inbred mouse strains, each with a unique genetic makeup and potential for variable responses to influenza infection. Using highly controlled infection studies, the response to influenza virus infection is classified on a spectrum from susceptible to resistant, reflecting severe morbidity and high mortality, to limited or no morbidity and no mortality. Although there have been a variety of studies establishing disparate pathogenesis amongst various murine strains, thus far, there is no consensus regarding the determinants of the outcome of infection. The goal of this review is to explore and discuss the differences in pathogenesis, as well as the innate and adaptive immune responses to influenza infection that have been described in susceptible and resistant mouse strains. Understanding how host genetics influences the response to influenza infection provides valuable insight into the variable responses seen in vaccine or drug efficacy studies, as well as indicates possible mechanisms contributing to increased disease severity in humans infected with influenza virus with no known risk factors.

## Introduction

Influenza A virus is a seasonal pathogen and a major public health concern. Each year, seasonal influenza virus infection causes between 200,000 and 500,000 deaths globally, predominantly in the very young, elderly, and individuals with identified risk factors (1, 2).Efficacious vaccines are available, however, overall effectiveness varies from year to year and was most recently estimated at 48% for the 2016-17 influenza season (3). Approximately 25% of primary influenza viral pneumonia cases occur in individuals without known risk factors such as obesity, hypertension, asthma, and heart disease (2, 4). This suggests that other features may be contributing to disease severity. Genetic factors that contribute to host susceptibility and disease pathogenesis is an area of extreme interest for research; however, human studies, while appealing, are difficult to complete and results are complicated by lifelong exposures to influenza, vaccination, and other variables. Studies of influenza disease severity in genetically defined inbred mouse strains provide a controlled and compelling approach to elucidate how minor genetic differences can influence influenza infection and disease.

## Variation in Infection and Pathogenesis

The mouse is an established model for influenza virus infection and has been used to study influenza disease pathogenesis, as well as vaccine safety and efficacy. Several studies have shown that disease pathogenesis in mice varies from susceptible (DBA/2) to resistant (C57BL/6 and BALB/c), and is dependent on the mouse strain used. This variation in susceptibility has been studied across a wide variety of influenza subtypes and species of origin, including human H1N1 and H3N2, avian H5N1 and H7N7, and swine H1 and H3 strains (5-12). Variation in murine susceptibility is not unique to influenza and has been demonstrated to occur in a variety of infectious diseases including bacteria (*Mycobacterium bovis* and *tuberculosis*, *Orientia tsutsugmushi*, *Chlamydia trachomatis*, and *Salmonella typhimurium*), parasites (*Plasmodium chabaudi*, *Leishmania donovani*, and *Toxoplasma gondii*), fungi (*Candida albicans*), and even with toxins (lethal factor produced by *Bacillus anthracis* (13-21).

For influenza virus infection, increased disease severity seen in DBA/2 mice compared to resistant strains has been illustrated by greater weight loss, reduced survival time, and enhanced pathogenesis (5, 7, 10, 22). The increased pathogenesis in DBA/2 has been described through increased lung weight by five days post-infection, pronounced lung pathology characterized by increased consolidation and necrosis, and increased blockage of airways (cellular debris and infiltrates) by two days post-infection as compared to resistant C57BL/6 mice (5, 23). Furthermore, increased percentages of granulocytes and decreased percentages of lymphocytes from total white blood cell counts have been shown to correlate with severity of disease (24). Finally, increased disease severity in DBA/2 mice has also been associated with higher lung viral load as early as 12 – 24 hours post-infection and greater viral spread into the alveolar regions of the lung compared to resistant mouse strains (5-8, 11). While these phenotypes have been ascribed to susceptible mouse strains, the mechanisms of increased disease are still in question.

Several potential mechanisms for the significant differences in disease and viral load between resistant and susceptible mouse strains have been proposed. One hypothesis is that a difference in sialic acid receptor expression within the respiratory tract supports increased infection in susceptible mouse strains. Human-origin influenza A viruses preferentially bind α-2,6 linked sialic acids, avian influenza viruses preferentially bind α-2,3 linked sialic acids, whereas swine-origin influenza viruses may bind either, all of which is dependent on the hemagglutinin (25, 26). A study by Pica *et al.* compared the virulence of a panel of influenza viruses, including human, avian, and swine origin influenza A viruses, mouse-adapted influenza A viruses, and influenza B viruses in DBA/2 and C57BL/6 mice. While their study consistently demonstrated increased disease severity in the DBA/2 mice, with pathogenicity correlating with lung virus titers, they found no correlation between disease severity and sialic acid binding specificity of the viruses (11). Although the presence of specific sialic acids was not assessed in the DBA/2 and C57BL/6 mice, the lack of difference between human and avian viruses suggests that differential receptor expression was not responsible for the increased disease severity in DBA/2 mice (11). Earlier research utilizing an *ex vivo* primary differentiated cell culture approach demonstrated that murine tracheal epithelial cells (mTECs)

derived from C57BL/6 mice predominantly express the avian α-2,3-linked sialic acid receptor (27). Subsequent analysis of the lung airways also showed only α-2,3-linked sialic acids on the ciliated epithelial cells, which were preferentially infected upon *in vivo* infection with mouse-adapted H1N1 influenza virus. In addition, a human-origin H1N1 failed to infect the mTEC cell cultures or C57BL/6 mice (27). Casanova *et al.* demonstrated small, albeit significantly increased expression of the α-2,3 linked sialic acids but no difference in α-2,6 linked sialic acid expression on alveolar macrophages and mTECs derived from DBA/2 mice compared to cells derived from C57BL/6 mice (23). In this study, infection of the DBA/2 mTECs with a mouse-adapted H1N1 virus also resulted in increased virus titers compared to the C57BL/6 mTECs, which was abrogated with neuraminidase treatment, suggesting that differences in receptor expression may partially explain the difference in viral load during early influenza infection (23). While this may in part explain the increased pathology and severity of disease in DBA/2 mice, the receptor specificities of the mouse-adapted viruses used in these studies are unclear. Moreover, increased α-2,3 linked sialic acid expression would not account for differences in disease severity seen with human and swine influenza viruses having α-2,6 linked sialic acid specificities. Other mechanisms of increased disease severity in the DBA/2 mice must play a role, i.e. the host innate immune response.

## Variation in the Immune Response Post Infection

### Innate Immune Response

The innate immune response to influenza infection in susceptible mouse strains has been categorized as hyper-inflammatory (9). Studies show increased infiltration of neutrophils and macrophages to the lung by two and six days post-infection in DBA/2 compared to C57BL/6 mice (5, 23). Furthermore, alveolar macrophages derived from DBA/2 mice were shown to have greater phagocytic activity, compared to C57BL/6 derived macrophages. In the same study, myeloperoxidase (MPO) activity, a marker for neutrophil activity in the lung, was increased in DBA/2 mice compared to C57BL/6 mice by day 4 post-infection. In addition, despite infecting C57BL/6 mice with an almost 100 times greater inoculum, MPO activity did not increase (23). In contrast, a previous study infected C57BL/6 with 100 times greater

inoculum resulting in CCL2 and TNFα production increasing to similar concentrations as DBA/2 mice given the lower inoculum dose (22). Together these data suggest that the hyper-inflammatory immune response in DBA/2 mice is only partially due to viral load, and host genetics regulating the innate immune response contributes as well. Several studies agree that in addition to greater infiltration of neutrophils and macrophages, greater production of pro-inflammatory chemokines and cytokines contribute to the hyper-inflammatory response seen in the influenza susceptible DBA/2 strain. Pro-inflammatory cytokines including TNFα, IL-6, and IFNγ have been demonstrated to be produced to greater levels in the lungs of DBA/2 mice anywhere from 1 – 7 days post-infection. Furthermore, these studies have also shown increased chemokine production, including MCP-1, KC, MIP2, IP-10 and G-CSF early during infection in the lungs of DBA/2 mice compared to C57BL/6, although akin to the cytokine responses, the exact kinetics is unclear (Table 1) (5-7, 22, 23, 28). Importantly, a variety of inoculums and influenza strains including reassortants of highly pathogenic avian H5N1 (HK213) and mouse-adapted H3N2 (X31) and H1N1 strains (swine and PR8) were used across these studies, which could contribute to the differences in kinetics and discrepancy in IL-1β production. Separate studies have compared influenza infection in A/J mice, another susceptible strain to C57BL/6 mice. In this susceptible versus resistant comparison, several proinflammatory cytokines and chemokines were increased in the susceptible A/J mice, including IL-6, TNFα, and IL-10, as well as interferon β (IFNβ) and GM-CSF (29). Importantly, an early study by Szretter *et al.* demonstrated a role for TNFα in morbidity during influenza virus infection (30), supporting the potential impact of early increased levels of TNFα production in the susceptible DBA/2 and A/J mouse strains. While a variety of studies support the role of early inflammatory cytokine responses, additional studies to refine the kinetics of specific cytokine and chemokine production are needed to clarify their contribution to susceptibility to infection and relate these studies to human disease

Adaptive Immune Response

The adaptive immune response has also been studied among resistant and susceptible mouse strains. Historically, many of the studies focusing on the T cell response to influenza infection in the murine

model used the more resistant C57BL/6 and BALB/c strains. In these strains, $CD8^+$ T cells begin to expand in the mediastinal lymph node (mLN) 3 – 5 days post-infection but are not detectable in the lung until at least day 5, peaking between days 9 – 11 days post-infection, and contracting over the following week (31-34). Studies analyzing the $CD4^+$ T cell response to influenza virus infection demonstrate similar kinetics, albeit a reduced magnitude of response (35, 36). Interestingly, a more recent study assessing $CD4^+$ T cell responses to H1N1 influenza virus infection in resistant and susceptible mouse strains (BALB/c and A/J mice, respectively) demonstrated both strain elicited robust $CD4^+$ T cell responses in the mLN and spleen. The precise epitopes differed as the BALB/c and A/J mice have distinct MHC haplotypes ($I\text{-}A^d$, $I\text{-}E^d$ and $I\text{-}A^k$, $I\text{-}E^k$, respectively) (37). However, while the A/J mice were more susceptible to primary infection, prior exposure to influenza (i.e. sub-lethal infection) elicited comparable immune memory and protection from subsequent lethal challenge, indicating immune memory is sufficient to protect even highly susceptible mouse strains from enhanced disease (37). Finally, while some mouse strains have been described as Th1 or Th2 biased, being predisposed to pro-inflammatory or anti-inflammatory adaptive immune responses, respectively (38), these descriptions do not translate to resistance or susceptibility. C56BL/6 and BALB/c mice are categorized as Th1- and Th2- biased, respectively, but are both resistant to influenza virus infection. In contrast, the susceptible DBA/2 strain is categorized as an intermediate phenotype between Th1 and Th2 (38). Ultimately, while there are differences in cellular adaptive immune responses in susceptible and resistant mouse strains, the proposed hyper-inflammatory response and higher viral load occur within the first few days of influenza infection, prior to development of the primary T cell response. Thus, differences in the T cell response are unlikely to contribute to the increased disease pathogenesis described in susceptible mouse strains.

Humoral immunity has also been considered as contributing factor to differences in influenza pathogenesis in resistant versus susceptible mouse strains. In addition to assessing the potential contribution of influenza virus receptor expression, Pica *et al*. assessed the antibody response to sub-lethal infection with influenza, measuring IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 isotypes. It was established that there was

no difference in antibody responses between DBA/2 and C57BL/6 mice (11). Serum complement proteins have also been considered. DBA/2 mice have a two base-pair deletion rendering them deficient in the fifth complement protein (C5), whereas both C57BL/6 and BALB/c strains are C5 sufficient (39), suggesting a potential mechanism for disease susceptibility. However, in a recent study, Casanova *et al.* administered compliment-sufficient serum by infusion to DBA/2 mice prior to influenza infection with no effect on survival or body weight (23). Together this data suggest that the humoral response does not contribute to the differences in influenza pathogenesis between DBA/2 and C57BL/6 mice; however, further studies are needed to dismiss the role of humoral immunity.

## Differentially Regulated Genes Post-infection

One major benefit of using the mouse model is the availability of a variety of in–bred strains, each with their own unique genetic profile. Although it has been established that influenza pathogenesis in the mouse model does not completely correlate with human disease, there has been some debate whether the genetic expression profile in response to infection closely mimics human infection. One study utilizing C57BL/6 mice determined poor genomic correlation between the murine model and human response to a variety of inflammatory stressors (40); however, weaknesses in the approaches and overly broad conclusions reduce the concerns raised by the report (41). In contrast, a second study utilizing both C57BL/6 and BALB/c mice demonstrated significant correlation between the mouse and human genetic response to inflammatory conditions. Furthermore, a recent study found that the collaborative cross founder strains including C57BL/6J, 129S1/Svlmj, CAST/EiJ, and PWK/PhJ resulted in gene signature profiles that closely mimicked the human response to influenza A virus (42). Mx is a gene that most inbred murine strains (including BALB/c, C57BL/6, A/J, and DBA/2), have a large deletion or nonsense mutation, resulting in a loss of function. In humans, the Mx gene is fully functional and capable of conferring resistance to influenza infection (43-46). The importance of interferon induced Mx resistance has been shown both *in vitro* and *in vivo*, by use of genetic crosses with A2G mice (an inbred mouse strain with an intact Mx gene) (45, 47, 48). A more recent study demonstrated even the reduced expression of the Mx gene in human monocytes and

macrophages is correlated with increased expression of influenza genes while Mx-1 sufficient mice are more resistant to influenza infection than their wild type counterparts (49). Although Mx confers protection against influenza infection, early and rapid replication of the virus can overcome this antiviral response (50). Moreover, while the data are compelling, Mx is only one gene out of many that contribute to the pathogenesis outcome of influenza infection in mice and in humans.

Studies have used a variety of methods and programs including quantitative trait locus (QTL), gene chip array, genome-wide linkage analysis, ingenuity pathway analysis (IPA), and gene ontology to connect the influenza pathogenesis phenotype to specific genes and molecular pathways that differ in resistant and susceptible murine strains. Using gene ontology, one study compared DBA/2 and C57BL/6 mice 1 – 4 days post influenza infection and established DBA/2 had an overall greater number of genes upregulated. Moreover, the genes upregulated in DBA/2 were associated with the immune response whereas the genes upregulated in C57BL/6 were generally associated with cell cycle and cell division. In addition, of the interferon and interferon related genes, only IFNβ1 and IFNγ were upregulated in DBA/2 but not C57BL/6, while among chemokines only CXC11, associated with lung inflammation, was remarkably increased in DBA/2 mice. With the use of IPA, various genes found to be upregulated in DBA/2 and not C57BL/6 upon influenza infection, were associated with eicosanoid signaling, apoptosis, and coagulation (9). Another study by Boon *et al.* used principal component analysis comparing DBA/2 and BALB/c mice. Genes upregulated in BALB/c mice 7 days post-infection were associated with T and B cell function, including cell adhesion molecules, and antigen processing and presentation. In contrast, susceptible strains, including DBA/2 mice, continued to upregulate cytokines. It was also noted that susceptible strains expressed similar proinflammatory cytokines as resistant strains, but to a considerably greater extent (22). These studies highlight that differential gene expression may drive aspects of the susceptible versus resistant phenotype, but the magnitude of the host response may also mediate resistance or susceptibility.

Several studies have concentrated on using gene loci to focus on a smaller cohort of genes that could be associated with susceptibility or resistance to influenza virus infection in mice. An early study by

Boon *et al*. used QTL mapping of a panel of recombinant inbred mouse lines to identify several loci on chromosomes 2, 7, and 17 associated with the resistant phenotype. Furthermore, they identified 30 candidate genes including Trim12, Trim34, Plekhb1, Prkrir, Trpc20, Med1, and Hc (6). Additional testing on the role of Hc (hemolytic complement, identified on chromosome 2) to validate the QTL analysis compared influenza infection in Hc competent and Hc knock out mice. Boon *et al.* found a dose-dependent resistant phenotype in Hc intact mice compared to Hc knockout mice (6). Subsequent studies by Boivin *et al.* suggest Hc-related susceptibility may be dominant in female mice, highlighting the complexities of resistance and susceptibility studies and the potential for sexual dimorphism (29). Loci on chromosome 5, 16, 17-1, 17-2, and 19 have also been associated with resistance to influenza-mediated disease, leading to approximately 30 candidate genes including Sik1, Eif2ak1, Itgb6, Ifih1, Robo1, Nrip1, and LST1, some of which regulate innate immune pathways (51). Further studies on the role of LST1 (leukocyte specific transcript 1) in influenza infection demonstrated increased weight loss and a slight increase in mortality in LST1 knockout mice compared to the C57BL/6 parental strain. Interestingly, DBA/2 mice have a deletion in the LST1 gene; however, there was no difference in the histopathology or immune cell infiltrates found in the lungs of wild type C57BL/6 compared to $LST1^{(-/-)}$ mice, suggesting that while LST1 may contribute to the susceptibility seen in DBA/2 mice, there are additional factors contributing to the phenotype (52). A single locus on chromosome 6 has recently been associated with greater inflammation as demonstrated by increased production of both TNFα and IFNα within 48 hours of infection. Comparing this locus in a variety of resistant and susceptible murine strains, genes *Sam9l*, *Slc25a13*, and *Ica1* all contained single nucleotide polymorphisms (SNPs). In this locus, the gene *Col28a1* contained an in-frame deletion as well. However, there was no significant difference in cytokine production, morbidity, or mortality resulting from influenza infection between a *Slc25a13* knockout and its parental strain (28). Much of the QTL analysis has been done using C57BL/6 and DBA/2 strains or a variety of BXD crosses (recombinant inbred strains derived from a cross of C57BL/6J ($H\text{-}2^b$) and DBA/2J ($H\text{-}2^d$)). However, a recent study utilized the collaborative cross inbred mouse panel (53) to assess influenza susceptibility and resistance against a diverse genetic background representative of the human population. This study found several loci contributing to the

disease phenotype, including a novel allele of *Mx1*, identified in the well-described influenza resistance locus on chromosome 16. Moreover, a novel locus on chromosome 7 potentially associated with weight loss was identified and includes candidate genes *Nox4* and *Il16*. Additionally, a locus on chromosome 1 associated with changes in pulmonary edema and a locus on chromosome 15, potentially associated with differences in neutrophil infiltrates in the airway were discovered (54). These findings suggest that the host pathways that drive increased inflammation and subsequent increases in morbidity and mortality are regulated by a complex network of genes and gene products having overlapping and sometimes competitive effects. Thus, connecting candidate genes found by transcriptional analysis and the innate immune pathways regulated by those genes is the next step in discerning this complicated web of interactions that can ultimately result in the difference between susceptibility and resistance to influenza infection.

## Conclusions and Future Perspectives

The immune response to influenza infection in the murine model can be categorized by strain on a scale from susceptible to resistant based on morbidity and mortality. Substantial weight loss, a high lung viral load, and a robust proinflammatory response characterize strains that are susceptible to influenza infection. The pro-inflammatory response includes increased neutrophil and macrophage recruitment and increased production of cytokines and chemokines within a few days of influenza virus infection. Thus, disease in susceptible strains reflects the acute and excessive pro-inflammatory, antiviral response. However, in some studies, a high virus load in the lung, seen within 48 hours of infection, may contribute to the increased inflammatory response seen in susceptible mouse strains. While several groups have postulated mechanisms for the increased viral load early during infection, there is still no definitive answer. Furthermore, there is still debate whether a higher viral load elicits a proinflammatory response in resistant strains similar to that found in susceptible strains. Other host factors are almost certainly playing a role. Elucidating what host factors contribute to susceptibility to influenza infection in the murine model, may reveal possible factors that modulate the immune response in humans.

This review has focused on the murine model; however, there are other established animal models of influenza virus infection. Ferrets are a well-established model for influenza infection and considered superior to mice by some researchers as they are susceptible to infection with most human influenza strains without prior adaptation and the symptoms mimic human disease. Importantly, ferrets can be used for both pathogenesis and transmission studies, whereas mice do not readily transmit influenza virus. However, there are few immunologic reagents available for the ferret and the genetics are as yet poorly defined, limiting mechanistic studies in this model. Other animals used in influenza research include hamsters, cotton rats, guinea pigs, swine, and non-human primates (reviewed in detail by Bouvier and Lowen (55)). Each of these animal models has specific benefits and drawbacks, but only the mouse model has the array of genetically defined strains and transgenic or knock out mice and robust tools for analyzing the host response to infection critical for dissection of determinants of influenza susceptibility.

The mouse model is a widely accepted animal model for influenza virus infection and particularly useful for interrogation of the immune response to infection. A variety of disease endpoints are commonly used, including weight loss, survival, lung virus titer, lung weight, and histopathology (55). However, these endpoints may not fully measure acute respiratory distress syndrome (ARDS) in humans (56), which is associated with viral pneumonia, the primary complication of influenza infection in humans (57). Many of the studies addressing susceptibility to influenza infection do not directly consider ARDS, which needs to be considered for translation to human disease.

The comparison of host responses to influenza virus infection in resistant (C57Bl/6) and susceptible (DBA/2) mice has established a useful model system for interrogating host determinants of disease. Consideration of endpoints more relevant to human disease (i.e. ARDS) when defining susceptibility and resistance will only strengthen this model. Future studies should continue to develop the network of host immune pathways involved in the response to influenza virus infection. Identification of determinants of susceptibility or resistance to influenza-associated disease is critical for risk assessment as well as development of effective treatments for individuals with severe influenza disease.

Table 3.1: Cytokines and chemokines increased in DBA/2 relative to C57BL/6 mice in response to influenza virus infection. The virus strain used in each study is indicated.

| | Days Post Infection | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| **CYTOKINES** | | | | | | | |
| **G-CSF (CSF3)** | - | - | - | - | X31 | | |
| | - | PR8 | | | - | - | - |
| | - | - | HK213 | - | - | - | - |
| **IFNα** | - | HK213 | HK213 | - | - | - | - |
| | SW H1N1* | SW H1N1* | - | - | - | - | - |
| **IFNβ** | - | - | HK213 | - | - | - | - |
| | SW H1N1* | - | - | - | - | - | - |
| **IFNγ** | - | - | - | - | X31 | | |
| | - | - | - | - | - | SW H1N1 | |
| **IL-1α** | - | PR8 | | - | - | - | - |
| **IL-1β** | - | - | - | - | X31* | | |
| | - | - | SW H1N1 | | | - | - |
| **IL-5** | - | PR8 | | | - | - | - |
| **IL-6** | X31 | - | X31 | - | X31 | | |
| | - | - | SW H1N1 | | | | |
| | - | PR8 | | | - | - | - |
| **IL-12** | - | - | - | PR8 | - | - | - |
| **TNFα** | - | - | X31 | - | X31 | | |
| | - | HK213 | HK213 | - | - | - | - |
| | - | - | - | SW H1N1 | | | |
| **CHEMOKINES** | | | | | | | |
| **IP-10** | X31 | - | X31 | - | X31 | | |
| | - | PR8 | | | - | - | - |
| **KC** | X31 | - | X31 | - | X31 | | |
| | - | PR8 | | | - | - | - |
| | - | - | SW H1N1 | | | | |
| **MCP-1 (CCL2)** | X31 | - | X31 | - | X31 | | |
| | - | PR8 | | | - | - | - |
| | - | HK213 | - | - | - | - | - |
| | - | - | - | SW H1N1 | | | |
| **MIG** | - | PR8 | | | - | - | - |
| **MIP1α** | X31 | - | X31 | - | X31 | | |
| | - | PR8 | | | - | - | - |
| | - | - | - | - | - | SW H1N1 | |
| **MIP2 (CXCL2)** | - | - | HK213 | - | - | - | - |
| | - | PR8 | | | - | - | - |
| | SW H1N1 | - | SW H1N1 | | | | - |
| **RANTES** | - | PR8 | | | - | - | - |

[a] X31 – A/Aichi/2/68 (H3N2) x31 (6:2 reassortment with PR8) (7, 58)

[b] PR8 – mouse-adapted A/Puerto Rico/8/34 (H1N1) (5)

[c] HK213 – reverse genetics A/Hong Kong/213/2003 (PB1 segment A/Chicken/Hong Kong/Y0562/2002) (H5N1) (28)

[d] HK213 – reverse genetics A/Hong Kong/213/2003 (PB1 segment A/Chicken/Hong Kong/Y0562/2002) (H5N1) (6, 22)

[e] SW H1N1 – mouse-adapted A/Swine/Iowa/4/1976 (H1N1) (23)

- No data available or data available lacks statistical significance

* C57BL/6 > DBA/2

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# CHAPTER 4

# SPECIFIC GENETIC CONSTELLATIONS OF SWINE INFLUENZA A VIRUSES ELICIT GREATER DISEASE IN THE MURINE MODEL[1]

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Abstract

Prior to the 2009 H1N1 pandemic (pdmH1N1), swine influenza A viruses (swIAV) endemic in North American swine herds contained an internal gene segment constellation referred to as the triple reassortment internal gene (TRIG) cassette, which supported a level of gene reassortment not seen prior to its emergence in the 1990's. After the 2009 pandemic, the pdmH1N1 virus was quickly introduced to swine populations, but in general did not become endemic in North American herds. However, the pdmH1N1 did contribute the matrix gene segment (pdmM) to the swIAVs circulating in the pig population; which has subsequently replaced the classical swine matrix gene (swM) found in the TRIG cassette. This suggested that the pdmM has a fitness benefit and in support of this, others have shown that swIAV containing the pdmM have greater neuraminidase activity and transmission efficiency compared to viruses containing the swM gene segment. We hypothesized that the origin of the matrix gene would also affect disease and utilized two murine models of IAV infection, the phenotypically resistant (BALB/c) and susceptible (DBA/2) mouse strains to assess swIAV replication and pathogenicity. We infected BALB/c and DBA/2 mice with a panel of H1 and H3 swIAV isolates containing either the swM or pdmM gene and measured lung virus titers, morbidity and mortality, and lung histopathology. H1 influenza strains containing the pdmM gene caused greater morbidity and mortality in both resistant and susceptible murine strains, while H3 swIAVs caused no clinical disease. However, both H1 and H3 swIAVs containing the pdmM replicated to higher viral titers in the lungs and pdmM H1 viruses induced greater histological changes in the lungs compared to swM viruses. While the surface glycoproteins clearly contribute to swIAV pathogenicity, and other gene segments may also enhance disease, these data suggest that the origin of the matrix gene may also contribute to pathogenicity of swIAV viruses in mammals.

## Introduction

Influenza is considered a major public health threat causing between 250 and 300 thousand deaths annually worldwide (1-3). Influenza A viruses (IAVs) are single stranded, negative sense RNA viruses with a segmented genome, with eight gene segments (4). These include the hemagglutinin (HA) and neuraminidase (NA) genes, which encode the surface glycoproteins forming the spikes on the outside of the virion and determine the virus subtype, and the internal protein genes which include three polymerase, polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA) genes, as well as the nucleoprotein (NP), nonstructural (NS) and the matrix (M) segments (4).

The M gene encodes two proteins, M1 and M2. M2, is a 97 amino acid (aa) well-characterized proton channel, which contributes to the release of viral RNA from the virion upon infection (5), virus assembly (6), and is able to interact with the host immune response including inflammasome activation (7) and possible inhibition of autophagy (8). M1, which is a 252 aa protein, plays an important role in the release of viral RNA from the virion upon infection, export of the viral genome out of the nucleus, inhibition of re-import of the nucleocapsid, and assembly of virus particles. The M1 protein is a determinant of the characteristic pleomorphic morphology of influenza virus particles. The relevance being that filamentous virus particles have been linked to increased neuraminidase activity, which can also affect transmission (9-11).

Similar to humans, IAVs have been enzootic in swine worldwide, causing respiratory disease outbreaks in herd, characterized by high morbidity, low mortality and significant economic losses (12). For almost eight decades, a single H1N1 IAV, known as “classical” swine influenza, was circulating in the North American swine population (13, 14). However, in the late 1990’s novel triple reassortant H1N1, H1N2 and H3N2 viruses emerged in pigs containing gene segments from classical swine, human, and avian origins (15, 16). The six internal genes, known as the triple reassortment gene cassette (TRIG) became predominant; such that, since 2000 most fully characterized swine influenza strains contain this internal protein gene combination (14, 15, 17-20). Since the pandemic of 2009, which was caused by a novel

reassortant swine-origin H1N1 virus with gene segments from North American swine viruses and avian-like Eurasian swine viruses (21), the necessity for surveillance of the spread and genetic diversity of swine influenza viruses has become evident. Recent studies have shown regional differences in circulating strains both in the United States, as well as in other countries, and a diversification of genetic constellations (22-30). At the same time, field surveillance has demonstrated that since 2011, in the North American swine IAVs the pandemic origin M gene segment has been gradually replacing the TRIG M gene segment, up to 70% by 2011 increasing to 100% by 2015 (28, 31-33). Furthermore, the pdmM gene has been linked to increased neuraminidase activity and increased transmissibility (11, 34). Other studies have demonstrated that the M gene segment is under selective pressure independent of other genes with sites potentially related to host tropism and immune response (35).

Predisposition to increased severity of disease and complications from IAV infection can be attributed to a variety of host factors including obesity, hypertension, and asthma, as well as host genetics (1, 36-38). The murine model is one of the most common animal models used for IAV pathogenesis research. Previous studies have shown a variation in pathogenesis between inbred strains of mice resulting in categorization on a continuum from susceptible to resistant (39). Susceptible strains, such as DBA/2, demonstrate higher morbidity, mortality and viral replication while their immune response is characterized by greater concentrations of proinflammatory cytokines and increased numbers of lung infiltrates in response to influenza infection compared to more resistant strains such as BALB/c (39). Therefore, we chose to use both susceptible and resistant murine strains to characterize the infection and disease of swine influenza isolates.

We hypothesize that infection with swine IAV strains containing the pandemic (pdm) origin matrix gene (pdmM) will result in greater morbidity and mortality and induce more severe lung lesions than infection with swine IAV strains of the same subtype and combination of gene segments containing the North American swine origin (swine, sw) matrix gene (swM) in the murine model. Furthermore, we hypothesize that the difference in disease and lesions in the lung will occur irrespective of HA clades; however, specific gene constellations will induce greater pathologic changes than others.

## Materials and Methods

### *Cell Culture and Virus Propagation*

Swine influenza viruses A/swine/Missouri/A01444644/2013, A/swine/North Carolina/A01394568/2013, and A/swine/Minnesota/A01125993/2012 (28) were obtained from the USDA National Veterinary Services Laboratories (NVSL) reagent resource. Swine influenza viruses A/swine/North Carolina/152702/2015, A/swine/North Carolina/154074/2015, and A/swine/North Carolina/157671/2015 were obtained from the NIAID Centers of Excellent in Influenza Research and Surveillance (CEIRS) network (26). Viruses were propagated in Madin-Darby canine kidney (MDCK) cells in Minimal Essential Media [MEM (GIBCO™)] with 0.002μg tosyl phenylalnyl chloromethyl ketone (TPCK). Viruses were passaged to achieve stock titers above $1x10^6$ pfu/ml. Influenza virus titers were assessed by plaque assay on MDCK cells as previously described (52). Briefly, a 24-well plate of MDCK cells was incubated with serial dilutions of virus at 37˚C and 5% CO2 for 1-2 hours. The supernatant was removed, 1ml of 1:1 2.4% Avicel solution and overlay [MEM (GIBCO™) with 1M HEPES (GIBCO™), 200mM mM GlutaMAX-I, 7.5% NaHCO3 (GIBCO™), and antibiotic/antimycotic (GIBCO™)] was added and the cells were incubated at 37˚C and 5% CO2 for 48 -72 hours prior to fixation with 80/20 methanol/acetone and staining with crystal violet.

### *Sequencing and Analysis*

Sequences of viral gene segments that were previously sequenced were acquired from GenBank (Table 1). For virus strains with gene segments not previously sequenced, viral RNA was isolated using RNAzol®RT (Sigma-Aldrich) as per manufacturer's protocol. cDNA synthesis and PCR were performed using SuperScript III One-step RT-PCR (Invitrogen) as per manufacturer's protocol. Primers used were as follows: All genes MBTUni-12 5'ACGCGTGATCAGCRAAAGCAGG3',

-13 5'ACGCGTGATCAGTAGAAACAAGG3',

PB2 Forward 5'AGCRAAAGCAGGTCAATTATATTCA3',

PB2 Reverse 5’AGTAGAAACAAGGTCGTTTTTAAACTA3’,

PB1 Forward 5’AGCRAAAGCAGGCAAACCATTTGAATG3’

PB1 Reverse 5’AGTAGAAACAAGGCATTTTTTCATGAA3’,

PA Forward 5’ AGCRAAAGCAGGTACTGATYCGAAATG3’, and

PA Reverse 5’AGTAGAAACAAGGTACTTTTTTGGACA3’. NGS was performed using the Illumina MiSeq platform. Sequences were aligned using MUSCLE alignment and comparison of predicted amino acids using Geneious (Biomatters Ltd.). Sequences of each of the genes of the isolates were compared to reference genes of pandemic origin, TRIG origin, and classical swine origin. Genes were categorized by the highest percentage similarity between the isolate gene and the reference sequence.

*Mice*

Female 6 – 8-week-old BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC). Female, 6 – 8-week-old DBA/2 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal studies were approved by the Animal Care and Use Committee of the University of Georgia.

*In vivo infections*

Mice were anesthetized using 3% Isoflurane (Patterson Veterinary) and intranasally inoculated with 50µl virus diluted in phosphate buffered saline (PBS). Control mice were inoculated with 50µl PBS.

*Lung virus titers*

Mice were humanely euthanized, lungs were collected at set time points, homogenized in 1 ml cold PBS, centrifuged, and supernatant was frozen down at -80˚C. Plaque assays were performed to assess lung viral titers.

*Histology*

Mice were humanely euthanized at 2 and 4 DPI and lungs were collected, inflated with air, and fixed by immersion in 10% buffered formalin. Fixed lungs submitted to the Histology Laboratory in the Department of Pathology at the University of Georgia for processing and sectioning. Briefly, lungs were embedded in paraffin so that all lung lobes could be evaluated, and 4 μ sections were cut and stained with hematoxylin and eosin. Lungs were scored as follows: Perivascular (bronchial tree) inflammation (0=none;1=mild, 1-2 cells wide; 2=moderate, 3-10 cells wide; 3=severe, >10 cells wide); percentage of bronchioles affected (0 = none; 1= <25%' 2=25-75%. 3=>75%); peribronchiolar inflammation (0 = none; 1 = mild; 2 = moderate; 3 = severe); severity of airway luminal exudate, epithelial necrosis and inflammation (0 = none; 1 = mild; 2 = moderate; 3 = severe); percentage of alveolar involvement (0 = none; 1 = <25%; 2 = 25-50%; 3 = >50%); severity of interstitial inflammation (0 = none; 1 = mild; 2 = moderate; 3 = severe); edema (0 = none; 1 = present); hemorrhage (0=none;1=present); type II cell hyperplasia (0 = none; 1 = present); vasculitis (0 = none; 1 = present). This resulted in scores ranging from 0 - 22. BALT (well defined aggregates of mixed lymphocytes or follicles) was scored separately as 0-3 (0 = none; 1 = mild; 2 = moderate; 3 = severe).

*Statistical Analysis*

Statistics were run using GraphPad Prism version 7.03. Statistical analysis included two-way analysis of variance (ANOVA) with Bonferroni post-hoc for weight loss, viral titers, and lung pathology scores of H3 swIAVs, Dunnett post-hoc for weight loss, viral titers, and lung pathology scores of H1 swIAVs or Kaplan-Meier Survival Curve for survival data. All results were considered significant at p-values $< 0.05$.

## Results

*The matrix gene contributes to morbidity and mortality of swine H1 influenza strains in mice*

In order to reflect the potential for different morbidity and mortality outcomes dependent on the host, we inoculated both resistant (BALB/c) and susceptible (DBA/2) murine strains (1) with swine H1 IAVs spanning a variety of HA and NA clades and containing either the swM or pdmM gene segment (Figure 1). H1 viruses containing the pdmM caused greater morbidity, as demonstrated by weight loss, and mortality (Figures 2A-D) in both resistant and susceptible murine strains. As previously shown by several other groups, H3 viruses of human or swine origin result in minimal morbidity or mortality in the murine model and generally require adaptation for research purposes (2, 3). To investigate whether the pandemic origin matrix gene can induce greater morbidity or mortality in mice infected with swine H3 influenza strains, we inoculated both resistant and susceptible murine strains with H3 influenza strains differing only in the matrix gene (Figure 1). Both resistant and susceptible murine strains showed no morbidity, as determined by weight loss (Figure 3A and B), and no mortality (data not shown) when infected with either H3 strains containing pdmM or swM gene segments.

*The matrix gene contributes to increased viral replication in the lungs in the resistant and susceptible mice*

Influenza-mediated morbidity and mortality in mouse models can be attributed to viral replication and/or immune mediated damage. In order to determine whether viral replication contributes to the greater morbidity and mortality caused by IAV strains containing the pdmM gene segment, we inoculated resistant and susceptible murine strains and assessed lung viral titers over the course of infection. H1 influenza strains containing the pdmM gene segment replicated to significantly higher viral titers by almost 4 logs at 2 days post infection (DPI) and maintained significantly higher viral titers by approximately 2 logs through 6 DPI in the resistant BALB/c mice (Figure 4A). Interestingly, the same viruses replicated only slightly better, 2 – 3 logs higher, with the swM-containing virus replicating to higher titers in the susceptible DBA/2 mouse strain. Furthermore, the closely matched influenza strains differing only in the origin of M gene

segment, 702/pdmM and MO/664/swM replicated to similar viral titers in the lungs of DBA/2 mice at 2 DPI (Figure 4B). Lung virus titers were also compared between H3 viruses containing pdmM or swM gene segments, in resistant and susceptible mouse strains over the course of the infection. While neither of the swine H3 influenza viruses caused clinical signs, the viruses did replicate mice, albeit not to the levels seen with the H1 swIAVs. The NC/671/pdmM not only replicated to higher viral titers by almost 2 logs at 2 and 4 DPI; virus continued to replicate in the lungs through 6 DPI. In contrast, MN/993/swM was cleared from the lungs by 6 DPI (Figure 4C). In the susceptible DBA/2 mouse strain, no significant differences were recorded in viral lung titers, although titers in the pdmM-containing H3N2 were consistently slightly higher until cleared after 6 DPI (Figure 4D). Notably, while the swine H3N2 viruses did replicate in mice without causing disease, we also assessed several human H3N2 viruses with HA and NA genes related to the swIAVs for potential replication and pathogenesis in resistant and susceptible mouse strains and none of them were able to replicate, with no detectable virus in the lung at 2 DPI (data not shown). The greater lung virus titers of both H1 and H3 influenza strains containing the pandemic origin matrix gene has the potential to induce greater pathology in the lungs; therefore, we next assessed the lungs of infected mice for histopathologic changes.

*The pandemic origin matrix gene contributes to greater disease and more severe histological changes in the lung.*

In order to understand the development of disease and evaluate the characteristics and extent of lesions induced in the lung, we inoculated both resistant (BALB/c) and susceptible (DBA/2) mice with the panel of swIAVs and assessed histopathologic changes in the lung at 2 and 4 DPI. Both strains of mice inoculated with MO/664/swM had mild pulmonary changes on 2 DPI characterized by a small number of bronchioles with mild segmental necrosis of the epithelium and minimal peribronchiolar infiltrations of lymphocytes admixed with neutrophils. In addition, a small number of vessels with mild perivascular infiltrations of lymphocytes admixed with fewer neutrophils, and rare foci of alveoli containing small numbers of neutrophils and macrophages were present. In the resistant mouse strain, changes were slightly

more severe on 4 DPI (Figure 5A), as well as in the susceptible, DBA/2, strain (data not shown). The number of involved bronchioles and vessels increased, but was still less than 25%, and there was an increased amount of epithelial necrosis and numbers of peribronchiolar and perivascular inflammatory cells. Mild to moderate interstitial changes were present in the DBA/2 mice, characterized by mild multifocal alveolar infiltrations of a small number of neutrophils and macrophages (data not shown).

In both susceptible and resistant mouse strains inoculated with NC/702/pdmM, NC/074/pdmM, or NC/568/pdmM pulmonary changes on 2 DPI were similar in character to those seen in mice inoculated with the swM-containing virus; however, they were more severe, ranging from mild to moderate overall, with larger numbers of involved bronchioles and vessels, more extensive epithelial necrosis, and increased numbers of perivascular and peribronchiolar lymphocytes admixed with neutrophils. Pulmonary changes increased from mild to moderate in the NC/702/pdmM mice from 2 to 4 DPI (Figure 5B). However, in the NC/074/pdmM and NC/568/pdmM mice, changes were already moderate on 2 DPI, with those in the NC/568/pdmM inoculated mice being the most severe of all of the groups. Both resistant and susceptible mouse strains inoculated with the pdmM-containing viruses had more extensive and severe interstitial involvement than seen in the swM-inoculated mice, which increased in extent and severity of involvement from 2 to 4 DPI, being most severe in mice inoculated with NC/568/pdmM. In NC/702/pdmM and NC/074/pdmM inoculated mice, interstitial involvement ranged from mild (<25%) on 2 DPI to moderate (25-50%) by 4 DPI and was characterized by foci with slightly thickened alveolar septa and small numbers of neutrophils, lymphocytes and macrophages in alveoli to foci where alveoli were filled with large numbers of inflammatory cells and necrotic debris (Figure 5B and C). In NC/568/pdmM inoculated mice, interstitial involvement was already moderate on 2 DPI with >50% involvement of the parenchyma by 4 DPI. Interstitial foci were similar to mice inoculated with NC/702/pdmM and NC/074/pdmM but also included foci with moderately thickened alveolar septa with mild epithelial hyperplasia. Mice inoculated with NC/568/pdmM by 4 DPI had multifocal interstitial hemorrhage that was more severe than with any of the other virus isolates (Figure 5D) and included the development of hyaline membranes in DBA/2 mice, which

suggests more extensive alveolar septal damage with the NC/568/2013 isolate. Overall, there was significantly greater pulmonary changes in mice inoculated with swIAVs containing the pdmM gene segment compared to the virus containing the swM gene segment (Figure 5E and F).

Resistant and susceptible mouse strains were also inoculated with H3 swIAVs containing either the pdmM or swM gene and assessed histopathologic changes in the lung at 2 and 4 DPI. On 2 DPI, lungs from DBA/2 and BALB/c mice inoculated with MN/993/swM or NC/671/pdmM had minimal changes that were not specific for influenza. However, by 4 DPI, while both murine strains inoculated with NC/671/pdmM had mild pulmonary changes consistent with influenza (Figure 4E, data not shown). These changes were slightly more severe in BALB/c mice and included minimal to mild lymphocytic infiltrations around a small number of bronchioles and vessels and minimal to mild segmental epithelial necrosis in a few larger apical bronchioles. Together these data suggest that the pandemic origin matrix gene induces greater pathologic changes in the lung resulting in greater severity of disease in both susceptible and resistant murine strains.

## Discussion

Multiple introductions of the 2009 pH1N1 virus into the swine population has led to recombination events with previously circulating swine IAVs resulting in previously unseen genetic constellations. Previous research has shown the matrix gene from the pandemic virus is replacing the classical swine origin matrix gene found in the TRIG cassette, suggesting an evolutionary advantage or fitness benefit. We used resistant and susceptible mouse strains to investigate whether infection with swine influenza isolates containing the pandemic origin matrix gene (pdmM) would induce greater disease compared to isolates containing the classical swine origin matrix (swM) gene. We chose influenza isolates reflecting the predominant strains found in North America between 2010 and 2016 (31). Infection with H1 influenza viruses containing the pdmM resulted in greater morbidity and mortality in both susceptible and resistant mouse strains. However, infection with H3 isolates containing either the pdmM or swM did not induce

morbidity or mortality, which is in agreement with previous studies that have shown a very high lethal dose for earlier swine H3 viruses and an inability to infect mice with human H3N2 strains (42).

We evaluated virus replication in the lungs of both susceptible and resistant mice. Both H1 and H3 swIAVs containing the pdmM replicated to higher viral titers in the lungs of susceptible and resistant mice compared to swIAVs containing the swM. Higher viral titers in the mouse lungs suggests that the pdmM gene confers increased replication efficiency within the mice at least to some degree. Furthermore, this data suggests that the higher viral load in the lungs is contributing to the greater morbidity and mortality induced by the pdmM-containing influenza viruses. The reduced, or lack of difference, in viral replication between the IAVs with different origin M genes in the susceptible murine model suggests that differences in the host immune response may also contribute to the greater morbidity and mortality caused by these viruses.

One possible explanation for the differences in virus replication demonstrated by these viruses in the murine model can be attributed to mutations in other genes including the HA and NA which are also known to effect virus replication. This explanation cannot be completely discounted; however, it is unlikely that mutations in HA and NA contributed significantly to the differences between pdmM-containing strains and the swM-containing strains, as the pdmM-containing viruses included both matching and different HA and NA gene segments to the swM-containing strains.

While one recent study correlated inflammation and development of pneumonia in human infections with swine-origin, pandemic H1N1 (43), other studies have related an altered immune response with IAVs containing the pdmM gene (44). Therefore, we assessed the development of microscopic changes in the lungs of IAV infected BALB/c and DBA/2 mice. Both H1 and H3 swIAVs containing the pdmM gene induced greater histologic changes, characterized by necrosis, increased infiltrates, thickening of the alveoli septa, and epithelial hyperplasia compared to viruses containing the swM gene. This was seen in both resistant and susceptible mouse strains. The increased severity of lesions in the lungs of mice infected with a H3 swIAV containing the pdmM gene compared to the H3 virus containing the swM, while not as severe as was seen with the H1 isolates, was evident despite a lack of significant difference in

replication in the susceptible murine strain. Together these data suggest that while greater viral replication by the swIAVs containing the pdmM gene contributes in part to the increased histopathologic lesions in the lungs, other viral and host factors contribute to the enhanced lung lesions and subsequent disease. Of note, the microscopic changes in the lung switched from bronchiolar to more interstitial over time in the mice inoculated with the H1 viruses containing the pdmM gene. In humans, interstitial damage along with the development of hyaline membranes has been associated with the development of severe influenza viral pneumonia (45). Furthermore, in mice as well as in humans, severe influenza viral pneumonia has been correlated to the dysregulation of inflammation of the airways (46-49). This suggests that the differential lung pathology may in part be due to the subsequent immune response to the infection, and the potential for infection with swine influenza isolates containing the pandemic matrix gene to generate an exacerbated immune response in the murine model.

Studies using reverse genetics viruses would be useful in ascertaining to what degree the pandemic matrix gene is able to influence disease progression and outcome. Other studies have shown the origin of viral segments such as PB1 and NS1 genes may also contribute to swine influenza strain virulence and disease (50, 51). These studies along with ours highlights the importance of understanding the possible effects individual genes can have on virus replication, transmission, and disease as we continue to track the evolutionary changes of influenza strains as they occur.

**Table 4.1.** NCBI taxonomy id and GenBank accession numbers for the virus strains used in this study

| Influenza Strain | NCBI Taxon ID | Accession number | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PB2 | PB1 | PA | HA | NP | NA | M | NS |
| A/swine/Missouri/A01444664/2013 (H1N2) | NCBI:txid1289201 | | | | KC562218 | | KC562217 | KC562216 | |
| A/swine/North Carolina/A01394568/2013 (H1N1) | NCBI:txid1425844 | | | | KF874274 | | KF874275 | KF874276 | |
| A/swine/Minnesota/A01125993/2012 (H3N2) | NCBI:txid1214862 | KX851891 | KX851892 | KX838371 | JX422257 | KX851893 | JX422256 | JX422255 | KX851894 |

**Table 4.2.** Percent homology based on nucleotide sequence

| Influenza Strain | Sequence comparison (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PB2 | PB1 | PA | HA | NP | NA | M | NS |
| A/swine/Missouri/A01444664/2013 (H1N2) | -- | -- | -- | 89.9 | -- | 89.6 | 96.9 | -- |
| A/swine/North Carolina/152702/2015 (H1N2) | 93.8 | 95.0 | 97.4 | 89.9 | 91.8 | 89.6 | 97.3 | 93.3 |
| A/swine/North Carolina/154074/2015 (H1N1) | 93.8 | 95.0 | 97.4 | -- | 91.8 | 97.9 | 97.3 | 93.3 |
| A/swine/North Carolina/A01394568/2013 (H1N1) | -- | -- | -- | -- | -- | 97.9 | 97.3 | -- |
| A/swine/Minnesota/A01125993/2012 (H3N2) | 93.8 | 95.0 | 97.4 | 93.4 | 91.8 | 97.2 | 96.9 | 93.3 |
| A/swine/North Carolina/157671/2015 (H3N2) | 93.8 | 95.0 | 97.4 | 93.4 | 91.8 | 97.2 | 97.3 | 93.3 |
| Consensus pdmM vs consensus swM* | | | | | | | 90.5 | |

* Alignment of the predicted amino acid sequences based off of a consensus of the pandemic matrix and swine matrix gene containing strains used in this study

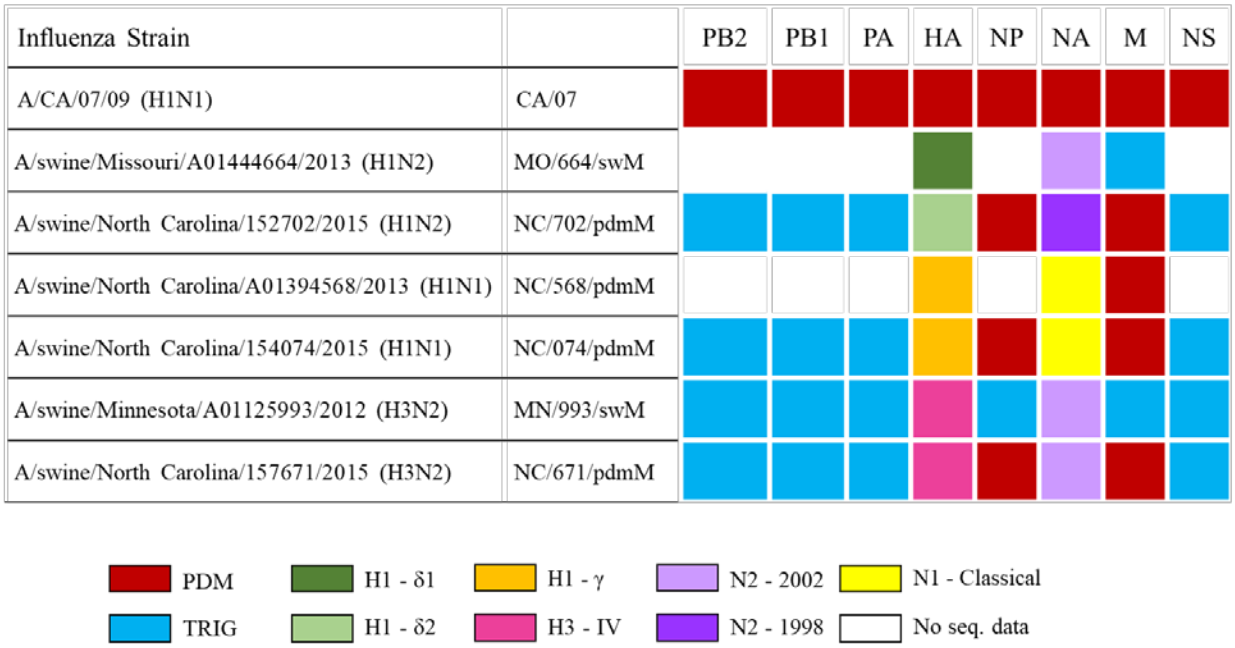


**Figure 4.1. Influenza viruses used and origin of their gene segments.** The origin of each gene segment is color coded according to the key. Abbreviations: PB2 polymerase basic 2; PB1 Polymerase basic 1; PA polymerase acidic; HA hemagglutinin; NP nuclear protein; NA neuraminidase; M matrix; NS nonstructural; pdm pandemic 2009 lineage; TRIG triple reassortment internal gene constellation

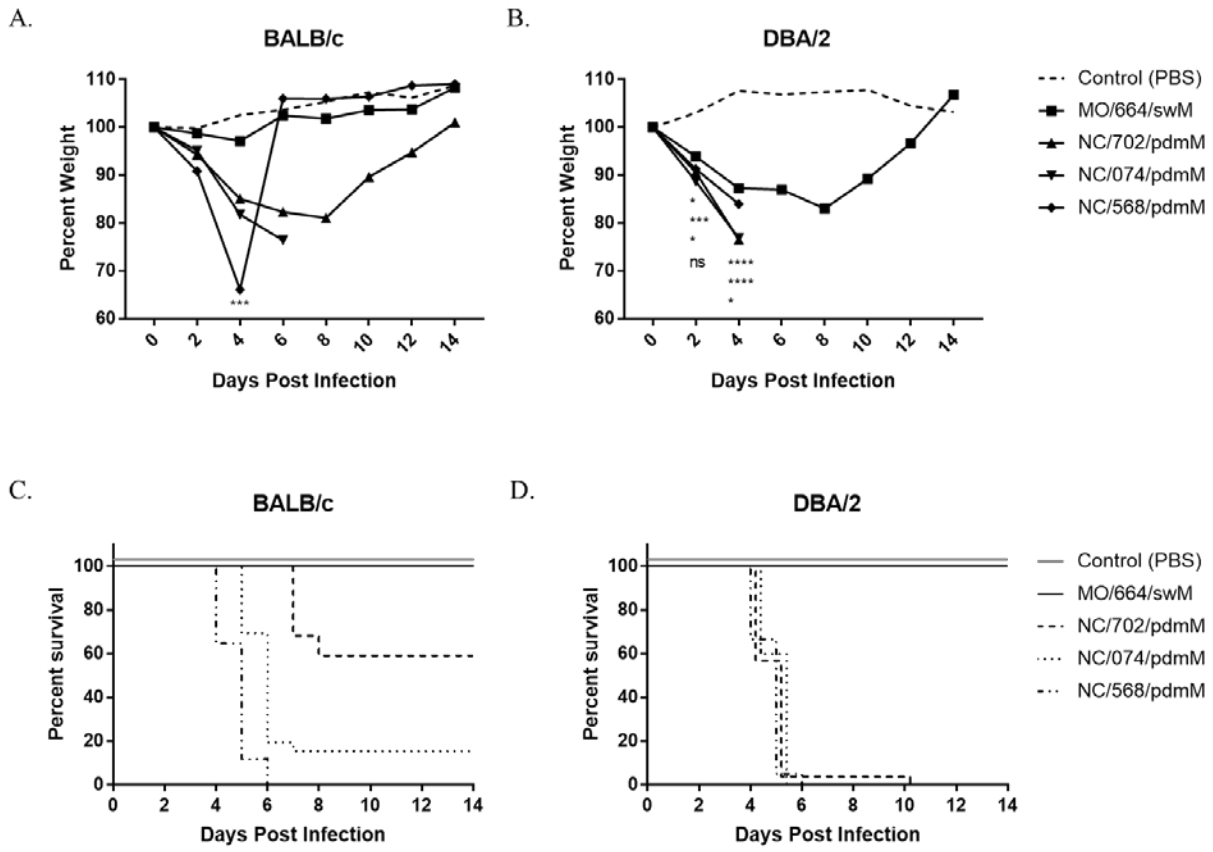


**Figure 4.2. Morbidity and mortality of mice infected with swine H1 influenza viruses.** BALB/c (A and C) and DBA/2 (B and D) were inoculated with $1x10^5$pfu of the indicated virus and weights were recorded every other day. A and B: Statistics were calculated between A/swine/Missouri/A01444664/2013 (H1N2) and the other viruses by two-way ANOVA with Dunnett post-hoc. * <0.05, **<0.005, *** <0.001, **** <0.0001. C and D: Kaplan-Meier survival curves.

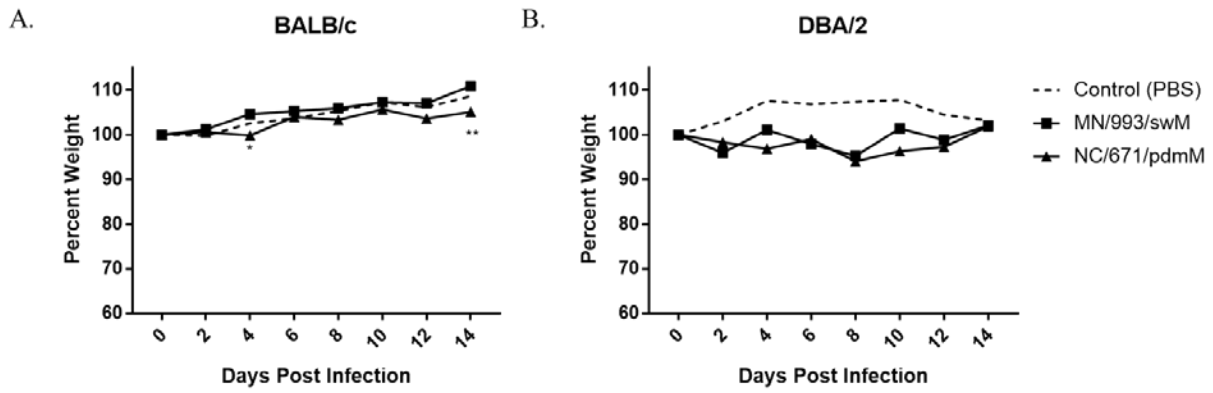


**Figure 4.3. Morbidity and mortality of mice infected with a swine H3 influenza viruses.** BALB/c (A and C) and DBA/2 (B and D) were inoculated with $1x10^5$pfu of the indicated viruses and weights were recorded every other day. A and B: Statistics were calculated between A/swine/Minnesota/A01125993/2012 (H3N2) and A/swine/North Carolina/157671/2015 (H3N2) by two-way ANOVA with Bonferroni post-hoc. * <0.05, **<0.005. C and D: Kaplan-Meier survival curves.Figure 4

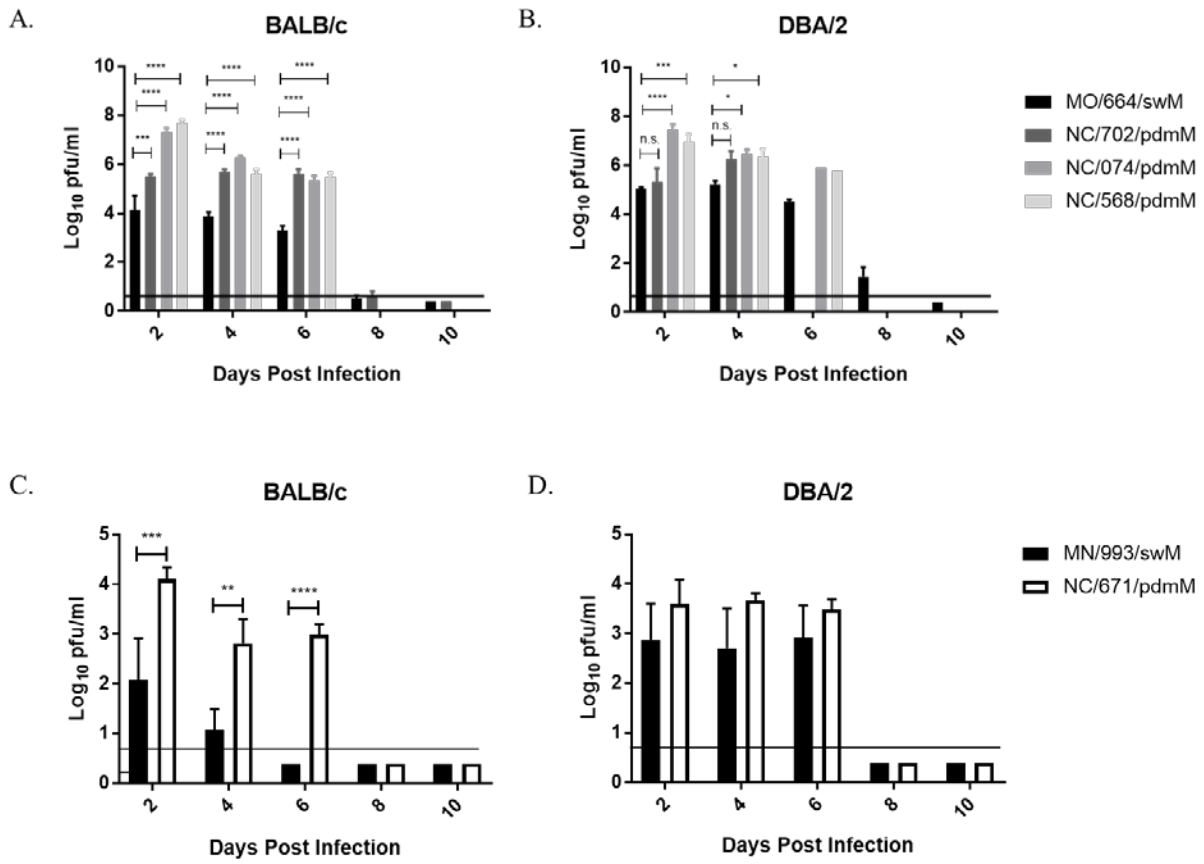


**Figure 4.4. Lung virus titers from mice infected with swine H1 and H3 influenza viruses.** BALB/c (A and C) and DBA/2 (B and D) were inoculated with $1x10^5$ pfu of the indicated viruses and lungs collected at set time points. Statistics were calculated using two-way ANOVA between (A and B) A/swine/Missouri/A01444664/2013 (H1N2) and the other viruses with Dunnett post-hoc or (C and D) A/swine/Minnesota/A01125993/2012 (H3N2) and A/swine/North Carolina/157671/2015 (H3N2) with Bonferroni post-hoc. * <0.05, **<0.005, *** <0.001, **** <0.0001.

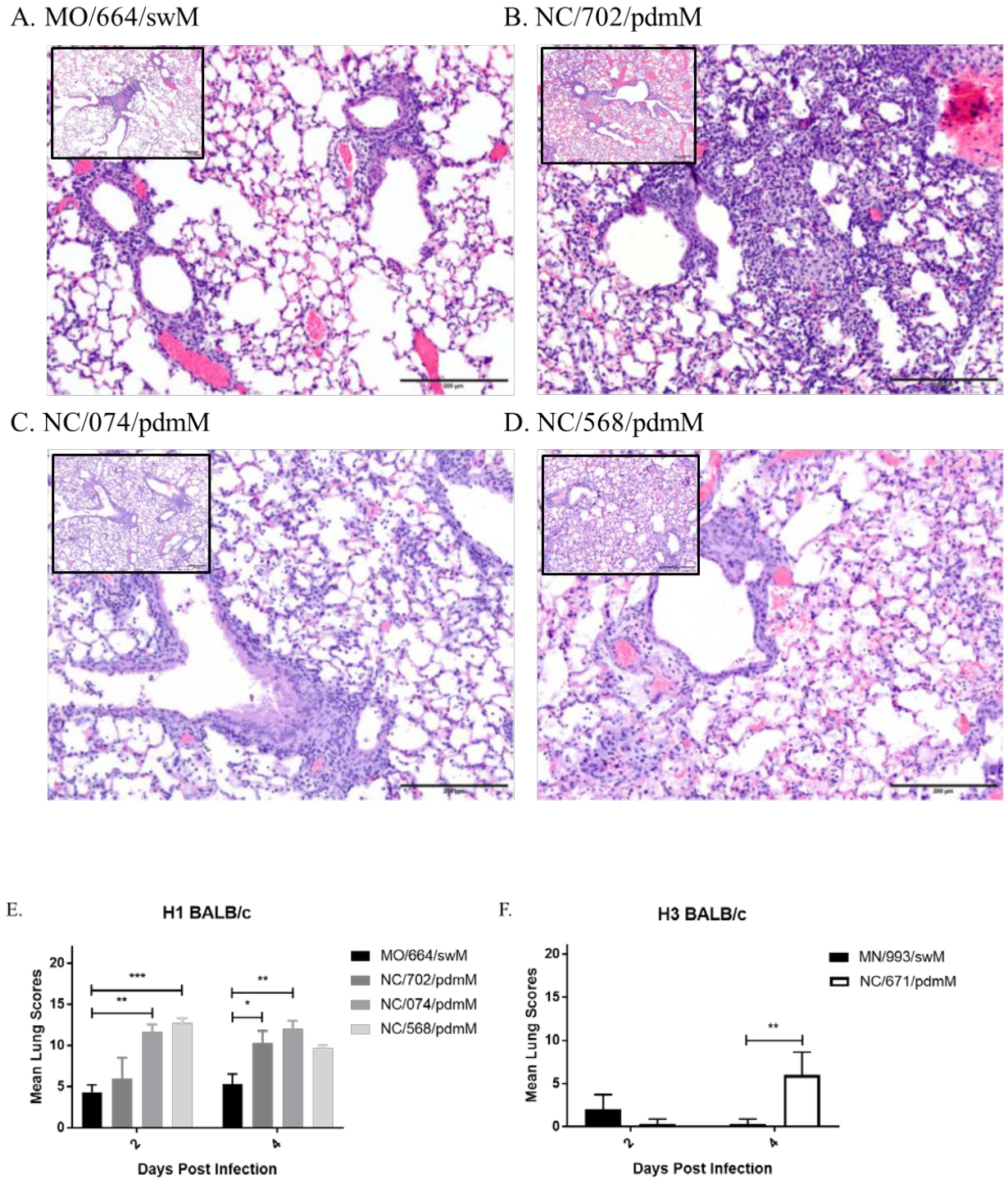


**Figure 4.5. Histological images and histologic lesion scores from mice infected with H1 and H3 swine influenza viruses.** BALB/c mice were inoculated with $1x10^5$ pfu of the indicated viruses, euthanized, and lungs fixed for histological analysis at 2 and 4 DPI. A – D: Images of lung sections at 20x and 10x (inset) for A/swine/Missouri/A01444664/2013 (H1N2) (A), A/swine/North Carolina/152702/2015 (H1N2) (B), A/swine/North Carolina/154074/2015 (H1N1) (C), and A/swine/North Carolina/A01394568/2013 (H1N1) (D). A. A/sw/MO/A01444664/2013 (H1N2): Mild perivascular and peribronchiolar infiltrations of mostly

lymphocytes and mild segmental necrosis of the bronchiolar epithelium are present, but there are no significant interstitial changes. Lesion score: 6. B. A/sw/NC/152702/2015 (H1N2): Bronchioles are dilated and there are moderate peribronchiolar and mild perivascular infiltrations of mostly lymphocytes and diffuse necrosis of the bronchiolar epithelium. Focally extending from the central bronchiole, alveolar septa are thickened and there are small numbers of inflammatory cells in the alveoli. Lesion score: 10. C. A/sw/NC/154074/2015 (H1N1): Bronchioles are slightly dilated with mild epithelial necrosis and sloughed epithelial cells and a few inflammatory cells in the lumen. There are mild to moderate peribronchiolar and perivascular infiltrations of mostly lymphocytes. Diffusely the alveolar septa are mildly thickened, and the alveoli contain small numbers of inflammatory cells. Lesion score: 13. D. A/sw/NC/A01394568/2013 (H1N1): Bronchioles are slightly dilated and lined by attenuated epithelium. There are mild peribronchiolar and perivascular infiltrations of mostly lymphocytes. Diffusely the alveolar septa are mildly thickened, and alveoli contain small numbers of inflammatory cells admixed with erythrocytes. Lesion score: 10. E. Histologic lesion scores out of 22 for BALB/c mice inoculated with either the H1 or H3 swine influenza isolates. Statistics were calculated using two-way ANOVA between (E) A/swine/Missouri/A01444664/2013 (H1N2) and the other viruses with Dunnett post-hoc or (F) A/swine/Minnesota/A01125993/2012 (H3N2) and A/swine/North Carolina/157671/2015 (H3N2) with Bonferroni post-hoc. * <0.05, **<0.005, *** <0.001, **** <0.0001.

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# CHAPTER 5

# SWINE INFLUENZA VIRUSES CONTAINING THE PANDEMIC MATRIX GENE DYSREGULATE THE HOST INNATE IMMUNE RESPONSE IN THE MURINE MODEL[1]

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## Abstract

The pandemic H1N1 (pdmH1N1) influenza A virus has repeatedly spilled back into North American swine populations since its emergence in 2009. While the pdmH1N1 virus did not become prevalent in North American swine, the genetic makeup of circulating swine influenza strains dramatically changed following its emergence. Since the late 1990's, swine influenza A viruses (swIAVs) endemic in North America contained an internal gene segment constellation of human, avian and swine IAV origin, referred to as the triple reassortment internal gene (TRIG) cassette, which quickly became dominant. However, reassortment events with the pdmH1N1 virus resulted in the pdmH1N1 origin matrix gene (pdmM) replacing the classical swine origin matrix gene (swM) previously found in the TRIG cassette, suggesting a fitness benefit. In other work we showed that the origin of the matrix gene correlates with disease severity during swIAV infection in a murine model of infection. We hypothesized that the swIAVs containing the pdmM gene induce more severe histological changes in the lungs of mice via the dysregulation of the host innate immune response. To confirm this, we infected BALB/c mice with a panel of H1 swIAVs containing either the pdmM or the swM gene segment. We assessed virus replication, lung pathology, cytokine and chemokine production, as well as cellular infiltration. Infection of mice with H1 swIAVs containing the pdmM gene resulted in an overall significantly greater proinflammatory and chemotactic response compared to swIAVs containing the swM gene. Moreover, plasmacytoid dendritic cells, eosinophils, and monocytes but no other innate immune cell types assessed were found to be disproportionately higher in the bronchoalveolar lavage (BAL) of mice infected with pdmM containing swIAVs. While other gene segments such as the NS1 or PB1-F2 traditionally antagonize the host innate immune response, our data suggest that the origin of the matrix gene contributes to an enhanced immune response against swIAVs in mammals.

## Introduction

Influenza A virus (IAV) is recognized by the host innate immune system via internal nucleic acid sensors including, retinoic acid induced gene I (RIG I), nod like receptor (NLR), NLRP3, and toll-like receptors (TLR) 3 and 7 (1, 2). Following respiratory epithelial cell infection with IAV, the infection can spread to resident immune cells including alveolar (AM) and plasmacytoid dendritic cells (pDCs) as well as circulating neutrophils and natural killer (NK) cells (3). The initiation of the antiviral response is the production of type I and type III interferons primarily by epithelial cells and pDCs (4-7). Interferon signaling results in the upregulation of interferon stimulated genes (ISGs) and the production of cytokines and chemokines that orchestrate the entire immune response. Cytokines and chemokines such as IL-6, IL-1α/β, MCP-1, and RANTES are regarded as proinflammatory, and are important for leukocyte and granulocyte recruitment as well as activation (8, 9). Although beneficial for controlling virus replication and priming the adaptive immune response, poorly controlled inflammation can also result in lung tissue damage (10).

The emergence of 2009 H1N1 pandemic influenza (pdmH1N1) virus resulted in an estimated 60 million infections, 274 thousand hospitalizations, and 12 thousand deaths in the Unites States alone (11). The pdmH1N1 strain was the result of a quadruple reassortment in swine between the circulating triple reassortment internal gene constellation (TRIG) lineage containing classical swine, human-like, and avian genes with a Eurasian avian-like swine H1N1 IAV (12, 13). This virus spread back into swine via multiple reverse-zoonotic events (14). Although the pdmH1N1 strain itself has not persisted in swine, several of its internal gene segments have become predominant in the current circulating swine IAV viruses in North America. Specifically, the pandemic origin matrix gene (pdmM) gene has gradually replaced the classical swine matrix gene (swM) from the TRIG lineage, becoming predominant in isolates sequenced in North America (15, 16). Moreover, studies have linked the pdmH1N1 strain and its gene segments to severe disease in humans and animal models of infection (17-21). Therefore, understanding how each of the gene segments and the virus as a whole can interact with the host immune response is of paramount importance.

Previously, we established a correlation between an increase in disease severity in the murine model and the pdmM gene in swIAVs. Here, we hypothesize that infection with swIAVs containing the pdmM gene compared to strains of the same subtype and combination of gene segments containing the swM gene, results in the dysregulation of the innate immune response and subsequent increase in disease severity in the murine model. Furthermore, we hypothesize that matrix protein 1 (M1) and matrix protein 2 (M2) derived from the pdmM gene directly induced greater activation of the innate immune response compared to those derived from the swM gene. Here we demonstrate that infection with pdmM-containing swIAVs induce greater production of proinflammatory cytokines and chemokines, greater infiltration of innate immune cells, and enhanced activation of innate immune pathways relative to infection with swM containing swIAVs in the murine model.

## Materials and Methods

### *Cell Culture and Virus Propagation*

Madin-Derby Canine Kidney (MDCK) cells were cultured in DMEM with 5% FBS. Swine influenza viruses A/swine/Missouri/A01444644/2013 and A/swine/North Carolina/A01394568/2013 were obtained from the USDA National Veterinary Services Laboratories (NVSL) reagent resource. Swine influenza viruses A/swine/North Carolina/152702/2015 and A/swine/North Carolina/154074/2015 were obtained from the NIAID Centers of Excellent in Influenza Research and Surveillance (CEIRS) network (52). Viruses were propagated in Madin-Darby canine kidney (MDCK) cells in Minimal Essential Media [MEM (GIBCO™)] with 0.002μg tosyl phenylalnyl chloromethyl ketone (TPCK). Viruses were passaged to achieve stock titers above $1x10^6$ pfu/ml. Influenza viral titers were assessed by plaque assay on MDCK cells as previously described (53). Briefly, a 24-well plate of MDCK cells was incubated with serial dilutions of virus at 37˚C and 5% CO2 for 1-2 hours. The supernatant was removed, 1ml of 1:1 2.4% Avicel solution and overlay [MEM (GIBCO™) with 1M HEPES (GIBCO™), 200mM mM GlutaMAX-I, 7.5% NaHCO3 (GIBCO™), and antibiotic/antimycotic (GIBCO™)] was added and the cells were

incubated at 37˚C and 5% CO2 for 48 -72 hours prior to fixation with 80/20 methanol/acetone and staining with crystal violet.

*Mice and in vivo infections*

Female 6 – 10-week-old BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC). All animal studies were approved by the Animal Care and Use Committee of the University of Georgia. Mice were anesthetized by 3% Isoflurane (Patterson Veterinary) and intranasally inoculated with 50µl virus diluted in phosphate buffered saline (PBS) or PBS alone for control. Weight loss was assessed every other day as a percent of weight at day 0.

*Bronchial Alveolar Lavage*

Mice were humanely euthanized and bronchial alveolar lavage (BAL) was performed as previously described (54). Briefly, 1ml of PBS was injected intratracheally into the lung and was used to wash three times. The BAL fluid was then collected and spun down at 500g for 7min and the cleared fluid and cells separated and collected for follow-up experiments.

*Cytokine Analysis*

From the BAL sample 500µl was concentrated using a 3K MWCO Pierce® Concentrator (Thermo Scientific) to 100µl. 50µl of concentrated sample was then run in duplicate using the murine 36-plex ProcartaPlex™ Multiplex Immunoassay (ThermoFisher Scientific) as per manufacturer's protocol. There was no detectable infectious virus left in the sample by the end of the protocol as detected by plaque assay described above. Samples were run using the Bio-Plex 200 Luminex system (BIO-RAD).

*MPO ELISA*

BAL samples were diluted in PBS 1:250. Samples were run using Mouse Myeloperoxidase DuoSet ELISA Kit (R&D Systems, Minneapolis MN) as per protocol. ELISAs were developed using TMB

substrate kit (Vector Laboratories, Burlingame CA) as per protocol and were analyzed at 450nm on Powerwave XS using Gen5 2.07 software (BioTek, Winooski VT).

*Flow Cytometry*

For each mouse, BAL was repeated for a total of 2ml and the cells collected. Red blood cells were lysed using ACK Lysis Buffer (Lonza, Basel Switzerland) for 10 minutes. 1ml PBS was added and cells centrifuged at 500g for 7 minutes. Cells were resuspended and incubated with FC Block (BioLegend®, San Diego, CA) for 15 – 30 minutes on ice followed by staining using a combination of the following antibodies: CD3 DX5 (ebioscience) CD45, CD115, CD11b, CD11c, Ly6G, Ly6C, and F4/80 (BioLegend®). Cells were fixed using BD Fixative (Beckton Dickinson, Franklin Lakes, NJ). Samples were run on a LSRII (BD Biosciences, San Jose CA) flow cytometer.

*Histology*

Mice were humanely euthanized at 6 DPI and lungs were collected, inflated and fixed by immersion in 10% buffered formalin. Fixed lungs were routinely processed and embedded in paraffin so that all lung lobes could be evaluated, and 4 μ sections were stained with hematoxylin and eosin. Lungs were scored as follows: Perivascular (bronchial tree) inflammation (0 = none; 1 = mild, 1-2 cells wide; 2 = moderate, 3-10 cells wide; 3 = severe, >10 cells wide); percentage of bronchioles affected (0 = none; 1 = <25%; 2 = 25-75%; 3 = >75%); peribronchiolar inflammation (0 = none; 1 = mild; 2 = moderate; 3 = severe); severity of airway luminal exudate, epithelial necrosis and inflammation (0 = none; 1 = mild; 2 = moderate; 3 = severe); percentage of alveolar involvement (0 = none; 1 = <25%; 2 = 25-50%; 3 = >50%); severity of interstitial inflammation (0 = none; 1 = mild; 2 = moderate; 3 = severe); edema (0 = none; 1 = present); hemorrhage (0 = none; 1 = present); type II cell hyperplasia (0 = none; 1 = present); vasculitis (0 = none; 1 = present). This resulted in scores ranging from 0-22. BALT (well defined aggregates of mixed lymphocytes or follicles) was scored separately as 0-3 (0 = none; 1 = mild; 2 = moderate; 3 = severe).

*Statistical Analysis*

Statistics were run using GraphPad Prism version 7.03. Statistical analysis included two-way analysis of variance (ANOVA) with Bonferroni post-hoc or Dunnett post-hoc. All results were considered significant at p-values $< 0.05$.

## Results

*Swine influenza strains containing the pandemic M gene induce greater histological changes in the lung despite minimal morbidity*

Previous work correlated an increase in disease severity and microscopic lung lesions in mice infected with swIAVs containing the pdmM gene compared to animals infected with similar swIAVs containing the swM gene segment. Since the H1 strains had a more discrete outcome between the different genetic constellations (Chapter 4), we chose these viruses to further elucidate the mechanism behind the escalation in disease pathogenesis (Table 1). BALB/c mice were inoculated at $1x10^3$ pfu virus/mouse and were assessed for weight loss and lung viral titers. Of the three swIAVs containing the pdmM gene, only one induced significant weight loss in the mice, and was lethal by 4 days post infection (DPI, Figure 1A). Due to the rapid lethality of the virus, NC/568/pdmM, was removed from the rest of the in vivo studies. Lung viral titers were significantly higher in the mice infected with strains containing the pdmM gene compared to strains containing the swM gene, despite minimal weight loss (Figure 1B). Histological analysis of the lungs was also performed to fully characterize the disease pathogenesis of these influenza strains at a lower inoculum dose than was previously used.

Mice infected with MO/664/swM had very mild changes characterized by mild lymphocytic infiltration around very few vessels and bronchioles, rare bronchioles with epithelial necrosis, and minimal to no interstitial involvement (Table 2). Changes in the lungs of mice infected with NC/702/pdmM (H1N2) or NC/074/pdmM (H1N1) were similar in severity and character and were more severe than those seen in the MO/664/swM (H1N2) mice. These mice had a moderate bronchointerstitial pneumonia characterized

by moderate perivascular and peribronchiolar infiltrations of predominately lymphocytes involving >75% of vessels and bronchioles with few to many of these bronchioles having epithelial necrosis and mild luminal accumulations of necrotic debris. In addition, the lungs of these mice had mild to moderate interstitial involvement arising from affected bronchioles, which included mild thickening of alveolar septa and alveoli containing small numbers of mixed inflammatory cells (lymphocytes, macrophages, and neutrophils) or filled with necrotic debris admixed with macrophages. Occasionally these lungs had perivascular edema, vasculitis of the cardiac-type vessels, or rarely small hemorrhages. Overall, mice infected with swIAVs containing the pdmM gene had significantly more severe histologic lesions in the lungs compared to mice infected with swine IAV containing the swM gene (Table 2). This is in agreement with previous studies that demonstrated higher severity of lung pathology with pdmH1N1 infection (22).

*Infection with swIAVs containing the pdmM gene induces a more acute and sustained interferon response in the lungs of mice compared to strains containing the swM gene*

The interferon response is the first line of defense against viral infection and can modulate the subsequent innate and adaptive immune responses. During influenza infection, both type I interferon α (IFNα) and type III interferon λ (IFNλ) are induced and aide in the control of virus replication (23, 24). Furthermore, a prolonged type I interferon response had been associated with greater disease severity (25). Therefore, we assessed interferon concentrations in the bronchiolar lavage fluid (BAL) at 4 and 6 DPI. There were significantly higher concentrations of IFNα and IFNλ at 4 and 6 DPI in the BAL of mice inoculated with swIAVs containing the pdmM gene compared to strains containing the swM gene (Figure 2A).

*Infection with swIAVs containing the pdmM gene induces greater cytokine and chemokine production in the lungs of mice compared to strains containing the swM gene*

Cytokine and chemokine production is key in orchestrating both the innate and adaptive immune responses. In order to elucidate differences in the development of the innate immune response to swIAVs,

we infected BALB/c mice with $1x10^3$ pfu/mouse swIAVs containing either the pdmM or the swM gene and analyzed the BAL for 36 different cytokines. Overall there was a significantly higher concentration of proinflammatory cytokines including: TNFα, IL-6, IL-1β, and IL-18 in the BAL of mice infected with strains containing the pdmM gene (Figure 2B). Furthermore, colony stimulating factors such as GM-CSF, G-CSF, and M-CSF along with other cytokines known to induce proliferation and activation of innate immune cells and their respective chemokines were also at significantly higher concentrations in BAL from pdmM containing strains (Figure 2B). These data suggest that infection with swIAVs containing the pdmM gene induce an overall greater cytokine and chemokine response in mice relative to strains containing the swM gene. Since the majority of cytokines are produced and can affect a multitude of cell types, we assessed the innate immune cell infiltrates in the BAL during the course of infection.

*Infection with swIAVs containing the pdmM gene induces disproportional infiltration of specific innate immune cells compared to strains containing the swM gene*

Mice were inoculated with $1x10^3$ pfu/mouse of swIAVs containing either the pdmM or swM gene. BAL was collected 4 and 6 DPI and the innate immune cell infiltrates assessed by flow cytometry. The cells were characterized as follows: neutrophils - $CD115^-$ $CD11b^+$ $Ly6G^+$; eosinophils - $CD115^-$ $CD11b^+$ $Ly6G^-$; alveolar macrophages - $CD115^+$ $CD11b^-$ $F4/80^+$ $CD11c^+$; interstitial macrophages - $CD115^+$ $CD11b^+$ $F4/80^+$ $CD11c^+$; plasmacytoid dendritic cells - $CD115^+$ $CD11b^-$ $Ly6c^+$ $Ly6G^-$ $F4/80^-$ $CD11c^{+/-}$; monocytes - $CD115^+$ $CD11b^+$ $F4/80^{+/-}$ $Ly6G^+$; natural killer cells – $CD45^+$ $CD3^-$ $DX5^+$. As expected, there was a significantly greater number of cells found in the BAL of mice infected with swIAVs containing the pdmM gene compared to viruses containing the swM gene (Figure 3A). Of the cell types characterized, only alveolar macrophages were found in lower proportion in the BAL of mice infected with pdmM containing strains. While the proportion of neutrophils and NK cells found in the BAL of mice were not significantly different between the swIAVs, the proportion of eosinophils, pDCs, and monocytes were significantly disproportionately greater in the lungs of mice infected with the pdmM-containing swIAVs (Figure 3B). Combined, these data suggest that infection with swIAVs containing the pdmM gene induces

not only a greater innate immune response as determined by cytokine and chemokine levels, but also a dysregulated immune response, in that not all innate immune cells are recruited equally to the site of infection.

*Infection with swIAVs containing the pdmM gene induces greater activation of innate immune cells in vivo*

Cytokines such as IL-15, IL-18, and type I interferons are known to induce the proliferation and activation of NK cells (26-28). When activated, NK cells produce IFNγ; additionally, early during influenza infection NK cells are the main producers of IFNγ rather than γδT cells or $CD4^+$ T cells (29, 30). Cytokines including TNFα, IL-1β, G-CSF, and GM-CSF, on the other hand are known to activate neutrophils (31). When activated, neutrophils, like other granulocytes, produce a variety of enzymes; however, myeloperoxidase (MPO) is a specific marker for neutrophil activation (32, 33). Therefore, we analyzed the activation of NK cells by IFNγ concentration and neutrophil activation by MPO concentration in the BAL of mice infected with swIAVs containing either the pdmM or swM gene. At both 4 and 6 DPI IFNγ concentrations and MPO concentrations were significantly higher in the BAL of mice infected with swIAVs containing the pdmM gene compared to strains containing the swM gene (Figure 4 A and B). This suggests that swIAVs containing the pdmM gene elicit greater activation of innate immune cells.

## Discussion

Between 2011 and 2015 the replacement of the classical swine matrix gene by the pdmM gene increased from 70% to 100% of all swIAV isolates sequenced in North America (15), suggesting a fitness advantage. The pdmM gene has been correlated with increased neuraminidase activity, a more filamentous shape, and increased transmission efficacy in guinea pigs as well as in swine (34-37). We previously correlated higher morbidity, mortality, and severity of histological changes in the lung with swIAVs containing the pdmM gene relative to strains containing the swM gene. We hypothesized that the severe lung pathology observed in murine infection of swIAVs containing the pdmM gene was correlated to a

dysregulation of the innate immune response; furthermore, this dysregulation could be specifically linked to the origin of the matrix gene.

We inoculated BALB/c mice with H1 swIAVs containing either the pdmM or swM gene segment and assessed morbidity, viral replication, and histological changes in the lungs. Only one strain containing the pdmM gene, NC/568/pdmM (H1N1), demonstrated significantly greater morbidity as determined by weight loss; however, due to the rapid lethality, this strain was removed from further in vivo experiments. Further research into the sequence differences between this strain and the NC/074/pdmM (H1N1) strain, a genetically similar but less lethal strain, would be extremely useful in elucidating potentially new virulence factors. Despite a lack of difference in morbidity in the mice at a $10^3$ pfu inoculum, infection with swIAVs containing the pdmM gene resulted in more severe histological changes in the lungs than with strains containing the swM gene. This is in congruence with our previous study; therefore, we analyzed the antiviral response along with cytokine and chemokine concentrations in the BAL to elucidate the mechanism(s) behind the histological changes.

Infection with swIAVs containing the pdmM gene significantly induced type I and type III interferon production both at 4 and 6 DPI compared to strains containing the swM gene. Furthermore, significantly higher levels of proinflammatory cytokines and chemokines were found in the BAL of mice infected with swIAVs containing the pdmM gene relative to strains containing the swM gene. This is in disagreement with a previous study by Osterlund *et al.* that demonstrated a reduced ability of the pdmH1N1 strain to induce an antiviral and proinflammatory response; however, this study compared the pandemic strain to A/WSN/33 and A/Udorn/72 (38). A/WSN/33 and A/Udorn/33 are two human origin influenza strains which have been passaged in the laboratory setting for many years, potentially allowing for the accumulation of mutations that could explain their relatively high induction of the innate immune response and the pdmH1N1 relatively low induction. Our data is in concordance with other studies which have also demonstrated greater cytokine induction by the pdmH1N1 strain in humans and in animal models (18, 22,

39, 40). Furthermore, this supports the mechanism behind our hypothesis, linking higher interferon and cytokine production with severity of disease as others have done with highly pathogenic H5N1 (41).

The significantly greater total cellular infiltrates in the BAL of mice infected with pdmM containing strains was in agreement with the more severe lung pathology and higher concentrations of chemokines. Interestingly not all cell types were proportionally elevated in these same mice. Alveolar macrophages made up a larger proportion of the total cellular infiltrate in the BAL of mice infected with swM containing strains. In contrast, pDCs, monocytes, and eosinophils made up a disproportionately larger amount of the total cellular infiltrates in the BAL of mice infected with pdmM containing strains. Together, this data further confirms the hypothesis that infection with swIAVs containing the pdmM gene results in the dysregulation of the innate immune response compared to strains containing the swM gene.

Myeloperoxidase, an enzyme specific to neutrophil function, although virucidal, has also been linked to inflammatory damage during influenza infection (32, 33, 42). Furthermore, neutrophils are able to augment NK cell activity resulting in greater IFNγ release (43). A recent study demonstrated the differential ability of the pdmH1N1 strain to alter the function of neutrophils (44). Therefore, we assessed the concentrations of MPO and IFNγ in the BAL of mice infected with swIAVs containing either the pdmM gene or the swM gene. Concentrations of both MPO and IFNγ were significantly greater in the BAL of mice infected with pdmM containing influenza strains, despite similar frequencies of neutrophils and NK cells. These data suggest that during infection with swIAVs containing the pdmM neutrophils and NK cells are more highly activated than infection with swIAVs containing the swM gene. This supports our hypothesis that swIAVs containing the pdmM gene induces dysregulation of the innate immune response resulting in more severe disease. Additional studies including in vivo infection with a reverse genetics system allowing for the replacement of the matrix gene with that of a pdmM origin or swM origin gene and assessing innate immune cell activation, can shed more light on the effect of the origin of the matrix gene on the dysregulation of the host innate immune response.

The dysregulation of the innate immune response by influenza is not unique. Several studies over the last few years have linked the severity of disease in highly pathogenic H5N1 and 1918 H1N1 infections to the dysregulation of the immune response (45-48). Up until now, the dysregulation of the immune response has been correlated to the NS1 protein, HA, NA, or to the replication ability of the polymerase and NP complex (41, 48-51). Here we show that the matrix gene of swIAVs contribute to the dysregulation of the host innate immune response. Furthermore, that the pdmM gene is correlated with high concentrations of interferons, cytokines, and chemokines which results in a greater recruitment of cellular infiltrates into the lungs. All together this corresponds to the severity of lung pathology. Further research is needed to determine the specific mechanisms of immune dysregulation that can be attributed to the M1 or M2 proteins derived from the pdmM gene. Understanding the mechanisms by which influenza A virus perturbs the host immune response can give us insight risk assessment of emerging viruses as well as into potential therapeutic drug targets to reduce the harmful inflammation induced in severe IAV infection.

**Table 5.1.** Swine IAVs used and the origin of their gene segments

| Influenza Strain | | NCBI Taxon ID | Gene Origin | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PB2 | PB1 | PA | HA | NP | NA | M | NS |
| A/swine/Missouri/A01444664/2013 (H1N2) | MO/664/swM | NCBI:txid1289201 | | | | H1δ1 | | N2-2002 | pdm | |
| A/swine/North Carolina/152702/2015 (H1N2) | NC/702/pdmM | | TRIG | TRIG | TRIG | H1δ2 | pdm | N2-1998 | Sw | TRIG |
| A/swine/North Carolina/154074/2015 (H1N1) | NC/074/pdmM | | TRIG | TRIG | TRIG | H1γ | pdm | N1-classical | sw | TRIG |
| A/swine/North Carolina/A01394568/2013 (H1N1) | NC/568/pdmM | NCBI:txid1425844 | | | | H1γ | | N1-classical | sw | |

pdm – pandemic, sw – classical swine, TRIG – triple reassortment internal gene constellation

**Table 5.2.** Histological analysis of lungs from mice infected with a panel of swIAVs

| Virus | Perivascular (bronchial tree) | Bronchioles | | | Alveoli | | | | | Vessels | Total Score |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Inflammation (0-3) | Percent extent affected (0-3) | Severity (0-3) | Peribronchiolar infiltration (0-3) | Percent extent of involvement (0-3) | Edema (0 or 1) | Hemorrhage (0 or 1) | Severity-Inflammation (0-3) | Hyperplasia (0 or 1) | Vasculitis | 22 ± SEM |
| **MO/2013** | 0.67 | 1.00 | 0.00 | 1.00 | 0.33 | 0.00 | 0.00 | 0.33 | 0.00 | 0.00 | 3.33 ± 0.33[A] |
| **NC/702/2015** | 1.67 | 3.00 | 1.33 | 1.67 | 1.33 | 0.00 | 0.00 | 1.67 | 0.00 | 0.33 | 11.0 ± 1.53[B] |
| **NC/074/2015** | 2.00 | 2.33 | 1.00 | 1.67 | 1.33 | 0.33 | 0.00 | 1.00 | 0.00 | 0.67 | 10.33 ± 2.89[B] |

Inflammation: 0 = none; 1 = mild (1-2 cells wide); 2 = moderate (3-10 cells wide); 3 = severe (>10 cells wide)

Percent affected: 0 = none; 1= <25%; 2 = 25-75%; 3 = >75%

Severity (Luminal exudate, epithelial necrosis, and inflammation): 0 = none; 1 = mild; 2 = moderate; 3 = severe

Peribronchiolar infiltration: 0 = none; 1 = mild (1-2 cells); 2 = moderate (3-10); 3 = severe (>10 cells thick)

Percent extent involvement: 0 = none; 1 = <25%; 2 = 25-50%; 3 = >50%

Edema / Hemorrhage / Hyperplasia / Vasculitis: 0 = absent / 1 = present

Severity Inflammation: 0 = none; 1 = mild (few); 2 = moderate (moderate numbers); 3 = severe (many)

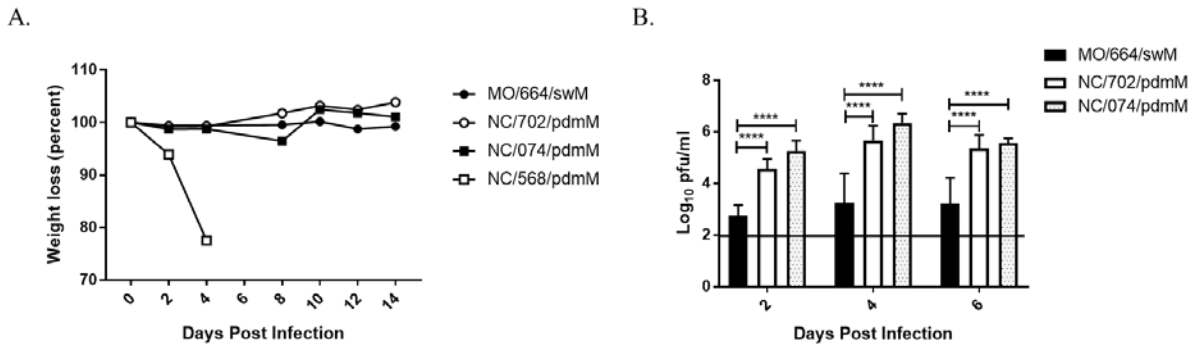


**Figure 5.1. Weight loss and lung viral replication of mice infected with H1 swIAVs.** BALB/c mice were inoculated intranasally with $1x10^3$ pfu/mouse of H1 swIAVs. Weight loss was assessed every other day (A) At 2, 4, and 6 DPI BAL and tissues were collected for cytokine analysis and virus titer, respectively. Black line is the limit of detection (B). Statistics were analyzed between A/swine/Missouri/A01444664/2013 (H1N2) and other influenza strains by two-way ANOVA followed by Dunnett post-hoc. * <0.05 ** <0.01 *** < 0.001 **** < 0.0001

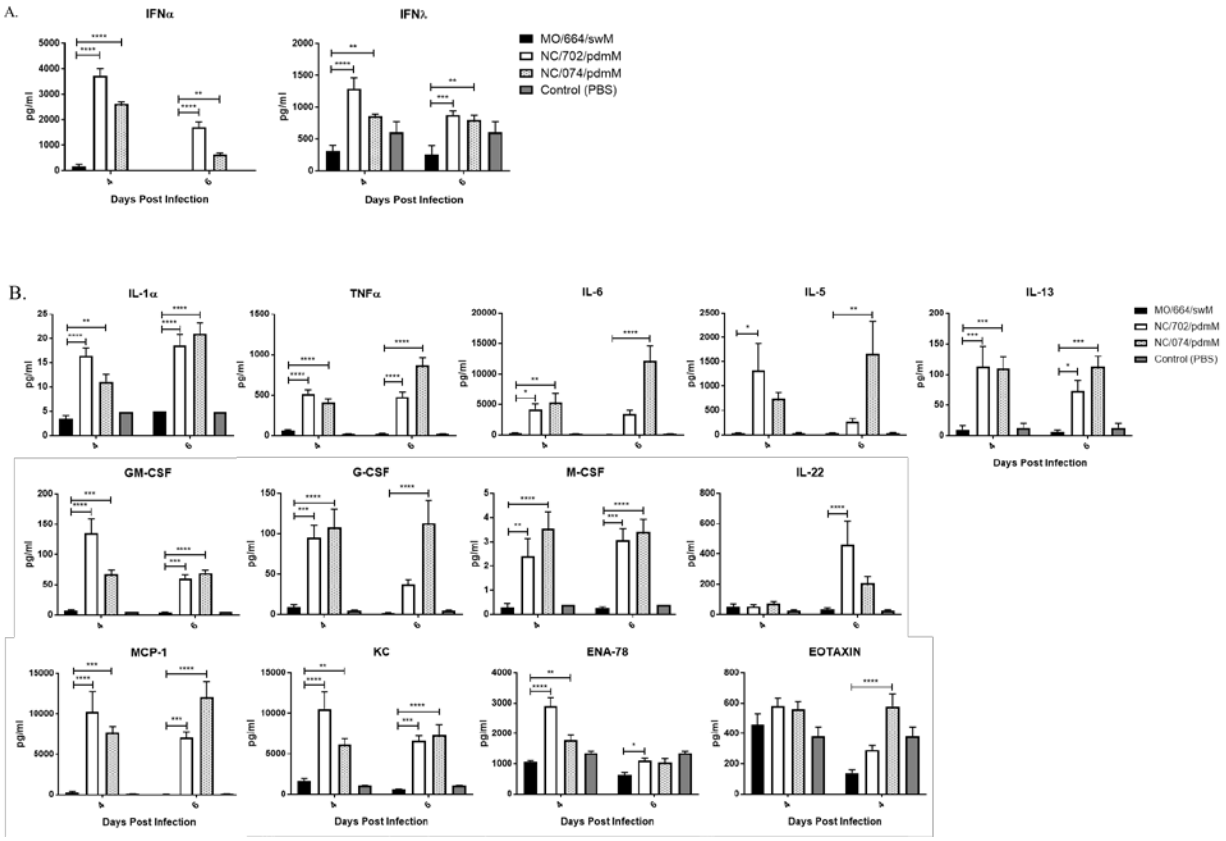


**Figure 5.2. Cytokine and chemokine production in mice infected with swIAV.** BALB/c mice were inoculated as described in Figure 1. 4 and 6 DPI BAL was collected, interferons (A), cytokines and chemokines (B) were assessed by Luminex bead array. Statistics were analyzed between A/swine/Missouri/A01444664/2013 (H1N2) and other influenza strains by two-way ANOVA followed by Dunnett post-hoc. * <0.05 **< 0.01 *** < 0.001 **** < 0.0001

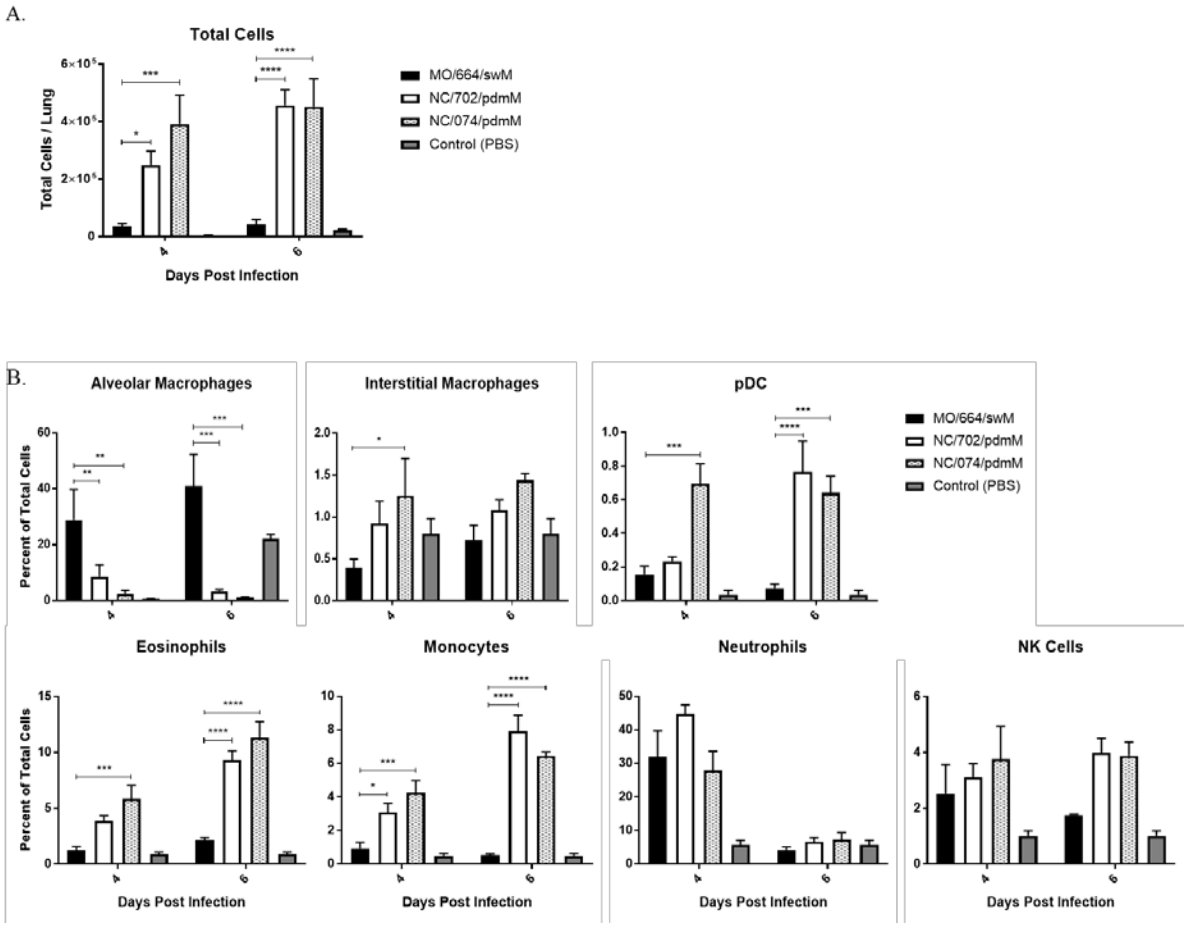


**Figure 5.3. Flow cytometric analysis of innate immune cells in mice infected with swIAVs.** BALB/c mice were inoculated as described in Figure 1. At 4 and 6 DPI BAL was collected, and cellular infiltrate analyzed by flow cytometry. Total cells (A). Alveolar macrophages, interstitial macrophages, pDCs, eosinophils, monocytes, neutrophils, and NK cells are calculated as percent of total cells counted for each individual sample (B). Statistics were analyzed between A/swine/Missouri/A01444664/2013 (H1N2) and other influenza strains by two-way ANOVA followed by Dunnett post-hoc. * <0.05 ** <0.01 *** < 0.001 **** < 0.0001

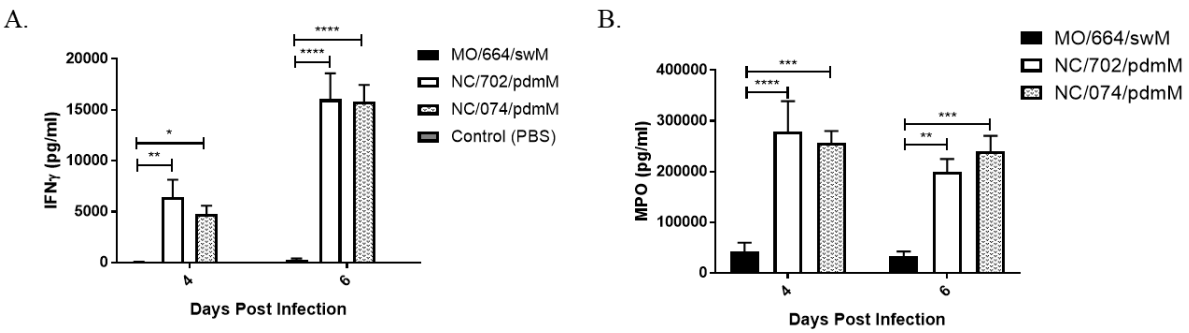


**Figure 5.4. Activation of NK cells and neutrophils in mice infected with swIAVs.** BALB/c mice were inoculated as described in Figure 1. At 4 and 6 DPI BAL was collected and IFNγ (A) and MPO (B) concentrations assessed. Statistics were analyzed between A/swine/Missouri/A01444664/2013 (H1N2) and other influenza strains by two-way ANOVA followed by Dunnett post-hoc. * <0.05 ** <0.01 *** < 0.001 **** < 0.0001

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# CHAPTER 6

# A STRONG PROINFLAMMATORY BUT LIMITED ANTIVIRAL INNATE IMMUNE RESPONSE CONTRIBUTES TO THE GREATER SUSCEPTIBILITY OF DBA/2 MICE TO INFLUENZA A INFECTION

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## Abstract

Host genetic factors contribute to increased risk for severe disease during influenza A infection in humans; and some of those potential host factors can be elucidated through the use of inbred mouse strains. Using phenotypically resistant and susceptible mice, BALB/c and DBA/2 respectively, the functional importance of interferon lambda (IFNλ) was assessed during influenza A infection. We established an influenza infection model minimizing the effects of increased replication in the susceptible DBA/2 mouse strain while retaining the established difference in morbidity relative to the resistant BALB/c mouse strain. Despite minimal differences in lung pathology, influenza-infected DBA/2 mice exhibited significantly greater concentrations of proinflammatory cytokines and lower concentrations of immune regulating cytokines. Furthermore, DBA/2 mice had a reduced capacity to elicit an antiviral response as indicated by reduced myeloperoxidase (MPO) and IFNγ concentrations. Additionally, high concentrations of type I and type III interferons in the bronchiolar alveolar lavage (BAL) with a limited subsequent ISG response in DBA/2 mice relative to BALB/c mice was also observed. Together these data suggest that in response to similar influenza infection kinetics, DBA/2 mice elicit an early proinflammatory, but limited antiviral response, compared to BALB/c mice, enabling the enhanced disease exhibited in the DBA/2 mouse strain. The divergence of IFN-λ response to influenza virus infection and subsequent signaling between BALB/c and DBA/2 mice may contribute to corresponding differences in disease.


## Introduction

Influenza is a major public health concern causing 250 – 300 thousand deaths annually worldwide (1-3). While there are several well-known host factors such as hypertension, obesity, asthma, and heart disease that predispose individuals to complications with influenza infection, up to 25% of pneumonia cases associated with influenza viral infection occur in individuals without identified risk factors (3-6). Studies have shown that, in the murine model, variation in susceptibility to influenza infection can be attributed to host genetic factors including genes related to cytokine signaling, cell cycle, cell adhesion molecules, and antigen processing and presentation (7, 8). Variation in murine susceptibility to infection is not unique to

influenza virus; but has also been observed in hepatitis C virus (HCV) and cytomegalovirus infections, as well as in bacterial infections including *Mycobacterium tuberculosis* (9-11). The relative susceptibility to influenza infection of a several of common inbred strains of mice has been ascertained; categorizing strains from resistant (BALB/c) to susceptible (DBA/2) (12). Susceptibility is associated with higher viral load, amplified expression of genes related to inflammation, higher leukocyte recruitment, increased production of proinflammatory cytokines, and subsequently greater lung damage (12-18).

Type III interferons, the interferon λ (IFNλ) family, are an important part of the innate immune response against influenza infection (19, 20). The IFNλ family consists of IFNλ1 (IL-29), IFNλ2 (IL-28A), IFNλ3 (IL-28B), and IFNλ4, the recently discovered IFNλ3 frameshift mutant (21). Unlike the human IFNλ family, murine IFNλ1 is a non-functional pseudogene, instead, murine IFNλ2 and IFNλ3 produce glycosylated proteins (22). Despite these differences, studies have shown that murine IFNλ signals via the same pathway and results in a similar antiviral response as has been shown in humans (23, 24).

Although they have unique receptors, type I and type III interferons signal via the same JAK-STAT pathway resulting in the upregulation of a wide variety of interferon stimulated genes (ISGs) (19, 20, 23, 25). All of the ISGs induced by IFNλ signaling, known to date, are also induced by type I interferons; however, the reverse may not be true (26). Potential functional differences between type I and type III interferons can be attributed to the kinetics of the signaling response, the variety of ISGs that are upregulated, and the restricted expression of the IFNλ receptor, IFNλRα, (26-33). Not only does IFNλ signaling result in ISG expression, IFNλ also induces monocytes and macrophages to produce IL-6, IL-8, and IL-10 (34). Furthermore, in response to IFNλ, monocyte derived macrophages produce chemokines including MIP1α, MIP1β, and RANTES, and increases TLR induced IL-12p40 (34-36).

We hypothesized that differences in IFNλ signaling result in a dysregulated innate immune response demonstrated by, a strong proinflammatory but weak antiviral immune response, consequently affecting the disease pathogenesis of influenza A infection in susceptible murine strains compared to resistant strains. Here we show that in DBA/2 mice, a susceptible strain, the innate immune response to

influenza A infection is proinflammatory but not antiviral, in contrast to the immune response in BALB/c mice, a resistant strain, which is mildly proinflammatory but also antiviral. Furthermore, the lack of an antiviral response in DBA/2 can be attributed to a reduced ISG response induced by IFNλ.

## Materials and Methods

### *Cell Culture and Virus propagation*

Murine lung epithelial cells, MLE-15 were cultured in HITES media [RPMI-1640 (Corning® cellgro®) media with 4% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Brach, GA), 10nM Hydrocortisone (Sigma-Aldrich, St. Louis, MO), 10nM β-estradiol (Sigma-Aldrich), 200 mM GlutaMAX-I (GlutaMAX™ GIBCO™), and 1% ITS (insulin-transferrin-selenium; GIBCO™, Carlsbad, CA)]. Influenza A strain A/WSN/33 (H1N1), kindly provided by Richard Webby (St. Jude Children's Research Hospital Memphis, TN), was propagated in the allantoic cavity of 9-day old embryonated chicken eggs at 37˚C for 72 hours. Influenza A strain A/CA/07/09, was obtained from the CDC international reagent resource and propagated in Madin-Darby canine kidney (MDCK) cells at 37˚C for 72 hours. Influenza viral titers were assessed by plaque assay on MDCK cells as previously described (37). Briefly, a 24-well plate of MDCK cells was incubated with serial dilutions of virus at 37˚C and 5% $CO_2$ for 1 hour. The supernatant was removed, 1ml of a 1:1 mixture of 2.4% Avicel solution and overlay media [MEM (GIBCO™) with 1M HEPES (GIBCO™), 200mM mM GlutaMAX-I, 7.5% $NaHCO_3$ (GIBCO™), and antibiotic/antimycotic (GIBCO™)] was added and the cells were incubated at 37˚C and 5% $CO_2$ for 48 hours prior to fixation with 80/20 methanol/acetone and staining with crystal violet.

### *Mice*

Female 6 – 8-week-old BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC). Female, 6 – 8-week-old DBA/2 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal studies were approved by the Animal Care and Use Committee of the University of Georgia.

*Plasmid Construction, Sequence Analysis and Protein Purification*

Primers were constructed to amplify the mouse IFNλ2/3 gene based on the predicted sequence accession #AY869695. These primers contained sequences homologous to murine IFNλ2/3 and sequences recognized by restriction endonucleases *Not*I and *Eco*RI or *Kpn*I to facilitate cloning. Mouse IFNλ2 genes were amplified by PCR using DreamTaq™ Hot Start PCR master mix (Thermo Scientific™) from the cDNA derived from the lungs of BALB/c mice 72 hours or DBA/2 mice 48 hours post influenza infection. Primer sequences were: IFNλ2/3 forward NotI (IFNλ-NotI) 5'-TTGCGGCCGCCATGCTCCTCC-3'; IFNλ2/3 reverse EcoRI (IFNλ-EcoRI) 5'-GAGAATTCCAGGTCAGACACACTGGTCTCC-3'; and IFNλ2/3 reverse with myc/His tag KpnI (IFNλ-KpnI) 5'-TTGGTACCGACACACTGGTCTCCACTGG-3'. The IFNλ2/3 gene was cloned into the pcDNA$^{TM}$3.1/*myc*-His (-) A (Invitrogen) vector with the use of the restriction endonucleases, constructing plasmids IFNλ-BALB/c and IFNλ-DBA/2 with and without His-tags. Plasmids were purified using QIAprep® Miniprep Kit (QIAGEN) and sequenced (Genewiz Inc., South Plainfield, NJ). Sequence comparisons of IFNλ2 and IFNλ3 between BALB/c and DBA/2 strains were done using MUSCLE alignment in Geneious (Biomatters Ltd.). Plasmids containing the IFNλ2 or IFNλ3 gene with a His tag from either BALB/c or DBA/2 were transfected into HEK293H cells. IFNλ2 and IFNλ3 proteins were then purified by nickel column followed by dialysis.

*Trachea epithelial cell (TEC) isolation*

Six-week-old BALB/c and DBA/2 mice were humanely euthanized, and the trachea removed. Attached connective tissue was removed and the trachea cut lengthwise, placed in media containing 0.15% pronase (Roche, Mannheim, Germany) and incubated at 4˚C for 18 – 24 hours. The next day FBS was added to a final concentration of 10%. The trachea was washed three times and the cells from the washes combined with the original pronase solution were pelleted by centrifugation at 390 x g for 10 min at 4˚C. The supernatant was aspirated, the cells resuspended in 200µl/ trachea DNase solution (0.5 mg/ml in media)

and incubated on ice for 10 min. Cells were pelleted by centrifugation, resuspended in 10 mL growth media (HITES media with 10%FBS and antibiotic/antimycotic), placed in a 6-well tissue culture plate, and incubated at 37˚C at 5% $CO_2$ for 3 – 5 hours for fibroblast attachment. Supernatants containing non-adhered cells were collected and cells were plated into a 24-well tissue culture plate at $1x10^5$ cells/well (approximately one well for every mouse trachea). Every two days, growth media was replaced and 4 – 10 days post plating, cells were useable as undifferentiated TECs for experiments.

*In Vivo Infections, Weight Loss, and Viral Titer Assessment*

BALB/c and DBA/2 mice were anesthetized by isoflurane or intraperitoneal injection of 250µl/mouse 2,2,2-tribromoethanol (Avertin). Mice were infected intranasally with 50µl/mouse of either A/WSN/33, A/CA/07/09, or PBS (mock infection). Percent body weight was calculated based on weight from Day 1. Mice were humanely euthanized, and lungs were removed and homogenized in 1ml PBS followed by centrifugation. Viral titer was assessed by plaque assay as described above.

*Cytokine Analysis*

For *in vivo* analysis, bronchiolar alveolar lavage (BAL) samples were collected by inserting a dulled needle into the trachea through a small incision and washing 3x with 1ml cold PBS. 500µl of each BAL sample or for ex vivo analysis, 1ml of supernatant, was concentrated using 3kMWC protein concentrator (Thermo scientific). Cytokine analysis was performed by Luminex technology using 36 murine ProcartaPlex panel (Thermo fisher) on Bio-Plex® 200 System (BioRad).

*Ex Vivo Stimulation*

For poly I:C or rIFN stimulation of TECs, cells were plated to confluency. 50µg/ml poly I:C HMW Poly I:C (InvivoGen, San Diego, CA), 1000 U/ml murine rIFNα (ebioscience), or 100 ng/ml murine rIFNλ (Pepro Tech, Rocky Hill, NJ) was diluted into growth media, added to cells, and incubated at 37˚C and 5% $CO_2$. Supernatants and RNA were collected at time points described.

*Ex Vivo Influenza A Infection*

TECs were plated to confluency overnight, supernatant was removed, and cells washed 2x with PBS. A/WSN/33 was diluted in HITES media with 10%FBS (infection media). Diluted virus was added to wells and incubated at 4˚C for 30 min followed by 37˚C and 5% $CO_2$ for 2 hours. Supernatant was removed, and cells washed 1x with infection media. Infection media was added back on and cells were incubated at 37˚C and 5% $CO_2$. Samples were collected at time points described. Virus titers were determined by plaque assay on MDCK cells as described previously.

*Quantitative RT-PCR (qPCR)*

Total RNA was isolated using RNAzol®RT per manufacturer's protocol. cDNA was synthesized using VERSO cDNA Synthesis Kit (Thermo Fisher Scientific) per manufacturer's protocol. Maxima Sybr Green/ROX qPCR master mix® (Thermo Fisher Scientific) was used per manufacturer's protocol and run on a MxPro 3000P (Strategene) thermocycler for 40 cycles. CT values were normalized to the house keeping gene GAPDH and analyzed by ΔΔCT method (38) relative to mock-infected or mock-stimulated controls.

*Statistical Analysis*

Statistics were run using GraphPad Prism version 5.04. Two-way analysis of variance (ANOVA) with Bonferroni post-hoc was used for weight loss, viral titers, cytokine responses, and interferon stimulated gene responses. All results were considered significant at p-values $< 0.05$.

## Results

*DBA/2 mice display greater disease severity independent of viral titer*

Previous research determining differences in the immune response to influenza infection between susceptible and resistant murine strains primarily used DBA/2 and C57BL/6 strains (respectively) and demonstrated a significantly higher viral load in DBA/2 as early as 24 hours post infection (7, 8). In order

to focus on differences in the early innate immune response due to host genetics, we established a unique comparative model between susceptible and resistant murine strains DBA/2 and BALB/c (respectively), using a moderate virus dose. Since A/WSN/33 is an extensively passaged, lab adapted human H1N1 virus, a more contemporary human influenza A strain, A/CA/07/09, a H1N1 pandemic strain, was used in addition to A/WSN/33. BALB/c and DBA/2 mice were infected intranasally with A/WSN/33 or A/CA/07/09 at $1x10^3$ pfu/mouse or PBS for control. Mice were monitored for weight loss and lungs assessed for viral titer. Influenza infection with A/WSN/33 did not cause greater disease in DBA/2 mice as measured by weight loss; however, infection with A/CA/07/09 did result in significantly greater weight loss in DBA/2 mice by 4 days post infection (DPI) relative to BALB/c (Figure 1A). Lung virus titers were not significantly different between DBA/2 and BALB/c mice at 4 and 6 DPI during infection with A/WSN/33. Although lung viral titers were significantly different during A/CA/07/09, the difference was relatively small, less than 1 $\log_{10}$, compared to previous studies which demonstrated 2 $\log_{10}$ difference (8) (Figure 1B). This is a unique comparative model which limits the effects of viral replication while retaining the established difference in morbidity by using both A/WSN/33 and A/CA/07/09 infections to assess differences in the innate immune response. To further characterize our model, we assessed the pathology induced in the lungs during influenza A infection for both DBA/2 and BALB/c mice.

*DBA/2 exhibit enhanced cytokine production during influenza A infection compared to BALB/c mice*

BALB/c and DBA/2 mice were inoculated intranasally with A/WSN/33 at $1x10^4$ pfu/mouse or PBS for control. At 2 and 4DPI mice were humanely euthanized, necropsied, and lungs were assessed for pathology by histochemistry; specifically scoring for edema, necrosis, and inflammation in the perivascular structure, bronchioles, alveoli, and septa. The most apparent differences in pathology were found in the bronchioles with greater amounts of exudate, inflammation, and slightly increased necrosis. Furthermore, a greater proportion of the bronchioles were affected in DBA/2 compared to BALB/c (data not shown). There were only minor differences in the pathology found in other areas of the lungs. This data correlates with previous studies in which the enhanced pathogenesis in DBA/2 mice was characterized by increased

consolidation and necrosis as well as blockage of airways by cellular debris and infiltrates (7, 15). To assess differences in cytokine production, BALB/c and DBA/2 mice were inoculated with either A/WSN/33 or A/CA/07/09 at $1x10^3$ pfu/mouse. At 4 and 6 DPI, the concentrations of 36 cytokines in the BAL were analyzed by Luminex bead array. IL-6, LIF, IL-17α, G-CSF, Eotaxin, MCP-1, IL-15, IL-3, and IL-12p70 had significantly increased concentrations in the BAL of DBA/2 mice compared to BALB/c at 4 DPI (Figure 2). In contrast, in the BAL of DBA/2 mice, IL-10, IL-27, and RANTES were significantly lower in concentration compared to BALB/c at 6 DPI (Figure 2). Similar results were seen with A/CA/07/09 (data not shown). This data is in concordance with previous studies comparing DBA/2 and C57BL/6 mice, a resistant mouse strain (8, 14, 15). Cytokines and chemokines not only affect the recruitment of innate immune cells but also their proliferation and function. Therefore, to further understand whether the differential cytokine response between BALB/c and DBA/2 mice affects the functional response of innate immune cells, we assessed the function of natural killer (NK) cells and neutrophils.

*DBA/2 have lower NK cell and neutrophil activity in response to influenza A infection compared to BALB/c*

Innate immune cells including NK cells and neutrophils are important for control of viral replication during influenza infection (39-41). The cytokines IL-15 and IL-3 together induce proliferation, activation, and recruitment of NK cells while IL-12 also activates NK cells resulting in IFNγ production (42-46). Although other cell types including $CD8^+$ T cells and γδT cells also produce IFNγ during influenza infection, NK cells are the main producers of IFNγ early during viral infection (47, 48). To assess NK cell activation, IFNγ concentration at 4 DPI was quantified in DBA/2 and BALB/c mice inoculated with either A/WSN/33 or A/CA/07/09 at $1x10^3$ pfu/mouse. At 4 DPI, the concentration of IFNγ in the BAL was significantly lower in DBA/2 mice than in BALB/c mice (Figure 3A). Several of the cytokines and chemokines mentioned above can induce proliferation, activation, and recruitment for more than just NK cells. Therefore, to determine whether the cytokine response is NK cell specific or whether other innate immune cells such as neutrophils are also affected, the concentration of myeloperoxidase (MPO), an enzyme produced primarily by neutrophils and a marker for neutrophil activation, was also assessed in

BAL. Similar to IFNγ, MPO concentrations in BAL were significantly lower in DBA/2 mice at 4 and 6 DPI compared to BALB/c (Figure 3B). Similarly, in response to A/CA/07/09 infection, DBA/2 mice exhibited reduced IFNγ and MPO concentrations in BAL relative to BALB/c mice (data not shown) Altogether these data support our hypothesis that DBA/2 exhibit a dysregulated innate immune response characterized by increased inflammation with reduced antiviral function.

*DBA/2 exhibit a sustained interferon response but limited antiviral response to influenza infection or stimulation compared to BALB/c*

The importance of the interferon response in controlling influenza replication early in infection has been well established (49-51). To determine whether differences in type I and/or type III interferons play a role in disease severity, we assessed the production of IFNα (type I) and IFNλ (type III) in response to influenza infection in both susceptible and resistant mice. BALB/c and DBA/2 mice were infected as aforementioned, BAL samples were collected at 4 and 6 DPI and cytokine concentrations analyzed by Luminex bead array. In response to both A/WSN/33 or A/CA/07/09, DBA/2 mice had significantly greater concentrations of IFNλ in BAL relative to BALB/c mice. In contrast, DBA/2 mice had significantly lower concentrations of IFNα at 4DPI in BAL relative to BALB/c. In DBA/2 mice, the IFNα response was sustained through 6 DPI in contrast to BALB/c mice where it was declining relative to 4DPI (Figure 4A and B). We evaluated the antiviral response, measuring expression of two ISGs: ISG15 and ISG56, which are known to be upregulated in response to influenza infection (52-54). RNA was isolated from lungs at 2 and 4 DPI from BALB/c and DBA/2 mice infected with A/WSN/33 and processed for qPCR analysis of IFNλ, ISG15, and ISG56. Despite higher expression of IFNλ in lungs of DBA/2 mice (data not shown), at 2 and 4DPI both ISG15 and ISG56 expression were lower relative to BALB/c (Figure 5A and B). Together these data suggest that while DBA/2 mice produce a robust and sustained type I and type III interferon response, the subsequent antiviral response is significantly muted compared to the response in BALB/c mice.

*Sequence analysis and glycosylation of IFNλ2 and IFNλ3 between BALB/c and DBA/2 strains*

Type I and III interferons induce redundant amplification loops that potentially result in cross-regulated feedback loops. However, IFNλ has been shown to be the predominant interferon induced in response to influenza infection (30, 49, 55, 56). Single nucleotide polymorphisms within the IFNλ promotor and untranslated regions have been documented within human populations. Furthermore, these mutations have been linked to altered viral clearance against HCV and cytomegalovirus (9, 11). In order to determine if differences in receptor signaling, as determined by ISG expression, correspond to differences in the IFNλ protein, we compared the mRNA sequence for IFNλ2 and IFNLλ3 from BALB/c and DBA/2 mouse strains. There was 100% amino acid identity between BALB/c and DBA/2 derived IFNλ2 or IFNλ3 and 97.3% sequence identity between IFNλ2 and IFNλ3 (data not shown). The sequence similarity between IFNλ2 and IFNλ3 is similar to what has been previously reported in human IFNλ2 and IFNλ3, at 96% amino acid identity (19). Murine IFNλ2 and IFNλ3 are known to be glycosylated at a single predicted glycosylation site (22). Upon deglycosylation of expressed IFNλ2 or IFNλ3, no difference was found between BALB/c and DBA/2 derived proteins (data not shown). Therefore, it is unlikely that the lack of a sustained antiviral response in DBA/2 mice is due to a difference in the lambda protein itself.

*Tracheal epithelial cells derived from DBA/2 elicit a lower antiviral response to stimulation or influenza infection compared to cells derived from BALB/c*

In order to elucidate the mechanism generating the reduced antiviral response in DBA/2, primary tracheal epithelial cells (TECs) were cultured from BALB/c and DBA/2 mice and infected with A/WSN/33 or stimulated with Poly I:C or recombinant IFN (rIFNλ and rIFNα). Stimulation with Poly I:C induced similar production of IFNλ by TECs derived from BALB/c and DBA/2 mice (Figure 6A). However, in response to influenza infection, IFNλ production at 2 and 3DPI was greater in TECs derived from DBA/2 compared to BALB/c, recapitulating the *in vivo* results (Figure 6B). Moreover, in response to rIFNλ, TECs derived from BALB/c mice had significantly higher expression of ISG15 compared to DBA/2 derived

TECs. In response to rIFNα stimulation, this trend remained, but was not significant (Figure 6 C and D). In response to influenza infection, ISG15 was significantly higher at later time points in TEC's derived from BALB/c relative to DBA/2 mice (Figure 6 E). These data suggest that the interferon signaling in DBA/2 induces a different antiviral response than in BALB/c, supporting the hypothesis that the lack of an antiviral response is attributed to interferon signaling.

## Discussion

Predisposition to increased severity of disease and complications from influenza infection can be attributed to a variety of host factors including host genetics (3-6). In susceptible mice, murine influenza infection elicits more severe disease manifesting in increased proinflammatory cytokines and chemokines and higher leukocyte recruitment to the lungs, likely caused by the higher virus titer in the lungs (12, 14, 15, 18). Although this variation in susceptibility to influenza infection has been linked to a variety of host genes and their pathways, the mechanism causing this hyper inflammatory response has yet to be elucidated (8, 13, 14, 16, 57). We hypothesized that the hyper inflammatory response in susceptible DBA/2 mice, was the result of early dysregulation of the innate immune response causing an increased proinflammatory, yet reduced antiviral immune response, relative to resistant BALB/c mice. Moreover, this dysregulation correlated with a difference in IFNλ signaling.

In order to eliminate the effects of differences in virus replication and peak titer on inflammatory responses, we developed an infection model that limited differences in viral replication while retaining the established difference in morbidity between BALB/c and DBA/2 mice. Characterization of this murine model demonstrated only minor differences in lung pathology by histological analysis. Previous research attributed the pronounced lung pathology in DBA/2 mice to increased necrosis and blockage of airways by cellular debris and infiltrates (8, 15). This is in contrast to our data; however, these studies correlated the increased disease severity in DBA/2 mice to higher lung viral load as early as 12 – 24 hours post-infection which is what our model was intentionally minimizing (7, 8, 14, 16, 57). Therefore, this model recapitulates

the differences previously established between resistant and susceptible strains, while remaining unique in focusing on the differences attributed to the host in the absence of differences in viral replication.

This model was further characterized comparing cytokine response between BALB/c and DBA/2 mice. Despite having limited differences in lung pathology, there were significantly higher concentrations of proinflammatory cytokines in the BAL of DBA/2 mice compared to BALB/c (Figure 2). In contrast, anti-inflammatory cytokines including IL-10, IL-27, and RANTES were significantly lower in DBA/2 mice. While higher concentrations of IL-6 and LIF have been associated with increased disease severity and the development of acute respiratory distress syndrome (ARDS), IL-10 and IL-27 have been shown to limit pathology from influenza infection (58-61). Previous research established the hyper inflammatory response in susceptible murine strains in response to influenza infection as an over production of proinflammatory cytokines and chemokines (7, 8, 12-15). While our data agrees with previous research, it also suggests a second mechanism for the development of the hyper inflammatory response: reduced production of cytokines which typically downregulate the inflammatory response in the DBA/2 mice compared to the resistant BALB/c mice.

The production of cytokines and chemokines induces the recruitment, proliferation and activation of innate immune cells including neutrophils and NK cells to control viral replication. We hypothesized that the dysregulation of the innate immune response in DBA/2 mice could extend to the activation and function of innate immune cells. Therefore, we assessed the activation of neutrophils by MPO concentration and NK cells by IFNγ concentrations in the BAL. The significantly lower concentrations of IFNγ and MPO in DBA/2 mice suggest either reduced activation or recruitment of these cell types (Figure 3). This data contrasts with previous research demonstrating much higher MPO production and IFNγ concentrations in the lungs of DBA/2 mice compared to resistant strains (14, 15). However, the studies demonstrating higher MPO production in DBA/2 mice also had higher viral loads in the lungs compared to resistant mouse strains, and in the study by Casanova et al. also higher neutrophil infiltration in the lungs which possibly masked the reduced activation of innate immune effector cells such as neutrophils and NK

cells (15). Minimal differences in the histological analysis of the lungs between BALB/c and DBA/2 mice in this study, along with the aforementioned studies, indicate the lower MPO and potentially IFNγ production is more likely due to a lack of activation rather than a lack of recruitment of these cell types (8, 15). A reduction in the activation of innate immune cells in DBA/2 mice supports the hypothesis of a dysregulated innate immune response exhibited by a weak antiviral response relative to the resistant BALB/c.

Type I (IFNα/β) and type III (IFNλ) interferons are produced in response to influenza infection. Studies have shown that both type I and type III interferons are able to modulate neutrophil and NK cell function (62-65). We hypothesized that the reduction in activation of innate immune cells would be correlated to the production and function of the interferon response. Therefore, we next assessed the concentrations of type I and type III interferons in the BAL of BALB/c and DBA/2 mice. IFNλ concentrations were higher in the BAL of DBA/2 mice at 4DPI (Figure 4A and B). Although IFNα concentrations were higher at 4 DPI, in the BAL of BALB/c mice compared to DBA/2 mice, by 6 DPI the reverse was true in the A/CA/07/09 infection. This is in concordance with previous studies that demonstrated greater IFNα and IFNβ concentrations in the lungs of DBA/2 mice compared to the more resistant C57BL/6 mice and the correlation of higher type I and type III interferon production with increased disease severity (7, 66). IFNα is known to regulate IL-10 signaling, in T cells specifically, promoting IL-10 signaling resulting in the suppression of TH17 cells which are the main producers of IL-17 (67, 68). IFNα is also known to regulate the expression of IL-27 which is also known to suppress Th17 effector function (69). The significantly higher concentrations of type I and type III interferons along with IL-17 and significantly lower concentrations of IL-10 and IL-27 in DBA/2 mice compared to BALB/c, suggests that the prolonged high interferon concentrations in the susceptible DBA/2 mice are inducing a continued pro-inflammatory response by inhibiting the normal mechanisms of control via IL-10. This may be one explanation for the previously reported increase in disease severity associated with prolonged type I interferon production (70).

The high interferon concentrations in DBA/2 mice is in seeming disagreement with the lower production of MPO and IFNγ in our model. We hypothesized that the lower production of MPO and IFNγ, presumably due to reduced activation, was a result of a lack of antiviral response in the DBA/2 despite higher interferon production. Consequently, the expression of ISG15 and ISG56 in the lungs was determined; both ISG15 and ISG56 have previously been shown to have direct antiviral effects on influenza infection (71, 72). ISG15 and ISG56 expression was higher in the lungs of BALB/c mice compared to DBA/2 mice both at 2 and 4DPI (Figure 5A and B), suggesting the antiviral response in DBA/2 mice is not sustained. To understand whether this is a functional difference in either IFNλ2 or IFNλ3, we analyzed the sequence and glycosylation of these proteins derived from BALB/c and DBA/2 mice. There was no difference in sequence or glycosylation patterns, suggesting that there is no functional difference between IFNλ produced in BALB/c and DBA/2 mice.

To confirm the lack of a sustained antiviral response in DBA/2 mice, we isolated primary tracheal epithelial cells from BALB/c and DBA/2 mice and assessed responses to stimulation. Poly IC stimulation induced similar levels of IFNλ production in cells from BALB/c and DBA/2 mice, although infection with A/WSN/33 induced greater IFNλ production only at later time points (Figure 6). Furthermore, ISG15 expression was significantly higher in TECs derived from BALB/c mice compared to DBA/2 in response to IFNλ stimulation and A/WSN/33 infection. Although the same trend occurred in response to IFNα stimulation, it was not significant. Since type I and type III interferons share positive feedback loops and are able to produce both type I and type III interferons, it is difficult to completely separate the effects of type I and type III interferons on ISG expression. Together this data suggests that compared to resistant, BALB/c mice, the induction of the antiviral response by IFNλ is less effective in DBA/2 mice. Whether the lack of an antiviral response, as demonstrated by reduced ISG expression, is directly associated with the reduced MPO and IFNγ production in DBA/2 mice, is still unknown. Together, the *ex vivo* and *in vivo* data suggest that DBA/2 mice exhibit a pro-inflammatory but minimal antiviral response to influenza infection. Therefore, we propose a model highlighting the difference between infection in the resistant BALB/c strain

demonstrating low severity of disease compared to infection in the susceptible DBA/2 strain, exhibiting high disease severity, a result of a lack of an antiviral response, which can at least in part be attributed to a lack of ISG induction by type I and type III interferons (Figure 7).

This study herein was unique in that it used a model of infection to assess the immune differences in susceptible and resistant murine strains to influenza infection in the absence of differences in viral titers. Early differences in viral replication, typical of influenza infection in susceptible versus resistant murine strains, complicates analysis of the results to determine the attributes due to host genetics versus effects of viral titer, highlighting the importance of our infection model. Our study has established another mechanism, the function of type III interferons, by which the host innate immune response to influenza infection is altered based on host genetics. Further analysis into the innate immune differences between susceptible and resistant hosts using this type of model, needs to be continued to better understand the role of host genetics and its contribution to disease severity with the prospect of applying these discoveries to humans. Recently it has become apparent that using antivirals to control influenza replication is not enough, but rather in the use of immune therapy in addition to the antivirals. Additional studies focusing on testing potential targets for immune therapy, such as modulating the IL-10 response, and finding new targets in order to limit disease pathogenesis and aid in the resolution of disease, is extremely important to the development of influenza therapeutics.

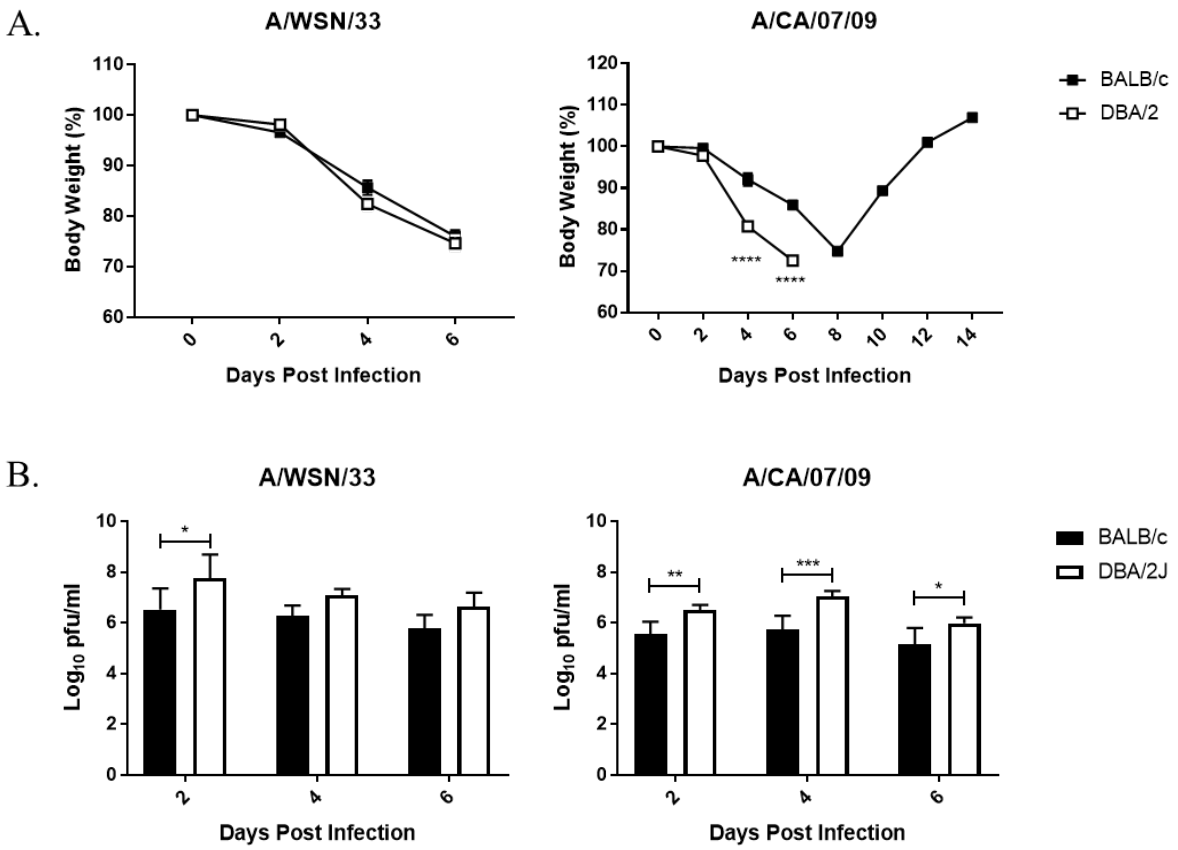


**Figure 6.1. Weight loss and lung viral titers in influenza A (H1N1) infection.** Mice were inoculated intranasally with $1x10^3$ pfu A/WSN/33 or A/CA/07/09. (A) Percent body weight. (B) Lungs were collected 2, 4, and 6 DPI. Virus titers were assessed by plaque assay from the supernatant of the lung homogenate. Statistics were calculated between BALB/c and DBA/2 strains at each time point by two-way ANOVA and Bonferroni post-hoc. (* $p<0.05$ ** $< 0.01$ ***$<0.001$ **** $p<0.0001$)

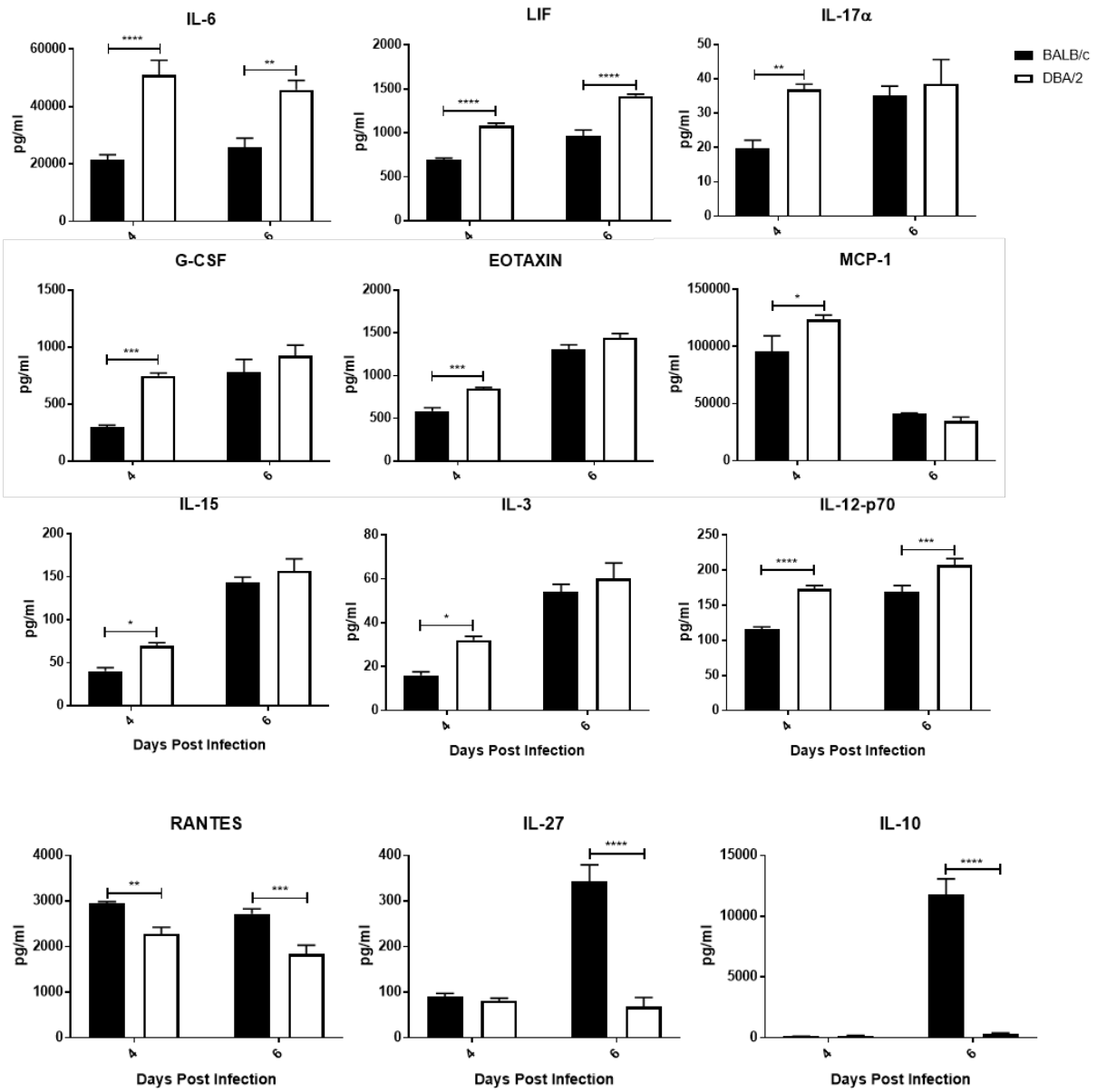


**Figure 6.2. Cytokine and chemokine production in response to A/WSN/33 infection.** Mice were inoculated intranasally at $1x10^3$ pfu/mouse. BAL was collected 4 and 6 DPI. Cytokine and chemokine concentrations in the BAL were analyzed by Luminex. Statistics were calculated between BALB/c and DBA/2 strains at each time point by two-way ANOVA and Bonferroni post-hoc. (* $p<0.05$ ** $< 0.01$ ***$<0.001$ **** $p<0.0001$)

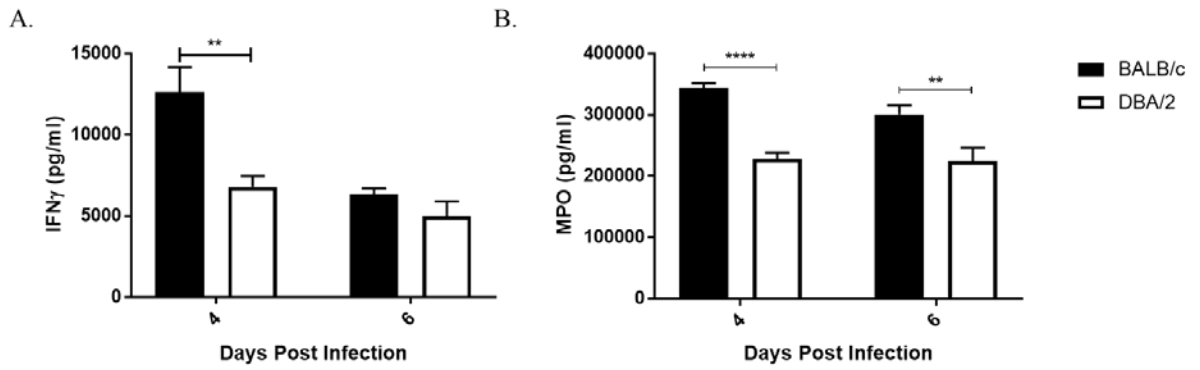


**Figure 6.3. IFNγ and MPO production in response to A/WSN/33 infection.** Mice were inoculated intranasally at $1x10^3$ pfu/mouse. BAL was collected 4 and 6 DPI. (A) IFNγ concentration in the BAL was analyzed by Luminex. (B) MPO production was analyzed by ELISA. Statistics were calculated between BALB/c and DBA/2 strains at each time point by two-way ANOVA and Bonferroni post-hoc. (* $p<0.05$ ** $< 0.01$ ***$<0.001$ **** $p<0.0001$)

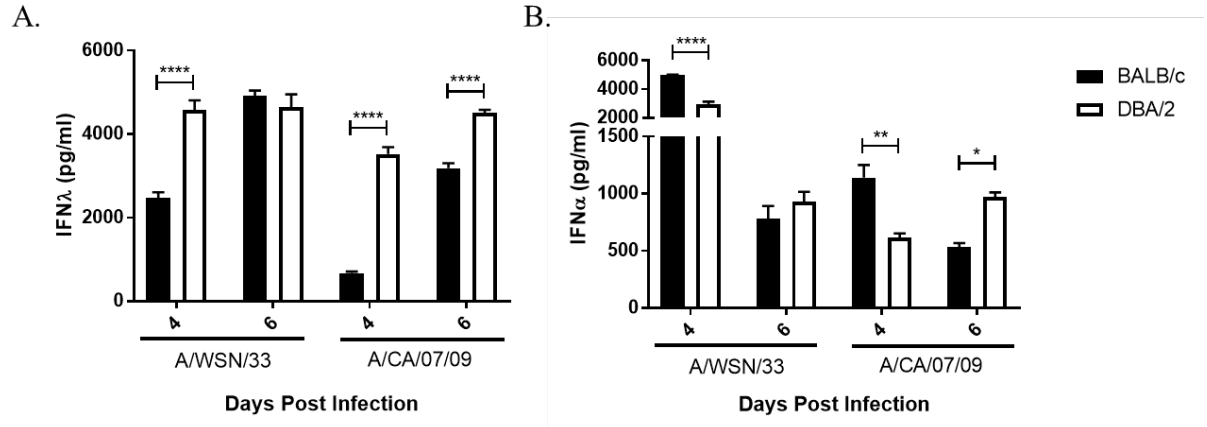


**Figure 6.4. Type I and Type III interferon production in response to A/WSN/33 or A/CA/07/09 infection.** Mice were inoculated as described in figure 3. BAL was collected at 4 and 6 DPI and (A) IFNλ and (B) IFNα concentrations analyzed by Luminex. Statistics were calculated between BALB/c and DBA/2 strains at each time point by two-way ANOVA and Bonferroni post-hoc. (* $p<0.05$ ** $< 0.01$ ***$<0.001$ **** $p<0.0001$)

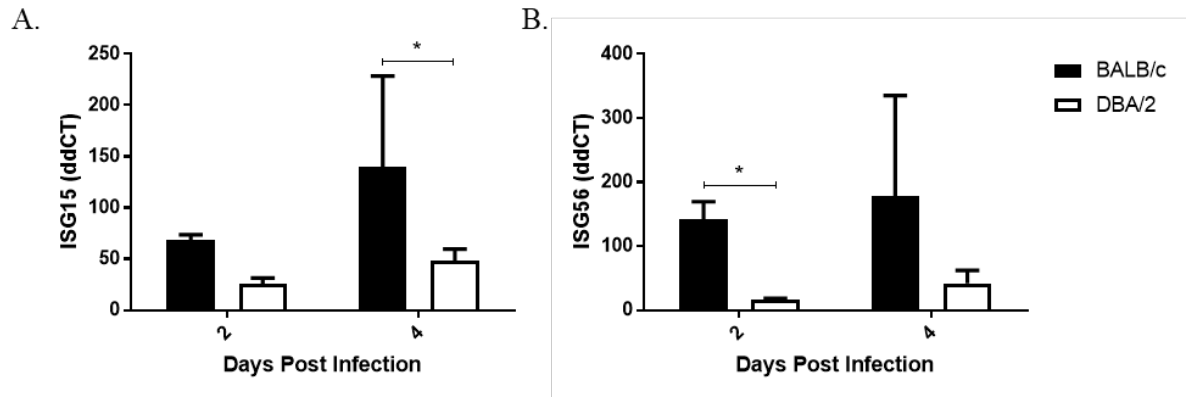


**Figure 6.5. ISG15 and ISG56 expression in response to A/WSN/33 infection.** Mice were inoculated as in figure 1. Lungs were collected 2 and 4DPI and samples were assayed by qPCR. ISG15 (A) and ISG56 (B) expression was normalized to GAPDH and relative to control (PBS). Statistics were calculated between BALB/c and DBA/2 strains at each time point by two-way ANOVA and Bonferroni post-hoc. (* $p<0.05$ ** $< 0.01$ ***$<0.001$ **** $p<0.0001$)

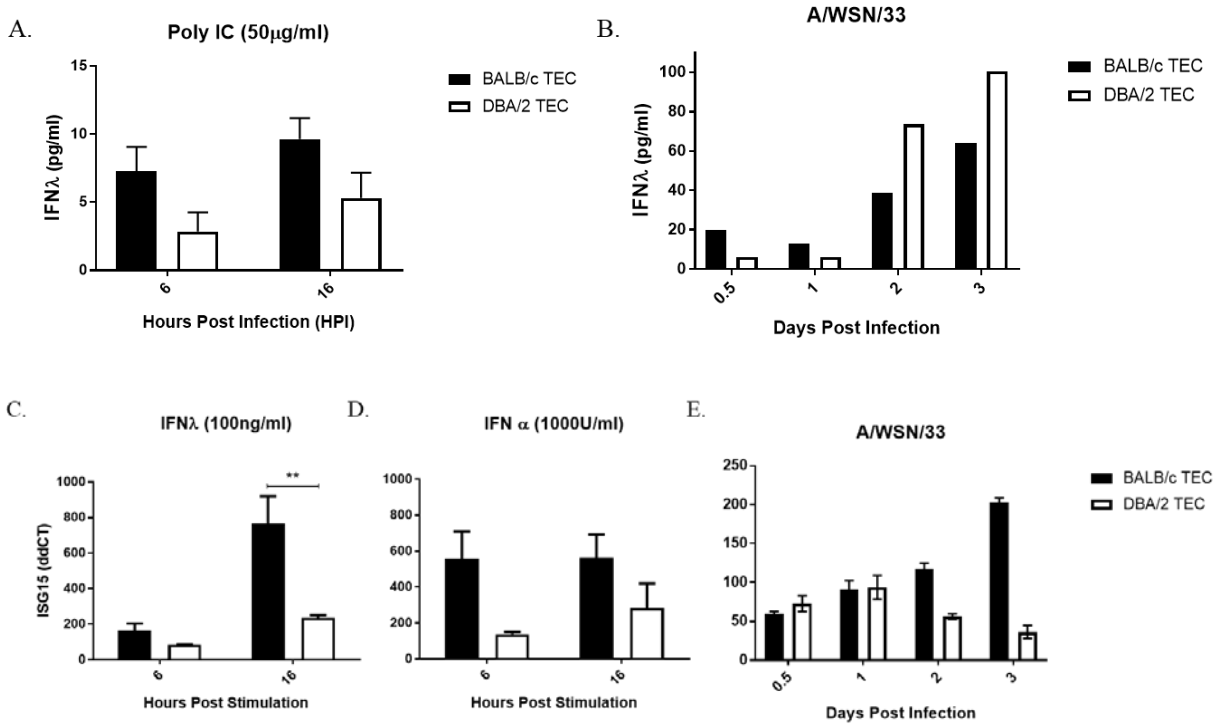


**Figure 6.6 Interferon λ production and ISG expression *ex vivo*.** Tracheal epithelial cells were derived from BALB/c and DBA/2 mice. Cells were stimulated with Poly I:C (A), recombinant IFNλ (C), or recombinant IFNα (D), or infected with A/WSN/33 (B and E). Supernatant was assessed for IFNλ production by Luminex (A and B). Cells were collected and assessed for ISG expression by qPCR. Fold change was normalized to GAPDH and calculated relative to control (unstimulated or uninfected) (C - E). A and C: Statistics were calculated between BALB/c and DBA/2 strains at each time point by two-way ANOVA and Bonferroni post-ho. (* $p<0.05$ ** $< 0.01$)

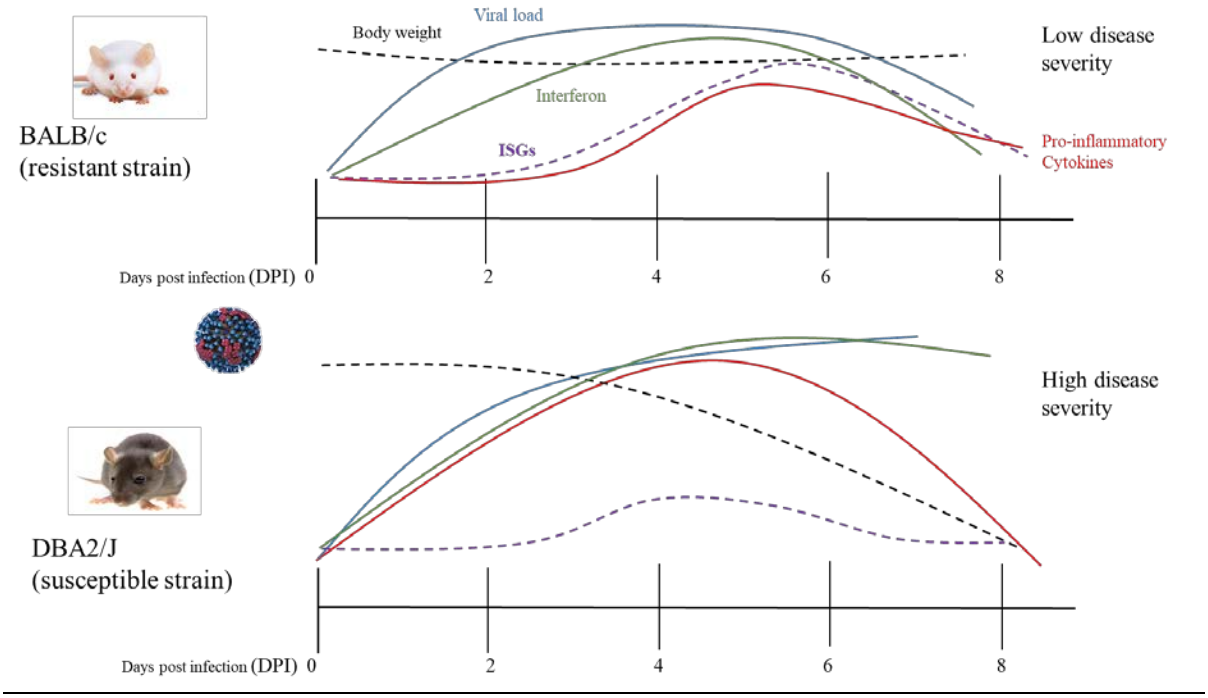


**Figure 6.7. Proposed model of disparity in immune response to influenza infection between resistant and susceptible murine systems and subsequent disease severity.**

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# CHAPTER 7

# SUMMARY AND CONCLUSIONS

Swine influenza A strains are constantly undergoing genetic drift and shift resulting in novel strains with the potential for zoonotic events resulting in pandemics. The matrix gene is highly conserved between influenza strains; therefore, when genetic changes to the matrix gene occur and persist in circulating influenza strains, such as happened with the 2009 pandemic H1N1 influenza strain, it suggests a fitness benefit. The matrix gene not only effects transmission of the virus but can also interact with the host immune response. The goal of this work was to evaluate the potential for swine influenza viruses containing the pandemic matrix gene to cause greater disease in the mouse model of infection. Since both viral and host factors can contribute to the severity of disease during influenza infection, the second goal of this work was to evaluate host innate immune factors affecting susceptibility to influenza virus infection.

To address the potential for influenza strains containing the pandemic origin matrix gene to cause greater disease, we used phenotypically resistant and susceptible mouse strains and compared the disease pathogenesis between influenza viruses that contained either the pandemic origin matrix gene or the swine origin matrix gene. A panel of H1 and H3 swine influenza viruses containing either the pandemic origin or swine origin matrix gene were sequenced and the origin of all their genes characterized. These viruses were then used to infect susceptible, DBA/2 and resistant, BALB/c mouse strains and virus replication, morbidity, mortality and pathology were assessed. The H1 swine influenza viruses containing the pandemic origin matrix gene caused greater morbidity and mortality, replicated to higher titers, and induced more severe histological changes in the lungs relative to strains containing the swine origin matrix gene (Chapter 4). We then assessed the immune response to infection with the same viruses.

Notably, the severity of histological changes in the lungs caused by infection with the different influenza viruses was associated with a dysregulation of the host innate immune response (Chapter 5).

Infection with swine influenza viruses containing the pdmM gene was characterized by a greater production of proinflammatory cytokines and chemokines and increased cellular recruitment to the lungs. Specific innate immune cells including plasmacytoid dendritic cells, eosinophils, and monocytes were disproportionately recruited to the lungs of mice infected with influenza strains containing the pdmM gene relative to strains containing the swM gene, suggesting a dysregulation of the innate immune response. Subsequent analysis of the activation of innate immune cells including neutrophils and NK cells correlated with enhanced activation of innate immune pathways. *In vivo* and *in vitro* analysis of inflammasome activation, known to be activated by the M2 protein, also correlated enhanced activation with the pdmM gene. This suggests that the matrix gene interacts with the host innate immune response, and that infection with influenza viruses that contain the pdmM gene elicit an enhanced antiviral response which becomes detrimental to the host as demonstrated by the difference in severity of disease relative to infection with viruses containing the swM gene. The severity of disease was associated with increased virus replication and potentially linked to the dysregulation of the host innate immune response by the matrix gene via multiple innate immune pathways. This gives precedent for further research into the interactions of the matrix gene with the host. One disadvantage to using swine influenza strains recently isolated from pigs is that the data produced in this work remains correlatory rather than definite. A complete contemporary swine influenza reverse genetics virus is not currently widely available and would be a useful tool for definitive *in vivo* and *in vitro* analysis on the effect of the replacement of the swine origin matrix gene with the pandemic origin the matrix gene in a rescued virus. This would enable assessment of disease pathogenesis and immune dysregulation in an isogenic system where only the matrix gene differed. This tool could also be used to identify virulence determinants in other influenza gene segments, as well as mapping specific mutations in the matrix gene responsible for the increase virulence associated with the pdmM gene. This would be of particular interest for A/swine/North

Carolina/A01394568/2013 (H1N1), which was extremely pathogenic in mice compared to other swine H1N1 viruses. This work was carried out using the murine model, which is useful, specifically for the availability of research tools to investigate the contribution of the host immune response to infection. Other animal models such as the ferret replicate human infection more closely, and this work provides rationale to explore the contribution of the matrix gene to disease pathogenesis in this and other models.

The 2009 H1N1 pandemic caused concern due to its rapid spread worldwide but also the severity of disease it caused in a subset of children and adults who were considered previously healthy. One goal of this work was to elucidate host factors that contribute to severity of disease using the murine model with the prospect of applying what we have learned to humans. To address this, we assessed differences in the host innate immune response between phenotypically resistant and susceptible murine strains. An infection model was developed which retained the established difference in morbidity between the resistant mouse strain, BALB/c, and the susceptible mouse strain, DBA/2, while minimizing the effects of higher viral replication typically seen in DBA/2 mice. This model retained some of the characteristic differences in lung pathology and proinflammatory cytokine and chemokine production previously reported by other groups. The susceptible DBA/2 strain exhibited a reduced production of cytokines associated with the control of inflammation, along with a reduced antiviral response despite higher interferon production relative to the resistant BALB/c strain (Chapter 6). This suggests that the hyper inflammatory response in DBA/2 mice previously established may originate from an interferon response which is less antiviral resulting in a dysregulated innate immune response relative to a more resistant strain. This study sets the precedent for further research into the potential interferon signaling differences between susceptible and resistant murine strains and its effects on influenza disease.

The effects of knocking out of both type I and type III interferon receptors in mice has been explored in influenza infection models and demonstrated enhanced lethality of influenza infection; however, knock out of their respective receptors on individual cell types and assessing the effects on the immune response to influenza infection has not been done. This focused interrogation of the interferon

response during influenza infection could shed light on the connection between a potentially reduced antiviral response from interferon signaling and the reduced production of cytokines associated with the regulation of inflammation. An advantage of the infection model used in our study is the enhanced capability of determining attributes due to host genetics which can be masked by the effects of higher virus replication. Whether the focus of the study is eliminating a host gene important to the antiviral response or swapping virus genes in a reverse genetics system for specific host-virus interactions, this model can be used in future studies to uncover nuances of the immune response that can have substantial effects on disease pathogenesis.

In conclusion, both viral and host factors contribute to the severity of disease during influenza virus infection. Elucidating viral factors, such as the origin of the matrix gene, that play a role in the development of infection and the subsequent immune response is important to assess the potential of new and emerging influenza viruses to cause substantial disease, either in the pigs they are circulating in, or in humans, in the case of zoonotic transmission. Identifying host factors, such as the nuance of interferon signaling, which effect the overall immune response to influenza infection can potentially be used to identify predisposition to severe disease in individuals, such as is already done with known factors, including heart disease and hypertension. By examining both viral and host factors that affect the severity of influenza disease and outcome, the potential burden of disease due to new pandemics or seasonal influenza may be mitigated.