ABSTRACT

Title of Document: **A DIFFERENCE IN HETEROSUBTYPIC IMMUNITY INDUCED BY A MODIFIED LIVE ATTENUATED AVIAN INFLUENZA BACKBONE IN MICE AND FERRETS**

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The unprecedented emergence of multiple avian influenza virus (AIV) subtypes with a broad host range poses a major challenge in the design of vaccination strategies that are effective against multiple subtypes of influenza. The present study focused on the protective effects of a modified AIV as a backbone for epidemic and pandemic influenza. In addition, the ability of this backbone to induce heterosubtypic immunity (Het-I) was also analyzed. Het-I is the ability of one influenza subtype to protect against a different influenza subtype. Previously, a live attenuated AIV with the internal backbone of A/guinea fowl/Hong Kong/WF10/99 (H9N2) (WF10), called WF10*att*, protected chickens against a lethal influenza challenge. To characterize the WF10*att* backbone as a master donor strain and determine its ability to induce Het-I, we evaluated its protective efficacy in mice and ferrets. Vaccinated mice were protected against homologous challenge with A/WSN/1933 (H1N1) (WSN), mouse-adapted A/California/04/2009 (pH1N1) and A/Vietnam/1203/2004 (H5N1) (HPAI

H5N1) viruses, and ferrets survived homologous challenge with HPAI H5N1. H7N2*att* vaccinated mice were protected against both H1N1 and HPAI H5N1 challenge; however, Het-I was observed in H9N2*att* vaccinated ferrets challenged with HPAI H5N1.

We found that both B and T cells are involved in the Het-I induced by our WF10*att* backbone. Cross-reactive non-neutralizing antibodies to viral proteins were detected. $JhD^{-/-}$ mice, which lack mature B-lymphocytes, were vaccinated with the recombinant vaccines and challenged with HPAI H5N1. None of the vaccinated mice survived challenge further suggesting a role for Het-I. In addition, cells isolated from the lungs of H7N2*att* vaccinated mice had cross-reactive antibody-secreting cells targeted to HPAI H5N1. Together, these results suggest a role for B cells in Het-I. Although B cells are important, T cells may also play a role in Het-I. Both IFN-γ and Granzyme B secreting cells were detected in lung and spleen cells isolated from H7N2*att* vaccinated mice and stimulated with HPAI H5N1 suggesting a role for T cells in Het-I. The ability of our WF10*att* backbone to induce Het-I depends on the surface glycoproteins expressed and the challenge virus subtype. In addition, WF10*att* uses both B and T cells to induce Het-I.

A DIFFERENCE IN HETEROSUBTYPIC IMMUNITY INDUCED BY A MODIFIED LIVE ATTENUATED AVIAN INFLUENZA BACKBONE IN MICE AND FERRETS

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Dedication

I dedicate my dissertation to my brother, Alfred Hickman, Jr., his wife LaKisha, and my niece Kiara. All of you have helped me survive this experience, and I do not know where I would be without your support. Thank you very much for always being there for me. Love you always!

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIV	avian influenza virus
att	attenuation
βPL	Beta-propiolactone
^{0}C	degrees Celsius
ca	cold-adapted
cDNA	complement DNA
dpc	days post challenge
dpv	days post vaccination
EID_{50}	50% egg infectious dose
FFU	fluorescent focus units
FDA	Food and Drug Administration
FPV	fowl plague virus
HA Assay	hemagglutinin assay
HEF	hemagglutinin esterase-fusion
HAI Assay	hemagglutinin inhibition assay
HAU	hemagglutinin units
Het-I	heterosubtypic immunity
HPAI	highly pathogenic avian influenza
LPAI	low pathogenic avian influenza
MDCK	Madin Darby Canine Kidney
MLD_{50}	mouse lethal dose 50
MN	microneutralization
mL	milliliter
mRNA	messenger RNA
NAIs	neuraminidase inhibitors
Neu5Ac	5-N-acetylneuraminic acid
NLS	nuclear localization signal
nt	nucleotide
PBS	phosphate-buffered saline
PHS	US Public Health Service
RBS	receptor binding site
RDE	receptor destroying enzyme
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	sialic acid
SPF	specific pathogen free
$TCID_{50}$	50% tissue culture infectious dose
TM	transmembrane
ts	temperature sensitive
µl	microliter
VLP	virus like particle
vRNP	viral ribonucleoprotein
vRNA	viral RNA
WHO	World Health Organization
WT	wildtype

Table of Influenza Genes: Genomic Size and Function

Gene	Segment	Size (nt)	Major Function
PB2	1	2341	Transcriptase: cap binding
PB1	2	2341	Transcriptase: elongation
PA	3	2231	Transcriptase: vRNA replication
HA	4	1765	Hemagglutinin: Host cell attachment, Receptor-binding, membrane fusion glycoprotein
NP	5	1565	Nucleoprotein: RNA binding, part of transcriptase complex, nuclear/ cytoplasmic transport of vRNA
NA	6	1465	Neuraminidase: receptor destroying enzyme/viral release
M	7	1027	Matrix: M1 major component of virion. M2 integral membrane protein-ion channel
NS	8	890	Nonstructural: NS1 cellular RNA transport, splicing, translation, anti-interferon protein. NS2 (NEP) nuclear export protein

Table of Letter Abbreviations for Amino Acids

G	Glycine	Gly
A	Alanine	Ala
V	Valine	Val
L	Leucine	Leu
I	Isoleucine	Ile
M	Methionine	Met
F	Phenylalanine	Phe
W	Tryptophan	Trp
P	Proline	Pro
S	Serine	Ser
T	Threonine	Thr
C	Cysteine	Cys
Y	Tyrosine	Tyr
N	Asparagine	Asn
Q	Glutamine	Gln
K	Lysine	Lys
R	Arginine	Arg
H	Histidine	His
D	Aspartic Acid	Asp
E	Glutamic Acid	Glu

Chapter 1: Introduction

1.1 General Introduction

Influenza viruses belong to the *Orthomyxoviridae*. Three types of influenza viruses, Type A, B and C, exist and are differentiated from each other by two viral proteins, nucleoprotein (NP) and matrix 1 (M1). There are key differences as well, including the length of RNA generated, the number of segments each virus contains, and the amount of variation within the virus segments. Both type A and B viruses have 8 RNA segments while type C has 7 RNA segments (Palese and Young 1982). Influenza A viruses have a broad host range, infecting birds, pigs, horses, sea mammals, dogs, civets and humans. Influenza B viruses infect only humans and seals (Osterhaus, Rimmelzwaan et al. 2000); influenza C viruses infect only humans and pigs (Guo, Jin et al. 1983). Influenza A viruses undergo more genetic variation than both influenza B and C viruses resulting in pandemics and seasonal epidemics while influenza B causes seasonal epidemics, and influenza C viruses are inconsequential to humans or pigs (Palese and Young 1982).

Influenza A viruses are further subdivided into subtypes; 16 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes have been described so far (Webster, Bean et al. 1992). Only two subtypes currently circulate in humans, H1N1 and H3N2. Previously, the H2N2 subtype circulated in humans from 1957 to 1968 (Nabel, Wei et al. 2011). Wild aquatic birds are considered the natural reservoir for influenza A viruses and contain all the HA and NA subtypes (Webster, Bean et al. 1992). Most influenza A viruses replicate in the cells lining the intestinal tract in

birds. The virus is transmitted among avian species through the fecal-oral route (Webster, Bean et al. 1992). Birds secrete high concentrations of virus (up to $10^{8.7}$ 50% egg infectious dose (EID_{50}) per gram) into the environment (Webster, Yakhno et al. 1978). Virus has been isolated from unconcentrated lake water as well as fecal material indicating that waterfowl efficiently transmit influenza A viruses by fecal contamination of the water supply (Webster, Bean et al. 1992). This transmission not only occurs between birds, but the virus can be transmitted to other species as well. In addition as a result of migration patterns, numerous young birds gather at specific areas each year and are exposed to the many viruses present, creating a high incidence of influenza infection among birds (Webster, Bean et al. 1992). The genetic diversity within this reservoir is sufficient to cause a pandemic because the genetic variability of influenza viruses is generated by 1) point mutations that occur during replication by the viral polymerase which has no proofreading capability (antigenic drift) and 2) genetic reassortment between two viruses infecting the same cell (antigenic shift) (Webster, Bean et al. 1992).

Disease signs caused by influenza A viruses vary in the avian species and depend on the age and species of the bird, the strain of the virus, the environment and the presence of bacterial infection. Some influenza strains are asymptomatic in birds, possibly due to the adaptation of these viruses to birds for centuries and are considered low pathogenic avian influenza viruses (LPAI). Other strains cause severe disease signs involving infection of the respiratory tract and central nervous system resulting in death within one week or less. These viruses are called highly pathogenic avian influenza viruses (HPAI) and only occur within the H5 and H7 subtypes. HPAI

H5N1 viruses are now endemic in many parts of the world including Asia, Europe, the Middle East, and some African countries. HPAI H5N1 has pandemic potential because it has infected additional species including leopards, tigers, cats, stone martens, and humans (Sandrock and Kelly 2007).

In May 1997, a previously healthy three-year-old boy had a febrile respiratory tract illness. HPAI H5N1 virus was isolated from the boy on day 10 of the illness and the boy died from complications on day 16 (Subbarao, Klimov et al. 1998). Similar to the 1918 pandemic virus, all genes from this HPAI H5N1 virus was of avian origin (Claas, Osterhaus et al. 1998; Horimoto and Kawaoka 2001). In November and December of 1997, there were 17 additional cases of laboratory-confirmed H5N1 in Hong Kong residents, making a total of 18 confirmed HPAI H5N1 cases, which resulted in 6 deaths in patients ranging from 1 to 60 years old. The clinical signs of the HPAI H5N1 infection were indistinguishable from H1N1 or H3N2 infections, however the rate of complications was higher (Subbarao and Katz 2000). During the human outbreaks of HPAI H5N1 in November and December of 1997, there were concomitant outbreaks of HPAI H5N1 in chickens in poultry markets and farms (Shortridge, Zhou et al. 1998). In 1999, 2001, 2002 and 2003, HPAI H5N1 outbreaks occurred in chickens in Hong Kong, and two human cases were reported in the 2003 outbreak. In December of 2003, HPAI H5N1 spread to poultry in Korea and China (Suarez 2010). From 2003 to 2006, the poultry outbreak spread throughout the world and resulted in 256 cases of human HPAI H5N1 infection and 151 fatalities (WHO 2011). As of October 10, 2011, 15 countries have 566 confirmed human cases of HPAI H5N1 resulting in 332 deaths (~60% mortality) and counting (WHO 2011).

Transmission of avian influenza viruses to humans is not restricted to the HPAI H5N1 viruses. In March 1999, H9N2 viruses were isolated from two children hospitalized in Hong Kong with mild respiratory disease. Both illnesses resolved without complication in 5-6 days (Peiris, Yuen et al. 1999). One patient was exposed to chickens in the weeks prior to her illness. H9N2 viruses were known to circulate amongst chickens and other avian species in live bird markets (Guan, Shortridge et al. 1999); however mild signs were seen in those birds. Since 1999, H9N2 viruses have been isolated in humans infrequently in China (Guo, Li et al. 1999; Butt, Smith et al. 2005). Recent serologic surveillance studies in both China and Iran have detected antibodies to H9N2 viruses in the sera of freshman entering a university in Guangxi, China (13.69%) (Chen, Ge et al. 2008), healthy poultry workers in Guangzhou, China (4.5%) (Wang, Fu et al. 2009), and the general population of Iran (2.5%) (Hadipour and Pazira 2011) suggesting that humans are exposed to H9N2 viruses frequently resulting in seroconversion. In contrast to H5N1 viruses, the illnesses associated with H9N2 viruses in both humans and birds are mild; however similar to H5N1 viruses, H9N2 viruses are endemic to poultry in parts of Asia and the Middle East. Therefore, both viruses remain a pandemic threat.

At the end of February 2003, outbreaks of H7N7 avian influenza occurred in commercial poultry farms in the Netherlands. The virus spread to 255 farms and resulted in the culling of an estimated 30 million chickens—28% of the total Netherlands chicken population (Koopmans, Wilbrink et al. 2004). The H7N7 virus that transmitted from poultry to humans was related to a LPAI detected in ducks during routine avian influenza surveillance in the Netherlands in 2000, and all

internal genes were of avian origin (Fouchier, Schneeberger et al. 2004). There have been previous reports of H7 associated conjunctivitis in humans caused by laboratory or occupational exposure (Webster, Hinshaw et al. 1981; Kurtz, Manvell et al. 1996; Alexander and Brown 2000). During the Netherlands outbreak in 2003, 86 people involved in the culling of infected chickens and 3 of their family members, with no contact with chickens, were infected with the H7N7 virus resulting in 1 fatality. Among these people, 78 had conjunctivitis, 5 had conjunctivitis and respiratory symptoms, and 2 had respiratory symptoms. The one individual who died was a veterinarian who visited many of the infected farms; he developed acute respiratory distress syndrome and fatal pneumonia (Belser, Bridges et al. 2009).

Most epidemiological studies have determined that the human cases of avian influenza came from contact with domesticated poultry. Contact includes consumption of undercooked or raw poultry products, handling of sick or dead birds without protection, or food processing at bird cleaning sites. These data suggest that interspecies transmission from birds to mammals occurs and tends to be self-limiting; the newly introduced viruses do not seem to be maintained through human-to-human transmission (Webster, Bean et al. 1992). However, limited human-to-human transmission has been reported previously with health care workers and family members (Katz, Lim et al. 1999; Buxton Bridges, Katz et al. 2000; Liem and Lim 2005; Ungchusak, Auewarakul et al. 2005). In the case of HPAI H5N1, since it has a high case-fatality associated with infection and the ability to mutate and adapt to other hosts, it remains a public health concern. However, any avian influenza has the possibility to cause the next pandemic.

There are two FDA approved human influenza vaccines, inactivated and live attenuated. Both are trivalent vaccines containing two type A influenza virus subtypes, H1N1 and H3N2, and one type B influenza virus. Inactivated vaccines are approved for use in commercial poultry. Although they are approved for use, inactivated vaccines are rarely used in developed countries. Usually, extraordinary circumstances are required before vaccines can be used. If inactivated vaccines are used in the field for commercial poultry, an oil emulsion vaccine can be formulated and used based on the field isolate circulating in the flocks. The vaccine is injected into birds subcutaneously or intramuscularly. The ability to mass administer poultry vaccines is extremely important and offers significant savings to poultry producers and would increase usage of the vaccines. Another important component of vaccines for poultry is the differentiation of infected from vaccinated animals, or DIVA strategy. The most common method is using a different neuraminidase gene than the circulating influenza virus is carrying. Not being able to determine if animals are vaccinated or infected could result in trade restrictions being imposed by other countries (Kapczynski and Swayne 2009).

Although there are approved vaccines available for humans and other animal species, improvements can be made to the current vaccines. A big downside of seasonal vaccines is the long production time required. Vaccines are produced in embryonated chicken eggs; one egg produces one to three doses of inactivated vaccine. Therefore, it usually takes 6 months for the vaccine to be produced and ready for use. A way to solve this dilemma is to use animal cells to produce vaccines. This could reduce the production time and allow officials more time to determine which

strains will be circulating during the influenza season. This in turn would result in closer matched strains in the vaccine that are more reflective of circulating viruses in nature. The biggest problem facing current vaccines is their inability to protect against antigenically different viruses, causing the need to produce new vaccines annually that contain a closer matched virus. This is what happened with the 2009 novel H1N1 pandemic that occurred in the spring of 2009. The H1N1 strain in the seasonal vaccine was unable to protect against the pandemic H1N1 virus; therefore, a monovalent pandemic vaccine was produced and available by December of 2009. More research is needed to develop more effective seasonal and pandemic vaccines that provide long-lasting immunity and broad protection against strains that differ antigenically from the vaccine viruses (Fiore, Bridges et al. 2009).

1.2 Research Objectives

The main goal of this dissertation was to determine a possible mechanism for the induction of heterosubtypic immunity (Het-I) induced by a live attenuated vaccine backbone. Current influenza vaccines for humans and other species are unable to protect against circulating influenza viruses that are antigenically different from the vaccine reference strains. Therefore, understanding the immunological components of a vaccine required to induce protection against antigenically distinct viruses will result in improved vaccines. To address this, we first sought to develop a live attenuated vaccine for poultry as one is currently not available. Thus, we decided to use A/guinea fowl/Hong Kong/WF10/1999 (H9N2) (WF10) virus as our backbone and transferred the cold-adapted (*ca*), temperature sensitive (*ts*) and attenuated (*att*) phenotype to WF10 creating the WF10*att* backbone. We found that this backbone

was protective in poultry against LPAI and HPAI H5N1 viruses (Song, Nieto et al. 2007). Subsequently, we focused on the ability of our avian influenza backbone, WF10*att*, to protect mammals, both mice and ferrets, from homotypic and heterosubtypic challenge, the induction of Het-I, and the mechanism for Het-I induction.

The research objectives were:

I. Determine if the WF10*att* backbone can be used as a master donor strain for live attenuated vaccines for epidemic and pandemic influenza in mammals, using the mouse and ferret models.

II. Determine if the WF10*att* backbone expressing H7N2 or H9N2 surface glycoproteins can induce Het-I in the mouse and ferret models.

III. Elucidate the possible mechanism(s) of Het-I induced by WF10*att* backbone in the mouse model.

1.3 Major Findings

I. WF10*att* expressing H1N1, H7N2, or H9N2 surface glycoproteins was protective in Balb/c mice against H1N1 challenge suggesting that WF10*att* can be used as a master donor.

II. Only Balb/c mice immunized with WF10*att* expressing ΔH5N1 or H7N2 surface glycoproteins and not H9N2 surface glycoproteins, were protected

against HPAI H5N1 challenge, indicating that depending on the surface proteins present Het-I can be induced by our backbone.

III. Ferrets immunized with WF10*att* expressing ΔH5N1 or H9N2 surface glycoproteins had increased survival against HPAI H5N1 challenge than ferrets immunized with WF10*att* expressing H7N2 surface glycoprotein, suggesting Het-I is species specific and depends on the subtypes used for challenge.

IV. Cross neutralizing antibodies against the heterosubtypic virus were not detected suggesting that other mechanisms are involved in Het-I.

V. Antibody secreting cells directed to βPL-inactivated ΔH5N1 virus were secreted from cells isolated from H7N2*att* immunized Balb/c mice and stimulated in vitro with βPL-inactivated ΔH5N1 virus further suggesting a role for antibodies in Het-I.

VI. IFN-γ was secreted from cells isolated from H7N2*att* immunized Balb/c mice and stimulated *in vitro* with βPL-inactivated ΔH5N1 virus suggesting a role for T cells in Het-I.

VII. Granzyme B was secreted from spleen cells isolated from H7N2*att* immunized Balb/c mice and stimulated in vitro with Concanavalin A strengthening the role for T or NK cells in Het-I.

Chapter 2: Influenza: Life cycle, Pandemics, Vaccination and Immunity

2.1 Influenza Virus

2.1.1 Discovery of Influenza

It is thought that influenza has existed for centuries. Hippocrates described a typical influenza epidemic in Greece in 412 BC (Klenk 2008). Influenza was originally named fowl plague by Perroncito in 1878 to describe a disease affecting poultry in Northern Italy (Perroncito 1878). Later, fowl plague spread from Italy to Austria and Germany, and later to Belgium and France (reviewed in (Lupiani and Reddy 2009)). Clinical signs displayed were distinct from fowl cholera, a bacterial disease, and infected fowl had hemorrhagic lesions in many organs including the brain (Klenk 2008). In 1901, Centanni characterized fowl plague as a virus (FPV) because the agent was able to pass through bacterial filters and because the agent could be passaged "indefinitely" in chickens (Centanni 1901). FPV was specific for different bird species; however, mammals were not susceptible to the disease. The virus was propagated in embryonated eggs, and Landsteiner and Berlinger determined the virus needed living cells in order to replicate (reviewed in (Klenk 2008)). By the mid 1900s, FPV infected most areas including most of Europe, Russia, North America, South America, the Middle East, Africa, and Asia (reviewed in (Lupiani and Reddy 2009)). In 1954, Schafer demonstrated FPV and influenza A virus were indistinguishable by physiochemical and serological assays (reviewed in (Klenk

2008)). Shope and Lewis isolated the first influenza virus during 1930, which was a swine influenza virus (Shope 1931). Three years later, the first virus was isolated from a human in 1933 (Smith 1933).

2.1.2 Virion Morphology and Genome Structure

The influenza A virion derives its lipid membrane from the plasma membrane of the host cell. The HA, neuraminidase (NA), and the matrix 2 (M2) proteins project from the virion (Fig 1); there are 4 HA molecules to every 1 NA molecule on the surface. The overall composition of the virion is 1% RNA, 5-8% carbohydrate, 20% lipid, and about 70% protein (Fields Virology 2007). The influenza virion is pleomorphic, but the spherical particles have a diameter of about 100 nanometers (nm) (Fields Virology 2007). Filamentous particles have been observed in fresh clinical isolates (Chu, Dawson et al. 1949) and have a length of 300 nm. The matrix (M) gene seems to be a major determinant of this difference in morphology although the HA and NA proteins my play a role as well (Varghese, Colman et al. 1997). Inside the virion is the matrix 1 (M1) protein that forms the scaffold just below the lipid membrane. Bound to the scaffolding are eight segments of single stranded negative sense RNA (Fields Virology 2007). Each RNA segment is coated with NP proteins and is associated with the components of the viral RNA polymerase—Polymerase basic protein 1 (PB1), Polymerase basic protein 2 (PB2), and the polymerase acidic protein (PA) (Fields Virology 2007).

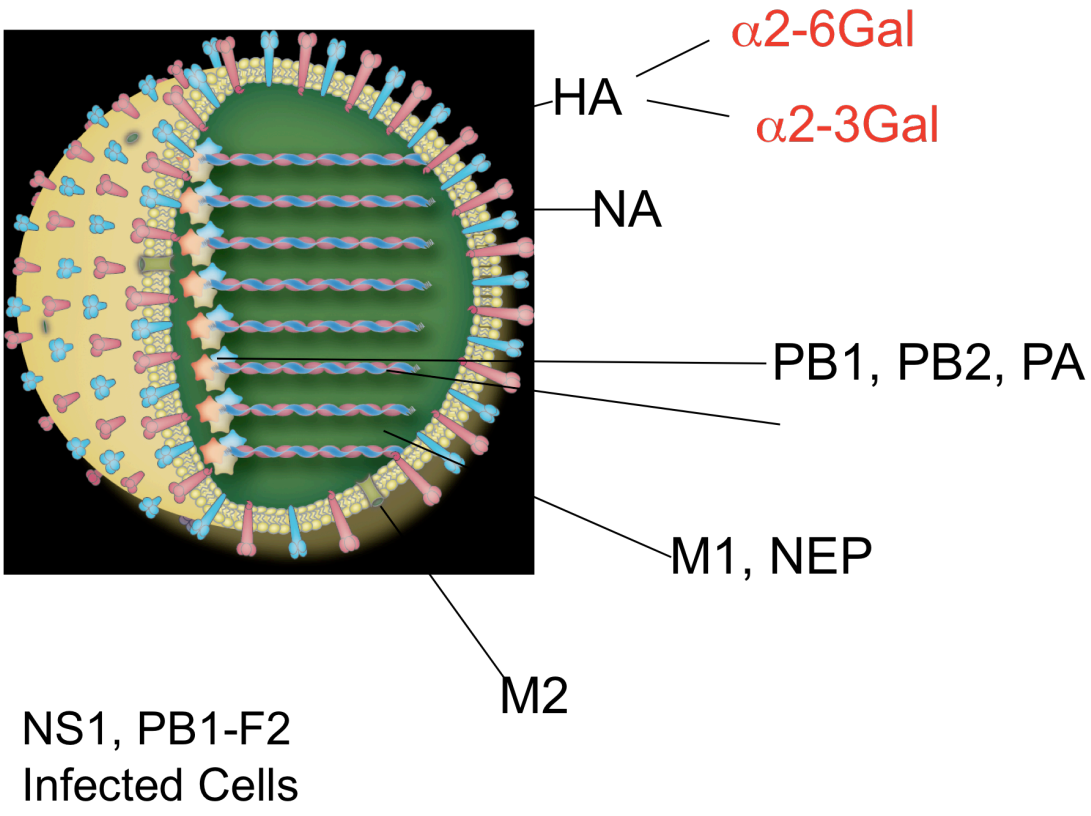

Figure 1. Influenza A virion. The virus contains a lipid bilayer derived from the host plasma membrane. Two surface glycoproteins, HA and NA, are the major antigenic determinants of the virus. The HA protein is responsible for binding sialic acid receptors on the host cell surface. Human influenza viruses bind preferentially sialic acids in an alpha 2,6 conformation (α2-6 gal), while those from avian species bind mostly to sialic acids in an alpha 2,3 conformation (α2-3 gal). The virion also contains several copies of an ion channel proton pump (M2) on the surface. Eight vRNA segments, each one of them associated to three polymerase subunits (PB2, PB1, and PA), and several copies of the nucleoprotein (NP) are located inside the virion protected by a protein mesh provided by the matrix protein (M1). In addition, the virus carries few copies of the virus encoded Nuclear Export Protein (NEP/NS2). In infected cells, the virus expresses NS1, which interferes with the antiviral state mounted by the cell. Some influenza strains express PB1-F2, a ~80 aa peptide, derived from the second open reading frame of segment 2 that encodes PB1. PB1-F2 has been shown to modulate apoptosis in certain cell types infected with influenza.

Adapted from (Sorrell, Ramirez-Nieto et al. 2007)

The eight RNA segments encode up to 11 proteins. Each viral segment contains noncoding regions at both the 5' and 3' ends, portions of these ends are conserved among all segments and a segment specific noncoding region follows this sequence. Segments 1, 2, and 3 encode components of the viral polymerase PB2, PB1, and PA, respectively (Fields Virology 2007). Depending on the virus, segment 2 can also encode PB1-F2, which is involved in the induction of host-cell apoptosis (Lamb and Takeda 2001). Segment 4 encodes the HA protein important for viral attachment to host receptors on the cell surface (Connor, Kawaoka et al. 1994). NP is encoded by segment 5 and interacts with the viral RNA to form ribonucleoproteins (RNPs) (Baudin, Bach et al. 1994). Segment 6 encodes the NA protein, which is responsible for cleaving sialic acids (SAs) resulting in the release of newly formed virions from the host cell (Fields Virology 2007). Segment 7 encodes the M1 and M2 proteins. As mentioned previously, the M1 protein provides rigidity to the virion (Fields Virology 2007), and the M2 is a ion channel that allows protons to enter and acidify the inside of the virion resulting in fusion of the virion with the endosome and release of RNPs into the cytosol (reviewed in (Lamb, Holsinger et al. 1994)). The final segment 8 encodes the nonstructural protein-1 (NS1) and nonstructural protein-2 (NS2) or nuclear export protein (NEP). NS1 is a multifunctional protein and plays a role in antagonizing the host's immune response to influenza. NS2 is responsible for exporting RNPs out of the nucleus and into the cytoplasm (Fields Virology 2007).

2.1.3 Orthomyxovirus Family

The *Orthomyxoviridae* is defined as viruses with negative sense, single stranded, and segmented RNA genomes and are named due to their ability to bind

mucus and separate them from the other negative stranded viruses, *Paramyxovirdae* (Fields Virology 2007). There are five genera in this family including Influenza A, B, C, Thogotovirus, and Isavirus (Table 1). The Thogotovirus genus consists of two viruses, Dhori virus and Thogoto virus, both isolated from ticks. The Isavirus genus consists of infectious salmon anemia virus (Fields Virology 2007).

Influenza A, B, and C viruses can be distinguished by antigenic differences in the nucleoprotein (NP) and matrix (M) proteins (Palese and Young 1982). In addition, type A viruses infect a wide variety of species including both bird species and mammals. Type B viruses infect only humans, and type C viruses are able to infect humans and pigs. There are also morphologic differences between the three types of influenza viruses (Palese and Young 1982; Fields Virology 2007). Influenza A viruses have increased sequence variability within the HA and NA proteins when compared to influenza B viruses. Influenza C viruses do not contain HA and NA proteins. In place of those proteins, influenza C viruses carry a hemagglutinin esterase-fusion (HEF) protein. Each of the genera expresses very similar proteins; however, they have different functions. Also, influenza A and B viruses have 8 RNA segments while influenza C has 7 RNA segments. This section will focus on influenza A viruses (Fields Virology 2007).

Table 1. ***Orthomyxovirdae***

Genus	Genome	Hosts
Influenza virus A	8 RNA segments	Birds, Humans, Pigs, Horses
Influenza virus B	8 RNA segments	Humans, Seals
Influenza virus C	7 RNA segments	Humans, Pigs
Isavirus	8 RNA segments	Salmon
Thogotovirus	6 or 7 RNA segments	Tick, Mosquitoes, Humans

2.1.4 Virus Entry

Influenza A viruses recognize sialic acids (SA) expressed on host cell surface proteins. Therefore, these SA receptors limit the viral cell and tissue tropism, interspecies transmission, and adaptation of influenza viruses to new hosts. Host cell surface receptors contain 5-N-acetylneuraminic acid (Neu5Ac), which is the prototypical SA. SAs are negatively charged 9 carbon sugars at the ends of oligosaccharide chains of glycoproteins and glycolipids. These Neu5Ac can be linked to the third or sixth carbon of the sugar galactose creating a α2,3 SA or α2,6 SA. Different strains of influenza viruses recognize different linkages of SAs. Generally, human influenza viruses recognize the α2,6 SAs and avian viruses recognize the α2,3 SAs (Matrosovich, Stech et al. 2009).

The globular head of the influenza HA contains the SA binding site, called the receptor-binding site (RBS); however, the RBS has a weak affinity for the SA. It takes many interactions between the amino acid residues of the HA and host cell receptor to gain tight binding. Many factors besides HA affinity for the receptor affect the ability of influenza viruses to bind their receptors including the abundance and availability of receptors on the host cell surface and the structure of oligosaccharide beneath the SA on the receptors. As a result, the receptor-binding properties of influenza viruses can be drastically changed by mutations to the amino acid residues resulting in altered glycosylation or electric charge inside the SA-binding pocket of the HA, on the pocket rim, or distant mutations (Matrosovich, Stech et al. 2009).

Once attached to its host receptor, influenza virus is taken up by endocytosis by four potential mechanisms: 1) via clathrin-coated pits, 2) via caveolae, 3) through

nonclatherin, noncaveolae pathways, or 4) through macropinocytosis. Clathrin-mediated endocytosis is the most discussed mechanism of influenza virus entry; however, other forms of internalization have been observed (Fields Virology 2007). Previous research has observed that influenza is still infectious when clathrin-mediated endocytosis is blocked, indicating that influenza can use an alternative pathway to enter the cell (Lakadamyali, Rust et al. 2004). The internalized virus is within an endosome where the pH becomes acidic as the endosome matures. Earlier in infection, the HA0 precursor is cleaved into HA1 and HA2. At low pH, the viral membrane fuses with the endosomal membrane when the HA2 undergoes a conformational change and the fusion peptide is exposed. Multiple HA2 fusion peptides and the transmembrane domains of the HA2 molecules open up a pore that releases the viral ribonucleoprotein (vRNP) into the cytoplasm. The timing and location of the release of the vRNPs, whether it is the early or late endosome, depends on the HA molecules involved (Stegmann, Morselt et al. 1987; Fields Virology 2007).

Weak bases such as ammonium chloride and chloroquine or ionophores are able to block the uncoating of influenza viruses. In addition, the M2 protein ion channel is also very important for uncoating because the protein is responsible for allowing the entrance of protons (H+) into the virion, which creates a low pH environment. A low pH disrupts protein-protein interactions therefore freeing vRNPs from M1 and allowing their release from the virion (Lamb, Holsinger et al. 1994). Antivirals, amantadine and rimantadine, block the M2 channel preventing viral uncoating (Fields Virology 2007).

Once the vRNPs are released into the cytoplasm, they must enter the nucleus where viral replication takes place. The vRNP consists of the viral RNA (vRNA) coated completely in nucleocapsid protein (NP) and is in a helical hairpin shape (Fields Virology 2007).

2.1.5 Viral Replication

All eight RNAs of influenza virus never exist as naked RNA; instead they are associated with the NP protein and the 3 subunits of the viral polymerase. The viral polymerase consists of the PB2, PB1, and PA subunits. PB2 is responsible for binding the cap of host pre-mRNA, which is necessary to prime viral mRNA synthesis. PB1 is the catalytic subunit that elongates the RNA, (Fields Virology 2007) and PA has endonuclease activity (Dias, Bouvier et al. 2009). In addition to endonuclease activity, mutational studies show a role for PA in cRNA synthesis (Bucher, Hemmes et al. 2004), cap snatching (Bui, Myers et al. 2002), cap binding and vRNA promoter recognition (Bui, Whittaker et al. 1996). Binding of the polymerase subunits to the 5' and 3' ends of the vRNA creates a panhandle structure (Hsu, Parvin et al. 1987). NP protein is essential for virus RNA transcription and replication as naked vRNA is not an efficient template. NP protein coats the vRNA and covers the sugar phosphate backbone while exposing the RNA bases (Fields Virology 2007).

Due to their large size, vRNPs are unable to diffuse into the nucleus from the cytoplasm of an infected cell. Therefore, all vRNAs contain a nuclear localization signal (NLS) to allow their association with the nuclear import machinery (Smith, Levin et al. 1987; Mukaigawa and Nayak 1991; Nieto, de la Luna et al. 1994; Wang,

Palese et al. 1997; Weber, Kochs et al. 1998). The NLS(s) are on NP and are sufficient and necessary for the import of the vRNPs (O'Neill, Jaskunas et al. 1995; Cros, Garcia-Sastre et al. 2005). Once the vRNPs reach the nucleus, the vRNAs are transcribed into mRNA. Viral mRNA synthesis is dependent on host RNA polymerase II activity. Prior to binding the vRNA, the polymerase complex is enzymatically inactive. Transcription occurs when the 5' end of the vRNA binds the PB1 subunit of the viral polymerase, which causes an allosteric change resulting in the activation of PB2 cap binding activity (Fields Virology 2007). PB2 binds the cap of the host pre-mRNAs; this binding causes a change in the polymerase structure that increases the affinity for the 3' end of the vRNA. PB1 binds the 3' end of the vRNA, which stabilizes the polymerase complex and causes an allosteric change that activates the endonuclease activity of PA. PA cleaves 10-13 nucleotides downstream of the host cap creating a primer for viral transcription (Dias, Bouvier et al. 2009). Nucleotides are added to the primer by the PB1 subunit. This continues until a stretch of approximately 5-7 uridine nucleotides are encountered by the viral polymerase, which causes the polymerase to stutter and add a string of adenosine nucleotides creating the polyadenylation at the end of the viral mRNA molecule (Fields Virology 2007).

Although vRNA is the template for both mRNA and cRNA, cRNA does not contain a cap or poly A tail, is not primer initiated, and is full-length positive strand versions of the vRNA. As a result, there are two steps needed to replicate vRNAs: 1) synthesis of template RNAs, which is cRNA and 2) copying the template into vRNAs (Fields Virology 2007). The mechanism of replicating vRNAs is unknown; however,

there are ideas of how the full-length cRNA is created. Soluble NP protein seems to play an important role in the antitermination of cRNA. NP binds the 5' end of the nascent RNA chain potentially covering up the stretch of 5-7 uridine nucleotides used to create the poly A tail for the viral mRNA (Fields Virology 2007). Covering these uridine residues prevents backward slipping by the viral polymerase; therefore, polymerase continues adding nucleotides until a full-length cRNA is created. Following replication, M1 protein and NEP/NS2 help export vRNPs from the nucleus to the cytoplasm (Fields Virology 2007).

2.1.6 Viral Assembly

Assembly and budding of influenza viruses occurs from the apical membrane of polarized cells, which results in a restricted tissue tropism of these viruses. HA, NA, and M2 proteins localize to the apical surface and are associated with lipid rafts present on the cell surface. Lipid rafts are nonionic detergent resistant lipid microdomains within (reviewed in (Rossman and Lamb 2011)) the plasma membrane that are rich in sphinoglipids and cholesterol (Ono and Freed 2005). They concentrate proteins within defined regions of the plasma membrane thus serving as functional domains (Lingwood and Simons). The M1 protein is the most abundant virion protein and is believed to make contact with both vRNPs and the cytoplasmic tails of the glycoproteins. The M1 protein is absolutely required for assembly, however very little is known about how the other viral components reach the assembly site and are packaged into the virion. It is hypothesized that M1 binds the vRNPs and by associating with the glycoproteins during their passage through the exocytic pathway (reviewed in (Rossman and Lamb 2011)). The M1 protein hitches a ride to the

assembly site where HA, NA, and M2 localize and brings along the vRNP-NEP/NS2 complex (Fields Virology 2007). An alternative model is the M1-vRNP complex uses the cytoskeleton to reach the assembly site because NP and M1 have been shown to interact with the cytoskeleton components (Avalos, Yu et al. 1997).

The mechanism by which exactly 8 RNA segments are packaged into the virion is not fully understood; however, there are 2 models. The first is the random incorporation model that hypothesizes that there are common structural features present on all the vRNPs that ensures their random incorporation into the virion (Fields Virology 2007). This model is supported by the fact that there exist virions containing more than 8 vRNPs, assuring the presence of a full complement of eight vRNPs in a significant percentage of virus particles (Enami, Sharma et al. 1991; Bancroft and Parslow 2002). The second model is the selective incorporation model that states each vRNP acts independently resulting in each segment being packaged individually because each segment has packaging signals. Coding regions of the NA (Fujii, Goto et al. 2003), HA (Watanabe, Watanabe et al. 2003), NS (Fujii, Fujii et al. 2005), PB2, PB1, and PA (Liang, Hong et al. 2005; Muramoto, Takada et al. 2006) segments have all been demonstrated to increase the ability of a reporter sequence to be incorporated within assembling virions. Segment specific packaging is thought to occur through RNA-RNA or protein-RNA interactions which allow diverging sequences of influenza to be packaged into the virion (Fields Virology 2007).

2.1.7 Viral Budding

Lipid rafts facilitate the budding of the viruses so influenza viruses preferentially bud from lipid rafts (Scheiffele, Rietveld et al. 1999; Zhang, Pekosz et

al. 2000). HA and NA associate with lipid raft domains. The 27 residue transmembrane (TM) domain of HA contains 1 palmitoylated cysteine residue and 2 palmitoylated cysteine residues are located in the cytoplasmic tail, which mediate lipid raft association. This association is important for viral replication (Chen, Takeda et al. 2005). According to previous research involving virus like particles (VLPs), HA was able to alter membrane curvature in the absence of other viral proteins. In the absence of HA and NA, M2 could also alter membrane curvature but to a lesser extent. Although HA is able to initiate budding, it seems as though the other viral proteins are required to complete the budding process. Deletion and mutational studies have shown deletion and mutation of the HA does not alter the number of virions budding, suggesting that other proteins are able to initiate budding such as NA. Mutational studies with the M2 protein indicate an essential role for M2 in the budding process. Lack of M2 prevents release of virions, therefore M2 is involved in the completion of budding but not initiation (reviewed in (Rossman and Lamb 2011)).

The size and shape of the influenza virus particle is determined by the extent the membrane is extruded before pinching off. Usually the particle will be either spherical or filamentous, and the shape is dependent on the M protein (Smirnov Yu, Kuznetsova et al. 1991; Roberts, Lamb et al. 1998; Bourmakina and Garcia-Sastre 2003; Elleman and Barclay 2004). After the virion has separated from the cell membrane during the budding process, the NA protein must actively release the virion. The HA protein binds SAs containing receptors on the cell surface keeping the virion associated with the host cell. NA removes the SAs from the carbohydrates on

the glycoproteins freeing the virion and preventing aggregation of the viruses on the cell surface (Fields Virology 2007).

2.1.8 History of Pandemics

Influenza epidemics are common annual events that are unpredictable in time and severity. They result from antigenic drift, which is the accumulation of point mutations in the hemagglutinin (HA) gene causing a change in antigenicity. However, pandemics result when two conditions occur: 1) an outbreak spreads throughout the world, a high percentage of individuals are infected, and there is an increase in mortality and 2) a new influenza subtype emerges that was not previously circulating in the human population, which is called antigenic shift. Many agree the first pandemic of influenza occurred in 1580 although there were previous reports of possible earlier pandemics (Potter 2001). It has been speculated that the first influenza pandemic occurred in 1173-74; (Hirsch 1883; Potter 2001) however, most believe this was just an outbreak. There are several reports from both the 14th and 15th centuries, however there is not enough information recorded to determine whether a pandemic occurred (Potter 2001). The first established pandemic in 1580 originated in Asia during the summer and spread to Africa and Europe (Pyle 1986). All of Europe was infected within 6 months, and the virus eventually spread to America (Pyle 1986; Beveridge 1991). During this pandemic, illness rates were high and thousands died (Beveridge 1991).

Although there are records indicating that influenza pandemics occurred during the 17th century, data from the 18th century is more definitive that an influenza pandemic occurred. The first agreed pandemic of the 18th century occurred in 1729

AD in Russia during the spring (Hirsch 1883; Finkler 1899; Pyle 1986; Patterson 1987). It quickly spread to Europe and affected the whole continent within 6 months, (Pyle 1986) and within 3 years the entire known world was infected with this virus resulting in high death rates. There were distinct waves of infection with the first waves being less severe than the latter (Brown 1932; Beveridge 1977; Patterson 1987). The next pandemic occurred over 40 years later in 1781-82 (Finkler 1899; Pyle 1986). This pandemic started in China during autumn and spread to Russia and then encompassed all of Europe within an 8-month period (Pyle 1984). The rate of infection was high among young adults (Thompson 1890). More pandemics were recorded during the 19th century, and the first started during the winter in China in 1830-33 and had similar severity to the famous 1918 Spanish flu (Beveridge 1977; Pyle 1986; Patterson 1987). This pandemic spread south to the Philippines, India, and Indonesia, and eventually moved to Russia and then Europe. The pandemic spread to North America in 1831-32 and then reoccurred in Europe at the same time and reoccurred in Europe again in 1832-33 (Pyle 1984). There was a high infection rate of 20-25% of the population; however, the mortality rate was relatively low (Patterson 1987).

Influenza pandemics were recoded four times during the 20th century (Jordan 1927; Burnet 1942; Pyle 1986; Patterson 1987). The greatest pandemic occurred in 1918-20 and was caused by an H1N1 virus with unknown origin. The first outbreaks occurred simultaneously in March 1918 in North America in Detroit, South Carolina, and San Quentin Prison in California (Crosby 1989). Infection spread outward and eastbound. World War I had an effect on the spread the virus resulting in its spread

from North America to Europe and later Russia by the transport of soldiers and supplies. By May 1918, North Africa was infected, and then the virus moved to other areas in Africa before spreading to China, New Zealand and the Philippines in June 1918. In each country, infection spread quickly for a few weeks then sharply declined. The events of March-July 1918 were not exceptional and the numbers of deaths recorded were comparable to previous pandemics. August 1918 saw a second wave of infection in Sierra Leone that was more virulent than the previous outbreaks resulting in a 10-fold increase in deaths. A second wave hit Europe and spread quickly throughout the continent and this virus was more virulent as well. This more virulent virus spread all over the world and resulted in millions of deaths mainly in adults aged 20-40 years (Potter 2001). It is estimated that 50% of the world's population was infected, 25% suffered a clinical infection and the total mortality was between 40-50 million (Crosby 1976).

The next pandemic occurred in 1957-58 when a reassortment between an avian and human influenza created the H2N2 Asian influenza virus, which replaced the previous circulating H1N1 virus (Tscherne and Garcia-Sastre 2011). This H2N2 virus, which harbored the PB1 segment from an avian virus (Potter 2001), originated in China during February and spread rapidly to Singapore, Taiwan, and Japan. Later it spread to the Southern hemisphere during the winter then spread to the Northern hemisphere during its winter. The pandemic was mainly transmitted among sea travel and within 6 months, the pandemic had spanned the globe. Deaths were estimated to be 1 in every 4000 individuals and primarily affected the elderly and the very young (Potter 2001). In 1968, a pandemic arose when human and avian influenza viruses

reassorted to generate an H3N2 virus that replaced the then circulating H2N2 virus (Tscherne and Garcia-Sastre 2011). In July 1968, the H3N2 virus was first isolated in Hong Kong, and by August, spread rapidly to Singapore, Thailand, Taiwan, and Vietnam. By September, Australia, Iran, India, and the US were all infected. The virus affected all age groups. In general, symptoms were mild and no increases in deaths were reported; however, some areas were hit harder than others. For example, the U.S. experienced an increase in deaths equaling mortality rates that were seen during the 1957-58 pandemic; this was unique to the 1968 pandemic (Vernick 2010).

The first pandemic of the 21st century originated in Mexico during March and April of 2009 and was caused by a H1N1 swine-origin influenza virus (WHO 2009). During the late 1990s, triple-reassortant H3N2 and H1N2 viruses evolved when North American avian and human H3N2 and H1N2 viruses exchanged genetic material (Dunham, Dugan et al. 2009; Garten, Davis et al. 2009). A swine triple reassortant virus then reassorted with a Eurasian avian-like swine virus to create the swine origin pandemic virus of 2009 (Tscherne and Garcia-Sastre 2011). The PB2 and PA genes from the pandemic virus are from an avian virus, PB1 is from a human seasonal H3N2 virus, the HA, nucleoprotein (NP), and nonstructural (NS) genes are from the classical swine virus (Dunham, Dugan et al. 2009; Garten, Davis et al. 2009). NA and M are from the European avian-like H1N1 lineage (Dunham, Dugan et al. 2009; Garten, Davis et al. 2009). In the spring, several areas in Mexico reported large numbers of patients displaying influenza-like symptoms, and by June 2009, 74 countries had detected the virus and the WHO declared a pandemic (Fraser, Donnelly et al. 2009; WHO 2009). By July 2009, 214 countries had confirmed cases of the

H1N1 virus. Infection with the pandemic H1N1 virus is indistinguishable from seasonal influenza except for the increase in the number of patients reporting gastrointestinal symptoms. In contrast to seasonal influenza, there was an increase in infection in the younger population as opposed to the elderly (Tscherne and Garcia-Sastre 2011). Since emerging in spring of 2009, the pandemic H1N1 virus is the predominant H1N1 subtype circulating in humans, replacing the seasonal H1N1 virus (WHO 2009).

2.2 Influenza A Virus Ecology

2.2.1 Influenza A Viruses in Birds

The ecology of influenza A viruses is extremely complicated involving various viral genes and many different species. Wild aquatic birds, especially migrating waterfowl, are considered the natural reservoir of influenza A viruses and contain all the 16 HA and 9 NA subtypes (Hinshaw, Webster et al. 1980). The clinical signs and disease observed in wild aquatic birds depend on the age of the bird, strain of the virus, environment, and presence of bacteria in the bird (Alexander and Brown 2000). Avian influenza viruses infect a limited number of cell types usually located in the gastrointestinal tract of birds resulting from the ingestion of the virus. In birds, the virus is transmitted by the fecal-oral route because birds excrete high amounts of virus in feces contaminating the water. Virus-contaminated water serves as a vector for the spread of the virus to other birds and other species. The migrating nature of birds allows for the dissemination of influenza viruses to different geographical areas, and the interaction of different bird species carrying all influenza subtypes along migration routes (Webster, Bean et al. 1992).

Some influenza infections are asymptomatic in the bird reservoir; on occasion when the species barrier is crossed, these viruses can cause severe disease in birds, especially domesticated birds, humans, pigs, and some other species. Although all 16 HA and 9 NA subtypes are found in wild aquatic birds, not all can replicate in domesticated poultry without prior adaptation. In gallinaceous poultry, influenza A viruses are designated low pathogenic avian influenza (LPAI) or highly pathogenic avian influenza (HPAI). LPAI cause mild to no disease in birds while HPAI have polybasic cleavage sites in the HA gene that facilitates systemic replication of these viruses. Systemic replication results in viruses that can kill birds in one week or less. Since 2002, HPAI H5N1 viruses that replicate more efficiently in the trachea and not the intestines of ducks have emerged. This change in replication can change the route of transmission of influenza between birds, switching from a fecal-oral route to inhalation, which is the mode of transmission of human influenza (Boyce, Sandrock et al. 2009).

Domestic poultry may be an important intermediate host of influenza virus. It has been established that aquatic birds contain a vast array of influenza variability; however, poultry may come in contact with these birds. On several occasions in the past, HPAI H5N1 and H7N7 influenza outbreaks in domestic poultry were transmitted to humans resulting in deaths (Subbarao, Klimov et al. 1998; Guan, Shortridge et al. 1999; Guo, Li et al. 1999; Koopmans, Wilbrink et al. 2004; Butt, Smith et al. 2005). In addition, LPAI H9N2 was also transmitted to humans (Guo, Li et al. 1999; Peiris, Yuen et al. 1999; Butt, Smith et al. 2005), and both the 1997 HPAI H5N1 and the 1999 H9N2 viruses contain internal genes that belong to the same

phylogenic lineage. Fortunately, human-to-human transmission has been limited and usually, humans have been dead end hosts after the virus has jumped the species barrier (Webster, Bean et al. 1992). However, limited human-to-human transmission has been reported previously in health care workers and family members (Katz, Lim et al. 1999; Buxton Bridges, Katz et al. 2000; Liem and Lim 2005; Ungchusak, Auewarakul et al. 2005).

Wild aquatic birds are considered the natural reservoir for influenza A viruses; however, viruses from the natural reservoir must mutate before they can cross the species barrier to infect humans because these viruses replicate poorly in humans (Webster, Yakhno et al. 1978; Hinshaw, Bean et al. 1980; Hinshaw, Webster et al. 1980). Because land-based poultry are farmed around the world and have been involved in the spread of avian influenza to humans resulting in deaths, it is important to understand the role of land-based poultry as potential intermediates of influenza reassortant viruses. Sorrell *et al* examined the role of quail in the generation of influenza viruses with increased host range and found an H2N2 virus adapted in quail mutated resulting in the transmission and replication of the virus in both chickens and quail, which was a increase in host range (Sorrell and Perez 2007). In addition, the virus did not lose its ability to infect and transmit in mallard ducks (Sorrell and Perez 2007). These results suggest that land-based birds may act as intermediate hosts in the spread of influenza A viruses.

H2N2 viruses are not the only subtype found to increase its host range when adapted in land-based poultry. Hossain et al adapted an H9N2 virus in quail and chickens, which produced viruses with increased host range (Hossain, Hickman et al.

2008). These adapted viruses were readily able to infect mice providing further evidence that land-based poultry can act as intermediated hosts (Hossain, Hickman et al. 2008). H9N2 viruses isolated from 1988 to 2003 were tested for their ability to replicate in the ferret model. The viruses were able to replicate, partially transmit to direct contacts, and were unable to transmit to respiratory droplet contacts (Wan, Sorrell et al. 2008). H9N2 avian-human reassortant virus containing the internal genes from a human H3N2 virus and the surface genes from an H9N2 virus was characterized in the ferret model. There was an increase in replication in multiple respiratory tissues of the ferret with the H9N2 avian-human reassortant virus when compared to wholly H9N2 virus and the reassortant virus efficiently transmitted to direct contacts; however, still no respiratory droplet transmission was observed (Wan, Sorrell et al. 2008). In addition, the H9N2 avian-human reassortant virus induced clinical signs similar to the parental wild type H3N2 virus suggesting the establishment and prevalence of H9N2 viruses in poultry pose a significant threat for humans (Wan, Sorrell et al. 2008).

Since H9N2 viruses are endemic in countries throughout the world and have transmitted to both humans and pigs, the question arises whether an avian-human H9N2 reassortment can gain the ability to transmit by respiratory droplet in mammals. An H9N2 avian-human reassortant virus containing the HA and NA from an H9N2 virus and the six internal genes from an H3N2 virus was adapted in ferrets resulting in a virus that transmitted by respiratory droplet. Minimal mutations were found in the adapted virus and a reassortant virus expressing the HA and NA genes from the adapted virus was able to maintain the respiratory droplet transmission

phenotype, suggesting currently circulating avian H9N2 viruses require little adaptation in mammals to create viruses that can transmit by aerosol (Sorrell, Wan et al. 2009). Therefore, aerosolized respiratory transmission is not exclusive to current human H1, H2, and H3 influenza subtypes (Sorrell, Wan et al. 2009).

2.2.2 Influenza A Viruses in other species

For an estimated 70 years, classical swine influenza (cH1N1) circulated as the predominant subtype in pigs in the U.S. until 1998 (Ma, Lager et al. 2009). Influenza was first observed in swine in 1918 and 1919 during the Spanish flu pandemic, and cH1N1 virus is antigenically similar to the 1918 pandemic H1N1 virus. Clinical signs of influenza in swine include nasal discharge, coughing, fever, labored breathing, and conjunctivitis (Shope 1931; Shope 1958). In 1998, a swine influenza-like outbreak occurred in North Carolina and spread to pig farms in Minnesota, Iowa, and Texas. An H3N2 influenza A virus was identified as the causative agent. The virus from North Carolina was a double reassortant containing genes from cH1N1 virus (PB2, PA, NP, M, NS) and genes from a recent human virus (PB1, HA, NA). However, the virus isolated from Minnesota, Iowa, and Texas was an A/H3N2 triple reassortant virus containing the genes from cH1N1 (NP, M, NS), genes from a human virus (PB1, HA, NA), and genes from an avian virus (PA, PB2) (Zhou, Senne et al. 1999). By the end of 1999, viruses antigenically similar to the triple reassortant were widespread in the U.S. swine population (Webby, Swenson et al. 2000).

Since then, H3N2 and cH1N1 viruses have coevolved, resulting in the identification of H3N2 (Webby, Swenson et al. 2000; Richt, Lager et al. 2003; Webby, Rossow et al. 2004), H1N2 (Choi, Goyal et al. 2002; Karasin, Landgraf et al.

2002), reassortant H1N1 (rH1N1) (Webby, Rossow et al. 2004), and H3N1 (Lekcharoensuk, Lager et al. 2006; Ma, Gramer et al. 2006) genotypes in pigs. H3N2, rH1N1, and H1N2 viruses are endemic in swine in the U.S. and Canada and continue to co-circulate. Recently, human H1-like viruses, which are distinct from cH1N1 viruses, have been introduced into swine in Canada (Karasin, Carman et al. 2006). However, all swine viruses maintain the original triple reassortant internal genes (PB2, PB1, PA, M, NP, NS) known as the triple reassortant internal gene (TRIG) cassette.

Pigs are considered a "mixing vessel" because they can be infected with both avian and human influenza viruses due to having α2,3 and α2,6 sialic acids in their respiratory tract (Ito, Couceiro et al. 1998). This allows for possible reassortment resulting in the generation of novel influenza viruses that may cause a pandemic. There are three parts to the mixing vessel hypothesis: 1) swine are susceptible to human and avian viruses, 2) reassortment of human and avian viruses occur within the swine, and 3) swine transmit influenza A to humans. Although swine are thought to be an intermediate host between birds and humans, the documentation of birds, especially domesticated birds transmitting influenza to humans, has complicated the interspecies transmission scenario.

Equine influenza (EI) is a very important respiratory disease of horses and was first isolated from horses in 1956 (Sovinova, Tumova et al. 1958). The disease is characterized by pyrexia, coughing, nasal discharge, loss of appetite, tracheobronchitis, and muscle soreness (Webster, Bean et al. 1992; van Maanen and Cullinane 2002). The virus is highly contagious among horses; however, the mortality

rate remains low unless viral infection is accompanied by bacterial infection, which is common, or horses are continuously worked.

The spread of EI in horses in controlled by vaccines, which were introduced in the 1960s in Europe and North America. Most vaccines are adjuvant-inactivated viruses or subunit vaccine and contain H7N7 (Equine 1 viruses) and H3N8 (Equine 2 viruses) subtypes; however, H7N7 viruses have not been isolated from horses since 1977 and are considered extinct (Webster 1993). EI viruses are more genetically stable than human and avian influenza viruses, but antigenic drift does occur and impacts vaccine efficiency. Several changes have occurred to the H3N8 viruses since the 1960s. H3N8 viruses were evolving as a single lineage (Kawaoka, Bean et al. 1989), until they diverged into a Eurasian and American lineage based on geographical distribution (Daly, Lai et al. 1996). Viruses from the American lineage predominated and spread internationally resulting in three sublineages—Argentina, Kentucky, and Florida (Lai, Chambers et al. 2001).

In the U.S., approximately 20% of harbor seals died of severe respiratory infection from 1979 to 1980. H7N7 virus (A/Seal/Massachusetts/1/1980) was found at high concentrations in the lungs and brain of these dead seals (Geraci, St Aubin et al. 1982). The virus was related to avian influenza viruses; however when tested experimentally, the virus replicated efficiently in mammalian animal models, including ferrets, cats, and pigs. In contrast, the virus replicated poorly in avian species and resulted in no clinical signs of disease and was not detected in the feces. In addition, humans were infected with the virus and only conjunctivitis occurred; infected individuals recovered with no complications (Webster, Geraci et al. 1981).

The mode of transmission to the seals is unknown—either virus was transmitted from birds or influenza viruses have been circulating undetected in seals. It is hypothesized that birds were the source of the virus because there is no serological evidence of influenza in the surviving animals, and there is no further evidence of influenza in seals since 1980s. In 1983, an H4N5 virus was isolated from dead seals on the New England coast and, as before, the virus was related to avian influenza; these two cases raised concern that some human or mammalian influenza viruses are derived from avian viruses.

2.3 Animal Models Used to Study Influenza Virus

2.3.1 Overview

Efforts remain to completely understand the pathogenesis of influenza, develop new vaccines to prevent influenza, and develop new treatments for influenza. As a result, it is imperative to have laboratory animal models that reflect human influenza virus infection (Barnard 2009). The ultimate goal of any animal model is to mimic disease outcomes in humans; therefore, an animal model must shed light on human disease. There are several animals models used to study influenza infection. The model used will depend on the focus of the study. The most common animal models include mice and ferrets; however, there are many more that are used including the guinea pig, Syrian hamster, chinchilla, hedgehog, many avian species, pigs, nonhuman primates, and the rat (Maher and DeStefano 2004; Barnard 2009). This section will focus on the two animal models used in my studies—mice and ferrets.

2.3.2 Mice as a model for influenza A virus

The mouse is commonly used as a model for influenza infection to study viral pathogenesis, and development of vaccines and antiviral agents due to its size and low cost. It allows researchers to use large numbers of animals, which are easy to house. In addition, there are numerous reagents to study the immune response to infection as well as knockout mice lacking immune components. Mice do not display all of the clinical symptoms observed in humans infected with influenza including fever, coughing, sneezing, increase in rectal temperature, and nasal discharge (Barnard 2009). However, mice do display some clinical signs similar to humans infected with influenza that allow researchers to assess the severity of the disease. These include reduced blood oxygen saturation levels, which are a measure of lung function (Barnard 2009). These levels are dramatically lower in mice approaching death. In addition, weight loss, cytokine levels, viral lung titers, and serum proteins can be measured to monitor the severity of the disease (Sidwell 2004; Barnard 2009). However, there are downsides to using mice, including the fact they are not a natural host of influenza (Maher and DeStefano 2004); most human influenza viruses do not cause disease in mice, and therefore, most strains need to be adapted to mice prior to experimentation, except for some highly pathogenic avian influenza (HPAI) H5N1 strains (Barnard 2009). Also, mice do not transmit the virus by respiratory droplet to neighboring animals, and most laboratory mouse strains lack the myxovirus resistance (Mx) gene, which plays a role in the innate host defense against influenza virus infection.

2.3.3 Ferrets as a model for influenza A virus

Ferrets are considered the ideal model for influenza vaccine efficiency assessments because they are naturally susceptible to human influenza viruses both A and B subtypes, and they have similar clinical signs, pathogenesis, and immunity displayed by humans infected with influenza. The ferret has been used in influenza research since 1933. As a result, many of the contemporary concepts of immunity to influenza virus have been established. The signs and clinical course of influenza infection in ferrets is similar to that observed in humans. In both ferrets and humans the clinical signs displayed depend on the age of the host, strain of the virus, environmental conditions, and the degree of secondary bacterial infection. Infection is restricted to the upper respiratory tract and both display clinical signs including sneezing, nasal discharge, malaise, anoxia, watery eyes, and fever. Also, the duration of the disease is acute in both species, lasting 3-5 days in cases where there are no complications. Infected ferrets are able to transmit the disease to other ferrets or humans through respiratory droplets, and the pathogenesis of influenza A virus in ferrets is similar to the pathogenesis in humans (Maher and DeStefano 2004). Ferrets are outbred animals unlike other animal models used to study influenza virus, which creates variability in immune responses. This variability may mimic humans' immune responses to disease. Also, ferrets are the only small animal model that develop fever to naturally occurring influenza A viruses similar to humans (Maher and DeStefano 2004). Although there are many positives to using ferrets as a model of influenza, there are many downsides including the fact that ferrets are expensive and are less responsive to infections with influenza B. They also require more housing space,

there is no inbred or specific-pathogen free (SPF) animals available, and there are limited or no reagents to study the immune response.

2.4 Influenza Vaccines and Antivirals

2.4.1 Overview

Current influenza vaccines are trivalent and contain representative influenza A (H3N2), influenza A (H1N1), and influenza B viruses anticipated to circulate that year using viral surveillance data (Fiore, Bridges et al. 2009). Each year the World Health Organization (WHO) and the US Public Heath Service (PHS) recommend the reference strains that should be part of the vaccine. Because the HA and NA undergo antigenic drift, the vaccines must be updated annually. Effectiveness of influenza vaccines is associated with age of the vaccinee, immune competence of the vaccinee, and the antigenic relatedness of the vaccine strains to circulating strains in nature. When vaccine strains are well matched, they are 70-90% effective in randomized, placebo-controlled trials conducted among children and healthy adults. However, this is not true among the elderly and immunocompromised individuals where effectiveness is much lower (Fiore, Bridges et al. 2009); vaccine efficiency drops to 40-60% in the elderly (Govaert, Sprenger et al. 1994). There are currently two types of vaccines approved for use in humans, inactivated vaccines and live attenuated cold-adapted vaccines (Fiore, Bridges et al. 2009). Vaccine viruses are created using the internal genes from A/Puerto Rico/8/34 (H1N1) (PR8) for inactivated vaccines and the cold-adapted A/Ann Arbor/6/60 (H2N2) for live attenuated vaccines, and the HA and NA from the three circulating wildtype viruses that make up the vaccine for that particular year. Reassortants are created in order to maximize growth of the

vaccines in eggs for mass production. B/Ann Arbor/1/66 is the master donor strain for B viruses (Kilbourne, Schulman et al. 1971; Fiore, Bridges et al. 2009).

Although there are vaccines available for influenza virus, improved vaccines are still needed. Vaccines need to be more efficacious in certain populations including young children, the elderly, and the immunocompromised. Children have the highest influenza infection rates, as high as 30-40% in an epidemic, and most need to be hospitalized (Cox, Brokstad et al. 2004). Influenza infection in adults >65 years old or the immunocompromised can result in severe illness, complications from infection, and even death (Cox, Brokstad et al. 2004). In addition to diminished efficacy in certain populations, there are other improvements needed for current influenza vaccines. The substrate used is a major disadvantage. The egg supply needed to grow the vaccine has to be prepared well in advance because large quantities of eggs are needed to grow large amounts of vaccine. One egg produces 1-3 doses of vaccine. Eggs are also susceptible to microbial contamination, which can delay production. There is a possibility that some human influenza viruses may not grow well in eggs, which would lead to further delays. Individuals may have egg allergies and are unable to be vaccinated with egg-derived vaccines (Fields Virology 2007; Fiore, Bridges et al. 2009). There have been studies that suggest that eggs may change the antigenic composition of the HA protein and introduce mutations that promote egg growth, which may lead to decreased immunogenicity of the vaccines (Katz, Naeve et al. 1987; Williams and Robertson 1993). Changing to tissue culture-based vaccine production may eliminate most of these hindrances (Glezen 2011). A problem facing

currently available vaccines is their inability to induce broad protection against multiple strains of influenza virus.

2.4.2 Inactivated Vaccines

The first commercial vaccine was approved for use in humans in the United States in 1945 (Fiore, Bridges et al. 2009). High growth reassortants are created using the internal gene segments from the master donor strain PR8 and the HA and NA gene from the circulating H1N1 and H3N2 influenza A viruses. The influenza seed viruses are replicated in eggs and then the vaccine virus is harvested from the allantoic fluid, purified, concentrated by zonal centrifugation or column chromatography, and then inactivated using either formalin (Fiore, Bridges et al. 2009). Purification techniques have greatly reduced the number of local and systemic reactions; however, the vaccine still contains trace amounts of endotoxin, egg-derived protein, free formaldehyde, and most have thimerosal preservative (Fiore, Bridges et al. 2009). Although these contaminants may be present, they do not appear to contribute to the reactogenicity or toxicity of the vaccine for humans. The monovalent vaccines containing the H1N1, H3N2, and B influenza are combined to form the seasonal trivalent vaccines. There are two types of inactivated vaccines—subvirion/split and whole virus vaccines (Fiore, Bridges et al. 2009). The subvirion or split inactivated vaccines have been the predominant vaccines produced since the 1970s and are prepared using a detergent that solubilizes the viral lipid envelope followed by chemical inactivation of residual virus. Whole inactivated vaccines are not used due to their reactogenicity (Fiore, Bridges et al. 2009).

Inactivated influenza vaccines are intramuscularly injected and contain 15μg of HA for each vaccine strain for individuals greater than or equal to three years old and 7.5μg of HA for each strain for individuals 6-35 months old (Fiore, Bridges et al. 2009). The Advisory Committee on Immunization Practices (ACIP) recommends the standard dose of HA used in the vaccine (CDC 2011). There is no standard quantity of NA in the vaccine because it is labile during the process of purification and storage (Fiore, Bridges et al. 2009). For children younger than nine years of age who have not previously been vaccinated, two doses, a priming dose followed by a booster dose four or more weeks after the first dose, are needed to induce a protective antibody response. A booster dose is recommended due to younger children's lack of exposure to prior influenza infection (Fiore, Bridges et al. 2009). The preparation of inactivated vaccines for the highly pathogenic H5N1 virus posed problems because the virus killed the embryo before enough virus was present to harvest. Creating reassortants with the master donor strain PR8 and removing the cleavage site from the H5 molecule solved this problem. In addition, due to the poor immunogenicity of the H5 protein, the standard dose of inactivated H5N1 is 90μg of HA per vaccine and multiple doses are necessary for protection (Enserink 2005).

Inactivated vaccines induce antibodies to the two major surface proteins HA and NA; however, HA is the main immunogen in inactivated vaccines (Fiore, Bridges et al. 2009). Antibodies against HA are the key determinant in protection from infection against antigenically similar viruses, and antibodies directed toward NA are important for reducing disease (Fiore, Bridges et al. 2009). Due to the relative rapid decline in antibody levels, protective immunity is short lived. As mentioned

previously, vaccines are usually 70-90% efficacious with the high value corresponding to protection against homologous virus and the low value corresponding to protection against viruses that have undergone antigenic drift (Fiore, Bridges et al. 2009). Resistance to circulating influenza viruses correlates with the level of hemagglutination inhibition (HAI) antibodies induced to the infecting strain (Fields Virology 2007). A titer of 1:32 or 1:40 is the benchmark for protection; however, these values are not absolute (Fiore, Bridges et al. 2009). In situations where the efficacy was low, the surface antigen of the epidemic virus differed from the vaccine virus or the vaccine was of low potency and did not stimulate high enough levels of antibodies to the epidemic virus. Inactivated vaccines have continued to be used for years due to their safety and efficacy (Fields Virology 2007).

Inactivated vaccines for humans are based on epidemiologic, molecular, and antigenic data; however in poultry, state government and agricultural authorities determine the use of vaccines based on risk and economical considerations (Kapczynski and Swayne 2009). In the case of poultry, there are three types of vaccines approved for use including inactivated whole avian influenza vaccines, recombinant fowl poxvirus vector with avian H5 insert, and recombinant Newcastle disease virus with avian H5 HA gene insert (Swayne 2009). Poultry influenza vaccines should be used in conjunction with biosecurity, culling, diagnostics, and surveillance to be effective. The use of vaccines in poultry varies by country. In most developed countries vaccines are not used unless extraordinary circumstances occur (Kapczynski and Swayne 2009; Swayne 2009). In contrast, Asia, the Middle East, Central America, and Africa routinely vaccinate poultry (Swayne 2009). The majority

of field-inactivated vaccines are oil emulsion inactivated whole avian influenza vaccines that are injected subcutaneously or intramuscularly. Each vaccine is custom made against the specific HA subtype circulating amongst the birds in the field (Kapczynski and Swayne 2009). Usually two doses are necessary for protection and subsequent boosting is usually needed in long-lived birds. The ability to mass administer poultry vaccines is extremely important and offers significant cost savings to poultry producers. Also, it would increase field usage as individual administration can be time-consuming (Kapczynski and Swayne 2009).

2.4.3 Live Attenuated Vaccines

Local immunity is thought to play a major role in resistance to respiratory pathogens. Immunization with live attenuated vaccines seems to be the most efficient method of stimulating such immunity. Infection of the respiratory tract stimulates both systemic and local immunity and also induces cell-mediated immunity (Fiore, Bridges et al. 2009). In order to develop effective live vaccines, vaccine manufacturers need to keep in mind that influenza viruses undergo significant antigenic variation. Therefore, it is not feasible to attenuate each new circulating variant of influenza that appears in nature by multiple passages within tissue culture. A strategy is needed in which attenuation can be achieved in a single step by transferring genes from an attenuated donor virus to each new epidemic or pandemic virus. The master donor strain A/Ann Arbor/6/60 (H2N2) was adapted to grow at a lower temperature by serially passaging the virus in primary chicken kidney cells while gradually lowering the temperature to 25°C until a mutant was recovered that efficiently replicated at 25°C (Fiore, Bridges et al. 2009). Wildtype influenza viruses

are restricted for growth at 25°C. A virus that replicates efficiently at 25°C has 1) a temperature sensitive (*ts*) phenotype (restricted growth at 38-39°C), 2) a cold-adapted (*ca*) phenotype (efficient replication at 25°C), and 3) an attenuated (*att*) phenotype (lack of replication in the lungs of ferrets) (Maassab 1967). The amino acid mutations for these characteristics have been mapped within A/Ann Arbor/6/60 and are located in PB2 (N256S), PB1 (K391E, E581G, A661T), and in NP (D34G) (Jin, Lu et al. 2003). The master donor strain used for influenza B viruses in the United States is B/Ann Arbor/1/66 (Fiore, Bridges et al. 2009).

New live attenuated reassortant viruses are created using the six internal genes from the master donor strain plus the HA and NA genes from the epidemic virus circulating in nature. Live vaccines are grown in embryonated eggs similar to inactivated viruses and are trivalent containing H1N1, H3N2, and influenza B viruses. The allantoic fluid is harvested, pooled, clarified by filtration, and concentrated by ultracentrifugation. The vaccine consists of $10^{6.5-7.5}$ fluorescent focus units (FFU) of each of the three strains (Fiore, Bridges et al. 2009). Live attenuated vaccines are administered intranasally and are recommended for use in children and non-pregnant individuals 2-49 years of age (Fiore, Bridges et al. 2009).

Live attenuated viruses protect against experimental or natural infection with influenza A virus in adult and pediatric subjects that were exposed to influenza previously. In addition, live vaccines are immunogenic in seronegative individuals suggesting the vaccine would be protective in this population. A previous study observed that children aged 15-71 months, who were given 1 or 2 does of the trivalent nasal spray vaccine had a an efficacy of 93% overall against culture confirmed

influenza A/H3N2 and B (Belshe, Gruber et al. 2000). In the elderly, co-administration of live vaccine and inactivated vaccine is more efficacious than inactivated vaccine administered alone (Tian, Buckler-White et al. 1985). Different vaccines and schedule of vaccination are necessary for different populations; as a result, the suggestions for yearly immunizations vary depending on the population. These suggestions include: 1) pediatric population older than 6 months should receive live vaccine because it is more immunogenic in this population, 2) previously primed children and adults <65 years old should receive live or inactivated vaccine because efficacies are comparable in these populations, and 3) the elderly (>65 years of age) should be co-administered live vaccine and inactivated vaccine because a combination is more efficacious in this population. Unlike inactivated vaccines, correlates of protection are less clear for live vaccines; therefore serum HAI antibody does not correlate with protective immunity (Clover, Crawford et al. 1991).

In contrast to humans, live attenuated vaccines are not approved for use in commercial poultry because it is feared that the vaccine could reassort with wildtype viruses circulating with the flocks, especially H5 and H7 subtypes, creating a new virulent strain. LPAI of the H5 and H7 subtypes have demonstrated the ability to mutate (gain polybasic cleavage site in HA) into HPAI. Live attenuated vaccines also have the ability to cause economic losses due to respiratory disease caused by virus replication and could spread to surrounding farms (Kapczynski and Swayne 2009). Until live attenuated vaccines are able to overcome these obstacles, inactivated vaccines will continue to be a vaccine option for poultry producers.

2.4.4 Antivirals

Since influenza has a short incubation period, with a mean of 1.9 days, and has a range of symptoms from asymptomatic to acute primary viral pneumonia, surveillance and quarantine alone are not effective strategies to contain a pandemic. Therefore, antivirals and vaccines are required in order to curb the effects of a pandemic. Pandemic plans in countries around the world contemplate the stockpiling of sufficient doses of vaccine candidates and courses of approved antivirals, which could be deployed in the event of a pandemic. Since it is plausible that the antigenic make up of the stockpiled vaccine candidate does not fully match the pandemic strain and thus may not be fully protective, the access to effective antivirals is essential. There are several parameters that would determine which antivirals are used in the event of a pandemic such as efficacy of prophylaxis, treatment, ease of administration, tolerability, and safety. The best use of antivirals may be as prophylactic treatments in instances were individuals are most likely exposed to infection and where transmission is at its highest risk (Hayden 2001). There has to be enough antivirals stockpiled and a distribution strategy must be created before a pandemic occurs in order for antivirals to be effective (Longini, Halloran et al. 2004). Because there are several steps within the influenza virus life cycle, there are several different approaches and agents that can be used to reduce or eliminate virus propagation. These steps include viral entry, uncoating, replication, viral protein translation, viral budding, and the signal cascade events triggered during influenza infection.

Two classes of drugs are approved for use in the U.S. for the treatment or

prophylaxis of influenza infection. These two classes consist of M2 inhibitors, amantadine (Symmetrel) and rimantadine (Flumadine); and neuraminidase inhibitors (NAIs), oseltamivir (Tamiflu) and zanamivir (Relenza). M2 inhibitors are anti-influenza drugs that target the viral M2 protein and are only effective against type A influenza viruses. Amantadine was first approved by the Food and Drug Administration (FDA) in 1966 and was used prophylactically during the 1968 H3N2 Hong Kong pandemic and 1977 H1N1 reappearance (Hayden 2001; Fields Virology 2007). Rimantadine was not approved until 1993 (Fields Virology 2007). The M2 protein is a unidirectional proton (H+) pump that promotes the acidification of the virus's interior during entry by endocytosis and allows the release of the viral genome into the cytoplasm (Pinto, Holsinger et al. 1992). Unfortunately, the use of M2 inhibitors leads to the emergence of resistant strains that remain fully infectious, which limits the use of these drugs (Jackson, Roberts et al. 2000; Hayden 2001; Bright, Medina et al. 2005; Bright, Shay et al. 2006; De Clercq 2006; Ong and Hayden 2007). The most common mutation found in these isolates is a substitution of a bulkier asparagine for serine at position 31 (N31S) (Ong and Hayden 2007), which prevents the binding of the drug while still maintaining the H+ activity. It has been observed that some H5N1 strains that emerged from Asia were resistant to M2 inhibitors (Smith, Naipospos et al. 2006; Hurt, Selleck et al. 2007) leading to the speculation that they arose as a consequence of the improper use of these drugs to contain outbreaks in poultry.

NA's main function is to cleave terminal SA residues on the receptors located on target cell surfaces allowing the release and spread of viral particles. It also helps

in virus entry by cleaving off SA residues present in mucopolysaccharides, which would otherwise prevent the virus from binding to the cell surface SA receptors. NAIs bind the NA at the cleavage site preventing the processing of SAs and the release of viral particles (Varghese, Smith et al. 1998; De Clercq 2006). Currently the United States has approved two NAIs, zanamivir and oseltamivir, for the use in influenza prophylaxis and/or treatment (Hayden 2001). Oseltamivir is administered orally at a dose of 75mg twice a day, and it is available in tablet and liquid forms (De Clercq and Neyts 2007). Some animal studies suggest that the virulence of the disease may require an adjustment of the dose and length of time that the drug is administered (Yen, Monto et al. 2005). A change in the route of administration (intravenous) may be used for more ill patients (Hayden 2001). Zanamivir administration is performed using an inhaler device. There are advantages and disadvantages with the use of both drugs: Oseltamivir is easier to administer but results in systemic distribution and the possibility to cross the blood brain barrier as demonstrated in a rat model (Sweeny, Lynch et al. 2000). Zanamivir has localized distribution to the upper respiratory tract and thus is less effective against influenza infections that are characterized by heavy involvement of the lower respiratory tract as occurs with H5N1 viruses (Shinya, Ebina et al. 2006; De Clercq and Neyts 2007). Resistant strains to oseltamivir have been observed for human influenza viruses and H5N1 viruses, which could limit its use if the new pandemic virus is naturally resistant to it or if resistant strains are quickly selected for during the early stages of a pandemic. Due to the structure of zanamivir, it is thought that resistant strains are less likely to develop than with oseltamivir; however, the former has not been as widely used as the latter, and

therefore, it is difficult to predict what could happen (Varghese, Smith et al. 1998). Resistant strains to NAIs have arisen through mutations within the globular head of the mushroom-shaped homotetrameric NA glycoprotein (Kiso, Mitamura et al. 2004). Mutations at positions 119 (E119V) in H3N2 viruses, 152 (R152K) and 198 (D198N) in influenza B viruses, 274 (H274Y) in H1N1 viruses, and 292 (R292K) in H3N2 and H4N2 viruses have been associated to resistance to NAIs (Kiso, Mitamura et al. 2004; De Clercq and Neyts 2007). A recent report compares sensitivity to NAIs of 55 influenza A (H5N1) virus isolates. The majority of the isolates are sensitive to NAIs, but two strains are less sensitive due to two mutations (residues 116 and 117) within a highly conserved region (Singer, Nunn et al. 2007). Two other NAIs are at different stages of development; peramivir (Sidwell, Smee et al. 2001) and A315675 (Kati, Montgomery et al. 2002). Both have been shown to be effective against zanamivir- and oseltamivir-resistant strains (Mishin, Hayden et al. 2005).

Although M2-inhibitors and NAIs decrease the symptoms associated with influenza infection, they have a limited time frame (48 h) from the onset of symptoms to be effective. Thus, due to the inherent shortcomings of the current available anti-influenza drugs there is a need to explore other viable alternatives.

2.5 Homotypic and Heterosubtypic Immunity

2.5.1 Homotypic immunity

Homotypic immunity is the ability of one strain of influenza virus to protect against another strain within the same subtype. Current seasonal influenza vaccines are able to induce homotypic immunity and protect against influenza strains that are closely related antigenically (Gerhard 2001). Seasonal vaccines are unable to protect

against antigenically distinct strains. Homotypic immunity is primarily mediated by antibodies to the HA and NA. These antibodies neutralize and prevent infection by the challenge virus (Gerhard 2001). Homotypic immunity is usually effective for four or five years following the initial infection (Couch 2003).

2.5.2 Heterosubtypic immunity

Heterosubtypic immunity (Het-I) is the ability of one subtype of influenza to protect against multiple subtypes of influenza and has been studied for nearly fifty years (Schulman and Kilbourne 1965). It is hypothesized that Het-I is mediated by T cells that recognize epitopes in conserved influenza proteins, such as NP (Doherty, Topham et al. 1997; Subbarao, Murphy et al. 2006). Het-I immunity does not prevent infection, but does lower viral titers, accelerate viral clearance, and reduce morbidity and mortality.

Vaccines that are able to induce Het-I are considered universal vaccines. Different strategies are used to develop universal vaccines. One strategy involves using conserved regions of the virus such as whole proteins or epitopes within viral proteins that can stimulate humoral or cell mediated immunity or both. Pharmaceutical companies are creating universal vaccines using a single conserved epitope. The influenza M2 protein forms a homotetramer and is a single-pass type III transmembrane protein within the viral lipid envelope responsible for allowing protons to enter the virion. The decrease in pH within the virion results in the uncoating of the virion and release of the RNPs into the cytoplasm during viral entry (Schnell and Chou 2008). Great attention is being paid to the M2 ectodomain (M2e) as a universal vaccine candidate. M2e is a 24 amino acid N-terminal domain of the

M2 viral protein; M2e protein is conserved in both avian and human flu viruses and induces antibodies that inhibit broad spectrum influenza A subtypes *in vitro* and *in vivo* (Neirynck, Deroo et al. 1999; Stanekova and Vareckova 2010). However, these M2e antibodies are ineffective against type B strains because the ion channel is mediated by different proteins. Although antibodies are generated to the M2e peptide, M2e is less immunogenic than the HA and NA viral proteins (Feng, Zhang et al. 2006); therefore, various approaches are being used to increase the immunogenicity of the M2e peptide (Neirynck, Deroo et al. 1999; Fiers, De Filette et al. 2004; De Filette, Ramne et al. 2006).

Another viral target to develop a universal vaccine is the HA2 domain. HA2 is the C-terminal portion of the HA protein that forms a stem-like structure that anchors globular HA1domain to the viral membrane. Although the HA2 domain is less accessible than the HA1 domain, HA2-specific antibodies are induced during natural infection in humans (Styk, Russ et al. 1979) as well as mice (Kostolansky, Mucha et al. 2002). These HA2-specific antibodies have broad cross reactivity with many influenza subtypes (Vareckova, Cox et al. 2002; Gerhard, Mozdzanowska et al. 2006; Vareckova, Mucha et al. 2008; Stropkovska, Mucha et al. 2009; Steel, Lowen et al. 2010); however, the antibodies do not neutralize influenza viruses and prevent binding of the virus (Becht, Huang et al. 1984; Russ, Polakova et al. 1987; Sanchez-Fauquier, Villanueva et al. 1987). HA2-specific antibodies are able to decrease influenza replication levels using multiple mechanisms: 1) binding antibodies can inhibit the fusion of viral and endosomal membranes, (Edwards and Dimmock 2000; Edwards and Dimmock 2001) 2) preventing the conformation change of HA induced

by low pH, (Okuno, Isegawa et al. 1993; Outlaw and Dimmock 1993; Imai, Sugimoto et al. 1998) or 3) by blocking the insertion of the fusion peptide into the endosomal membrane (Vareckova, Mucha et al. 2003; Vareckova, Wharton et al. 2003). In addition, removing the HA1 portion to create headless HA2 trimers that are more accessible to B cells than the native HA. Vaccination of mice with these headless HA2 elicited antibodies cross-reactive to several subtypes of influenza and protected mice from lethal challenge (Steel, Lowen et al. 2010).

Other methods are being used to create universal influenza vaccines as well. Pharmaceutical companies have created vaccines using whole virus or proteins including the Gamma Flu vaccine, which is a whole inactivated virus (WIV) vaccine inactivated by gamma irradiation. Gamma irradiation destroys the genetic material in the virus (prevents replication); however, it leaves surface proteins intact. These intact proteins stimulate cytotoxic T cells and protects against multiple strains of influenza (Rudolph and Yedidia 2011). In pre-clinical trials, this strategy was shown to stimulate T cells (Furuya, Chan et al. 2010).

Several pharmaceutical companies have products in pre-clinical and Phase I trials including in 2008 when VaxInnate reported positive results from a Phase I trail with their M2e-flagellin combined vaccine (VaxInnate 2008). Multi epitope vaccines, which are vaccines containing multiple conserved epitopes, are also being investigated for use as universal vaccines. The Dynavex vaccine containing an M2e epitope + NP + TLR9 immuno-stimulating sequence (ISS) provides protection against divergent strains by eliciting both T and B cell immunity (Dynavex 2010). A Phase 1 trial for the multi epitope vaccine was started in late June 2010 (Dynavex

2010). Another company, Inovio, is in pre-clinical trials with its VGX™-3400 vaccine, which is a prophylactic DNA vaccine that combines conserved regions of NA + M2e-NP + HA from H1, H2, H3, and H5 strains (Inovio 2010). Another approach used by Juvaris is to use synthetic tetrameric M2e-peptide antigens plus their JVRS-100 adjuvant. This vaccine is in pre-clinical trials and has been shown to induce significant Th1 biased antibody responses that are highly protective (Juvaris Biotherapeutics 2009). Other companies trying variations on the multiple epitope approach including SEEK, which designed a vaccine Flu-v that contains 6 highly conserved CTL epitopes and they are in Phase 1 trials (SEEK 2010). Immune Targeting Systems has a Phase I trial with FP01 which is comprised of 6 long (35 amino acid) $CD4^+$ and $CD8^+$ conserved T cell epitopes administered as synthetic fluropeptides forming stable, immunogenic nanoparticles (Innovation 2010). Biondvax pharmaceuticals created a vaccine that stimulates both humoral and cell mediated immunity. The vaccine is in Phase II trials and consists of 9 conserved epitopes from HA, NP, M1 combined in a single recombinant protein expressed in E. coli (Ben-Yedidia and Arnon 2007; Biondvax 2010). The vaccine was tested in adults 18-49 and older adults 55-75 found to be safe and successful; Also the vaccine protects against both influenza type A and B viruses (Ben-Yedidia and Arnon 2005; Biondvax 2010).

Although there are several approaches underway to generate universal vaccines, the basic mechanism for Het-I is still unknown; according to the current literature, a variety of mechanisms are involved in Het-I. Once these mechanisms are elucidated, it will be easier to generate effective universal vaccines.

2.5.3 Role of T cells in heterosubtypic immunity

Both natural infection and previous vaccination generate neutralizing antibodies that bind antigenically similar viruses and prevent them from entering cells of the respiratory tract. However, these antibodies are typically inadequate at protecting against serologically distinct strains (Cox, Brokstad et al. 2004; Thomas, Keating et al. 2006). Early reports demonstrated that mice and ferrets were protected from heterosubtypic influenza challenge in the absence of cross-reactive antibodies indicating that T cells may result in this protection (Tumpey, Renshaw et al. 2001; Kreijtz, Bodewes et al. 2009; Perrone, Ahmad et al. 2009). The surface glycoproteins undergo constant antigenic drift leading to antigenically distinct viruses. However, the internal genes are usually conserved between different influenza viruses. Therefore, it is commonly believed that T cells play an important role in heterosubtypic immunity (Het-I). T cells recognize viral peptides associated with major histocompatibility complex (MHC) I and II molecules. These T cells recognize highly conserved internal proteins, especially NP and M proteins. Pre-existing influenza-specific T cells do not prevent infection; however, they do help clear the virus and reduce pathology. It is believed that $CD8^+$ cytotoxic T lymphocytes (CTLs) play the most significant role because they eliminate virus-infected cells by releasing perforin and/or inducing the Fas/Fas ligand apoptotic pathway (Furuya, Chan et al. ; Topham, Tripp et al. 1997; Trapani and Smyth 2002), and result in the release of cytokines, IFN-γ and TNF-α. In addition, $CD4^+$ T cells are thought to play a role in Het-I; they may be involved in supporting and enhancing $CD8^+$ T cell functions and also secreting cytokines that help B-cell responses (Epstein, Lo et al. 1997).

Early studies demonstrated previous influenza infection resulted in cross-reactive T cells, and showed these cross-reactive T cells were involved in Het-I. T cells isolated from the spleens of infected mice were transferred into naïve mice and decreased lung viral titers and increased survival upon heterosubtypic challenge (Yap and Ada 1978). This protection correlated with the cytotoxic activity of the transferred splenic T cells (Yap and Ada 1978). Later techniques were developed to culture $CD8^+$ T cells *in vitro*, and it was shown that transferred $CD8^+$ T cells protected mice from heterosubtypic challenge (Lin and Askonas 1981; Lukacher, Braciale et al. 1984).

Most studies focusing on the mechanisms of Het-I have used live virus priming followed by heterosubtypic live virus challenge. Liang, *et al* performed depletion studies in mice primed with A/Puerto Rico/8/34 (H1N1) (PR8) and then challenged with A/Aichi/2/68 (H3N2) (X31). X31 is a reassortant virus containing the internal genes from PR8 and the surface glycoproteins from an H3N2 virus. These studies found that both $CD8^+$ and $CD4^+$ T cells were equally important in the induction of Het-I; however, the Het-I response was relatively short lived lasting 4-5 months (Liang, Mozdzanowska et al. 1994). Benton, et al confirmed the role of $CD8^+$ and $CD4^+$ T cells in Het-I by priming $Ig^{-/-}$ mice with PR8 and challenging with H3N2 (Benton, Misplon et al. 2001). Other studies have focused on the role that $CD4^+$ T cells play in helping $CD8^+$ T cells. Without $CD4^+$ T cells, $CD8^+$ T cells are impaired in their ability to clear the virus, clonally expand, and be recruited to the lungs from the spleen (Riberdy, Christensen et al. 2000).

There have also been reports that different immunization strategies use T cells to induce Het-I. Earlier studies immunized outbred and inbred mice with a recombinant chimeric protein fusing the NS1 viral protein and HA2 subunit. These mice were challenged with a heterosubtypic virus and were protected from the challenge. Importantly, deleting both $CD8^+$ and $CD4^+$ T cells eliminated this protection (Kuwano, Scott et al. 1989; Mbawuike, Dillion et al. 1994). In addition to peptides, DNA immunizations have also induced Het-I (Ulmer, Donnelly et al. 1993; Epstein, Kong et al. 2005; Lo, Wu et al. 2008). Epstein, *et al* immunized mice *i.n.* or *i.m*. with recombinant Adenovirus (rAd) vectors expressing either viral NP protein or NP protein together with M protein. They found that mice were protected from heterosubtypic challenge with H1N1, H3N2, and HPAI H5N1, and this protection was also mediated by T cells (Price, Soboleski et al. ; Price, Soboleski et al. 2009).

Few studies have focused on the ability of live attenuated vaccines to induce Het-I (Mak, Zhang et al. 1982; Tannock and Paul 1987). Powell, et al found depleting $CD8^+$ T cells alone resulted in the death of Balb/c mice primed with a cold-adapted H3N2 virus and challenged with PR8 virus. In contrast, mice depleted of $CD4^+$ T cells survived heterosubtypic challenge, indicating a role for $CD8^+$ T cells in Het-I induced by a live attenuated virus (Powell, Strutt et al. 2007).

Although many reports suggest an important role for both $CD8^+$ and $CD4^+$ T cells in Het-I, T cells may not be absolutely required for Het-I as some studies suggest a role for other immune cells such as NK cells and γδ T cells; previous studies showed that $CD8^+$ T cells may be dispensable for Het-I (Nguyen, van Ginkel et al. 2001; Tumpey, Renshaw et al. 2001) and suggest that $CD8^+$, $CD4^+$, NK cells,

and γδ T cells have overlapping functions. Elimination of one population may not completely impair Het-I because another cell type can compensate for the loss (Benton, Misplon et al. 2001). Therefore, more experiments are needed to tease apart the role of the different cellular components in Het-I.

2.5.4 Role of B cells in heterosubtypic immunity

Most neutralizing antibody responses are directed toward the influenza HA and bind the exposed loop of HA1 that surrounds the receptor-binding site (RBS). These loops are variable between strains, and as a result, antibodies are usually strain specific. Influenza HAs cluster into two distinct groups on the basis of their primary sequence and major structural features (Air 1981; Nobusawa, Aoyama et al. 1991; Russell, Gamblin et al. 2004; Fouchier, Munster et al. 2005). Group 1 consists of 10 of the 16 subtypes including H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16. Group 2 consists of H3, H4, H7, H10, H14, and H15; due to these groupings, it is possible for cross-reactive antibodies to appear. Although a majority of antibodies are generated to the HA, antibodies specific for all the other viral proteins have been detected (LaMere, Lam et al. ; Zhang, Zharikova et al. 2006). Although the standard dogma is that T cells are important for Het-I because they can be directed toward conserved epitopes found in many influenza subtypes, some reports have demonstrated a role for B cells in Het-I (Nguyen, van Ginkel et al. 2001; Tumpey, Renshaw et al. 2001).

As a mechanism for Het-I, some groups argue that B cells may be more important than $CD8^+$ T cells (Nguyen, van Ginkel et al. 2001; Tumpey, Renshaw et al. 2001). Antibodies can exist as the result of previous influenza virus infection or vaccination,

which could be cross-reactive and bind the conserved regions of M2, HA or NA proteins from different subtypes of influenza. Non-neutralizing antibodies to the conserved epitopes on the different viral proteins such as the NP protein may play a role in Het-I. These antibodies could bind and help eliminate infected cells or free virions (Rangel-Moreno, Carragher et al. 2008). Researchers have observed heterosubtypic antibody responses that are non-neutralizing and are directed towards conserved regions of the HA molecule. These non-neutralizing, heterosubtypic HA specific antibodies may assist in antibody-dependent cell-mediated cytotoxicity and/or clearance of antigen-antibody complexes mediated by macrophages. Therefore, they may aid in the resolution of infection or reduction of morbidity and mortality (Sambhara, Kurichh et al. 2001; Goy, Von Bibra et al. 2008), and therefore Het-I.

Tumpey, *et al* found no role for $CD8^+$ or $CD4^+$ T cells in their challenge studies using $\beta 2M^{-/-}$ mice (mice that lack MHC class I) and T cell depletion studies. However, B cell deficient mice (IgH-$6^{-/-}$ mice) were not protected against heterosubtypic challenge. In addition, cross-reactive antibodies to the challenge virus were found. These results indicate a role of B cells in Het-I (Tumpey, Renshaw et al. 2001). Others have also reported a more significant role for B cells than T cells in Het-I including Quan et al who found heat stable components (most likely antibodies) provided protection from Het-I when using inactivated influenza A virus with cholera toxin as an adjuvant (Quan, Compans et al. 2008). Using a sublethal dose of a live virus priming followed by a heterosubtypic challenge, Nguyen et al observed B cell deficient mice were unable to survive heterosubtypic challenge indicating a key role

for B cells in Het-I (Nguyen, van Ginkel et al. 2001), and further showed a diversified antibody repertoire is also needed for Het-I (Nguyen, Zemlin et al. 2007). In contrast, there is the possibility that T cells and B cells together can induce Het-I. Memory T cells can help naïve B cells differentiate and produce new antibodies that may help clear the challenge infection (Rangel-Moreno, Carragher et al. 2008).

Even with the continued analysis of Het-I, the field remains conflicted, however the importance of elucidating Het-I remains crucial especially after the 2009 swine-origin pandemic and the inability of the seasonal H1N1 and H3N2 vaccine to protect against the pandemic virus. What would happen if a more virulent pandemic occurs in the future with novel HA and NA genes? While some studies have focused on the role of T or B cells in Het-I, others believe Het-I is multifactorial and persists despite knocking out or depleting one or another immune component, highlighting the redundancy of the immune system, which is a key strength to build upon for vaccine strategies. Therefore, it is possible for CD4 and CD8 T cells, non-neutralizing antibodies, natural killer cells, and γδ T cells to be involved. Different effectors may be important under different circumstances. For example in the absence of B cells, $CD8^+$ and $CD4^+$ T cells play a key role in Het-I. Experimental systems highlighting the contribution of particular components allow us to determine the immune components that are important under specific circumstances, and which components should be monitored in vaccine trials. Future vaccines must be designed to elicit as many effector mechanisms as possible in hopes of inducing Het-I and providing the greatest breadth of protection.

Chapter 3: Protective efficacy and heterosubtypic immunity conferred by a modified live attenuated avian influenza A backbone in mice and ferrets

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3.1 Abstract

The unprecedented emergence in Asia of multiple avian influenza virus (AIV) subtypes with a broad host range poses a major challenge in the design of vaccination strategies that are both effective and available in a timely manner. The present study focused on the protective effects of a genetically modified AIV as a backbone for the preparation of vaccines for epidemic and pandemic influenza. It has previously been shown that a live attenuated AIV vaccine based on the internal backbone of influenza A/guinea fowl/Hong Kong/WF10/99 (WF10) (H9N2), called WF10*att*, protects chickens against low- and high-pathogenic influenza strains. More importantly, this live attenuated virus provided effective protection when administered *in ovo*. In order to further characterize the WF10*att* backbone for use in epidemic and pandemic influenza vaccines, we evaluated its protective effects in two mammals, mice and ferrets, and the ability of the WF10*att* backbone to induce heterosubtypic immunity (Het-I). Het-I is the ability of one virus subtype to protect against a different subtype. Intranasal inoculation of the modified attenuated virus in Balb/c mice provided

adequate protective immunity against homologous lethal challenges with both the wildtype influenza A/WSN/1933 (H1N1) (WSN), mouse-adapted A/California/04/2009 (H1N1) (pH1N1) and A/Vietnam/1203/2004 (H5N1) (HPAI H5N1) viruses. C57BL/6 mice were partially protected from homologous challenge with HPAI H5N1. In addition, the modified attenuated virus protected ferrets against a homologous challenge with HPAI H5N1. Het-I was observed in mice vaccinated with modified attenuated virus carrying H7N2 surface proteins against both WSN and HPAI H5N1 challenge, and in ferrets vaccinated with the modified attenuated virus carrying the H9N2 surface proteins when challenged with HPAI H5N1. The results presented suggest that the internal genes of a genetically modified AIV confer a difference in Het-I depending on the surface proteins expressed and the animal model used.

3.2 Introduction

3.2.1 Live attenuated avian influenza backbone

The emergence of highly pathogenic H5N1 avian influenza virus (AIV) in Asia, with an unusually broad host range and the ability to infect and kill humans, has raised concerns that an H5N1 virus could cause a pandemic (Horimoto and Kawaoka 2001). Vaccines are undoubtedly a major resource that can greatly reduce the impact of a pandemic. Currently, two types of vaccine are commercially available for the prevention of seasonal influenza in the USA: inactivated split virion and live attenuated vaccines (Belshe 2004; Harper, Fukuda et al. 2004; Zangwill and Belshe 2004). Murphy et al. and Subbarao et al. developed alternative approaches for the generation of live attenuated vaccines for humans using reassortants between avian

and human influenza A viruses (Murphy, Sly et al. 1982; Subbarao, Webster et al. 1995; Murphy, Park et al. 1997). The main concept behind these latter approaches was based on the host-range restriction shown by AIVs. Thus, viruses carrying genes derived from an AIV would be attenuated in humans, whereas the presence of the human hemagglutinin (HA) and neuraminidase (NA) surface proteins would elicit a protective immune response against circulating influenza A viruses. These experimental vaccines showed great promise in pre-clinical studies and in clinical studies in adults and older children (Sears, Clements et al. 1988; Steinhoff, Halsey et al. 1990). Unfortunately, some of these vaccines caused reactions in young children and infants, resulting in high fever and other flu-like symptoms. In addition, the consistent failure to obtain some of the reassortant viruses made these approaches impractical (Steinhoff, Halsey et al. 1990; Steinhoff, Halsey et al. 1991).

The advent of reverse genetics has opened up new alternatives for the development of live attenuated vaccines (Neumann and Kawaoka 2001). This is particularly important considering the unprecedented emergence of multiple strains of AIVs with unexpectedly broad host ranges (Capua and Alexander 2004). If one of these strains developed the ability to spread among a broad range of animal species, there would be major health, economic and ecological consequences. It is unrealistic to consider the preparation of multiple vaccine formulations specifically tailored for multiple animal species if such a strain were to emerge (Capua and Alexander 2002; Capua and Alexander 2004; Capua and Marangon 2004). Thus, our laboratory has previously analyzed an AIV backbone that has shown a broad host range, influenza A/guinea fowl/Hong Kong/WF10/99 (H9N2) (WF10), for its potential as a suitable

virus vaccine donor that could be used in multiple animal species, including humans (Song, Nieto et al. 2007).

H9N2 viruses of the same lineage as the WF10 virus have been shown to effectively infect multiple domestic poultry species, including ducks, turkeys, chickens and quail, as well as mice, without prior adaptation (Peiris, Yuen et al. 1999; Guan, Shortridge et al. 2000; Lin, Shaw et al. 2000; Peiris, Guan et al. 2001; Perez, Lim et al. 2003; Perez, Webby et al. 2003; Choi, Ozaki et al. 2004; Xu, Fan et al. 2004). Viruses phylogenetically related to the WF10 virus have also been isolated from pigs (Xu, Fan et al. 2004), and we have shown that the WF10 virus has many biological features similar to human influenza viruses, including the ability to infect non-ciliated cells in cultures of human airway epithelial cells (Wan and Perez 2007). Thus, WF10 potentially represents an ideal candidate for the preparation of live vaccines applicable to multiple animal species.

3.2.2 Heterosubtypic immunity

In June 2009, the WHO declared the swine-origin H1N1 virus a new pandemic (WHO 2011). With the threat of this and future pandemics looming, there is a need to develop vaccines that are able to protect against multiple subtypes of influenza viruses. Het-I will not prevent infection but will result in earlier clearance of the virus (Yetter, Lehrer et al. 1980), and reduced morbidity, mortality, and transmission. In order to develop these vaccines, we need to further understand the underlying mechanisms of Het-I and the induction of these immune mechanisms through vaccination. Although Het-I has been studied for several decades in animals (Schulman and Kilbourne 1965), the role of antibodies, T cells, and other immune

cells in Het-I remains unclear. Most studies have focused on the ability of natural infections to induce heterosubtypic immunity (Schulman and Kilbourne 1965; Yetter, Barber et al. 1980; Yetter, Lehrer et al. 1980; Liang, Mozdzanowska et al. 1994; Epstein, Lo et al. 1997; Benton, Misplon et al. 2001; Nguyen, van Ginkel et al. 2001; Droebner, Haasbach et al. 2008; Kreijtz, Bodewes et al. 2009) or inactivated vaccines with or without adjuvants (McLaren and Potter 1974; Quan, Compans et al. 2008; Alsharifi, Furuya et al. 2009), but few studies have analyzed the ability of live attenuated influenza vaccines containing different surface glycoproteins to induce Het-I. In addition, many studies have focused on the same influenza virus subtypes/strains and their ability to induce Het-I, which include A/Puerto Rico/8/34 (H1N1) (PR8) and X31 strain (a reassortant strain containing the internal genes from PR8 and the surface genes from A/Aichi/2/68 (H3N2)). Therefore, it is important to determine whether the conclusions formed from these studies are consistent for other strains of influenza.

In previous studies, we showed that the WF10*att* backbone was attenuated in birds and provided protection against both a low- and high-pathogenic challenge (Song, Nieto et al. 2007). In this study, we expand on the characterization of the genetically modified WF10*att* backbone in mammals, mice and ferrets. Our results show that genetically modified WF10*att* backbone induced protective immunity against the homologous challenge with lethal H1N1 or HPAI H5N1 in Balb/c mice, C57BL/6 and ferrets. Furthermore, Het-I was induced in both the mouse and ferret models. However, there was a difference in Het-I depending on the surface proteins (H7N2 or H9N2) expressed with the WF10*att* backbone. These studies highlight the

potential of a genetically modified AIV backbone as a donor for influenza vaccines for avian and mammalian species.

3.3 Materials and methods

3.3.1 Cells and viruses

Human embryonic kidney 293T and Madin–Darby canine kidney (MDCK) cells were maintained as described previously (Song, Nieto et al. 2007). The WF10 and the mouse-adapted influenza A/WSN/1933 (H1N1) (WSN) viruses were kindly provided by Dr. Robert Webster, St Jude Children's Research Hospital, Memphis, TN, USA. The highly pathogenic AIV A/Vietnam/1203/2004 (H5N1) (HPAI H5N1) was obtained from the repository at the Centers for Disease Control and Prevention, Atlanta, GA, USA. The A/chicken/Delaware/VIVA/2004 (H7N2) virus was kindly provided by Dr. Dennis Senne, National Veterinary Services Laboratory, APHIS-USDA, Ames, IA, USA. The mouse-adapted pandemic A/California/04/2009 (pH1N1) was created first by intranasally infecting DBA/J2 with wildtype Ca/04 virus (5.4×10^5 $TCID_{50}$). Lungs were collected from the infected DBA/J2 mice and were homogenized in PBS with antibiotics. After centrifugation at 6,000 rpm for 10 min, 50µl of supernatant from the homogenate was used to infect naive Balb/c mice. Lungs from these infected Balb/c mice were then homogenized and inoculated into MDCK cells to prepare the pH1N1 virus stock. Stock virus titers were measured by plaque assay on MDCK cells at 37°C or 32°C or by 50% egg infectious dose (EID_{50}) as described previously (Reed 1938). All studies using HPAI H5N1 virus were performed in an enhanced Biosafety Level 3 plus (BSL-3+) facility approved by the U.S. Department of Agriculture (USDA).

3.3.2 Generation of recombinant viruses by reverse genetics

The H7 and N2 genes of the H7N2 virus, ΔH5 (deletion of polybasic amino acids of the cleavage site) and N1 genes of the HPAI H5N1 virus, and the H1 and N1 genes of pH1N1 were cloned as described by Song et al. (Song, Nieto et al. 2007). Recombinant viruses were generated by transfection of plasmid DNA into co-cultured 293T and MDCK cells as described previously (Hoffmann, Neumann et al. 2000). Recombinant virus stocks were amplified in the allantoic cavity of 10-day-old embryonated chicken eggs. The pH1N1 vaccine virus was serially passaged in eggs seven times to increase the stock titer. For each virus, RT-PCR and full-length sequencing were performed for each viral segment to verify their identity. Sequences were generated using specific primers, Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Calsbad, CA) and a 3100 Genetic Analyzer (Applied Biosystems, Calsbad, CA), according to the manufacturer's instructions.

3.3.3 Immunization and challenge of mice

To evaluate the protective capacity of the recombinant vaccines, ΔH5N1*att*, H7N2*att*, and H9N2*att*, five-week-old female specific pathogen free (SPF) BALB/c or C57BL/6 mice (National Cancer Institute (NCI), Fredrick, MD) were anesthetized with isofluorane administered using Vetequip mobile anesthesia system (Vetequip, Inc, Pleasanton, CA). Then the mice were immunized intranasally (*i.n.*) with 10^6 PFU of WSN H1N1*att*, or 10^6 EID_{50} of ΔH5N1*att*, H7N2*att,* or H9N2*att*, or 10^6 $TCID_{50}$ of pH1N1*att* in 50µl PBS; all mock-immunized mice received 50µl PBS. On day 3-post vaccination, six mice per vaccine group were anesthetized with isofluorane and sacrificed by cervical dislocation. For 3 mice per group, lungs were collected,

homogenized in PBS, and stored at -70ºC until processed. Virus titers in lung homogenates were determined by EID_{50} at 35ºC. For the remaining 3 mice in each group, lungs were inflated inside the mouse cavity (in-situ) with 10% neutral buffered formalin (VWR, Radnor, PA) and dropped in formalin for histology. A certified pathologist examined all slides and generated a report and images.

The 50% mouse lethal dose (MLD_{50}) for the challenge viruses, WSN, pH1N1, and HPAI H5N1, was calculated using groups of four mice inoculated *i.n.* with virus doses ranging from 10^0 to 10^6 PFU, EID_{50}, or $TCID_{50}$. The Reed and Muench method was used to calculate the MLD_{50} (Reed 1938). Clinical signs, body weight and mortality of mice were monitored and recorded for 14 or 21 days.

To assess vaccine efficacy, at 21 days post vaccination (dpv), all the immunized mice were anesthetized with isofluorane and challenged *i.n.* with 20 MLD_{50} in 50µl PBS of WSN, HPAI H5N1, or 100 MLD_{50} pH1N1 virus. At 3, 6, 9, or 12 days post challenge (dpc), 3 mice per group were sacrificed, and lungs were collected to measure virus titers. Lung homogenates were prepared in PBS and frozen at -70ºC until processed. Virus titers in lung homogenates were determined by $TCID_{50}$ on MDCK cells at 37ºC, and titers were determined using the Reed and Muench method. At 9 dpc, 3 mice per group were sacrificed, and lungs were inflated inside the mouse cavity (in-situ) with 10% neutral buffered formalin (VWR, Radnor, PA) and dropped in formalin for histology. A certified pathologist examined all slides and generated a report and images. Animal studies using WSN were performed under BSL-2 conditions; studies using pH1N1 were performed under BSL-2+ conditions. HPAI H5N1 experiments were performed under BSL-3+ conditions with USDA

approval. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park.

3.3.4 Immunization and challenge of ferrets

Four-month-old female Fitch ferrets (*Mustela putorius furo)* (Triple F Farms, Sayre, PA) were lightly anesthetized with ketamine (20 mg/kg) and xylazine (1 mg/kg) via an intramuscular injection before vaccination and challenge. A subcutaneous temperature transponder (Bio Medic Data Systems, Seaford, DE) was placed in each ferret for identification and temperature readings. Temperatures were recorded daily post vaccination and post challenge. Ferrets (5 per group) were inoculated *i.n.* with 10^6 EID_{50} or $TCID_{50}$/ferret of recombinant vaccines in 0.5 mL PBS, 0.25 mL per nostril. All mock-immunized ferrets received 0.25 mL of PBS per nostril. Nasal washes were taken on days 1, 3, 5, and 7-post vaccination to titer vaccine viruses in the upper respiratory tract of the ferret. Three days post vaccination (dpv), three ferrets per group were sacrificed and lungs were collected to titer the vaccine virus. Vaccine viral titers were determined by $TCID_{50}$ on MDCK cells at 37ºC. Nasal washes were collected by anesthetizing the ferrets as described above and 0.5 mL of PBS was expelled into each nostril to induce sneezing onto a petri dish. Blood was drawn on days 0, 7, 14, and 21-post vaccination.

Twenty-one dpv, the remaining two ferrets were infected with $2x10^5$ PFU of HPAI H5N1 because this dose is lethal in ferrets by 9 dpi (Mahmood, Bright et al. 2008). On days 1, 3, 5, and 7-post challenge, nasal washes were collected and blood was taken on days 7 and 14 post-challenge. All nasal wash samples were resuspended

in a total volume of 1 mL and frozen at -70ºC until processed. Virus titers in nasal washes were determined by $TCID_{50}$ on MDCK cells at 37ºC using the Reed and Muench method. Clinical signs, body weight and mortality of ferrets were monitored daily and recorded for 14 dpc. Three independent ferret challenge experiments were performed totaling six ferrets per group; two ferrets were challenged in each experiment. Animal studies using HPAI H5N1 were performed under BSL-3+ conditions with USDA approval. Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park.

3.4 Results

3.4.1 Genetically modified WF10*att* viruses are attenuated in both Balb/c and C57BL/6 mice

A/Ann Arbor/6/60 (H2N2) strain is the master donor strain for live attenuated influenza A viruses (MDV-A) currently in clinical use. The temperature sensitive (*ts*) phenotype of influenza has been mapped to three amino acid mutations in PB1 (K391E, E581G, A661T), one in PB2 (N265S), and one in NP (D34G) (Jin, Zhou et al. 2004). We previously showed that the *ts* loci in the PB2 and PB1 genes of the MDV-A strain can be transferred to the WF10 backbone producing a similar *ts* phenotype (Song, Nieto et al. 2007) and that the addition of an HA tag at the C-terminus of the PB1 gene provided an attenuated (*att*) phenotype in chickens and quail. To further characterize the biological properties of attenuated viruses using the WF10*att* backbone and to determine their potential as a universal master donor strain for animals and humans, we created additional recombinant viruses containing the

WF10*att* internal genes carrying different surface glycoproteins as follows: H1N1 from WSN or pH1N1, ΔH5N1 from HPAI H5N1 (deletion of the poly basic cleavage site), H7N2 from A/chicken/Delaware/04, and H9N2 from WF10 (Table 2). We rescued five recombinant viruses, called 2H1N1:6WF10*att* from WSN or pH1N1, 2ΔH5N1:6WF10*att*, 2H7N2:6WF10*att*, and 2H9N2:6WF10*att*. All virus names were abbreviated as shown in Table 2.

Table 2. Recombinant Vaccines

Surface	Backbone	Abbreviation
2H1N1:6WF10*att*[a]	WF10*att*	WSN H1N1*att*
2pH1N1:6WF10*att*[b]	WF10*att*	pH1N1*att*
2ΔH5N1:6WF10*att*[c]	WF10*att*	ΔH5N1*att*
2H7N2:6WF10*att*[d]	WF10*att*	H7N2*att*
2H9N2:6WF10*att*[e]	WF10*att*	H9N2*att*

Surface proteins (HA and NA) from

[a]A/WSN/1933 (H1N1)

[b]Mouse-adapted A/California/04/2009 (pH1N1)

Egg passage 7 (P7)

[c]A/Vietnam/1203/2004 (HPAI H5N1)

[d]A/chicken/Delaware/VIVA/2004 (H7N2)

[e]A/guinea Fowl/Hong Kong/WF10/1999 (H9N2)

Five-week-old Balb/c and C57BL/6 mice were *i.n.* inoculated with recombinant vaccines in 50µl of PBS. pH1N1*att* P1 grows very slowly, therefore the virus had to be passaged six times in eggs creating pH1N1*att* P7 stock. Serial passaging the vaccine increased the titer such that mice could be inoculated with 10^6 $TCID_{50}$. On day 3-post vaccination, 3 mice per group were sacrificed, and lungs were collected to titer the vaccine virus (Table 3 and Table 4). Only pH1N1*att* and H7N2*att* were detected in the lungs of Balb/c mice (Table 3) while both H7N2*att* and H9N2*att* were detected in the lungs of C57BL/6 mice (Table 4). Also at 3 dpv, 3 mice per group were sacrificed, and lungs were prepared for histology (Figures 2 and 3). Only mild lung pathology was observed in the lungs of the vaccinated Balb/c and C57BL/6 mice. ΔH5N1*att* caused lymphocyte cuffing in the lungs of both Balb/c and C57BL/6 mice while H7N2*att* caused hyperplasia resulting in inflammation in Balb/c mice lungs. Both H7N2*att* and H9N2*att* caused bronchiolar necrosis in the C57BL/6 mice; H9N2*att* caused lymphocyte cuffing in Balb/c mice (Figures 2 and 3). The mice were monitored for 21 dpv for body weight loss and clinical signs, and no disease signs were observed indicating that the vaccines were attenuated in both strains of mice, Balb/c and C57BL/6.

Table 3. Replication of Recombinant vaccines in lungs of Balb/c mice.

Vaccine	Immunization dose (PFU[b]/, $TCID_{50}$[c]/, or EID_{50}[d]/mouse)	Titer in log_{10} EID_{50}/g at 3 dpv
PBS Control	--	BLD[e]
WSN H1N1*att*	10^{6a}	BLD[e]
pH1N1*att* P7[a]	10^{6b}	4.9±0.5
ΔH5N1*att*	10^{6c}	BLD[e]
H7N2*att*	10^{6c}	2.8±0.8
H9N2*att*	10^{6c}	BLD[e]

[a]P7 is egg passage 7

[e]BLD is below limit of detection

Table 4. Replication of recombinant vaccines in the lungs of C57BL/6 mice.

Vaccine	Immunization dose (EID_{50}/mouse)	Titer in log_{10} EID_{50}/g at 3 dpv
PBS Control	--	BLD[a]
ΔH5N1*att*	10^6	BLD[a]
H7N2*att*	10^6	3.8±1.6
H9N2*att*	10^6	1.8±1.6

[a]BLD is below limit of detection

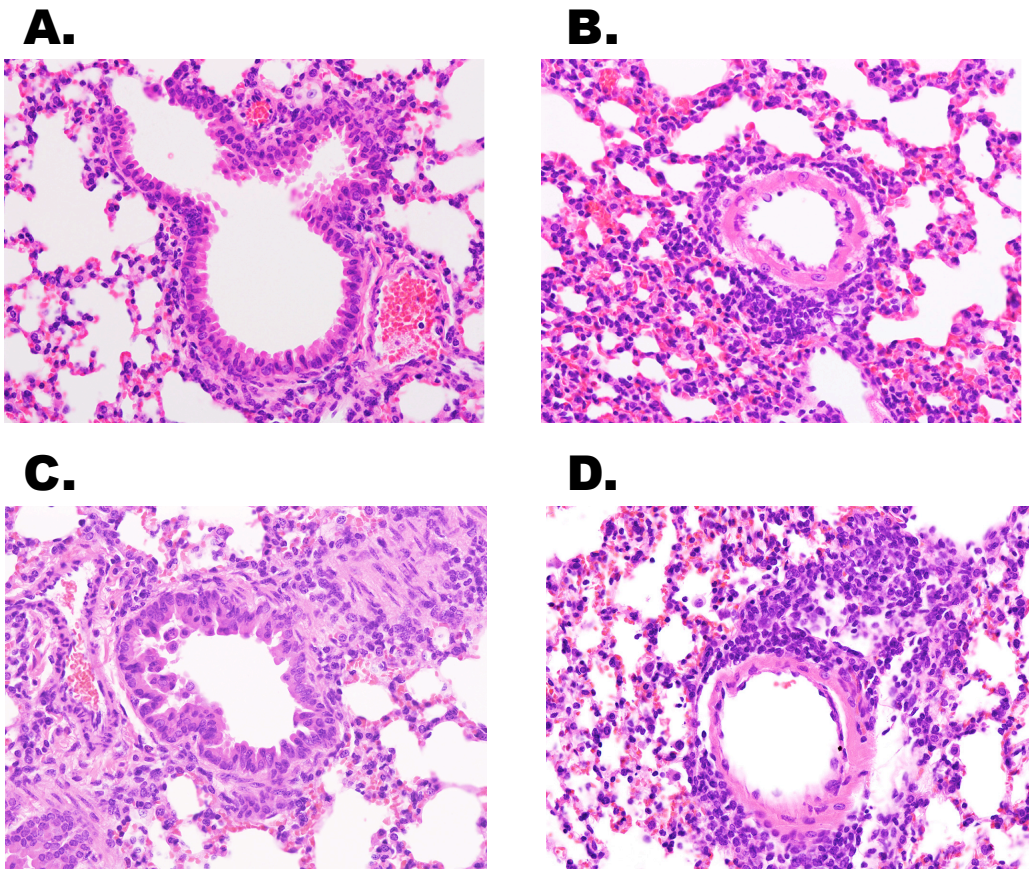

Figure 2. Histology of Balb/c mouse lung tissue at 3 dpv. Five-week-old female Balb/c mice were immunized with 10^6 EID_{50} in 50µl PBS of ΔH5N1*att*, H7N2*att*, or H9N2*att*. Control mice received 50µl PBS. At 3 dpv, lungs were collected for histology. No obvious pathology was found with the (A) PBS control mice. Only mild pathology was found after immunization with the different vaccines. With the (B) ΔH5N1*att* immunized mice lymphocyte cuffing was observed, with (C) H7N2*att* immunized mice mild inflammation was observed, and with (D) H9N2*att* immunized mice lymphocyte cuffing was observed. Magnification is 400X.

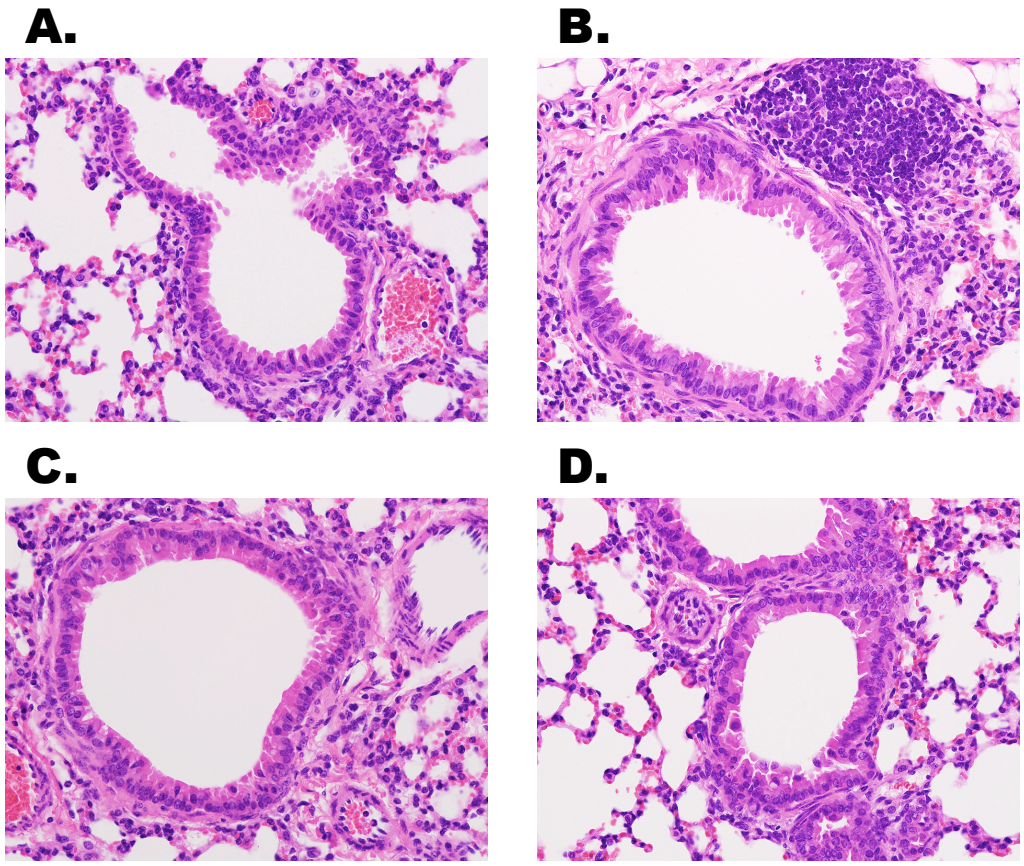

Figure 3. Histology of C57BL/6 mouse lungs at 3 dpv. Five-week-old female Balb/c mice were immunized with 10^6 EID_{50} in 50µl PBS of ΔH5N1*att*, H7N2*att*, or H9N2*att*. Control mice received 50µl PBS. At 3 dpv, lungs were collected for histology. No pathology was found with the (A) PBS control mice. Only mild pathology was found after immunization with the different vaccines. With the (B) ΔH5N1*att* immunized mice lymphocyte cuffing was observed, with (C) H7N2*att* immunized mice mild bronchiolar necrosis was observed, and with (D) H9N2*att* immunized mice lymphocyte cuffing was also observed. Magnification is 400X.

3.4.2 The WF10*att* backbone protects Balb/c mice against lethal H1N1 challenge

Five-week-old Balb/c and C57BL/6 mice were *i.n.* inoculated with vaccine viruses and control mice received PBS (Figure 4). Twenty-one days post vaccination, all mice were challenged with 20 MLD_{50} of WSN virus. The mice were monitored for 21 days post challenge for disease signs including decrease in body weight (Figure 5), lack of grooming, decrease in activity, respiratory signs, and dehydration. At 3 and 6 dpc, 3 mice per group were sacrificed and virus titers in the lungs were determined by $TCID_{50}$ (Table 5). The mice vaccinated with WSN H1N1*att* were completely protected from lethal challenge (Figure 5). No disease signs or decrease in body weight were observed. In addition, no challenge virus was detected in the lungs at either 3 or 6 dpc (Table 5). Both the H7N2*att* and H9N2*att* vaccinated mice showed body weight loss and displayed mild to moderate disease signs. The H7N2*att* vaccinated mice lost more body weight (~15%) and had more pronounced disease signs including lack of grooming and slight hunched posture, while the H9N2*att* vaccinated mice showed a lack of grooming (Figure 5). Both H7N2*att* and H9N2*att* vaccinated mice had similar levels of challenge virus in the lungs at 3 dpc, however both groups of vaccinated animals were able to clear the challenge virus from the lungs by 6 dpc (Table 5). All vaccinated mice survived lethal challenge with WSN indicating that the WF10*att* backbone induces both homotypic and heterosubtypic immunity in Balb/c mice.

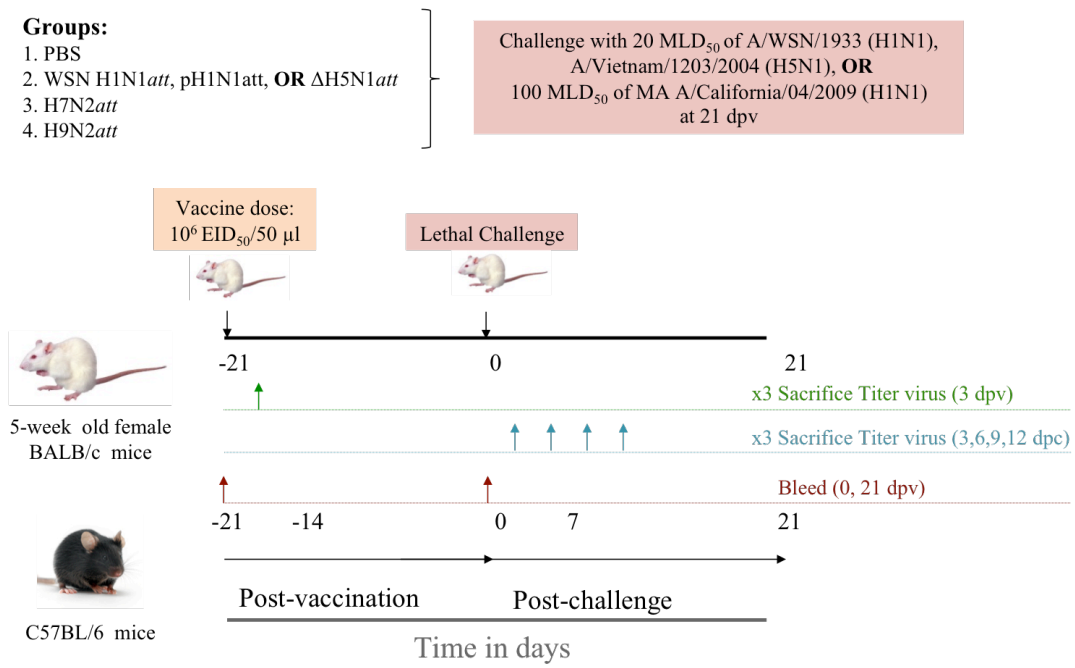

Figure 4. Mouse Vaccination Strategy. Five-week-old female Balb/c or C57BL/6 mice were vaccinated *i.n.* with 10^6 EID_{50} of ΔH5N1*att*, H7N2*att*, or H9N2*att* vaccine in 50µl PBS. Controls received 50µl of PBS. Mice were bled on day 0 and 21 post vaccination to collect sera. Twenty-one days post vaccination, mice were challenged with 20 MLD_{50} of WSN, HPAI H5N1, or 100 MLD_{50} pH1N1. Three mice per group were anesthetized and sacrificed by cervical dislocation at 3, 6, 9, or 12 dpc as indicated to titer the challenge virus in the lungs. Mice were monitored for disease signs for 21 dpc.

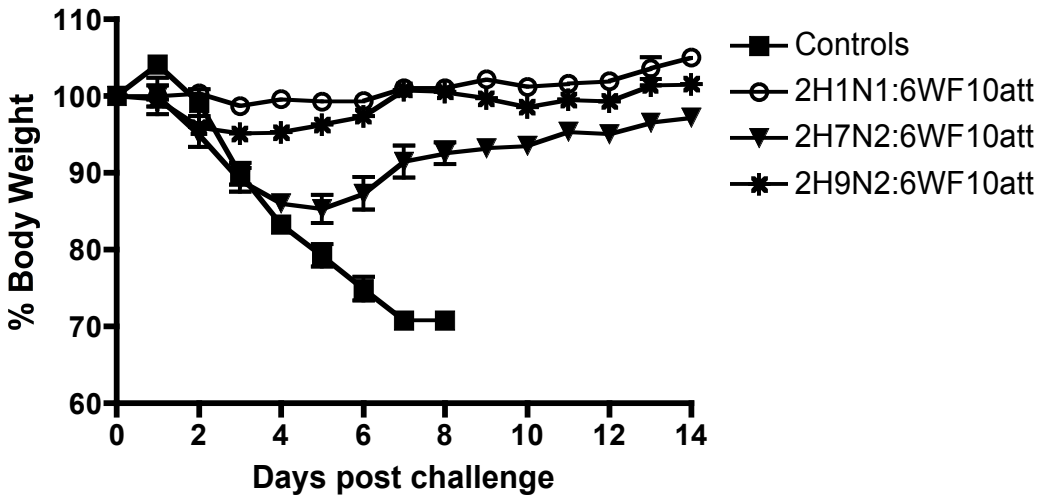

Figure 5. Percent body weight loss in vaccinated Balb/c mice challenged with A/WSN/1933 (H1N1). Vaccinated female Balb/c mice were challenged with 20 MLD_{50} of lethal WSN virus. All control mice died or had to be humanely sacrificed by 8 dpc. WSN H1N1*att* vaccinated mice did not lose body weight and displayed no disease signs post challenge. H9N2*att* vaccinated mice lost less than 5% of their body between days 3-6 post challenge, however they were able to regain the weight and displayed mild disease signs. H7N2*att* vaccinated mice lost 15% of their body weight and displayed moderate disease signs. All vaccinated mice survived the lethal WSN challenge. Data represents two independent experiments.

Table 5. Clearance of A/WSN/33 (H1N1) challenge virus in the lungs of Balb/c mice immunized with recombinant vaccines.

Vaccine	Immunization dose (PFU[a] or EID_{50}[b]/mouse)	Challenged with 20 MLD_{50} of	Challenge virus titer in log_{10} (PFU[a] or $TCID_{50}$[c])/g at 3 dpc	Challenge virus titer in log_{10} (PFU[a] or $TCID_{50}$[c])/g at 6 dpc
PBS	--	WSN	6.1±0.1[c]	5.4±0.4[c]
WSN H1N1*att*	10^{6a}	WSN	BLD[a,d]	BLD[a,d]
H7N2*att*	10^{6b}	WSN	5.9±0.7[c]	BLD[c,d]
H9N2*att*	10^{6b}	WSN	5.1±0.1[c]	BLD[c,d]

[d]BLD, below limit of detection

Although all vaccinated Balb/c mice were protected against lethal WSN challenge, we sought to determine if this finding was strain specific. WSN is a laboratory strain that is highly adapted to mice. Thus, we examined if the WF10*att* backbone would induce both homotypic and heterosubtypic immunity when a more contemporary challenge virus was used at a higher dose. To address this hypothesis, we vaccinated 5-week-old female Balb/c mice with 10^6 $TCID_{50}$ or EID_{50} of pH1N1*att*, H7N2*att*, or H9N2*att* in 50µl PBS. Control mice received 50µl PBS. Twenty-one days post vaccination, all mice were challenged with 100 MLD_{50} of pH1N1. On days 3 and 6 post challenge, 3 mice per group were sacrificed to titer the challenge virus in the lungs (Table 6). Similar to our findings with WSN, all vaccinated mice survived pH1N1 challenge. The H1N1*att* vaccinated mice lost less than 10% of their body weight and showed a lack of grooming; however, these mice regained their body weight by 10 dpc and survived (Figure 6). A small amount of challenge virus was detected in the lungs at 3 dpc although by 6 dpc no virus was isolated from the lungs (Table 6). Both the H7N2*att* and H9N2*att* vaccinated mice lost greater than 15% of their body weight by 6 dpc and displayed disease signs including a lack of grooming, respiratory symptoms, and inactivity. Also virus titers similar to the control mice were detected in the lungs of both groups at 3 dpc. By 6 dpc, both the H7N2*att* and H9N2*att* mice reduced the challenge virus in the lungs (Table 6) and survived challenge (Figure 6). These results indicate that the WF10*att* backbone is able to induce homotypic and heterosubtypic immunity when challenged with both older and contemporary strains of H1N1.

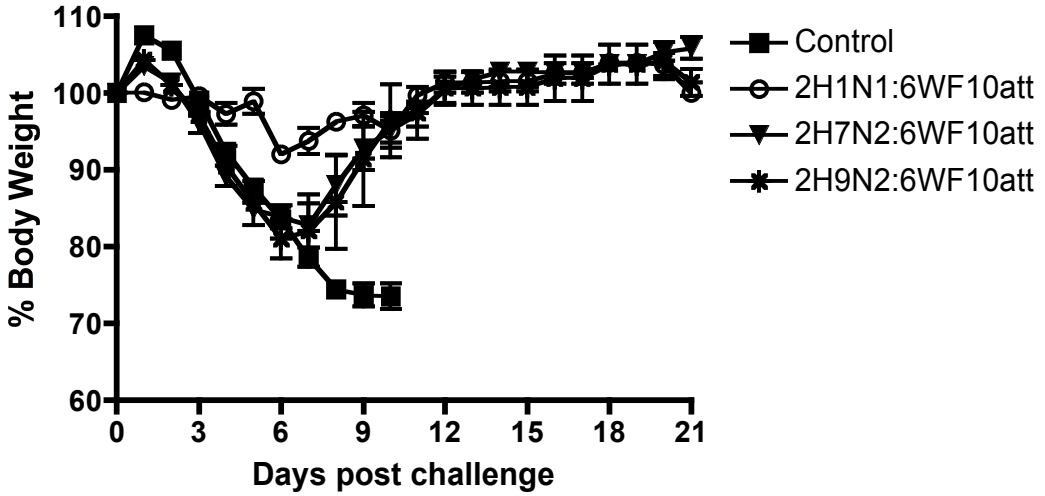

Figure 6. Percent body weight loss in vaccinated Balb/c mice challenged with Mouse-adapted A/California/04/2009 (pH1N1). Vaccinated female Balb/c mice were challenged with 100 MLD_{50} of pH1N1. pH1N1*att* vaccinated mice lost less than 10% of their body and displayed mild disease signs including lack of grooming. Both H7N2*att* and H9N2*att* vaccinated mice lost approximately 20% of their body weight and displayed moderate disease signs including rough coat, hunched posture and inactivity. All control mice died or had to be humanely sacrificed by 10 dpc, while all vaccinated mice survived challenge.

Table 6. Clearance of Mouse-adapted A/California/04/2009 (pH1N1) challenge virus in the lungs of Balb/c mice immunized with recombinant vaccines.

Vaccine	Immunization dose (EID_{50}/mouse)	Challenged with 100 MLD_{50} of	Titer in log_{10} $TCID_{50}$/g at 3 dpc	Titer in log_{10} $TCID_{50}$/g at 6 dpc
PBS Control	10^6	pH1N1	5.7±0.3	6.0±0.3
pH1N1*att* P7[a]	10^6	pH1N1	1.6±0.5	BLD[b]
H7N2*att*	10^6	pH1N1	5.7±0.5	3.6±0.7
H9N2*att*	10^6	pH1N1	6.0±0.2	4.3±1.1

[a]P7 is egg passage 7

[b]BLD, below limit of detection

3.4.3 Surface glycoproteins influence the degree of heterosubtypic immunity conferred to Balb/c mice vaccinated by WF10*att* backbone upon HPAI H5N1 challenge

Since the WF10*att* backbone induced both homotypic and heterosubtypic immunity during H1N1 challenges, we wanted to determine if the same is true for a different subtype, HPAI H5N1. The emergence of HPAI H5N1 virus in Asia, with an unusually broad host range and the ability to infect and kill people, has raised concerns that it may cause a pandemic. Therefore, five-week-old female Balb/c mice were vaccinated *i.n.* with 10^6 EID_{50} of ΔH5N1*att*, H7N2*att*, or H9N2*att* in 50µl PBS; control mice received 50µl of PBS. Twenty-one days post vaccination, all mice were challenged with 20 MLD_{50} of HPAI H5N1. On days 6, 9, and 12 post challenge, 3 mice per group were sacrificed to titer the challenge virus in the lungs. As observed previously, mice vaccinated with ΔH5N1*att*, lost less than 5% of their body weight and displayed few disease signs. Only a lack of grooming was observed between 4-7 dpc. By 8 dpc, mice regained their body weight and survived challenge (Figure 7). These mice had high titers of challenge virus in the lung at 6 dpc, however they were able to clear the challenge virus by 9 dpc (Table 7). The H7N2*att* mice lost 10% of their body weight and displayed disease signs including a rough coat, hunched posture, and respiratory signs between 4-10 dpc. These vaccinated mice were able to regain their body weight and survived challenge (Figure 7). The H7N2*att* mice also had high titers of challenge virus in their lungs at 6 dpc, however one mouse was able to clear the challenge virus by 9 dpc and the other mice were able to clear the challenge virus by 12 dpc (Table 7). In contrast, the H9N2*att* vaccinated mice began

losing weight at 4 dpc and displayed disease signs including rough coat, hunched posture, inactivity, labored breathing, dehydration, and some mice displayed neurological signs until they succumbed to the infection by 13 dpc (Figure 7). These animals had high levels of challenge virus in the lungs until death (Table 7).

These data were corroborated with the histology data (Figure 8). PBS controls had interstitial lesions in addition to bronchiolar necrosis while ΔH5N1*att* immunized mice had mild pathology with lymphocyte cuffing; however, the bronchioles and alveoli were normal (Figure 8). Although the H7N2*att* immunized mice were protected from challenge, these mice displayed lung pathology including lymphocyte cuffing, bronchiolar necrosis, hyperplasia, and interstitial inflammation. The H9N2*att* immunized mice had severe lung pathology including lymphocyte cuffing and interstitial inflammation consistent with the high viral titers detected post challenge (Table 7). Therefore, there was a clear difference in the degree of heterosubtypic immunity with HPAI H5N1 challenge; all the H7N2*att* vaccinated mice survived challenge while the H9N2*att* vaccinated mice succumbed to the infection.

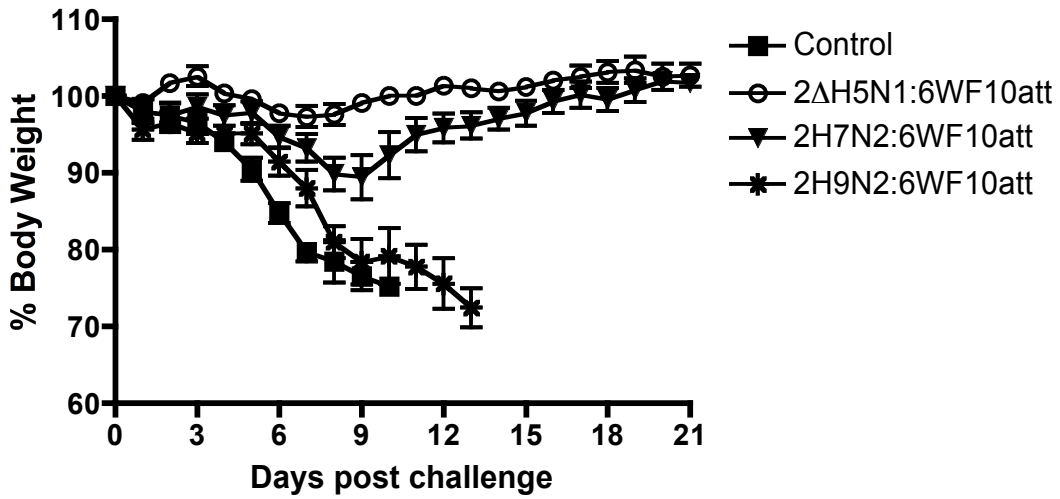

Figure 7. Percent body weight loss in vaccinated Balb/c mice challenged with A/Vietnam/1203/2004 (HPAI H5N1). Vaccinated female Balb/c mice were challenged with 20 MLD_{50} of HPAI H5N1 virus at 21 dpv. All control mice died or had to be humanely euthanized by 10 dpc. The ΔH5N1*att* immunized mice did not lose body weight and displayed no disease signs. The H7N2*att* immunized mice lost 10% of their body weight and displayed disease signs including rough coat, hunched posture, and respiratory signs between days 4-10 post challenge. The H9N2*att* immunized mice began losing weight at 4 dpc and continued until death, which occurred by 13 dpc. These mice also displayed disease signs including rough coat, hunched posture, inactivity, labored breathing, dehydration, and neurological signs. Data represents two independent experiments.

Table 7. Clearance of A/Vietnam/1203/2004 (HPAI H5N1) challenge virus in the lungs of Balb/c mice immunized with recombinant vaccines.

Vaccine	Challenged with 20 MLD_{50} of	Titer in log_{10} $TCID_{50}$/lung at 6 dpc	Titer in log_{10} $TCID_{50}$/g at 9 dpc	Titer in log_{10} $TCID_{50}$/g at 12 dpc
PBS Control	HPAI H5N1	7.1±0.3	5.3±0.1	--
ΔH5N1*att*	HPAI H5N1	4.9±0.1	BLD	BLD
H7N2att	HPAI H5N1	5.1±0.3	2.2±0.0*	BLD
H9N2*att*	HPAI H5N1	5.7±0.3	5.2±0.0	--

*One mouse was able to clear the challenge virus completely

--, indicates all mice died or had to be humanely euthanized before collection date

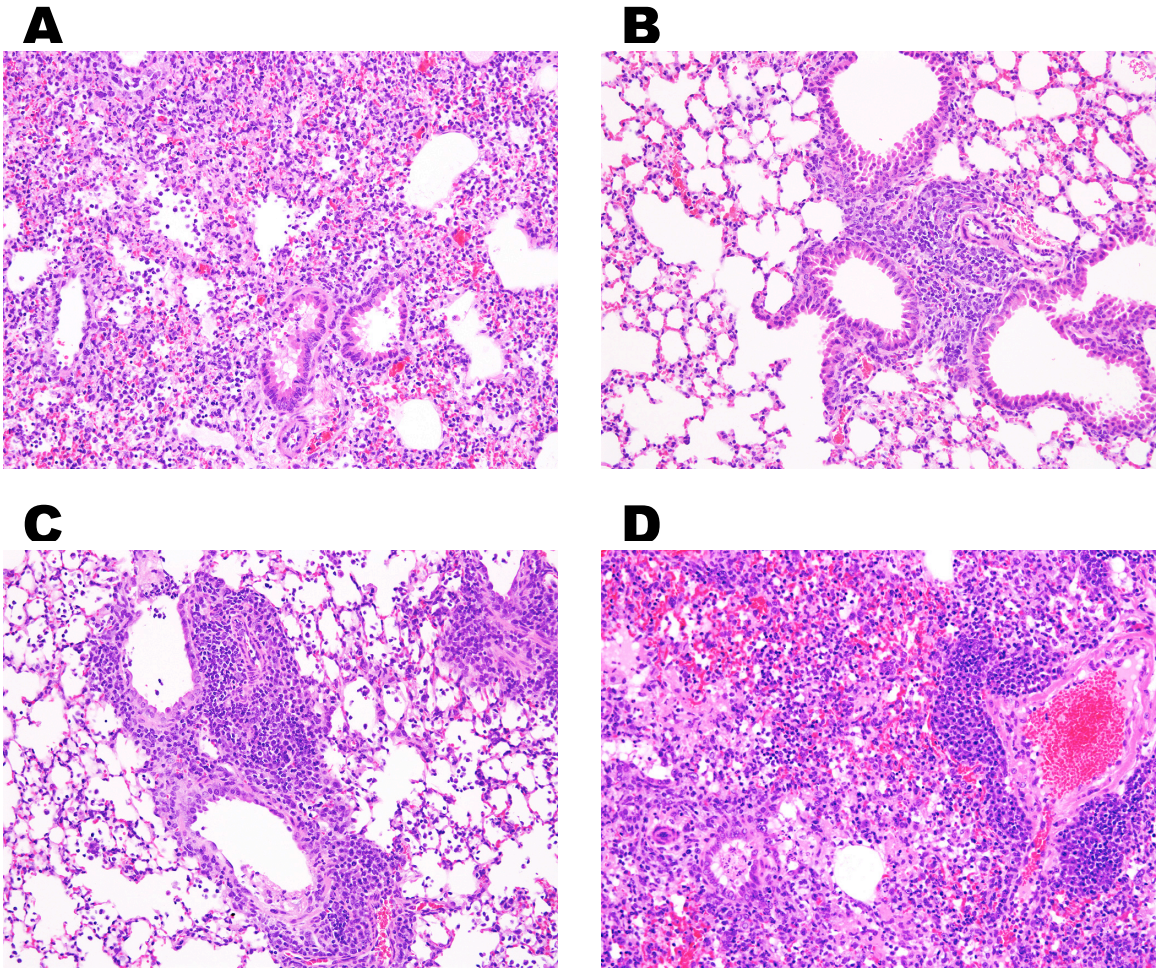

Figure 8. Histology of Balb/c mouse lung tissue at 9 dpc. Five-week-old female Balb/c mice were immunized with 10^6 EID_{50} in 50µl PBS of ΔH5N1*att*, H7N2*att*, or H9N2*att*. Control mice received 50µl PBS. At 21 dpv, mice were challenged with 20 MLD_{50} of HPAI H5N1. At 9 dpc, lungs were collected for histology. (A) PBS controls had interstitial lesions in addition to bronchiolar necrosis. (B) ΔH5N1*att* immunized mice had mild pathology with lymphocyte cuffing observed, however the bronchioles and alveoli were normal. (C) H7N2*att* immunized mice displayed lymphocyte cuffing, bronchiolar necrosis, hyperplasia, and interstitial inflammation, and (D) H9N2*att* immunized mice displayed lymphocyte cuffing and interstitial inflammation. Magnification is 200X.

3.4.4 Surface glycoproteins influence the degree of heterosubtypic immunity conferred to C57BL/6 mice vaccinated with WF10*att* backbone and challenged with HPAI H5N1

After observing a difference in the protection induced by the WF10*att* backbone expressing different surface proteins in Balb/c, we sought to determine if this finding was mouse strain specific. Furthermore, as an initial step towards determining the mechanism of heterosubtypic immunity, we vaccinated and challenged C57BL/6 mice because most immune deficient knockout mice are in the C57BL/6 background. The same vaccination and challenge strategy used for the Balb/c mice was used for the C57BL/6 mice (Figure 4). Interestingly, only 50% of the homotypically challenge C57BL/6 mice were completely protected from challenge although these mice had similar levels of virus in the lung as Balb/c mice (Figure 9 and Table 8). In regards to the heterosubtypic challenged mice, the H7N2*att* vaccinated mice were slightly more protected than the H9N2*att* vaccinated mice (Figure 9). Both groups of mice had high levels of challenge virus in the lungs at 3 and 6 dpc (Table 8).

These results are consistent with the histology data (Figure 10). PBS controls displayed lymphocyte cuffing, bronchiolar necrosis, fibrin, and interstitial inflammation. While the ΔH5N1*att* immunized mice, which had some level of protection, displayed mild pathology with lymphocyte cuffing observed, however the bronchioles and alveoli were normal. H7N2*att* immunized mice displayed lymphocyte cuffing and interstitial inflammation, and H9N2*att* immunized mice displayed lymphocyte cuffing, bronchiolar hyperplasia and necrosis, and alveolar inflammation (Figure 10). These results suggest that protection may be related to the

genetic background of the host, as the H7N2*att* surface protected Balb/c mice, but did not induce high levels of heterosubtypic immunity in C57BL/6 mice. However, the ΔH5N1*att* vaccine did provide 50% protection (Figure 9b). In C57BL/6 mice similar to Balb/c mice, the H7N2*att* vaccinated mice had increased survival when compared to H9N2*att* vaccinated mice challenged with HPAI, suggesting a difference in heterosubtypic immunity in C57BL/6 mice. Given the degree of heterosubtypic immunity was different in two mice strains, we questioned whether this phenotype would occur in ferrets, a closer model to human influenza infection.

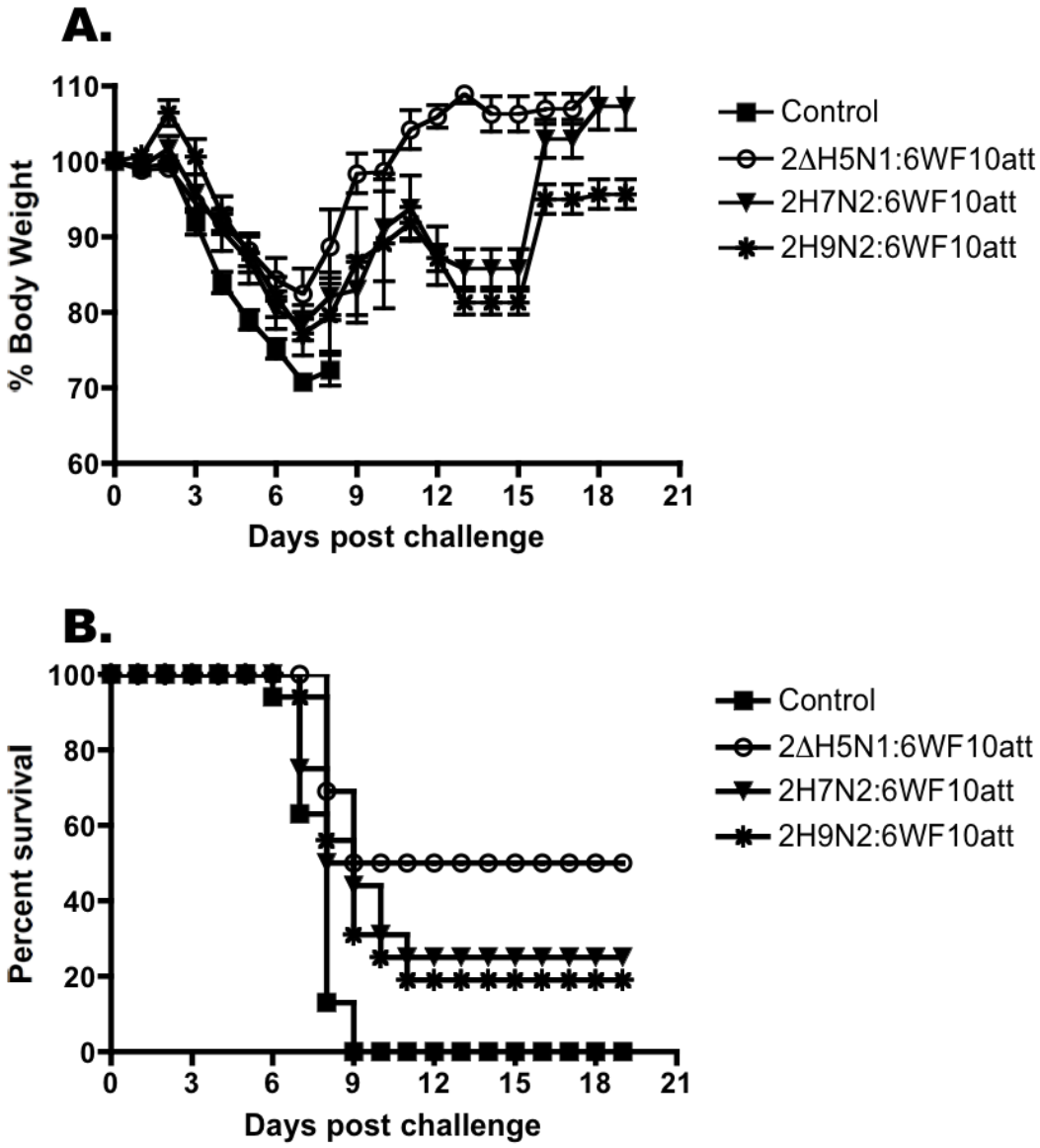

Figure 9. Percent body weight and percent survival of vaccinated C57BL/6 mice challenged with A/Vietnam/1203/2004 (HPAI H5N1). Vaccinated female C57BL/6 mice were challenged with 20 MLD_{50} of HPAI H5N1. (A) **Percent body weight loss.** The ΔH5N1*att* immunized mice lost approximately 15% of their body weight and only 50% of the mice were able to regain the weight and survived challenge. The H7N2att immunized mice lost 20% of their body weight and 25% were able to regain the weight and survived. The H9N2*att* immunized mice also lost 20% of their body weight and 19% of these mice survived. All control mice died or were humanely euthanized by 8 dpc. Data represents two independent experiments. (B) **Percent survival.** Fifty percent of the ΔH5N1*att* immunized mice were protected against homotypic challenge with HPAI H5N1. Twenty-five percent of H7N2*att* immunized mice survived challenge while only 19% of H9N2*att* immunized mice survived challenge. Data represents two independent experiments.

Table 8. Clearance of A/Vietnam/1203/2004 (HPAI H5N1) challenge virus in the lungs of C57BL/6 mice immunized with recombinant vaccines.

Vaccine	Immunization dose (EID_{50}/mouse)	Challenged with 20 MLD_{50} of	Titer in log_{10} $TCID_{50}$/g at 3 dpc	Titer in log_{10} $TCID_{50}$/g at 6 dpc
PBS Control	--	HPAI H5N1	4.7±0.6	6.2±0.0
ΔH5N1*att*	10^6	HPAI H5N1	4.5±1.2	3.4±0.7
H7N2*att*	10^6	HPAI H5N1	4.9±0.3	4.7±0.6
H9N2*att*	10^6	HPAI H5N1	5.2±0.4	5.6±0.2

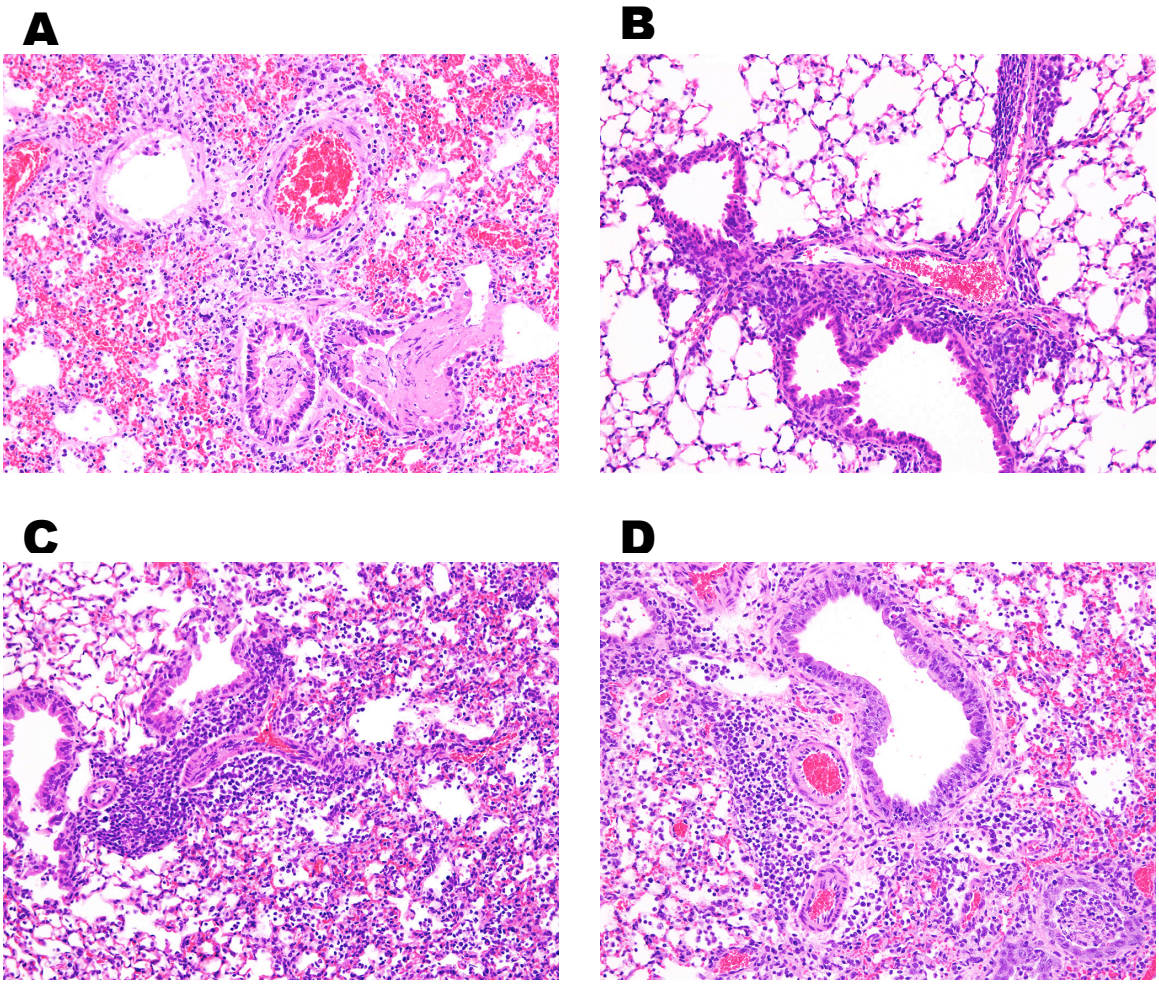

Figure 10. Histology of C57BL/6 mouse lung tissue at 9 dpc. Five-week-old female C57BL/6 mice were immunized with 10^6 EID_{50} in 50µl PBS of ΔH5N1*att*, H7N2*att*, or H9N2*att*. Control mice received 50µl PBS. At 21 dpv, mice were challenged with 20 MLD_{50} of HPAI H5N1. At 9 dpc, lungs were collected for histology. (A) PBS controls displayed lymphocyte cuffing, bronchiolar necrosis, fibrin, and interstitial inflammation. (B) ΔH5N1*att* immunized mice had mild pathology with lymphocyte cuffing observed, however, their bronchioles and alveoli were normal. (C) H7N2*att* immunized mice displayed lymphocyte cuffing and interstitial inflammation and (D) H9N2*att* immunized mice displayed lymphocyte cuffing, bronchiolar hyperplasia and necrosis, and alveolar inflammation. Magnification is 200X.

3.4.5 Boost dose protects Balb/c mice against HPAI challenge

After observing a difference in the protection induced by the WF10*att* backbone expressing different surface proteins in Balb/c, we sought to determine if a boost dose of WF10*att* would protect the H9N2*att* immunized mice from HPAI H5N1 challenge. Balb/c mice were immunized with 10^6 EID_{50} of ΔH5N1*att*, H7N2*att*, or H9N2*att* in 50µl PBS. Control mice received 50µl PBS. At 21 dpv, all mice received a second dose of 10^6 EID_{50} of each vaccine or PBS for controls. At 21 days after the boost dose, mice are challenged *i.n.* with 20 MLD_{50} of HPAI H5N1. On days 3 and 6 post challenge, 3 mice per group were sacrificed to titer the challenge virus in the lungs. Mice vaccinated with two doses of ΔH5N1*att* lost no body weight, displayed no disease signs post challenge, and survived challenge (Figure 11). These mice had high titers of challenge virus in the lung at 3 dpc; however, they were able to reduce the challenge virus by 6 dpc (Table 9). The H7N2*att* mice lost less than 5% of their body weight and displayed mild disease signs including lack of grooming between 5-9 dpc. These vaccinated mice were able to regain their body weight and survived challenge (Figure 11). The H7N2*att* mice also had high titers of challenge virus in their lungs at both 3 and 6 dpc (Table 9). Surprisingly, the H9N2*att* vaccinated mice lost about 15% of their body weight and displayed disease signs including rough coat, hunched posture, and respiratory signs. However with two doses of vaccine, the animals were able to regain their body weight and survived challenge (Figure 11). These animals had similar levels of challenge virus in their lungs as the H7N2*att* vaccinated mice. The results show that two doses of vaccine are needed for the H9N2*att* group of mice to be protected against H5N1.

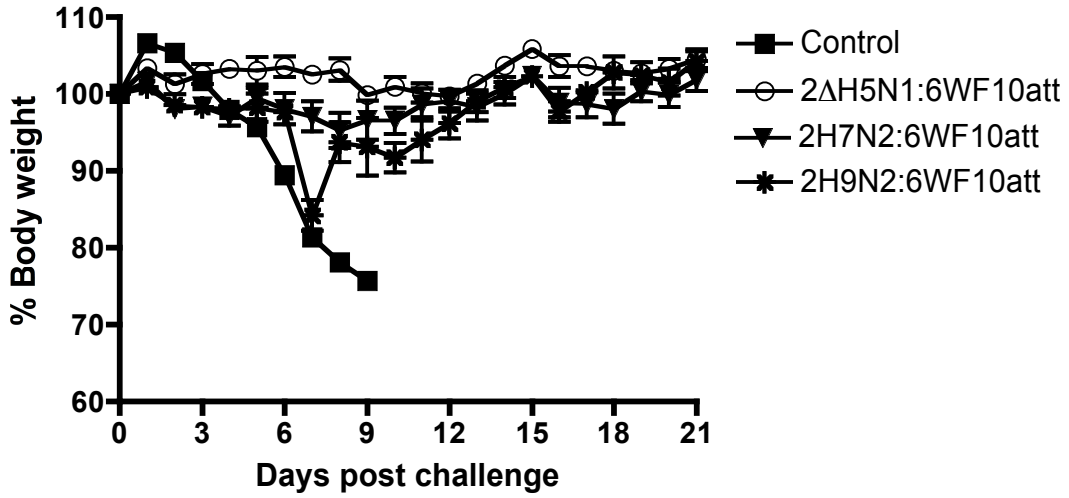

Figure 11. Percent body weight of Balb/c mice vaccinated with two doses of recombinant vaccine and challenged with A/Vietnam/1203/2004 (HPAI H5N1). Mice vaccinated with two doses of ΔH5N1*att* lost no body weight, displayed no disease signs post challenge, and survived challenge. The H7N2*att* mice lost less than 5% of their body weight and displayed mild disease signs including lack of grooming between 5-9 dpc. These vaccinated mice were able to regain their body weight and survived challenge. Surprisingly, the H9N2*att* vaccinated mice lost about 15% of their body weight and displayed disease signs including rough coat, hunched posture, and respiratory signs. However with two doses of vaccine, the animals were able to regain their body weight and survived challenge.

Table 9. Clearance of A/Vietnam/1203/2004 (HPAI H5N1) challenge virus in the lungs of Balb/c mice immunized with two doses of recombinant vaccines.

Vaccine	Immunization dose (EID_{50})	Challenged with 20 MLD_{50} of	Titer in log_{10} $TCID_{50}$/gram at 3 dpc	Titer in log_{10} $TCID_{50}$/gram at 6 dpc
PBS Control	--	HPAI H5N1	5.7±0.3	7.2±0.04
ΔH5N1*att*	10^6	HPAI H5N1	6.8±0.2	2.3±0.1
H7N2*att*	10^6	HPAI H5N1	5.8±0.2	5.8±0.8
H9N2*att*	10^6	HPAI H5N1	5.7±0.4	5.5±0.9

3.4.6 Genetically modified WF10*att* viruses are attenuated in ferrets.

Since we observed a difference in heterosubtypic immunity in both Balb/c and C57BL/6 mice, we wanted to determine if our WF10*att* backbone would also induce a difference in heterosubtypic immunity in ferrets. Importantly, the ferret model of influenza closely resembles influenza infection in humans. The definition of an *att* virus is the lack of replication in the lungs of ferrets; however, replication is detected in the upper respiratory tract where the temperature is lower. Therefore, ferrets were vaccinated with 10^6 EID_{50} or $TCID_{50}$ of ΔH5N1*att*, H7N2*att*, or H9N2*att* vaccine viruses in 0.5 mL PBS; controls animals received 0.5 mL PBS (Figure 12). At 3 dpv, 3 ferrets per group were sedated with a mixture of xylazine and ketamine and sacrificed. Lungs were collected and vaccine virus was titered by $TCID_{50}$. None of the vaccine viruses were detected in the lung of ferrets 3 dpv indicating that our WF10*att* backbone is attenuated regardless of the surface protein subtype expressed. At 1, 3, 5, and 7 dpv, nasal washes were taken to titer the vaccine viruses in the upper respiratory tract of ferrets. ΔH5N1*att* was not detected in the nasal washes at any day post vaccination, while both H7N2*att* and H9N2*att* replicated to high titers in the upper respiratory tract (Figure 13). Although some of the recombinant vaccines replicated to high titers in the upper respiratory tract of ferrets, no clinical signs were observed post vaccination indicating that the vaccines were attenuated in ferrets.

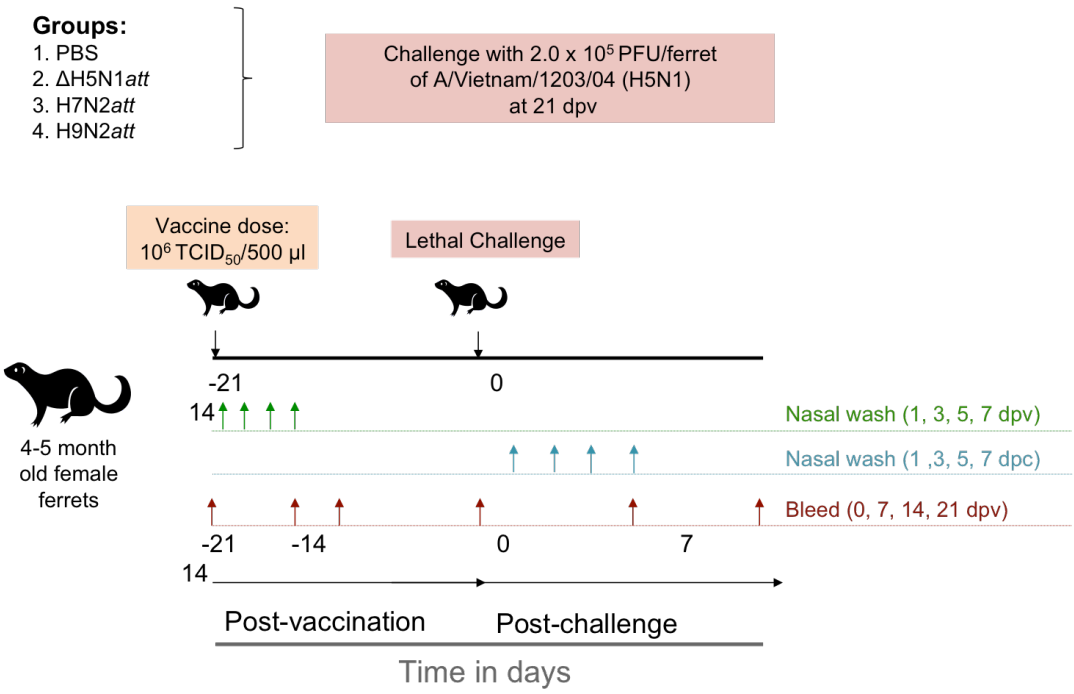

Figure 12. Ferret Vaccination Strategy. Four-five month old female ferrets (2 per group) were immunized *i.n.* with 10^6 EID_{50} or $TCID_{50}$/ferret of recombinant vaccines in 0.5 mL PBS, 0.25 mL per nostril. All mock-immunized ferrets received 0.25 mL of PBS per nostril. Nasal washes were taken on days 1, 3, 5, and 7-post vaccination to titer vaccine viruses in the upper respiratory tract of the ferret. Blood was drawn on days 0, 7, 14, and 21 post vaccination. Twenty-one dpv, ferrets were infected with $2x10^5$ PFU of HPAI H5N1. On days 1, 3, 5, and 7-post challenge, nasal washes were collected to titer the challenge virus. Blood was taken on days 7 and 14-post challenge. Clinical signs, body weight and mortality of ferrets were monitored and recorded for 14 dpc. Three independent ferret vaccine experiments were performed totaling six ferrets per group.

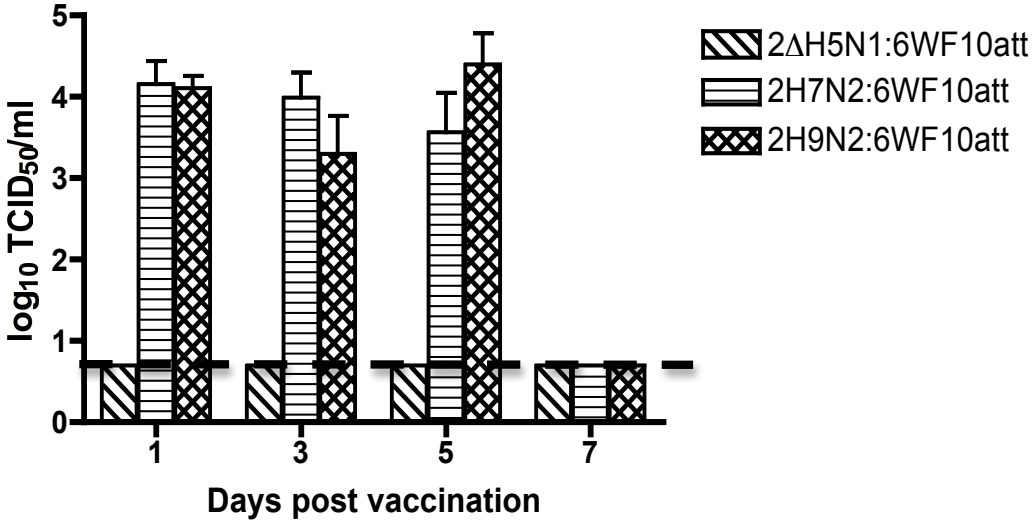

Figure 13. Replication of recombinant vaccines in nasal washes (upper respiratory tract) of ferrets. Ferrets were vaccinated *i.n.* with ΔH5N1*att*, H7N2*att*, or H9N2*att* in 0.5 mL PBS. On days 1, 3, 5, and 7-post vaccination, nasal washes were collected and vaccine virus was titered by $TCID_{50}$. The ΔH5N1*att* vaccine was not detected in the nasal wash of ferrets at any day post vaccination. Both H7N2*att* and H9N2*att* were detected on 1, 3, and 5 days post vaccination and were cleared by 7 dpv. Data combined from three independent experiments. Data are mean ± SD of nasal washes (log_{10} $TCID_{50}$/mL) for 6 ferrets per group.

3.4.7 Surface glycoproteins influence the degree of heterosubtypic immunity conferred to ferrets vaccinated with WF10*att* backbone upon HPAI H5N1 challenge.

Although two of the vaccine viruses replicated to high titers in the upper respiratory tract of ferrets, no virus was detected in the lungs and no clinical signs were observed for 21 dpv. Therefore, given this attenuated phenotype we next evaluated the protective capacity of our WF10*att* backbone in ferrets. Three independent experiments were performed with 2 ferrets per group for a total of 6 ferrets per group. At 21 dpv, ferrets were challenged *i.n.* with a lethal dose of 2×10^5 PFU of HPAI H5N1 virus in 0.5 mL PBS. On days 1, 3, 5, and 7-post challenge, nasal washes were performed to titer the challenge virus. Ferrets were observed for clinical signs for 14 dpc. The ΔH5N1*att* vaccinated ferrets survived challenge (Figure 14b) and lost less than 5% body weight (Figure 14a); all the H9N2*att* vaccinated ferrets survived challenge (Figure 14b). However, they lost as much as 10% of their body weight (Figure 14a). In contrast, the H7N2*att* vaccinated ferrets were partially protected from challenge and lost up to 15% of their body weight (Figure 14a). Only 50% of these animals survived (Figure 14b). The challenge virus replicated to high titers in the nasal washes of all ferrets on days 1 and 3-post challenge (Figure 15). By day 5 post challenge, ΔH5N1*att* vaccinated ferrets were able to clear the challenge virus while the surviving H7N2*att* and H9N2*att* vaccinated ferrets were able to reduce challenge virus titers by 5 dpc and completely clear it by 7 dpc (Figure 15). These results suggest that our WF10*att* backbone is protective in multiple species against homotypic challenge; however, the induction of Het-I is species specific.

H7N2*att* vaccinated Balb/c mice were completely protected from HPAI H5N1 while only 50% of H7N2att vaccinated ferrets were protected from HPAI H5N1.

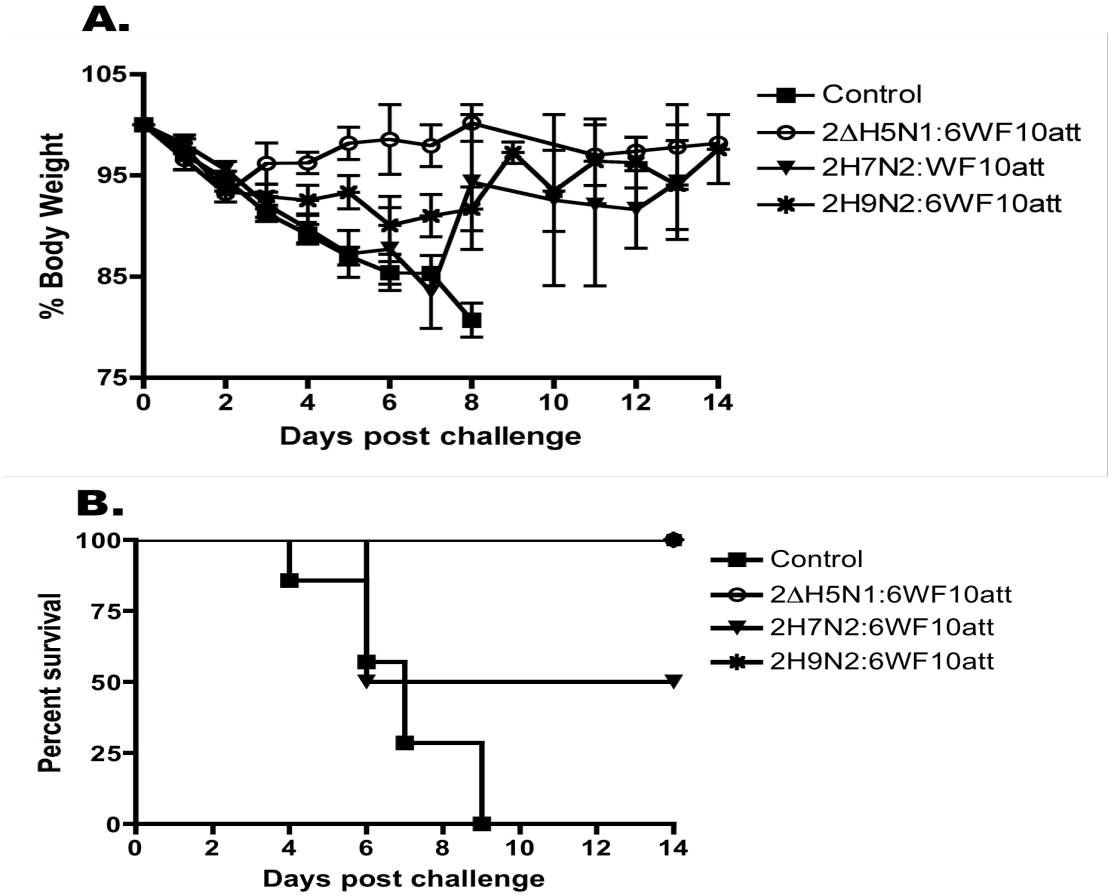

Figure 14. Percent body weight and survival of vaccinated ferrets challenged with A/Vietnam/1203/2004 (HPAI H5N1). Ferrets were challenged *i.n.* with lethal dose of 2 x 10^5 PFU of HPAI H5N1. Body weights were recorded and clinical signs were observed for 14 dpc. (A) **Body weight loss.** The ΔH5N1*att* immunized ferrets lost less than 5% of their body weight and displayed no disease signs. The H9N2*att* immunized ferrets lost 10% of their body weight and displayed a range of disease signs from mild to moderate. The H7N2*att* immunized ferrets lost 15% of their body weight and displayed moderate disease signs. (B) **Percent survival.** All of the ΔH5N1*att* and H9N2*att* vaccinated ferrets survived challenge, while only 50% of the H7N2*att* vaccinated ferrets survived challenge. Data represents three independent experiments. Mild clinical signs include decreased activity (animals are alert but playful only when stimulated), nasal rattling, less than 5% body weight loss, or no increase in body temperature. Moderate clinical signs include decreased activity (alert but not playful), nasal discharge, sneezing, ocular discharge, diarrhea, inappetence, ≥10% body weight loss, or an increase in body temperature no more than 2°. Severe clinical signs include no activity (animals are neither alert or playful when stimulated), mouth breathing, labored breathing, wheezing, inappetence, ≥15% body weight loss, or neurological symptoms (ataxia and hind-limb paralysis).

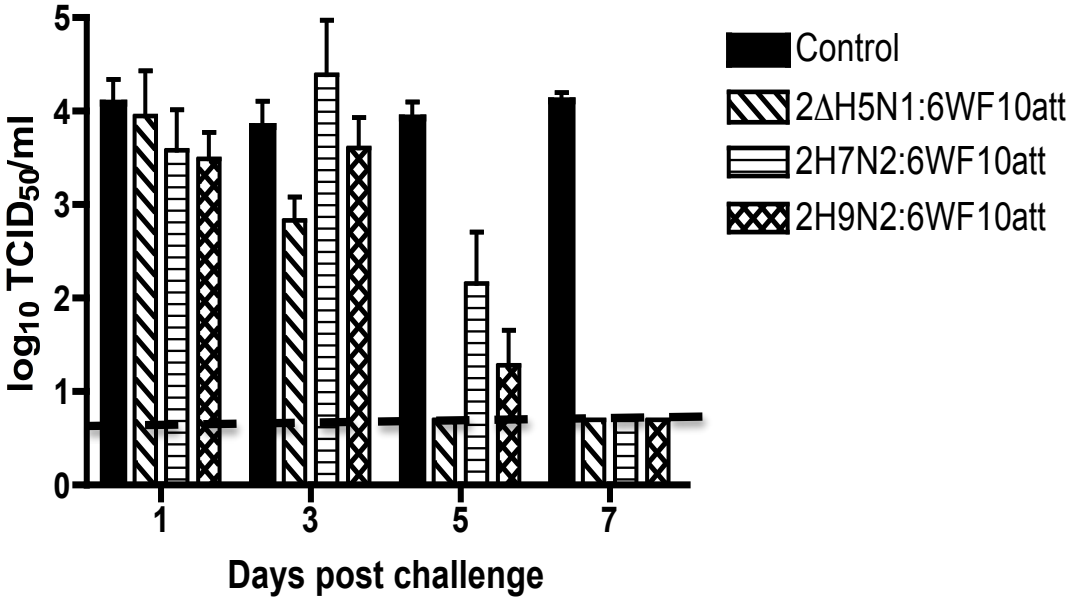

Figure 15. Clearance of A/Vietnam/1203/2004 (HPAI H5N1) challenge virus in ferrets immunized with recombinant vaccines. Vaccinated ferrets were challenge with 2 x 10^5 PFU of HPAI H5N1. On days 1, 3, 5, and 7 post-challenge, nasal washes were collected and challenge virus was titered by $TCID_{50}$. The challenge virus was detected in the nasal washes of both the control ferrets and the vaccinated ferrets. ΔH5N1*att* vaccinated ferrets cleared the virus by day 5 post challenge. Both the H7N2*att* and H9N2*att* vaccinated ferrets cleared the challenge virus by day 7 post-challenge. The challenge virus titers remained high in the control ferrets. Data combined from three independent experiments. Data are mean ± SE of nasal washes (log_{10} $TCID_{50}$/mL) for 6 ferrets per group.

3.5 Discussion

Recent studies have indicated that transferring the *ts* amino acid signature of the master donor strain A/Ann Arbor/6/60 (H2N2) (MDV-A) into different human influenza strains results in a *ts* phenotype *in vitro* and attenuation in ferrets (Jin, Zhou et al. 2004). Due to the transferable nature of the *ts* mutations of the MDV-A virus, we sought to determine whether such mutations would impart a similar phenotype to an AIV in mammals. For this purpose, we chose a virus that has demonstrated a broad host range in order to generate an attenuated virus backbone that could be used for the development of a universal vaccine for multiple animal species (i.e. from poultry to humans). We chose the internal genes of the AIV A/guinea fowl/Hong Kong/WF10/99 (H9N2), which replicates and transmits efficiently in birds, causes respiratory disease in mice without adaptation and replicates efficiently in ferrets (Choi, Ozaki et al. 2004; Wan, Sorrell et al. 2008; Sorrell, Wan et al. 2009). We successfully generated attenuated H1N1, H5N1, H7N2, and H9N2 reassortant viruses with the internal genes from the WF10*att* virus backbone. Previously, we showed the WF10*att* backbone was able to replicate in the upper respiratory tract of chickens and very little virus was found in the lungs or cloaca, suggesting attenuation in chickens (Song, Nieto et al. 2007). In these current studies, we have shown that the WF10*att* backbone is also attenuated in both mice and ferrets (Figure 2, 3, 13 and Tables 3 and 4). In addition to being attenuated, the vaccines were protective against homotypic challenge with an H1N1 or HPAI H5N1 challenge in both animal models (Figures 5, 6, 7, 9, 11, and 14). Importantly, our WF10*att* backbone was able to induce Het-I

depending on the surface proteins expressed and the challenge viruses used. In the mouse model, both H7N2*att* and H9N2*att* vaccinated mice were protected against H1N1 challenge with either an old (Figure 5) or contemporary virus (Figure 6). Surprisingly in both mice and ferrets, during HPAI H5N1 challenge, we found a difference in Het-I depending on the surface glycoproteins expressed in our vaccine. The H7N2*att* vaccine was protective in mice (Figure 7) and the H9N2*att* vaccine was protective in ferrets (Figure 14) against HPAI H5N1 challenge. In the mouse model, two doses of H9N2*att* vaccine was needed for protection against HPAI H5N1 challenge (Figure 11). Given our observed differences, it will be interesting to determine the mechanisms involved in inducing Het-I in these two models especially since influenza infection in ferrets more closely resembles human influenza infection. In addition, the genetic variability in ferrets is also reflective of the human population.

With the declaration of the first pandemic in over forty years in 2009, the swine-origin H1N1 pandemic highlights the need for influenza vaccines that induce broader protection against all subtypes of influenza. Few studies have focused on the ability of live attenuated influenza vaccines to induce Het-I in both the ferret and mouse models, and even fewer studies have examined the ability of various influenza surface glycoproteins to induce Het-I outside of the H1 and H3 subtypes. To study the mechanisms of Het-I, predominately two virus strains are used in the literature, A/Puerto Rico/8/1934 (H1N1) (PR8) and A/Hong Kong/X31/1968 (H3N2) (X31). X31 is a reassortant virus containing the internal genes from PR8 and H3N2 surface glycoproteins from A/Hong Kong/X31/1968. Therefore, it remains unknown whether

the information observed in the literature correlates with other subtypes of influenza. Our study is unique because it focuses on the induction of Het-I by a live attenuated avian backbone. Here we show a live attenuated avian backbone expressing different surface glycoproteins differentially induces Het-I in the mouse and ferret models. Furthermore, we developed a model system to study the mechanism of Het-I because we have a vaccine that induces Het-I and another vaccine that does not. It will be interesting to uncover the immune response generated by our WF10*att* backbone expressing different surface glycoproteins in both mice and ferrets. The only recombinant vaccine that replicated in the mouse lung was H7N2*att* (Table 2), which is the vaccine that induced Het-I in HPAI H5N1 challenge (Figure 7). It is possible that replication alone may be responsible for the induction of Het-I at least in mice. The picture in ferrets is less clear; Both the H7N2*att* and H9N2*att* vaccines replicated in the nasal turbinates, however none of our vaccines replicated in the lungs of ferrets. Therefore, it is likely that factors other than pulmonary replication are involved in the induction of Het-I.

Previous studies have indicated a role for T cells specific to conserved regions of the virus in Het-I (Furuya, Chan et al.; Benton, Misplon et al. 2001; Alsharifi, Furuya et al. 2009). We plan to determine the quality of the immune response generated post vaccination using T cell ELISPOTs. Any differences in the quality of immune response between the vaccines would depend on the surface proteins or the compatibility of the surface proteins with the internal WF10*att* genes. In addition, others have found a role for B cells in Het-I (Nguyen, van Ginkel et al. 2001; Tumpey, Renshaw et al. 2001; Nguyen, Zemlin et al. 2007; Droebner, Haasbach et al.

2008; Quan, Compans et al. 2008). We also plan to perform microneutralization and ELISA assays on the immune sera collected from the vaccinated mice and ferrets to see if any cross-reactive neutralizing or non-neutralizing antibodies are present, and lastly we plan to perform vaccination and challenge studies in $JhD^{-/-}$ mice, which lack antibodies, to determine the role of antibodies in Het-I observed with our WF10*att* backbone.

There are obvious limitations in the preparation of influenza vaccine stocks for a pandemic due to the rapid mutability of the virus. Thus, it is not possible to predict whether the antigenic make-up of the vaccine seed stock would confer protective immunity against the pandemic strain. Therefore, it would be advantageous for the vaccine seed stocks to protect against many subtypes of influenza. Understanding the mechanisms of Het-I will allow the development of vaccines that provide broader cross protection.

Table 10. Summary survival table of all challenge studies performed.

		Challenge Virus		
Strain	**Vaccine**	**WSN H1N1**	**pH1N1**	**HPAI H5N1**
Balb/c mice	WSN H1N1*att*	+++		
	pH1N1*att*		+++	
	ΔH5N1*att*			+++
	H7N2*att*	+++	+++	+++
	H9N2*att*	+++	+++	--
C57BL/6 mice	ΔH5N1*att*			++
	H7N2*att*			+
	H9N2*att*			+
Ferrets	ΔH5N1*att*			+++
	H7N2*att*			++
	H9N2*att*			+++

+++, 100% survival

++, 50% survival

+, <50% survival

--, 0% survival

Empty, not done

Chapter 4: Mechanisms of heterosubtypic immunity induced by a modified live attenuated avian influenza A backbone

4.1 Abstract

Current seasonal influenza vaccines are unable to protect against antigenically distinct viruses; therefore, it is extremely important to understand the mechanisms involved in the induction of heterosubtypic immunity (Het-I), the ability of one subtype to protect against a different subtype. We developed a modified live attenuated avian influenza A backbone using the virus strain A/guinea fowl/Hong Kong/WF10/1999 (H9N2) (WF10) and transferring the temperature sensitive (*ts*), cold-adapted (*ca*), and attenuated (*att*) mutations into this virus. In addition, a HA tag was placed in-frame at the C-terminus of the PB1 gene. This recombinant virus is designated WF10*att*. We found that our WF10*att* backbone expressing H7N2 surface glycoproteins was protective in Balb/c mice against A/Vietnam/1203/2004 (HPAI H5N1) challenge. We were interested in determining the mechanisms involved in the Het-I. We found no role for IFN-γ, IL-1β, and TNF-α induction post vaccination or cross-reactive neutralizing antibodies in the induction of Het-I. However, we found that B cells are involved in Het-I. Cross-reactive non-neutralizing antibodies to non-hemagglutinin viral proteins were detected. In addition, naïve Balb/c mice were passively transferred by intraperitoneal injection with sera collected at 21 days post vaccination (dpv) from vaccinated Balb/c mice. Three out of ten mice that received ΔH5N1*att* sera were protected from challenge with HPAI H5N1 and one out of ten

mice receiving H7N2*att* sera were protected from challenge with HPAI H5N1. These results were confirmed using $JhD^{-/-}$ mice, which lack mature B-lymphocytes. $JhD^{-/-}$ mice vaccinated with the recombinant vaccines and challenged with HPAI H5N1 did not survive challenge further suggesting a potential role for B cells in Het-I. Also cells isolated from the lungs of H7N2*att* vaccinated mice had antibody-secreting cells targeted to HPAI H5N1. Together these results further suggest a role for B cells in the induction of Het-I with our WF10*att* backbone. Although B cells are important, our results suggest a role for other immune cells in Het-I. Both IFN-γ and Granzyme B secreting cells were detected in lung and spleen cells isolated from H7N2*att* vaccinated mice and stimulated with HPAI H5N1. Our modified live attenuated avian influenza A backbone uses multiple immune cell populations to induce Het-I.

4.2 Introduction

4.2.1 Proposed mechanisms of heterosubtypic immunity

Heterosubtypic immunity (Het-I) is the ability of one subtype of influenza to protect against a different subtype of influenza. Current seasonal vaccines are unable to induce Het-I and protect against antigenically distinct viruses; this was recently demonstrated by the novel H1N1 pandemic (pH1N1) in 2009. A monovalent vaccine containing the pH1N1 was prepared and available later in the influenza season. It is crucial that researchers elucidate the mechanism of Het-I so that seasonal vaccines can protect against subtypes not contained in the vaccine. This may decrease the severity of a pandemic; Het-I does not prevent infection however viral titers and lung pathology are reduced allowing recovery from the disease. This may save lives and allow time to create a vaccine containing the pandemic virus. Therefore, the

mechanisms of Het-I have been analyzed for >40 years (Schulman and Kilbourne 1965). Many researchers observed a substantial role for T cells in Het-I. It is thought T cells directed to the internal genes of influenza, specifically the NP and M genes, mediate Het-I because these genes are the most conserved between different subtypes of influenza A virus.

Perrone and colleagues immunized mice with virus like particles (VLPs) containing the HA, NA, and M genes from the 1918 Spanish flu and observed protection against HPAI H5N1. No neutralizing antibodies were detected suggesting a role for T cell in this Het-I (Perrone, Ahmad et al. 2009). Similar results were observed when mice were primed with a live H3N2 virus and challenged with HPAI H5N1. Cross-reactive $CD8^+$ T cells to the HPAI H5N1 were detected in splenocytes isolated from mice primed with H3N2 virus (Kreijtz, Bodewes et al. 2009). In addition, splenic transfer experiments in mice demonstrated that $CD8^+$ T cells were responsible for heterosubtypic protection (Grebe, Yewdell et al. 2008). Also Benton, *et al* used knockout mice and found antibodies ($IgA^{-/-}$, $Ig^{-/-}$) and natural killer T cells (NKT) are not necessary for Het-I; however, both $CD8^+$ and $CD4^+$ T cells were important in the induction of Het-I (Benton, Misplon et al. 2001).

In contrast, antibodies to the surface glycoproteins, HA and NA, are extremely important in homotypic immunity; however, it is thought that antibodies to HA and NA have no role in Het-I. Some researchers have found a prominent role for HA- and NA-specific antibodies in Het-I. Quan and colleagues found an increase in heat stable components (most likely neutralizing antibodies) occurred in mice when immunized with inactivated virus and administered with an adjuvant. Mice deficient in $CD8^+$ and

$CD4^+$ T cells were immunized with the same vaccine and adjuvant were also protected from heterosubtypic challenge (Quan, Compans et al. 2008) suggesting B and not T cells are important for protection against heterosubtypic challenge. Nguyen, *et al* also found antibodies played a role in Het-I when they used $TdT^{-/-}$ (B cells are less polyreactive and T cells are more promiscuous) and ΔD-iD (impaired B cell development and antibody production) knockout mice. The majority of these mice did not survive heterosubtypic challenge although they had similar cytotoxic T cell activity as wildtype mice (Nguyen, Zemlin et al. 2007) suggesting humoral immunity is needed for protection against heterosubtypic challenge.

There are studies that suggest multiple immune cells are involved in Het-I. Epstein, *et al* explored the roles of different T cell subsets and antibodies in Het-I using knockout mice primed with live virus of one subtype and challenged with another subtype. They found that a lack of $CD8^+$ or $CD4^+$ T cells alone had no effect on mice surviving Het-I challenge. Passive transfer experiments using the serum from primed mice found that naïve mice that received the serum did not survive challenge suggesting that antibodies have no role in Het-I. In addition mice lacking transported IgA were protected from heterosubtypic challenge suggesting no role for transported IgA (Epstein, Lo et al. 1997). These results implicate a role for redundancy in the immune system. Other immune cell populations present can compensate for the lack of one or more cell subsets. Other researchers observed mice deficient in γδ T cells in addition to $CD4^+$ and $CD8^+$ T cells were not protected against heterosubtypic challenge (Benton, Misplon et al. 2001; Grebe, Yewdell et al. 2008). Therefore, it is possible that not just one immune cell population (T cells or antibodies) is responsible

for Het-I. A better understanding of the specific immune cells involved in the induction of Het-I by influenza vaccines, both inactivated and live attenuated, is important in the development of improved vaccines that are able to protect against multiple influenza subtypes and possibly protect against a pandemic influenza strain.

4.3 Methods and materials

4.3.1 Microneutralization Assay

Blood was collected from specific pathogen free (SPF) Balb/c mice (NCI, Fredrick, MD) at 21 days post vaccination (dpv) from the submandibular vein; blood was collected from ferrets at 7, 14, and 21 dpv from the vena cava. The blood was allowed to clot overnight at 4°C. To collect the sera, the blood was centrifuged at 2,000 rpm for 5 minutes. The serum was removed, aliquoted into new tubes, and stored at -20°C until processed. All sera were diluted 1:3 with receptor-destroying enzyme (RDE) from *Vibrio cholerae* (Fisher Scientific, Pittsburgh, PA) and incubated at 37°C overnight to destroy nonspecific serum inhibitor activity. The next day, the sera were transferred to a 56°C water bath and incubated for 45 min to inactivate the RDE. Then the sera were diluted 1:10 and 0.1 mL were placed in the first row of a 96-well flat-bottom plate. The sera were serially diluted two-fold in PBS within the 96-well flat-bottomed plate (50µl per well). Following the addition of 50µl containing 100 $TCID_{50}$ of virus diluted in PBS into each well, the plates were mixed and incubated at 37°C for 1 h. Subsequently, the serum:virus mixture (0.1 mL) was added to a monolayer of MDCK cells in a 96-well plate. The plate was incubated at 4°C for 15 min and then transferred to 37°C for 45 min. After incubation, the serum:virus mixture was removed from the cells and 0.2 mL Opti-MEM I (Invitrogen, Carlsbad,

California) with 1μg TPCK-trypsin/mL (Sigma, St. Louis, MO) was added. The cells were incubated at 37°C for 3 days and an HA assay was performed on the supernatant. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution of the sample that completely inhibited hemagglutination. HA assays were performed following the recommendations of WHO/OIE (WHO 2011).

4.3.2 ELISA

Serum from immunized Balb/c mice (NCI, Fredrick, MD) collected at 4, 7, 14, and 21 dpv were tested by ELISA for the presence of antiviral immunoglobulins. The sera was pooled from four individual mice. All sera were diluted 1:3 with RDE and incubated at 37°C overnight to destroy nonspecific serum inhibitor activity. Nunc immuno plates (Thermo Scientific, Rochester, NY) were coated with 1000 HA units (HAU) of β-Propiolactone (βPL)-inactivated A/Vietnam/1203/2004 (ΔH5N1) virus or 1.0μg of H5 protein from A/Vietnam/1203/2004 (HPAI H5N1) virus (Protein Sciences Corporation, Meriden, CT) per well in 100μl bicarbonate carbonate coating buffer. The ΔH5N1 virus has the polybasic cleavage site removed from the HA protein so that the virus is no longer a HPAI virus and can be used at BSL-2 levels. Control wells received either allantoic fluid or FLAG peptide (Sigma, St. Louis, MO). The ELISA plate was incubated at 4°C overnight. The next day, the sera were transferred to a 56°C water bath and incubated for 45 min to inactivate the RDE. Prior to adding the sera diluted 1:20 to the plate, the plate was blocked with 5% Non-fat milk (BioRad, Hercules, CA) for 1 h. The diluted sera samples were added to the plate and incubated at 37°C for 2 h. After the incubation, the plate was washed three times with PBS + 0.05% Tween-20 (PBS-T). Goat anti-mouse Ig conjugated to

horseradish peroxidase (1:5000) (SouthernBiotech, Birmingham, AL) was added to the plate and incubated at room temperature for 1 h. Excess antibody was washed away using PBS-T. The last wash was PBS and then 0.1 mL of a 1:1 mixture of TMB Peroxidase substrate and Peroxidase substrate solution B (KPL, Gaithersburg, MD) was added to each well. The reaction was stopped after two minutes with TMB Blue stop solution (KPL, Gaithersburg, MD). The plate was read on a plate reader at 630 nm.

4.3.3 Passive transfer of sera from vaccinated Balb/c mice

To determine the role of B cells in heterosubtypic immunity (Het-I), five-week-old female specific pathogen free (SPF) Balb/c mice (10 per group) were passively immunized by intraperitoneal (*i.p.*) injection with 100µl of sera from ΔH5N1*att*, H7N2*att*, or H9N2*att* immunized mice collected at 21 dpv. Control animals received injections of day 0 pre-vaccinated sera. There was a total of 3 *i.p.* injections of sera administered 24 h apart, 3 days before challenge. Twenty-four hours after the third injection, mice were administered isofluorane using Vetequip mobile anesthesia system (Vetequip, Inc, Pleasanton, CA) and challenged intranasally (*i.n.*) with 5 MLD_{50} of HPAI H5N1. The body weights and disease signs of the mice were monitored and recorded daily for 21 dpc. HPAI H5N1 challenge was performed under BSL-3+ conditions with USDA approval and according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park.

4.3.4 Immunization and challenge of $JhD^{-/-}$ mice.

To further evaluate the role of antibodies in Het-I, four to six-week-old female

SPF $JhD^{-/-}$ mice (Taconic Farms, Inc, Hudson, NY) were anesthetized with isofluorane administered using Vetequip mobile anesthesia system (Vetequip, Inc, Pleasanton, CA) before *i.n* immunization. Mice were immunized *i.n.* with 10^6 EID_{50} of the recombinant vaccines in 50µl PBS; all mock-immunized mice received 50µl PBS. The 50 % mouse lethal dose (MLD_{50}) for the challenge virus, HPAI H5N1, was calculated using groups of four mice inoculated *i.n.* with various doses ranging from 10^0 to 10^5 EID_{50}, and the Reed and Muench method was used to calculate the MLD_{50} (Reed 1938). Clinical signs, body weight and mortality of mice were monitored and recorded for 21 dpv. At 21 dpv, the immunized mice were challenged with 20 MLD_{50} of HPAI H5N1 virus *i.n.* in 50µl PBS. Clinical signs, body weight and mortality of mice were monitored and recorded for 21 dpc. HPAI H5N1 challenge was performed under BSL-3+ conditions with USDA approval and according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park.

4.3.5 Spleen and lung cell isolation from mice

Total cells were isolated from the spleen and lungs of immunized female Balb/c mice. Five-week-old female SPF Balb/c mice (NCI, Fredrick, MD) were anesthetized and immunized as described above. At 7, 14, or 21 dpv, mice were bled, anesthetized, and sacrificed by cervical dislocation. Spleens and lungs were removed aseptically and placed in separate 70-µM cell strainers over 50 mL tubes. The organs were completely mashed using a syringe plunger; ten milliliters of complete RPMI were used to wash the cell strainer and wash through all the cells. The cells were centrifuged at 300g for 5 min at 4°C and treated with lysis buffer

(Fisher scientific, Pittsburgh, PA) to lyse the red blood cells. The cells were resuspended in complete RPMI media and used for subsequent assays.

4.3.6 B cell ELISPOT

ELISPOT plate (Millipore, Billerica, MA) was pre-wet with 35% ethanol and washed three times with PBS. Then the plate was coated with 30,000 HAU per mL of βPL-inactivated ΔH5N1 virus (50µl per well) and incubated overnight at 4°C. Prior to seeding the wells, the plate was blocked with complete RPMI media for 2 h at room temperature. Cells from the spleen and lungs of vaccinated Balb/c mice were isolated at 7, 14, and 21 dpv. Lung cells were added at a concentration of 3.0×10^5 cells per well and 3.0×10^6 per well for spleen cells. To determine the background level of spots, wells with no cells added were included. Cells were incubated overnight (~20 h) at 37°C. Wells were washed with PBS containing Tween-20 (PBS-T) and mouse antibodies were probed using goat anti-mouse IgG conjugated to biotin (1:1500) (Sigma, St. Louis, MO) and incubating overnight at 4°C. After washing with PBS-T, horseradish peroxidase strepavidin (1:2000) (Vector Laboratories, Burlingame, CA) was added and incubated at room temperature for 2 h. After incubation, the plate was washed with PBS-T and then PBS. AEC substrate (BD Biosciences, San Jose, CA) was added to develop the spots. Plates were sent to Zellnet Consulting, Inc (Fort Lee, NJ) to count the spots per well.

4.3.7 Cytokine profile of vaccinated Balb/c mice

To determine whether there is a difference in the cytokine profile induced by the recombinant vaccines, the levels of IFN-γ, IL-1β, and TNF-α post vaccination was determined. Five-week-old female SPF Balb/c mice (NCI, Fredrick, MD) immunized

i.n. with 10^6 EID_{50} of ΔH5N1*att*, H7N2*att*, or H9N2*att* in 50µl PBS; all mock immunized control mice received 50µl PBS. At three and five days post vaccination, mice were bled, anesthetized, and sacrificed by cervical dislocation. Lungs were collected and homogenized. Homogenates were clarified by centrifugation and stored at -80°C until they were shipped to the University of Maryland Cytokine Core Laboratory (Baltimore, MD) where a Luminex 100 system was used to determine the levels of IFN-γ, IL-1β, and TNF-α cytokines.

4.3.8 IFN-γ ELISPOT

To determine the level of IFN-γ secreting cells post vaccination, an ELISPOT plate (Millipore, Billerica, MA) was pre-wet with 35% ethanol and washed three times with PBS. The plate was then coated with anti-mouse IFN-γ (1:200) (BD Biosciences, San Jose, CA) and incubated overnight at 4°C. The plate was blocked with complete RPMI media after washing with PBS and incubated at room temperature for 2 h. Cells from the spleen and lungs of immunized Balb/c mice were isolated at 7 and 21 dpv. Lung cells were added to each well at a concentration of 3.0 x 10^5 cells and 3.0 x 10^6 cells per well for spleen cells. βPL-inactivated virus was added to the cells for stimulation. To determine the background spot levels, media alone was added to cells and wells containing no cells were added for negative controls. Cells plus virus stimulant were incubated at 37°C for ~20 h. The next day wells were washed with PBS-T and anti-mouse IFN-γ conjugated to biotin (1:2000) (BD Biosciences, San Jose, CA) was added and incubated overnight at 4°C. After incubation, the plate was washed with PBS-T and then horseradish peroxidase strepavidin (1:250) (BD Biosciences, San Jose, CA) was added and incubated for 2 h

at room temperature. After washing with PBS-T, AEC substrate (BD Biosciences, San Jose, CA) was added to visualize the spots. Plates were sent to Zellnet Consulting, Inc (Fort Lee, NJ) to count the spots per well.

4.3.9 Granzyme B ELISPOT

ELISPOT plate (Millipore, Billerica, MA) was pre-wet with 35% ethanol and washed three times with PBS. The plate was then coated with 1μg of anti-mouse Granzyme B purified antibody (eBiosciences, San Diego, CA). The plate was incubated overnight at 4°C. The next day the plate was blocked with complete RPMI media after one wash with PBS and incubated at room temperature for 2 h. Cells from the spleen and lungs of immunized Balb/c mice were isolated at 14 and 21 dpv. Lung cells were added to each well at a concentration of 3.0×10^5 cells and 3.0×10^6 per well for spleen cells. Concanavalin A (ConA) (Sigma, St. Louis, MO) was added to the cells for stimulation. To determine background levels, media alone was added to cells and wells containing no cells were included as negative controls. Cells plus stimulant were incubated at 37°C for 20 h. The next day wells were washed with PBS-T and anti-mouse Granzyme B conjugated to biotin (1:2000) (BD Biosciences, San Jose, CA) was added to each well. The plate was incubated overnight at 4°C. After incubation, the plate was washed with PBS-T and then horseradish peroxidase strepavidin (1:2000) (Vector Laboratories, Burlingame, CA) was added and incubated for 2 h at room temperature. After washing with PBS-T and PBS alone, AEC substrate (BD Biosciences, San Jose, CA) was added to visualize the spots. Plates were sent to Zellnet Consulting, Inc (Fort Lee, NJ) to count the spots per well.

4.4 Results

4.4.1 Variations in the ability of recombinant WF10*att* viruses to induce neutralizing antibodies in Balb/c mice

To determine whether neutralizing antibodies play a role in protection from heterosubtypic challenge, we performed microneutralization assays using vaccinated mouse sera. Mice were vaccinated *i.n.* with the recombinant vaccines. A microneutralization assay was performed using the homologous vaccine as well as the challenge virus to see if any cross-reactive antibodies were detected in sera pooled from four individual mice. Although H7N2*att* vaccinated Balb/c mice were protected from challenge with HPAI H5N1, we were unable to detect cross-reactive neutralizing antibodies to WSN and HPAI H5N1, which suggests that the Het-I induced by our WF10*att* backbone is independent of neutralizing antibodies (Table 11). Neutralizing antibodies to the homologous virus were detected in all the vaccinated mice except ΔH5N1*att* vaccinated mice where no homologous neutralizing antibodies were detected at 21 dpv (Table 11). However, the ΔH5N1*att* vaccinated mice were completely protected from challenge with HPAI H5N1.

Table 11. Microneutralization (MN) antibody titers in Balb/c sera against homologous and heterologous viruses

Immunization group	Immunization dose	MN titers against homologous virus	MN titers against WSN virus	MN titers against H5N1 virus
PBS Control	-	<10	<10	<10
WSN H1N1*att*	10^6	160	160	<10
ΔH5N1*att*	10^6	<10	<10	<10
H9N2*att*	10^6	10	<10	<10
H7N2*att*	10^6	40	<10	<10

<10, indicates undetectable levels of antibodies

4.4.2 Variations in the ability of recombinant WF10*att* viruses to induce neutralizing antibodies in ferrets

To determine whether neutralizing antibodies play a role in protection from heterosubtypic challenge, we performed microneutralization assays on the vaccinated ferret sera. Ferrets were vaccinated *i.n.* with 10^6 EID_{50} or $TCID_{50}$ of ΔH5N1*att*, H7N2*att*, or H9N2*att* in 0.5 mL PBS; control ferrets received 0.5 mL PBS. At 7, 14 and 21 dpv, ferrets were bled from the vena cava, and sera were collected. A microneutralization assay was performed using the homologous virus as well as the challenge virus to see if any cross-reactive neutralizing antibodies were detected. Although the H9N2*att* vaccinated ferrets were completely protected from challenge with HPAI H5N1, we were unable to detect cross-reactive antibodies to the challenge virus HPAI H5N1, which suggests that neutralizing antibodies are not involved in Het-I (Table 12). Neutralizing antibodies to the homologous virus were detected in all the vaccinated ferrets except ΔH5N1*att* vaccinated ferrets where no neutralizing antibodies were detected post vaccination (Table 12). However, 100% of these ferrets survived homotypic challenge with HPAI H5N1.

Table 12. Microneutralization (MN) antibody titers in ferret sera pre-challenge against homologous and heterologous viruses.

Immunization group	Immunization dose	Days post vaccination	MN titers against homologous virus	MN titers against H5N1 virus
PBS Control	-	21	<10	<10
Δ H5N1*att*	10^6	7	<10	<10
		14	<10	<10
		21	<10	<10
H7N2*att*	10^6	7	160, 160, 10, 10, 20, 10	<10
		14	320, 320, 160, 40, 320,160	<10
		21	640, 160, 160, 320, 320, 160	<10
H9N2*att*	10^6	7	≥1280, 320, 160, 320, 80, 80	<10
		14	≥1280, ≥1280, 640, ≥1280, ≥1280, 640	<10
		21	≥1280, ≥1280, 640 ≥1280, ≥1280, ≥1280	<10

<10, indicates undetectable levels of antibodies

Each number represents titer for each individual ferret

4.4.3 Determining the role of non-neutralizing antibodies in Het-I induced by the WF10*att* backbone in Balb/c mice.

Because no cross-reactive neutralizing antibodies to the heterosubtypic challenge virus were detected in the sera of immunized mice, we sought to determine the role of non-neutralizing antibodies in Het-I. An ELISA assay was performed using the sera from PBS, ΔH5N1*att*, H7N2*att*, or H9N2*att* immunized mice (n=4). The first ELISA was coated with a recombinant A/Vietnam/1203/2004 H5 protein. ΔH5N1*att* sera collected at 14 and 21 dpv were positive for antibodies directed to the H5 protein (Figure 16a). H9N2*att* sera collected at 21 dpv had a small amount of antibodies that cross-reacted with the H5 protein; however, H7N2*att* sera had similar levels to the PBS control group indicating no cross-reactive antibodies were present to the H5 protein although 100% of these animals were protected from HPAI H5N1 (Figure 16a). Next we examined the presence of cross-reactive antibodies to other viral proteins. An ELISA plate was coated with βPL-inactivated ΔH5N1 virus, and sera from mice immunized with the recombinant vaccines were added. Similar to the H5 protein ELISA, ΔH5N1*att* had the highest levels of non-neutralizing antibodies to the ΔH5N1 virus at 14 and 21 dpv (Figure 16b). Both H7N2*att* and H9N2*att* had similar low levels of cross-reactive non-neutralizing antibodies at 14 and 21 dpv to ΔH5N1 viral proteins suggesting a potential role for non-neutralizing antibodies to non-HA viral proteins in Het-I (Figure 16b).

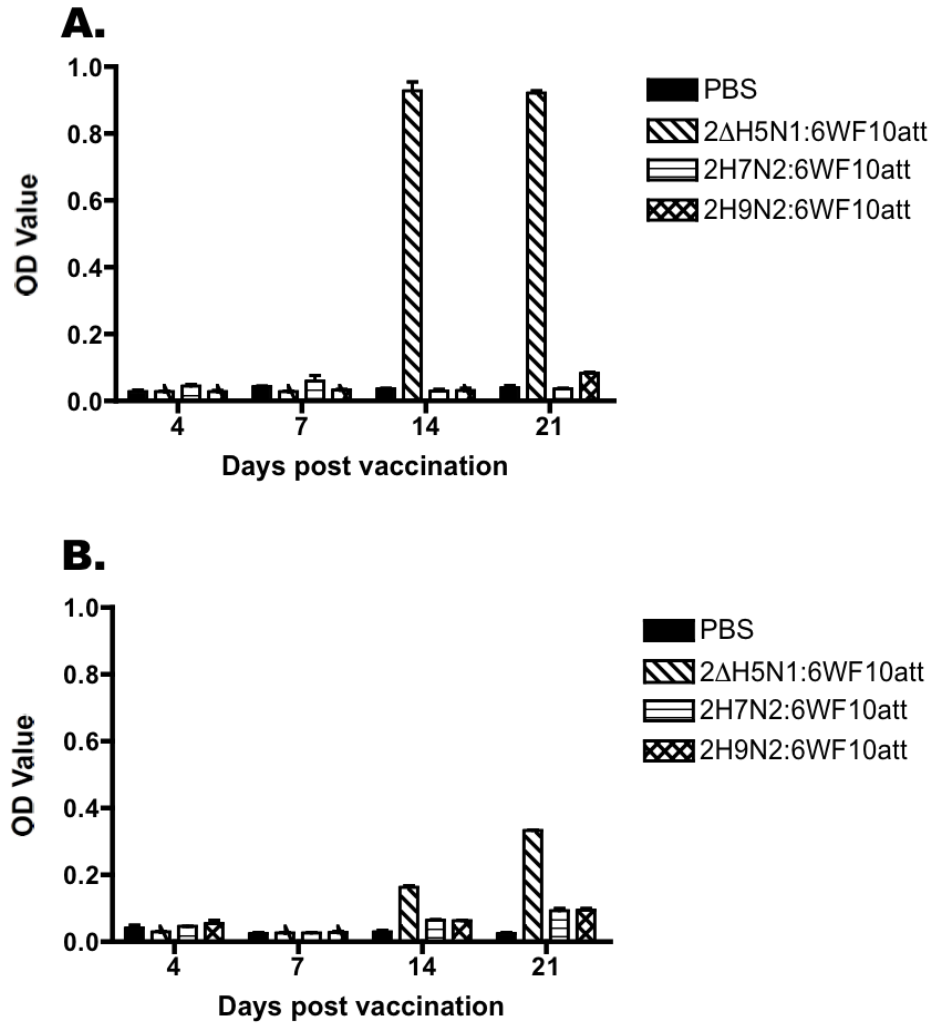

Figure 16. Serum antibody response to H5 HA protein from A/Vietnam/1203/2004 (HPAI H5N1) virus or βPL-inactivated ΔH5N1 virus after vaccination with recombinant vaccines. Sera samples from immunized Balb/c mice were tested in an ELISA to determine the level of non-neutralizing HA specific proteins and non-HA specific proteins to HPAI H5N1 virus. (A) **ELISA plate coated with H5 recombinant protein.** At days 4 and 7 post vaccination, no non-neutralizing antibodies were detected in any of the four groups. At days 14 and 21 dpv, the ΔH5N1*att* sera had high levels of antibodies to the H5 protein. At 21 dpv, H9N2*att* sera had a small level of cross-reactive non-neutralizing antibodies to the H5 protein; however, H7N2*att* sera had no cross-reactive non-neutralizing antibodies to the H5 protein at 4, 7, 14, or 21 dpv. (B) **ELISA plate coated with βPL-inactivated ΔH5N1 virus.** At days 4 and 7 post vaccination, no non-neutralizing antibodies were detected in any of the four groups. At days 14 and 21 dpv, the ΔH5N1*att* sera had high levels of antibodies to the ΔH5N1 virus. At 14 and 21 dpv, H7N2*att* and H9N2*att* sera had a small level of cross-reactive non-neutralizing antibodies to the ΔH5N1 virus. Data are mean ± standard deviation (SD) of OD values for 4 individual mouse serum pooled per group done in triplicate.

4.4.4 Determining the role of B cells in Het-I induced by the WF10*att* backbone in Balb/c mice.

After detecting cross-reactive non-neutralizing antibodies to HPAI H5N1 in the sera of heterosubtypically vaccinated mice, we wanted to further determine the role for B cells in Het-I. Five-week-old naïve female Balb/c mice were passively immunized by intraperitoneal (*i.p.*) injection with 100µl of sera from ΔH5N1*att*, H7N2*att*, or H9N2*att* immunized mice collected at 21 dpv. Naïve mice received pre-immune sera collected at day 0. All mice received a total of three *i.p.* injections of 100µl of sera 24 h apart prior to challenge. Twenty-four hours after the third injection, mice were challenged with 5 MLD_{50} of HPAI H5N1. Mice were monitored daily for clinical signs of disease including body weight, grooming, activity, and respiratory signs. Control mice that received sera from unimmunized mice started losing weight at 6 dpc and displayed a rough coat, hunched posture, inactivity, and respiratory signs until most mice died by 10 dpc and the last mouse died at 15 dpc (Figure 17a). The mice that received ΔH5N1*att* sera started losing body weight at 6 dpc and a lack of grooming was evident. As days progressed, mice continued to lose weight and displayed rough coat, hunched posture, respiratory signs, and inactivity until they died at 11 dpc. However, 3 of the 10 mice regained their body weight by 11 dpc and survived the challenge (Figure 17a, 17b). Both groups of mice receiving H7N2*att* and H9N2*att* sera began losing weight at 6 dpc similar to the other two groups, and their condition deteriorated over time and most mice (9 out of 10) died by 17 dpc. However, one out of 10 mice did survive challenge (Figure 17a, 17b), which could suggest a potential role for antibodies in both homotypic and heterosubtypic challenge when immunizing with the WF10*att* backbone.

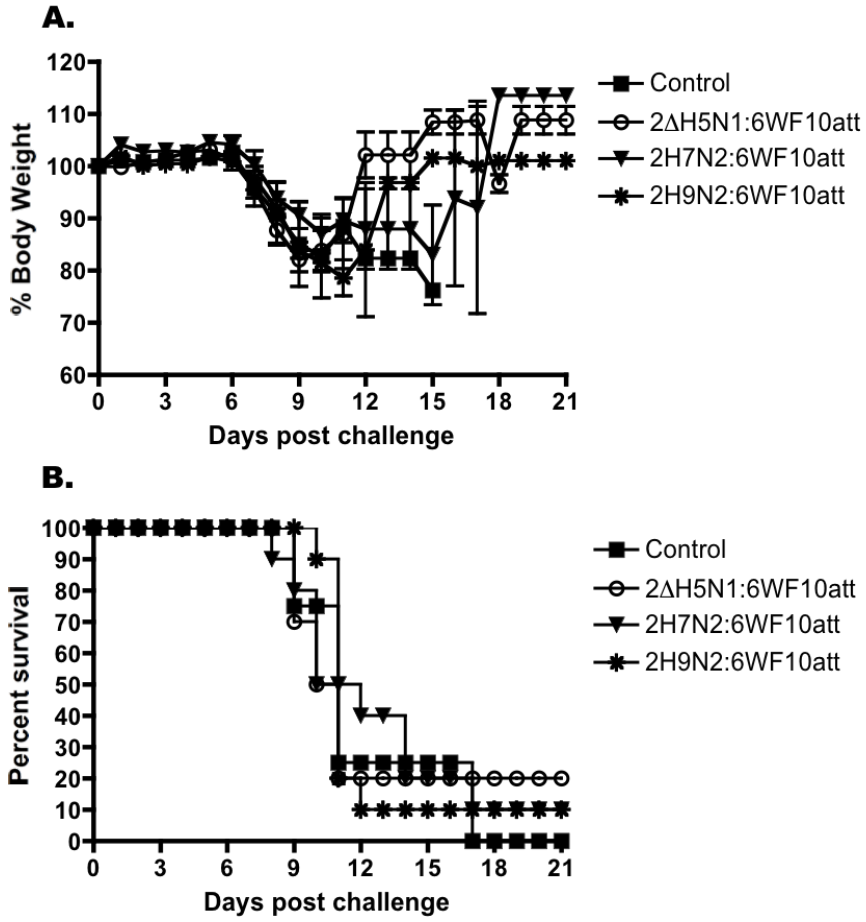

Figure 17. Percent body weight and survival of Balb/c mice passively immunized with sera and challenged with A/Vietnam/1203/2004 (HPAI H5N1). Naïve 5-week-old female Balb/c mice were passively transferred interperitoneally (*i.p.*) with 100µl sera from mice previously immunized with ΔH5N1*att*, H7N2*att*, or H9N2*att* collected at 21 dpv. Control mice received *i.p.* injection of 100µl from pre-immunize sera. Each mouse received 3 *i.p.* injections with 100µl of sera 24 hours apart prior to challenge. Twenty-four hours after the third *i.p.* injection, mice were challenged with 5 MLD_{50} of HPAI H5N1. (A) **Body weights.** All groups of mice started losing weight at 6 dpc. Mice receiving the ΔH5N1*att* sera lost a little less than 20% of their body weight until 9 dpc then the surviving (3 mice out of 10 mice) animals started regaining weight and survived challenge. Mice receiving the H7N2*att* lost approximately 15% of their body weight until 9 dpc and then the surviving animal regained its body weight and survived challenge. Mice receiving the H9N2*att* sera lost a little more than 20% of their body weight until 11 dpc and the surviving animal regained its body weight and survived challenge. (B) **Percent survival.** Three of the ten mice receiving the ΔH5N1*att* sera survived the HPAI H5N1 challenge. With the heterosubtypic challenge, only 1 of 10 mice receiving the H7N2*att* or H9N2*att* sera survived challenge.

The ELISA and passive transfer studies suggested a small role for antibodies in Het-I induced by our WF10*att* backbone, we sought to confirm this using B cell deficient mice. $JhD^{-/-}$ mice, which carry a deletion of the endogenous murine J segments of the Ig heavy chain locus resulting in no mature B-lymphocytes in the spleen, bone marrow, lymph nodes, peripheral blood or peritoneum, were immunized with the different recombinant vaccines and challenged with 20 MLD_{50} HPAI H5N1. Five-week-old female $JhD^{-/-}$ mice (n=10) were *i.n.* inoculated with 10^6 EID_{50} of ΔH5N1*att*, H7N2*att*, or H9N2*att* in 50µl of PBS; control animals received 50µl of PBS. No decreases in body weight or clinical signs were observed in the mice post vaccination indicating that the recombinant viruses were attenuated in these immunodeficient mice (Figure 18a). Since the recombinant viruses were attenuated in these mice, the mice were challenged with 20 MLD_{50} of HPAI H5N1 and monitored daily for disease signs and survival. All immunized and control animals began losing weight at 3 dpc and clinical signs such as lack of grooming and decrease in activity were observed. As days progressed, all mice continued to lose weight and displayed more clinical signs of disease such as rough coat, hunched posture, inactivity, and respiratory signs (Figure 18b). Control mice and H9N2*att* immunized mice died by 8 dpc while ΔH5N1*att* and H7N2*att* immunized mice died by 9 dpc (Figure 18c). These results further suggest a role for antibodies in both homotypic and heterosubtypic immunity induced by our WF10*att* backbone.

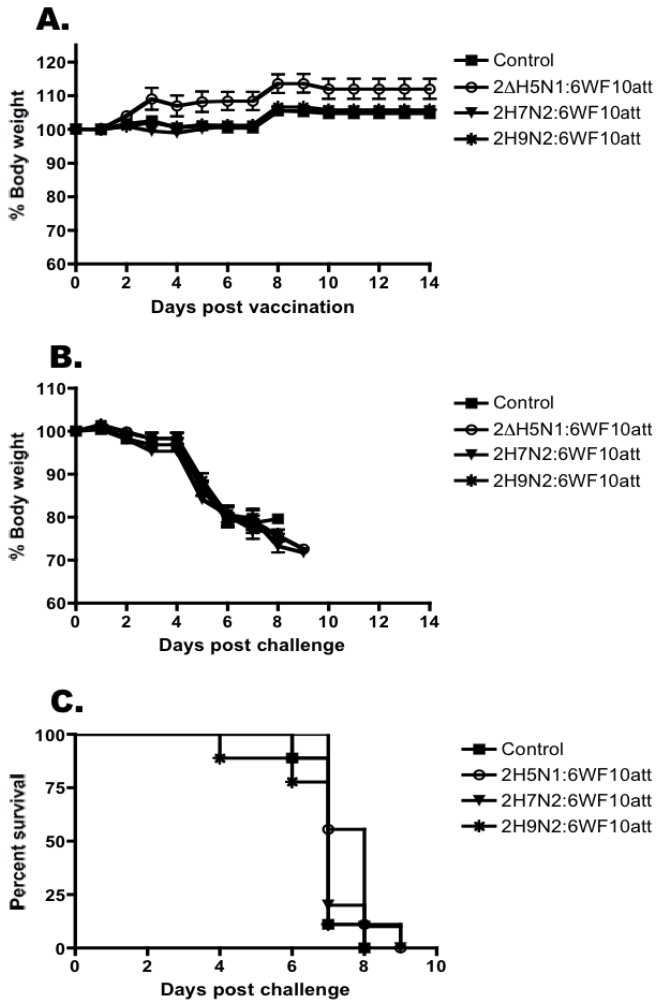

Figure 18. Percent body weight and survival of JhD$^{-/-}$ mice immunized with recombinant vaccines and challenge with A/Vietnam/1203/2004 (HPAI H5N1). Five-week-old female JhD$^{-/-}$ mice were immunized with 10^6 EID_{50} of ΔH5N1*att*, H7N2*att*, or H9N2*att* in 50µl PBS. Control mice received 50µl PBS. (A) **Post vaccination percent body weight.** Mice were monitored daily for disease signs for 14 dpv. ΔH5N1*att* immunized mice gained 10% more body weight post vaccination while the other three groups, PBS control, H7N2*att*, and H9N2*att* immunized mice, gained approximately 5% of their body weight, and no disease signs were detected post vaccination. (B) **Post challenge percent body weight** and (C) **Percent survival.** JhD$^{-/-}$ mice immunized with the recombinant vaccines were challenged twenty-one days post vaccination with 20 MLD_{50} of HPAI H5N1. All groups of mice started losing weight at 4 dpc, and a lack of grooming was observed. Control and H9N2*att* immunized JhD$^{-/-}$ mice continued to lose weight and displayed disease signs including rough coat, hunched posture, inactivity, and respiratory signs until all mice died by 8 dpc. Both the ΔH5N1*att* and H7N2*att* immunized mice continued to lose weight and displayed the same disease signs as the other two groups until their death at 9 dpc. No animals survived HPAI H5N1 challenge.

Lungs are the major site of antibody-secreting cells (ASCs) following wildtype virus infection (Joo, He et al. 2008); therefore, we decided to determine the number of ASCs in both the lungs and spleens of mice immunized with the different recombinant vaccines using a B cell ELISPOT. It is possible that the frequency of ASCs post vaccination may play a role in Het-I immunity. B cell ELISPOT plates were coated with βPL-inactivated A/Vietnam/1203/2004 (ΔH5N1) virus. The number of ASCs from total cells from the lung or spleen was determined at 7, 14, and 21 dpv. At 14 dpv, cells from H7N2*att* immunized mice had approximately 87 ASCs per 3.0 x 10^5 cells in the lungs while ΔH5N1*att* and H9N2*att* cells had 24 and 30 ASCs, respectively (Figure 19a). At day 21 post vaccination, the number of ASCs in the lungs increased for cells isolated from both ΔH5N1*att* and H9N2*att* to 109 and 138 ASCs, respectively (Figure 19a). Cells isolated from H7N2*att* had 75 ASCs directed to the ΔH5N1 virus (Figure 19a). No ASCs from any of the cells isolated from immunized mice spleens were detected (Figure 19b) suggesting the recombinant vaccines induced a local response. The cross-reactive ASCs detected in the lungs corroborate previous data and suggest a role for cross-reactive antibodies in Het-I.

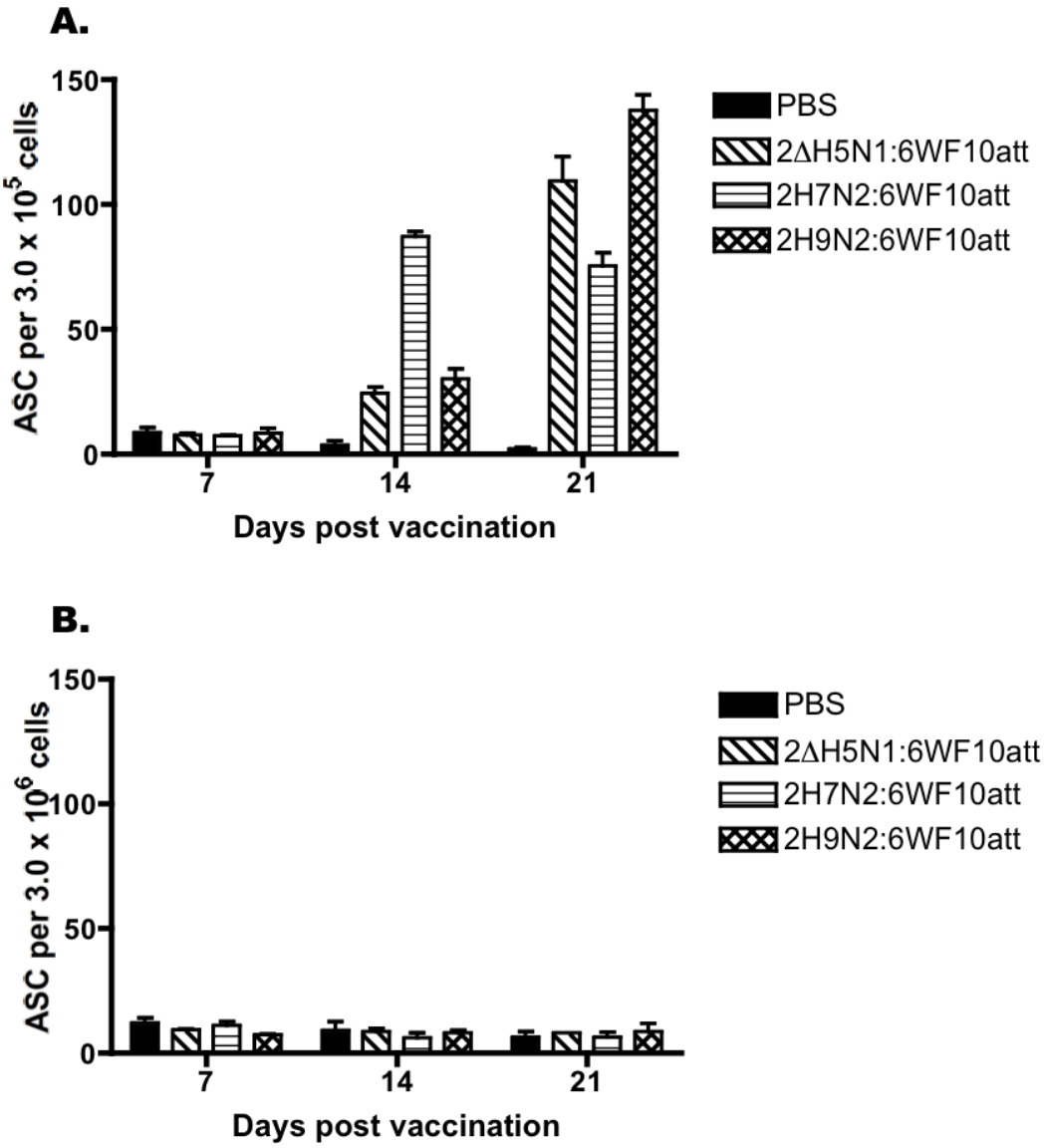

Figure 19. The induction of antibody-secreting cells (ASCs) in the lungs and spleens of mice immunized with recombinant vaccines. Five mice per vaccine group were sacrificed at days 7, 14, and 21 post vaccination, and the number of antibody-secreting cells (ASCs) to βPL-inactivated A/Vietnam/1203/2004 (ΔH5N1) virus were determined in the (A) **lungs** and (B) **spleens**. At 14 and 21 dpv, the ΔH5N1*att* immunized group secreted antibodies to the ΔH5N1 virus. Both the heterosubtypic immunized groups, H7N2*att* and H9N2*att*, secreted antibodies to the ΔH5N1 virus. No antibody-secreting cells to the ΔH5N1 virus were detected in the spleens of the immunized mice. Data are mean ± SD of ASCs per 3.0 x 10^5 cells in the lungs or 3.0 x 10^6 cells in the spleen for 5 mice per group done in triplicate.

4.4.5 Determining the role of cytokines in Het-I induced by the WF10*att* backbone

Previous results have shown a role for B cells in Het-I induced by our WF10*att* backbone. We sought to determine if our WF10*att* backbone expressing different surface glycoproteins induced different cytokine profiles post vaccination and if a difference in cytokine profile plays a role in Het-I. H7N2*att* was detected in the lungs of immunized mice at 3 dpv; in contrast, ΔH5N1*att* and H9N2*att* did not replicate in the lungs. Therefore, we thought the ability of this vaccine to replicate in the lungs resulted in an increased immune response—evidenced by an increase in the secretion of proinflammatory cytokines. A difference in cytokine profile may contribute to the ability of the H7N2*att* immunized mice to survive HPAI H5N1 challenge. Therefore, five-week-old female Balb/c mice were vaccinated with 10^6 EID_{50} of the recombinant vaccines, and control animals received PBS only. At three and five days post vaccination, mice were sacrificed. Lungs were collected and homogenized in PBS. The homogenates were analyzed by the Luminex 100 system for three cytokines, IFN-γ, IL-1β, and TNF-α. No difference in the level of all three cytokines, IFN-γ, IL-1β, and TNF-α (Figure 20a, b, c) was found suggesting a difference in cytokine profile is not responsible for the difference in Het-I.

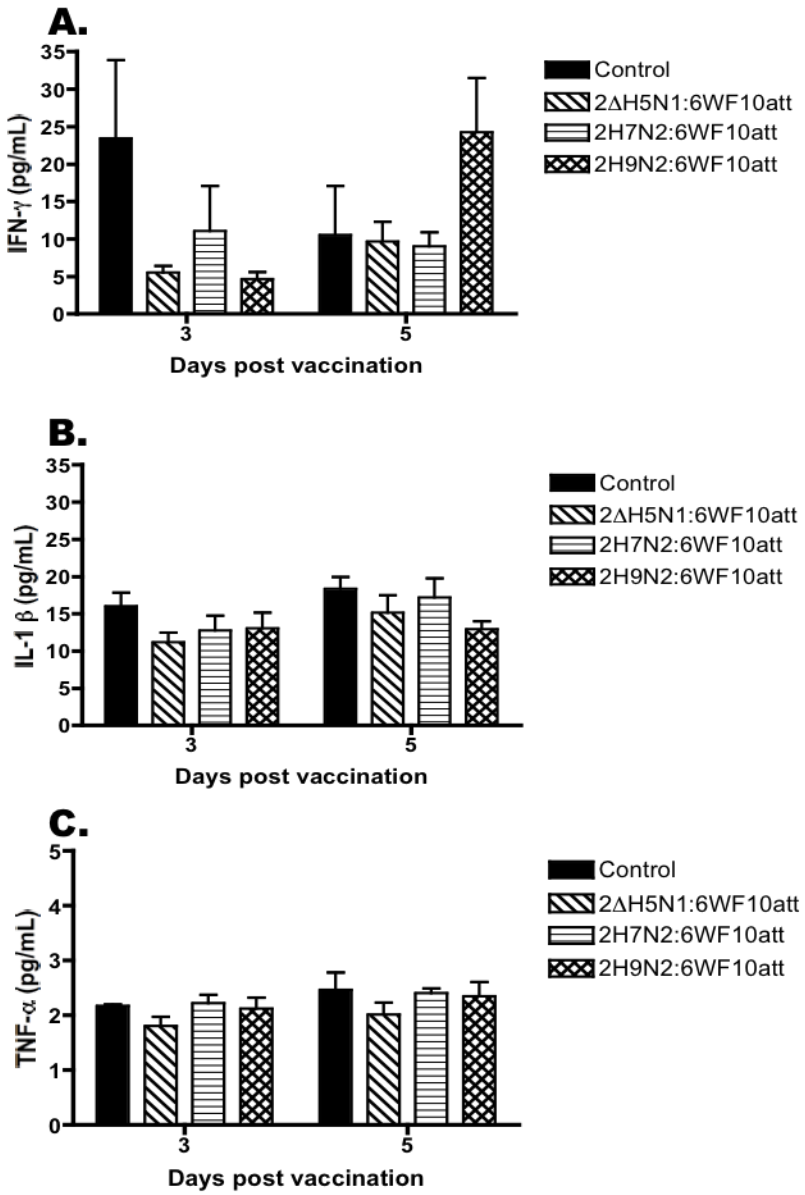

Figure 20. Cytokine levels in lung homogenates of Balb/c mice immunized with recombinant vaccines. Five-week-old female Balb/c mice were immunized with the recombinant vaccines or PBS. At three and five days post vaccination, mice were bled, anesthetized with isofluorane, and sacrificed by cervical dislocation. Lungs were collected and homogenized in PBS. The lung homogenates were analyzed for cytokines by the Luminex 100 system. (A) **IFN-γ**, (B) **IL-1β**, and (C) **TNF-α**. No difference in any three of the cytokines was observed at 3 or 5 dpv between the ΔH5N1*att*, H7N2*att*, or H9N2*att* immunized groups and the PBS controls. Data are mean ± SD of cytokine concentrations (pg/mL) for 5 mice per group. IFN, interferon, IL, interleukin, and TNF, tumor necrosis factor.

4.4.6 Determining the role of T cells in Het-I induced by the WF10*att* backbone.

To this point, B cells seem to play a role in Het-I with our WF10*att* backbone in mice. The question remains whether T cells play a role in Het-I; therefore, we isolated cells from Balb/c mice immunized with the three recombinant vaccines, ΔH5N1*att*, H7N2*att*, or H9N2*att*; control animals received PBS. We used an ELISPOT coated with an IFN-γ capture antibody to determine the frequency of IFN-γ secreting cells isolated from both lung and spleen and stimulated with ΔH5N1 virus. At both 7 and 21 dpv, all three vaccine groups had cells that secreted IFN-γ in the lungs (Figure 21a). At 7 dpv, spleen cells from H7N2*att* immunized mice had detectable levels of IFN-γ secreting cells. However, at 21 dpv both cells isolated from ΔH5N1*att* and H7N2*att* had a larger number of IFN-γ secreting cells compared to H9N2*att* (Figure 21b). These results suggest a role for T cells in Het-I with our WF10*att* backbone.

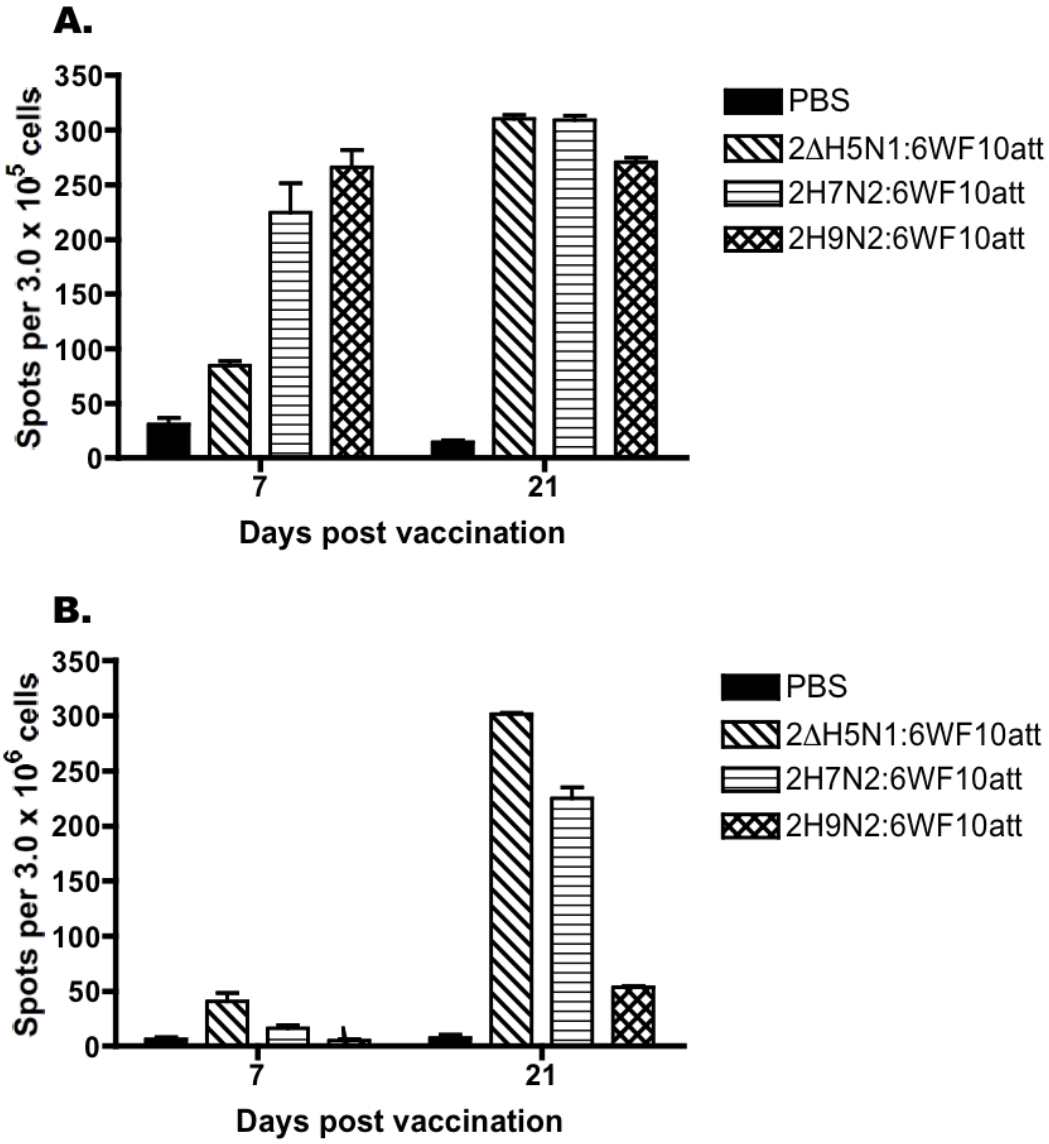

Figure 21. IFN-γ production from lung and spleen cells in response to immunization with recombinant vaccines in Balb/c mice. (A) **Lung** and (B) **Spleen** cells were isolated from Balb/c mice immunized with the recombinant vaccines, ΔH5N1*att*, H7N2*att*, or H9N2*att*, at 7 and 21 dpv. The cells were added to ELISPOT plates coated with IFN-γ capture antibody and stimulated with A/Vietnam/1203/2004 (ΔH5N1) virus. At 7 and 21 dpv, lung cells isolated from all the immunized mice produced IFN-γ. At 7 dpv, the spleen cells isolated from H7N2*att* had cells secreting IFN-γ and by 21 dpv all vaccine groups had cells secreting a high amount of IFN-γ. Data are mean ± SD of spots per 3.0 x 10^5 cells from the lung or 3.0 x 10^6 cells from the spleen for 5 mice per group done in triplicate.

After analyzing the ability of the recombinant vaccines to induce IFN-γ secretion, we analyzed the ability of the recombinant vaccines to induced other immune cells that are able to lyse ΔH5N1 infected cells, which could play a role in Het-I. Therefore, we used an ELISPOT assay to determine the frequency of cells secreting Granzyme B. Granzyme B is a member of the granzyme family, which contains serine proteases found in cytotoxic granules secreted by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Kam, Hudig et al. 2000; Smyth, Kelly et al. 2001). Granzyme B is able to access the target cell cytosol through holes made by perforin and induces cell death. Cells were isolated from both lungs and spleen. These cells were added to an ELISPOT plate coated with Granzyme B capture antibody and stimulated overnight with βPL-inactivated ΔH5N1 or ConA. We were unable to detect any Granzyme B secreting cells from the lungs when stimulated with either ΔH5N1 virus or ConA. Also no spots were detected from cells isolated from the spleen and stimulated with ΔH5N1 virus. However, higher levels of Granzyme B secreting cells were detected in spleen cells isolated from Balb/c mice immunized with ΔH5N1*att* and H7N2*att* when compared to H9N2*att*; these cells were stimulated with ConA (Figure 22). These Granzyme B secreting cells in the spleen may play a role in the ability of mice immunized with a single dose of H7N2*att* to be protected against lethal challenge with HPAI H5N1.

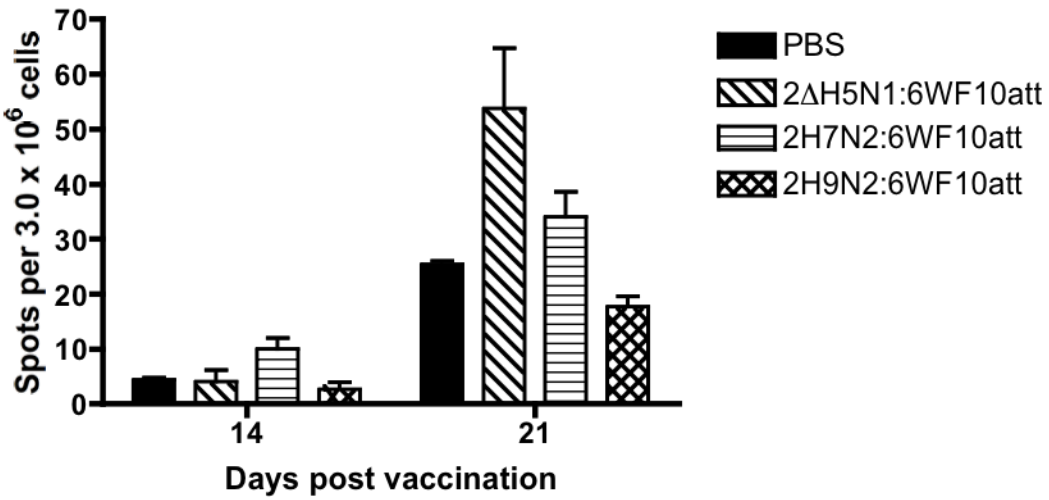

Figure 22. Induction of Granzyme B from spleen cells isolated from Balb/c mice immunized with recombinant vaccines. Spleen cells were isolated from Balb/c mice immunized with the recombinant vaccines, ΔH5N1*att*, H7N2*att*, or H9N2*att*; controls cells were isolated from mice immunized with PBS. The spleen cells were isolated at 14 and 21 dpv. The cells were added to an ELISPOT plate coated with Granzyme B capture antibody and stimulated with Concanavalin A (ConA). At 21 dpv, higher levels of Granzyme B secreting cells were detected in spleen cells isolated from Balb/c mice immunized with ΔH5N1*att* and H7N2*att* when compared to H9N2*att*. Data are mean ± SD of spots per 3.0 x 10^6 cells from the spleen for 5 mice per group done in triplicate.

4.5 Discussion

This is the first time a live attenuated vaccine has shown a difference in Het-I depending on the surface glycoproteins expressed and the species vaccinated. These findings suggest that Het-I is species specific and the ability of a vaccine to induce Het-I depends on the surface glycoproteins expressed by both the vaccine and challenge virus. We vaccinated mice with the same H7N2*att* and H9N2*att* vaccines and challenged them with either an H1N1 virus or HPAI H5N1. Both H7N2*att* and H9N2*att* vaccinated mice were protected from lethal challenge with 2 different H1N1 strains. However, only H7N2*att* vaccinated mice were protected from HPAI H5N1. We suspected a role for the surface glycoproteins because our recombinant vaccines have the same attenuated internal genes from WF10*att*, or there was a difference in the interaction between the different surface glycoproteins and the internal genes. However, depending on the surface glycoproteins expressed on the vaccine and the challenge virus used, our WF10*att* backbone induces different levels of Het-I.

We were unable to detect cross-reactive neutralizing antibodies to the HPAI H5N1 in the sera of H7N2*att* and H9N2*att* immunized mice or ferrets using a microneutralization assay (Hickman, Hossain et al. 2008) (Table 11 and 12). Previous research has observed the presence of Het-I in the absence of serum neutralizing antibodies (Perrone, Ahmad et al. 2009). Also in an ELISA, no cross-reactive non-neutralizing antibodies to the HPAI H5 recombinant protein were detected in the H7N2*att* sera; however, cross-reactive non-neutralizing antibodies to the H5 protein were observed in H9N2*att* sera (Figure 16a). This was not a surprise because

influenza hemagglutinin proteins are separated into two groups based on their primary sequence. H1, H5 and H9 are in group 1 while H7 is in group 2. Therefore, cross-reactive non-neutralizing antibodies from H9N2*att* to H5 protein were expected. However, these non-neutralizing antibodies are not involved in protection because the H9N2*att* vaccinated mice did not survive protection from HPAI H5N1 challenge. When we used an ELISA coated with βPL-inactivated ΔH5N1 virus, we were able to detect non-neutralizing antibodies to viral proteins other than HA in both H7N2*att* and H9N2*att* sera (Figure 16b) suggesting a potential role for non-neutralizing antibodies to non-HA viral proteins in the protection of H7N2*att* vaccinated mice from HPAI H5N1 challenge. Tumpey, *et al* also detected non-neutralizing antibodies in the sera and lung washes of mice immunized with an inactivated X-31 vaccine and found a role for B cells in protection against heterosubtypic challenge with HPAI H5N1 (Tumpey, Renshaw et al. 2001). Others have found a role for antibodies directed to the conserved internal proteins such as NP (Carragher, Kaminski et al. 2008) and M2 (Neirynck, Deroo et al. 1999; Fan, Liang et al. 2004; Tompkins, Zhao et al. 2007). However, in our system non-neutralizing antibodies cannot be the whole story because both heterosubtypic challenged mice (H7N2*att* and H9N2*att*) contained non-neutralizing antibodies to the challenge virus; however, only H7N2*att* mice were protected from challenge suggesting more immune mechanisms are involved.

We further examined the role of antibodies in Het-I induced by our WF10*att* backbone because previous studies have found a substantial role for B cells and not T cells in Het-I (Nguyen, van Ginkel et al. 2001; Tumpey, Renshaw et al. 2001;

Nguyen, Zemlin et al. 2007; Quan, Compans et al. 2008). However, most of these studies have focused on the ability of two subtypes, H1N1 and H3N2, to induce Het-I. In addition, most studies have been performed with either live virus or inactivated vaccines. Therefore, there is a lack of information within the literature regarding the ability of live attenuated vaccines and different influenza subtypes, besides H1N1 and H3N2, to induce Het-I. Our study focused on the ability of a live attenuated vaccine expressing either H7N2 or H9N2 to protect against HPAI H5N1. Because it has been shown B cells have a role in Het-I and our vaccines contain that same internal genes from WF10*att*, we focused on the role of B cells in the ability of our WF10*att* live attenuated avian backbone to induce Het-I. We performed three different assays to examine the role of B cells in Het-I including serum passive transfer study, challenge study using B-cell knockout mice, and B cell ELISPOT.

When naïve Balb/c mice were *i.p.* injected three times with serum collected 21 dpv from mice immunized with the recombinant vaccines and challenged twenty-four hours after the final injection, only three mice receiving the ΔH5N1*att* serum survived challenge with HPAI H5N1, and one mouse survived challenge from each of the H7N2*att* or H9N2*att* sera receiving groups (Figure 17) suggesting a potential role for B cells in both homotypic and heterosubtypic challenge with our WF10*att* backbone. In addition, no $JhD^{-/-}$ B cell knockout mice vaccinated with either of the recombinant vaccines and challenged with HPAI H5N1 survived challenge (Figure 18). This data is in contrast to a previous report that immunized $JhD^{-/-}$ with a live attenuated H3N2 vaccine. These mice were protected from challenge with lethal dose of PR8 virus (Powell, Strutt et al. 2007). It is important to remember that different

vaccines and challenge viruses were used when comparing our study to the Powell, *et al* study. Our previous results suggest different vaccines subtypes can induce a difference in Het-I, which may be the reason these reports are conflicting. Furthermore, we then performed a B cell ELISPOT using plates coated with βPL-inactivated ΔH5N1 virus to confirm the level of cross-reactive antibodies induced by our H7N2*att* vaccine. Lung cells isolated from mice immunized with H7N2*att* or H9N2*att* had cross-reactive antibody-secreting cells targeted to the ΔH5N1 virus (Figure 19a). No ASCs were detected in the spleen for any of the vaccinated mice, suggesting that our WF10*att* backbone induces a local immune response in the lungs post vaccination and not a systemic antibody immune response (Figure 19b). More studies need to be performed to determine the specificity of these heterosubtypic antibodies and determine the role they may play in protecting H7N2*att* immunized mice from HPAI H5N1 challenge. However, together all these results further indicate a role for antibodies in Het-I.

Similar results were observed with both the H7N2*att* and H9N2*att* vaccinated mice indicating a role for other immune mechanisms. The passive transfer study should be repeated using convalescent sera as a positive control and serum should be injected through the tail vein. Also the $JhD^{-/-}$ challenge study should be repeated using lower doses of challenge virus including 0.5, 1.0, 5, and 10 MLD_{50} of HPAI H5N1 in order to confirm the role of antibodies in Het-I. This will allow us to better understand the role of B cells in Het-I induced by our WF10*att* backbone.

Although our results suggest a role for B cells in Het-I, other immune mechanisms seem to play a role as well. As mentioned previously few reports have

focused on the ability of live attenuated vaccines to induce Het-I. It is hypothesized that the internal influenza antigens, specifically the NP and M genes, play a major role in Het-I because internal genes are more conserved between different subtypes. It is also thought that $CD8^+$ T cells play a major role in Het-I because they target conserved viral proteins, specifically the NP and M proteins (Townsend, Rothbard et al. 1986). Therefore, we focused on the role of T cells in Het-I. Previous research has examined the mechanism of Het-I and found an important role for both $CD8^+$ and $CD4^+$ T cells (Benton, Misplon et al. 2001; Droebner, Haasbach et al. 2008; Kreijtz, Bodewes et al. 2009; Perrone, Ahmad et al. 2009). In contrast, others have found that CTLs are not necessary for Het-I and found that antibodies are more important (Nguyen, van Ginkel et al. 2001; Droebner, Haasbach et al. 2008; Perrone, Ahmad et al. 2009).

We were unable to find a difference in levels of proinflammatory cytokines IFN-γ, IL-1β, or TNF-α post vaccination with the recombinant vaccines (Figure 20), which was surprising. The H7N2*att* vaccine replicated in the lungs of Balb/c mice at 3 dpv while both the ΔH5N1*att* and H9N2*att* vaccines were not detected. We thought the ability of this vaccine to replicate would induce an immune response including a difference in cytokine expression that may result in the protection from HPAI H5N1 challenge. There potentially could be a difference in the level of cytokines in lung homogenates at different time points earlier post vaccination, either 1 dpv or 2 dpv. Also if we examine other compartments such as draining lymph nodes or bronchoalveolar lavage, we may find a difference in the cytokine levels that may explain the difference in protection against HPAI H5N1.

To further determine the role of other immune cells in Het-I, we performed an IFN-γ ELISPOT using the lung and spleen cells isolated from Balb/c mice immunized with the recombinant vaccines. We observed IFN-γ secreting cells from all of the vaccine groups within the lung cells at both 14 and 21 dpv, (Figure 21) indicating a local immune response in the lungs was induced by all the recombinant vaccines. In contrast, spleen cells from both ΔH5N1*att* and H7N2*att* immunized mice secreted IFN-γ at 21 dpv (Figure 21) suggesting that both recombinant vaccines are able to induce a systemic immune response post vaccination. Also we detected higher levels of Granzyme B secreting cells in the spleen cells isolated from H7N2*att* immunized mice than the H9N2*att* mice when these cells were stimulated with ConA. Again suggesting the H7N2*att* recombinant vaccine is able to induce a systemic response post vaccination, which may play a role in the induction of Het-I. The Granzyme B data suggest that the H7N2 surface glycoproteins induce increased levels of CTLs post vaccination, which could be a result of the ability of this vaccine to replicate or there could be a difference in the cell types infected by the vaccines. Also the increased level of Granzyme B could result in the killing of infected cells by antibody-dependent cell mediated cytotoxicity (ADCC). In ADCC, infected cells are eliminated when Fc receptor-bearing NK cells recognize antibody-coated infected cells and secret perforins and granzymes that kill the targeted cell (Jegerlehner, Schmitz et al. 2004). This may result in the induction of Het-I.

Understanding the mechanisms in Het-I remains a top priority as influenza viruses continue to circulate and change. We are unable to predict when the next pandemic will occur and what subtype will cause it. Therefore, there needs to be a

concerted effort to analyze the ability and mechanisms used by all subtypes of influenza viruses to induce Het-I. In addition, more studies are needed to determine the ability of live attenuated vaccine to induce Het-I. Over time, vaccines have become more prevalent and it remains unknown if they can protect against different subtypes. Therefore, our studies focusing on the ability of H7N2 and H9N2 avian live attenuated vaccines to induce Het-I are needed because they expand our understanding of the molecular mechanisms regulating Het-I. Our results suggest a role for both B and T cells in Het-I induced by our live attenuated avian influenza backbone WF10*att*. More studies are needed to truly understand how our H7N2*att* vaccine protects mice against HPAI H5N1. The information gained will impact the development of a universal influenza vaccine for humans.

Chapter 5: Conclusions

5.1 Conclusions from dissertation research

5.1.1 Avian WF10*att* backbone as a master donor for live attenuated vaccines in mammals and the induction of heterosubtypic immunity

The primary goal of this project was to determine if our WF10*att* backbone would provide protection for mice and ferrets. Previously, we observed that the WF10*att* backbone was protective against low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) in chickens using a LPAI H7N2 and HPAI H5N1 challenge. The question arose whether this backbone could be used as a master donor for live attenuated vaccines for epidemic and pandemic influenza. Therefore, we needed to know if the backbone was protective in mammals. We decided to use both the mouse and ferret models to determine if this backbone could be used as a master donor stain. In addition, current influenza vaccines are unable to induce heterosubtypic immunity (Het-I); therefore, we sought to determine if our WF10*att* backbone could induce Het-I using vaccines expressing H7N2 or H9N2 surface glycoproteins.

First, we determined that the recombinant WF10*att* vaccines carrying the surface glycoproteins, H1N1, ΔH5N1, H7N2, or H9N2 are all attenuated in female Balb/c and C7BL/6 mice. We observed Balb/c mice vaccinated with H1N1*att*, H7N2att, or H9N2*att* and challenged with an H1N1 lethal virus all survived the challenge. Therefore, with an H1N1 challenge, the WF10*att* backbone protects against both homotypic and heterosubtypic challenge. Surprisingly, this was not the

case with a lethal HPAI H5N1 challenge. Both Balb/c and C57BL/6 mice vaccinated with ΔH5N1*att* were at least partially protected from homotypic challenge with HPAI H5N1. However, mice vaccinated with a single dose of H7N2*att* were protected from HPAI H5N1 while mice vaccinated with a single dose of H9N2*att* were not protected. H9N2*att* vaccinated mice needed two doses of vaccine to survive the HPAI H5N1 challenge.

Since we saw a difference in Het-I depending on the surface glycoprotein expressed on the WF10*att* vaccine backbone, we sought to determine if this difference in Het-I was species dependent. We used the ferret model because ferrets are considered the best model for human influenza. We vaccinated ferrets with the same recombinant WF10*att* vaccines expressing different surface glycoproteins, ΔH5N1, H7N2, or H9N2 and challenged them with HPAI H5N1. We found that ferrets vaccinated with ΔH5N1*att* were protected against lethal challenge with HPAI H5N1. However, in the ferret model there was a difference in Het-I when compared to the mouse model. H9N2*att* survived challenge against HPAI H5N1 (100% survival) while the H7N2*att* vaccinated ferrets (50% survival) were partially protected from challenge. The data from the mouse and ferret models confirm that the WF10*att* backbone can be used as a master donor strain, similar to the current live attenuated master donor strain A/Ann Arbor/6/60 H2N2, for epidemic and pandemic influenza vaccines. The backbone protected mice against homotypic challenge with both H1N1 and H5N1 viruses, and ferrets were protected against homotypic challenge with HPAI H5N1. Het-I was different depending on the surface glycoproteins expressed on the WF10*att* vaccine and the challenge virus used. These

results further highlighted the idea that the surface glycoproteins influence the induction of Het-I, which has been shown in the literature previously (Rutigliano, Morris et al. 2010). One new discovery was that the induction of Het-I is different depending on the species vaccinated. This information has not been reported in the literature previously. The question remains what mechanisms result in the induction of Het-I.

5.1.2 Possible mechanisms of Het-I induced by WF10*att* vaccine backbone in mouse model

After observing a difference in Het-I in both the mouse and ferret models, we decided to determine the mechanisms of Het-I induced by our WF10*att* vaccine backbone using the mouse model due to the numerous reagents available to analyze the immune response. We first examined the role of cross-reactive antibodies in Het-I. Since the H7N2*att* vaccinated mice were protected from challenge with both H1N1 and HPAI H5N1, it was possible the mice had cross-reactive neutralizing antibodies to the challenge virus. However, when we performed a microneutralization assay, we were unable to detect any cross-reactive neutralizing antibodies in the heterosubtypic-vaccinated mice. In the absence of cross-reactive neutralizing antibodies, non-neutralizing antibodies to the challenge virus may be involved in Het-I. Therefore, an ELISA was performed that was coated with the A/Vietnam/1203/2004 H5 protein. Similar to the microneutralization assay, no cross-reactive non-neutralizing antibodies to the H5 protein were detected in the H7N2*att* sera. However, the H9N2*att* sera had low levels of non-neutralizing antibodies targeted to the H5 protein when compared to ΔH5N1*att* sera. This was expected because both H5 and H9 belong to group 1 of

influenza HAs, which is based on primary sequence. When an ELISA coated with β-propiolactone (βPL) inactivated ΔH5N1 virus, we detected similar levels of non-neutralizing antibodies directed to non-HA viral proteins in both H7N2*att* and H9N2*att* sera. These results suggest a role for non-neutralizing antibodies directed to non-HA viral proteins in Het-I.

We sought to further determine the role of antibodies in Het-I, we performed a passive transfer study using sera from Balb/c mice vaccinated with recombinant vaccines and collected at 21 dpv. Three out of the ten naïve mice that received the ΔH5N1*att* sera by intraperitoneal (*i.p.*) injection survived challenge with HPAI H5N1 while one out of ten of the naïve mice that received the H7N2*att* or H9N2*att* sera survived the challenge suggesting a small role for antibodies in both homotypic and heterosubtypic challenge. Also we vaccinated and challenged $JhD^{-/-}$ mice, mice that lack mature B-lymphocytes, and found that no mice survived challenge with HPAI H5N1 further indicating a role for antibodies in Het-I. The role of B cells in Het-I was confirmed using a B cell ELISPOT that was coated with βPL-inactivated ΔH5N1 virus. Similar to the ELISA assay, we observed cross-reactive antibodies being secreted from the B cells isolated from the lungs of H7N2*att* vaccinated mice further suggesting a role for antibodies in Het-I with our WF10*att* backbone in Balb/c mice.

After determining a role for B cells in Het-I, there was a possibility that T cells are involved in Het-I induced by our WF10*att* backbone. We next examined the role of cytokines induced post vaccination. H7N2*att* was the only recombinant vaccine detected in the lungs of Balb/c mice 3 dpv. Therefore, it was possible that replication alone may be responsible for the induction of Het-I. We analyzed the

cytokine profile in the lung homogenates of vaccinated Balb/c mice at 3 and 5 dpv. We analyzed IFN-γ, IL-1β, and TNF-α and observed no difference between any of the three cytokines between each of the vaccine groups. These results suggest the cytokine profile is not involved in Het-I. We also performed IFN-γ and Granzyme B ELISPOTs on spleen and lung cells isolated from vaccinated mice and found that IFN-γ and Granzyme B spots were detected in the vaccinated mice suggesting a role for T cells or NK cells in Het-I. With regards to our WF10*att* backbone, our results suggest a role for both B and T cells in the induction of Het-I. Further studies are necessary to pin point the exact role of the different immune cells in Het-I.

5.2 Future prospects

5.2.1 Immune components involved in heterosubtypic immunity

The results in this thesis suggest a role for B cells in Het-I induced by our WF10*att* backbone, however further studies are needed to confirm their role. The serum passive transfer studies should be repeated using convalescent sera from HPAI H5N1 challenge as a positive control. Also the naïve mice should receive multiple injections of the sera through the tail vein, and varying doses of HPAI H5N1 challenge virus (0.5, 1, 5, 10, and 20 MLD_{50}) should be used in order to observe subtle differences in the role antibodies in Het-I. In addition to passive transfer studies, the immunization and challenge studies in $JhD^{-/-}$ mice should be repeated using varying doses of HPAI H5N1 challenge virus (0.5, 1, 5, 10, and 20 MLD_{50}) to tease out the role of antibodies in Het-I. These experiments may help confirm a role for B cells in Het-I.

We only performed IFN-γ and Granzyme B ELISPOTs, which suggest a role for T cells or NK cells in Het-I; however more needs to be done to confirm a role for other immune cells. Performing T cell passive transfer studies are needed to confirm a role for T cells—transfer at least 2.0 x 10^6 cells per mouse isolated from vaccinated mice into the tail vein of naïve Balb/c mice and challenge with varying doses of HPAI H5N1 (0.5, 1, 5, 10, and 20 MLD_{50}). This type of study will provide a better understanding of the role of T cells in Het-I. Intracellular cytokine staining and IFN-γ ELISPOTs should be performed post challenge with the different vaccines to see if there is a difference in the cytokine profiles and IFN-γ secreting cells between the vaccines. This information may shed light on why the H7N2*att* vaccine is able to protect against HPAI H5N1 with a single dose and H9N2*att* is unable to protect against HPAI H5N1 in mice. In addition, performing experiments in ferrets is also important to determine why there is a difference in Het-I between the mouse and ferret models.

5.2.2 Analysis of different surface glycoproteins expressed by WF10*att* backbone

Previous reports have observed a difference in protection depending on the priming subtype and the challenge virus subtype (Rutigliano, Morris et al. 2010). Our results further support this idea using avian influenza subtypes. It remains unclear whether different influenza subtypes infect different cells types. Experiments need to be performed that determine the cell types infected with our WF10att backbone expressing the different surface glycoproteins, H1N1, ΔH5N1, H7N2, or H9N2. We developed a shuffled influenza virus that is able to express GFP; therefore, flow cytometry can be used to determine the cell types that are expressing GFP. This

information could shed light on the ability of influenza viruses to transmit if there is a difference in cell infected by different influenza viruses.

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