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Studies on the Vaccine Protection and Transmission of A(H3N2)v viruses in ferrets

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Abstract

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By Katherine Virginia Houser

The emergence of novel influenza A virus subtypes with the capacity to spread efficiently among humans can result in a pandemic. Multiple influenza A subtypes of zoonotic origin have resulted in human infections, but most do not display the capacity for efficient human-to-human transmission. Sustained transmission among humans is a critical feature for a pandemic virus and understanding the changes responsible for human adaptation of zoonotic influenza viruses is of vital importance for pandemic preparedness. To discover the contributions of individual genes towards transmission and pathogenesis of avian H5N1 viruses we developed a reverse genetic plasmid system. A novel zoonotic influenza virus of the H3N2 subtype with limited human-to-human transmission capability began to emerge from the swine population in 2011.

The swine-origin H3N2 viruses continue to circulate in swine, resulting in occasional transmission from infected pigs to humans, referred to as H3N2 variant virus [A(H3N2)v] infections. To date, there have been over 300 laboratory-confirmed human cases of A(H3N2)v virus infection, however there has been no evidence of sustained human-to-human transmission. Conversely, previous studies have shown that A(H3N2)v virus transmitted efficiently in the naïve ferret, an animal model that generally recapitulates the inherent ability of influenza viruses to transmit among humans. Serologic studies have shown that cross-reactive antibodies to A(H3N2)v are present at varying levels in most age groups in humans, with young adults displaying the highest frequency and young children the lowest. We investigated if the presence of cross-reactive immunity generated through seasonal influenza virus vaccination or infection could account for the limited transmission of A(H3N2)v viruses. We found that vaccination failed to substantially reduce A(H3N2)v virus shedding and subsequent transmission to naïve ferrets. However, prior seasonal H3N2 virus infection resulted in reduced virus shedding following A(H3N2)v virus challenge which blunted transmission to naïve ferrets. We also observed an increase in IgG and IgA antibody titers in both the sera and respiratory tracts of ferrets after prior seasonal H3N2 virus infection compared to vaccination. The data demonstrate in ferrets that the efficiency of A(H3N2)v transmission is disrupted by preexisting immunity to seasonal H3N2 virus induced by natural infection.

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Chapter 1.
Introduction

1.1 Influenza History and Pathogenesis

Influenza viruses have been responsible for illness and death in humans throughout history. Documents dating from 417 BC describe epidemics suggestive of influenza, while more recent and detailed accounts indicate that pandemics of influenza have been occurring as far back as 1580 (1, 2). In modern times seasonal outbreaks occur on a yearly basis in temperate climates, while the virus is isolated year-round from tropical regions (3). In contrast, pandemic strains emerge sporadically but infect a large number of people in a short time span. Seasonal influenza viruses infect about five to twenty percent of the population every year, leading an average of 226,000 hospitalizations (range of 55,000 to 431,000) and 21,600 deaths (range of 3,300 to 48,600) associated with influenza infections in the US alone (4, 5). Infection rates are typically highest among children under the age of seventeen, but hospitalizations and deaths are highest among infants and adults over sixty-five years of age (5, 6). In the United States, the influenza vaccine is currently recommended for everyone 6 months or older (7, 8).

The modes of transmission of influenza are not well understood but are believed to include spread by inhalation of airborne respiratory droplets that are formed by infected persons when coughing or sneezing or by direct or indirect contact with virally laden fomites (9, 10). The illness typically consists of sudden onset of high fever, malaise, headache, chills, joint pain, coughing, sore throat and/or runny nose (11, 12). The incubation period for human influenza viruses is typically two days (with a range of one to four days) before symptom onset (11, 13). The acute illness lasts about five to seven days before symptoms begin to dissipate (14). Influenza viruses are capable of

causing mild to severe and sometimes fatal illness in humans, most often in the very young and older persons, or those with underlying health conditions. The virus continues to be a public health burden on society involving losses in productivity, increases in hospital and doctor visits, and school closures, with total annual economic burden of epidemics estimated to be $87.1 billion (15). More research is needed to better understand modes of influenza transmission in epidemics or pandemics, to understand the viral and host properties that contribute to severe disease, and to identify improved approaches for the control of the virus among human populations.

1.2 Influenza A Virus Subtypes and Host Range

Influenza viruses are enveloped viruses of the family *Orthomyxoviridae* that contain a genome of single-stranded, negative sense RNA (16). There are five genera of *Orthomyxoviridae* viruses, with three of these genera being influenza A, B, and C viruses. The genomes of *Orthomyxoviridae* viruses are segmented, with seven to eight unique strands (16). While influenza A viruses are capable of infecting many animal species, infection with influenza B and C viruses are mainly restricted to humans (17). Influenza A and B viruses are responsible for seasonal epidemics in human populations and are both included in the seasonal influenza vaccine. The research involved here will focus entirely on influenza A viruses, and therefore the other genera will not be discussed further.

The genera of influenza A contain eight unique genetic segments (16, 18). The viral genes exist in the virion in a panhandle conformation through interactions in the terminal untranslated regions of the genes (19). These segments encode for at least

thirteen unique viral proteins (20-22). Several of the viral genes have the ability to produce multiple proteins from the same gene segment through the use of splicing and multiple start sites for translation which will be discussed in subsequent sections (16).

Influenza A viruses are further divided into subtypes based on antigenic differences in the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (16, 23). Seventeen subtypes of HA and ten subtypes of NA in influenza A viruses have been described (24-27). Only a select few subtypes of HA (H1, H2, and H3) have caused sustained epidemics of disease in humans to date. There are several additional subtypes (H5, H6, H7, H9, and H10) that have resulted in human infections, but have yet to display efficient human-to-human transmission. Among these only H5 and H7 subtypes have caused outbreaks of human disease.

Influenza A subtypes are found naturally circulating in wild water birds, the reservoir for most influenza viruses. The exception to this involves the most recently discovered subtype, H17N10, which has only been identified in bats (26). Most infections are asymptomatic in wild water birds and replication occurs in the intestinal tract (28). The spread of virus between these birds may occur through the fecal-oral route as high viral titers are found in fecal samples (25). Viruses from wild aquatic birds frequently spread into domesticated waterfowl or land-based avian species. Although the viruses are relatively genetically stable while within the wild water bird population, they may rapidly evolve once they transfer into other avian species. Stable lineages of influenza A viruses have also been documented in canines, horses, and swine (25).

1.3 Influenza A Virion Structure

Influenza virions are enveloped particles enclosed by a host-derived lipid membrane with viral glycoproteins present on the surface (16, 25). These proteins include the HA, NA, and one of the membrane splice variants, matrix 2 (M2) protein (see Figure 1). The viral genes are contained in the interior of the envelope, in ribonucleoprotein (RNP) complexes composed of the nucleoprotein (NP), the viral genomic RNA, and polymerase proteins (29). The NP proteins bind evenly along the inside of the vRNA while allowing the viral polymerases access to the outside of the vRNA (30). One polymerase complex is attached to each RNP and is composed of the three polymerase proteins- PB1, PB2, and PA (16). These RNP complexes are then attached to the inner lipid membrane of the virion through an interaction with the matrix 1 (M1) protein, which is believed to associate with the cytoplasmic tails of the surface glycoproteins (31, 32). The nuclear export protein (NEP) is also a minor part of the virion, and associates with the M1 protein (33). Several additional viral proteins are not components of the virions but are synthesized in the infected cell.

The virions themselves are pleomorphic in appearance, with both spherical and filamentous shaped particles (16). The filamentous forms are mainly found in clinical isolates of influenza (16). The virions are around 100nm in diameter and can be over 20μm in length when in filamentous form. The M gene has been shown to play a role in determining the shape of the virion particles (34, 35).

1.4 The Viral Hemagglutinin

The HA is the most abundant surface glycoprotein on the virion and is also the major antigen recognized by the host immune system during an infection (16). The HA proteins are present on the virion surface in the form of homotrimers. A single HA protein is composed of two distinct sections including a long stalk region at the base and a globular head above that contains the receptor-binding site (23).

The HA protein is originally produced as a single transcript, HA0 (23). The HA0 subunit is the inactive form of the protein, but contains a cleavage site for further processing by cellular proteases. The protease cleavage generates the active form of the protein, composed of the HA1 and HA2 subunits (25). The type of cleavage site available affects the type of protease that is capable of cleaving the HA, as well as the location where the cleavage takes place during infection. A single basic arginine residue is susceptible to trypsin-like proteases located in the extracellular environment (23). A multi-basic cleavage site is found in some avian influenza viruses of the H5 and H7 subtype that are capable of high pathogenicity and is composed of several adjacent basic residues in the form of an -RRRKK- sequence. These multi-basic cleavage sites are susceptible to furin-like proteases (36). Unlike trypsin-type proteases which are generally restricted to the respiratory tract, furin-like proteases are more ubiquitously expressed in multiple organs, enabling systemic replication and disease of highly pathogenic avian influenza (HPAI) in infected birds, in particular domestic poultry (37). The H7 subtype have also displayed non-homologous recombination occurring between the HA and other viral genes, including M and NP, which introduces additional amino acids into the

cleavage site of the HA, creating a larger site and adapting the virus for furin-like protease cleavage (38, 39).

The viral HA is capable of binding to glycoproteins that contain particular terminal sialic acid moieties present on the surface of cells. A subset of the amino acids in the receptor binding site of the HA protein determine the binding preference of the virus and are a crucial determinant of host range for the viruses (40, 41). Human-adapted influenza viruses generally bind a subset of residues that contain N-acetylneuraminic acid attached to the penultimate galactose sugar through an α2,6 linkage (Neu5Acα(2,6)-Gal) while avian subtypes typically prefer a subset of α2,3 linked (Neu5Acα(2,3)-Gal) sialic acid residues (42). Human-adapted viruses display a preference for nonciliated cells in the human respiratory tract while avian viruses predominantly infect the minor population of ciliated cells (43).

The subtype of terminal sialic acid moieties that the virus binds to also affects the transmissibility of the virus among humans (42, 44, 45). Several influenza viruses are capable of binding both α2,3 and α2,6 sialic acid residues, but not all of these viruses display efficient transmission in animal models (44). Both α2,3 and short α2,6 sialic acid residues are capable of forming a "cone-like topology" where the terminal end of the sialic acid are critical for HA binding. Long α2,6 sialic acids are capable of forming an "umbrella-like topology" with the viral HA that not only includes the terminal sialic acids, but also the oligosaccharide length and branches (42). The ability of viruses to bind to long α2,6 linkages at low concentrations appears critical for transmissibility (45).

A number of conserved amino acid residues on the globular head of the HA molecule function as a receptor-binding site and interact with sialic acid residues on the

surface of host cells (46). Mutations that alter the binding preferences of the HA protein in H1, H2, and H3 subtypes have been defined. The amino acids at residues 190 and 225 (based on H3 numbering) determine the binding preference for the H1 subtype of viruses, while the amino acids at 226 and 228 govern binding preference for H2 and H3 viruses (16, 47-49). Recently two papers have described binding mutations involved in creating H5N1 viruses with altered binding preferences that resulted in increased α2,6 binding (50, 51). Both studies included mutations in the binding site of the HA from an H5N1 subtype of virus that allowed for efficient transmission of the virus between ferrets via respiratory droplet spread. This topic will be discussed in more detail in the next chapter.

1.5 Influenza A Replication Cycle

The infection of a cell by an influenza virus is a multi-step process that takes place in both the cytoplasm and the nucleus of a cell (see Figure 2). The HA proteins are responsible for viral binding and entry into susceptible cells. Experiments measuring binding strength have shown that the affinity of an individual HA protein for sialosides is rather low, indicating that for the strong binding seen during infection a large number of HA molecules must bind simultaneously (23, 45). Once the HA proteins bind their preferred sialic acid moieties, the viruses undergo clathrin-mediated endocytosis to enter the host cell (52). Other forms of endocytosis have also been described for viral entry (53).

As the virion-containing endosome moves into the cell, the pH inside begins to lower. This results in two modifications to the virion within. The M2 channel acts as a proton transporter, leading to a flow of H^+ ions into the virion (54). This lower internal

pH allows for the separation of the RNP complexes and the M1 proteins from the inner membrane surface of the virion, allowing for the release of the viral genomic material from the virion once the lipid membrane becomes disrupted (16). This disruption occurs with the second viral modification from the lower internal pH, where the HA proteins undergo a conformational change that exposes the fusion peptide of the HA (55). The fusion peptide inserts and anchors itself into the endosomal membrane (56). This anchor allows the virus to pull the viral and endosomal membranes together to form a fusion pore. This pore structure then creates a passage for the contents of the virion to spill into the cytoplasm of the host cell (16, 55).

The next stage of viral replication occurs in the nucleus of the infected cell. All of the viral proteins included in the RNP complex contain nuclear localization signals (NLS) that allow the viral gene complexes to become actively imported into the nucleus by cellular machinery at nuclear pores (57-59). Once inside the nucleus the virus is able to halt cellular replication and commandeer the cellular machinery for its own uses. Several viral proteins are responsible for the hijacking of cellular machinery once the viral genes have relocated into the nucleus. The viral polymerase complex is an RNA-dependent RNA polymerase (RdRp) which transcribes viral RNA through the use of cellular RNA polymerase II (60). The heterotrimeric complex of the RdRp is formed with PB1 in the center interacting with both PB2 and PA (61). The transcriptional activity of the polymerase begins with the 5` end of a viral gene binding to the PB1 protein. This binding causes an alteration in the shape of the complex, allowing the PB1 protein to also bind the 3` end of the gene (62). Once the gene is held at both ends the PB2 protein has the ability to bind to the 5` cap of a cellular pre-mRNA transcript

containing a m^7GpppX^m methylated cap structure (16). The endonuclease activity of the polymerase, located in the PA subunit, allows the complex to "snatch" this 5' capped oligonucleotide from a cellular transcript (63-65). With the cellular 5`cap cleaved, and both ends of the viral gene are held in place by the PB1 protein, transcription of the viral mRNA can begin (66, 67). The PB1 protein is responsible for the elongation of the newly emerging viral mRNAs by adding the corresponding amino acids to the nascent mRNA (16, 68). As transcription occurs the 3` end of the vRNA detaches from the polymerase structure, but the 5` end remains bound (16). This eventually leads to steric hindrance, as the polymerase cannot read through the bound section of vRNA. The structural impediment creates a stuttering effect at a stretch of uridine bases and leads to the production of the poly-A tail of the mRNA (69, 70).

To manufacture viral proteins inside the infected cell, the nascent viral mRNA transcripts utilize cellular ribosomal machinery in the cytoplasm or cytoplasmic face of the endoplasmic reticulum (in the case of the surface glycoproteins) to translate the mRNA into proteins (55). In the cytoplasm the NS1 protein induces preferential translation of viral transcripts over cellular mRNAs through interaction with cellular machinery (71). Once translated and properly glycosylated, the HA and NA proteins assemble in lipid raft regions in the apical cellular membrane along with M2 proteins (72, 73).

The viral RdRp is also responsible for the replication of the viral genome through production of cRNA. The cRNAs exist as complete copies of the viral genome without splicing or polyadenylation (16). The cRNAs differ from viral mRNAs by not requiring

a capped primer to begin transcription and are encapsidated by NP. The positive-sense cRNAs then act as transcripts for the negative sense viral genome copies.

Once the negative sense viral genes are produced in the nucleus they must be transported out of the nucleus to the interior side of the lipid membrane at the cell surface for assembly into virions. Two of the viral proteins that are involved with the nuclear export of the genes include M1 and NEP. Once an infection is ongoing in a cell the M1 proteins in the nucleus bind to newly copied viral gene/NP complexes to direct them towards nuclear export via NEP (74). NEP has been shown to directly associate with cellular proteins that aid in export of protein complexes from the nucleus (75, 76).

For an infectious influenza particle to be produced, all eight of the individual genes must be included in a progeny virion. The gene segments contain segment specific packaging sequences in their noncoding regions (77). Electron microscopy has shown the viral genes become incorporated in a characteristic pattern of 7 gene segments surrounding a central gene (78). Whether this inclusion occurs randomly or whether the genes are added selectively is still unknown. An argument for the selective addition theory includes a study that shows at least PB2 must be included into a virion before any other gene becomes added (79). Within a formed virion, the M1 protein is the most abundant viral protein and associates with both the viral genes as well as with the cytoplasmic tails of the surface glycoproteins (16). The mechanism of viral budding is also not known, although the HA protein displays the ability to affect membrane curvature and budding off of membrane in a VLP system (80). In a virus-infected cell however, it does appear additional proteins are required, including NA and M2 (81).

Once the virion has successfully budded from the infected cell, virus egress cannot take place until the sialic acid moieties present on the surface of the infected cell are removed. In order for the virus to become separated from the initial infected cell these sialic acid residues must be removed by the viral NA protein (16, 55). The neuraminidase is a type II glycoprotein present on the cell surface in homotetramers and contains a sialidase activity (16). Viral egress occurs once the sialic acids are no longer present and the virions then spread to infect additional cells.

1.6 Viral Strategies for Multiple Protein Production

As previously mentioned, several of the viral genes from influenza are capable of creating multiple vRNAs from the same genetic segment. The virus is adept at utilizing the host cellular splicing machinery to accomplish this goal (82). The NS and M genes of the virus utilize host cellular proteins in order to produce the NEP and M2 transcripts. Splicing of viral transcripts is an inefficient process, and only occurs in about 10% of the total viral transcripts for a particular gene (16). The rate of export of the proteins from the nucleus is a large determining factor in the efficiency of splicing for both proteins (16, 82).

Influenza viruses also make use of splicing-independent methods in order to produce multiple transcripts from the same gene, including multiple ATG start codons and alternative reading frames (16). As an example, the PB1 segment contains an alternate ATG site in the 5' end of the message in an alternate reading frame (20). When transcription occurs at this alternate start site, the protein PB1-F2 is created. The PB1-F2 protein has been shown to result in apoptosis of alveolar macrophages and increased viral

virulence during infection (83, 84). There have also been publications involving another translated protein in PB1 (N40), which is downstream of the PB1-F2 start site (85, 86). The N40 protein appears to affect the regulation of viral gene expression during viral replication (85).

More recently the PA gene has been described as having multiple polypeptides, including the full-length PA and PA-X (87). PA-X is transcribed by the second splicing-independent method known as ribosomal frameshifting (21). Ribosomal frameshifting occurs when a ribosome moves into an alternate reading frame during the translation process, resulting in two proteins with distinct C terminals (82). The PA-X protein is created from the amino-terminal 191aa of the PA protein with an additional 61aa after the frameshift occurs, and has been implicated in the modulation of influenza virulence during viral infection (21, 88).

1.7 Influenza A Mutation and Host Adaptation

Influenza viruses continually mutate throughout replication, creating quasispecies within an individual during an infection (89). The viral RdRp encoded by the influenza virus lacks any proofreading capacity, which results in an error-prone phenotype and helps account for the high mutation rate (25). The virus has the ability to evolve and undergo mutation by two methods. The first type is antigenic drift, which occurs during viral replication as random amino acid changes are inserted into the genome. These alterations can lead to antibody escape variants that are able to avoid neutralization by the antibodies produced during a prior infection or vaccination; and occur in H3 viruses at a rate of approximately 3 amino acids changes per year (16, 90). These changes eventually

lead to the emergence of variant viruses with mutations accumulated in the HA glycoprotein. The new variants replace viruses against which there is immunity present in the human population and cause seasonal epidemics (90).

Alternatively, a virus can acquire new genetic material when two different influenza A viruses infect the same cell, in a process called reassortment (16). With both viruses replicating in the nucleus of a cell the two genomes can reassort to form new distinct progeny viruses, with a possibility of 256 unique combinations. Antigenic shift occurs when reassortment leads to the introduction of a novel HA into a human population that lacks immunity to this new subtype. As discussed in a later section, a novel subtype is characteristic of pandemic viruses. Swine serve as a platform for reassortment between avian and mammalian influenza viruses as their tracheas contain receptors for both, giving them the capacity to act as both a host for the viruses as well as a mixing vessel (91). The three most recent pandemic strains have arisen from reassortment, demonstrating the importance of antigenic shift to escape immune pressure in the human population.

Several mutations have been found to allow for influenza virus adaptation and alterations in virulence in humans. While a complete list of mutations is beyond the scope of this introduction, several key examples of changes known to affect the pathogenicity of an influenza virus are discussed below. One such change is located in the PB2 protein at the amino acid site 627. The change E627K allows for the virus to replicate more efficiently at 33°C *in vitro* and has been indicated as a virulence factor in mice (92, 93). The ability of a virus to replicate at a lower temperature improves the capacity to replicate in the upper respiratory tract (URT) of humans; potentially

increasing their ability to transmit (94). There also appears to be a compensatory mutation that can result in the recovery of viral transmissibility at 701D in the PB2 protein after the introduction of 627E into seasonal strains (95). Another mutation in the influenza virus is located in the PB1-F2 protein at amino acid 66. The amino acid alteration N66S is associated with increased virulence in 1918 and H5N1 viruses, both of which have exhibited heightened virulence in humans (96). The total length of the translated PB1-F2 protein also contributes to the ability of the protein to affect virulence during infection. The protein exists as an 87 or 90aa protein when full-length, but is truncated in some strains to only 57aa and in the 2009 pandemic strain to 11aa (97). The truncated form is inactive, and is present in many human-adapted strains, especially H1N1 subtypes. The full-length form is present in avian subtypes of virus. Full-length PB1-F2 has been shown to induce apoptosis in alveolar macrophages (20). PB1-F2 can also inhibit IFN production by binding and inactivating mitochondrial antiviral signaling protein (MAVS) (98-100). As mentioned previously, the cleavage site in the HA protein can influence the virulence and extrapulmonary spread of an influenza virus after infection. The extent of glycosylation on the HA can impact the virulence of a virus, demonstrated by attenuation of both the A(H1N1)pdm and 1918 strains after the addition of sugars to their HA proteins (101, 102).

1.8 Innate Immune Response to Influenza A

Multiple cell types of the respiratory tract are susceptible to influenza virus infection. However, the respiratory tract contains several innate barriers the virus will encounter before reaching cells. Collectins (such as surfactant proteins A and D) are

collagen-like lectins secreted from cells that bind and neutralize influenza viruses in the airway prior to infection (103). Mucins are glycoproteins secreted by the mucosal epithelial present in the airways that bind and inhibit the virus from infecting cells (104). Cilia present on cell surfaces beat and expel the mucin/virus complexes back out of the airway, preventing infection.

Once a virus enters a host cell, the intercellular innate immune system recognizes the viral components as foreign and becomes activated. Cellular pattern recognition receptors (PRR) recognize viral pathogen-associated molecular patterns (PAMP) in the influenza virus and trigger cellular pathways that lead to the production of antiviral genes and molecules (104). Some of these cellular products activate a local antiviral state to help limit the spread of the virus. Other molecules, such as chemokines, act at a further distance, by attracting cells of the immune system to the infected area.

Once viral infection of cells in the respiratory tract occurs, innate sensors in the cells react to the presence of viral RNA in both the endosome and the cytosol. Toll-like recognition protein 3 (TLR3) and TLR7 are located in the endosomes of epithelial cells. TLR3 recognizes viral-derived dsRNA while TLR7 recognizes ssRNA (105, 106). Upon activation, TRL3 and TLR7 both signal through MyD88-dependent pathways, leading to the production of inflammatory cytokines and type I interferons (105). Interferons help to inhibit viral synthesis and limit replication, and to activate local innate immune cells (104). In the mouse model, animals lacking the interferon (α/β) receptor display more morbidity and succumb to infection more rapidly than wildtype mice (107).

After the virus escapes the endosome, the cell has additional innate immune detectors in the cytoplasm. The cytosolic innate sensor proteins retinoic acid-induced

gene I (RIG-I) and melanoma differentiation-associated protein 5 (Mda5), part of the RIG-I like receptor (RLR) family, are able to detect viral RNA in the cytosol. RIG-I is believed to recognize single-stranded 5'-triphosphate RNA, while Mda5 reacts to longer double-stranded RNA (108). Once activated the RIG-I proteins lead to further cytokine production and type I interferon expression.

Interferons are important cytokines for regulation and control of influenza infections. They can act in either an autocrine or paracrine fashion to create an antiviral state (109). These cytokines lead to the production of interferon-stimulated genes (ISGs), which are involved in creating antiviral conditions. The produced ISGs result in increased innate responses, regulating host cell transcription/translation, regulation of apoptosis, and attraction of additional immune cells to the site of infection (109). HPAI H5N1 and some A(H1N1)pdm strains viruses display a greater attenuation of the type I interferon response compared to seasonal strains, resulting in lower ISG levels in Calu-3 cells and may contribute to the greater virulence observed in these infections compared to seasonal strains (110-112).

The NLRP3 inflammasome in the cytoplasm has also shown to be involved in viral detection (113, 114). Recognition of viral components by NLRP3 produces active forms of the inflammatory cytokines IL-1β and IL-18. Additionally, detection of dsRNA produced during viral replication activates RNA-dependent protein kinase (PKR) in the cytosol on infected cells (115). PKR expression is induced by interferons, and its activation leads to translational arrest in the cell, which prevents both cellular and viral protein synthesis. Epithelial cells also recruit additional innate immune cells (monocytes) to the site of infection through the production of CCL2 (116).

Several resident innate immune cells within the respiratory tract are vital to recognition of influenza infection. These cells initiate the activation of the innate and regulation of the adaptive immune response against the virus. Alveolar macrophages are one of the first cells of the innate immune system to encounter the viruses during infection. These cells reside in the alveoli of the lungs and act as regulatory cells during homeostasis (117). Once activated, alveolar macrophages actively phagocytose infected cells and produce inflammatory cytokines, including IL-6 and TNF-α (118). Macrophages also display high levels of tumor necrosis factor-related apoptosis-induced ligand (TRAIL), leading to apoptosis of infected epithelial cells (119). They can also contribute to immunopathology through production of NOS2 and TNFα (120, 121).

Natural killer (NK) cells contribute to viral clearance through antibody dependent cell-mediated cytotoxicity (ADCC) and cytokine production. ADCC lyses cells after receptors on the surface of the NK cell recognize the Fc portions of antibodies bound to the surface of infected cells. Natural cytolytic receptors NKp46 and NKp44 lead to cell lysis after detecting viral HA protein present on the surface of infected cells (122, 123). NK cells can produce the cytokines IFNγ, TNFα, and MIP-1α to help control an infection (124, 125). NK cells are also reported to modulate and enhance both dendritic cell and T cell responses (126, 127).

Dendritic cells (DCs) are resident cells located beneath the epithelial barrier in the airway, with dendrites extending into the lumen of the airway through tight junctions between cells. There are multiple types of DCs in the respiratory tract that can contribute to an immune response. Conventional DCs (cDCs) predominantly utilize TLR3 and the RLRs for viral detection while plasmacytoid DCs (pDCs) primarily detect antigens

through TLR7 and PKR and are the primary producers of type I interferons during infection (105, 128, 129). DCs can be activated either through direct infection by the virus, or through phagocytosis of viral antigens (130, 131). Activated DCs upregulate MHC complexes and costimulatory markers on their surface, and produce multiple inflammatory cytokines, including IL-6, IL-12, TNF-α, IL-8, IP-10, RANTES, MIP-1β, and type I interferons (132). After activation the DC displays CCR7 on its surface and migrates to the draining lymph node to act as an APC to influenza-specific T cells (133). They can present viral antigens through either the MHCI or MHCII pathway based on how the antigens were processed after phagocytosis, and can therefore activate either CD8 or CD4 cells, respectively (104).

The levels and location of cytokine expression produced by innate immune responses have been shown to correlate with the transmissibility and pathogenicity of influenza viruses in the ferret model (134). Seasonal strains that transmitted efficiently displayed a high upregulation of proinflammatory cytokines in the UTR including TNF-α, IL-6, IFN-α/β compared to avian viruses that failed to transmit. These results agreed with human studies that examined cytokines produced in the UTR after infection with seasonal influenza strains (135-137). Viruses that display a high level of pathogenicity display greater upregulation of proinflammatory cytokines and chemokines in the lower respiratory tract (LTR) (134). These higher levels of chemokines can attract a greater concentration of immune cells into the infected area and create a larger immune response, resulting in greater inflammation and more immunopathology (134, 138, 139).

1.9 Adaptive Immune Response to Influenza

Once antigen-presenting cells (APCs) travel into the draining lymph node, the circulating lymphocytes specific to influenza are able to detect their cognate viral antigen and become activated. T cells require both recognition of the specific MHC molecule and expression of costimulatory molecules for activation to occur (104). T cells proliferate in the draining lymph node following activation by the APCs. $CD4^+$ T cells move into the germinal center areas to activate B cells and shape the antibody response to infection, while $CD8^+$ T cells will migrate to the site of infection for their effector functions (140, 141).

$CD4^+$ T cells activated during an immune response to influenza infection are mainly helper T cells, although they can have cytolytic activity (142). The $CD8^+$ T cells act as cytotoxic T lymphocytes (CTLs) after activation by DCs and lyse infected cells (141). Infected cells are recognized by CTLs by the viral antigens displayed in the MHCI on the surface of the cell. CTLs eliminate infected cells to prevent further viral replication through several methods. These methods include the release of granzyme B and perforin granules as well as a Fas-FasL mediated apoptosis (141). They also produce cytokines including IFNγ and TNFα and can result in substantial immunopathology (143). The major targets of CTL receptors in human influenza are NP and M1 epitopes (144, 145). These epitopes are more highly conserved and therefore more cross-reactive against multiple subtypes compared to epitopes recognized by antibodies (146, 147).

B cells are also activated through infection to become plasma cells that produce large amounts of influenza specific antibodies. This activation also occurs in the draining lymph node through interactions with APCs. B cell activation that is Th-dependent

occurs in the germinal centers of the lymph node and creates long-lived immunity (140). $CD4^+$ T cell help during B cell activation results in antibody class-switching and affinity maturation of the antibodies produced (148). There are also B cells activated through Th-independent means in extra-follicular areas, but these cells are short-lived and create an inferior memory response (149).

The main target of antibodies are the surface glycoproteins of the virus, especially the HA protein. The presence of antibodies against the HA is currently the best-known correlate of protection against the virus (150, 151). Antibodies against the globular head of HA can be neutralizing by blocking the ability of the virus to bind to and enter susceptible cells or by inhibiting virus replication post-entry (151). There are five known antibody recognition sites on the globular head region of the HA (152). The antibodies can also be specific to the stem region of the HA protein. Antibodies against the stem region of HA can inhibit viral fusion during entry and tend to be more broadly reactive than those against the head region (151, 153-155). Antibodies against the NA protein inhibit the sialidase activity of the NA and reduce viral spread (25, 151). While antibodies against the M2 protein tend to occur at low concentrations, they are broadly cross-reactive and have been the target of recent attempts at creating a universal influenza vaccine (156, 157). Antibodies raised against NP have also been shown to induce ADCC, although the mechanism is not well understood (158).

Multiple classes of antibodies are produced during infection with influenza. IgM antibodies are produced after a primary infection (104, 159). These antibodies tend to have a lower affinity against their antigen, but their shape allows for a high avidity binding to the viral antigens. IgM antibodies are very proficient at inducing complement-

mediated lysis of infected cells once bound to the surface. IgA antibodies are produced quickly after infection (160). These antibodies are produced locally within the respiratory tract, and are transported along the mucus. They are capable of neutralizing virus intracellularly (161). IgG antibodies are produced systemically, but can transudate into the respiratory tract, and can provide long-term protection (162). IgG antibodies produced against influenza surface glycoproteins are also capable of facilitating ADCC and complement-mediated lysis through the Fc tail portion of the antibody (104, 163).

1.10 Memory Response to Influenza

After an influenza infection has been cleared, the cells of the immune system undergo a massive contraction phase. Most of the influenza-specific T-cells and B-cells die, although a small portion is long-lived and can be reactivated during secondary infection. These cells are present in higher numbers, persist longer, and respond more rapidly than naïve cells (104). Both arms of the adaptive immune system respond more rapidly and specifically to a subsequent influenza infection. With the immune response being composed of responses to multiple epitopes and proteins from the virus protection can persist even after antigenic drift has occurred.

Once influenza-specific memory has been established the response to a subsequent influenza infection is more effective. Memory cells respond to lower concentrations of viral antigens, are less dependent on costimulatory signaling, and are capable of producing a broad range of cytokines once activated (164, 165). $CD4^+$ T cells have been shown to be vital to the production of an effective memory response to influenza infection. Memory $CD4^+$ cells help regulate the innate and adaptive memory

responses during subsequent infections. Memory $CD4^+$ T cells rapidly activate APCs and result in enhanced cytokine production (166). While the primary $CD8^+$ T cell response is not strongly affected by the absence of $CD4^+$ T cells, the memory response is reduced and delayed (167, 168). Memory $CD8^+$ T cells exist in two subtypes, classified as effector (Tem) and central memory (Tcm) (169). The Tem cells are a transitory cell population while Tcm contain the full memory phenotype of self-renewal and rapid reactivation (170). Memory $CD8^+$ cells can rapidly activate and recover cytolytic activities once reintroduced to antigen.

Two forms of long-lived B cells exist after influenza infection: memory B cells and plasma cells (171). Both of these cell types differ from naïve cells by containing high-affinity isotype-switched antibodies (172). The long-lived plasma cells reside in niches of the bone marrow and secrete continuous low-levels of antibody (173). Memory B cells preferentially localize in site of antigen entry in the body, and can encounter antigen more rapidly than naïve cells located in follicles (174). After restimulation memory B cells are capable of producing large amounts of antibodies much faster than naïve cells.

The influenza virus that causes the first exposure can affect the memory recall response to a heterologous influenza virus infection. This effect on a subsequent infection is known as original antigenic sin (OAS). Even after antigenic drift has occurred shared epitopes between two strains can remain. During the infection with an antigenically drifted virus, both memory cells against shared epitopes as well as naïve cells against novel epitopes should become activated. OAS occurs when the memory response dominates over the response against novel epitopes in the drifted virus. This

can result in low seroconversion rates to the antigenically drifted virus (175, 176). OAS has been observed in humans as well as in animal models of influenza (177, 178). Studies in mice have shown that prior infection creates a much stronger OAS effect compared to vaccination (179).

1.11 Influenza A Immune Evasion

In addition to evading the host adaptive humoral response through antigenic drift or shift, influenza viruses have evolved multiple ways to evade the immune system and impede the immune response mounted against the virus. Many of these techniques require the viral NS1 protein. Once produced inside an infected cell, this protein is capable of binding multiple cellular proteins to interrupt numerous cellular response pathways (180). NS1 binds to viral RNA to help mask the genetic material from cellular TLRs and RIG-I proteins. NS1 further prevents RIG-I activation by binding tripartite motif-containing protein 25 (TRIM25), a protein which ubiquitinylates the CARD domain of RIG-I and is required for signaling (181). NS1 additionally binds to and inhibits 2'-5' oligoadenylate synthase activation (180). The pathway involving RNA-dependent protein kinase (PKR) is inhibited by NS1 binding to and sequestering viral dsRNA to keep PKR from activating. NP can further interfere with this pathway by binding $P58^{IPK}$, the inhibitor of PKR (182). $P58^{IPK}$ is usually present in cells in a complex with heat shock protein 40 (hsp40) but NP protein is capable of binding this complex and causing dissociation of $P58^{IPK}$ from hsp40, which then allows $P58^{IPK}$ to bind with PKR and block its activation.

NS1 also interferes with general cell functions and is involved with regulation of viral replication. The viral protein interferes with the splicing of pre-mRNA transcripts by blocking 3' polyadenylation of cellular transcripts by interacting with cleavage- and polyadenylation-specific factor (CPSF30) (183, 184). NS1 interferes with cellular export machinery as well, which decreases mRNA transport out of the nucleus and concentrates the mRNA for cap-snatching by the viral polymerase (185).

NS1 and NP are not the only influenza proteins with known immune regulation properties. As mentioned previously, PB1-F2 and PA-X proteins can both affect the immune response. PB1-F2 has an effect when in its full-length form by inhibiting IFN production and mutation of PA-X creates an exacerbation of the immune response to influenza virus (21, 98-100).

1.12 Influenza Vaccines and Antivirals

Beginning in 2010, the Advisory Committee for Immunization Practices began recommending everyone 6 months and older get vaccinated annually with the most recent composition of influenza vaccine (7, 8). Populations considered to have high risk of complications from influenza infection include young children, persons ≥65 years of age, pregnant women, and immunocompromised individuals (8). There are several different formulations of the annual vaccine offered, with varying recommended target groups.

The most widely used formulation of the seasonal influenza vaccine in the US is the trivalent inactivated influenza vaccine (TIV). This vaccine contains antigens from the three most widespread forms of influenza currently circulating in humans: H1N1, H3N2, and B. A quadrivalent inactivated vaccine is also now available, with the strains of the

two antigenically divergent B sub-lineages included. The makeup of the TIV vaccine is reevaluated annually, to include the strains that are expected to be the most prevalent during the approaching influenza season. The vaccine growth strains contain the surface glycoproteins of the circulating virus, with the internal genes of A/Puerto Rico/8/34, to increase growth ability in eggs (186). They are primarily created by traditional reassortment methods and then inactivated through treatment with chemical agents. Once inactivated the lipid membranes of the virions are disrupted, or split, by a detergent and the surface HA proteins are selectively purified. This split vaccine has a lower immunogenicity than a whole virion version but also has a lower reactogenicity and is therefore safer (187). The HA amounts from each virus is determined by single radial immunodiffusion (SRID) and are then diluted to the proper concentration (188). Higher concentration TIV vaccines with four times the normal HA antigen dose are available to improve immune responses in older adults (8). Split vaccines developed in cell cultures also exist to avoid egg allergies and improve manufacturing capacity (189-191).

Depending on the purification processes during manufacture, the other viral components may be present in the TIV in varying amounts, including NA, M, and NS, as well as viral RNA. This may have a beneficial effect on vaccine immunogenicity, as it's been shown that viral RNA in a vaccine can trigger an immune response through TLR7 in mice (192). The TIV is effective at eliciting a serum IgG response against the HA antigens. However, intramuscular delivery is not particularly successful at stimulating local immune response within the respiratory tract. The TIV has been shown to be poor at stimulating a secretory IgA or CTL response in naïve populations but more effective at boosting preexisting memory responses in ferrets and humans (159, 193, 194).

The FDA has recently approved a recombinant HA protein vaccine, called Flublok, which is produced by baculovirus expression in insect cells (195). This system creates a vaccine that can be produced quickly in the case of another pandemic and avoids the use of eggs during manufacturing (196). The vaccine is administered intramuscularly like the TIV and has been approved for use in adults. Another recent FDA approval involves the Fluzone intradermal vaccine that delivers a much smaller amount of HA antigens into the dermis layer of skin through hollow microneedles, resulting in seroconversion rates similar to the intramuscular delivery method (197, 198).

An important addition to the collection of approved influenza vaccines is the live attenuated influenza vaccine (LAIV). The LAIV is delivered through intranasal spray, a more natural route of administration than the intramuscular route of the TIV. This vaccine provides much better mucosal IgA antibody and cell-mediated response, especially for naïve individuals (199). The live attenuated vaccine also appears to provide better protection against antigenically drifted strains compared to the inactivated TIV in ferrets (200). The currently licensed LAIV use a cold-adapted master donor strain as the basis of attenuation. Strains such as A/Ann Arbor/6/60 have been previously cold-adapted and grow best at temperatures much lower than 37°C (201). This adaptation includes stable mutations in multiple internal genes (202). Other unlicensed attenuated strains in development contain NS genes with either mutations or truncation (203). Without the NS gene to modulate the immune response, the virus is less able to combat the human immune response.

Other experimental vaccines have displayed effectiveness in animal models and clinical trials, but have yet to gain approval for human use in the US. An additional type

of vaccination targeting the response against the HA protein involve the use of solid microneedles for inoculation (204). Recombinant HA molecules are coated on solid or dissolving microneedles that are placed onto the skin and puncture the dermis to place the antigen. Once applied to the skin the coated viral antigen dissolves off the microneedles (or with the dissolving microneedles) and diffuses into the dermal layer. Virus-like particles (VLPs) are another promising form of vaccination, with viral surface antigens but no internal genetic material. These VLPs are unable to replicate but appear to create a broader immune response than traditional TIV (205, 206). Techniques that target cell-mediated immunity are also currently being investigated. DNA-based vaccines try to avoid the antigenic drift that occurs with the surface glycoproteins by targeting conserved epitopes in the internal genes, targeting T cell immunity. These epitopes are preserved across subtypes, so these vaccines have the possibility of creating broad heterosubtyptic immunity. The M and NP genes are the current focus for these vaccines since they are the major T cell targets during infection (207). These approaches are often referred to as universal vaccines. Another approach at a universal vaccine involves VLP vaccines with the HA2 stem-portions of the hemagglutinin proteins (155). The stem regions of the HA proteins are highly conserved and protected mice from a lethal heterologous challenge. Adjuvants are additional vaccine components that are known to have stimulatory effects on the immune system. Adjuvants are included in vaccines in several countries around the world, but the United States still only licenses unadjuvanted influenza vaccines (191).

Antiviral drugs have been shown to decrease influenza virus shedding and shorten the duration of illness when taken early in the course of infection. Prescription antivirals in use currently target two influenza proteins. Amantadine and Rimantadine target the

M2 transmembrane channel in influenza A viruses to prevent the ion flux into the virus in the endosome of the cell. Zanamivir and Oseltamivir antivirals target the active site of the NA protein of both A and B strains of virus, to inhibit viral release from infected cells. Antivirals taken prophylactically can reduce transmission between household contacts, as shown with inhaled zanamivir (208, 209). The drugs differ in routes of administration, doses, and recommended age groups. Also, resistance has developed against some of these drugs, especially for the adamantanes, making them less effective against some seasonal strains of virus (210). Mutations resulting in antiviral resistance may lead to a decrease in transmissibility and overall fitness in the resulting strain (211, 212).

1.13 Animal Models for Influenza A Viruses

Several animal models exist for the study of influenza virus infection. Each model contains both advantages and limitations, and different models are preferred over others based on the focus and approach of the research. The breadth of reagents available differs greatly between models. The cost and space for the housing and care of the animals also must be a consideration. The most frequently utilized models are compared and the most common uses for them are discussed in detail below.

Mice are the most widely used animal model for influenza pathogenesis (213). This model has the largest number of reagents available for analysis, as well as the availability of transgenic animals for study. Almost every aspect of the disease can be analyzed during the infection, whether it involves morbidity of the disease, immune response of the animal, or memory/recall responses to the virus (214). Mice have also

been bred to be genetically homogenous and can be studied in large numbers, making the process of showing statistical significance easier (215). They are also low-cost, and easy to care for and house during experiments. However, mice are a relatively poor animal model for the study of virus transmission. Furthermore, seasonal influenza viruses often require prior adaptation in order to cause disease in mice (216).

Guinea pigs can be used in transmission experiments of influenza virus infection, and have been shown to transmit virus to naïve contact animals (217). Influenza viruses do not require prior adaptation before causing infection in this animal model (218). However, guinea pigs are a poor model when studying pathogenesis, as they fail to display many clinical signs of disease during infection and do not recapitulate the virulence of some influenza viruses including H5N1 viruses in humans (215). Guinea pigs are also more difficult and expensive to house during experiments than mice.

Non-human primates are a very useful model for comparison to human infections and immune responses to influenza virus. However, the cost and size of housing, and ethical considerations make these animals a less than optimal model for most studies (219). Non-human primates display multiple clinical symptoms following infection, including coughing, fever, anorexia, and lethargy (220). H5N1 viral infections are typically non-lethal in this animal model and lack extrapulmonary spread for H5N1 infections in both cynomolgus macaques and rhesus macaques (221-223). However, this species has been utilized for analyzing vaccine efficacy and transcriptomic analyses (224-226).

The ferret animal model can be utilized for both transmission and pathogenicity studies. Influenza A viruses do not require prior adaptation in order to cause disease in

these animals (215). The transmissibility of viruses in this model generally corresponds to human cases, and the ability of the viruses to transmit appears to correlate with the presence of sneezing during infection (134). Ferrets display many of the clinical symptoms seen in human cases of infection, including lethargy, fever, nasal discharge, and weight loss (227). While replication of seasonal influenza A viruses is generally restricted to the upper respiratory tract following intranasal inoculation, 2009 H1N1 and H3N2v viruses can be detected in upper and lower respiratory tract tissues with occasional spread to the gastrointestional tract (228, 229). Several HPAI viruses have further been shown to have the capacity to spread outside of the respiratory tract in these animals, and have been found in systemic organs including the intestines, spleen, and brain within three days of inoculation (230). The reagents available for study of the immune response to infection are more restricted in this model, although they are becoming the focus of renewed interest and research (215, 218, 231). The animals in this model are outbred, which allows for the study of complex virus-host interactions though it increases the chance of genetic variability compared to inbred animals (232). The cost and size of the housing and bio-isolators required for these animals limit the number of animals that can be included in an experiment. These factors make confirmation of statistical significance more challenging compared to a mouse model (233). As the ferret represents the most relevant small mammalian model to study both virus pathogenicity and transmissibility, the studies contained within this dissertation utilize the ferret animal model for all experiments.

1.14 Influenza A Pandemic History

In order to cause a pandemic, a newly emerging virus must be capable of causing illness in humans and display sustained human-to-human transmission (234, 235). The HA protein must also be antigenically distinct from those recently circulating among human populations (236). The lack of preexisting antibodies in the population increases the ability for the virus to infect and spread among humans.

The influenza pandemic of 1918 (H1N1), the worst pandemic in recent history, killed an estimated 675,000 people in the United States and up to 50 million people worldwide (237). Although historical evidence is limited, it has been suggested that all of the genes in the pandemic strain came from avian influenza strains that adapted directly to human hosts (238, 239). The pandemic virus in 1957 was of the H2N2 subtype, and was responsible for the deaths of an estimated 2 million people worldwide. This virus contained the HA, NA, and PB1 genes of an avian virus that were introduced into a human strain through antigenic shift (240, 241). The H3N2 subtype pandemic virus of 1968 also arose through antigenic shift, where the HA and PB1 genes were derived from an avian source (239). The 1968 outbreak was responsible for the death of an estimated 1 million people worldwide.

The most recent pandemic, also of the H1N1 virus subtype, occurred in early 2009. This virus, which emerged from swine, was estimated to have caused over 18,000 deaths worldwide in over 200 countries from April 2009 to August 2010 (242). The novel H1N1 quadruple reassortant virus contained individual gene segments from humans, birds, and swine origin (243). The 2009 H1N1 pandemic virus was a reassortant virus with the PB1 gene from a seasonal H3N2 virus, the PB2 and PA genes originating from

an avian influenza virus, the HA, NP, and NS genes from the North American swine lineage, and the NA and M genes from the Eurasian swine lineage (244). The 2009 pandemic strain also lacked all of the known virulence factors and human adaptations seen in other influenza A viruses, including the amino acid 627 in PB2 or a functional PB1-F2 protein (244).

The inability to predict this most recent emergence of a pandemic strain highlights the need to understand the additional factors involved in the development of pandemic strains, as well as a need for improved virologic surveillance in animal species. Although this most recent pandemic strain originated from a non-avian source through a swine intermediate, it does not negate the importance of studying avian influenza viruses. Both swine and avian populations require constant monitoring and sampling to keep track of the contemporary strains circulating in those groups. They both share the same zoonotic origin, and continue to display the capacity to mutate and infect humans. As such the potential of influenza viruses to develop into new pandemic strains remains a constant threat.

1.15 Avian Subtypes of Influenza A Virus

A subset of the H5 and H7 viruses has shown the capability of becoming a highly pathogenic virus once the virus is introduced into a land-based domestic avian species (245). HPAI viruses are defined as being capable of causing lethality in six to eight of eight experimentally infected chickens within 10 days post-infection with infectious virus, and are capable of replicating in cell culture in the absence of trypsin (246). A key molecular signature of H5 and H7 HPAI viruses is the presence of a highly cleavable HA

protein as described in a previous section. The classification of HPAI is separate from the pathogenicity observed in mammalian animal models (235, 247). Detection of influenza infections in the domesticated avian population has led to massive cullings in facilities to attempt to avoid human outbreaks.

Since the late 1990s, select avian subtypes (H5, H7, and H9) of influenza A viruses have caused documented human disease but do not exhibit the ability for sustained human-to-human transmission (25). Avian influenza virus infections of humans occur primarily from direct or indirect contact with infected poultry and have resulted in cases of both high and low severity. Infections with H5N1 viruses can lead to severe pathology including respiratory distress syndrome and rarely encephalopathy (248, 249). Conjunctivitis may also occur, especially in infections caused by H7 subtype viruses (250)(251).

One of the avian subtypes, H5, was first documented in birds in the 1960s. The subtype H5N1 first appeared in humans in Hong Kong in 1997 (252). This outbreak represents the first time that avian influenza A viruses were recognized to cause severe respiratory disease and death in humans (253). The initial outbreak led to eighteen infections and six deaths in humans; culling of all poultry in Hong Kong eventually contained the human outbreak (252). Isolation of this virus subtype in poultry continued between 2000 and 2003 in Hong Kong, but the virus did not reemerge in humans until 2003 (254). Since then, H5N1 virus has since become endemic in 15 countries, with 628 cases and 374 deaths in humans reported to date (248). The H5N1 viruses that have reappeared display a higher virulence than those in the original outbreak, with a mortality rate of almost 60% (248, 255). H5N1 virus has been found in extrapulmonary sites such

as the blood, neurons, the small intestine, and bone marrow among severe H5N1 cases (256, 257). The higher virulence may be potentially due to the fact the modern H5N1 viruses contain a different gene constellation than the 1997 isolates, due to further reassortments occurring in avian species (258). These viruses naturally show inefficient transmission among humans, although isolated transmission events have occurred within familial clusters (259-261). While fewer in number, human infections with H7 and H9 viruses have also been documented with only limited evidence for human-to-human transmission in the case of H7 viruses (250, 262). HPAI H7N7 and H7N3 viruses have caused human infections, whereas low pathogenic avian influenza (LPAI) H9 viruses have been more rarely detected in humans (262, 263). Recently, the H7N9 subtype resulted in over 130 human infections and more than 40 lethal cases in the spring of 2013 (248). Some avian subtypes, including H6, have been shown experimentally to gain a HPAI phenotype when a multibasic cleavage site is inserted in the HA protein, although whether such viruses would arise in nature remains unknown (264). Nevertheless, this HPAI ability further underscores the pandemic potential and risk of human illness with all avian virus subtypes. The continuing evolution of avian influenza viruses and frequent infections they cause among the human population highlights the need for continued surveillance and characterization of these viruses. The genetic changes these viruses must undergo to become transmissible among humans needs further urgent research and the appearance of such genetic changes in natural viral isolates should be closely monitored.

1.16 Swine-Origin Influenza A Viruses

Influenza viruses were first isolated from the swine population in the 1930s (265, 266), although a swine influenza-like disease had already been well documented, as far back as the 1918 pandemic (267). Humans were shown to have antibodies that cross-reacted to the swine isolates, demonstrating the closeness between human and swine lineages of virus. Swine display illness in the respiratory tract during an infection with an influenza virus, similar to humans. They can exhibit symptoms including fever, lethargy, weight loss, coughing, dyspnea, and nasal or ocular discharge (17, 267). Infected swine are generally sick for 3-7 days, but illness can also lead to a more severe condition, termed porcine respiratory disease complex (267). Cells in swine respiratory tracts contain both α2,6- as well as α2,3-linked sialic acid residues. This allows for an increased possibility of production of reassortant viruses between avian and human-adapted viruses if simultaneous infections were to occur. For this reason swine have been termed a "mixing-vessel" for influenza viruses of various subtypes. Within the last twenty years there have been multiple examples of antigenic shift occurring in the swine populations in multiple countries around the world (268, 269).

Surveillance of swine influenza has been sparse in the 20th century, although it was enhanced after the A(H1N1)pdm09 virus emerged from the swine population (244). For most of the 1900s, the major subtype that swine contained antibodies against was H1N1, with less than 1% of pigs displaying antibodies against the H3 subtype (270, 271). This changed in the late 1990s when the incidence of infections with the H3 subtype began to appear with higher prevalence, with over 8% of pigs showing antibodies to that subtype due to the introduction of HA and NA genes from human viruses (272, 273).

Reassortant viruses between the two subtypes also began to emerge with H1N2 isolates (274).

The triple reassortant internal gene (TRIG) cassette emerged in swine from reassortment events in the late 1990s. A variation of this genetic constellation was present among the A(H1N1)pdm09 viruses (244). These genes came from a variety of sources, including human, avian, and swine (275). The internal genes of these viruses originated as follows: PB2 and PA genes were of avian origin, the PB1 gene from humans, and the M, NP, and NS genes from the classical swine lineage (244). The A(H1N1)pdm09 virus varied slightly from the traditional TRIG cassette, as the NA and M genes were from a distinct Eurasian swine lineage.

In 2011 a new variant of triple-reassortant virus arose from the swine population and caused 12 human infections and exhibited some limited human-to-human transmission (276). Some of these transmission events are believed to have occurred in familial clusters, although some occurred among children in a daycare setting (276). Swine influenza viruses that result in a human infection are termed variant viruses (277). These variant viruses of the H3N2 subtype [A(H3N2)v] included a TRIG constellation of genes, but also contained the M gene from the A(H1N1)pdm09 virus. In 2012, A(H3N2)v virus caused human illness in 309 additional documented cases (8). The majority of these cases appeared to occur from direct contact with swine, primarily at pig exhibitions associated with agricultural fairs. Experimentally, these viruses displayed efficient replication in human airway epithelial cells, and efficient respiratory droplet transmission in the ferret animal model (228). A(H3N2)v virus was also found to be antigenically distinct from the current seasonal H3N2 viruses included in the trivalent

seasonal vaccines (278, 279). As of August 5th, 15 additional human cases have been confirmed in 2013.

1.17 Aims of the project

Novel influenza viruses continue to emerge from animal reservoirs to infect humans. Knowledge of the role of preexisting immunity against seasonal influenza viruses in protecting against infection and/or transmission of novel influenza viruses is needed to better understand the pandemic potential of these emerging viruses. Since 2011, A(H3N2)v viruses containing the M gene from the A(H1N1)pdm09 virus have caused a growing number of human infections, as a result of direct or indirect exposure to swine primarily at agricultural fairs. While these viruses have been shown to transmit efficiently by respiratory droplets in the ferret animal model, sustained person-to-person transmission has not been observed during the human outbreaks. The overall aim of this project is to evaluate whether prior seasonal H3N2 influenza vaccination or infection affects virus replication and transmission of A(H3N2)v virus in the ferret animal model. Such information will provide an understanding of whether the infection/vaccination history present in the human population accounts for the limited human-to-human transmission of A(H3N2)v viruses.

Aim I: Determine the efficacy of the seasonal (2011-2012) trivalent inactivated influenza vaccine (TIV) against A(H3N2)v virus. Studies have shown A(H3N2)v viruses to be antigenically distinct from contemporary seasonal H3N2 viruses, including the A/Perth/16/2009-like virus that was included in the 2011-2012 TIV. Limited serological data suggest that only a portion of the population have cross-reactive

antibodies against these viruses, mostly in the young adult age group (10-39 yrs) (278-280). Little to no cross-reactive antibody has been found in children under the age of approximately 10 years and immunization with the 2010-2011 TIV had no impact on inducing cross-reactive antibody titers (278). We hypothesize that the seasonal TIV will provide little to no protection against a A(H3N2)v virus infection *in vivo*. To study the efficacy of recent seasonal vaccines *in vivo*, we will immunize ferrets with the 2011-2012 seasonal TIV. Once the ferrets display sufficient antibody titers against the three homologous viruses in the 2011-2012 TIV, we will then compare them to naïve animals following viral challenge. Viral titers and clinical symptoms such as weight loss and fever will be evaluated in both groups. These results will help to determine whether a vaccine specific for these A(H3N2)v viruses is required to increase cross-protection in the population.

Aim II: Determine if inactivated influenza vaccines limit transmission of A(H3N2)v virus. The H3N2 virus subtype entered into the swine population in the mid 1990's, leading to a divergent evolution from that of the human seasonal H3N2 viruses. It is believed that exposure to historical H3N2 viruses and the seasonal influenza vaccines from the early 1990's may explain the cross-reactive antibodies evident in the adult population against these recently isolated A(H3N2)v viruses. These cross-reactive antibodies may also be the reason sustained human-to-human transmission has not been seen during the reported outbreaks. The only observed post infection ferret serum that displays HI titers against A/Indiana/08/2011 virus is from infection with A/Beijing/32/92, a seasonal H3N2 virus included in the TIV for the 1993-1994 season (281). We

hypothesize historical seasonal H3N2 strains will provide greater protection against A(H3N2)v viruses compared to contemporary seasonal strains, and that inactivated vaccines will decrease transmission of A(H3N2)v in the ferret animal model. By creating whole-virus monovalent vaccines from pertinent timeframes (modern and historical seasonal H3N2 viruses, and a homologous A(H3N2)v strain), we will determine the effect the various vaccines have on transmission of the A(H3N2)v virus A/Indiana/08/11 in the respiratory droplet ferret model. Transmission will also be examined following vaccination with the 2011-2012 seasonal TIV. Viral transmission to naïve contacts through respiratory droplet spread will be verified by detection of viral titers in the contacts and seroconversion against the challenge virus.

Aim III: Determine the effect of prior infection with seasonal H3N2 virus on transmission of A(H3N2)v virus. Natural infection with seasonal influenza viruses leads to a strong production of cell-mediated responses as well as a robust local mucosal immune response. Cell-mediated and mucosal immune responses are not always effectively created following immunization with inactivated vaccines in ferrets (193). Therefore we wanted to examine the possible difference between vaccination and prior infection on a subsequent challenge with A(H3N2)v viruses. We hypothesize that prior infection by H3N2 seasonal viruses will limit transmission during a subsequent challenge with A(H3N2)v virus, with possibly greater protective efficacy compared to vaccination. Ferrets will be infected with A/Perth/16/2009 virus and be allowed to create a memory immune response prior to viral challenge with either a homologous seasonal H3N2 or A(H3N2)v virus. Viral titers and clinical symptoms will be evaluated and transmission

will be studied in the respiratory droplet model. Furthermore, IgG and IgA antibodies titers in the respiratory tract and serum of both vaccinated and previously infected ferrets will be analyzed to compare the humoral immune responses generated.

Aim IV: Determine the mutations required for efficient transmission of avian H5N1 viruses in the ferret animal model. To date H5N1 viruses have displayed inefficient transmission during human infections. However, the documented cases have a mortality rate of almost 60%. If an HPAI H5N1 virus gained the ability to spread efficiently among the human population then a pandemic would most likely occur. However, the mutations that would result in a transmissible phenotype in this subtype in mammals are unknown. We hypothesize both an alteration in the HA binding as well as the addition of other human genes will be required for adaptation of the H5N1 viruses towards a transmissible phenotype in mammalian populations. We will establish a reverse genetics system to analyze the amino acid changes in the HA protein that will result in an alteration from the avian α2,3 binding preference to a human α2,6 preference. We will also examine the contribution of individual genes from both a human seasonal H1N1 and an avian H5N1 on transmission and pathogenesis of recombinant viruses in a ferret model.

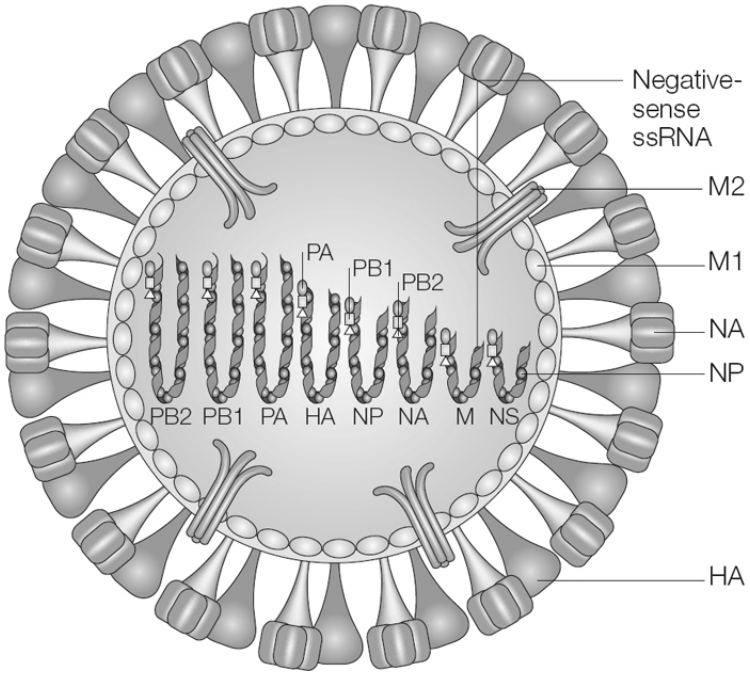

Figure 1. Schematic diagram of an influenza A virus virion. From: Horimoto, T. *et al.* Influenza: lessons from past pandemics, warnings from current incidents. *Nature Reviews Microbiology* 3, 592. The hemagglutinin (HA) and neuraminidase (NA) glycoproteins are located on the surface of the virion, with the membrane (M2) protein protruding through the host-derived lipid membrane. The membrane (M1) proteins are located on the internal side of the lipid membrane, providing the link between the lipid membrane and viral genetic components. The viral genes are contained in the virion, bound by viral ribonucleoprotein (RNP) complexes. The RNP complexes are composed of the polymerase proteins (PB1, PB2, and PA) and the nucleoprotein (NP).

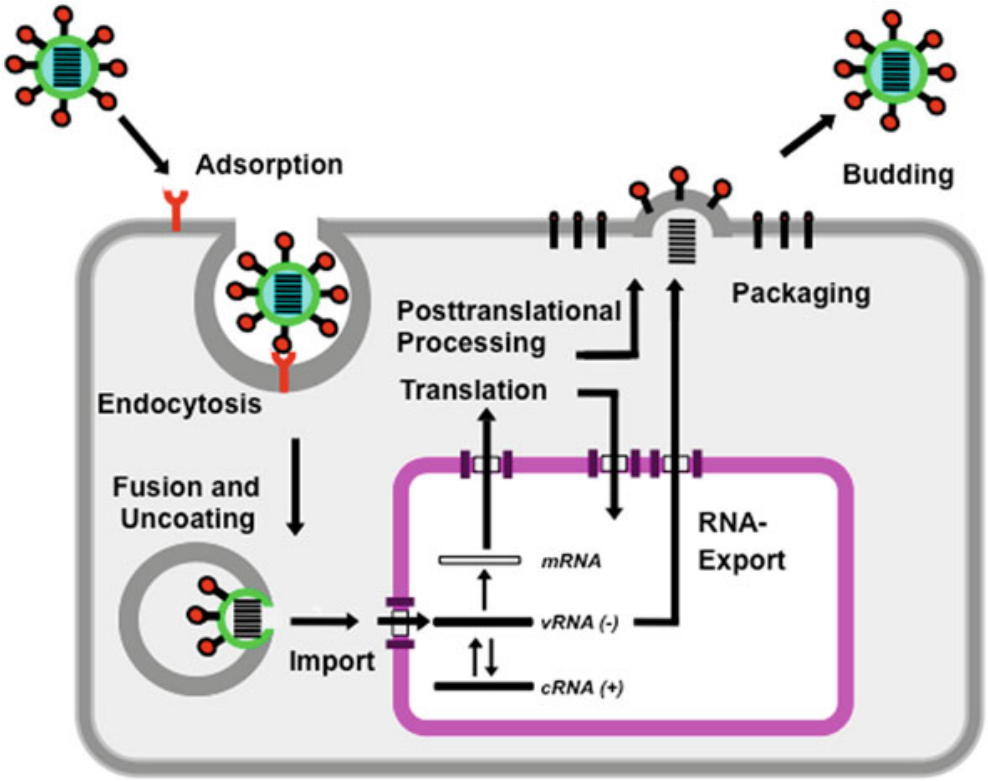

Figure 2. Influenza A virus replication cycle. From: *Fields Virology*, Ed 5. Chapter 47-Orthomyxoviridae: the Viruses and Their Replication. See text (Section 1.5) for detailed description of the model.

Chapter 2.

Seasonal Trivalent Inactivated Influenza Vaccine Does Not Protect Against Newly Emerging Variants of Influenza A (H3N2v) virus in Ferrets

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Adapted from a short-form paper format

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Contribution of other authors to this body of work: TT assisted with vaccination and handling of ferrets during animal studies.

2.1 Abstract

The recent increase in human cases with influenza A H3N2 variant virus [A(H3N2)v] highlights the need to assess whether seasonal influenza vaccination provides cross-protection against A(H3N2)v virus. Our data demonstrate that the 2011-2012 trivalent inactivated influenza vaccine (TIV) protected ferrets against homologous H3N2 virus challenge, but provided minimal to no protection against A(H3N2)v virus. The complete absence of specific hemagglutination-inhibition antibody response to A(H3N2)v is consistent with the poor cross-protection observed among TIV-immune animals.

2.2 Introduction

Influenza A viruses have been isolated from swine since 1930 (266), and have been known to spread and cause disease in this species since the 1918 pandemic (267). The classical H1N1 virus was the predominant subtype isolated from U.S. swine until the late 1990s when human H3N2 viruses infected this species and subsequently spread widely in North American pigs (282). Since that time, multiple reassortment events that have presumably occurred in swine have resulted in the emergence of an H3N2 virus with a triple reassortant internal gene (TRIG) cassette (283). The TRIG cassette, which share host gene-lineage origins with the A(H1N1)pdm09 virus, highlights the public health threat posed by swine-origin influenza subtypes (244).

Until recently, transmission of novel variants of H3N2 [A(H3N2)v] from swine to humans was rare, with only 7 confirmed cases documented in 2009-10 (284-286). In 2011, public health laboratories in the U.S. detected an additional 12 cases of human infection (287, 288), caused by a novel A(H3N2)v virus that had acquired the M gene from A(H1N1)pdm09 virus (286). Since July 2012, there has been a substantial increase of swine to human transmission of A(H3N2)v virus (277). As of October 19, 2012, there have been 307 additional confirmed cases (including hospitalizations) among 11 U.S. states (289). Clinical characteristics of the A(H3N2)v cases have been generally consistent with signs and symptoms of seasonal influenza and there is no evidence at this time that sustained human-to-human transmission is occurring. However, rare instances of probable human-to-human transmission associated with A(H3N2)v cases have occurred and findings from an experimental study suggest that A(H3N2)v viruses have the capacity for efficient replication and transmission in mammals (228).

Vaccination is the most effective measure to control influenza. The seasonal H3N2 vaccine component present in the 2010-2011 and 2011-2012 trivalent inactivated influenza vaccine (TIV) are A/Perth/16/2009 (Perth/09; H3N2)-like viruses (290). Although serological studies indicate that Perth/09 (H3N2) and A(H3N2)v viruses are antigenically distinct from each other (286), the efficacy of seasonal influenza vaccination against A(H3N2)v has not been adequately evaluated *in vivo*.

2.3 Materials and Methods

2.3.1 Viruses. The stock virus for A/Indiana/08/2011 (IN/11) was grown in Madin-Darby kidney (MDCK) cells. The seasonal human H3N2 virus A/Perth/16/2009 (Perth/09) stock was grown in the allantoic cavities of ten-day-old embryonating hens' eggs at 34°C for 48-72 hrs as previously described (Zeng, 2007 #527). Pooled allantoic fluid was clarified by centrifugation and stored at -70°C. Virus stocks were titrated in a standard plaque assay and expressed as plaque forming units (pfu).

2.3.2 Measurement of Antibody Responses by hemagglutination inhibition (HI) assay. HI assays were performed using the WHO influenza reagent kit (according to instructions) obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention (Atlanta, GA). All sera were initially diluted in receptor-destroying enzyme (RDE) from Vibrio cholerae (Denka Seiken, Tokyo, Japan). The enzyme was inactivated by 30 min. incubation at 56^{o}C followed by addition of six parts PBS for a final dilution of 1/10. HAI assays were performed in V-bottom 96-well plates using four hemagglutinating units (HAU) of virus and 0.5% turkey red blood cells. Ferret sera were tested for HI antibody titers against A/California/07/2009, Perth/09, B/Brisbane/60 (WHO kit) and IN/11 virus. The HI titer is expressed as the reciprocal of the highest dilution which inhibits 4 hemagglutinating units of virus. The HI titers are also presented as the geometric mean titers (GMT) from vaccinated or control ferrets.

2.3.3 Vaccination and challenge. Adult male Fitch ferrets, five to twelve months of age (Triple F Farms, Sayre, PA), serologically negative by hemagglutination inhibition (HI) assay for currently circulating influenza viruses, were used in this study. For vaccinations, ferrets were each injected 3 times (3-4 weeks between injections) intramuscularly (i.m.) with an adult human dose (0.5 ml) of the 2011-2012 seasonal inactivated split-product TIV or PBS (controls). To determine viral replication in nasal washes as well as clinical signs of infection, ferrets were randomly assigned to be challenged with IN/11 virus (6 TIV-inoculated and 6 PBS controls), Perth/09 virus (5 TIV-inoculated and 6 PBS controls).

Prior to initial vaccination, vaccine boosts and viral challenge, all ferrets were bled for collection of serum. Following anesthesia with an i.m. injection of a ketamine-xylazine-atropine cocktail, ferrets were challenged intranasally with 10^6 pfu of virus in a total volume of 1 mL (500 μL per nostril) diluted in PBS(Pearce, 2011 #29998). Challenge with IN/11 or Perth/09 virus occurred four to five weeks after final vaccine boost. Following challenge, ferrets were monitored daily for changes in body weight and temperature as well as clinical signs of illness(Maines, 2005 #728). Statistical significance of weight loss and virus titers (nasal washes and tissues) between vaccinated and control animals were determined by Student's t-test. All experiments were performed in biosafety level 3 laboratories with enhancements (BSL3+) as outlined in the Biomedical Microbiological and Biomedical Laboratory(Chosewood, 2009 #30085). Animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee in an Association for

Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

2.4 Results

In this study, we evaluated whether the 2011-2012 TIV protected ferrets against A(H3N2)v A/Indiana/08/2011 (IN/11) virus challenge. Male Fitch ferrets (Triple F Farms, Sayre, PA), 8 to 12 months of age and seronegative against currently circulating human influenza H1, H3 and type B viruses, were vaccinated and twice boosted (3-4 weeks between injections) intramuscularly with an adult human dose (0.5 ml) of the 2011-2012 seasonal inactivated split-product TIV or PBS (controls) (291). Prior to vaccine boost and viral challenge, ferret sera were collected to assess hemagglutination-inhibition (HI) antibody responses against IN/11 virus and the three homologous viruses in the 2011-2012 TIV. As shown in Table 1, all TIV vaccinated ferrets displayed HI antibody titers of ≥ 80 against all three homologous viruses present in the TIV, however cross-reactive HI antibodies to A(H3N2)v IN/11 virus were not observed.

We first determined the level of protection, induced by seasonal TIV against seasonal homologous Perth/09 (H3N2) virus challenge. The Perth/09 virus stock was grown in the allantoic cavities of ten-day-old embryonated hens' eggs at 34°C for 48 hrs and titrated in a standard plaque assay expressed as plaque forming units (pfu). Ferrets were challenged intranasally with 10^6 pfu of virus and vaccine protection was measured by reduction in fever, weight loss, and upper respiratory tract virus replication (291). Viral challenge with the seasonal Perth/09 virus resulted in minimal morbidity among vaccinated and control ferrets, causing 3.4% and 3.9% maximum weight loss, respectively (Table 1). No significant differences in body temperatures were detected between TIV-immune and unimmunized control ferrets observed for 14 days post-challenge (p.c.), although there was a trend toward reduced fevers among TIV immune

animals. The extent of virus replication in the upper respiratory tract was determined by titrating nasal wash samples collected on alternating days p.c. The TIV did not provide sterilizing immunity against homologous viral challenge, as seen previously (291), and viral titers were observed in all TIV immunized ferrets and control ferrets (Figure 1). However, in comparison to control ferrets, TIV immunized ferrets displayed a significant reduction in viral titers on every day analyzed (Day 2 p=0.007, Day 4 p=0.03, and Day 6 p=0.04), until viral clearance was observed in both groups on day 8 p.c.

Next, we assessed the degree of cross-protection against the A(H3N2)v IN/11 virus conferred by seasonal TIV. Ferrets were challenged intranasally with 10^6 pfu of the IN/11 stock virus, which was grown in Madin Darby canine kidney (MDCK) cells. Overall, ferrets challenged with IN/11 virus displayed higher temperatures and greater weight loss compared to ferrets challenged with Perth/09 virus (Table 2). On day 2 p.c., all unimmunized control ferrets exhibited an early spike in body temperature, ranging from 0.5 to 1.8°C over baseline (mean maximum = 1.2°C) (Table 2). Similarly, TIV immune animals also displayed an early spike in body temperature, ranging from 0.75 to 1.8°C over baseline (mean maximum = 1.5°C). Moreover, in comparison to control animals, TIV-immune ferrets did not display significant differences in weight loss and virus titers on peak days (2 – 6 p.c.) of replication (Table 2 and Figure 1). However, TIV-immune ferrets showed a modest reduction in viral titers on day 8 (p=0.02), perhaps due to a low level of anti-N2 neuraminidase cross-reactive antibodies induced by the TIV (292).

2.5 Discussion

The results of this study suggest that previous immunization with the commercially available seasonal TIV may provide minimal to no cross-protection against A(H3N2)v virus. These data are consistent with human serologic studies demonstrating that immunization with the 2010–11 TIV has no impact on the level of cross-reactive A(H3N2)v antibodies in immunologically naive children (aged <3 years) and failed to substantially improve the level of cross-reactive antibodies in adults (278, 279). Because the majority of the population lacks specific immunity against this new virus variant, an A(H3N2)v–specific vaccine is needed for optimal protection for all ages.

2.6 Acknowledgements

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Table 2.1. Serum Hemagglutination Inhibition (HI) antibody responses to TIV immunization in ferrets

	HI antibody titers[a]			
	A/California/07/2009	A/Perth/16/2009	B/Brisbane/60/2008	A/Indiana/08/2011
Pre-Vaccination	<10[b]	<10	<10	<10
Pre-first Booster Vaccination	80-1280 (202)[c]	80-640 (302)	80-1280 (453)	<10
Pre-second Booster Vaccination	160-1280 (453)	80-1280 (761)	640-1280 (1140)	<10
Pre-Challenge	80-2560 (320)	160-1280 (498)	160-5120 (823)	<10

[a] Titers generated by HI with 0.5% turkey RBCs, against A/Indiana/08/2011 A(H3N2)v and viruses in the TIV 2011-12 formulation; A/California/07/2009 (H1N1), Perth/09 (H3N2) and B/Brisbane/60/2008. Assays were performed using the WHO influenza reagent kit (according to instructions) obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention (Atlanta, GA).

[b] <10, below the limit of detection in this assay.

[c] Range of antibody titers shown for each ferret group, geometric mean in parentheses. 11 ferrets per group. All sera were initially diluted in receptor-destroying enzyme (RDE) from Vibrio cholerae (Denka Seiken, Tokyo, Japan) for a final dilution of 1/10.

Table 2.2. Clinical symptoms observed in TIV-immune ferrets challenged with homologous and A(H3N2)v virus

Challenge Group	Mean % maximum weight loss[a]	Mean temp increase over baseline°C[b]	Mean Peak Viral Titer[c]
A/Perth/16/2009			
TIV	3.4	0.9	5.5 ± 0.65
PBS	3.9	1.2	6.7 ± 0.42
A/Indiana/08/2011			
TIV	9.5	1.5	5.6 ± 0.79
PBS	9.6	1.2	6.8 ± 0.42

[a] Mean maximum weight loss percentage values shown (Day 7 post-challenge) for 6 ferrets per group.

[b] Temperature increases over ferret baseline of 38.3 ± 0.5 °C, all maximum temperatures from day 2 p.c.

[c] Mean peak titer shown as $\log_{10}$ PFU/ml including standard deviation. All peak titers from day 2 p.c.

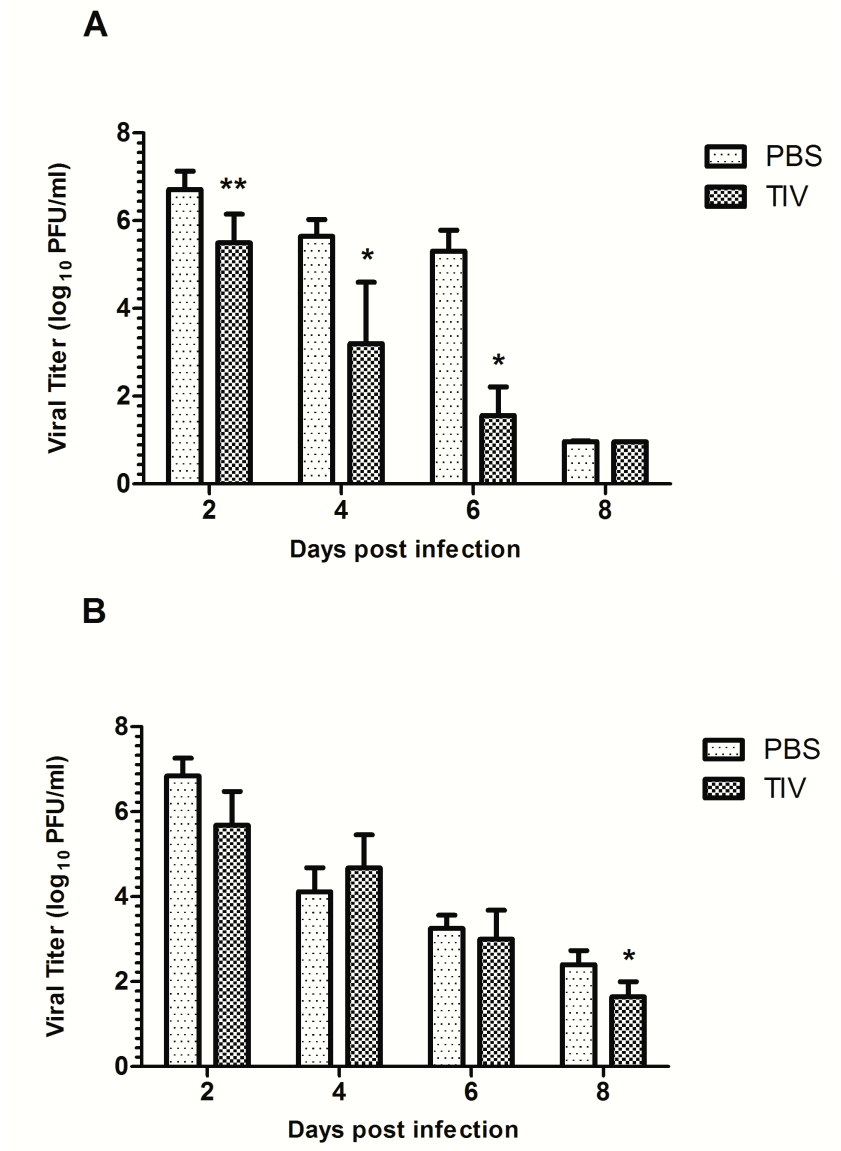

Figure 2.1 TIV vaccine efficacy following challenge with A/Perth/16/2009 (Perth/09) or A/Indiana/08/2011 (IN/11) virus. Nasal washes were collected with 1ml of PBS on even numbered days post challenge. Titers were determined by standard plaque assay on MDCK cells from challenge with A/Perth/16/2009 **(A)** or A/Indiana/08/2011 **(B)** virus. Bars display average values with standard deviation shown as error bars, with 5-6 ferrets per group. Differences in viral titers were analyzed by the Student's *t*-test (* $p \leq 0.05$, ** $p \leq 0.01$).

Chapter 3.

Impact of prior seasonal H3N2 influenza vaccination or infection on protection and transmission of emerging variants of influenza A(H3N2)v virus in ferrets

Submitted to *Journal of Virology*

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Contribution of other authors to this body of work: MBP and TT assisted with vaccination and handling of ferrets during animal studies. MBP and TT aided with mucosal wash sample collection.

3.1 Abstract

Influenza H3N2 viruses continue to circulate in swine and rarely infect humans, resulting in outbreaks of variant influenza A H3N2 [A(H3N2)v]. It has been previously demonstrated in ferrets that A(H3N2)v viruses transmit as efficiently as seasonal influenza viruses, raising concern over the pandemic potential of these viruses. However, A(H3N2)v viruses have not acquired the ability to transmit efficiently among humans, which may be due in part to existing cross-reactive immunity to A(H3N2)v viruses. Although current seasonal H3N2 and A(H3N2)v viruses are antigenically distinct from one another, historical seasonal H3N2 viruses share some antigenic similarity to A(H3N2)v viruses and previous exposure to these viruses may provide a measure of immune protection sufficient to dampen A(H3N2)v virus transmission. Here, we evaluated whether prior seasonal H3N2 influenza vaccination or infection affects virus replication and transmission of A(H3N2)v virus in the ferret animal model. We found that the seasonal trivalent inactivated influenza vaccine (TIV) or a monovalent vaccine prepared from an antigenically related 1992 seasonal influenza H3N2 (A/Beijing/32/92) virus failed to substantially reduce A(H3N2)v (A/Indiana/08/2011) virus shedding and subsequent transmission to naïve hosts. Conversely, ferrets primed by seasonal H3N2 virus infection displayed reduced virus shedding following A(H3N2)v virus challenge which blunted transmission to ferrets. A higher level of specific IgG and IgA titers antibodies detected among infected versus vaccinated ferrets was associated with the degree of protection offered by seasonal H3N2 virus infection. The data demonstrate in ferrets that the efficiency of A(H3N2)v transmission is disrupted by pre-existing immunity induced by seasonal H3N2 virus infection.

3.2 Introduction

Swine-origin influenza A H3N2 variant [A(H3N2)v] viruses have been responsible for numerous transmissions from pigs to humans since 2011. To date, there have been over 330 laboratory-confirmed human cases of A(H3N2)v virus infection and recently the first A(H3N2)v cases of 2013 were reported on June 28, 2013 (293). Although A(H3N2)v viruses generally induce mild symptoms similar to those normally seen with seasonal influenza, there have been 16 hospitalizations and one death reported in the U.S. since July, 2012 (293). An investigation of one of the first A(H3N2)v influenza cases detected at a U.S. state agricultural fair in 2011 identified 3 virologically confirmed cases, 4 seropositive probable cases and a further 82 suspected cases with respiratory illness associated with a fair visit, suggesting that only a minority of A(H3N2)v influenza cases are laboratory-confirmed (275, 283). In a retrospective cohort study conducted among children of an agricultural club who attended a fair, the risk for suspected case status increased with increasing exposure to swine (275).

The A(H3N2)v viruses that have infected humans originated in pigs following the introduction of hemagglutinin (HA) H3 and neuraminidase N2 genes from human seasonal H3N2 influenza viruses that circulated globally in the mid-1990s (275). The introduction of seasonal H3N2 virus into pigs also contributed to multiple reassortment events resulting in the emergence of a swine H3N2 virus with a triple reassortant internal gene (TRIG) cassette, containing a combination of avian, swine, and human influenza genes (275, 283). The H3 HA of human and swine influenza viruses followed divergent evolutionary pathways resulting in antigenically distinct influenza H3N2 viruses (284, 286, 294). Experimentally, the seasonal 2011-2012 trivalent inactivated influenza vaccine

(TIV) failed to generate a cross-reactive antibody response to A(H3N2)v virus in ferrets and offered no protection from A(H3N2)v virus challenge (295). However, A(H3N2)v viruses retained a low degree of serologic cross-reactivity with human H3N2 viruses that circulated in the early 1990's (284, 286). This is supported by human serology studies showing that young adults aged 18-39 years possess substantial levels of pre-existing cross-reactive antibodies to A(H3N2)v viruses (278-280). In contrast, children younger than 12 years of age, have little to no pre-existing cross-reactive antibodies to A(H3N2)v viruses (280). The observed immunity may exist in this population due to exposure to H3N2 influenza virus antigens through natural infection and/or vaccination during the 1990s.

The ferret model recapitulates the efficient transmission of seasonal influenza viruses and the poor transmission of avian influenza viruses in humans (296). Using this model, previous studies have shown that A(H3N2)v virus transmitted efficiently to naïve ferrets by respiratory droplets (228). However, there has been no evidence of sustained human-to-human transmission of A(H3N2)v virus and most of the rare, limited human-to-human transmission has occurred between children within familial clusters or within daycare settings (276, 297). It is conceivable that the lack of efficient A(H3N2)v virus transmission in humans is partly due to the presence of pre-existing cross-reactive immunity to this virus in the human population.

The impact of prior virus infection or vaccination on influenza virus transmission has been evaluated in only a limited number of studies. The ferret and guinea pig models have been used to evaluate the protection against A(H1N1)pdm virus transmission conferred by previous exposure to a seasonal influenza virus (298-300). Our laboratory

showed that prior immunization with seasonal (2008-2009) live attenuated influenza vaccine (LAIV) or infection with former seasonal H1N1 virus provides some cross-protection against A(H1N1)pdm virus challenge, but had no significant effect on the transmission efficiency of A(H1N1)pdm virus in ferrets (301). To date, animal studies have not been performed to assess the effect of seasonal H3N2 influenza vaccination or infection on the transmission of A(H3N2)v virus. Here, we used the ferret model to determine if the degree of protection offered by previous exposure to seasonal human viruses (historical or contemporary) could account for the limited capacity of these viruses to transmit among the human population. We demonstrate that prior H3N2 virus infection, but not immunization with seasonal TIV, substantially reduced A(H3N2)v virus shedding and subsequent transmission to naïve ferrets. Specific IgA and IgG antibody responses in serum and mucosal secretions were also measured to help explain the observed differences in protection induced by prior H3N2 infection versus seasonal influenza vaccination.

3.3 Materials and Methods

3.3.1 Viruses. The seasonal H3N2 viruses, A/Beijing/32/92 (Beijing/92) and A/Perth/16/2009 (Perth/09) were propagated in the allantoic cavity of ten-day-old embryonated eggs at 34°C for 48 h. The A(H3N2)v virus, A/Indiana/08/2011 (IN/11) was grown in Madin-Darby kidney (MDCK) cells for at 37°C for 48 h. IN/11 is a representative of H3N2v virus containing the M gene from A(H1N1)pdm09 virus (228). All virus stocks were clarified by centrifugation after collection and stored at -80°C. Stocks were titrated in a standard plaque assay on MDCK cells and expressed as plaque forming units (pfu) (110).

3.3.2 Whole-virus vaccine preparation. Virus was concentrated from large volumes of allantoic fluid by ultracentrifugation at 17,000 rpm for 3 h at 4°C. Concentrated viruses were purified over 30–60% sucrose cushion as previously described (302). The HA gene of each vaccine stock was confirmed by sequencing to ensure no inadvertent mutations were introduced during propagation of new vaccine stocks. Concentrated viruses were inactivated with 0.025% formalin at 4°C for 3 days. The treatment resulted in the complete loss of infectivity of virus, as determined by titration of vaccine preparations in eggs. The vaccine doses used are expressed as amount of total protein measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Evaluation of the HA protein content of purified vaccines was determined using Coomassie gel system as previously described (303). The HA protein was estimated to make up 40-55% of the total protein of purified vaccines.

3.3.3 Ferret immunizations. All animal experiments were performed in biosafety level 3 laboratories with enhancements (BSL3+) as outlined in Biosafety in Microbiological and Biomedical Laboratories (304). Animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. Adult male Fitch ferrets, aged five to eight months of age (Triple F Farms, Sayre, PA), serologically negative by hemagglutination-inhibition (HI) assay (described below) for currently circulating influenza viruses, were used in this study. Ferrets were vaccinated twice (3-5 weeks between vaccinations) intramuscularly (i.m.) with an adult human dose (0.5 ml prefilled syringe) of the seasonal (2011-2012) commercially available split-product TIV. In some experiments, ferrets were injected i.m. with 15 μg of inactivated whole-virus vaccine prepared from Beijing/92, Perth/09 or IN/11 virus. Prior to initial vaccination, vaccine boost and viral challenge, all ferrets were bled for collection of serum and analyzed by HI assay. For pre-challenge antibody titers, sera were collected 5 weeks following the final vaccination. For Perth/09 virus infections, ferrets were first anesthetized with ketamine-xylazine-atropine cocktail, given i.m. followed by a 1 ml intranasal (i.n.) inoculation (500μl per nostril) of 10^6 pfu of infectious virus diluted in PBS. Nasal wash titers were collected (described below) at day 3 post-infection (p.i.), and sera were collected at 6 weeks post-inoculation (p.i.) to confirm viral replication and seroconversion, respectively.

3.3.4 Virus challenge and transmission experiments. Baseline serum, temperature, and weight measurements were obtained before virus inoculations. Temperatures were

measured with a subcutaneous (s.c.) implantable temperature transponder (BioMedic Data Systems, Seaford, DE). For A(H3N2)v virus challenges, ferrets were inoculated with 10^6 pfu of virus as described above. Virus challenges occurred 5 weeks after the final vaccine boost, and 6 weeks after prior infection. For respiratory droplet transmission experiments, naïve contact animals were placed in adjacent cages 24 h after challenge using a previously established model (94, 296). Following inoculation, animals were monitored daily for changes in body weight and temperature as well as clinical signs of illness. Nasal washes were collected on alternate days post challenge (p.c.) with 1 ml PBS containing BSA and antibiotics (94, 296). Viral titers of the nasal washes were determined by plaque assay and sera were collected from the contact ferrets approximately 21 days after contact was established to check for seroconversion via HI.

3.3.5 Hemagglutination-Inhibition. HI assays were performed using infectious virus stocks or the World Health Organization (WHO) influenza reagent kit (according to instructions) obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention (Atlanta, GA). Sera were initially treated with receptor-destroying enzyme (RDE) from Vibrio cholerae (Denka Seiken, Tokyo, Japan) overnight at 37°C. The enzyme was then inactivated at 56°C for 30 min, and PBS was added to the sera for a final dilution of 1:10. The sera were then absorbed to turkey RBCs (tRBCs) for 30 min at 4°C to remove any nonspecific agglutinins. The HI assay was performed in V-bottom 96-well plates using 4 HAU of virus and 0.5% tRBCs. Ferret sera were tested for HI antibody titers against a selection of the following antigens:

Beijing/92, Perth/09, Ind/11, A/California/07/2009 (pH1N1), and B/Brisbane/60/2008 (Influenza B).

3.3.6 Ferret mucosal wash collection. Both upper respiratory tract (URT, sampled by tracheal/nasal wash) and lower respiratory tract (LRT, bronchoaveolar (lung) wash samples) were collected 5 weeks after prior infection or final vaccination using a novel protocol adapted from protocols established for mice (305). Ferrets were exsanguinated by cardiac puncture under ketamine anesthesia to best avoid blood contamination of the mucosal samples. The chest cavity was opened to expose the lungs and an incision was made to expose the trachea. The trachea was cut just below the larynx and clamped before the entire respiratory tract was carefully removed with the heart. For LRT washes, a volume of 10 mL of PBS containing antibiotics was infused slowly down the trachea into the lung using a pipette. After filling, the lungs were gently massaged, inverted and the fluid was allowed to drip back through the trachea collected in 35-mm petri dishes. Approximately 6-8 mL of fluid was retrieved and immediately kept on ice. The URT washes from the same animals were collected by flushing 5 ml of PBS with antibiotics through the open tracheal incision and forward into the nasal passages 3 times. Supernatant from lavage fluid was collected following centrifugation at 200g at 4°C for 10 min and frozen at -20°C until analyzed by ELISA. Any sample with blood contamination was excluded from analysis.

3.3.7 ELISA. Ferret IgG and IgA antibodies were detected using a modified procedure of enzyme-linked immunosorbent assay (ELISA) as previously described (200, 302).

Flat-bottom 96-well microtiter plates (Immulon II; Dynatech Laboratories, Chantilly, VA) were coated with 100 hemagglutinating units per well of purified Perth/09 inactivated virus overnight at 4°C. Serum samples were added to the plate in 4-fold serial dilutions starting at 1:100 for IgG and 1:20 for IgA. LRT/URT samples were added at starting dilutions of 1:10 for IgG and 1:2 for IgA. Bound antibody was detected by IgG HRP conjugated antibody (Bethyl Laboratories, Montgomery, TX) or by a goat anti-ferret IgA followed by a rabbit anti-goat HRP conjugated antibody (Bethyl). The absorbance was measured at 490 nm following addition of OPD in citrate buffer (Sigma). Titers are expressed as the highest dilution that yielded an optical density greater than the mean plus two standard deviations of similarly diluted PBS control sera.

3.3.8 Statistics. Statistical significance was determined by one of the following methods: student's t-test, 2-way ANOVA with a Bonferroni post-test, or Area Under the Curve (AUC) with a Mann-Whitney post-test.

3.4 Results

3.4.1 Transmission of A(H3N2)v and seasonal H3N2 virus.

To examine whether seasonal H3N2 vaccination or prior infection affects the transmission efficiency of A(H3N2)v or homologous seasonal H3N2 viruses, we first determined the transmission efficiency of these viruses in naïve ferrets. For respiratory droplet transmission, ferrets were housed in adjacent cages with a perforated side wall, allowing air exchange between ferrets in the absence of direct or indirect contact (94, 296). Ferrets were inoculated with 10^6 pfu of Perth/09, representative of H3N2 viruses circulating in the 2011-2012 northern hemisphere season. Nasal washes were collected every other day and clinical signs were monitored daily from both inoculated and contact ferrets. All Perth/09 virus inoculated ferrets exhibited a spike in temperature over baseline (Table 1) and shed high titers of infectious virus as early as day 1 that were sustained at titers of $\geq 10^{4.0}$ pfu/ml for 5 days p.i. (Figure 1A). Although viral titers were detected in the nasal washes of only 2 of the 3 contact ferrets, all three animals displayed body temperature elevation and seroconverted to Perth/09 virus (Table 1). Thus, consistent with the experimental transmission data obtained with other seasonal human influenza viruses (94, 296), Perth/09 H3N2 virus exhibited efficient respiratory droplet transmission.

Ferrets inoculated with A(H3N2)v (IN/11) virus exhibited modest weight loss and a maximum temperature increase of 2.2°C on day 2 p.i. (Table 1). Similar to the results described for the seasonal Perth/09 virus, IN/11 virus replicated efficiently in the upper respiratory tract of inoculated ferrets and spread efficiently to contact ferrets (Figure 1B).

The results are consistent with earlier ferrets studies which found that A(H3N2)v viruses isolated since 2010 exhibited efficient respiratory droplet transmission (228).

3.4.2 Transmission of A(H3N2)v virus following seasonal TIV immunization.

To test whether seasonal influenza vaccination reduces the transmission of A(H3N2)v virus to naïve ferrets, experiments were set up in which TIV-immune ferrets were challenged with IN/11 virus and the subsequent transmission to naïve animals was analyzed. Prior to virus challenge, all six vaccinated ferrets displayed HI titers of ≥40 to each of the three influenza HA antigens present in the 2011-2012 TIV, but lacked detectable cross-reactive HI antibodies to IN/11 virus antigen (Table 2). Seasonal TIV immunization did not protect ferrets from weight loss, fever (Table 1) or virus replication (Fig. 2A) following IN/11 virus challenge. As late as 5 days p.i., virus titers of $\geq 10^{2.6}$ PFU/ml were still present in nasal washes of all vaccinated ferrets and A(H3N2)v virus spread to six of nine naïve contact ferrets (Fig. 2A). Virus transmission occurred with kinetics similar to that observed in naïve ferrets with the majority of contacts displaying viral titers in their nasal washes by day 3 p.c.

A separate group of TIV-immune ferrets were challenged with homologous Perth/09 virus for comparison. Similar to IN/11 virus challenge of TIV-immune ferrets, the inactivated influenza vaccine did not markedly reduce the level of homologous virus replication and all of the Perth/09 inoculated ferrets displayed relatively high viral titers ($\geq 10^{2.1}$ PFU/ml) in their nasal washes for 5 days p.i. (Fig. 2B). Despite the pre-challenge HI titers of ≥40 to homologous virus, respiratory droplet transmission of Perth/09 virus

still occurred. Of the nine total respiratory droplet pairs, four inoculated ferrets transmitted to their naïve contacts resulting in seroconversion to Perth/09 virus (Table 2).

3.4.3 Impact of vaccination of a historical seasonal H3N2 virus on A(H3N2)v virus transmission.

Serologic studies have previously demonstrated that the young adult population has the highest percentage of individuals with pre-existing cross-reactive antibodies to A(H3N2)v virus (278-280). The individuals within this age range would have encountered antigenically related H3N2 viruses through virus infection or vaccination in the 1990s. We first performed antigenic analyses of 6 representative seasonal H3N2 viruses (spanning the years 1990-2013) by HI assay and the use of reference ferret antisera. The only seasonal H3N2 virus that displayed limited cross-reactivity with antisera to A(H3N2)v virus was A/Beijing/32/92 (Beijing/92) virus, a component in the 1993-1994 seasonal TIV. Ferret antisera to Beijing/92 virus infection reacted to low titers (HAI titer = 20) to IN/11 virus. These results are consistent with previous antigenic characterization which demonstrated that A(H3N2)v retain antigenic properties more similar to human seasonal viruses from the early 1990s than from currently circulating human A(H3N2) viruses (284).

We next assessed the ability of vaccination with inactivated whole-virus monovalent vaccines prepared from Beijing/92, Perth/09 and IN/11 viruses to limit infection and transmission of A(H3N2)v. Groups of ferrets were vaccinated i.m. with 15 μg of formalin-inactivated vaccine and two boosts in order to obtain HI titers of ≥40 against the homologous viral strain. HI antibody titers of ≥ 40 have been associated with

protection from influenza illness in up to 50% in adult populations (306-308). The vaccines failed to elicit cross-reactive HI antibodies against heterologous H3N2 or A(H3N2)v viruses using the standard starting dilution of 1:10 serum (Table 2). However, a repeat of the HI test using a starting dilution of 1:4 revealed that Beijing/92-immune sera gave HI titers of 4-8 to IN/11 virus, whereas Perth/09 immune sera had undetectable (<4) HI titers to IN/11 virus (not shown). Five weeks after the final vaccine boost, ferrets were set up in transmission cages and vaccinated ferrets were challenged with 10^6 pfu of IN/11 virus. As indicated in Fig. 3A, relatively high viral titers were detected in the nasal washes of Perth/09-vaccinated ferrets and transmission of IN/11 virus occurred in 2 of the 3 contact animals. Furthermore, Perth/09-vaccinated ferrets displayed weight loss and fever consistent with that observed in unimmunized PBS controls (Table 1). Despite the low HI cross-reactivity to IN/11 virus, Beijing/92 vaccinated ferrets also shed high titers of infectious virus and full transmission of IN/11 virus was observed to naïve contacts (Fig. 3B). All three of the contacts began to shed virus by day 3 p.c., and seroconverted to A(H3N2)v virus (Table 1). Consistent with immunized controls, Beijing/92-vaccinated ferrets displayed a mean maximum weight loss of 7.5% and a fever spike of 1.3°C (Table 1). For comparison, the transmission efficiency of IN/11 virus was performed among homologous IN/11-vaccinated ferrets. In contrast to the lack of protection against IN/11 virus transmission conferred by heterologous vaccination, we found that homologous virus challenge of IN/11-vaccinated ferrets blunted transmission to the naïve contact ferrets (Fig. 3C). Viral titers were only detected in the nasal washes of the inoculated ferrets on day 1 p.c. and vaccinated animals displayed marginal weight loss (1.6 %) and reduced fever (0.8°C) (Table 1). Taken together, monovalent vaccines

prepared from Perth/09 virus or an antigenically related seasonal influenza H3N2 (Beijing/92) virus failed to substantially reduce A(H3N2)v virus shedding and subsequent transmission to naïve hosts. Furthermore, the vaccine prepared from the 1992 seasonal H3N2 virus (Beijing/92) offered no added benefit over a contemporary seasonal H3N2 vaccine.

3.4.4 The effect of prior seasonal H3N2 virus infection on cross-protection and transmission of A(H3N2)v virus.

Next, we evaluated the level of cross-protection that develops from infection with a seasonal H3N2 virus. Although contemporary seasonal viruses are antigenically distinct from the A(H3N2)v viruses, prior influenza virus infection generally induces an effective mucosal immune response and may provide greater cross-protection compared to that induced by inactivated influenza vaccines given parenterally (217, 299). Ferrets were inoculated i.n. with 10^6 pfu of Perth/09 virus, or PBS for controls, and challenged 6 weeks later with either Perth/09 or IN/11 virus. Following homologous virus challenge with Perth/09, prior infection resulted in significantly lower viral titers in their nasal washes compared to PBS controls on all days p.c ($p=0.004$, $p=0.004$, and $p=0.003$ for days 2, 4, and 6 respectively) (Fig. 4A). Of the six ferrets in the prior infection group, 5 displayed a complete lack of detectable virus in nasal washes. Immunization with Perth/09 virus also significantly lowered viral titers in nasal washes following heterologous IN/11 virus infection ($p=0.002$, $p=0.004$, and $p=0.003$ for days 2, 4, and 6 respectively) (Fig. 4B). Moreover, prior infection lowered the mean maximum weight

loss by more than half (5.9% in PBS controls compared to 2.3% for Perth/09 immune ferrets) (Table 3).

Next we assessed the effect of H3N2-immunity on transmission of A(H3N2)v (IN/11) virus to naïve animals. For comparison, a group of H3N2-immune ferrets received homologous Perth/09 virus challenge. No virus shedding was detected from any of the six Perth/09 challenged animals on any day post challenge. Moreover, Perth/09-immune ferrets displayed minimal weight loss (maximum weight loss = 2.1%) and fever (maximum temperature increase = 0.7°C). Furthermore, none of the contact ferrets shed virus or showed a serological response to Perth/09 virus (Fig. 4C) (Table 1). Following heterologous virus challenge, IN/11 virus was detected in the nasal washes of Perth/09-immune ferrets, but only through day 3 p.c. (Figure 4D). Only one of six contact ferrets shed infectious virus and seroconverted to IN/11 virus. Taken together, the results show that prior seasonal virus infection effectively limited virus growth and blunted transmission of A(H3N2v) virus.

3.4.5 IgG and IgA in upper and lower respiratory tracts of vaccinated and previously infected ferrets.

To elucidate the differences observed in transmission efficiency of A(H3N2)v virus following virus infection versus vaccination, pre-challenge sera and mucosal washes were collected and tested for antiviral IgG and IgA antibodies. Non-immune and immunized ferrets were necropsied for collection mucosal washes from the upper respiratory tract (URT) and lower (LRT, bronchoaveolar - lung). In general, IgG and IgA responses among vaccinated and previously infected ferrets were increased compared to

naïve animals. As shown in Table 4, the highest antibody titers were IgG titers observed in serum and LRT of ferrets that received prior infection. Lung IgG titers were approximately 10,000-fold higher than those induced by TIV immunization. Serum IgA titers induced by either mode of immunization were similar, however prior infection induced IgA responses in the URT that were 16-fold higher than those elicited by vaccination. Taken together, Perth/09 virus infection resulted in consistently higher IgG and URT IgA titers compared to TIV immunization.

3.5 Discussion

New reports of human infections with A(H3N2)v in 2013 continue to raise public health concerns and highlight the need to better understand the potential of these viruses to transmit efficiently among humans (293). A paradox exists with regard to the transmission efficiency of A(H3N2)v virus in humans versus that observed in the naïve ferret model. Sustained or community-wide transmission of A(H3N2v) has not occurred, whereas these viruses transmit efficiently between immunologically naïve ferrets (228). We hypothesized that pre-existing immunity to seasonal H3N2 viruses present in the general population accounts for the contrasting results. Vaccination is currently the best public health measure to prevent influenza infection and human epidemiological studies have clearly established that immunization of those who are most likely to acquire and transmit influenza (usually children) can reduce the risk of secondary transmission of influenza within families (306, 307). However, the impact of prior seasonal influenza vaccination on influenza virus transmission has not been extensively modeled in animals (299). In this study, ferrets were used to model transmission of A(H3N2)v virus following pre-exposure by seasonal H3N2 virus infection or vaccination. The data presented suggest that vaccination with inactivated seasonal influenza vaccines would not provide any significant protection against A(H3N2)v virus infection and subsequent transmission of the virus. Conversely, prior infection with a contemporary seasonal H3N2 virus effectively limited virus shedding and blunted subsequent transmission of A(H3N2v) virus. The cross-protective response induced by influenza virus infection was associated with higher levels of virus-specific IgG and IgA antibodies compared to ferrets that received inactivated influenza vaccines.

In inability of seasonal TIV (2011-2012) immunization to block transmission of A(H3N2)v virus to naïve contact animals prompted us to prepare a monovalent vaccine to a seasonal influenza H3N2 virus, Beijing/92, that showed some, albeit low antigenic similarity with A(H3N2)v virus using post-infection ferret antisera (284, 286). For comparison, a monovalent Perth/09 vaccine, the H3N2 virus component of the seasonal (2011-2012) TIV was similarly tested. Although Beijing/92 virus is more closely related antigenically to A(H3N2)v (IN/11) virus, as an inactivated vaccine it failed to provide greater protection than that observed in ferrets vaccinated with monovalent Perth/09 vaccine. Thus, in our hands, a distinction could not be made between the historical and contemporary H3N2 inactivated vaccines administered parenterally in ferrets. It is conceivable that the H3N2 whole-virus vaccines induced non-HA immune responses that masked any differences that would have been observed from vaccinating with HA only. A different vaccine formulation, such as virus-like particle (VLP) or recombinant HA proteins, in which the HA proteins are the primary antigens, may yield differences in protection against A(H3N2)v virus challenge in ferrets. Alternatively, a vaccine dose–response study would have revealed vaccine efficacy differences between the historical and contemporary seasonal H3N2 vaccines.

In contrast to results obtained with inactivated vaccines, we found that ferrets previously exposed to seasonal H3N2 influenza virus exhibited stronger immune protection and transmission was blocked following subsequent challenge with homologous or heterologous virus. Prior infection with Perth/09 virus dramatically reduced transmission rates from 100% in naïve ferrets to 0% following homologous virus challenge, and 17% for heterologous challenge. However, the impact of prior influenza

virus infection on limiting transmission appears to be dependent on the transmission model and/or the mode of viral transmission. Using a direct contact transmission (co-housed) model, Ellebedy *et al.* reported that prior H1N1 virus infection was unable to alter transmission in ferrets following challenge with A(H1N1)pdm virus (299). These results are consistent with a study by Laurie *et al.* showing that prior infection with A(H1N1)pdm virus did not significantly alter A(H1N1)pdm virus transmission in a direct contact ferret model (309). Interestingly, the latter study demonstrated that multiple pre-infections of seasonal H1N1 virus were required to reduce A(H1N1)pdm virus transmission. Using guinea pigs in a direct contact model, two studies found that transmission of influenza virus could be disrupted when guinea pigs had prior exposure to seasonal virus (299). However, unlike our study, these studies did not assess test the impact of prior influenza infection on respiratory droplet (or airborne) transmission. More of an effect on transmission may have been observed in our studies since the respiratory droplet model is a more stringent test and inhibits any direct or indirect contact transmission from occurring between the ferrets.

To understand the immunologic bases conferring protection by virus infection, we compared the IgG and IgA antibody levels present in serum and respiratory tract washings. Both routes (vaccination or infection) of immunization induced high and similar levels of antibodies in the serum; however virus infection induced consistently higher specific IgG and IgA antibody titers in the respiratory tract compared to the respiratory tracts of TIV immunized ferrets. The differences in IgA and IgG antibodies between the immunization groups were not evident using the standard HI assay. These results are consistent with the ability of a live attenuated influenza vaccine to elicit higher

levels of nasal wash IgA and greater reduction of virus shedding compared with an inactivated influenza vaccine (200, 302). Although the precise immunological mechanism(s) involved in viral clearance was not addressed in the current study, it is reasonable to speculate that raised antibody levels in the ferret respiratory tract of virus infected ferrets would have a greater capacity to neutralize virus infectivity and blunt transmission.

Because of the paucity of immunological reagents for ferrets, the role of T cell immunity and cytokines involved in conferring protection by infection was not addressed in the current study. Parenterally administered (unadjuvanted) vaccines, such as TIV do not effectively induce influenza-specific $CD8^+$ T cells or mucosal immune responses, two immune effectors capable of protecting the host (310, 311). $CD8^+$ T-cells responses are important in heterologous protection, and children have been shown to have weaker heterologous responses following vaccination compared to adults (312). Although non-HA components in the TIV have been shown previously to contribute to the immune response and these components may contain conserved T cell epitopes (192, 313), such cross-reactive immunity was not sufficient to provide protection against A(H3N2)v virus transmission. The effects of this improved immune response following seasonal H3N2 infection on subsequent viral transmission need further investigation.

The potential of A(H3N2)v virus to mutate or undergo additional reassortment events, and thus improve its transmissibility is of concern. Determining the level of immunity required to blunt transmission of novel influenza viruses, such as A(H3N2)v could deepen our understanding of how pandemic influenza subtypes arise and spread in humans. It is apparent from this study and others (discussed above) that influenza virus

transmission is influenced by the infection history of the host. The current data suggest that inactivated vaccines would only be effective at reducing A(H3N2)v virus shedding and subsequent transmission if the vaccine were A(H3N2)v-specific. Whereas prior infection with infectious virus, capable of inducing protective mucosal immunity, can generate heterologous protection in the absence of specific A(H3N2)v antibody. One caveat to our studies is that the immune response to vaccination was only studied in naïve ferrets, while the TIV has been shown to boost preexisting immunity in humans and result in limited mucosal antibody production (314). There are certainly additional unknown factors contributing to the poor transmission of A(H3N2)v virus in humans, especially in light of the fact that the largest concentration of confirmed A(H3N2)v infections have been in children that have the least previous exposure to influenza, and the most limited pre-existing serological and heterologous $CD8^+$ T-cell responses against these viruses (278, 279).

3.6 Acknowledgements

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Table 3.1. Clinical symptoms of inoculated and contact ferrets

Treatment	Challenge virus	Maximum mean weight loss (%)[a]		Maximum mean temp increase (°C)[b]		Contact Seroconversion[c]
		Inoculated	Contacts	Inoculated	Contacts	Contacts
None						6/6
	Perth/09	4.3	2.0	1.0	0.9	3/3 (640)
	IN/11	5.8	2.8	2.2	1.6	3/3 (1015)
TIV						10/18
	Perth/09	6.3	3.0	1.3	1.0	4/9 (1076)
	IN/11	7.3	6.0	1.1	1.5	6/9 (640)
Monovalent Vaccine						5/9
Perth/09 Vaccine	IN/11	8.1	6.3	1.3	1.1	2/3 (453)
Beijing/92 Vaccine	IN/11	7.5	6.0	1.3	1.2	3/3 (640)
IN/11 Vaccine	IN/11	1.6	NA	0.8	NA	0/3 (<10)
Perth/09 Infection						1/12
	Perth/09	2.1	NA	0.7	NA	0/6 (<10)
	IN/11	3.8	3.5	1.1	1.7	1/6 (1280)

[a] Percentage of mean maximum weight loss shown for each ferret group.

[b] Mean maximum mean increases calculated as rise over ferret baseline of 38.3 ±0.5 °C. All maximum temperatures observed on days 1-2 p.c.

[c] Seroconversion measured by HI from sera collected on 21 p.c. Geometric mean titer of seroconverted contact ferrets in parentheses. <10 is below limit of detection for the HI assay.

Table 3.2. Pre-challenge Hemagglutination-Inhibition (HI) antibody responses to immunization in ferrets

Treatment	Challenge virus	Challenge Experiment [a]	Ferrets per group	HI antibody titer [b]			
TIV		**Transmission**	18	**pH1**	**H3**	**B**	**IN/11**
	Perth/09		9	80-640 (235)	40-640 (118)	40-320 (86)	<10[c]
	IN/11		9	40-640 (127)	40-640 (109)	20-160 (50)	<10
Monovalent Vaccine		**Transmission**	9	**Perth/16**	**Beijing/32**	**IN/11**	
Perth/09 Vaccine	IN/11		3	160-320 (254)	<10	<10	
Beijing/92 Vaccine	IN/11		3	<10	40-80 (50)	<10	
IN/11 Vaccine	IN/11		3	<10	<10	80-160 (127)	
Perth/09 Infection		**Protection**	12	**Perth/16**	**IN/11**		
	Perth/09		6	640-1280 (905)	<10		
	IN/11		6	640-2560 (1280)	<10		
Perth/09 Infection		**Transmission**	12	**Perth/16**	**IN/11**		
	Perth/09		6	320-1280 (507)	<10		
	IN/11		6	640-1280 (806)	<10		

[a] Each experiment was divided into groups based on vaccine tested and by challenge virus.

[b] HI titers per virus separated by experiment. Antibody titer range within each group of ferrets shown. Geometric mean included in parentheses.

[c] <10, below the limit of detection in this assay

Table 3.3. The effect of prior seasonal H3N2 virus infection on protection against homologous and heterologous challenge

Treatment	Challenge Virus	Max. mean weight loss (%)[a]	Max. mean temp increase (°C)[b]	Mean Peak Viral Titer[c]
Perth/09 Protection				
Perth/09 Infection	Perth/09	1.7	1.1	1.2±0.6
PBS controls	IN/11	1.9	1.9	5.9±0.7
Perth/09 Infection	Perth/09	2.3	2.0	2.7±0.4
PBS controls	IN/11	5.9	1.8	5.2±0.5

[a] Percentage of mean maximum weight loss shown for each ferret group.

[b] Mean maximum termperature increases calculated as rise over ferret baseline of 38.3 ±0.5 °C. All maximum temperatures observed on days 1-2 p.c.

[c] Mean peak titer shown as $\log_{10}$ PFU/ml including standard deviation. All peak titers from day 2 p.c.

Table 3.4. Antiviral IgG and IgA antibody responses among vaccinated and previously infected ferrets

	Serum		URT Wash		LRT Wash	
Antibody	Infected	Vaccinated	Infected	Vaccinated	Infected	Vaccinated
IgG	409600[1]	102400	160	ND	409600	40
IgA	1280	1280	128	8	8	2

[1]Titers from 4 ferrets per group are expressed as largest dilution of sample having a mean optical density greater than the mean plus two standard deviations (SD) of similarly diluted control (unimmunized) sample.

ND= not detected, no dilution read above the mean+2SD of the control sample.

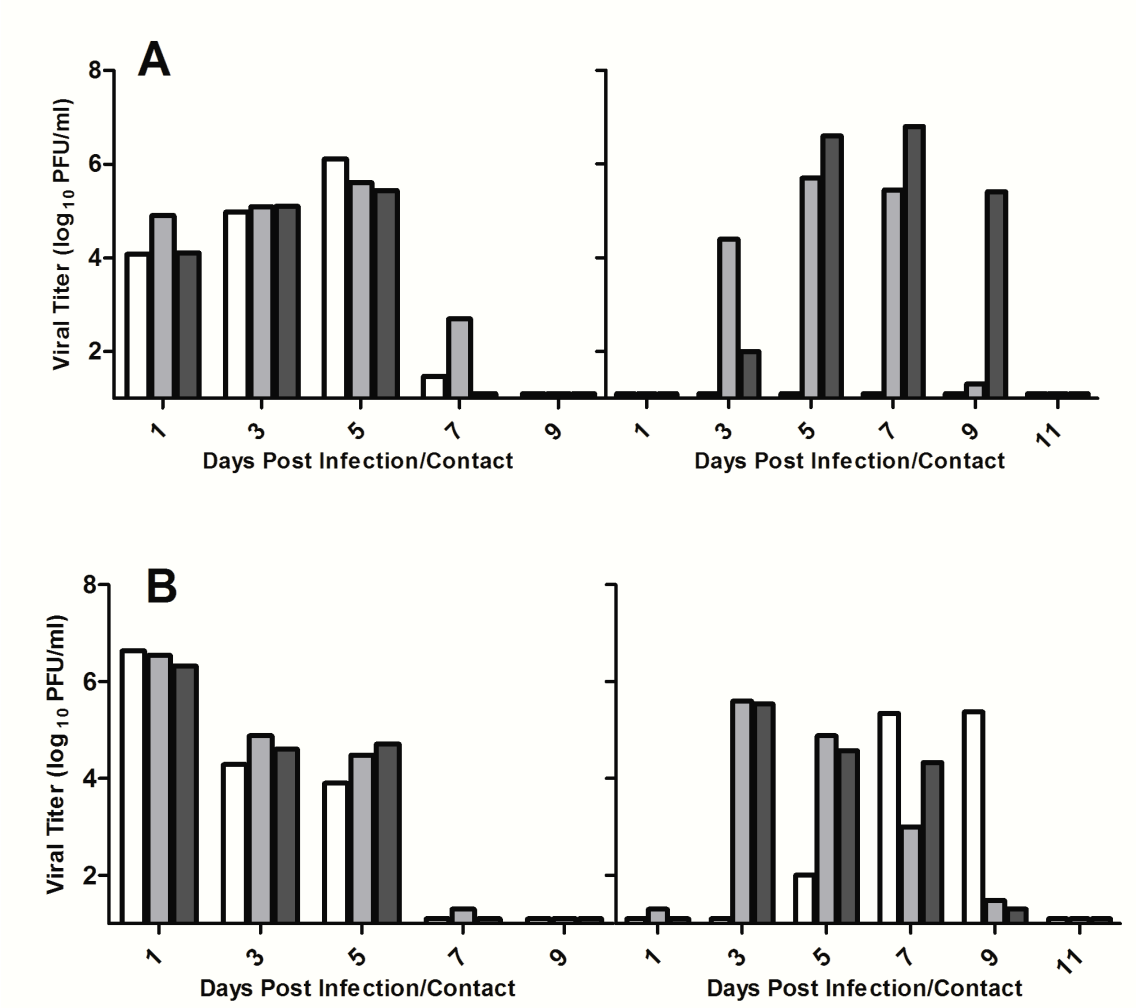

Figure 3.1. Transmissibility of seasonal H3N2 and A(H3N2)v influenza viruses among naïve ferrets in the respiratory droplet model. Ferrets were inoculated i.n. with 10^6 pfu of virus with 3 transmission pairs per experiment. Contact animals were placed in adjacent cages with perforated sidewalls 24 hours post inoculation. (A) Transmission of seasonal H3N2 virus, Perth/09. (B) Transmission of A(H3N2)v virus, IN/11. Each bar represents the viral titer detected in nasal washes of inoculated ferrets (left half of figure) and their corresponding contact animal (right half). Limit of virus detection 1 $\log_{10}$ PFU/ml.

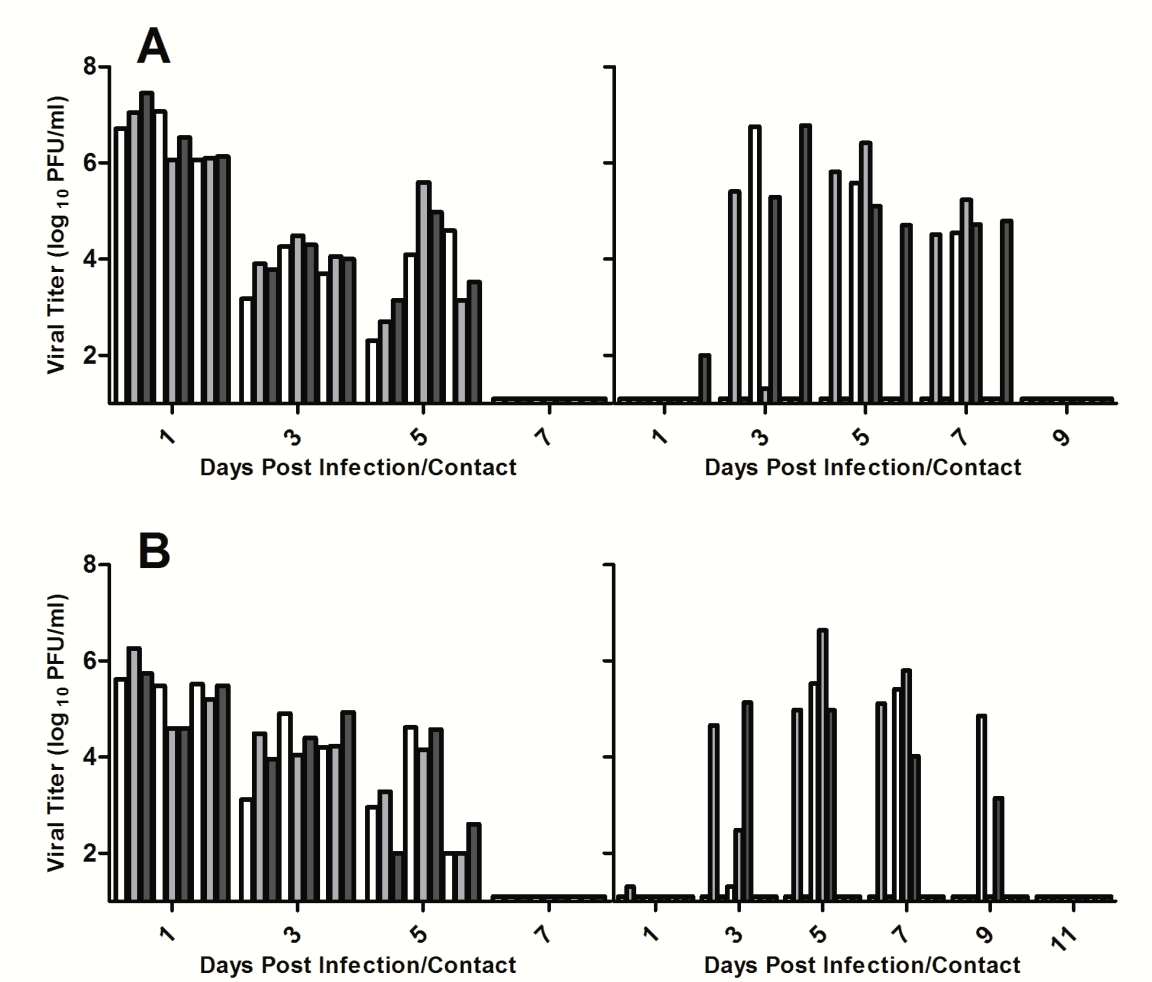

Figure 3.2. Transmission of seasonal H3N2 and A(H3N2)v viruses following vaccination with the seasonal TIV. Five weeks after final vaccine boost, TIV-immune ferrets (were challenged with heterologous IN/11 virus (A) or homologous seasonal Perth/09 virus (B). Each bar represents the viral titer detected in nasal washes of inoculated ferrets (n =6) and their corresponding contact animal (n =6). Limit of virus detection 1 $\log_{10}$ PFU/ml.

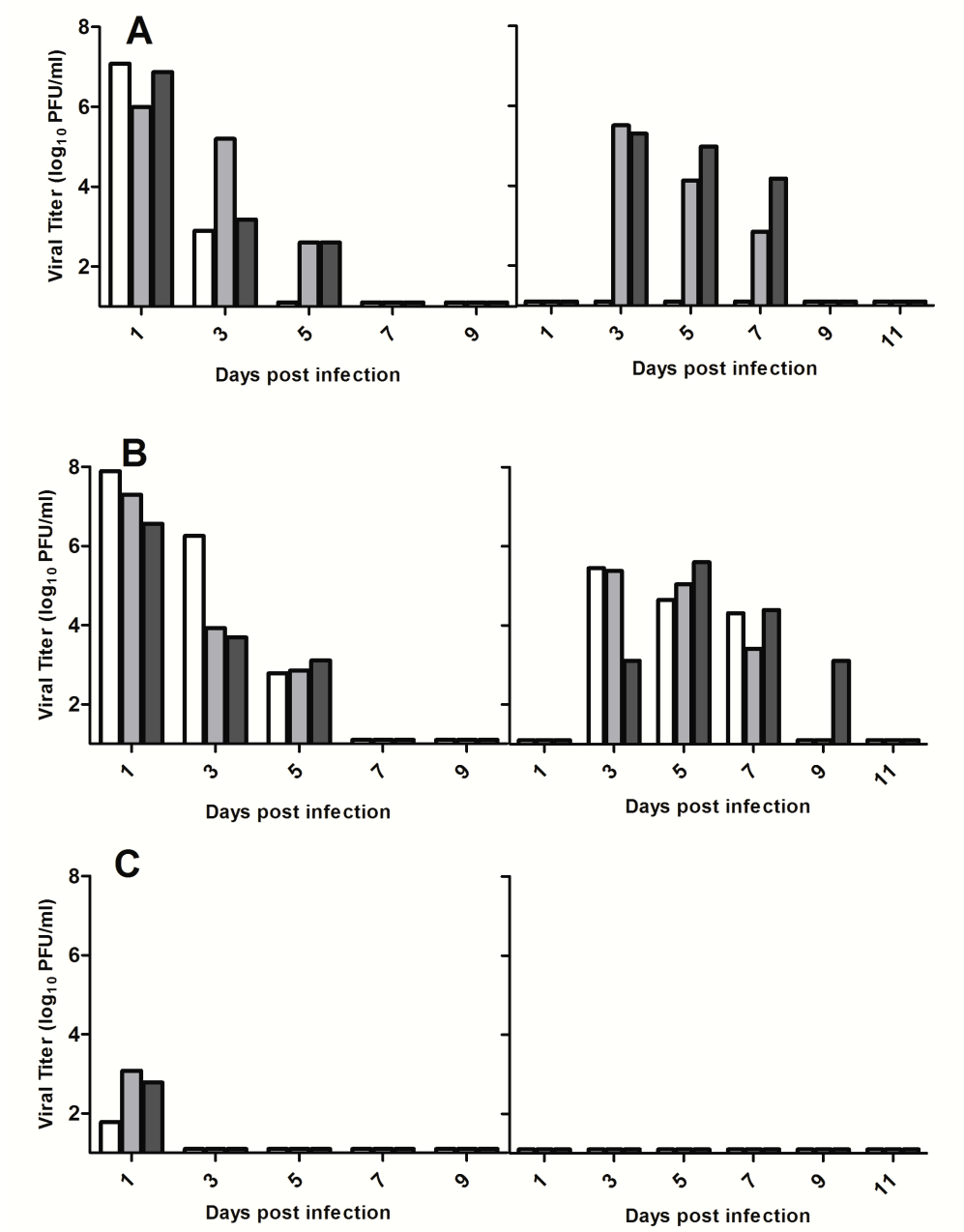

Figure 3.3. Impact of vaccination of a historical seasonal H3N2 virus on A(H3N2)v virus transmission. Five weeks after final vaccine boost, monovalent vaccinated ferrets were placed into transmission cages and challenged with IN/11 virus. Groups of ferrets were vaccinated with inactivated vaccines prepared from Perth/09 (A), Beijing/92 (B) or IN/11 (C). Transmission from inoculated (vaccinated) ferrets to naïve contacts was determined. Limit of virus detection 1 $\log_{10}$ PFU/ml.

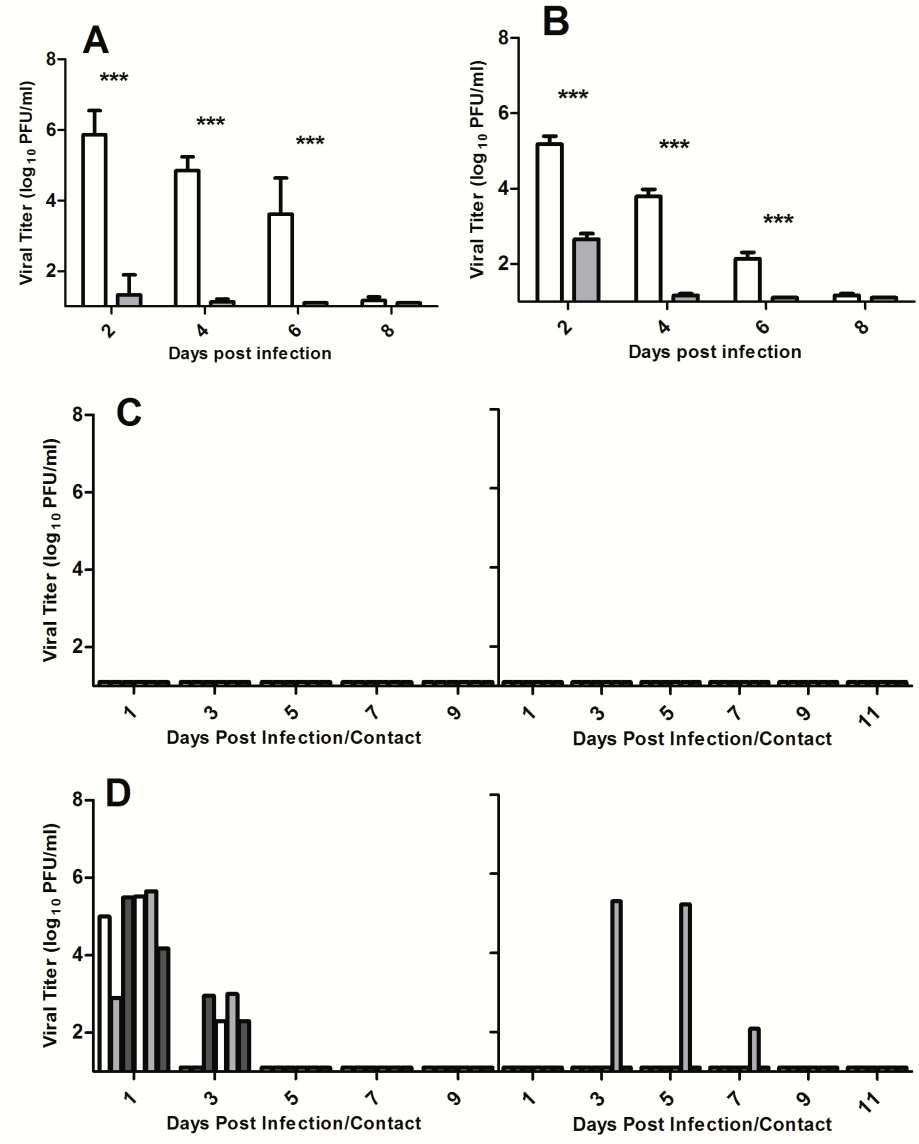

Figure 3.4. The effect of prior seasonal H3N2 virus infection on cross-protection and transmission of A(H3N2)v virus. Six weeks after prior seasonal H3N2 virus infection, Perth/09-immune ferrets were placed into transmission cages and challenged with homologous Perth/09 (A and C) or heterologous IN/11 (B and D) virus. Protection against challenge measured (A and B) represent virus titers in nasal wash samples of naïve ferrets (white bars), and Perth/09-immune (grey bars). Error bars correspond to standard deviation. *** $p \leq 0.005$ by Mann-Whitney nonparametric t-test. Transmission (C and D) from inoculated (Perth/09-immune) ferrets to naïve contacts was determined. Limit of virus detection 1 $\log_{10}$ PFU/ml.

Chapter 4.

Determination of the molecular mechanisms of influenza pathogenesis and transmission of avian H5N1-human H1N1 recombinant viruses

Unpublished data

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Contribution of other authors to this body of work: TT helped with project design and sample collection during the ferret studies. HZ assisted with the Calu-3 studies and immunofluorescence staining. CP aided with the donation of universal primers and the creation of the 1918 and DK/NY plasmids.

4.1 Abstract

Avian H5N1 influenza viruses continue to pose a pandemic threat. Highly pathogenic avian influenza H5N1 viruses can cause severe disease in humans, but do not readily transmit from person to person. If H5N1 viruses acquire the ability to easily spread among humans, a new pandemic virus could emerge that is capable of causing high mortality rates. To better understand the molecular determinants that could contribute to the emergence of a mammalian transmissible H5N1 virus, we used a reverse genetics approach to determine the specific contributions of individual influenza genes from both a seasonal H1N1 virus and an avian H5N1 virus towards a transmissible phenotype in a mammalian model. Specific mutations of the H5 hemagglutinin (HA) protein that facilitates binding to human-type receptors and confers efficient transmission also were examined. Recombinant H5 HA mutant viruses were assessed for their replication ability in human airway epithelial cells. Studies to analyze the pathogenesis and transmissibility of these viruses in the ferret animal model were planned; however, a moratorium on H5N1 virus "gain-of-function" experiments occurred before the viruses that were created could be assessed.

4.2 Introduction

Novel influenza A viruses that arise by genetic reassortment between human and avian influenza viruses have the potential to cause a pandemic if they are capable of spreading rapidly and causing widespread disease in immunologically naïve populations (235, 236). Each of the pandemic viruses that appeared in the last hundred years contained influenza genes of avian origin. The PB2 and PA genes in the A(H1N1)pdm09 virus originated from an avian source, as did the HA and PB1 genes in the 1968 H3N2 pandemic virus, and the HA, NA, and PB1 genes in the 1957 H2N2 pandemic virus (237, 240, 241, 244). The 1918 pandemic strain may have been composed entirely of avian genes that directly infected the human population without prior adaptation (238, 239). Non-pandemic strains of H5, H6, H7, H9, and H10 influenza virus subtypes of avian origin have infected humans and are capable of causing severe disease resulting in death, but remain unable to transmit efficiently and in a sustained manner among humans (248, 315, 316).

The first documented emergence of highly pathogenic avian influenza (HPAI) H5N1 viruses in humans occurred in 1997 in Hong Kong, infecting 18 individuals and causing 6 deaths (253). These early H5N1 viruses were eradicated by the mass slaughter of poultry in Hong Kong, but in 2001-2004 new genetic lineages of H5N1 emerged in domestic and subsequently wild birds, providing an ongoing source for occasional human infections. This subtype has led to 633 confirmed human cases and 377 deaths in 15 countries since 2003, according to the World Health Organization (248). Many H5N1 viruses are highly pathogenic in humans following infection from contact with infected poultry or contaminated environments (317-319). Unlike seasonal influenza viruses,

which mainly infect the upper respiratory tract, avian HPAI H5N1 viruses are capable of spreading to and replicating in extrapulmonary organs of mammals. This systemic spread of infection is due in part to the multibasic cleavage site in HPAI H5 HA proteins, which is recognized by a broader range of non-tissue specific proteases (256, 257). H5N1 viruses currently lack the ability to spread efficiently from person to person, despite the detection of virus in the upper respiratory tract of humans during infection (320). However, this subtype continues to cross the species barrier to infect humans, thereby increasing the likelihood of adaptation and the subsequent generation of H5N1 viruses that transmit efficiently in humans.

Studies in ferret model revealed that simply replacing the external genes of an avian H5N1 strain with the HA and NA genes from an H3N2 virus was not sufficient to allow the avian virus to transmit (296). Previous studies in our laboratory involving non-H5N1 viruses by Van Hoeven et al have used human H1N1 (A/SC/1/18) and avian H1N1 (A/Dk/NY/15024-21/96) recombinant viruses to demonstrate that the human HA, NA, and PB2 genes (1918HANAPB2:Dk/NY) were sufficient for respiratory droplet (RD) transmission of the recombinant avian virus (94). However, this research did not investigate whether the human NA gene was necessary for transmission to occur. These studies indicate that other alterations (such as point mutations of the HA) and additional human influenza virus genes may be required for efficient transmission of avian H5N1 viruses in mammals. Determination of these genes for the avian H5N1 viruses may shed light not only on the pandemic potential of the H5N1 subtype specifically but also provide new knowledge on the origins of pandemic viruses in general and help in future pandemic responses.

One of the molecular changes believed to be necessary for the H5N1 subtype to become a pandemic virus is for the HA protein to switch its binding preference from α2,3 to α2,6 sialic acid residues (235). Avian influenza viruses display high binding preferences to sialic acid glycans containing the α2-3 linkage, whereas human-adapted viruses show a preference for α2,6 sialic acids. Longer α2,6 glycans appear to be especially important for transmission of human-adapted influenza viruses, and are present in large quantities in the upper airways of the human respiratory tract (42, 45). Modification of the binding preference of the 1918 pandemic virus from α2,6 preference to a dual α2,3/α2,6 binding with a lower affinity for long α2,6 glycans, greatly decreased the capacity of the virus to transmit through respiratory droplets (44, 45). Further alteration in the HA protein created a virus that only bound to α2,3 sialic acids and completely abolished viral transmission by this route. These studies show that molecular manipulation of the receptor binding capabilities of viruses is an important approach to better understand characteristics of transmission.

Reverse genetics methods were used in this study to characterize virus binding, transmission, and pathogenicity of H5N1 mutant viruses (321, 322). This investigation was undertaken to elucidate the contribution of individual viral genes that confer transmission and virulence of avian H5N1 influenza viruses. The mutations required to switch the binding properties of the H5 HA protein were explored through site-directed mutagenesis. Once the mutations in H5 HA that resulted in a α2,6 binding preference were identified, rescued viruses were generated and compared to wild-type control viruses for their ability to replicate in Calu-3 cells, derived from human bronchial epithelium. Additional experiments were planned to assess the transmissibility of the

mutant viruses in the ferret model. However, during the course of these studies two other groups reported their work on the gain of transmission function of H5N1 viruses in the ferret respiratory droplet model. This resulted in an international debate on the ethical and biosecurity grounds of such studies and led to a voluntary moratorium on H5N1 virus "gain of function" research. Described in this Chapter are the studies that were performed up until the moratorium took place.

4.3 Materials and Methods

4.3.1 Generation of viral gene plasmids for SI/06 and VN/04. vRNA was obtained from A/Solomon Islands/03/2006 (SI/06) virus. RT-PCR was performed on the vRNA by AccessQuick (Promega) using universal primers encoding the UTR sequences corresponding to A/WSN/33 (WSN) virus containing an external SapI restriction enzyme digest sequence. After SapI digestion, the PCR products were inserted into pPol plasmid backbones (kindly provided by Adolfo García-Sastre, Mount Sinai School of Medicine, New York). The pPol A/Vietnam/1203/2004 (VN/04) plasmids were also obtained from Adolfo García-Sastre. Plasmids were transformed in XL-10 Gold cells and grown in maxipreps (Quiagen). The viral genes were sequenced and compared to the wild-type stocks at the Centers for Disease Control and Prevention (CDC).

Mutations were introduced in the plasmids by QuikChange Site-Directed Mutagenesis kit (Stratagene). Mutagenesis primers were designed with help from the QuikChange Primer Design program (http://www.genomics.agilent.com/primerDesignProgram.jsp). Plasmids were sequenced after mutagenesis to ensure that no additional mutations had occurred.

4.3.2 Rescue of viruses by reverse genetics (rg). Viruses (rgSI/06, rgVN/04, and rg1918HAPB2:Dk/NY) were rescued by transfection into 293T cells using Lipofectamine 2000 in OPTI-MEM. pPol transfection required the 8 viral gene plasmids with an additional 4 helper plasmids (WSN pCAGGS). OPTI-MEM was removed after overnight incubation and replaced with DMEM media with PenStrep. Following transfection, 1918 and H5N1 rescues were transferred into BSL3+ facilities, where the

remaining work was conducted in accordance with the NIH and CDC guidelines and the requirements by the USDA/CDC Select Agent program (323).

To rescue the egg-grown stocks, culture supernatants with positive HAU titers were inoculated (200ul) into 9-10 day old eggs, and incubated at 35-37°C for 24 to 48 hrs. Stocks were grown from rescued viruses with an additional passage in eggs at a dilution range of 1:2000 to 1:8000 of allantoic fluid. Virus was clarified by centrifugation and stored at -80°C. The 50% egg infectious dose (EID_{50}) titer was determined and calculated by the Reed and Muench method (324).

4.3.3 Hemagglutination inhibition (HI) assays. Sera collected throughout the study were treated overnight at 37°C with receptor-destroying enzyme (RDE, Denka Seiken, Tokyo, Japan) and then inactivated at 56°C for 30 minutes. PBS was added to the serum for a final dilution of 1:10. Virus was adjusted to 4 hemagglutination units (HAU) per 25 µl and incubated with sera for 15 minutes (SI/06) or 30 minutes (VN/04). Red blood cells added to the virus-sera mix for 30 minutes (0.5% turkey RBCs for SI/06) or 1 hour (1% horse RBCs for VN/04) before inhibition of viral agglutination was visually examined (325).

4.3.4 Resialation assays for determination of viral binding preference. Virus binding specificity was examined through the production of altered turkey red blood cells (tRBCs). Natural sialic acids were removed from the surface of tRBCs by a neuraminidase enzyme (Roche Molecular Diagnostics, Indianapolis, IN). α2,3 or α2,6 sialic acids were then added back to the cells by α2,3 or α2,6 specific sialyltransferase

enzyme (Sigma Aldrich, St. Louis, MO) (326, 327). Appropriate controls were also examined to determine proper functioning of the enzymes.

4.3.5 Examination of viral kinetics in Calu-3 Cells. The human bronchial epithelial cell line, Calu-3, obtained from American Type Culture (ATCC, Manassas, VA) were grown on transwells in Eagle's minimal essential medium (MEM) supplemented with 10% FBS (110). Once confluent with a measured transepithelial resistance of >1,000 $\Omega \cdot cm^2$, cells were inoculated apically with the indicated influenza viruses at a multiplicity of infection (MOI) of 0.01 for 1 hr at 37°C in triplicate. Supernatants were collected from apical and basal sides of the transwells at specific time points post-inoculation (p.i.). Virus titers from supernatants were determined by a standard plaque assay using MDCK cells. In each experiment, the initial infection rate at 8 h p.i. was determined by fixing cells with 2% formaldehyde for 30 min. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 20 min, blocked with 3% BSA, and stained with mouse anti- nucleoprotein (NP) monoclonal (A3) antibody (CDC's Influenza Reagent Resources, IRR). This was followed by a FITC-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Immunostained cells were mounted with VECTASHIELD mounting medium with 4′,6-Diamidino-2-phenyindole (DAPI; Roche) and examined under a Zeiss Axioskop 2 fluorescent microscope (110).

4.3.6 Inoculation of ferrets for pathogenesis and transmission studies. Male Fitch ferrets, 6-10 months old and serologically negative against currently circulating influenza viruses were housed in cages within a Duo-Flo BioClean environmental enclosure (Lab

Products, Seaford, DE) throughout each experiment. Ferrets were anesthetized with a ketamine-xylazine-atropine cocktail before intranasal (i.n.) inoculation with 10^6 EID_{50} of virus. In direct contact (DC) transmission experiments the contact animals are placed in the same cage as the inoculated animals 24 hours p.i, as described (296). In respiratory droplet (RD) transmission experiments, the ferrets are placed in adjacent modified cages, containing perforated sides 24 hours p.i. While in separate cages, the ferrets share the same air while unable to come into contact with each other, or share food and water.

On alternate days p.i., nasal wash samples were collected in 1ml PBS with PenStrep, BSA, and Gent (230). Virus titers were determined by serial titration in eggs. Blood was collected at 21 days post-contact (p.c.) for isolation of serum and confirmation of transmission. Seroconversion to the inoculated virus was examined by standard hemagglutination-inhibition (HI) assay using tRBCs.

4.4 Results

4.4.1 Transmission of 1918HAPB2:DkNY virus

Previously our laboratory showed that unlike human H1N1viruses, avian H1N1 viruses do not transmit efficiently by either respiratory droplets (RD) or direct contact (DC) in the ferret model, consistent with the transmission data obtained with contemporary avian influenza H5N1 viruses (94). Furthermore, Van Hoeven et al found that the HA, NA, and PB2 genes of the 1918 (H1N1) pandemic virus, A/SC/1/18, was sufficient for RD transmission of a virus containing the remaining genes of an avian H1N1 virus, A/Duck/NY/15024-21/96 (1918HANAPB2:Dk/NY) (94). To test if the 1918 NA was required for RD transmission to occur in the ferret animal model, a recombinant virus was rescued that included only the HA and PB2 genes from SC/18 virus with the remaining genes from Dk/NY (1918HAPB2:Dk/NY) virus. Transmission cages were set up and three ferrets were inoculated i.n. with 10^6 EID50 of 1918HAPB2:Dk/NY virus. The inoculated ferrets all displayed lethargy following infection for several days, and had a mean maximum weight loss of 17% and an average maximum temperature increase of 2.0°C above baseline (Table 1). Due to excessive weight loss, one of the inoculated ferrets was humanely euthanized on day 6 p.i. The remaining two ferrets cleared infectious virus by day 9 p.i. and recovered. The 1918HAPB2:Dk/NY virus transmitted efficiently to all three contact ferrets, which shed virus in their nasal wash samples by day 5 p.c. and seroconverted to the virus (Figure 1). The contacts exhibited an average weight loss of 6.3% and showed a fever of 1.4°C over baseline. These results suggest that the 1918 HA and PB2 contribute to the

transmissibility of the 1918 pandemic influenza virus and that the 1918 NA protein is not required for RD transmission of the avian H1N1 virus.

4.4.2 Rescue and replication efficiency of A/Vietnam/1203/2004 (VN/04) virus

pPol plasmids containing the individual genes from VN/04 were each checked for functionality by replacing a single gene from WSN virus (with known function) in the transfection protocol with the corresponding gene from the H5N1 virus. 48-72 hrs post-transfection, culture supernatants were assayed for hemagglutination activity to detect the initial presence of rescued virus. Once the plasmids were confirmed as functional, the entire set of rescue plasmids for the VN/04 genome was transfected into 293T cells. An HAU titer was detected in the supernatant of the viral transfections within a few days. After virus was detected, a virus stock was grown in eggs and harvested 24 hours later. The stock was sequenced and compared to wild-type virus prior to further testing to ensure that no inadvertent mutations occurred during virus rescue and propagation.

The replication efficiency of rgVN/04 virus was compared to wild-type VN/04 virus as previously described (255). Calu-3 cells grown on transwells were inoculated with virus at an MOI of 0.01. Supernatants were collected from the apical and basal sides of the transwells. Additional plates were set up and stained for anti-NP to confirm that both VN/04 viruses displayed similar infection rates (data not shown). Calu-3 cells were fixed and compared at 72 hrs p.i. for levels of visual CPE. No significant difference was observed in the replication kinetics of the rescued (rgVN/04) virus compared to the wild-type VN/04 virus throughout the time course in either the apical or basal sides of the transwells (Figure 2A and data not shown).

4.4.3 Pathogenesis and transmission of rgVN/04 virus

Since the rgVN/04 virus matched the wild-type characteristics in human airway cells, the virus was further examined for transmission and for its ability to cause severe disease in the ferret model. Since avian viruses do not transmit efficiently by respiratory droplets, the direct contact (DC) model was utilized for these transmission studies. The DC transmission model was set up and 3 ferrets were inoculated i.n. with 10^6 PFU of rgVN/04 virus. The next day, contact animals were added into the same cage as the inoculated ferrets and nasal washes were collected on alternate days p.i./p.c.

The rgVN/04 virus caused severe lethargy and replicated to high titers in the three inoculated ferrets (Figure 2B, and data not shown). All of the inoculated ferrets succumbed to infection by day 7 p.i. due to neurological symptoms. Shortly before euthanasia rgVN/04-inoculated ferrets displayed mean maximum weight loss of 14.5% and a temperature spike of 1.9°C over baseline (Table 1). At day 3 p.i., extrapulmonary spread of the virus to organs including the brain, spleen, liver, and intestines was detected (Figure 2D), consistent with previous findings (255). Virus infection with rgVN/04 also resulted in a high level of lymphopenia by day 3p.i. (Figure 2C).

In the DC transmission model, the rgVN/04 virus transmitted to one of three contact ferrets, which succumbed to infection on day 8 p.c. The two remaining contacts did not seroconvert to VN/04 virus. The day 5 p.c. nasal wash of the infected contact ferret was sequenced and no detectable mutations were observed. All euthanized ferrets displayed high virus titers in all brain samples collected, including the rgVN/04 positive contact (Figure 2E). This experiment demonstrated the rgVN/04 rescued virus is capable of recapitulating the high pathogenicity of the wild-type VN/04 strain.

4.4.4. Rescue of A/Solomon Islands/03/2006 virus

Next, influenza genes of a seasonal H1N1 virus were cloned and a reverse genetics system established. The vRNA from the A/Solomon Islands/03/2006 (SI/06) viral stock obtained at the CDC was isolated and incorporated into the pPol plasmid backbone. After cloning and transformation, the plasmids were sequenced. Several nucleotide changes were detected in several UTR regions (in NP, M, and NS) that were traced back to the universal primers used during the initial PCR cloning reactions. Alterations in the UTRs of influenza affect the replication and pathogenesis of the resulting virus (328, 329), therefore mutagenesis was initiated to exchange the mutations for the correct the H1N1 wild-type UTR sequence.

Other studies have observed amino acid variations in the receptor-binding site of the HA protein in various SI/06 virus sequences available in the public domain. The majority population of our CDC SI/06 virus stock was found to have changes at HA residues 190D and 226R that can lead to altered receptor-binding preference of the virus from α-2,6-linked to α-2,3-linked sialic acid (102, 330). This virus did not transmit to contact animals by means of respiratory droplets (data not shown). A minority of our SI/06 virus stock contained HA residue changes 190A and 226Q, but was purified out during the cloning and rescue process so that the initial rgSI/06 virus contained only HA 190D and 226R. Site-directed mutagenesis was performed to alter the bases D-190 to A and R-226 to Q to confer α2,6 binding preference. Sequencing confirmed that no additional mutations occurred during cloning or following the mutagenesis.

The gene plasmids were tested individually for function by single replacement in the WSN/33 genome. The entire SI/06 genome was then rescued in 293T cells;

hemagglutination activity was detected in the supernatant within the first few days following transfection and an egg-grown stock was produced by inoculation of eggs with 1:2000 dilution of supernatant.

Replication of the rgSI/06 virus stock was tested and compared to the wild-type SI/06 stock utilizing the Calu-3 *in vitro* cell system. The Calu-3 cells were inoculated at a MOI of 0.01. No significant difference was observed in the viral titers collected from the apical side of rgSI/06 inoculated cultures compared to the wild-type SI/06 virus at any time point (Figure 3A). Similarly no virus was detected in the basal supernatants of either virus cultures. Taken together, these data suggest that no significant differences exist between the rescued virus and the wild-type virus *in vitro*.

4.4.5. Transmission of A/SI/03/2006 virus in ferrets

The wild-type SI/06 virus has been previously shown to transmit efficiently between ferrets during RD transmission (94, 328). rgSI/06 virus was inoculated into three ferrets at 10^6 EID_{50} to examine the *in vivo* characteristics of the rescued virus. Three naïve contact ferrets were introduced into adjacent cages 24 hours following inoculation. The virus replicated efficiently in the 3 inoculated ferrets, causing an average weight loss of 6.4% and a mean temperature increase of 1.0°C (Table 1). The virus transmitted to 2 of the 3 contacts resulting in seroconversion (Figure 3B). The infected contact ferrets exhibited an average weight loss of 5.2% and a maximum temperature increase of 0.8°C over baseline (Table 1). The alteration of amino acids at positions 190 and 226 resulted in a transmissibility phenotype of the rgSI/06 strain that matches the wild-type strain.

4.4.6. Mutagenesis of the receptor-binding site of VN/04 virus

Once both the complete seasonal H1N1 and avian H5N1 genomes were cloned into the pPol backbone and the rescued viruses recapitulated the wild-type virus characteristics, mutations could then be introduced into the H5N1 virus genome to alter the transmissibility of the avian virus. Previous studies have focused on mutations created in the HA protein in order to increase the ability of the virus to bind human-type (α2,6 sialic acids) receptor. The preferential binding of α2,6 sialic acids appears to be a molecular determinant critical for transmission among mammals (44). The literature has highlighted the generation of the following single amino acid changes; S137A, E190D, T192I, R216E, S221N, Q226L, S227N, and G228S (based on H3 numbering). These amino acids are located in or around the receptor-binding site, and have been shown to affect binding preferences in other influenza viruses (47, 331-334). A study using several H5 viruses showed that mutations at residues E190D, K193S, and G225D eliminated most α2,3 binding without increasing the α2,6 binding capacity (335). However, the same study also showed increases in α2,6 binding for avian viruses with mutations in residues S137A and T192I. It was recently reported that removing a glycosylation site that is present on H5 viruses at amino acid 160 which hinders HA binding to α2,6 sialic acid residues may improve the binding of these viruses to α2,6 linkages (333). Double mutants have also been created to mimic the alterations shown to affect the binding of H1 and H2/H3 subtype influenza viruses, including E190D G225D and Q226L G228S (LS) respectively. Several mutants were created involving multiple bases, including the E190D Q226L G228S (DLS) and E190D K193S Q226L G228S (DSLS) mutants from a Maines et al study (336). In that study a VN/04 HA mutant with changes at amino acids

193, 226, and 228 displayed binding to both α2,3 and α2,6 sialosides, however the overall binding intensity was reduced compared to wild-type virus. The T160A mutation was also added to both the LS and DSLS mutants, as the removal of the glycosylation eliminates steric hindrance and was shown to increase the flexibility of the receptor-binding site (333).

In the current study, the aforementioned 14 mutant viruses were rescued in the rgVN/04 plasmid backbone, made into stocks, and tested for their binding preference in a resialation assay (Table 2). Turkey red blood cells (tRBCs) were stripped of their natural surface sialic acids after incubation with a neuraminidase. Either α2,3 or α2,6 sialic acids were preferentially added back to the cells by the use of specific sialyltransferase enzymes. Both sets of altered tRBCs cells were confirmed to function by binding viruses of known receptor preferences (Table 2). Several mutations did not have a detectable effect on the binding preference of the virus, including: S137A, T192I, and G228S. Other mutations, including S227N, LS, and ALS, caused only a two-fold decrease in the α2,3 specific binding compared to the normal tRBC binding. Several mutations resulted in a decrease of four-fold or more in α2,3 binding preference: S221N, E190D, RLS, DLS, and ADSLS. A few of the mutations resulted in complete abolishment of binding to any of the RBCs, even the normal tRBCs: Q226L, E190D G225D, and DSLS. Only one of the rgVN/04 mutants displayed any detectable α2,6 binding- ALS. The binding was low (HAU titer of 8), and there was still detectable α2,3 binding (HAU=32). The binding effects seen here follow the observations found in other mutational studies (47, 334, 336), although more α2,6 binding has been observed for the LS mutant in other studies (334, 337).

4.4.7. Replication of LS and ALS mutant viruses

Since other publications have shown the LS mutant in a VN/04 background results in an increase of α2,6 binding (337), the LS and ALS mutants were studied further for possible alterations in *in vitro* virus replication before testing *in vivo*. Calu-3 cells were infected at an MOI of 0.01 and culture supernatants were collected up to 72 hrs p.i. Both mutant viruses displayed efficient replication in the bronchial epithelial cell line that was comparable to wild-type control viruses (Figure 4A). Both mutant viruses reached a maximum viral titer at 48 hrs with a titer of around 10^8 $\log_{10}$ PFU/ml, similar to the rgVN/04 strain. The NP staining also displayed similar levels of viral replication in both mutants at 24 hours p.i. compared to the non-mutated viruses (Figure 4B-I). None of the amino acid changes introduced into the LS and ALS rgVN/04 mutant viruses appeared to alter the replication efficiencies of the rescued viruses in the Calu-3 cells.

The future research plans included testing these mutants in a respiratory droplet model of transmission, as well as serially passaging the viruses from ferret to ferret to attempt to allow the replication within the ferrets to result in any additional required adaptations. However, as will be explained in the discussion, these experiments were unable to be conducted.

4.5 Discussion

The Van Hoeven et al study demonstrated that the HA protein of the 1918 virus was sufficient for direct contact transmission of an avian influenza virus, and the PB2 allowed for respiratory droplet transmission between ferrets (94). However, in those studies the 1918 NA protein was also rescued among the H1N1 recombinant viruses tested. Data from the study described here demonstrates that the NA protein is not necessary for RD transmission of the avian influenza H1N1 virus to occur.

Currently, while H5N1 virus strains continue to result in human infections, they lack the capacity for sustained human-to-human transmission. For the H5N1 subtype to become a pandemic threat, efficient transmission is an essential characteristic (235). The ultimate goal for the reverse genetics system developed here was to identify the molecular determinants required for transmissibility and severe disease in ferrets, which might suggest the viral genetic changes that are required for adaptation of avian H5N1 viruses in humans.

In December of 2011, while these studies were ongoing, the NSABB (National Science Advisory Board for Biosecurity) reviewed two scientific manuscripts concerning similar studies examining H5N1 transmission potential (51, 338). Initially, the NSABB recommended redacting the studies due to bioterror concerns and the risk of an unintentional release of the viruses used in the two ferret studies. Following review of the manuscripts influenza researchers worldwide enacted a voluntary moratorium on H5N1 research until concerns of information misuse could be addressed (339). Ultimately, the manuscripts were published after the authors more fully addressed the potential public health benefits to the studies, and described the safety precautions in

place to protect the public from the created virus strains. The moratorium has since been lifted and new strict guidelines for H5N1 "gain of function" research are in place. Currently, any H5N1 proposed projects that may result in a transmissible virus require additional review prior to funding approval. Due to these new regulations the studies described here will remain uncompleted.

In one of the H5N1 ferret transmission study, Imai et al rescued the H5 HA from A/Vietnam/1203/2004 on a virus that contained all other genes from the A(H1N1)pdm09 virus backbone (51). The HA from VN/04 virus contained four mutations (N158D N224K Q226L T318I) shown to bind α2,6-linked SA with increased affinity and resulted in seroconversion of all six contact animals in a respiratory droplet model. While virus was detected in brain samples of infected ferrets, the transmitting virus was not lethal. A reassortant H5 HA/H1N1 with only three mutations in the HA (N158D N224K Q226L) was also shown to seroconvert 5 of 6 contacts without lethality. In the second study, Herfst et al used a somewhat different approach to achieve the same goal as Imai et al.; they rescued and tested a virus containing all 8 gene segments derived from A/Indonesia/5/2005 (H5N1) virus (338). Ultimately, the group created a virus with several targeted mutations (E627K in the PB2, and Q226L G228S in the HA by H3 numbering) and serially passaged the virus through ferrets, inoculating the animals intranasally with supernatant from homogenized nasal turbinates. Following 10 passages, the resulting virus transmitted to respiratory droplet contact animals, and consistently contained two additional HA mutations (T160A and H109Y). The resulting H5N1 virus was attenuated in ferrets and did not result in lethality in the contact animals.

For the studies described here, the parental viruses were initially characterized and compared to the wild-type viruses to demonstrate that no alteration of the viral phenotypes occurred during the reverse genetics processes. Once the parental rescued H5N1 and H1N1 viruses were generated, only viruses containing the H5 HA were considered for subsequent mutations. Since mutation of H5 HA alone would not have been sufficient for transmission to occur, the addition of internal human genes in the recombinant viruses would have been analyzed. The PB2 gene appears to be particularly important for transmission potential as a mutation at PB2 residue 627 from E to K alters the virus's ability to replicate at 33°C (94, 340, 341). This change may increase the capacity of the virus to spread efficiently in mammals; coincidentally it is already present in A/Vietnam/1203/2004 virus (92). The entire polymerase complex (PB1, PB2 and PA) would have been initially rescued in H5N1 recombinant viruses, and if a transmissible phenotype was seen the specific gene responsible would be narrowed down with individual polymerase gene replacements.

Several similarities exist between the findings described in this Chapter and the two published studies. While the Imai et al study had not focused as deeply on the HA changes at amino acid position 228, the VN/04 mutant in that study still shared the change at 226, as well as the removal of the glycosylation site around amino acid 160 (the same effect occurs whether the mutation is created at 158 or 160). Although the Herfst et al paper had utilized the A/Indonesia/5/2005 H5N1 virus instead of VN/04, the ALS mutant created in this Chapter was only missing one mutation from the transmissible Ind/05 virus created during the studies. VN/04 already contains the E627K mutation, and therefore only the amino acid change at HA position 109 was absent.

Both the Herst et al and Imai et al studies identified structural features in the HA protein that are important for changing the receptor binding properties of the H5N1 viruses, as well as determining areas of the protein that are important for stabilization. Additional studies are needed to determine which mutations with these structural features confer RD transmissibility in mammals for different H5N1 virus clades and subclades. The possibility exists that a fully avian version of VN/04 virus would never result in a transmissible virus. Although the A/Indonesia/5/2005 (clade 2.1) virus was capable of RD transmission, the differences between the clade 2.1 and clade 1 (to which VN/04 belongs) may be too great to make direct comparisons.

H5N1 viruses are not alone among avian influenza viruses that have the capacity of causing severe or fatal disease in humans requiring close monitoring. The recent H7N9 outbreak in China highlights the ability of this avian subtype to jump the species barrier to infect humans. In total, there have been over 130 confirmed illnesses with over 40 deaths within the span of a few months in 2013. Influenza viruses have also entered into the human population from swine, with recent examples including A(H1N1)pdm09 and A(H3N2)v viruses.

Based on the available information, every pandemic virus that has entered into the human population has done so with an avian influenza virus component. The adaptations that occur to allow these avian virus genes to thrive within the human populace remain largely unknown. The pandemic virus that appeared around the world in the spring of 2009 did not contain any of the known human adaptations, and highlights the fact that many genetic alterations remain undiscovered. In order for us to fully understand how

pandemic viruses emerge, the mutations that allow for human-adaptation must be further explored.

4.6 Acknowledgements

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Table 4.1. Clinical observations following viral challenges

	Weight Loss (%)[a]		Temperature (°C)[b]		Contacts	
Viruses	**Inoculated**	**Contacts**	**Inoculated**	**Contacts**	**Sneezing**	**Seroconversion**
1918HAPB2:Dk/NY	**17.0**	**6.3**	**2.0**	**1.4**	**3/3**	**3/3**
rgVN/04	**14.5**	**18**	**1.9**	**1.1**	**1/3**	**0/2***
rgSI/06	**6.4**	**5.2**	**1.0**	**0.8**	**2/3**	**2/3**

[a] Maximum mean weight loss percentage shown for each ferret group, values found days 6-8 post challenge.

[b] Maximum mean temperature increases calculated as rise over ferret baseline of 38.3 ±0.5 °C, all maximum temperatures in inoculated animals found on day 2 p.c.

NA= not applicable. Data could not be determined without transmission.

*seroconversion not determined in the contact that was euthanized.

Table 4.2 Binding preference of rgVN/04 constructs in resialation assay

	Binding Strength (HA titer)			
VN/04 mutant	**tRBC[a]**	**Desialated tRBC[b]**	**α2,3**	**α2,6**
α2,3 control (Dk/NY/96)	256	-	256	-
α2,6 control (Dk/NY/96-DD)	128	-	-	128
wild-type (wtVN/04)	256	-	128	-
rescued (rg/VN/04)	256	-	256	-
S221N	256	-	64	-
Q226L	-	-	-	-
S227N	32	-	16	-
G228S	64	-	64	-
S137A	16	-	16	-
T192I	32	-	32	-
E190D	256	-	32	-
R216E	256	-	64	-
Q226L G228S (LS)	32	-	16	-
E190D G225D	-	-	-	-
T160A Q226L G228S (ALS)	64	-	32	8
E190D Q226L G228S (DLS)	64	-	4	-
E190D K193S Q226L G228S (DSLS)	-	-	-	-
T160A E190D K193S Q226L G228S (ADSLS)	256	-	64	-

[a] All RBCs were diluted to 0.5% concentration by volume in PBS with 0.5% BSA before use. Resialated cells were made and used on the same day.

[b] - indicates no binding was observed. Limit of detection for HA assay is 10.

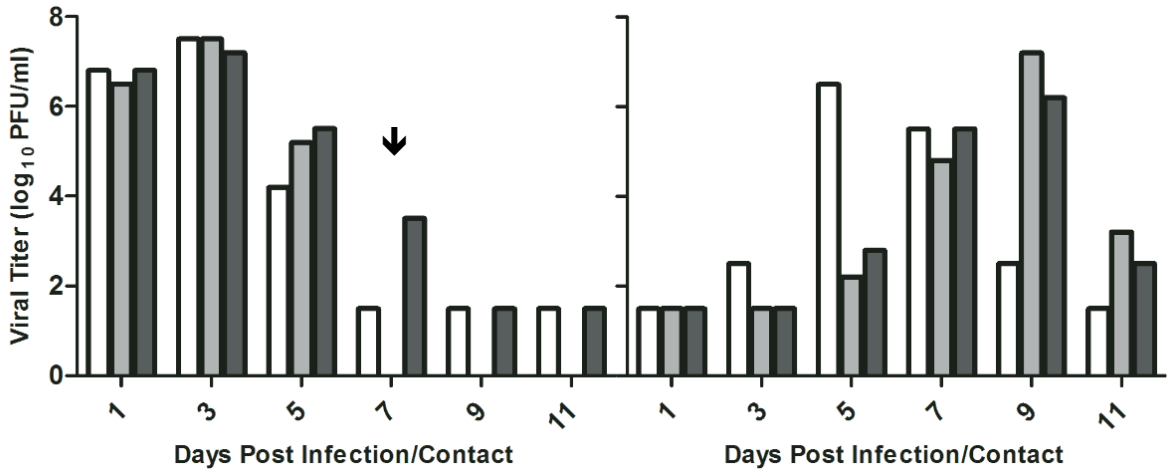
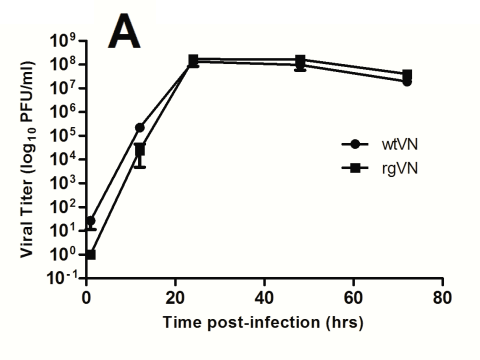
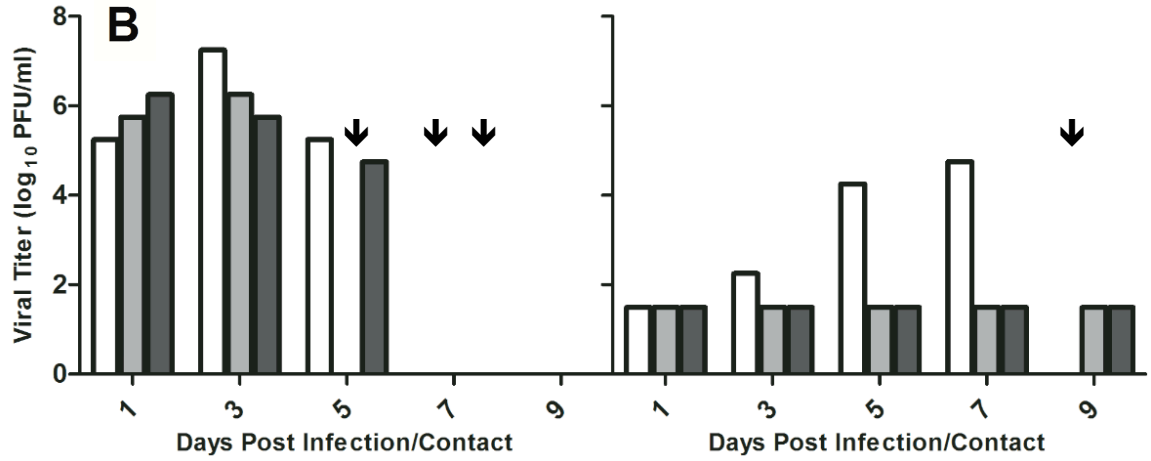
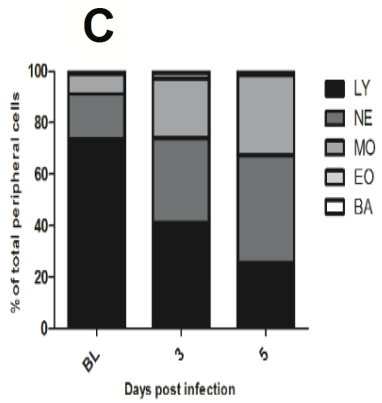
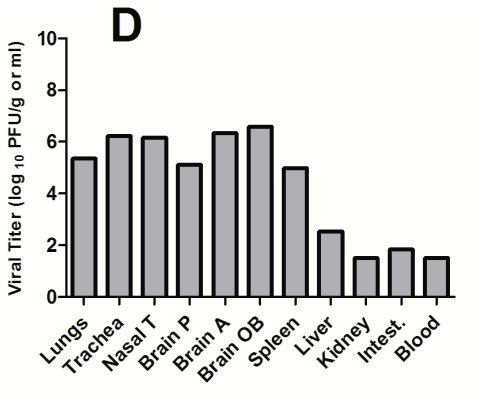


Figure 4.1. Transmissibility of 1918HAPB2:DkNY among ferrets in the respiratory droplet model. Three ferrets were inoculated with 10^6 PFU of virus intranasally. Each bar represents the viral titer isolated from a nasal wash of an individual inoculated ferret (left side of graph) and their corresponding contact ferret (right side of graph). ↓ indicates when a ferret succumbed to infection.


A
Viral Titer (log10 PFU/ml)
Time post-infection (hrs)
wtVN
rgVN


B
Viral Titer (log10 PFU/ml)
Days Post Infection/Contact
Days Post Infection/Contact


C
% of total peripheral cells
LY
NE
MO
EO
BA
BL
3
5
Days post infection


D
Viral Titer (log10 PFU/g or ml)
Lungs
Trachea
Nasal T
Brain P
Brain A
Brain OB
Spleen
Liver
Kidney
Intest.
Blood

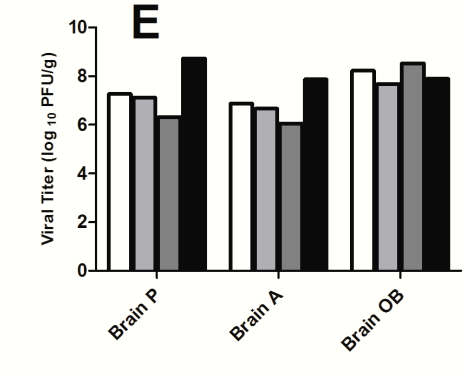

E
Viral Titer (log10 PFU/g)
Brain P
Brain A
Brain OB

Figure 4.2. *In vitro* and *in vivo* testing of rgVN/04 stock. (A) Replication kinetics of rgVN/04 in Calu-3 cells from apical side. Cells were inoculated with virus at an MOI of 0.01. Supernatants were collected at time points up to 72 hours post infection. The titer value represents an average of three independent wells. (B) Transmissibility of rgVN/04 among ferrets in the direct contact model. Three ferrets were inoculated with 10^6 PFU of virus intranasally. Each bar represents the viral titer isolated from a nasal wash of an individual inoculated ferret (left side of graph) and their corresponding contact ferret (right side of graph). ↓ indicates when a ferret succumbed to infection. (C) Hemavet data from infected ferrets, compared to pre-infection baseline. Levels of lymphocytes (LY), neutrophils (NE), monocytes (MO), eosinophils (EO), and basophils (BA) measured. (D) Viral titers collected from multiple systemic organs on day 3 p.c. (E) Viral titers from the brain sections of all infected ferrets during the transmission experiment.

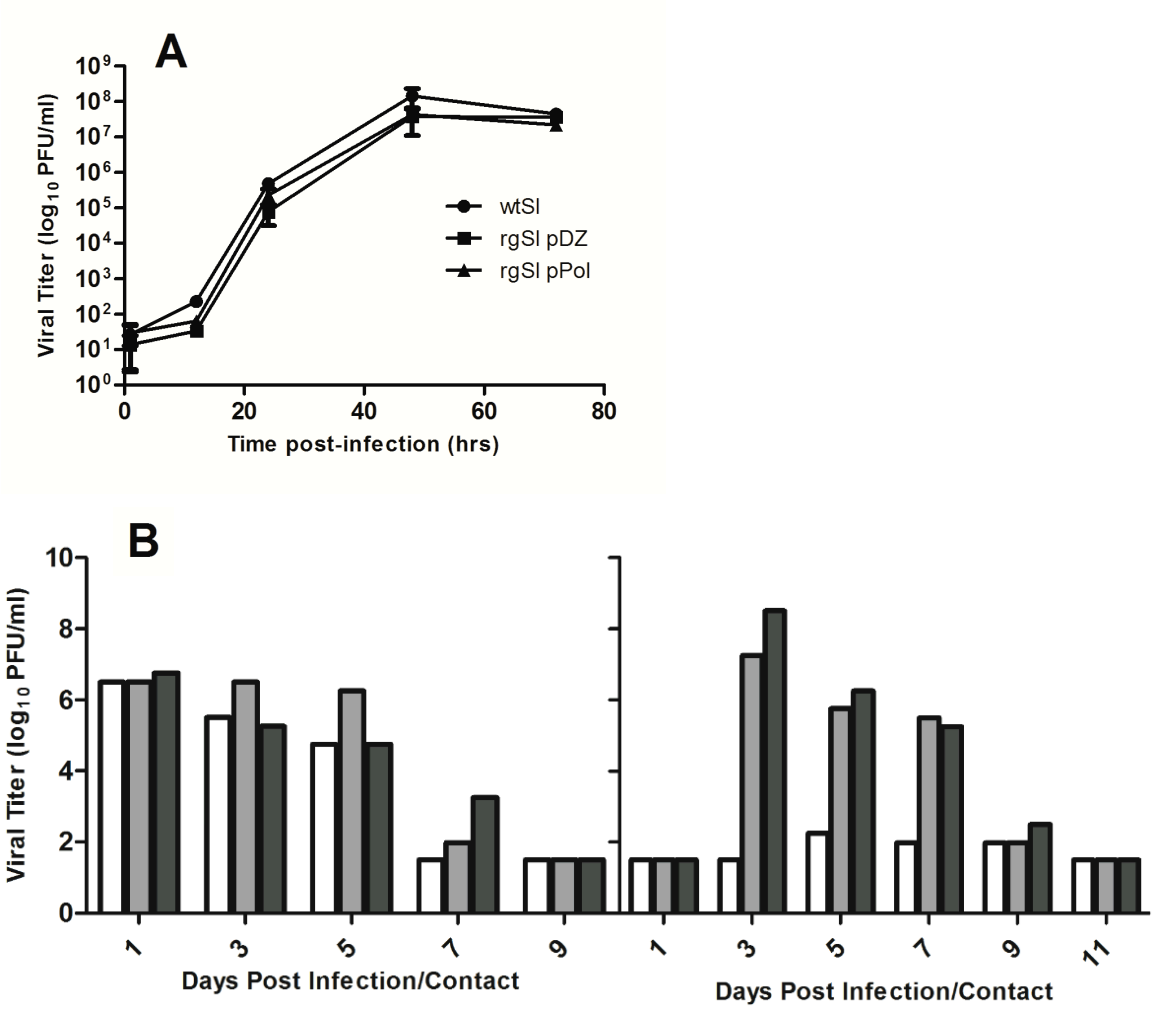


Figure 4.3. *In vitro* and *in vivo* testing of rgSI/06 stock. (A) Replication kinetics of SI/06 influenza viruses in Calu-3 cells from apical side. Cells were inoculated with virus at an MOI of 0.01. Supernatants were collected at time points up to 72 hours post infection. The titer value represents an average of three independent wells. (B) Transmissibility of rescued SI/06 viruses among ferrets in a respiratory droplet model. Three ferrets were inoculated with 10^6 PFU of virus intranasally. Each bar represents the viral titer isolated from a nasal wash of an individual inoculated ferret (left side of graph) and their corresponding contact ferret (right side of graph).

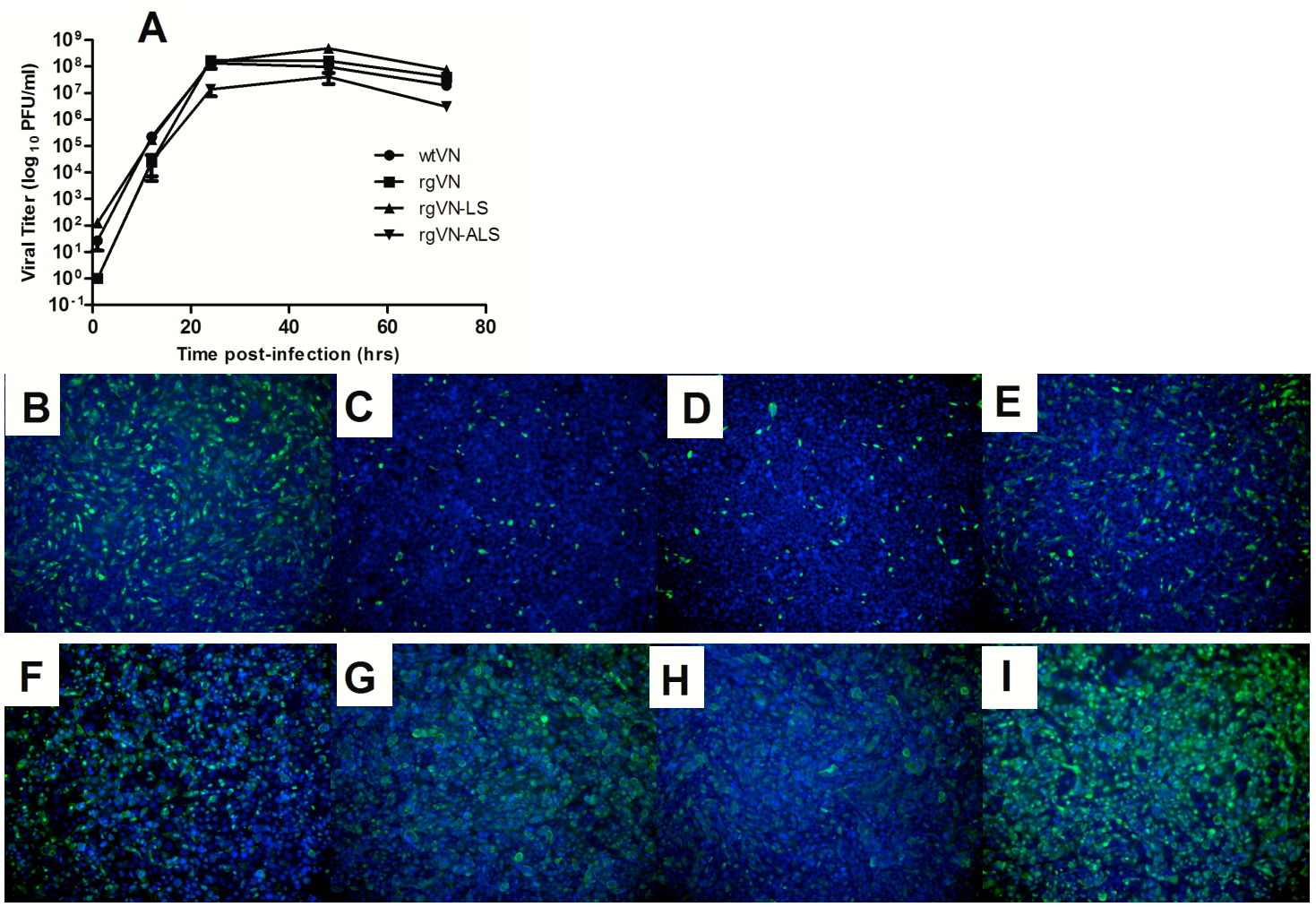

A
Viral Titer (log 10 PFU/ml)
Time post-infection (hrs)
wtVN
rgVN
rgVN-LS
rgVN-ALS
B
C
D
E
F
G
H
I

Figure 4.4. Replication kinetics for VN/04 mutant viruses in Calu-3 cells from apical side. Cells were inoculated with virus at an MOI of 0.01. Supernatants were collected at time points up to 72 hours post infection. The titer value represents an average of three independent wells. (A) Viral titers collected from wtVN/04, rgVN/04 and two rgVN/04 mutants at timepoints up to 72 hours post infection. (B-I) Cells fixed with 3.7% formaldehyde, and labeled with antibody against influenza NP virus (green). Cell nuclei labeled with DAPI (blue). (B) wtVN/04, (C) rgVN/04, (D) rgVN/04-LS, or (E) rgVN/04-ALS at 8hrs post infection and (F-I) corresponding 24 hrs post infection. Images at 10x.

Chapter 5.

Conclusions

An influenza pandemic occurs when an influenza A virus with a novel HA protein emerges with the capacity to cause widespread disease in immunologically naïve populations. Influenza viruses from zoonotic (mainly swine and avian) sources have been detected in humans with increasing frequency for the last 20 years. Avian influenza subtypes including H5, H6, H7, H9, and H10 have appeared with phenotypes of both low and high pathogenicity (246). These viruses have spread sporadically to humans from poultry but continued to display a lack of efficient transmission among humans. The most recent example involves the H7N9 subtype that emerged from an avian source to cause human infections in China in the spring of 2013. This subtype had never been previously detected in humans but led to over 130 infections with the majority of cases occurring during the month of April (248). Some of the infections were mild, but many led to severe respiratory illness within the patient and over 40 cases were fatal (248). Within the ferret animal model these viruses displayed efficient replication within the respiratory tracts but inefficient transmission via respiratory droplets (342-344). Several influenza A subtypes have also emerged from the swine population to infect humans; including H1N1, H1N2, and H3N2 (288). Although the majority of zoonotic influenza viruses, including the recent A(H3N2)v viruses, have displayed only limited human-to-human transmission, the A(H1N1)pdm09 virus from swine caused the first pandemic of the twenty-first century. These public health events highlight the diversity of pandemic threats and the need to understand the steps required for human adaptation to occur for different subtypes emerging from animal reservoirs.

The studies described here utilized the ferret animal model for both transmission and pathogenesis studies. The ferret is the premiere animal model for the study of

influenza infection as it recapitulates many features of influenza virus pathogenicity and transmissibility observed in humans (227). The transmission studies described here focused on the respiratory droplet model, as the ability of influenza viruses to transmit through aerosols and respiratory droplets is an important prerequisite of a pandemic strain (234).

A highly virulent H5N1 virus that gained the capacity for efficient spread amongst the human population would have devastating consequences for public health. Even within countries where H5N1 viruses are endemic, seroprevalence studies suggest that there is little to no preexisting antibody to H5 viruses in the population (345). As a consequence, any human transmissible H5N1 virus that emerged would meet little immunological resistance from the human population. Two recent papers described mutations that allowed for avian H5N1 viruses from two distinct subclades to transmit between ferrets in a respiratory droplet model (51, 338). One of the transmissible H5N1 viruses was a reassortant with an A(H1N1)pdm09 strain that is still circulating widely in the human population, while the other was composed entirely of H5N1 viral genes. We had been examining similar mutations in Chapter 4 while utilizing the same H5N1 strain as the Imai et al study and were close to examining the rescued viruses *in vivo* before the research moratorium was put in place (51). None of the resulting transmissible strains were lethal in the contact ferrets in either publication, although one of the intranasally inoculated ferrets died following inoculation in the Herfst et al study (338). The possibility exists that all mutations that confer transmission in HPAI H5N1 viruses will attenuate the virulence similarly. This attenuation may be caused by an alteration in the tropism of these viruses. The preference for α2,3 sialic acids and preferred tropism of

avian viruses for cells located predominately within the lower respiratory tract (LRT) is thought to be one reason of the enhanced virulence observed during human infections (346). Changing the binding preference of the viruses to α2,6 would likely alter the preferred tropism to the upper respiratory tract (URT) of the infected animals, and may cause a reduction in virulence. It is also possible the mutations that confer transmissibility and sustain the highly pathogenic phenotype have not yet been identified. The recently discovered mutations for H5N1 viruses should be tested further in H7 and H9 viruses to see if the adaptations that allow for transmission are universal for avian viruses, or if they are H5 subtype or even clade specific. Understanding the mutations responsible for human adaptation of avian influenza viruses is of vital importance for pandemic preparedness, and for monitoring of circulating strains for the emergence of such mutations.

While the emergence of H5N1 viruses with genetic changes that result in human transmissibility could be devastating to the population, a similar outcome would be unlikely for the recent A(H3N2)v viruses. Several seroprevalence studies showed that most age groups have preexisting antibodies that are capable of cross-reacting to the A(H3N2)v viruses (278-280). The young adult population contains the highest frequency of cross-reactive antibodies titers with a HI titer of ≥40, a threshold serological titer generally associated with a reduction in the risk from influenza virus infection in adult populations, while children under the age of ten have the lowest frequency. This young age group is also accountable for the majority of confirmed cases (276, 278, 289, 347). In addition, the seroprevalence studies demonstrated that vaccination with the 2010-2011 seasonal TIV failed to induce cross-reactive antibody in young children (278). We show

in Chapter 2 that this lack of serologic cross-reactivity also occurs following vaccination with the 2011-2012 TIV in ferrets. While the ferrets produced antibodies against the three HA antigens contained in the seasonal TIV, no cross-reactive antibodies were detected against the A(H3N2)v virus. Although the previously mentioned serological studies cannot directly prove if the cross-reactive antibodies in the population correlate with protection against infection with A(H3N2)v viruses, our studies are able to directly show a lack of protection against A(H3N2)v viruses following vaccination with a seasonal 2011-2012 TIV. We observed a decrease in viral titers after challenge with the homologous H3N2 strain, but the same trend was not seen after challenge with the A(H3N2)v virus. Vaccinated ferrets had viral titers similar to naïve animals throughout the days of peak viral replication. Based on these data, no cross-protection against A(H3N2)v was observed in ferrets vaccinated with the 2011-2012 seasonal TIV, and a vaccine specific for a A(H3N2)v virus will be required to increase the level of protective antibodies in the population. A vaccine strain, A/Minnesota/11/2010, has been created in case such a need arises (278, 286).

While the A(H3N2)v viruses have displayed the capacity for efficient transmission among ferrets, only limited person-to-person spread has been observed in the documented human cases (228, 276). The ferret model typically recapitulates the inherent transmission properties of a virus observed in a naïve human host. One drawback to our system is that the ferrets do not mirror the complex immune history present in the human population, but can be a close comparison for younger children who tend to be more immunologically naïve against influenza viruses. Young children are the age group that has displayed the most limited human-to-human transmission of the

A(H3N2)v viruses (276, 278, 289, 347). In Chapter 3 we explored the reason behind the differences between the ferret model and human cases to determine if the varying levels of preexisting immunity found in the various age groups were responsible. The data showed that seasonal vaccination failed to substantially reduce A(H3N2)v virus shedding and subsequent transmission to naïve ferrets. Unlike vaccination, prior infection resulted in a block in transmission following both homologous and heterologous viral challenge. No viral titers were obtained from the inoculated ferrets following homologous viral challenge. We also observed a decrease in morbidity in ferrets with prior influenza infection, but not prior vaccination. While the serum HI titers obtained from the previously infected ferrets overlapped with the range acquired from the TIV inoculated ferrets, the immune response from the previous infection resulted in a more effective and protective immune response. This improved response was at least partially provided by an enhanced local IgG and IgA response detected within the respiratory tracts of the previously infected ferrets compared to the vaccinated animals.

Influenza-specific antibodies located within the mucosa of the respiratory tract are an important primary line of defense against subsequent infections. In mice, IgA antibodies provide protection within the URT, while IgG antibodies are more beneficial in the LRT (348, 349). Both vaccination and prior infection resulted in increases in IgA antibodies in the URT and IgG titers in the LRT of the ferrets. However, prior infection resulted in higher increases in both cases, indicating a possible explanation for the superior protection observed in these animals. Studies in mice have also shown the passive transfer of IgG antibodies from the serum into the mucosa can also result in protection during a viral infection (350). The vaccinated ferrets displayed high titers of

IgG antibodies in the serum, which may have been able to compensate for the low titers observed in the respiratory tract samples. The complete lack of detectable IgA in the upper respiratory tract of the vaccinated ferrets may explain why transmission still occurred in these animals following viral challenge, as mucosal IgA has been shown to decrease viral spread in guinea pigs (351).

Studies investigating prior vaccination or infection in mice have suggested that cell-mediated responses also contribute to heterologous protection (352, 353). $CD8^+$ and $CD4^+$ cell-mediated responses were associated with heterosubtypic immunity in mice (147). In ferrets prior seasonal H3N2 infection was shown to reduce replication and symptoms from H5N1 virus through the development of cross-reactive T-cells (354). T-cell responses formed following TIV vaccination are typically weak given that the TIV is a split virion vaccine that is incapable of replication. Without viral replication fewer antigens are presented on MHC molecules for $CD8^+$ T-cell activation. As the A(H3N2)v contains an internal mixture of avian, swine, and human genes, the numbers of cross-reactive T-cell epitopes with seasonal human strains may be low (284, 286). However, a recent immuno-informatics study analyzing the HA and M genes of the A(H3N2)v viruses show a high conservation of T-cell epitopes with contemporary seasonal H3N2 and H1N1 viruses (355). However, the M gene from A/PuertoRico/8/34, contained in the reassortant viruses in the seasonal TIV was not analyzed for epitope conservation in this study for comparison. While we did not analyze cell-mediated responses for the studies described here, they should be investigated in this model during future studies.

We also examined in Chapter 3 whether vaccination with a historical seasonal H3N2 virus resulted in greater protection than a contemporary seasonal influenza

vaccine. The H3 HA of human and swine influenza viruses followed divergent evolutionary pathways resulting in antigenically distinct influenza H3N2 viruses (286). Sequence studies have shown the HA1 region of A(H3N2)v (A/Indiana/08/2011) to be most similar to A/Wuhan/359/95 (H3N2) virus, but some antigenic cross-reactivity exists between the A(H3N2)v and A/Beijing/32/92 viruses (284). However, using whole-virus inactivated vaccines we observed similar protection following challenge with A(H3N2)v virus from both the historical and the contemporary vaccines. Neither vaccine composed of a standard 15μg dose of protein was capable of blocking transmission of A(H3N2)v viruses to naïve contacts, and no protection against weight loss or fever was observed. It is conceivable that the H3N2 whole-virus vaccines induced non-HA immune responses that masked any differences that would have been observed from vaccinating with HA only. A different vaccine formulation, such as virus-like particle (VLP) or recombinant HA proteins, in which the HA proteins are the primary antigens, may yield differences in protection against A(H3N2)v virus challenge in ferrets.

While both H3N2 vaccination and infection of ferrets led to detectable immune responses against influenza, prior virus infection appeared to result in a more potent protective immune response in our ferret studies. Previous studies in ferrets have examined the effects of prior infection or vaccination with a seasonal H1N1 virus on a subsequent A(H1N1)pdm09 virus challenge (298, 299). In one study, H1N1 vaccination did not provide protection against viral replication, while prior H1N1 virus infection lowered clinical symptoms and improved viral clearance but did not affect transmission in the direct contact model (299). Interestingly, multiple virus infections led to an increase in the production of broadly cross-reacting antibodies in ferrets and resulted in

blunting transmission to naïve contacts (309, 356, 357). The addition of adjuvants into vaccines have resulted in more broadly neutralizing antibodies in mice and ferrets, have been shown to be effective at preventing infection with A(H1N1)pdm09 viruses in humans, and should be studied further for their effect on heterologous virus challenge and subsequent transmission (358, 359).

For the transmission studies conducted, we only tested virus spread in one direction; from an animal with preexisting immunity to an immunologically naïve contact ferret. We chose that direction based on a previous study in guinea pigs that found that arrangement to result in the largest effect of prior vaccination or infection on transmission (360). Future studies should investigate whether transmission in the opposite direction (from a challenged naive ferret to a ferret with preexisting immunity) displays similar results, or if transmission remains unaffected by the immune history of the animal in that experimental design. Although less of an effect would be expected, such studies should be addressed as an alternative of modeling influenza transmission in humans.

Avian subtypes, such as H5 and H7, have been poorly immunogenic in previous human vaccine trials, which further hinder the efforts to create effective pandemic responses against these viruses (361). Multiple doses with high concentrations of HA protein are typically required in humans for unadjuvanted inactivated vaccine formulations (362). The addition of adjuvants into H5N1 vaccines have proven effective at increasing the seroconversion rate and geometric mean antibody titers, and results in moderate cross-reactivity against other H5N1 subclades in both animal models and humans (358, 363). VLP vaccines have shown promising results in mice and ferrets

across multiple subclades of H5N1 viruses (364). Several commercial VLP vaccine products have shown the induction of robust HI antibody responses following vaccination without serious adverse reactions, the Novavax A(H1N1)pdm09 vaccine being one example (365). Studies such as the ones described in this dissertation provide needed context and further understanding of the immune responses that are required for a vaccine to be optimally effective against novel influenza subtypes emerging in humans.

The relative lack of vaccine protection against virus shedding and subsequent transmission following vaccination with the TIV is likely due to the inability of inactivated vaccines to induce potent mucosal or cell-mediated immune responses in naïve hosts. As mentioned above, the TIV is an inactivated split or subunit vaccine, which induces a less efficient cell-mediated response compared to the live attenuated influenza vaccine (LAIV) (366). Also, as the vaccination is delivered intramuscularly, the antigens are not presented well to cells within the respiratory tract and thus do not produce an efficient local immune response (367, 368). We observed this effect (Chapter 3) as vaccinated ferrets displayed a weaker mucosal IgG and IgA antibody response compared to the ferrets with prior infection history. It should be noted that while TIV is less efficient at generating immune responses in naïve ferrets, it has been shown to help boost preexisting memory responses in humans (369, 370). The ability for the TIV to affect transmission in ferrets with prior infection history should also be examined in future studies, as a greater effect may be observed.

The human population contains variable levels of preexisting cross-reactive immunity against A(H3N2)v viruses, by antibody and predicted cell-mediated responses (278-280, 355). Antibodies cross-reactive to the A(H1N1)pdm09 virus were also present

in the population prior to the pandemic, but were limited to the elderly age group (371). This resulted in the majority of the population lacking preexisting immunity against the virus. While it is unknown if A(H3N2)v will evolve into a pandemic virus, these studies demonstrate the importance of prior H3N2 virus infection on the production of broadly cross-reactive immune responses. In ferrets, other modes of vaccination, such as the LAIV, have been shown to result in the induction of multiple levels of immune responses, including systemic and local humoral responses as well as cell-mediated immunity (301). Human studies with LAIV have shown heterosubtypic immunity lasts through several influenza seasons and protects from drifted strains of seasonal H3N2 virus (372). Unfortunately, the LAIV vaccine was not available during this study for comparison, but should be added for comparison in the future. Additional vaccine formulations that are not yet FDA approved, such as VLPs, have shown strong heterosubtypic protection in animal models, but the effect on transmission has not been explored (205, 373, 374). These vaccines should also be examined in this model for their effect on transmission.

With the increased detections of novel subtypes infecting the human population, universal vaccines that result in protection from multiple subtypes of influenza are a necessary public health goal. The pandemic in 2009 proved that the timeframe required for the production of the traditional inactivated vaccine is inefficient during a pandemic response. We have shown here that the seasonal TIV is not capable of producing a strong immune response against A(H3N2)v heterologous virus in previously naïve ferrets, and does not completely inhibit transmission even against homologous strains. Vaccines that

produce immune responses capable of producing broad protection against heterologous viruses and decreased virus transmission warrant future investigation.

References:

1. **Potter CW.** 2001. A history of influenza. Journal of applied microbiology **91:**572-579.
2. **Hoehling A.** 1961. The Great Epidemic. Little, Brown & Co., Boston.
3. **Wong CM, Yang L, Chan KP, Leung GM, Chan KH, Guan Y, Lam TH, Hedley AJ, Peiris JS.** 2006. Influenza-associated hospitalization in a subtropical city. PLoS medicine **3:**e121.
4. **CDC.** 2010. Estimates of deaths associated with seasonal influenza --- United States, 1976-2007. MMWR Morb Mortal Wkly Rep **59:**1057-1062.
5. **Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, Fukuda K.** 2004. Influenza-associated hospitalizations in the United States. JAMA : the journal of the American Medical Association **292:**1333-1340.
6. **Glezen WP.** 1996. Emerging infections: pandemic influenza. Epidemiologic reviews **18:**64-76.
7. **Fiore AE, Uyeki TM, Broder K, Finelli L, Euler GL, Singleton JA, Iskander JK, Wortley PM, Shay DK, Bresee JS, Cox NJ, Centers for Disease C, Prevention.** 2010. Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control **59:**1-62.
8. **CDC** 2013, posting date. CDC - Seasonal Influenza: Flu Basics. [Online.]
9. **Bridges CB, Kuehnert MJ, Hall CB.** 2003. Transmission of influenza: implications for control in health care settings. Clin Infect Dis **37:**1094-1101.
10. **Brankston G, Gitterman L, Hirji Z, Lemieux C, Gardam M.** 2007. Transmission of influenza A in human beings. The Lancet infectious diseases **7:**257-265.
11. **WHO** 2013, posting date. Influenza Fact Sheet. [Online.]
12. **Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K.** 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA : the journal of the American Medical Association **289:**179-186.
13. **Carrat F, Vergu E, Ferguson NM, Lemaitre M, Cauchemez S, Leach S, Valleron AJ.** 2008. Time lines of infection and disease in human influenza: a review of volunteer challenge studies. American journal of epidemiology **167:**775-785.
14. **Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J.** 2000. Clinical signs and symptoms predicting influenza infection. Arch Intern Med **160:**3243-3247.
15. **Molinari NA, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E, Bridges CB.** 2007. The annual impact of seasonal influenza in the US: measuring disease burden and costs. Vaccine **25:**5086-5096.

16. **Palese P. SML.** 2007. Orthomyxoviridae: The Viruses and Their Replication, p. 1647-1689. *In* Knipe D.M. HPM (ed.), Field's Virology, 5 ed. Lippincott Williams & Wilkins, Philadelphia.
17. **Easterday BC.** 1975. Animal Influenza, p. p. 449-481, The influenza viruses and influenza. Academic Press, Orlando.
18. **McGeoch D, Fellner P, Newton C.** 1976. Influenza virus genome consists of eight distinct RNA species. Proc Natl Acad Sci U S A **73:**3045-3049.
19. **Hsu MT, Parvin JD, Gupta S, Krystal M, Palese P.** 1987. Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc Natl Acad Sci U S A **84:**8140-8144.
20. **Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW.** 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nature medicine **7:**1306-1312.
21. **Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A, Efstathiou S, Atkins JF, Firth AE, Taubenberger JK, Digard P.** 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science **337:**199-204.
22. **Lamb RA, Krug RM.** 1996. Orthomyxoviridae: The Viruses and Their Replication p. 1353-1395. *In* Fields BN, Knipe DM, Howley PM (ed.), Fields Virology. Lippincott - Raven Publishers, Philadelphia.
23. **Skehel JJ, Wiley DC.** 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem **69:**531-569.
24. **Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD.** 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal of virology **79:**2814-2822.
25. **Wright PF, Naumann G., Kawaoka Y.** 2007. Orthomyxoviruses, p. 1691-1740. *In* Knipe D.M. HPM (ed.), Fields Virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia.
26. **Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO.** 2012. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A **109:**4269-4274.
27. **Zhu X, Yang H, Guo Z, Yu W, Carney PJ, Li Y, Chen LM, Paulson JC, Donis RO, Tong S, Stevens J, Wilson IA.** 2012. Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. Proc Natl Acad Sci U S A.
28. **Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG.** 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology **84:**268-278.
29. **Lamb RA, Choppin PW.** 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P

polypeptides, from RNA produced by primary transcription. Virology **74:**504-519.

30. **Baudin F, Bach C, Cusack S, Ruigrok RW.** 1994. Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent. The EMBO journal **13:**3158-3165.
31. **Nayak DP, Hui EK, Barman S.** 2004. Assembly and budding of influenza virus. Virus research **106:**147-165.
32. **Patterson S, Gross J, Oxford JS.** 1988. The intracellular distribution of influenza virus matrix protein and nucleoprotein in infected cells and their relationship to haemagglutinin in the plasma membrane. The Journal of general virology **69 (Pt 8):**1859-1872.
33. **Yasuda J, Nakada S, Kato A, Toyoda T, Ishihama A.** 1993. Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. Virology **196:**249-255.
34. **Smirnov Yu A, Kuznetsova MA, Kaverin NV.** 1991. The genetic aspects of influenza virus filamentous particle formation. Archives of virology **118:**279-284.
35. **Bourmakina SV, Garcia-Sastre A.** 2003. Reverse genetics studies on the filamentous morphology of influenza A virus. The Journal of general virology **84:**517-527.
36. **Walker JA, Molloy SS, Thomas G, Sakaguchi T, Yoshida T, Chambers TM, Kawaoka Y.** 1994. Sequence specificity of furin, a proprotein-processing endoprotease, for the hemagglutinin of a virulent avian influenza virus. Journal of virology **68:**1213-1218.
37. **Steinhauer DA.** 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1-20.
38. **Suarez DL, Senne DA, Banks J, Brown IH, Essen SC, Lee CW, Manvell RJ, Mathieu-Benson C, Moreno V, Pedersen JC, Panigrahy B, Rojas H, Spackman E, Alexander DJ.** 2004. Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerging infectious diseases **10:**693-699.
39. **Pasick J, Handel K, Robinson J, Copps J, Ridd D, Hills K, Kehler H, Cottam-Birt C, Neufeld J, Berhane Y, Czub S.** 2005. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. The Journal of general virology **86:**727-731.
40. **Ito T, Kawaoka Y.** 2000. Host-range barrier of influenza A viruses. Veterinary microbiology **74:**71-75.
41. **Rogers GN, Paulson JC.** 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology **127:**361-373.
42. **Chandrasekaran A, Srinivasan A, Raman R, Viswanathan K, Raguram S, Tumpey TM, Sasisekharan V, Sasisekharan R.** 2008. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nature biotechnology **26:**107-113.

43. **Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD.** 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A **101:**4620-4624.
44. **Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A.** 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science **315:**655-659.
45. **Srinivasan A, Viswanathan K, Raman R, Chandrasekaran A, Raguram S, Tumpey TM, Sasisekharan V, Sasisekharan R.** 2008. Quantitative biochemical rationale for differences in transmissibility of 1918 pandemic influenza A viruses. Proc Natl Acad Sci U S A **105:**2800-2805.
46. **Martin J, Wharton SA, Lin YP, Takemoto DK, Skehel JJ, Wiley DC, Steinhauer DA.** 1998. Studies of the binding properties of influenza hemagglutinin receptor-site mutants. Virology **241:**101-111.
47. **Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA.** 2006. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science **312:**404-410.
48. **Connor RJ, Kawaoka Y, Webster RG, Paulson JC.** 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology **205:**17-23.
49. **Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC.** 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature **304:**76-78.
50. **Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, Herfst S, van Boheemen S, Linster M, Schrauwen EJ, Katzelnick L, Mosterin A, Kuiken T, Maher E, Neumann G, Osterhaus AD, Kawaoka Y, Fouchier RA, Smith DJ.** 2012. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. Science **336:**1541-1547.
51. **Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y.** 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature **486:**420-428.
52. **Lakadamyali M, Rust MJ, Zhuang X.** 2004. Endocytosis of influenza viruses. Microbes and infection / Institut Pasteur **6:**929-936.
53. **Sieczkarski SB, Whittaker GR.** 2002. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. Journal of virology **76:**10455-10464.
54. **Pinto LH, Holsinger LJ, Lamb RA.** 1992. Influenza virus M2 protein has ion channel activity. Cell **69:**517-528.
55. **Pleschka S.** 2012. Overview of Influenza Viruses. Curr Top Microbiol Immunol.
56. **Han X, Bushweller JH, Cafiso DS, Tamm LK.** 2001. Membrane structure and fusion-triggering conformational change of the fusion domain from influenza hemagglutinin. Nature structural biology **8:**715-720.

57. **Akkina RK, Chambers TM, Londo DR, Nayak DP.** 1987. Intracellular localization of the viral polymerase proteins in cells infected with influenza virus and cells expressing PB1 protein from cloned cDNA. Journal of virology **61:**2217-2224.
58. **Cros JF, Garcia-Sastre A, Palese P.** 2005. An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein. Traffic **6:**205-213.
59. **O'Neill RE, Jaskunas R, Blobel G, Palese P, Moroianu J.** 1995. Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. The Journal of biological chemistry **270:**22701-22704.
60. **Hay AJ, Lomniczi B, Bellamy AR, Skehel JJ.** 1977. Transcription of the influenza virus genome. Virology **83:**337-355.
61. **Gonzalez S, Zurcher T, Ortin J.** 1996. Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. Nucleic acids research **24:**4456-4463.
62. **Lee MT, Klumpp K, Digard P, Tiley L.** 2003. Activation of influenza virus RNA polymerase by the 5' and 3' terminal duplex of genomic RNA. Nucleic acids research **31:**1624-1632.
63. **Plotch SJ, Bouloy M, Ulmanen I, Krug RM.** 1981. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell **23:**847-858.
64. **Yuan P, Bartlam M, Lou Z, Chen S, Zhou J, He X, Lv Z, Ge R, Li X, Deng T, Fodor E, Rao Z, Liu Y.** 2009. Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. Nature **458:**909-913.
65. **Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, Cusack S, Ruigrok RW.** 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature **458:**914-918.
66. **Hagen M, Chung TD, Butcher JA, Krystal M.** 1994. Recombinant influenza virus polymerase: requirement of both 5' and 3' viral ends for endonuclease activity. Journal of virology **68:**1509-1515.
67. **Krug RM, Broni BA, Bouloy M.** 1979. Are the 5' ends of influenza viral mRNAs synthesized in vivo donated by host mRNAs? Cell **18:**329-334.
68. **Braam J, Ulmanen I, Krug RM.** 1983. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. Cell **34:**609-618.
69. **Poon LL, Pritlove DC, Fodor E, Brownlee GG.** 1999. Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. Journal of virology **73:**3473-3476.
70. **Zheng H, Lee HA, Palese P, Garcia-Sastre A.** 1999. Influenza A virus RNA polymerase has the ability to stutter at the polyadenylation site of a viral RNA template during RNA replication. Journal of virology **73:**5240-5243.

71. **Park YW, Katze MG.** 1995. Translational control by influenza virus. Identification of cis-acting sequences and trans-acting factors which may regulate selective viral mRNA translation. The Journal of biological chemistry **270:**28433-28439.
72. **Scheiffele P, Rietveld A, Wilk T, Simons K.** 1999. Influenza viruses select ordered lipid domains during budding from the plasma membrane. The Journal of biological chemistry **274:**2038-2044.
73. **Zhang J, Pekosz A, Lamb RA.** 2000. Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. Journal of virology **74:**4634-4644.
74. **Martin K, Helenius A.** 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell **67:**117-130.
75. **Neumann G, Hughes MT, Kawaoka Y.** 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. The EMBO journal **19:**6751-6758.
76. **O'Neill RE, Talon J, Palese P.** 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. The EMBO journal **17:**288-296.
77. **Gog JR, Afonso Edos S, Dalton RM, Leclercq I, Tiley L, Elton D, von Kirchbach JC, Naffakh N, Escriou N, Digard P.** 2007. Codon conservation in the influenza A virus genome defines RNA packaging signals. Nucleic acids research **35:**1897-1907.
78. **Noda T, Sagara H, Yen A, Takada A, Kida H, Cheng RH, Kawaoka Y.** 2006. Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature **439:**490-492.
79. **Muramoto Y, Takada A, Fujii K, Noda T, Iwatsuki-Horimoto K, Watanabe S, Horimoto T, Kida H, Kawaoka Y.** 2006. Hierarchy among viral RNA (vRNA) segments in their role in vRNA incorporation into influenza A virions. Journal of virology **80:**2318-2325.
80. **Chen BJ, Leser GP, Morita E, Lamb RA.** 2007. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. Journal of virology **81:**7111-7123.
81. **Rossman JS, Jing X, Leser GP, Lamb RA.** 2010. Influenza virus M2 protein mediates ESCRT-independent membrane scission. Cell **142:**902-913.
82. **S. J. Flint LWE, V. R. Racaniello, A. M. Skalka.** 2003. Principles of Virology : Molecular Biology, Pathogenesis, and Control of Animal Viruses. ASM Press.
83. **Coleman JR.** 2007. The PB1-F2 protein of Influenza A virus: increasing pathogenicity by disrupting alveolar macrophages. Virol J **4:**9.
84. **Zamarin D, Ortigoza MB, Palese P.** 2006. Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. Journal of virology **80:**7976-7983.
85. **Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, Howard W, Anderson EC, Barclay WS, Digard P.** 2009. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. Journal of virology **83:**8021-8031.

86. **Tauber S, Ligertwood Y, Quigg-Nicol M, Dutia BM, Elliott RM.** 2012. Behaviour of influenza A viruses differentially expressing segment 2 gene products in vitro and in vivo. The Journal of general virology **93:**840-849.
87. **Muramoto Y, Noda T, Kawakami E, Akkina R, Kawaoka Y.** 2012. Identification of novel influenza A virus proteins translated from PA mRNA. Journal of virology.
88. **Yewdell JW, Ince WL.** 2012. Virology. Frameshifting to PA-X influenza. Science **337:**164-165.
89. **Nowak MA.** 1992. What is a quasispecies? Trends in ecology & evolution **7:**118-121.
90. **Bean WJ, Schell M, Katz J, Kawaoka Y, Naeve C, Gorman O, Webster RG.** 1992. Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. Journal of virology **66:**1129-1138.
91. **Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y.** 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. Journal of virology **72:**7367-7373.
92. **Hatta M, Gao P, Halfmann P, Kawaoka Y.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840-1842.
93. **Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y.** 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS pathogens **3:**1374-1379.
94. **Van Hoeven N, Pappas C, Belser JA, Maines TR, Zeng H, Garcia-Sastre A, Sasisekharan R, Katz JM, Tumpey TM.** 2009. Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A **106:**3366-3371.
95. **Steel J, Lowen AC, Mubareka S, Palese P.** 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS pathogens **5:**e1000252.
96. **Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P.** 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS pathogens **3:**1414-1421.
97. **Zell R, Krumbholz A, Eitner A, Krieg R, Halbhuber KJ, Wutzler P.** 2007. Prevalence of PB1-F2 of influenza A viruses. The Journal of general virology **88:**536-546.
98. **Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P.** 2005. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS pathogens **1:**e4.
99. **Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, Fernandez-Sesma A, Palese P.** 2011. The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. PLoS pathogens **7:**e1002067.
100. **Dudek SE, Wixler L, Nordhoff C, Nordmann A, Anhlan D, Wixler V, Ludwig S.** 2011. The influenza virus PB1-F2 protein has interferon antagonistic activity. Biological chemistry **392:**1135-1144.

101. **Medina RA, Stertz S, Manicassamy B, Zimmermann P, Sun X, Albrecht RA, Uusi-Kerttula H, Zagordi O, Belshe RB, Frey SE, Tumpey TM, Garcia-Sastre A.** 2013. Glycosylations in the Globular Head of the Hemagglutinin Protein Modulate the Virulence and Antigenic Properties of the H1N1 Influenza Viruses. Science translational medicine **5:**187ra170.
102. **Sun X, Jayaraman A, Maniprasad P, Raman R, Houser KV, Pappas C, Zeng H, Sasisekharan R, Katz JM, Tumpey TM.** 2013. N-linked Glycosylation of the hemagglutinin protein influences virulence and antigenicity of the 1918 pandemic and seasonal H1N1 influenza A viruses. Journal of virology.
103. **Hawgood S, Brown C, Edmondson J, Stumbaugh A, Allen L, Goerke J, Clark H, Poulain F.** 2004. Pulmonary collectins modulate strain-specific influenza a virus infection and host responses. Journal of virology **78:**8565-8572.
104. **Ken Murphy PT, Mark Walport** 2007. Janeway's Immunobiology, 7th ed. Garland Science.
105. **Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C.** 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science **303:**1529-1531.
106. **Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M, Si-Tahar M.** 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. Journal of immunology **178:**3368-3372.
107. **Szretter KJ, Gangappa S, Belser JA, Zeng H, Chen H, Matsuoka Y, Sambhara S, Swayne DE, Tumpey TM, Katz JM.** 2009. Early Control of H5N1 Influenza Virus Replication by the Type I Interferon Response in Mice. Journal of virology.
108. **Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G.** 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science **314:**994-997.
109. **Randall RE, Goodbourn S.** 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. The Journal of general virology **89:**1-47.
110. **Zeng H, Goldsmith C, Thawatsupha P, Chittaganpitch M, Waicharoen S, Zaki S, Tumpey TM, Katz JM.** 2007. Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type i interferon response in polarized human bronchial epithelial cells. Journal of virology **81:**12439-12449.
111. **Patel JR, Vora KP, Tripathi S, Zeng H, Tumpey TM, Katz JM, Sambhara S, Gangappa S.** 2011. Infection of lung epithelial cells with pandemic 2009 A(H1N1) influenza viruses reveals isolate-specific differences in infectivity and host cellular responses. Viral immunology **24:**89-99.
112. **Zeng H, Pappas C, Katz JM, Tumpey TM.** 2011. The 2009 pandemic H1N1 and triple-reassortant swine H1N1 influenza viruses replicate efficiently but elicit an attenuated inflammatory response in polarized human bronchial epithelial cells. Journal of virology **85:**686-696.
113. **Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, Guthrie EH, Pickles RJ, Ting JP.** 2009. The NLRP3 inflammasome mediates

in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity **30:**556-565.

114. **Ichinohe T, Lee HK, Ogura Y, Flavell R, Iwasaki A.** 2009. Inflammasome recognition of influenza virus is essential for adaptive immune responses. The Journal of experimental medicine **206:**79-87.
115. **Meurs E, Chong K, Galabru J, Thomas NS, Kerr IM, Williams BR, Hovanessian AG.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell **62:**379-390.
116. **Herold S, von Wulffen W, Steinmueller M, Pleschka S, Kuziel WA, Mack M, Srivastava M, Seeger W, Maus UA, Lohmeyer J.** 2006. Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. Journal of immunology **177:**1817-1824.
117. **Holt PG.** 1978. Inhibitory activity of unstimulated alveolar macrophages on T-lymphocyte blastogenic response. The American review of respiratory disease **118:**791-793.
118. **Becker S, Quay J, Soukup J.** 1991. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. Journal of immunology **147:**4307-4312.
119. **Herold S, Steinmueller M, von Wulffen W, Cakarova L, Pinto R, Pleschka S, Mack M, Kuziel WA, Corazza N, Brunner T, Seeger W, Lohmeyer J.** 2008. Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. The Journal of experimental medicine **205:**3065-3077.
120. **Lin KL, Suzuki Y, Nakano H, Ramsburg E, Gunn MD.** 2008. CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. Journal of immunology **180:**2562-2572.
121. **Jayasekera JP, Vinuesa CG, Karupiah G, King NJ.** 2006. Enhanced antiviral antibody secretion and attenuated immunopathology during influenza virus infection in nitric oxide synthase-2-deficient mice. The Journal of general virology **87:**3361-3371.
122. **Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, Davis DM, Strominger JL, Yewdell JW, Porgador A.** 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature **409:**1055-1060.
123. **Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O.** 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. European journal of immunology **31:**2680-2689.
124. **Trinchieri G.** 1989. Biology of natural killer cells. Advances in immunology **47:**187-376.
125. **Tay CH, Szomolanyi-Tsuda E, Welsh RM.** 1998. Control of infections by NK cells. Curr Top Microbiol Immunol **230:**193-220.
126. **Gerosa F, Gobbi A, Zorzi P, Burg S, Briere F, Carra G, Trinchieri G.** 2005. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic

cells profoundly affects innate resistance functions. Journal of immunology **174:**727-734.

127. **He XS, Draghi M, Mahmood K, Holmes TH, Kemble GW, Dekker CL, Arvin AM, Parham P, Greenberg HB.** 2004. T cell-dependent production of IFN-gamma by NK cells in response to influenza A virus. The Journal of clinical investigation **114:**1812-1819.
128. **Barchet W, Krug A, Cella M, Newby C, Fischer JA, Dzionek A, Pekosz A, Colonna M.** 2005. Dendritic cells respond to influenza virus through TLR7- and PKR-independent pathways. European journal of immunology **35:**236-242.
129. **Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S.** 2005. Cell type-specific involvement of RIG-I in antiviral response. Immunity **23:**19-28.
130. **Bhardwaj N, Bender A, Gonzalez N, Bui LK, Garrett MC, Steinman RM.** 1994. Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells. The Journal of clinical investigation **94:**797-807.
131. **Albert ML, Sauter B, Bhardwaj N.** 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature **392:**86-89.
132. **Banchereau J, Steinman RM.** 1998. Dendritic cells and the control of immunity. Nature **392:**245-252.
133. **GeurtsvanKessel CH, Lambrecht BN.** 2008. Division of labor between dendritic cell subsets of the lung. Mucosal immunology **1:**442-450.
134. **Maines TR, Belser JA, Gustin KM, van Hoeven N, Zeng H, Svitek N, von Messling V, Katz JM, Tumpey TM.** 2012. Local innate immune responses and influenza virus transmission and virulence in ferrets. The Journal of infectious diseases **205:**474-485.
135. **Gentile D, Doyle W, Whiteside T, Fireman P, Hayden FG, Skoner D.** 1998. Increased interleukin-6 levels in nasal lavage samples following experimental influenza A virus infection. Clinical and diagnostic laboratory immunology **5:**604-608.
136. **Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, Straus SE.** 1998. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. The Journal of clinical investigation **101:**643-649.
137. **Skoner DP, Gentile DA, Patel A, Doyle WJ.** 1999. Evidence for cytokine mediation of disease expression in adults experimentally infected with influenza A virus. The Journal of infectious diseases **180:**10-14.
138. **Zeng H, Goldsmith CS, Maines TR, Belser JA, Gustin KM, Pekosz A, Zaki SR, Katz JM, Tumpey TM.** 2013. Tropism and infectivity of influenza virus, including highly pathogenic avian H5N1 virus, in ferret tracheal differentiated primary epithelial cell cultures. Journal of virology **87:**2597-2607.
139. **Cameron CM, Cameron MJ, Bermejo-Martin JF, Ran L, Xu L, Turner PV, Ran R, Danesh A, Fang Y, Chan PK, Mytle N, Sullivan TJ, Collins TL, Johnson MG, Medina JC, Rowe T, Kelvin DJ.** 2008. Gene expression analysis

of host innate immune responses during Lethal H5N1 infection in ferrets. Journal of virology **82:**11308-11317.

140. **Wolniak KL, Shinall SM, Waldschmidt TJ.** 2004. The germinal center response. Critical reviews in immunology **24:**39-65.
141. **Topham DJ, Tripp RA, Doherty PC.** 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. Journal of immunology **159:**5197-5200.
142. **Soghoian DZ, Streeck H.** 2010. Cytolytic CD4(+) T cells in viral immunity. Expert review of vaccines **9:**1453-1463.
143. **Moskophidis D, Kioussis D.** 1998. Contribution of virus-specific CD8+ cytotoxic T cells to virus clearance or pathologic manifestations of influenza virus infection in a T cell receptor transgenic mouse model. The Journal of experimental medicine **188:**223-232.
144. **Yewdell JW, Bennink JR, Smith GL, Moss B.** 1985. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc Natl Acad Sci U S A **82:**1785-1789.
145. **Gotch F, Rothbard J, Howland K, Townsend A, McMichael A.** 1987. Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2. Nature **326:**881-882.
146. **Zweerink HJ, Courtneidge SA, Skehel JJ, Crumpton MJ, Askonas BA.** 1977. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. Nature **267:**354-356.
147. **Hillaire ML, van Trierum SE, Kreijtz JH, Bodewes R, Geelhoed-Mieras MM, Nieuwkoop NJ, Fouchier RA, Kuiken T, Osterhaus AD, Rimmelzwaan GF.** 2011. Cross-protective immunity against influenza pH1N1 2009 viruses induced by seasonal influenza A (H3N2) virus is mediated by virus-specific T-cells. The Journal of general virology **92:**2339-2349.
148. **Yu D, Vinuesa CG.** 2010. The elusive identity of T follicular helper cells. Trends in immunology **31:**377-383.
149. **Lee BO, Rangel-Moreno J, Moyron-Quiroz JE, Hartson L, Makris M, Sprague F, Lund FE, Randall TD.** 2005. CD4 T cell-independent antibody response promotes resolution of primary influenza infection and helps to prevent reinfection. Journal of immunology **175:**5827-5838.
150. **De Jong JC, Rimmelzwaan GF, Fouchier RA, Osterhaus AD.** 2000. Influenza virus: a master of metamorphosis. The Journal of infection **40:**218-228.
151. **Gerhard W.** 2001. The role of the antibody response in influenza virus infection. Curr Top Microbiol Immunol **260:**171-190.
152. **Wilson IA, Cox NJ.** 1990. Structural basis of immune recognition of influenza virus hemagglutinin. Annual review of immunology **8:**737-771.
153. **Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA.** 2009. Antibody recognition of a highly conserved influenza virus epitope. Science **324:**246-251.
154. **Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA.** 2009. Structural and

functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nature structural & molecular biology **16:**265-273.

155. **Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, Haye K, Garcia-Sastre A, Palese P.** 2010. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio **1**.
156. **Slepushkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ.** 1995. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. Vaccine **13:**1399-1402.
157. **Song JM, Wang BZ, Park KM, Van Rooijen N, Quan FS, Kim MC, Jin HT, Pekosz A, Compans RW, Kang SM.** 2011. Influenza virus-like particles containing M2 induce broadly cross protective immunity. PloS one **6:**e14538.
158. **Sambhara S, Kurichh A, Miranda R, Tumpey T, Rowe T, Renshaw M, Arpino R, Tamane A, Kandil A, James O, Underdown B, Klein M, Katz J, Burt D.** 2001. Heterosubtypic immunity against human influenza A viruses, including recently emerged avian H5 and H9 viruses, induced by FLU-ISCOM vaccine in mice requires both cytotoxic T-lymphocyte and macrophage function. Cellular immunology **211:**143-153.
159. **el-Madhun AS, Cox RJ, Soreide A, Olofsson J, Haaheim LR.** 1998. Systemic and mucosal immune responses in young children and adults after parenteral influenza vaccination. The Journal of infectious diseases **178:**933-939.
160. **Rothbarth PH, Groen J, Bohnen AM, de Groot R, Osterhaus AD.** 1999. Influenza virus serology--a comparative study. Journal of virological methods **78:**163-169.
161. **Mazanec MB, Coudret CL, Fletcher DR.** 1995. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. Journal of virology **69:**1339-1343.
162. **Murphy TF.** 1992. Haemophilus influenzae and viruses 100 years later: from influenza to human immunodeficiency virus. The American journal of medicine **92:**583-586.
163. **Mozdzanowska K, Maiese K, Furchner M, Gerhard W.** 1999. Treatment of influenza virus-infected SCID mice with nonneutralizing antibodies specific for the transmembrane proteins matrix 2 and neuraminidase reduces the pulmonary virus titer but fails to clear the infection. Virology **254:**138-146.
164. **Croft M, Bradley LM, Swain SL.** 1994. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. Journal of immunology **152:**2675-2685.
165. **McKinstry KK, Golech S, Lee WH, Huston G, Weng NP, Swain SL.** 2007. Rapid default transition of CD4 T cell effectors to functional memory cells. The Journal of experimental medicine **204:**2199-2211.
166. **Strutt TM, McKinstry KK, Dibble JP, Winchell C, Kuang Y, Curtis JD, Huston G, Dutton RW, Swain SL.** 2010. Memory CD4+ T cells induce innate responses independently of pathogen. Nature medicine **16:**558-564, 551p following 564.

167. **Riberdy JM, Christensen JP, Branum K, Doherty PC.** 2000. Diminished primary and secondary influenza virus-specific CD8(+) T-cell responses in CD4-depleted Ig(-/-) mice. Journal of virology **74:**9762-9765.
168. **Belz GT, Wodarz D, Diaz G, Nowak MA, Doherty PC.** 2002. Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. Journal of virology **76:**12388-12393.
169. **Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A.** 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature **401:**708-712.
170. **Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R.** 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nature immunology **4:**225-234.
171. **McHeyzer-Williams MG, Ahmed R.** 1999. B cell memory and the long-lived plasma cell. Current opinion in immunology **11:**172-179.
172. **McHeyzer-Williams MG, Nossal GJ, Lalor PA.** 1991. Molecular characterization of single memory B cells. Nature **350:**502-505.
173. **Minges Wols HA, Underhill GH, Kansas GS, Witte PL.** 2002. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. Journal of immunology **169:**4213-4221.
174. **Dunn-Walters DK, Isaacson PG, Spencer J.** 1995. Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells. The Journal of experimental medicine **182:**559-566.
175. **Francis T, Jr., Salk JE, Quilligan JJ, Jr.** 1947. Experience with vaccination against influenza in the spring of 1947; a preliminary report. American journal of public health and the nation's health **37:**1013-1016.
176. **Davenport FM, Hennessy AV, Francis T, Jr.** 1953. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. The Journal of experimental medicine **98:**641-656.
177. **Webster RG.** 1966. Original antigenic sin in ferrets: the response to sequential infections with influenza viruses. Journal of immunology **97:**177-183.
178. **Davenport FM, Hennessy AV.** 1956. A serologic recapitulation of past experiences with influenza A; antibody response to monovalent vaccine. The Journal of experimental medicine **104:**85-97.
179. **Kim JH, Skountzou I, Compans R, Jacob J.** 2009. Original antigenic sin responses to influenza viruses. Journal of immunology **183:**3294-3301.
180. **Hale BG, Randall RE, Ortin J, Jackson D.** 2008. The multifunctional NS1 protein of influenza A viruses. The Journal of general virology **89:**2359-2376.
181. **Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, Garcia-Sastre A.** 2009. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell host & microbe **5:**439-449.

182. **Sharma K, Tripathi S, Ranjan P, Kumar P, Garten R, Deyde V, Katz JM, Cox NJ, Lal RB, Sambhara S, Lal SK.** 2011. Influenza A virus nucleoprotein exploits Hsp40 to inhibit PKR activation. PloS one **6:**e20215.
183. **Lu Y, Qian XY, Krug RM.** 1994. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. Genes & development **8:**1817-1828.
184. **Nemeroff ME, Barabino SM, Li Y, Keller W, Krug RM.** 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs. Molecular cell **1:**991-1000.
185. **Qiu Y, Krug RM.** 1994. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). Journal of virology **68:**2425-2432.
186. **Baez M, Palese P, Kilbourne ED.** 1980. Gene composition of high-yielding influenza vaccine strains obtained by recombination. The Journal of infectious diseases **141:**362-365.
187. **Cate TR, Couch RB, Parker D, Baxter B.** 1983. Reactogenicity, immunogenicity, and antibody persistence in adults given inactivated influenza virus vaccines - 1978. Reviews of infectious diseases **5:**737-747.
188. **Williams MS, Mayner RE, Daniel NJ, Phelan MA, Rastogi SC, Bozeman FM, Ennis FA.** 1980. New developments in the measurement of the hemagglutinin content of influenza virus vaccines by single-radial-immunodiffusion. Journal of biological standardization **8:**289-296.
189. **FDA** 2012, posting date. Approved Products- Flucelvax. [Online.]
190. **Halperin SA, Smith B, Mabrouk T, Germain M, Trepanier P, Hassell T, Treanor J, Gauthier R, Mills EL.** 2002. Safety and immunogenicity of a trivalent, inactivated, mammalian cell culture-derived influenza vaccine in healthy adults, seniors, and children. Vaccine **20:**1240-1247.
191. **Lambert LC, Fauci AS.** 2010. Influenza vaccines for the future. The New England journal of medicine **363:**2036-2044.
192. **Jeisy-Scott V, Kim JH, Davis WG, Cao W, Katz JM, Sambhara S.** 2012. TLR7 Recognition Is Dispensable for Influenza Virus A Infection but Important for the Induction of Hemagglutinin-Specific Antibodies in Response to the 2009 Pandemic Split Vaccine in Mice. Journal of virology **86:**10988-10998.
193. **Cheng X, Zengel JR, Suguitan AL, Jr., Xu Q, Wang W, Lin J, Jin H.** 2013. Evaluation of the humoral and cellular immune responses elicited by the live attenuated and inactivated influenza vaccines and their roles in heterologous protection in ferrets. The Journal of infectious diseases **208:**594-602.
194. **McMichael AJ, Gotch F, Cullen P, Askonas B, Webster RG.** 1981. The human cytotoxic T cell response to influenza A vaccination. Clinical and experimental immunology **43:**276-284.
195. **FDA** 2013, posting date. Approved Products- Flublok. [Online.]
196. **Sciences P** 2011, posting date. Flublok. [Online.]
197. **FDA** 2012, posting date. Approved Products- Fluzone Intradermal. [Online.]
198. **Leroux-Roels I, Vets E, Freese R, Seiberling M, Weber F, Salamand C, Leroux-Roels G.** 2008. Seasonal influenza vaccine delivered by intradermal microinjection: A randomised controlled safety and immunogenicity trial in adults. Vaccine **26:**6614-6619.

199. **Sealy R, Webby RJ, Crumpton JC, Hurwitz JL.** 2012. Differential localization and function of antibody-forming cells responsive to inactivated or live-attenuated influenza virus vaccines. International immunology.
200. **Gustin KM, Maines TR, Belser JA, van Hoeven N, Lu X, Dong L, Isakova-Sivak I, Chen LM, Voeten JT, Heldens JG, van den Bosch H, Cox NJ, Tumpey TM, Klimov AI, Rudenko L, Donis RO, Katz JM.** 2011. Comparative immunogenicity and cross-clade protective efficacy of mammalian cell-grown inactivated and live attenuated H5N1 reassortant vaccines in ferrets. The Journal of infectious diseases **204:**1491-1499.
201. **Maassab HF.** 1969. Biologic and immunologic characteristics of cold-adapted influenza virus. Journal of immunology **102:**728-732.
202. **Cha TA, Kao K, Zhao J, Fast PE, Mendelman PM, Arvin A.** 2000. Genotypic stability of cold-adapted influenza virus vaccine in an efficacy clinical trial. Journal of clinical microbiology **38:**839-845.
203. **Zhou B, Li Y, Belser JA, Pearce MB, Schmolke M, Subba AX, Shi Z, Zaki SR, Blau DM, Garcia-Sastre A, Tumpey TM, Wentworth DE.** 2010. NS-based live attenuated H1N1 pandemic vaccines protect mice and ferrets. Vaccine **28:**8015-8025.
204. **Kim YC, Park JH, Prausnitz MR.** 2012. Microneedles for drug and vaccine delivery. Advanced drug delivery reviews **64:**1547-1568.
205. **Wu CY, Yeh YC, Chan JT, Yang YC, Yang JR, Liu MT, Wu HS, Hsiao PW.** 2012. A VLP vaccine induces broad-spectrum cross-protective antibody immunity against H5N1 and H1N1 subtypes of influenza A virus. PloS one **7:**e42363.
206. **Giles BM, Ross TM.** 2012. Computationally optimized antigens to overcome influenza viral diversity. Expert review of vaccines **11:**267-269.
207. **Price GE, Soboleski MR, Lo CY, Misplon JA, Pappas C, Houser KV, Tumpey TM, Epstein SL.** 2009. Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. Vaccine **27:**6512-6521.
208. **Monto AS, Pichichero ME, Blanckenberg SJ, Ruuskanen O, Cooper C, Fleming DM, Kerr C.** 2002. Zanamivir prophylaxis: an effective strategy for the prevention of influenza types A and B within households. The Journal of infectious diseases **186:**1582-1588.
209. **Monto AS, Robinson DP, Herlocher ML, Hinson JM, Jr., Elliott MJ, Crisp A.** 1999. Zanamivir in the prevention of influenza among healthy adults: a randomized controlled trial. JAMA : the journal of the American Medical Association **282:**31-35.
210. **Centers for Disease C, Prevention.** 2010. Update: influenza activity--United States, August 30, 2009-January 9, 2010. MMWR Morb Mortal Wkly Rep **59:**38-43.
211. **Hamelin ME, Baz M, Bouhy X, Beaulieu E, Dube K, Mallett C, Boivin G.** 2011. Reduced airborne transmission of oseltamivir-resistant pandemic A/H1N1 virus in ferrets. Antiviral therapy **16:**775-779.

212. **Govorkova EA.** 2013. Consequences of resistance: in vitro fitness, in vivo infectivity, and transmissibility of oseltamivir-resistant influenza A viruses. Influenza and other respiratory viruses **7 Suppl 1:**50-57.
213. **Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM.** 1999. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. Journal of virology **73:**5903-5911.
214. **Novak M, Moldoveanu Z, Schafer DP, Mestecky J, Compans RW.** 1993. Murine model for evaluation of protective immunity to influenza virus. Vaccine **11:**55-60.
215. **Tripp RA, Tompkins SM.** 2009. Animal models for evaluation of influenza vaccines. Curr Top Microbiol Immunol **333:**397-412.
216. **Hirst GK.** 1947. Studies on the Mechanism of Adaptation of Influenza Virus to Mice. The Journal of experimental medicine **86:**357-366.
217. **Lowen AC, Mubareka S, Tumpey TM, Garcia-Sastre A, Palese P.** 2006. The guinea pig as a transmission model for human influenza viruses. Proc Natl Acad Sci U S A **103:**9988-9992.
218. **Bodewes R, Rimmelzwaan GF, Osterhaus AD.** 2010. Animal models for the preclinical evaluation of candidate influenza vaccines. Expert review of vaccines **9:**59-72.
219. **O'Donnell CD, Subbarao K.** 2011. The contribution of animal models to the understanding of the host range and virulence of influenza A viruses. Microbes and infection / Institut Pasteur **13:**502-515.
220. **Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD.** 2001. Pathogenesis of influenza A (H5N1) virus infection in a primate model. Journal of virology **75:**6687-6691.
221. **Itoh Y, Ozaki H, Tsuchiya H, Okamoto K, Torii R, Sakoda Y, Kawaoka Y, Ogasawara K, Kida H.** 2008. A vaccine prepared from a non-pathogenic H5N1 avian influenza virus strain confers protective immunity against highly pathogenic avian influenza virus infection in cynomolgus macaques. Vaccine **26:**562-572.
222. **Chen Y, Deng W, Jia C, Dai X, Zhu H, Kong Q, Huang L, Liu Y, Ma C, Li J, Xiao C, Liu Y, Wei Q, Qin C.** 2009. Pathological lesions and viral localization of influenza A (H5N1) virus in experimentally infected Chinese rhesus macaques: implications for pathogenesis and viral transmission. Archives of virology **154:**227-233.
223. **Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD.** 2003. Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (Macaca fascicularis). Veterinary pathology **40:**304-310.
224. **Fan S, Gao Y, Shinya K, Li CK, Li Y, Shi J, Jiang Y, Suo Y, Tong T, Zhong G, Song J, Zhang Y, Tian G, Guan Y, Xu XN, Bu Z, Kawaoka Y, Chen H.** 2009. Immunogenicity and protective efficacy of a live attenuated H5N1 vaccine in nonhuman primates. PLoS pathogens **5:**e1000409.
225. **Li Y, Li J, Belisle S, Baskin CR, Tumpey TM, Katze MG.** 2011. Differential microRNA expression and virulence of avian, 1918 reassortant, and reconstructed 1918 influenza A viruses. Virology **421:**105-113.

226. **Shinya K, Gao Y, Cilloniz C, Suzuki Y, Fujie M, Deng G, Zhu Q, Fan S, Makino A, Muramoto Y, Fukuyama S, Tamura D, Noda T, Eisfeld AJ, Katze MG, Chen H, Kawaoka Y.** 2012. Integrated clinical, pathologic, virologic, and transcriptomic analysis of H5N1 influenza virus-induced viral pneumonia in the rhesus macaque. Journal of virology **86:**6055-6066.
227. **Reuman PD, Keely S, Schiff GM.** 1989. Assessment of signs of influenza illness in the ferret model. Journal of virological methods **24:**27-34.
228. **Pearce MB, Jayaraman A, Pappas C, Belser JA, Zeng H, Gustin KM, Maines TR, Sun X, Raman R, Cox NJ, Sasisekharan R, Katz JM, Tumpey TM.** 2012. Pathogenesis and transmission of swine origin A(H3N2)v influenza viruses in ferrets. Proc Natl Acad Sci U S A **109:**3944-3949.
229. **Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, Zeng H, Gustin KM, Pearce MB, Viswanathan K, Shriver ZH, Raman R, Cox NJ, Sasisekharan R, Katz JM, Tumpey TM.** 2009. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science **325:**484-487.
230. **Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM.** 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. Journal of virology **76:**4420-4429.
231. **Cheng X, Zengel JR, Suguitan AL, Jr., Xu Q, Wang W, Lin J, Jin H.** 2013. Evaluation of the Humoral and Cellular Immune Responses Elicited by the Live Attenuated and Inactivated Influenza Vaccines and Their Roles in Heterologous Protection in Ferrets. The Journal of infectious diseases.
232. **van der Laan JW, Herberts C, Lambkin-Williams R, Boyers A, Mann AJ, Oxford J.** 2008. Animal models in influenza vaccine testing. Expert review of vaccines **7:**783-793.
233. **Nishiura H, Yen HL, Cowling BJ.** 2013. Sample Size Considerations for One-to-One Animal Transmission Studies of the Influenza A Viruses. PloS one **8:**e55358.
234. **Belser JA, Maines TR, Tumpey TM, Katz JM.** 2010. Influenza A virus transmission: contributing factors and clinical implications. Expert reviews in molecular medicine **12:**e39.
235. **Tumpey TM, Belser JA.** 2009. Resurrected pandemic influenza viruses. Annual review of microbiology **63:**79-98.
236. **Miller MA, Viboud C, Balinska M, Simonsen L.** 2009. The signature features of influenza pandemics--implications for policy. The New England journal of medicine **360:**2595-2598.
237. **Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A.** 2005. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science **310:**77-80.
238. **Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG.** 2005. Characterization of the 1918 influenza virus polymerase genes. Nature **437:**889-893.

239. **Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y.** 1992. Evolution and ecology of influenza A viruses. Microbiological reviews **56:**152-179.
240. **Gething MJ, Bye J, Skehel J, Waterfield M.** 1980. Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature **287:**301-306.
241. **Kawaoka Y, Krauss S, Webster RG.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. Journal of virology **63:**4603-4608.
242. **WHO** 2010, posting date. Pandemic (H1N1) 2009 - update 112. [Online.]
243. **Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM.** 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. The New England journal of medicine **360:**2605-2615.
244. **Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr., Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ.** 2009. Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans. Science **325:**197-201.
245. **Capua I, Alexander DJ.** 2004. Human health implications of avian influenza viruses and paramyxoviruses. Eur J Clin Microbiol Infect Dis **23:**1-6.
246. **(OIE) OIdE.** 1996. Highly pathogenic avian influenza (fowl plague). p. 155-160, Manual of standards for diagnostic tests and vaccines,, 3 ed, vol. 2013. OIE, Paris.
247. **Suarez DL.** 2010. Avian influenza: our current understanding. Animal health research reviews / Conference of Research Workers in Animal Diseases **11:**19-33.
248. **WHO** 2013, posting date. Influenza at the Human-Animal Interface (HAI). [Online.]
249. **CDC**, posting date. CDC - Avian Influenza (Bird Flu). [Online.]
250. **Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD.** 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A **101:**1356-1361.
251. **Belser JA, Lu X, Maines TR, Smith C, Li Y, Donis RO, Katz JM, Tumpey TM.** 2007. Pathogenesis of avian influenza (H7) virus infection in mice and

ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. Journal of virology **81:**11139-11147.

252. **Centers for Disease C, Prevention.** 1997. Isolation of avian influenza A(H5N1) viruses from humans--Hong Kong, May-December 1997. MMWR Morb Mortal Wkly Rep **46:**1204-1207.
253. **Neumann G, Chen H, Gao GF, Shu Y, Kawaoka Y.** 2010. H5N1 influenza viruses: outbreaks and biological properties. Cell research **20:**51-61.
254. **Centers for Disease C, Prevention.** 2004. Outbreaks of avian influenza A (H5N1) in Asia and interim recommendations for evaluation and reporting of suspected cases--United States, 2004. MMWR Morb Mortal Wkly Rep **53:**97-100.
255. **Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P, Chittaganpitch M, Waicharoen S, Nguyen DT, Nguyen T, Nguyen HH, Kim JH, Hoang LT, Kang C, Phuong LS, Lim W, Zaki S, Donis RO, Cox NJ, Katz JM, Tumpey TM.** 2005. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. Journal of virology **79:**11788-11800.
256. **Gu J, Xie Z, Gao Z, Liu J, Korteweg C, Ye J, Lau LT, Lu J, Gao Z, Zhang B, McNutt MA, Lu M, Anderson VM, Gong E, Yu AC, Lipkin WI.** 2007. H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet **370:**1137-1145.
257. **Zhang Z, Zhang J, Huang K, Li KS, Yuen KY, Guan Y, Chen H, Ng WF.** 2009. Systemic infection of avian influenza A virus H5N1 subtype in humans. Human pathology **40:**735-739.
258. **Neumann G, Green MA, Macken CA.** 2010. Evolution of highly pathogenic avian H5N1 influenza viruses and the emergence of dominant variants. The Journal of general virology **91:**1984-1995.
259. **Yang Y, Halloran ME, Sugimoto JD, Longini IM, Jr.** 2007. Detecting human-to-human transmission of avian influenza A (H5N1). Emerging infectious diseases **13:**1348-1353.
260. **Wang H, Feng Z, Shu Y, Yu H, Zhou L, Zu R, Huai Y, Dong J, Bao C, Wen L, Yang P, Zhao W, Dong L, Zhou M, Liao Q, Yang H, Wang M, Lu X, Shi Z, Wang W, Gu L, Zhu F, Li Q, Yin W, Yang W, Li D, Uyeki TM, Wang Y.** 2008. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. Lancet **371:**1427-1434.
261. **Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, Uiprasertkul M, Boonnak K, Pittayawonganon C, Cox NJ, Zaki SR, Thawatsupha P, Chittaganpitch M, Khontong R, Simmerman JM, Chunsutthiwat S.** 2005. Probable person-to-person transmission of avian influenza A (H5N1). The New England journal of medicine **352:**333-340.
262. **Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF.** 1999. Human infection with influenza H9N2. Lancet **354:**916-917.

263. **Belser JA, Bridges CB, Katz JM, Tumpey TM.** 2009. Past, present, and possible future human infection with influenza virus A subtype H7. Emerging infectious diseases **15:**859-865.
264. **Munster VJ, Schrauwen EJ, de Wit E, van den Brand JM, Bestebroer TM, Herfst S, Rimmelzwaan GF, Osterhaus AD, Fouchier RA.** 2010. Insertion of a multibasic cleavage motif into the hemagglutinin of a low-pathogenic avian influenza H6N1 virus induces a highly pathogenic phenotype. Journal of virology **84:**7953-7960.
265. **Shope RE.** 1934. The Infection of Ferrets with Swine Influenza Virus, p. 49-62.
266. **Shope RE.** 1931. Swine Influenza : I. Experimental Transmission and Pathology. The Journal of experimental medicine **54:**349-359.
267. **Olsen CW.** 2002. The emergence of novel swine influenza viruses in North America. Virus research **85:**199-210.
268. **Krueger WS, Gray GC.** 2012. Swine Influenza Virus Infections in Man. Curr Top Microbiol Immunol.
269. **Lycett SJ, Baillie G, Coulter E, Bhatt S, Kellam P, McCauley JW, Wood JL, Brown IH, Pybus OG, Leigh Brown AJ, Combating Swine Influenza Initiative CC.** 2012. Estimating reassortment rates in co-circulating Eurasian swine influenza viruses. The Journal of general virology **93:**2326-2336.
270. **Chambers TM, Hinshaw VS, Kawaoka Y, Easterday BC, Webster RG.** 1991. Influenza viral infection of swine in the United States 1988-1989. Archives of virology **116:**261-265.
271. **Hinshaw VS, Bean WJ, Jr., Webster RG, Easterday BC.** 1978. The prevalence of influenza viruses in swine and the antigenic and genetic relatedness of influenza viruses from man and swine. Virology **84:**51-62.
272. **Vincent AL, Lager KM, Ma W, Lekcharoensuk P, Gramer MR, Loiacono C, Richt JA.** 2006. Evaluation of hemagglutinin subtype 1 swine influenza viruses from the United States. Veterinary microbiology **118:**212-222.
273. **Olsen CW, Carey S, Hinshaw L, Karasin AI.** 2000. Virologic and serologic surveillance for human, swine and avian influenza virus infections among pigs in the north-central United States. Archives of virology **145:**1399-1419.
274. **Karasin AI, Landgraf J, Swenson S, Erickson G, Goyal S, Woodruff M, Scherba G, Anderson G, Olsen CW.** 2002. Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. Journal of clinical microbiology **40:**1073-1079.
275. **Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S, Webster RG.** 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. Journal of virology **73:**8851-8856.
276. **CDC.** 2011. Limited Human-to-Human Transmission of Novel Influenza A (H3N2) Virus - Iowa, November 2011. MMWR Morb Mortal Wkly Rep **60:**1615-1617.
277. **Centers for Disease C, Prevention.** 2012. Notes from the Field: Outbreak of Influenza A (H3N2) Virus Among Persons and Swine at a County Fair - Indiana, July 2012. MMWR Morb Mortal Wkly Rep **61:**561.

278. **Centers for Disease C, Prevention.** 2012. Antibodies cross-reactive to influenza A (H3N2) variant virus and impact of 2010-11 seasonal influenza vaccine on cross-reactive antibodies - United States. MMWR Morb Mortal Wkly Rep **61:**237-241.
279. **Skowronski DM, Janjua NZ, De Serres G, Purych D, Gilca V, Scheifele DW, Dionne M, Sabaiduc S, Gardy JL, Li G, Bastien N, Petric M, Boivin G, Li Y.** 2012. Cross-reactive and vaccine-induced antibody to an emerging swine-origin variant of influenza A virus subtype H3N2 (H3N2v). The Journal of infectious diseases **206:**1852-1861.
280. **Waalen K, Kilander A, Dudman SG, Ramos-Ocao R, Hungnes O.** 2012. Age-dependent prevalence of antibodies cross-reactive to the influenza A(H3N2) variant virus in sera collected in Norway in 2011. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin **17**.
281. **Centers for Disease C.** 1993. Prevention and control of influenza: Part I, Vaccines. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control **42:**1-14.
282. **Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon KJ, Krauss S, Webster RG.** 2000. Emergence of H3N2 reassortant influenza A viruses in North American pigs. Veterinary microbiology **74:**47-58.
283. **Vincent AL, Ma W, Lager KM, Janke BH, Richt JA.** 2008. Swine influenza viruses a North American perspective. Advances in virus research **72:**127-154.
284. **Shu B, Garten R, Emery S, Balish A, Cooper L, Sessions W, Deyde V, Smith C, Berman L, Klimov A, Lindstrom S, Xu X.** 2012. Genetic analysis and antigenic characterization of swine origin influenza viruses isolated from humans in the United States, 1990-2010. Virology **422:**151-160.
285. **Cox CM, Neises D, Garten RJ, Bryant B, Hesse RA, Anderson GA, Trevino-Garrison I, Shu B, Lindstrom S, Klimov AI, Finelli L.** 2011. Swine Influenza Virus A (H3N2) Infection in Human, Kansas, USA, 2009. Emerging infectious diseases **17:**1143-1144.
286. **Lindstrom S, Garten R, Balish A, Shu B, Emery S, Berman L, Barnes N, Sleeman K, Gubareva L, Villanueva J, Klimov A.** 2012. Human infections with novel reassortant influenza A(H3N2)v viruses, United States, 2011. Emerging infectious diseases **18:**834-837.
287. 2012. Update: Influenza A (H3N2)v Transmission and Guidelines - Five States, 2011. MMWR Morb Mortal Wkly Rep **60:**1741-1744.
288. **Centers for Disease C, Prevention.** 2012. Update: influenza activity - United States, 2011-12 season and composition of the 2012-13 influenza vaccine. MMWR Morb Mortal Wkly Rep **61:**414-420.
289. **Centers for Disease C, Prevention.** 2012. Evaluation of rapid influenza diagnostic tests for influenza A (H3N2)v virus and updated case count--United States, 2012. MMWR Morb Mortal Wkly Rep **61:**619-621.

290. **Centers for Disease C, Prevention.** 2011. Update: influenza activity--United States, 2010-11 season, and composition of the 2011-12 influenza vaccine. MMWR Morb Mortal Wkly Rep **60:**705-712.
291. **Pearce MB, Belser JA, Gustin KM, Pappas C, Houser KV, Sun X, Maines TR, Pantin-Jackwood MJ, Katz JM, Tumpey TM.** 2012. Seasonal trivalent inactivated influenza vaccine protects against 1918 Spanish influenza virus infection in ferrets. Journal of virology **86:**7118-7125.
292. **Powers DC, Kilbourne ED, Johansson BE.** 1996. Neuraminidase-specific antibody responses to inactivated influenza virus vaccine in young and elderly adults. Clinical and diagnostic laboratory immunology **3:**511-516.
293. **CDC** July 5 2013, posting date. Case Count: Detected U.S. Human Infections with H3N2v by State since August 2011. [Online.]
294. **Richt JA, Lager KM, Janke BH, Woods RD, Webster RG, Webby RJ.** 2003. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. Journal of clinical microbiology **41:**3198-3205.
295. **Houser KV, Katz JM, Tumpey TM.** 2013. Seasonal trivalent inactivated influenza vaccine does not protect against newly emerging variants of influenza A (H3N2v) virus in ferrets. Journal of virology **87:**1261-1263.
296. **Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, Ortin J, Falcon A, Nguyen TH, Mai le Q, Sedyaningsih ER, Harun S, Tumpey TM, Donis RO, Cox NJ, Subbarao K, Katz JM.** 2006. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A **103:**12121-12126.
297. **Epperson S, Jhung M, Richards S, Quinlisk P, Ball L, Moll M, Boulton R, Haddy L, Biggerstaff M, Brammer L, Trock S, Burns E, Gomez T, Wong KK, Katz J, Lindstrom S, Klimov A, Bresee JS, Jernigan DB, Cox N, Finelli L, Influenza AvVIT.** 2013. Human Infections With Influenza A(H3N2) Variant Virus in the United States, 2011-2012. Clin Infect Dis **57 Suppl 1:**S4-S11.
298. **Pascua PN, Song MS, Lee JH, Park KJ, Kwon HI, Baek YH, Hong SP, Rho JB, Kim CJ, Poo H, Ryoo TS, Sung MH, Choi YK.** 2009. Evaluation of the efficacy and cross-protectivity of recent human and swine vaccines against the pandemic (H1N1) 2009 virus infection. PloS one **4:**e8431.
299. **Ellebedy AH, Ducatez MF, Duan S, Stigger-Rosser E, Rubrum AM, Govorkova EA, Webster RG, Webby RJ.** 2011. Impact of prior seasonal influenza vaccination and infection on pandemic A (H1N1) influenza virus replication in ferrets. Vaccine **29:**3335-3339.
300. **Steel J, Staeheli P, Mubareka S, Garcia-Sastre A, Palese P, Lowen AC.** 2010. Transmission of pandemic H1N1 influenza virus and impact of prior exposure to seasonal strains or interferon treatment. Journal of virology **84:**21-26.
301. **Pearce MB, Belser JA, Houser KV, Katz JM, Tumpey TM.** 2011. Efficacy of seasonal live attenuated influenza vaccine against virus replication and transmission of a pandemic 2009 H1N1 virus in ferrets. Vaccine **29:**2887-2894.

302. **Tumpey TM, Renshaw M, Clements JD, Katz JM.** 2001. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. Journal of virology **75:**5141-5150.
303. **Harvey R, Wheeler JX, Wallis CL, Robertson JS, Engelhardt OG.** 2008. Quantitation of haemagglutinin in H5N1 influenza viruses reveals low haemagglutinin content of vaccine virus NIBRG-14 (H5N1). Vaccine **26:**6550-6554.
304. **HHS.** 2009. Biosafety in Microbiological and Biomedical Laboratories, 5th ed, vol. CDC 21-1112. HHS Publication, Atlanta, Georgia.
305. **Katz JM, Lu X, Young SA, Galphin JC.** 1997. Adjuvant activity of the heat-labile enterotoxin from enterotoxigenic Escherichia coli for oral administration of inactivated influenza virus vaccine. The Journal of infectious diseases **175:**352-363.
306. **Cox NJ, Subbarao K.** 1999. Influenza. Lancet **354:**1277-1282.
307. **Ghendon YZ, Kaira AN, Elshina GA.** 2006. The effect of mass influenza immunization in children on the morbidity of the unvaccinated elderly. Epidemiology and infection **134:**71-78.
308. **Hannoun C, Megas F, Piercy J.** 2004. Immunogenicity and protective efficacy of influenza vaccination. Virus research **103:**133-138.
309. **Laurie KL, Carolan LA, Middleton D, Lowther S, Kelso A, Barr IG.** 2010. Multiple infections with seasonal influenza A virus induce cross-protective immunity against A(H1N1) pandemic influenza virus in a ferret model. The Journal of infectious diseases **202:**1011-1020.
310. **Forrest BD, Pride MW, Dunning AJ, Capeding MR, Chotpitayasunondh T, Tam JS, Rappaport R, Eldridge JH, Gruber WC.** 2008. Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. Clin Vaccine Immunol **15:**1042-1053.
311. **Gorse GJ, Otto EE, Powers DC, Chambers GW, Eickhoff CS, Newman FK.** 1996. Induction of mucosal antibodies by live attenuated and inactivated influenza virus vaccines in the chronically ill elderly. The Journal of infectious diseases **173:**285-290.
312. **He XS, Holmes TH, Mahmood K, Kemble GW, Dekker CL, Arvin AM, Greenberg HB.** 2008. Phenotypic changes in influenza-specific CD8+ T cells after immunization of children and adults with influenza vaccines. The Journal of infectious diseases **197:**803-811.
313. **Richards KA, Chaves FA, Alam S, Sant AJ.** 2012. Trivalent inactivated influenza vaccines induce broad immunological reactivity to both internal virion components and influenza surface proteins. Vaccine **31:**219-225.
314. **Clements ML, Murphy BR.** 1986. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. Journal of clinical microbiology **23:**66-72.
315. **Arzey GG, Kirkland PD, Arzey KE, Frost M, Maywood P, Conaty S, Hurt AC, Deng YM, Iannello P, Barr I, Dwyer DE, Ratnamohan M, McPhie K, Selleck P.** 2012. Influenza virus A (H10N7) in chickens and poultry abattoir workers, Australia. Emerging infectious diseases **18:**814-816.

316. **ProMED-mail** 2013, posting date. Avian Influenza: Taiwan, H6N1 human case. International Society for Infectious Diseases. [Online.]
317. **Alford RH, Kasel JA, Gerone PJ, Knight V.** 1966. Human influenza resulting from aerosol inhalation. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y **122:**800-804.
318. **Bean B, Moore BM, Sterner B, Peterson LR, Gerding DN, Balfour HH, Jr.** 1982. Survival of influenza viruses on environmental surfaces. The Journal of infectious diseases **146:**47-51.
319. **Lidwell OM.** 1974. Aerial dispersal of micro-organisms from the human respiratory tract. Society for Applied Bacteriology symposium series **3:**135-154.
320. **Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soeroso S, Uyeki TM.** 2008. Update on avian influenza A (H5N1) virus infection in humans. The New England journal of medicine **358:**261-273.
321. **Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A **97:**6108-6113.
322. **Bergmann M, Garcia-Sastre A, Palese P.** 1992. Transfection-mediated recombination of influenza A virus. Journal of virology **66:**7576-7580.
323. **Chosewood LC WD, Centers for Disease Control and Prevention (US), and National Institutes of Health (US).** 2009. Biosafety in microbiological and biomedical laboratories, 5th ed. U.S. Department of Health and Human Services, Public Health Services, Centers for Disease Control and Prevention, National Institutes of Health, Washington, DC.
324. **Reed LJ, Muench H.** 1938. A simple method for estimating 50 percent endpoints. Am J Hyg **27:**493-497.
325. **Stephenson I, Wood JM, Nicholson KG, Charlett A, Zambon MC.** 2004. Detection of anti-H5 responses in human sera by HI using horse erythrocytes following MF59-adjuvanted influenza A/Duck/Singapore/97 vaccine. Virus research **103:**91-95.
326. **Glaser L, Zamarin D, Acland HM, Spackman E, Palese P, Garcia-Sastre A, Tewari D.** 2006. Sequence analysis and receptor specificity of the hemagglutinin of a recent influenza H2N2 virus isolated from chicken in North America. Glycoconjugate journal **23:**93-99.
327. **Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, Tumpey TM, Basler CF, Taubenberger JK, Palese P.** 2005. A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. Journal of virology **79:**11533-11536.
328. **Fodor E, Pritlove DC, Brownlee GG.** 1995. Characterization of the RNA-fork model of virion RNA in the initiation of transcription in influenza A virus. Journal of virology **69:**4012-4019.
329. **Kim HJ, Fodor E, Brownlee GG, Seong BL.** 1997. Mutational analysis of the RNA-fork model of the influenza A virus vRNA promoter in vivo. The Journal of general virology **78 (Pt 2):**353-357.

330. **Xu Q, Wang W, Cheng X, Zengel J, Jin H.** 2010. Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. Journal of virology **84:**4936-4945.
331. **Stevens J, Blixt O, Chen LM, Donis RO, Paulson JC, Wilson IA.** 2008. Recent avian H5N1 viruses exhibit increased propensity for acquiring human receptor specificity. Journal of molecular biology **381:**1382-1394.
332. **Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y.** 2006. Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature **444:**378-382.
333. **Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y, Li Z, Guan Y, Tian G, Li Y, Shi J, Liu L, Zeng X, Bu Z, Xia X, Kawaoka Y, Chen H.** 2009. Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS pathogens **5:**e1000709.
334. **Chutinimitkul S, van Riel D, Munster VJ, van den Brand JM, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA, de Wit E.** 2010. In vitro assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity. Journal of virology **84:**6825-6833.
335. **Yang ZY, Wei CJ, Kong WP, Wu L, Xu L, Smith DF, Nabel GJ.** 2007. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. Science **317:**825-828.
336. **Maines TR, Chen LM, Van Hoeven N, Tumpey TM, Blixt O, Belser JA, Gustin KM, Pearce MB, Pappas C, Stevens J, Cox NJ, Paulson JC, Raman R, Sasisekharan R, Katz JM, Donis RO.** 2011. Effect of receptor binding domain mutations on receptor binding and transmissibility of avian influenza H5N1 viruses. Virology **413:**139-147.
337. **Ilyushina NA, Govorkova EA, Gray TE, Bovin NV, Webster RG.** 2008. Human-like receptor specificity does not affect the neuraminidase-inhibitor susceptibility of H5N1 influenza viruses. PLoS pathogens **4:**e1000043.
338. **Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA.** 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science **336:**1534-1541.
339. **Fouchier RA, Garcia-Sastre A, Kawaoka Y.** 2012. Pause on avian flu transmission studies. Nature **481:**443.
340. **Massin P, van der Werf S, Naffakh N.** 2001. Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. Journal of virology **75:**5398-5404.
341. **Subbarao EK, London W, Murphy BR.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. Journal of virology **67:**1761-1764.

342. **Belser JA, Gustin KM, Pearce MB, Maines TR, Zeng H, Pappas C, Sun X, Carney PJ, Villanueva JM, Stevens J, Katz JM, Tumpey TM.** 2013. Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. Nature.
343. **Zhu H, Wang D, Kelvin DJ, Li L, Zheng Z, Yoon SW, Wong SS, Farooqui A, Wang J, Banner D, Chen R, Zheng R, Zhou J, Zhang Y, Hong W, Dong W, Cai Q, Roehrl MH, Huang SS, Kelvin AA, Yao T, Zhou B, Chen X, Leung GM, Poon LL, Webster RG, Webby RJ, Peiris JS, Guan Y, Shu Y.** 2013. Infectivity, transmission, and pathology of human-isolated H7N9 influenza virus in ferrets and pigs. Science **341:**183-186.
344. **Watanabe T, Kiso M, Fukuyama S, Nakajima N, Imai M, Yamada S, Murakami S, Yamayoshi S, Iwatsuki-Horimoto K, Sakoda Y, Takashita E, McBride R, Noda T, Hatta M, Imai H, Zhao D, Kishida N, Shirakura M, de Vries RP, Shichinohe S, Okamatsu M, Tamura T, Tomita Y, Fujimoto N, Goto K, Katsura H, Kawakami E, Ishikawa I, Watanabe S, Ito M, Sakai-Tagawa Y, Sugita Y, Uraki R, Yamaji R, Eisfeld AJ, Zhong G, Fan S, Ping J, Maher EA, Hanson A, Uchida Y, Saito T, Ozawa M, Neumann G, Kida H, Odagiri T, Paulson JC, Hasegawa H, Tashiro M, Kawaoka Y.** 2013. Characterization of H7N9 influenza A viruses isolated from humans. Nature.
345. **Van Kerkhove MD, Mumford E, Mounts AW, Bresee J, Ly S, Bridges CB, Otte J.** 2011. Highly pathogenic avian influenza (H5N1): pathways of exposure at the animal-human interface, a systematic review. PloS one **6:**e14582.
346. **Maines TR, Szretter KJ, Perrone L, Belser JA, Bright RA, Zeng H, Tumpey TM, Katz JM.** 2008. Pathogenesis of emerging avian influenza viruses in mammals and the host innate immune response. Immunological reviews **225:**68-84.
347. **Wong KK, Greenbaum A, Moll ME, Lando J, Moore EL, Ganatra R, Biggerstaff M, Lam E, Smith EE, Storms AD, Miller JR, Dato V, Nalluswami K, Nambiar A, Silvestri SA, Lute JR, Ostroff S, Hancock K, Branch A, Trock SC, Klimov A, Shu B, Brammer L, Epperson S, Finelli L, Jhung MA.** 2012. Outbreak of influenza A (H3N2) variant virus infection among attendees of an agricultural fair, Pennsylvania, USA, 2011. Emerging infectious diseases **18:**1937-1944.
348. **Ito R, Ozaki YA, Yoshikawa T, Hasegawa H, Sato Y, Suzuki Y, Inoue R, Morishima T, Kondo N, Sata T, Kurata T, Tamura S.** 2003. Roles of anti-hemagglutinin IgA and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia. Vaccine **21:**2362-2371.
349. **Renegar KB, Small PA, Jr., Boykins LG, Wright PF.** 2004. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. Journal of immunology **173:**1978-1986.
350. **Mbawuike IN, Pacheco S, Acuna CL, Switzer KC, Zhang Y, Harriman GR.** 1999. Mucosal immunity to influenza without IgA: an IgA knockout mouse model. Journal of immunology **162:**2530-2537.

351. **Seibert CW, Rahmat S, Krause JC, Eggink D, Albrecht RA, Goff PH, Krammer F, Duty JA, Bouvier NM, Garcia-Sastre A, Palese P.** 2013. Recombinant IgA is sufficient to prevent influenza virus transmission in Guinea pigs. Journal of virology **87:**7793-7804.
352. **O'Neill E, Krauss SL, Riberdy JM, Webster RG, Woodland DL.** 2000. Heterologous protection against lethal A/HongKong/156/97 (H5N1) influenza virus infection in C57BL/6 mice. The Journal of general virology **81:**2689-2696.
353. **Weinfurter JT, Brunner K, Capuano SV, 3rd, Li C, Broman KW, Kawaoka Y, Friedrich TC.** 2011. Cross-reactive T cells are involved in rapid clearance of 2009 pandemic H1N1 influenza virus in nonhuman primates. PLoS pathogens **7:**e1002381.
354. **Bodewes R, Kreijtz JH, Geelhoed-Mieras MM, van Amerongen G, Verburgh RJ, van Trierum SE, Kuiken T, Fouchier RA, Osterhaus AD, Rimmelzwaan GF.** 2011. Vaccination against seasonal influenza A/H3N2 virus reduces the induction of heterosubtypic immunity against influenza A/H5N1 virus infection in ferrets. Journal of virology **85:**2695-2702.
355. **Duvvuri VR, Marchand-Austin A, Eshaghi A, Patel SN, Low DE, Gubbay JB.** 2012. Potential T cell epitopes within swine-origin triple reassortant influenza A (H3N2) variant virus which emerged in 2011: an immunoinformatics study. Vaccine **30:**6054-6063.
356. **Wei CJ, Yassine HM, McTamney PM, Gall JG, Whittle JR, Boyington JC, Nabel GJ.** 2012. Elicitation of broadly neutralizing influenza antibodies in animals with previous influenza exposure. Science translational medicine **4:**147ra114.
357. **Carter DM, Bloom CE, Nascimento EJ, Marques ET, Craigo JK, Cherry JL, Lipman DJ, Ross TM.** 2013. Sequential seasonal H1N1 influenza virus infections protect ferrets against novel 2009 H1N1 influenza virus. Journal of virology **87:**1400-1410.
358. **Clegg CH, Roque R, Van Hoeven N, Perrone L, Baldwin SL, Rininger JA, Bowen RA, Reed SG.** 2012. Adjuvant solution for pandemic influenza vaccine production. Proc Natl Acad Sci U S A **109:**17585-17590.
359. **Skowronski DM, Janjua NZ, De Serres G, Hottes TS, Dickinson JA, Crowcroft N, Kwindt TL, Tang P, Charest H, Fonseca K, Gubbay JB, Bastien N, Li Y, Petric M.** 2011. Effectiveness of AS03 adjuvanted pandemic H1N1 vaccine: case-control evaluation based on sentinel surveillance system in Canada, autumn 2009. Bmj **342:**c7297.
360. **Lowen AC, Steel J, Mubareka S, Carnero E, Garcia-Sastre A, Palese P.** 2009. Blocking interhost transmission of influenza virus by vaccination in the guinea pig model. Journal of virology **83:**2803-2818.
361. **Couch RB, Decker WK, Utama B, Atmar RL, Nino D, Feng JQ, Halpert MM, Air GM.** 2012. Evaluations for in vitro correlates of immunogenicity of inactivated influenza a H5, H7 and H9 vaccines in humans. PloS one **7:**e50830.
362. **Belshe RB, Frey SE, Graham I, Mulligan MJ, Edupuganti S, Jackson LA, Wald A, Poland G, Jacobson R, Keyserling HL, Spearman P, Hill H, Wolff**

M, National Institute of A, Infectious Diseases-Funded V, Treatment Evaluation U. 2011. Safety and immunogenicity of influenza A H5 subunit vaccines: effect of vaccine schedule and antigenic variant. The Journal of infectious diseases **203:**666-673.

363. **Moris P, van der Most R, Leroux-Roels I, Clement F, Drame M, Hanon E, Leroux-Roels GG, Van Mechelen M.** 2011. H5N1 influenza vaccine formulated with AS03 A induces strong cross-reactive and polyfunctional CD4 T-cell responses. Journal of clinical immunology **31:**443-454.
364. **Giles BM, Ross TM.** 2011. A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets. Vaccine **29:**3043-3054.
365. **Lopez-Macias C, Ferat-Osorio E, Tenorio-Calvo A, Isibasi A, Talavera J, Arteaga-Ruiz O, Arriaga-Pizano L, Hickman SP, Allende M, Lenhard K, Pincus S, Connolly K, Raghunandan R, Smith G, Glenn G.** 2011. Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine in a blinded, randomized, placebo-controlled trial of adults in Mexico. Vaccine **29:**7826-7834.
366. **Hoft DF, Babusis E, Worku S, Spencer CT, Lottenbach K, Truscott SM, Abate G, Sakala IG, Edwards KM, Creech CB, Gerber MA, Bernstein DI, Newman F, Graham I, Anderson EL, Belshe RB.** 2011. Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. The Journal of infectious diseases **204:**845-853.
367. **Cox RJ, Brokstad KA, Ogra P.** 2004. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scandinavian journal of immunology **59:**1-15.
368. **Brokstad KA, Eriksson JC, Cox RJ, Tynning T, Olofsson J, Jonsson R, Davidsson A.** 2002. Parenteral vaccination against influenza does not induce a local antigen-specific immune response in the nasal mucosa. The Journal of infectious diseases **185:**878-884.
369. **Sasaki S, Jaimes MC, Holmes TH, Dekker CL, Mahmood K, Kemble GW, Arvin AM, Greenberg HB.** 2007. Comparison of the influenza virus-specific effector and memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccines. Journal of virology **81:**215-228.
370. **Lambe T, Spencer AJ, Mullarkey CE, Antrobus RD, Yu LM, de Whalley P, Thompson BAV, Jones C, Chalk J, Kerridge S, Hill AVS, Snape MD, Pollard AJ, Gilbert SC.** 2012. T-Cell Responses in Children to Internal Influenza Antigens, 1 Year After Immunization With Pandemic H1N1 Influenza Vaccine, and Response to Revaccination With Seasonal Trivalent-inactivated Influenza Vaccine. Pediatric Infectious Disease Journal **31:**E86-E91.
371. **Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Gargiullo PM, Brammer TL, Cox NJ, Tumpey TM, Katz JM.** 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. The New England journal of medicine **361:**1945-1952.

372. **Belshe RB, Nichol KL, Black SB, Shinefield H, Cordova J, Walker R, Hessel C, Cho I, Mendelman PM.** 2004. Safety, efficacy, and effectiveness of live, attenuated, cold-adapted influenza vaccine in an indicated population aged 5-49 years. Clin Infect Dis **39:**920-927.

373. **Galarza JM, Latham T, Cupo A.** 2005. Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge. Viral immunology **18:**244-251.

374. **Wang BZ, Xu R, Quan FS, Kang SM, Wang L, Compans RW.** 2010. Intranasal immunization with influenza VLPs incorporating membrane-anchored flagellin induces strong heterosubtypic protection. PloS one **5:**e13972.