THE DEVELOPMENT AND CHARACTERIZATION OF COMPUTATIONALLY OPTIMIZED BROADLY REACTIVE ANTIGEN (COBRA) FOR H5NX INFLUENZA VIRUSES

by

IVETTE ARIELA NUNEZ

(Under the Direction of TED M ROSS)

ABSTRACT

Avian influenza viruses pose a constant threat to the human population and continuously circulate around the globe in wild water fowl species. These hosts commonly come into contact with susceptible species including domestic chicken, turkeys and ducks. Close contact with such species leaves poultry farmers and live-market patrons at risk for contracting this deadly disease. Current strategies to contains such spill-over events can become problematic, especially those concerning culling and have negative economic impacts. Multiple countries, including the United States, depend on the poultry industry as a main source of economical income. When avian influenza viruses spill over into the human population, the results are catastrophic, ultimately resulting in systemic infection and ultimately death. Vaccines currently stockpiled for such an outbreak are poorly immunogenic and are obsolete facing the rapidly changing antigenic nature of influenza viruses. Combating this problematic virus is centered around developing an avian influenza vaccine that is able to induce cross-reactive antibodies against multiple viral clades, is highly immunogenic in all pre-immune backgrounds, and involves a dose-sparing technique. The studies discussed in this body of work investigate the molecular epitopes responsible for antibody

elicitation, design of new immunogenic vaccines, and explores the importance of pre-immune status on avian influenza virus infection. The importance of glycosylation of hemagglutinin-based vaccines was explored through site-directed mutagenesis, and was thoroughly tested in a mouse pre-clinical model of disease. We demonstrated that glycosylation of the hemagglutinin determines immunogenicity and effectiveness of eliciting cross-reactive antibodies against multiple viral clades. Furthermore, new vaccines were constructed using avian isolates and were mapped for immunogenic epitopes prior to vaccination. These studies showed that epitope mapping was closely correlated with hemagglutinin antibody responses and survival in a mouse model. Lastly, imprinting and pre-immune status was investigated in the context of avian influenza infection in a ferret model. Influenza virus group imprinting determined the severity of avian influenza H5N1 virus infection, and could be curtailed with specific antigen vaccination. This research highlights the importance of pandemic avian influenza vaccine design in the context of immunogenicity, cross protection, and pre-immune status in preparation for a potential human outbreak.

INDEX WORDS: avian influenza virus, H5N1, avian influenza vaccines, influenza vaccines

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DEDICATION

I dedicate this dissertation to all the people I grew up with who were not able to overcome the debilitating institutionalized racism and poverty that surrounded our everyday living. The people who were drawn into the circles of gangs and drugs, who died too young. I dedicate this dissertation to all the people who were undermined by their professors because of the color of their skin, because of the language they spoke, because of their dark eyes and dark features, or lack of clean clothes and nice shoes. I dedicate this composition of science to all of my brothers and sisters who were led to believe they were never good enough to get out of the ghetto, to go to college, and contribute to society that extended pass picking up the trash on the side of the road. To the women who were constantly told girls "just aren't good at math", or were sexually harassed and were subsequently blamed for "wanting it". I dedicate this to all the lives lost in the hands of our "police force", for the inhuman killing, strangling and shooting of every black, Hispanic or indigenous individual. In my darkest moments, in times when I thought I could never move on, I remembered the voices of the people who told me "you can't do it", and that's what kept me going.

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CHAPTER 1

INTRODUCTION

Avian influenza viruses naturally circulate in wild aquatic birds, such as ducks, geese, swans, gulls, shorebirds and terns [1]. The avian influenza viruses of the subtype H5N1 are often highly pathogenic avian influenza (HPAI) that was originally discovered in geese in China's Guangdong province in 1996. Several outbreaks occurred in farmed geese in Sanshui, a small town 50 miles outside the capital of Guangdong with a mortality rate of more than 40% [2]. By 1997, the A/goose/Guangdong/1/1996-like viruses spilled over into the live poultry markets in Hong Kong with high rates of mortality. Simultaneously, there were 18 confirmed human cases of HPAI infection, 6 of whom died [3]. There was a large degree of homology between the avian isolates and the viral isolates collected from these human infections indicating that these viruses were being transmitted from birds to human hosts [3].

The 1997 outbreak was contained through culling or "stamping out" all of poultry in Hong Kong. However, avian influenza viruses continued to circulate in healthy duck populations in surrounding areas. Its re-emergence in 2003 resulted in the infection of two human cases caused by novel H5N1 genetic variants that continued to circulate and evolve into 10 phylogenetic clades (0-9) [4]. At the end of 2017, there were 860 laboratory confirmed cases of H5N1 influenza virus infection from 16 different countries, resulting in 454 deaths [1]. Infection of humans with avian influenza viruses are rare, but sporadic infections can occur due to direct contact with infected birds or through contaminated environments [5]. According to the Food and Agriculture Organization of the United

Nations (FAO), China has ~64% of the world's domesticated ducks and 95% of the domesticated goose population breeding in live poultry markets alongside other poultry and swine. These conditions allow the these markets to become breeding grounds for H5Nx influenza virus circulation [6]. Outbreaks caused by avian influenza viruses have devastated of live poultry markets in Asia and have a substantial negative impact on the United States economy [6]. In this review, H5Nx viruses will be discussed for their replication, infection, evolution and threat to the poultry industry, with emphasis on the need for a broadly reactive vaccine to protect the human population.

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CHAPTER 2

LITERATURE REVIEW

Influenza Virus Replication Cycle

Influenza viruses fall into the *Orthomyxioviridae* family which consists of six genera, *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, *Isavirus* and *Quarajavirus*, these are classified by serological cross reactivity to the nucleoprotein and matrix proteins. Of the 3 types of influenza viruses, Influenza A have the most genetic variation and the broadest host range [1]. Influenza viruses are further categorized into subtypes by the hemagglutinin (HA) and neuraminidase (NA) genes they have. As of now, there are 18 HA and 11 NA types. Phylogenetic analyses of viral genes indicate that these viruses have a long-established history of infections in avian hosts. The nomenclature system for avian influenza viruses is established by World Health Organization (WHO), the World Organization for Animal Health (OIE) and the FAO [2]. Phylogenetic analysis is performed on HA sequences that have evolved from A/goose/Guangdong/1996 H5N1 virus. Viruses are grouped into virus "clades" based on their phylogenetic characterization and sequence homology of the HA gene [2]. The average percentage pairwise nucleotide distances between clades is >1.5% and is <1.5% within clades [2]. As these viruses evolve throughout time, new subclades emerge. Influenza A viruses contain negative-sense, single stranded segmented genomes that encode for 10 viral proteins: hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), nucleoprotein (NP), non-

structural protein 1 (NS1), nuclear export protein (NEP), viral polymerase basic protein 1(PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA) [3]. The 10 viral proteins are encoded by eight segmented genomic strands [4], which are coated with NP, have a double-helical hairpin structure and carry one polymerase heterotrimer consisting of PB1, PB2 and PA, (vRNPs) [5, 6].

Upon entry into a cell, the HA protein on the surface of the virion recognizes and binds to sialic acid on the surface of host cells (Figure 2.1, Step 1). After binding, the virus enters the cell through receptor mediated endocytosis [7]. The exact mechanism of endocytosis is not known, but it has been speculated that influenza can use both clathrin dependent and independent mechanisms to enter the cell [7]. Upon entry, the endosome travels into the cell and undergoes a change in pH, progressively becoming more acidic [8]. This acidification process causes an irreversible conformational change in the HA molecule on the influenza virus and exposes the hydrophobic fusion peptide [9]. The fusion peptide inserts into the endosomal membrane that causes the fusion of the viral and endosomal membrane [10]. The viral M2 protein forms a tetramer in the virion, where its transmembrane domains acts as a pore for the M2 channel [11-13]. The M2 protein functions as an ion channel that modulates the intra-virion pH, pumping free hydrogen atoms into the viral core, causing the dissociation of the vRNPs from the M1 matrix proteins (Figure 2.1, Step 2) [13, 14]. This mechanism allows the inner contents of the viral core to be released into the cytoplasm and subsequently enter the nucleus (Figure 2.1, Step 3) [13]. After dissociation from M1, vRNPs are translocated to the host nucleus where viral replication and transcription occurs (Figure 2.1, Steps 3 and 4) [6, 13]. Influenza viruses are one of the few RNA viruses that can replicate in a host nucleus, due to the need of a cap sequence in order for the RNA polymerase to

perform transcription (Figure 2.1, Step 4) [15, 16]. Influenza viral RNA segments do not contain a 5' cap in order for the RNA dependent RNA polymerase (RDRP) to perform transcription, so the PB1, PB2 and PA component perform "cap-snatching" of host DNA in order to complete this process [15-18]. Cap-containing viral mRNA is released into the cytoplasm to be translated by host ribosome machinery. Surface proteins such as HA and NA are translated into the rough endoplasmic reticulum and are then translocated into the golgi apparatus for post-translational modifications [19]. (Figure 2.1, Steps 5 and 6).

The viral nuclear export protein NS2 is critical to nuclear export of viral RNPs, viruses that lack NS2 resulted in reduced viral growth [20]. After genomic replication, transcription and protein synthesis, NS2 and M1 help escort the new viral proteins to the host cell membrane, where they assemble and bud newly synthesized virions (Figure 2.1, Step 7) [21, 22]. As the nascent virions bud from the host cell, NA cleaves the sialic acid residues on the host cell membrane [23], which allows the viruses to escape the host membrane (Figure 2.1, Step 8) [24]. Antiviral drugs that block NA activity result in influenza viruses accumulating at the membrane and cannot further disseminate can cause infection of neighboring cells [25].

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Highly Pathogenic Avian Influenza Hemagglutinin.

The hemagglutinin protein is located on the surface of the influenza virus and it facilitates viral entry into the host cell by binding to sialic acid on the host cell surface [26]. Avian adapted strains of influenza virus preferentially bind to N-acetylneuraminic acid with an α-2,3-sialic acids [27]. These sialic acids are located in the gut and the digestive tract of avian species and in the lower respiratory tract of humans [28, 29]. HA is synthesized as a polypeptide chain encoded domains

HA1 and HA2, the HA is co-translationally translocated into the lumen of the endoplasmic reticulum and eventually to the surface [30]. The HA protein contains a cleavage site between the HA1 and HA2 domain, cleavage is essential for infectivity and allows the HA molecule to undergo an irreversible conformation change in acidic endosomes. This cleavage is performed by cellular proteases to create two subunit HA1 and HA2 domains linked by disulfide bonds [31]. The cleavage nature of H5 HA proteins is achieved when virions are incubated with trypsin. This results in the conversion of HA to HA1 and HA2. The cleavage of HA can be blocked by a protease inhibitor [30]. The HA is expressed on virion as a trimeric protein that is stabilized by residues on the HA2 region. The HA ectodomain is composed of two regions, a stem region and a globular head region [32]. Neutralizing antibodies directed to the globular head of hemagglutinin are critical for reducing viral infection and disease [33]. An important factor affecting viral pathogenicity depends upon the sequence of the amino acids in the HA0 cleavage site [34, 35]. HA proteins from HPAI contains a multi basic cleavage site that is cleaved by the ubiquitous furin cellular protease [35, 36]. In contrast, low pathogenic avian influenza viruses, that contain only one basic amino acid, the cleavage of HA is tissue specific that results in a lower clinical manifestation in poultry.

The polybasic cleavage site on HA is a strong determinant for high pathogenicity of H5 viruses, however insertion of polybasic sequences into a low pathogenic avian HA does not always result in a lethal phenotype as tested in chickens [37]. Other influenza proteins such as PB2, PB1, and NP may increase pathogenicity of an influenza virus. Pathogenic phenotype of H5 viruses is not HA dependent. Pathogenicity and efficiency of replication can also be dependent on PB2, NP, NA and M genes [38]. The deleted stalk region of NA found in HPAIV also confers pathogenicity,

where rescue of the NA stalk region leads to a decreased pathogenesis in chickens [38]. Deletion of the stalk region increases lethality and transmission compared to the wild type viruses that display a lower lethality. In addition to H5 viruses, this same NA stalk deletion abrogates H2N2 virus replication in ducks, but shifts the virus tropism from the intestinal tract to the respiratory tract in chickens [39]. Although, the presence of the polybasic HA cleavage site was sufficient enough to induce viral neurotropism [37].

The dominant circulating avian influenza strains that arose since 2015 are comprised of viruses in the clade 2.3.4.4, which includes reassortant viruses in the H5N6, H5N8 and H5N2 subtypes. However, the strains within this clade that has crossed over into the human population most often have been associated with the H5N6 subtype. The ability of H5N6 viruses to spill-over into the human population may be associated with mutations in the HA molecule that affect the specific RBC binding preference of HA. These H5N6 viruses preferentially bind to different sialic acids depending on the host each virus was isolated. Strains present in chickens have preferential binding to α-2,6-sialic acid residues, whereas H5N6 strains isolated from ducks preferentially bind to α-2,6 sialic acids [40]. Thus, H5N6 viruses that infect chickens may have a higher affinity binding α-2,6 sialic acids and therefore these viruses may be more prone to infect humans, due to the fact that a humans upper respiratory tract predominantly expresses α-2,6 sialic acids [40]

Neuraminidase in H5Nx Viruses

Characterization of low pathogenic or high pathogenic viral infections in poultry usually refers to the pathogenicity of the virus during infection and whether the virus contains a poly-basic cleavage site in its HA molecule (as reviewed above). However other proteins, such as the neuraminidase,

can add to the virus pathogenic nature. To date, the newly circulating strains of avian influenza in China are H5N6, H5N8 and H5N2 [41]. These viral reassortments can result in a dominant NA molecule that increases the pathogenicity and release of viral particles. Overall, this can increase viral transmission between hosts. The role of non-HA viral gene products and how these proteins contribute to viral tissue tropism and virulence is still not well understood. Multiple passages of H5N3 viruses in poultry results in a mutation in the catalytic site of the neuraminidase, which increases the virulence of these viruses in poultry [42]. Mutations in internal genes, such as PB2, have also been linked to increased viral pathogenicity in H5Nx viruses. Multiple passages of an H5N5 viruses in mice resulted in a substitution in amino acid position 627 from glutamic acid to lysine (E627K in the HA protein [43]. This adaptive mutation increased the pathogenicity of these viruses in mice by 1000x and enhanced viral replication *in vivo* and *in vitro* [43]. There were significant structural and functional differences in the NA proteins (N6, N8 and N2) from several viruses associated with the clade 2.3.4.4 [42]. The HA/NA interplay may be age dependent, whereas non-functional H5 viruses results in the death of day old chickens, but infection with the same virus in week old chickens showed no signs of clinical illness at all [44]. This seems to be an H5 specific phenomenon, whereas H7 viruses were less dependent on a functional NA to cause illness. the dominant avian influenza viruses that infected humans have been associated with are H5N6 viruses from clade 2.3.4.4. Out of the 17 human infections with H5N6 virus, 16 of the viruses contain a NA stalk deletion. Recombinant H5N6 viruses containing a 10 amino acid NA stalk deletion (amino acid 58 to the 68) had an increase of viral replication in mammalian cell lines compared to the intact NA of H5N6 viruses. These viruses containing the NA stalk deletion also showed an increased viral replication in avian CEF cells, whereas H5N2 virus had lower titers in these cells [45]. This recombinant virus with the NA deletion (ΔH5N6) did not infect neural tissue

in mice, whereas the full length H5N6 recombinant virus was neurotropic [45]. Wild-type H5N6 viruses had higher rates of viral transmission and were more lethal to poultry compared to the ΔH5N6 virus. Wild-type H5N6 viruses were 100% lethal to chickens. All birds died within 10 days post-infection. Whereas, only 85% of the ΔH5N6 challenged chickens died by day 14 post-infection. This data suggests that the NA stalk region in H5N6 viruses plays an important role in pathogenicity in mammalian hosts and displayed a decreased pathogenicity in chicken cells.

Avian Influenza Virus Infection in birds

Domesticated birds, such as chickens and turkeys, may become infected with avian influenza viruses through direct contact with infected waterfowl or infected poultry. Avian influenza viruses infects over 105 bird species across the globe, but the natural reservoirs for this virus resides in aquatic fowl such as gulls, terns and shorebirds [46]. Waterfowl can transmit avian influenza virus to other avian species such as terrestrial poultry. Infection of poultry with LPAI virus can result in little to no disease. Clinical signs of LPAI viral infection are ruffled feathers and a drop in egg production [47]. Infection of birds with LPAI virus can result in the virus to mutate and adapt to the unsusceptible bird, possibly creating a HPAI virus in these birds [46]. Adaptation of the virus to increase replication efficacy can result in a LPAI virus to transform into a HPAI virus with addition of basic amino acids inserted into the cleavage site on HA [47]. Natural LPAI virus infections in wild birds do not present with clinical signs of infection or tissue lesions [48, 49]. The H5N1 associated response in chickens includes a massive influx of cytokines, antiviral cytokines and interferons which should inhibit viral replication [50]. However, some cytokines that are activated like IFN TNF-α, IL-8and IL-6 may be responsible for influenza induced pathology [51].

Wild birds, such as ducks, are more resistant to H5N1 HPAI virus infection compared to gallinaceous poultry [52]. The rapid disease progression seen in infected chickens is not observed in ducks [53]. Ducks are able to maintain H5 infections without developing severe disease and continue to spread the H5 virus into susceptible chicken populations [54]. Infection with H5N1 in susceptible birds results in systemic infection, leading to multiple organ failure, damage to cardiovascular and nervous system and ultimately death [46, 47]. HPAI H5 viruses replicate in the respiratory and gastrointestinal tracts of birds [52-54]. Clinical signs of infection include loss of appetite, lack of energy, loss of coordination, discoloration and swelling of body parts, diarrhea, nasal discharge, coughing, sneezing, and misshapen eggs [47]. Diagnosis of avian influenza in birds are done by taking throat swabs of birds, in wild birds, a fecal sample is taken instead and is tested through PCR analysis. Positive PCR results then leads to virus isolation and growth of the virus in an embryonated chicken egg [55].

The Evolution and Spread of H5 Viruses

The A/goose/Guangdong/1/96 virus was initially detected in wild birds in southeast Asia, but shortly thereafter was detected in several areas in Asia, Europe, Africa and recently North America [56]. Sequence analysis of the H5N1 viral gene segments isolated from poultry in Hong Kong revealed that there were two distinguished groups of viruses circulating in domestic poultry [57]. During infection, the HA segment did not undergo mutation to adapt to the human hosts. However, The PA, PB2, NP and M2 gene products had multiple amino acid changes after replicating in human hosts [57]. Those mutated amino acids were similar to those in viruses that commonly infect the human population [57]. There are nine different types of H5 viruses in wild bird

populations (H5N1, H5N2, H5N3, H5N4, H5N5, H5N6, H5N7, H5N8, and H5N9). These H5 subtypes can present considerable risk to the human population.

This rapid expansion of the A/goose/Guangdong/1/1996-like viruses was driven by reassortment with other avian influenza viruses [58]. Following the goose/Guangdong virus emergence, H5N1 viruses continued to circulate in China with a seasonal pattern peaking from October to March when the temperature is below 20°C [58]. In 2003, the H5N1 outbreaks in humans revealed that the goose/Guangdong-like viruses had diverged into eight genotypes. Several of these genotypes survived, while others went extinct [58]. Specific adaptations to the viral genome led to an increased fitness of select strains that continued to circulate in Southern China [38]. A series of genetic reassortment events led to the initial human outbreak in Hong Kong in 1997, which can be directly traced back to a viral genotype in chickens and ducks named "Z". Strains of the genotype Z replaced the genotypes A-E, X and Y in 1997 and then became dominant in aquatic fowl and terrestrial poultry. The overall prevalence of H5 viruses has increased since 2010. The most prevalent avian influenza viruses (AIVs) are viruses in the H5N6 and H5N8 subtype circulating in domestic waterfowl in China [59].

Since year 2008, HPAI subtypes H5N5, H5N2 and H5N8 viruses have caused outbreaks in poultry across Asia, Europe and North American [60-63]. By 2014, outbreaks of novel reassortants viruses such as H5N6 and H5N8 were reported in Asia [41, 63]. Viral reassortants of H5N1 and H5N2 variants have also been isolated from chickens and waterfowl and have caused outbreaks in chickens in Hebei Province during December, 2013 [59]. The H5N2 viruses expressing the HA gene from clade 2.3.2, 2.3.4 or 7.2 have been isolated with internal genes from both clade 7.2

H5N1 viruses and H9N2 viruses. H5N2 viruses contain gene segments from multiple viral clades including HA,PA, M1, PB2, NS1 from 2.3.4.4 and 3 American wild birds lineage genes NA, NP and PB1 [64, 65]. These H5N2 viruses were the source of an outbreak in Hubei, Shandong, and Henan Provinces in China. Low pathogenic H5N2 influenza viruses naturally infect wild birds [66]. However, these LPAI H5N2 viruses can transform into a HPAI isolate by adding either adding basic amino acids to its cleavage site or by loss of a N-glycosylation at amino acid site 11 [67]. These viruses rapidly adapted to replicate in terrestrial poultry by adding an additional glycosylation site on the hemagglutinin molecule, as well as deleting 19 amino acids in the neuraminidase (NA) stalk region [66].

By September 2016, there were 4 new human cases of H5 infections, with one fatal case [68]. The A/goose/Guangdong/1/1996-like viruses are still being detected in poultry and wild birds in many countries. The majority of these isolates are classified as clade 2, including the subclades 2.2.1.2 in Egypt and 2.3.2.1a in Bangladesh, Bhutan and India [68]. H5 viruses designated in the clade 2.3.2.1c have been detected in wild bird populations in China, Southeast Asia and Africa [68]. H5 influenza viruses of the clade 2.3.4.4 have been detected in wild birds across around the world. The clade 2.3.4.4 viruses from Africa and Europe are primarily of the H5N8 subtype, whereas those viruses isolated in Asia are in the H5N6 subtype, and those strains isolated in in the United States are classified as H5N2 isolates [68]. Clade 2.3.4.4 H5 influenza viruses caused 1537 outbreaks in birds in 14 countries over the past decade [69]. These outbreaks have been associated with H5N6, H5N8, H5N2 and H5N3 isolated. These viruses emerged through multiple reassortment events with the H5N1 subtype and now routinely circulate in domestic poultry and waterfowl [69]. The first 2.3.4.4 virus that emerged was classified as in the H5N6 subtype and it

circulated throughout China and traveled to Southeast Asia causing the death of 457 birds in Laos [70]. The H5N6 subtype is the only 2.3.4.4 clade virus that has infected people [71]. The H5N6 subtype arose from a reassortment event with hemagglutinin genes from H5N1 and neuraminidase genes from the H6N6 virus [70]. The H5N8 subtype caused an outbreak in 2014 in South Korea leading to a distinction of two different H5N8 virus subgroups, group A and group B [72]. Group A comprises a set of H5N8 isolates and is referred to as the intercontinental group A (icA) group. The icA H5 viruses further evolved into 3 different subgroups, icA1, icA2, icA3. The icA1 subtypes group contains viruses that were isolated from Europe, Russia, and Japan. The icA2 subgroup in composed of H5N8 and HPAIV reassortants H5N2 and H5N1 from North America in 2014. The icA3 subgroup is composed of H5N8 viruses isolated in Japan and in Korea [72]. In 2014, H5N8 Eurasian subtypes emerged in Canada, Germany, The Netherlands, United Kingdom and East Asia and was concurrently detected in the U.S. state of Washington in captive falcons, wild birds and poultry. The spread of these intercontinental-like viruses coincided with the bird migration out of Russia and most likely spread by migratory birds [72]. Clade 2.3.4.4 viruses are less pathogenic in wild waterfowl, which increases its chances of being spread through migratory birds. In late 2014, H5N8 viruses were identified in Canada and in Washington State in the United States where it caused infection in captive falcons, wild birds and poultry and spread across mid-Western and North Central States causing devastation to the poultry industry. Over 48 million chickens and turkeys were culled and led to a loss of $1.6 billion USD [73].

Transmission of HPAIV into swine

The ability of an avian influenza virus to transmit amongst humans may rely on the ability of the virus to replicate efficiently in swine cells. Swine are often the host for in viral reassortment and

generating novel viruses in the ecosystem (Figure 2) [74]. Swine cells in the respiratory tract have both α-2,3 and α-2,6 sialic acid receptors making them a "mixing pot" for generation of novel pathogenic viruses [75]. Swine are able to transmit influenza A viruses to the United States in 1998 which contained segments from avian, swine and human viruses [76, 77]. Three major influenza species spillover events from human to swine have been documented in the United States [76]. (Figure 2.2) The ability of H5Nx viruses to replicate and reassort in pigs is still under investigation. As of now, there have been 32 submissions of H5Nx swine infection uploaded to the GISAID (https://www.gisaid.org) website between 1968 and 2017, with 23 of the HA sequences being unique. Few pigs are naturally infected with H5 viruses (0.25%) as determined by antibody titers to these viruses [78]. However, these viruses were able to replicate in pigs and cause some weight loss, but do not transmit to naïve, contact pigs [78]. The potential for reassortment of avian influenza viruses and mammalian influenza viruses in pigs is still unknown, but swine are permissive hosts for viral replication of avian influenza viruses [79-81]. Newly characterized clade 2.3.4.4 H5Nx viruses do not readily infect trachea cell extracts collected from pigs [81]. There are no clinical symptoms of disease in pigs following infection with LPAI or HPAI viruses [79-83]. Microscopic pathological analysis does reveal some cellular infiltrates, along with minor macroscopic lesions, but replication in these hosts does not permit transmission to naïve pigs [79]. In addition, co-housing poultry that were infected with highly pathogenic avian influenza were not able to transmit the virus to naïve pigs [84]. Pigs infected with H5Nx influenza viruses replicated in the lower respiratory tract, since nasal swabs were negative in RT-PCR assays, but bronchoaveolar lavage fluid (BALF) contained viral titers post infection [79] demonstrating that avian influenza viruses replicate in the lower respiratory tract in pigs. This site of replication in the lungs is consistent with the location of the 2,3 alpha receptors found in the lower respiratory

tract of pigs and not present in the upper respiratory tract [75]. Serum samples on 16 swine farms in southern China determined that no pigs or humans were HAI positive for H5N1 IAV [85]. Therefore, pigs are not easily susceptible to H5Nx infection and that those that do get infected, the virus does not easily transmit to naïve companions. However, there is always the concern that due to the lack of H5N1 virus induced clinical manifestations that an H5Nx and seasonal H1N1/H3N2 influenza viruses could co-infection the same animal resulting in a reassortment event that leads to a human transmissible H5 influenza virus.

Avian Influenza: Human Transmissible Agent

HPAIV infection in humans occurs sporadically in Asia, Africa, Europe and the Middle East [86]. As of now, there have not been any reported cases of human H5N1 infection in the United States, but one case was reported in Canada in 2014 from a person who recently traveled from China [87]. HPAIV H5 virus infection in humans can initially present as an uncomplicated seasonal influenza infection with clinical signs of fever, body aches, upper respiratory tract symptoms [86, 88, 89] [90]. However, the infection eventually progresses into a lower respiratory tract infection. The infection can progress to become severe pneumonia, multi-organ failure, encephalitis and septic shock [86, 88, 89]. The incubation period for H5N1 virus infection is estimated to be 7 days, but is more commonly 2-5 days after exposure. In the rare cases where human to human transmission occurred the incubation period varies from 3-4 day to 2-10 days. Variability in this incubation period can possibly reflect upon the level of viral shedding, exposure and immunological factors [91]. A reported pediatric case with H5N1 virus infection presented with fever, diarrhea and seizures, which progressed to a coma and eventually developed into encephalitis. The patient had contracted the virus from his sister two weeks earlier who also suffered from encephalitis and died.

Neither patient presented with any respiratory symptoms [92]. The severity of the illness varies with the virus clade, age of the patient, and other genetic factors that are unknown. Rapid disease progression following avian influenza infection occurs in the majority of human cases. Extrapulmonary complications that arise from H5 viral infections include cardiac failure, kidney failure, encephalitis, muti-organ failure, and intravascular coagulation [90, 93-95]. Human to human transmission of avian H5N1 virus has been recorded in several households, but is often limited [88, 89]. Transmission usually results from close intimate contact and or care of a family member infected with H5N1 viruses. Surveillance by RT-PCR of contact patients led to the detection of mild cases of infection in older adults and increased number of seropositive families in northern Vietnam [96].

Elevated levels of pro-inflammatory cytokines are found in clinical specimens from patients infected with H5N1 influenza viruses [97, 98]. Autopsy studies revealed alveolar damage and high levels of circulating chemokines and cytokines in the peripheral blood of the patient [98]. H5N1 viral infections in humans induces a higher transcription of pro-inflammatory cytokines than seasonal H3N2 or H1N1 influenza viruses [99]. The cytokines TNF-α and interferon-β are significantly elevated following H5N1 viral infection [99]. This is partially due to the non-structural (NS1) gene segment of the H5N1 viruses [100], and may contribute to the overall severity of H5N1 influenza virus infections in humans [99].

Since its first emergence in the human population in 1997, the HPAI virus have caused a total of 860 confirmed cases and resulted in 454 deaths worldwide [101]. The reported number of human infections has been decreasing since 2003. Although there have been few cases of documented

human to human transmission, these viruses are not easily transmitted in humans. This may reduce the threat of H5Nx viruses causing wide-spread infections in people and becoming a pandemic issue in the human population. However, the virus only needs to accumulate minor mutations in order for viruses with H5 HA proteins to transmit easily between mammalian hosts [102]. One factor that may increase the pandemic potential of avian influenza viruses are the ability of these viruses to be transmitted through aerosolized particles [103]. This is largely dependent on the ability of the virus to replicate in the upper respiratory tract of mammalian cells. Distribution of sialic acid receptors between human and avian hosts differ, with α-2,6 sialic acids being predominant in the upper respiratory tract of humans, and α-2,3 sialic acids being more predominant in the lower respiratory track of humans. Whereas avian species have α-2,3 sialic acid receptors predominantly expressed in avian species, in both respiratory tract and digestive tract. Therefore, sustained human to human transmission of avian influenza may also include the switch from α-2,3 sialic acid binding to preferentially α-2,6 sialic acid binding to ensure dissemination through aerosolized droplets from the upper respiratory tract [103]. Sialic acid preference is not solely responsible for the pathogenicity of avian influenza in mammalian cells [104]. Avian influenza virus mutants that preferentially bind to α-2,6 sialic acid receptors have lower viral replication rates compared to wild type viruses [104]. Efficient transition for avian influenza virus to replicate in human hosts is dependent on more than receptor bind preference. Replication and release of these infectious particles is also critical when discussing the switch of infection from birds to mammals. A specific mutation in the polymerase protein PB2 in site 627 from a glutamic acid to a lysine (E627K) increases the pathogenicity of influenza virus infection in mammalian models [105-107]. Influenza viruses that readily infect humans hosts predominantly display a lysine in site 627 in the PB2 protein. In avian species, the PB2 protein 627 site is glutamic

acid. The function of PB2 is restricted in mammalian cells when these avian influenza viruses contain glutamic acid (E) in site 627. These viruses do not assemble into ribonucleoprotein complexes and have decreased genome transcription and virus production [105]. The lysine (K) amino acid substitution in the PB2 protein allows the virus to replicate in lower temperatures of the upper respiratory tract of mammals (33°C), as opposed to the higher temperature (41°C) in the gastrointestinal tracts of avian species [108]. H5N1 viruses that contain the PB2 E627K substitution have increased viral replication in the nasal passages of mice compared to viruses with the E627 wild-type virus [108]. Other changes in PB2 such as the D701N mutation have been associated with an increased animal tropism, increased polymerase activity, and enhanced pathogenicity in mammals. The SR polymorphism in PB2 allows the polymerase to escape species specific restriction factors that targets polymerases from avian viruses [108]. These mutations increase the overall viral replication of avian influenza viruses in mammalian hosts cells and ensure a high replicative titer and infection. Viral reassortments containing replicative proteins from multiple species can help avian influenza viruses expand their tropism and adapt to new hosts [109].

PB1-F2 is a small protein that is encoded from the same vRNA segment as PB2 from a +1 reading frame. PB1-F2 protein is an important virulence factor in influenza viruses can have three roles in viral infection: 1) regulate antiviral innate immunity, 2) enhance Viral polymerase activity, and 3) induces cellular apoptosis [110]. Viruses isolated from the 1997 H5N1 pandemic contained an amino acid at position N66S that correlated with high pathogenicity in mice [111]. When an S66N mutation is introduced into the PB1-F2 protein of an H5N1 viruses, there is an attenuation of disease in mice. Viruses that contain a N66S mutation have increased disease severity and cytokine

production [111]. The truncated version of PB1-F2 does not increase virulence in mice or their predisposition to co-infection by bacterial strains [112]. However, the PB1-F2 protein that lacks an ATG start site was 1000 fold more pathogenic than the full length PB1-F2 containing an ATG site [113]. The ability of H5Nx viruses to successfully replicate in human hosts are dependent on the adaptation of multiple viral RNA segments and are not restricted to HA and NA activity.

H5N1 avian influenza vaccines

Preparing for pandemic vaccines is extremely problematic when dealing the H5Nx viruses. Due to the fast rate of mutagenesis and reassortment events, vaccine development against H5 viruses can be complicated and constant surveillance and analysis is necessary to produce antigen reference strains. The current standard for producing vaccines against pandemic viruses is performed by government associations such as the World Health Organization (WHO) and the Center for Disease Control and Prevention (CDC) [114, 115]. The WHO and CDC annually identifies influenza strains circulating in wild water fowl populations in order to create candidate vaccine viruses (CVV). This is accomplished this by performing a multistep process which includes the following: 1) Production of a CVV 2) Quality assessment, 3) Determination of attenuation 4) request for USDA select Agent Exclusion and 5) Distribution of the CVV vaccine manufacture and other stakeholders [114]. These CVVs are kept available for possible outbreaks and can be used to seed a large manufacturing process for mass distribution. Theses vaccines are monovalent, inactivated viral stocks that are release to match the current circulating pandemic strain. The creation of inactivated monovalent vaccine will be described below.

Inactivated vaccines

The HA surface glycoprotein is a main target for vaccine antigens to produce protective antibody responses against H5Nx infection. The first H5N1 vaccines were made using similar protocols to that of seasonal influenza vaccines, where a virus strain is chosen to propagated in embryonated chicken eggs 2) the virus is purified 3) the virus is inactivated with formaldehyde of beta-propiolactone, 4) inactivating reagent are removed 5) the virus is split with detergent or mechanically splitting [116, 117]. The time from strain selection to distributions is at least 6-months in an ideal setting [118]. However, theses manufacturing processes are susceptible to changes and setbacks. Growth of CVV H5Nx viruses also require the virus to be genetically modified to decrease pathogenicity for growth and safety reasons [119]. Reverse genetics are often employed to ensure the attenuation of H5N1 by replacing the six internal genes with a donor virus A/Puerto Rico/8/1934. Decreasing the pathogenicity of the virus not only enables the safe handling by laboratory staff, but also ensures the viral growth titers are higher by attenuating the pathogenesis in chicken eggs [120]. These genetic modifications take time and can also decrease the immunogenicity of the viral vaccine strain [121]. A study performed by Subbarao et al showed that a recombinant live attenuated H5 vaccine with a modified HA had decreased immunogenicity in chickens, mice and ferrets, and restoring the poly-basic cleavage site increased viral replication and efficacy [121].

Following the production of the CVV, a quality assessment is performed to ensure the mutations have been properly performed and that the virus does not cause lethality in the embryonated chicken eggs [119]. Along with testing its multi-basic cleavage sites have properly been replaces with a low-path cleavage site, impurity testing and exclusivity testing ensures there are no

contaminants either bacteria or viral. The virus is also tested antigenically to ensure its antigenic hemagglutination characteristics are similar to those of the wild type virus, and it will in fact produce a similar antibody response against the wild-type strain [119]. The CVV is then tested for is pathogenicity in poultry and in ferret models to ensure the virus is indeed attenuated [119]. Although these steps are multi-faceted and cumbersome, they are required to produce a safe and non-pathogenic vaccine.

An issue that is presented when producing inactivated influenza virus vaccines is that they have proven to be poorly immunogenic against pandemic strains of H5N1 c [118, 122-125]. To overcome this low immunogenicity without adjuvant use, multiple vaccinations and large antigen dosages are commonly required. A study performed by Sanofi Pasteur in the USA indicated that two high doses of 90 μg of HA antigen were required to induce immune responses in adult recipients [126]. Healthy adults ranging from the ages 18-64 received unadjuvanted vaccination doses of 90, 45, 15, or 7.5 μg of H5 HA antigen [126]. They found that people who received doses of 45 μg or 90 μg had the highest serum antibody titers, and those who received two doses of 90 μg had neutralization titers of 1:40 in 58% of the study participants [126]. These high levels of HA antigen required to only bring half of the population up to 1:40 neutralization titer does not account for dose sparing technique during a pandemic outbreak.

Whole inactivated viruses that are used for vaccines have been shown to elicit a higher serological response in people [127]. Wu et al. studied the comparison of an alum adjuvanted inactivated split virus vaccine and an egg grown whole virus vaccine in a human trial. They tested adjuvanted inactivated and whole viral vaccines in children aged 3-11 and 12-17 years old. They found that

whole-virion vaccination and a lower HA μg dosage (5 μg compared to 30 μg) induced a high seroconversion rate children [127]. However, they also discovered that local and systemic reactions were seen in children of the years 3-11 who received a 30ug dose of split-virion vaccine, and children in the age groups of 13-17 experience adverse effects with 10μg-15μg dose of the whole viral vaccines [127]. Most of the adverse effects cause by the vaccine were mild fevers, pain at the injection sight, fatigue and myalgia. Inactivated monovalent vaccine adjuvanted with aluminum hydroxide was tested in a randomized control study where they found that 10 μg of adjuvanted HA antigen had the highest antibody responses compared to 1 dose of 25 μg and 2 does of 5 μg vaccinated groups [128]. It is believed that lower doses of whole-virion vaccines can avoid systemic reactions while still eliciting protected antibody responses and is more adaptable to the antigen sparing strategy.

Recombinant Protein HA vaccines

In order to produce recombinant proteins for influenza vaccination, there are three systems that are commonly used, the Baculovirus system, a plant derived system and the human cell line system. The Baculovirus expression system uses an expression vector that infects insect cells, specifically lepidopteran species of insects [129]. Baculoviruses commonly infect insect species but are unable to replicate in mammalian cells, therefore making it a safe model for vaccine development. During the infection of insect cells, the baculovirus makes large amounts of a viral protein polyhedrin, which has been found to not be a necessary product for viral infection [129]. This large level of expression is utilized by replacing the polyhedrin gene with the foreign gene HA from the influenza virus gene of interest [130, 131]. Viral proteins can be expressed by recombinant baculovirus expression in insect cells to produce large amounts of HA protein for influenza

vaccination. Recombinant baculovirus expressed recombinant HA (rHA) vaccines have been used in mouse animal models of disease for H5N1 vaccination and challenge. Mice vaccinated orally with baculovirus derived rHA have proven to be safe and efficacious in multiple pre-clinal animal models of disease and in human clinical trials [132]. A study performed by Treanor et al revealed the immunogenicity of recombinant Baculovirus-expressed H5 HA in healthy adults and had varying antigen doses including 25, 45 and 90 μg of rHA from a clade 0 H5 virus [132]. Intramuscular vaccination was well tolerated but only 52% of the individuals developed neutralizing antibody titers after two doses of the highest antigen does of 90 μg of rHA [132]. Currently, there is a recombinant trivalent HA vaccine produces using the Baculovirus system that is available for human consumption called FluBlok™. The recombinant protein vaccine was developed to use an alternative to the egg based system for people who contain allergies to egg derived proteins [133]. It contains 3x the amount of HA compared to the contemporary inactivated influenza vaccine [133].

Recombinant protein can also by produced by plant strains of viruses. This system, like the Baculovirus system enables large and rapid scale production of HA proteins. Transgenic plants can produce HA antigens efficiently and for long time periods and can be efficacious when considering a production of a pandemic vaccine outbreak [134]. Like in other protein expression systems, the HA molecule is modified to remove the transmembrane domain and signal peptide in order for the protein to become soluble [134]. Histidine tags are added for purification. The expression vector used for plant derived protein expression is the tobacco mosaic virus vector [134]. This vector is then transduced into a agrobacterium tumefaciens strain of bacteria and were then transduced into aerial parts of *Nicotiana benthamiana* [135] plants. Leaf tissue is then

harvested and homogenized to extract the H5HA and is purified using chromatography techniques [136]. Studies have shown that plant derived rHA vaccine are safe and efficacious in animal models. A study performed by Major et al at the NIH showed that intranasal vaccination with plant derived H5 HA protects both mice and ferrets form lethal challenge with HPAI virus [137]. Ferrets who were vaccinated with 45 µg of rHA formulated with the adjuvant c-di-GMP had 100% survival rates compared to non-adjuvanted and mock vaccinated ferrets (71% and 50% respectively)[137].

Mammalian protein expression systems have also been proven to be safe and efficacious in pre-clinical animal models of influenza infection. Mammalian cell lines such as Madin Darby canine kidney cells (MDCK) [138] and human embryonic retinal cells (PER.C6) [139], monkey kidney cells (Vero) [140], and human embryo kidney cells (HKE293) [141] have been utilized for the production of recombinant influenza proteins. Soluble recombinant HA protein derived from the pandemic H1N1 virus was shown to elicit cross-protective antibodies from an H5N1 heterologous strain of virus [142]. In this study performed by Yamada et al, recombinant protein was generated using the mammalian cell line Expi293F and the expression vector pCAGGS and was purified using agarose chromatography and female BALB/c mice were vaccinated on a prime-boost-boost regimen with the adjuvant addavax [142]. Mice were challenged with an H1N1pdm09 virus, HPAI v H5N1 and H3N2, they found that vaccinations with the H1N1pdm09 HA elicited protective antibodies against both pdm09H1N1 and H5N1 viral challenge [142]. Vaccination however did not elicit protection against the H3N2 challenge and all the mice succumbed to infection 9 days following challenge [142]. There are currently 3 mammalian cell produced vaccines that are approved by the FDA for human consumption: Optaflu/Flucelvax, Preflucel and Celvapan [143].

Optaflu or Flucelvax is a trivalent subunit vaccine produced in a suspension line of MDCK cells [144, 145]. The trivalent subunit vaccine contains one H1N1 strain, one H3N2 and one Influenza B strain [146]. Preflucel and Celvapan are expressed by Verocells that are grown in a microcarrier, however, Celvapan is used primarily for pandemic strains of viruses, most notably pandemic H5N1 or H1N1 . All three of these vaccines are inactivated, purified and are licensed to use in EU. Although immortalized animal cell lines are less robust protein producers, they include animal like post-translational modifications to proteins. This method also includes setbacks such as the high price of transfection medium, constant CO2 and culture medium.

Although both virus and plant derived expression systems are able to perform post-translational modifications of protein, synthesis of N-linked glycans differ from mammalian systems and can be potentially immunogenic to humans [147]. Insect cells add shorter N-linked glycans and plant cells typically have glycans that contain fucose and xylose residues [147]. Therefore, considering the production of safe and immunogenic proteins for human consumption should be limited to mammalian cell culture systems.

Virus-Like Particles

Virus-like particles (VLP) are vaccines that express viral glycoproteins on the surface of membrane bound empty virus-like structures. They are multiprotein structures that mimic the composite of viruses but lack virus genomes therefore making it a safer vaccine candidate [148]. GVLPs can activate antigen-presenting cells such as dendritic cells to present antigens to both T and B lymphocytes [149]. There are vaccines in VLP format that are licensed throughout the work including vaccines for human papilloma virus (HPV) and Hepatitis B. there are many VLP

platforms that are undergoing testing for human use such as parvovirus, Ebola, enterovirus, HIV and influenza viruses [148]. VLP vaccine platforms can be extremely useful due to the fact that they do not have to be inactivated and therefore maintains the structural integrity of viral proteins. The VLP platform is also known to express a large amount of antigen on their surface, inducing a strong B-cell response in the absence of adjuvant. The structures of VLPs contain highly repetitive glycoproteins which has been shown to increase IgG antibodies [150, 151]. The high amount of antigen presentation allows crosslinking of B-cell receptors, initiating B-cell proliferation and IgM production in the absence of T-cells [151]. Unless there is a very high amount of antigen present, the switch of B-cell production from IgM to IgG will not occur unless they are aided by Th-cell-dependent signals[150, 151].

Influenza VLPs resemble intact influenza viruses and contain functional HA and NA proteins with are structurally similar to those of wild-type viruses. The self-assembly of VLPs allows for the conformational epitopes of the HA protein to be available to the immune system [152]. VLPs made for pandemic H5N1 use have been used for preclinical and clinical models of disease. Novavax (MD, USA) developed a pandemic H5N1 VLP for preclinical and clinical Phase I and phase IIa trials. Pre-clinical vaccine trial in ferrets showed that baculovirus derived VLPs containing HA, NA, and M1 proteins developed neutralizing antibody titers against homologous and heterologous clade of H5N1 viruses [153], and protected against lethal viral challenge [153]. Novavax has also developed VLP H5N1 vaccine for human clinical trials which was reported to be well tolerated and induced neutralizing antibodies against H5N1 and induced cross-reactive antibodies to heterologous strains of H5N1 viruses [154]. The study included 230 healthy adults who were vaccinated on a prime-boost regimen and showed a dose-dependent immune response to

vaccination. The highest responders belonged to the 90 µg vaccinated individuals, which showed a four-fold increase in neutralizing antibody titers in comparison to lower doses. [154]

Adjuvants

Due to the low immunogenicity of inactivated influenza vaccine trials, adjuvants have been recruited in the use of pandemic H5N1 vaccines. Formulating a pandemic vaccine with an adjuvant usually targets to 1) improve immune responses in elderly and young populations, 2) boost immunogenicity of an antigen, 3) accelerate response to a vaccine, 4) enable dose sparing 5) enhance the quality of the immune response and 6) enable mucosal delivery of vaccines [155]. The current stockpiled vaccines available in the United States for pandemic distribution are non-adjuvanted, monovalent inactivated vaccine stockpiles. This vaccine regimen requires 2 vaccinations to obtain a serological response detected by HAI titer. However, in the case of a pandemic, large scale vaccination will be urgent and the requirement for multiple vaccinations will introduce compliance issues. In the case that a new vaccine will have to be made using one of the many CVVs available by the WHO, this will have to be mass produced and largescale antigen production will be limiting [156]. The use of adjuvants promotes antigen sparing and increases immunogenicity, removing the need for secondary vaccination and large antigen delivery.

The first appearance of adjuvant was the use of aluminum salts (alum) in the use of tetanus and diphtheria toxins in 1920s . The current hypothesis of alum derived immunogenicity is the depot effect of antigen that is stored in the dermis for an extended time after injection [155]. This theory was tested through vaccination and skin graph removal and transplant in in Guinea pigs, where localized high antigen concentration stimulates immune cells and induces local granulomas [157].

However, it is known that this antigen depot mechanism does not solely explain the immunogenicity elicited by the alum adjuvant [158]. Alums apparent mode of mechanism is in conjunction with the activation of inflammasome. Alum leads to local inflammation at the site on injection by signaling the recruitment of neutrophils, , dendritic cells [159], macrophages [160], and has a Th1 Helper phenotype in humans [161].

Currently, the adjuvants that are licensed vaccine use are aluminum slat (alum), and the squalene oil-n-water adjuvant MF59 and AS03. Current pandemic monovalent preparations of inactivated H5N1 virus have been used in conjunction with AS03 [129]. There have been multiple attempts of using Alum based H5N1 vaccines, however this adjuvant did not show an enhanced response compared to non-adjuvanted vaccines [124, 162]. The use of oil-in-water adjuvants have shown increased injection site symptoms compared to non-adjuvanted vaccines but also show higher antibody response with a lower antigenic dose [163]. The exact pathway of adjuvant enhancement is unknown; however, studies show that squalene-based adjuvants are naturally metabolized and activates cell recruitment and antigen uptake by antigen presenting dendritic cells and leads to recruitment monocytes [164]. Adjuvants such as MF59 have been seen to be more effective than CpG and aluminum salts, and is a stronger inducer of cytokines, cytokine receptors, adhesions molecules involved in leukocyte migrations and upregulation of antigen presenting genes [165] . The production of immunogenic H5N1 vaccines require the use of adjuvants to ensure dose sparing and cross-reactive antibody responses. Multiple studies have shown that the use of oil-in-water adjuvants with monovalent H5N1 vaccines increased HAI detectable antibody response and microneutralizing antibody production in randomized clinical trials [123, 124, 162].

Incorporating adjuvants into a vaccine can also promote immune modulation. Adjuvants use can increase specific T-cell based recruitment and increase T-cell memory responses compared to vaccines that do not contain adjuvants. Previous studies have shown that one dose of MF59-H5N1 vaccination can activate and expand CD4+ T lymphocytes that are Th1-effort memory cell based [166]. Individuals who received two doses of vaccination had an increased B cell memory repertoire and had high levels of neutralizing antibody titers [166]. The investigators also found that CD4+ T cell production was also correlated with neutralizing antibody response, and two-doses of vaccination resulted in high microneutralizing antibody titer [166]. Influenza oil-n-water adjuvanted vaccines have also been show to elicit strong antibody responses in both elderly populations and in young children, who are the populations at risk [167-170].

The use of adjuvants in pandemic H5 vaccines can also increase the speed of the immune response. A study performed in pre-vaccinated individuals showed that people who were primed with a MF59 adjuvanted vaccine developed a more robust antibody response upon secondary vaccination [171]. Those who were primed with an unadjuvanted vaccine had a lower antibody response and did not develop a cross-protective antibody response as those who were primed with the MF59 adjuvanted vaccine [171]. Most importantly, the investigators found that the development of neutralizing antibody titers were present 7-days following the boost vaccination compared to the non-adjuvanted vaccine [171].

Along with increase neutralizing antibody titers, adjuvants have also been shown to increase antibody breadth across different viral strains of H5 [171-173]. MF59-adjuvanted HA vaccine from a clade 1 H5 virus was used to vaccine primed individuals who received the vaccine 6-years

prion they found that the MF59 adjuvant helped induce polyclonal immune responses and increased heterologous antibodies [174]. The polyclonal sera generated against the HA1 fragment of the HA protein were significantly higher than sera obtained from unadjuvanted primed individuals [174]. They concluded that oil-in-water adjuvanted pandemic vaccines were able to elicit long term memory B cell responses against multiple potential variant pandemic viruses [174].

Conclusions

The current strategies put in place to protect the population from a possible H5Nx pandemic are not sufficient. The stockpiled vaccines the government has mass produced are monovalent, unadjuvanted formulas which have been shown to be poorly immunogenic not only in healthy populations, but less so in elderly and immunocompromised populations. Although studies have shown the use of AS03 in pandemic vaccine trials to be both immunogenic and safe, the United States government has not licensed the use of adjuvanted vaccines for at risk populations. In order to combat the evolution and divergence of avian influenza, a universal avian influenza vaccine should be developed (UAIV). A UAIV would be broadly protective against multiple viruses that span throughout several clades and against multiple mismatched NA (H5N1, H5N2, H5N6, H5N8). This vaccine should provide protective antibody or cell mediated response against viral challenge with one vaccine dose. Multiple or boost vaccination has been attempted with heterologous prime-boost strategy in avian influenza vaccination studies [175-177] . Although these types of vaccine protocols have proven successful, in the event of an epidemic, single dose adjuvanted vaccination would be less time consuming and more cost effective. In the event that a single dose is substantial, a greater portion of the population would have access to the vaccine compared to a two dose strategy where perhaps only half the population would receive the dose

[178]. Protective efficacy would also span across multiple age groups, taking into account pre-immunity to seasonal influenza strains such as H1N1 and H3N2. Although these qualifications for a UAIV may seem too farfetched, there has been progress in recent years. Our group has reported on a computationally optimized broadly reactive antigen (COBRA) adjuvanted vaccine that provides protection against heterologous challenges in mice, ferrets and non-human primate models [179-183], and is being analyzed for potential vaccine candidacy.

The molecular basis of HPAI H5Nx viruses to induce pathogenicity, evolution, as well as the potential for these viruses to adapt to the humans was reviewed. The segmented genome of the influenza virus facilitates reassortment in hosts that are infected with more than one strain of influenza virus. These reassortment events in birds and swine have generated new and novel strains of avian influenza viruses, including the divergent 2.3.4.4 clade viruses that contain avian NA proteins. Influenza viruses with genomic RNA segments coding for internal and non-structural proteins such as PB1, PB2, PA and NS1 have increased viral replication and overall enhanced pathogenicity. Reassortment of avian influenza virus RNA segments with seasonal human influenza virus strains in swine by coinfection may not factor into the evolution of novel avian viruses. Swine that are infected with avian influenza viruses have no clinical signs of infection and do not transmit nascent virions to immunologically naïve hosts. However, these animals may have a subclinical infection that could in fact promote reassortment events upon co-infection.

The next influenza pandemic is inevitable and most likely will emerge from a novel subtype that the human population has no pre-existing immunity. The ability of an HPAI to accumulate the appropriate mutations or reassorting with an efficient human or swine virus resulting in a human

transmissible virus is possible and could initiate a new pandemic. Therefore, continued scientific studies and surveillance of avian influenza viruses are essential to develop vaccines and therapeutics, not only for the poultry industry, but also to prepare for the next avian influenza virus emergence into the human population.

Figures

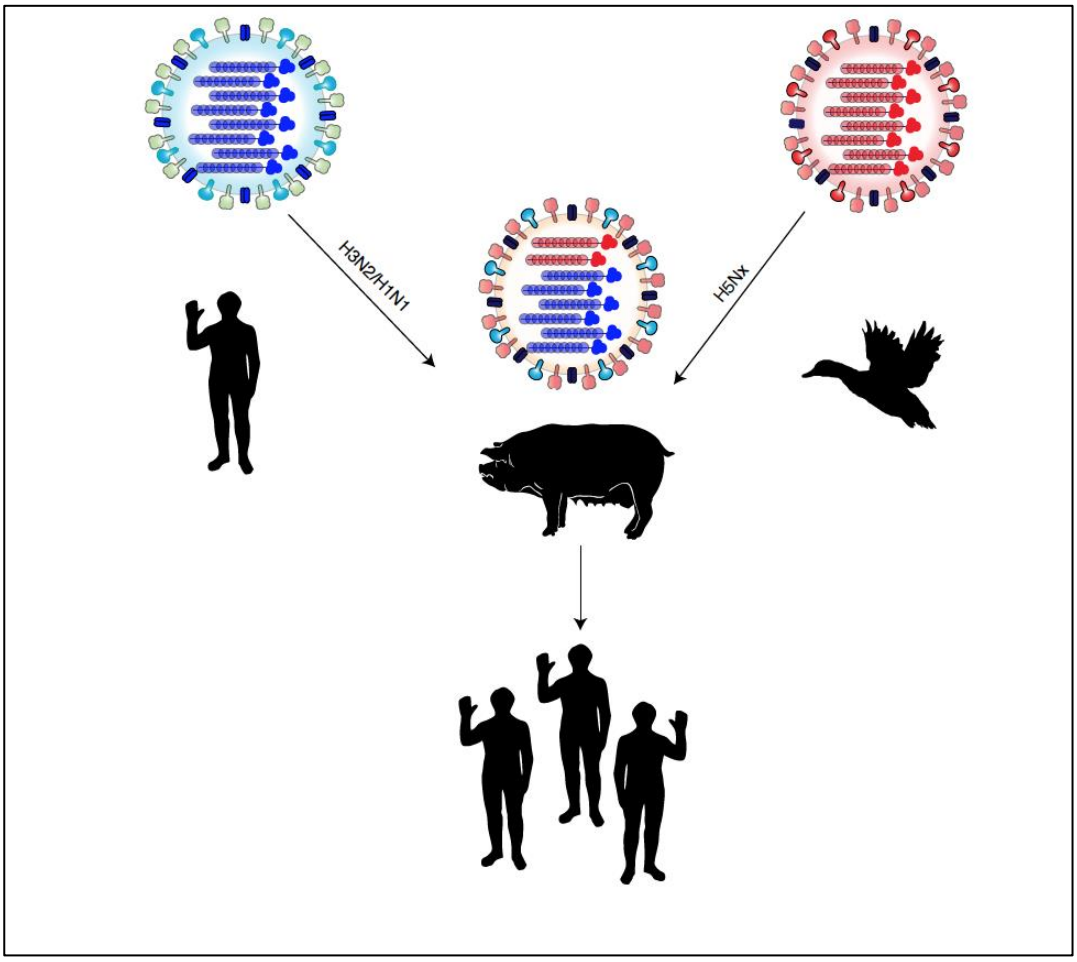

Figure 2.1: Cartoon depiction of the replication cycle of influenza viruses. 1.) Viral entry into host cell 2.) virus endocytosis into host endosome and acidification, leading to conformational change of HA molecule exposing fusion peptide and fusion of viral and host membrane. M2 protein pumps H+ atoms into the viral core, casuing the dissocation of M1 and release of vRNP. 3.) Release of vRNP into cytoplasm and translocation into the nucleus. 4.) vRNP replication and transcription, and cap snatching mechanisms occur in the nucleus. Viral proteins such as M1 and NS2 chaperone vRNP out of nucleus and into cytoplasm to be packaged into viral particles. 5.) Structural proteins are translated by host ribosomes and are transported to ER for proper folding. 6.) properly folded viral proteins are release from the ER and are directed towards the plasma membrane or to golgi for modifications prior to release. 7.) Movement of modified proteins from golgi network to plasma membrane for viral budding. 8.) release of infectious viral progeny.

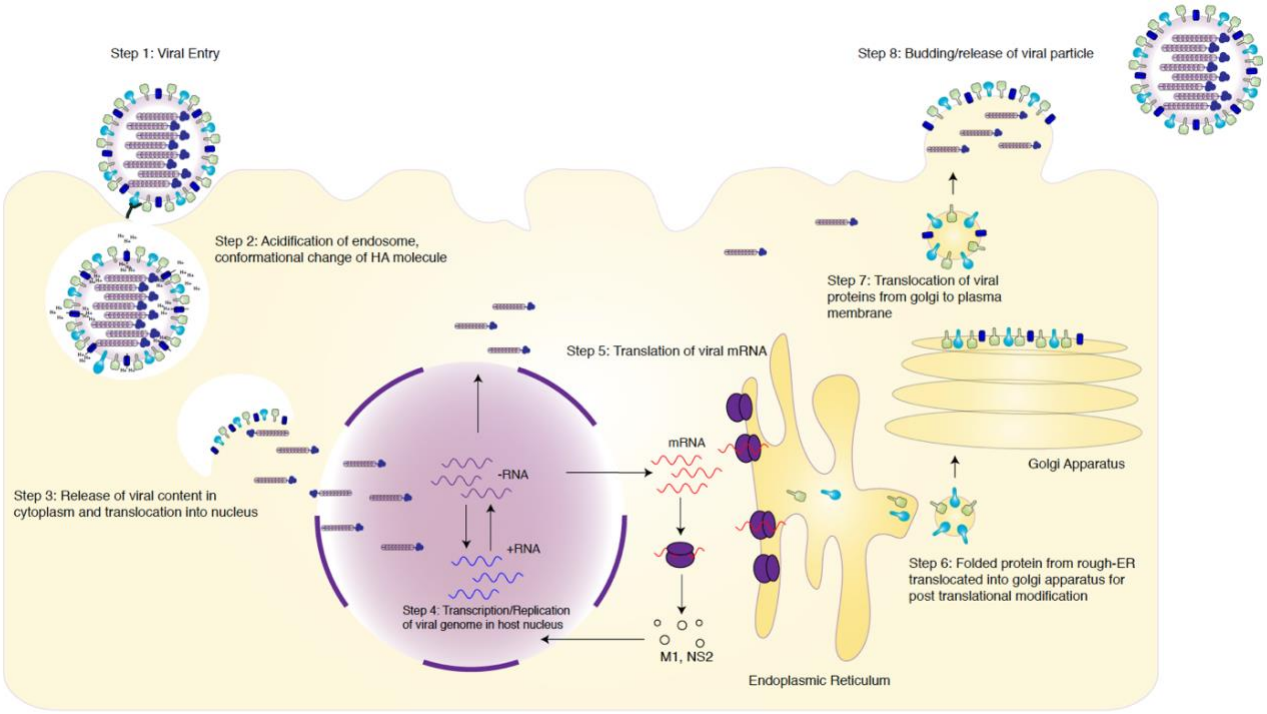

Figure 2.2: Depiction of swine reassortants. Seasonal influenza from human hosts (Left) can be transmitted into susceptible swine. Concurrent infection by avian strains (H5Nx) with seasonal influenza strains can lead to reassortants, novel influenza viruses that contain genetic segments from both humans and avian viruses. These novel viruses can then be transmitted into susceptible human populations, possibily leading to a pandemic outbreak.

Human outline: By Linda Salzman Sagan (original artwork); Tompw (GIF version); User:Holek (SVG) - Cut from File:Pioneer10-plaque.jpg, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=2647647
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CHAPTER 3

HUMAN COBRA 2 VACCINE CONTAINS TWO MAJOR EPITOPES THAT ARE RESPONSIBLE FOR ELICITING NEUTRALIZING ANTIBODY RESPSONSES AGAINST HETEROLOGOUS CLADES OF VIRUSES

Nuñez, IA, Ross, TM. Human COBRA 2 vaccine contains two major epitopes that are responsible for eliciting neutralizing antibody responses against heterologous clades of viruses. Vaccines. 2020; 38(4):830-839. Reprinted here with permission of publisher.

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ABSTRACT

Highly pathogenic H5N1 influenza viruses continue to spread around the globe and reassort with low pathogenic avian influenza viruses often resulting in morbidity and mortality to not only waterfowl, but also poultry. Our group previously developed two hemagglutinin (HA) based vaccines using a methodology termed computationally optimized broadly reactive antigen (COBRA). Each of these HA antigens, Human COBRA 2 (Hu-CO) and Human-Avian COBRA 2 (Hu-Av CO) elicit antibodies with hemagglutination-inhibition (HAI) activity against viruses from various clades, but not always the same viruses. Here, we have sought to identify residues in these two HA molecules that are critical for differential HAI activity against various H5Nx influenza viruses. The two HA antigens are similar in the globular head region, except for 4 residues at amino acids 140, 141, 155, and 156. By mutating these amino acids in each HA antigen, chimeric HA proteins were used to elicit immune responses in mice. When the Asn-Thr pair at position 155-156 in the Hu-CO HA was converted to the Ser-Ala residues found in the Hu-Av CO HA, the elicited antibodies lost HAI activity against clade 2.3.2.1 H5Nx viruses, such as A/Hubei/01/2010. When this Asn-Thr motif was added at these positions in the Hu-Av CO HA molecule, HAI activity in the elicited sera against A/Hubei/01/2010 was significantly increased which also correlated with survival data. We speculate that a putative N-linked glycosylation at this location in the Hu-CO HA antigen is a key driver in the elicitation of antibodies with HAI activity to different locations on wild-type H5 HA molecules resulting in differential neutralization of viral infection and protection *in vivo* against H5 influenza virus induced disease.

INTRODUCTION

Currently, there are no effective vaccines that can elicit protective immune responses against highly pathogenic H5N1 influenza A strains from multiple clades and subclades of the Goose/Guangdong (Gs/GD) lineage. These viruses circulate in wild bird populations, particularly in waterfowl, and have spread throughout the eastern hemisphere. In 2015, an outbreak of H5N2 avian influenza was detected in chickens and turkeys in North America resulting in over 50.5 million birds being affected [1] but to our knowledge there were no reports of people who were infected and died. However, as of May, 2018, 860 people have been infected with H5N1 HPAI virus resulting in 454 deaths [2].

H5N1 influenza viruses have been categorized into 10 different groups or clades based on the sequence diversity of the hemagglutinin (HA) glycoprotein. These viruses have further been grouped into subclades and sub-subclades as the virus has evolved over time. Therefore, developing a broadly protective H5 influenza vaccine that can provide coverage of antigenically distinct co-circulating viruses, as well as future drift variants is highly desirable. In order to develop novel vaccines to address the diversity in H5N1 influenza viruses, we invented a methodology termed computationally optimized broadly reactive antigen (COBRA) [3]. Full length sequences from H5N1 clade 2 human infections from 2004 to 2006 were acquired and used for consensus sequence generations. For each round of consensus generation, multiple alignment analysis was applied and the consensus sequence was generated. The final amino acid sequence, termed computationally optimized broadly reactive antigen (COBRA), was optimized for

expression in 293T mammalian cell line for protein expression [4]. A second COBRA HA antigen was designed using full length HA sequences from clade 2 viruses isolated from both humans and birds during 2004-2008 and was termed Human-Avian COBRA-2 (Hu-Av CO) [5]. Both HA proteins, when expressed on the surface of a virus-like particle (VLP) elicited antibodies with receptor-blocking, hemagglutination-inhibition (HAI) activity against a panel of viruses representing various clades isolated from 2005-2008 [4]. These vaccines elicited antibodies that protected mice, ferrets, monkeys, and chickens against the H5N1 isolate, A/whooper swan/Mongolia/244/2005 (ws/Mo/05) HPAI virus (clade 2.2) [4, 6-8]. Animals vaccinated with these vaccines show more efficient viral clearance and elicited broader antibody responses against different clades and sub-clades than animals vaccinated with VLP expressing wild-type HA proteins [3, 9, 10]. However, the Hu-CO HA VLP vaccine was able to elicit protective antibodies against the 2011 H5N1 isolate, A/duck/Vietnam/NCVD-672/2011 (clade 2.3.2.1B), but the Hu-Av CO HA vaccine could not [11]. In order to explore why one COBRA HA-based vaccine was able to elicit more broadly protective antibodies and the other COBRA HA vaccine was not, site directed mutagenesis was performed to determine which specific epitopes in HA were responsible for this phenotype. The two COBRA HA antigens only differed by 4 amino acids in the HA globular head and we hypothesized that one or more of these amino acids were responsible for an HA structure that allowed for the elicitation of these broadly protective antibodies. Exchange of these 4 amino acids between the two COBRA HA antigens was performed and the modified HA proteins were used to vaccinate mice for evaluation of the elicited antibodies. Challenge studies were also performed to access the vaccine induced survival in mice.

MATERIALS AND METHODS

Design of mutant vaccines and characterization of HA proteins

Sequence alignment of Hu-CO and Hu-Av CO was performed to determine differences between the two vaccine strains. Alignments showed that there are 6 amino acid differences in the HA1 head including sites: 140, 141, 155, 156, 282 and 355. Four of these sites 140, 141, 155, 156 are located near the receptor binding site (RBS) and were determined to be of more importance than sites 282 and 355, which are located farther from the RBS. Paired amino acids on Hu-CO were swapped out to reflect the amino acids located on Hu-Av CO, and the same was done for Hu-Av CO. Two amino acid pair substitutions were chosen to determine the specific globular head antigenic epitope containing multiple amino acid residues was most essential for eliciting protective antibodies against H5N1 strains. Western blot analysis determined that amino acid substitutions into COBRA backbone did not alter the protein expression in 293T cells. The HA mutants used in this study are: Human COBRA 2 (Hu-CO), Human COBRA 2 SP140-141KS (Hu SP140-141KS), Human COBRA 2 NT155-156SA (Hu NT155-156SA), Human Avian COBRA 2 (Hu-Av CO), Human Avian COBRA 2 KS140-141SP (Hu-Av KS140-141SP), Human Avian COBRA 2 SA155-156NT (Hu-Av SA155-156NT). HA activity was determined by hemagglutinin inhibition assay (HAI), HA activity in horse red blood cells was robust.

HA antigens were expressed in a TR600 plasmid backbone containing a kanamycin resistance gene, eukaryotic cytomegalovirus (CMV) promotor, a bacterial origin of replication (Ori), and bovine growth hormone (BGH) polyadenylation signal (REF). Influenza HA nucleotide sequences were inserted into TR600 using HindIII and BamHI restriction enzyme sites (Genewiz, South Plainfield, NJ, USA). Plasmids were amplified in Escherichia coli (strain DH-alpha) and purified

using QIAGEN plasmid maxi kit, according to the manufactures protocol. Plasmids were verified using restriction enzyme digests and was resolved on a 1% agarose gel, viewed with SYBR safe under UV light.

Viruses

Viruses were obtained through the Influenza Reagents Resource (IRR) and passaged once in embryonated chicken eggs as per the instructions provided the WHO [12]. Virus lots were titered with horse erythrocytes and made into aliquots for single-use applications. The H5NX vaccine panel includes the following reassortants viral strains containing internal genes from the mouse adapted strain A/Puerto Rico/8/1934: A/Vietnam/1203/2004 (Vn/04), A/Indonesia/5/2005 (In/05), A/Whooper swan/Mongolia/244/2005 (ws/Mo/05), A/Anhui/1/2005 (An/05), A/Egypt/321/2007 (Eg/07), A/chicken/Vietnam/NCVD-16/2008 (ck/Vn/08), A/Hubei/1/2010 (Hu/10), A/Egypt/N03072/2010 (Eg/10), A/Guizhou/1/2013 (Gu/13), A/Cambodia/X0810301/2013 (Cm/13), A/Sichuan/26221/2014 (Si/14), A/gyrfalcon/Washington/41088-6/2014 (gyr/WA/14). VLPs were also used to test HAI assay, HAs from: A/chicken/Egypt/CAL3-RLQP/2017 (ck/Eg/17), A/duck/Egypt/S78-RLQP/2017 (dk/Eg/17).

Vaccine preparation

Human embryonic kidney (HEK) 293T cells were transfected with 10 µg of COBRA or wild-type HA, neuraminidase (NA, A/Thailand/1(KAN-1)/2004, a clade 1, H5N1 virus), and HIV GAG with Lipofectamine 3000 according to the manufacturer's protocols (Thermo Fisher Scientific, Waltham, MA). After incubating cells with DNA/Lipofectamine mixture for 4 days at 37 °C, supernatants were collected and were filtered through a 0.22 µM sterile vacuum filter.

Concentrated VLPs were collected via ultracentrifugation at 100,000xg through 20% glycerol (weight per volume). Pellets were suspended in sterile phosphate buffered saline PBS, pH 7.2 and were stored at -80 °C until use. Protein concentration was determined by the MicroBCA™ Protein Assay Kit (Thermo Fisher Scientific; Pittsburgh, PA, USA). Hemagglutination activity of each preparation of VLPs was determined by adding equal volume horse red blood cells (RBCs) to a V-bottom 96-well plate and incubating with serially diluted volumes of VLPs for a 30 min incubation at RT. The highest dilution of VLP with full agglutination of RBCs was considered the endpoint HA titer.

Determination of HA content

Protein concentration was determined by the MicroBCA™ Protein Assay Kit (Thermo Fisher Scientific; Pittsburgh, PA, USA). HA concentration was determined by western blot and densitometry. Purified VLPs were prepared in standard total protein amounts and were electrophoresed on 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with anti-HA clade 1 influenza A viruses (Immune Technology Corporation; New York, NY, USA) monoclonal antibody. HA-antibody complexes were detected using a goat anti-mouse IgG conjugated to horse radish peroxidase (HRP) (Southern Biotech; Birmingham, AL, USA). HRP was detected by chemiluminescent substrate Clarity™ Western ECL substrate (Bio-Rad Laboratories; Hercules, CA, USA). Density of WT HA bands were used to calculate a standard curve and the density of the purified VLPs was interpolated using the results from the WT HA. Experiments were performed in duplicates. Density of bands was determined using myImageAnalysis™ Software (Thermo Fisher Scientific; Waltham, MA, USA).

COBRA VLP vaccination of mice

BALB/c mice (*Mus musculis*, female, 6-8 weeks) were purchased from Envigo (Indianapolis, IN, USA) and house in microisolator unites and were allowed free access to food and water and were cared for under USDA guidelines for laboratory animals. Mice (n=11/group) were vaccinated with purified VLPs (1 µg based upon HA content from the densitometry assay) via intramuscular injection at week 0 and then were boosted with the same dose at week 4. Vaccines were formulated with an oil-in-water emulsion adjuvant, according to the manufactures protocol. Mice were bled prior to vaccination to determine seronegative status and were bled again at weeks 4, 6, and 8. Blood was collected via submandibular bleeding using a lancet and transferred to a microfuge tube. Tubes were incubated at room temperature for at least 30 minutes prior to centrifugation, sera were collected and frozen at -20 °C ± 5 °C.

Influenza virus Challenge

In the challenge studies, female BALB/c mice were purchased from Evigo (Indianapolis, IN, USA) and house in microisolator unites and were allowed free access to food and water and were cared for under USDA guidelines for laboratory animals. Mice (n=8/group) were vaccinated with purified VLPs (1 µg based upon HA content from the densitometry assay) via intramuscular injection at week 0 and then were boosted with the same dose at week 4. Vaccines were formulated with an oil-in-water emulsion adjuvant, according to the manufactures protocol. Mice were bled prior to vaccination to determine seronegative status and were bled again at weeks 4, 6, and 8. Blood was collected via submandibular bleeding using a lancet and transferred to a microfuge tube. Tubes were incubated at room temperature for at least 30 minutes prior to centrifugation, sera were collected and frozen at -20°C ± 5°C. Four weeks following the final vaccination, mice

were briefly anesthetized and were intranasally challenged with either A/Hubei/01/2010 ($2.0x10^6$ pfu/mouse) or A/Sichuan/26221/2014 ($2.0x10^7$ pfu/mouse) dosage determined by preliminary LD50 study (data not shown). All procedures were in accordance with the NRC Guide for Care and Use of Laboratory Animals, the Animal Welfare act, and the CDC/NIH Biosafety and Microbiological and Biomedical Laboratories.

Plaque Forming Assay (PFA)

Viral titers were determined in BALB/c mice using a plaque forming assay as previously described [4, 6, 7, 13, 14] using $1x10^6$ Madin-Darby Canine Kidney (MDCK) cells. Mice were euthanized (n=3/group) 3 days post-infection, lungs were taken and snapped frozen and kept at -80 °C until processing. Lungs were diluted (10^0 to 10^6) and overlaid onto confluent MDCK cell layers for 1 hour in 200 µL of DMEM supplemented with penicillin-streptomycin. Cells were washed after 1-hour incubation and DMEM was replaced with 4 mL of L15 and 2.4% Avicel (FMC BioPolymer; Philadelphia, PA) (1:1). Cells were incubated for 72 hours at 37 °C with 5% CO_2. Avicel and L15 media was removed and washed 2x with sterile PBS, cells were fixed with 10% buffered formalin and stained for 15 mins with 1% crystal violet. Cells were washed with tap water and allowed to dry. Plaques were counted and the plaque forming units calculated (PFU/mL)

Hemagglutination-Inhibition (HAI) assay

Hemagglutinin inhibition assay (HAI) assay was used to assess receptor-blocking antibodies to the HA protein to inhibit agglutination of horse erythrocytes. The protocol is taken from the CDC laboratory influenza surveillance manual. To inactivate non-specific inhibitors, mouse sera was treated with receptor destroying enzyme (RDE, Denka Seiken, Co., Japan) prior to being tested.

Three parts of RDE was added to one-part sera and incubated overnight at 37 °C. The RDE was inactivated in 56 °C for 30 minutes, when cooled, 6 parts of sterile PBS was added to the sera and was kept at 4 degrees C until use. RDE treated sera was two-fold serially diluted in v-bottom microtiter plates. 25 µL of virus at 8 HAU/50 µL was added to each well (4 HAU per 25 µL). Plates were covered and incubated with virus for 20 minutes at room temperature before adding 1% Horse red blood cells (HRBC) (Lampire Biologicals, Pipersville, PA, USA) in PBS. Red blood cells were washed and stored at 4° C and used within a week of preparation. The plates were mixed by agitation and covered, the RBCs were allowed to settle for 1 hour at room temperature. HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Negative and positive serum controls were included for each plate. All mice were negative (HAI<1:10) for pre-existing antibodies to currently circulating human influenza viruses prior to vaccination.

Focus Reduction Assay (FRA)

The focus reduction assay (FRA) used in this study was initially developed by the World Health Organization Collaborating Centre in London, United Kingdom [15, 16], and modified by the U.S. Centers for Disease Control and Prevention (CDC) (Thomas Rowe, personal communication) as previously discussed [17]. MDCK-SIAT1 cells were plated at $2.5 \times 10_5$ to $3 \times 10_5$ cells/ml (100 µl/well in a 96-well plate) the day before the assay was run and were incubated in 37°C for 24 hours before the assay in (DMEM) containing 5% heat-inactivated fetal bovine serum and antibiotics. Cells were 95 to 100% confluent at the time of the assay. Cell monolayers were rinsed with 0.01 M PBS pH 7.2 followed by the addition of 2-fold serially diluted RDE-treated serum at 50 µl per well, starting with a 1:20 dilution in virus growth medium containing 1 µg/ml

tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin, termed VGM-T (DMEM containing 0.1% BSA, penicillin-streptomycin, and 1 μg/ml TPCK-treated trypsin [Sigma, St. Louis, MO, USA]). 50 μl of virus for all FRAs shown here was standardized to 1.2×10^4 focus-forming units (FFU)/ml (corresponds to 600 FFU/50 μl), and VGM-T was added to each plate or cell control wells. The virus stocks were standardized by previous titration in the FRA. Following 2 h of incubation at 37°C with 5% CO_2, the cells in each well were overlaid with 100 μl of equal volumes of 1.2% Avicel RC/CL [15] (type RC581 NF; FMC Health and Nutrition, Philadelphia, PA, USA) in 2× modified Eagle medium containing 1 μg/ml TPCK-treated trypsin, 0.1% BSA, and antibiotics. Plates were incubated for 18 to 22 h at 37°C, 5% CO_2. Overlays were removed from each well and the monolayer was washed once with PBS to remove any residual Avicel. The plates were fixed with ice-cold 4% formalin in PBS for 30 min at 4°C, followed by a PBS wash and permeabilization using 0.5% Triton X-100 in PBS-glycine at room temperature for 20 min. Plates were washed three times with wash buffer (PBST) and incubated for 1 h with a monoclonal antibody against influenza A nucleoprotein [18] (IRR) in ELISA buffer (PBS, 10% horse serum, 0.1% Tween 80). Cells were then washed three times with PBS-Tween, and were incubated with goat anti-mouse peroxidase-labeled IgG (SeraCare, Inc., Milford, MA) in ELISA buffer for 1 hour at RT. Plates were washed three times with PBST, and infectious foci (spots) were visualized using TrueBlue substrate (SeraCare, Inc., Milford, MA USA) containing 0.03% H_2O_2 and incubated at room temperature for 10 to 15 min. The reaction was stopped by washing five times with distilled water. Plates were dried and foci enumerated using a CTL BioSpot analyzer with ImmunoCapture 6.4.87 software (CTL, Shaker Heights, OH). The FRA titer was reported as the reciprocal of the highest dilution of serum corresponding to 50% focus reduction compared to the virus control minus the cell control. In order for a plate to pass quality control, both the average of the octuplet

virus control wells (VC) as well as the average of the octuplet cell control wells (CC) must pass. The virus controls initially were between 150 to 650 foci (spots) and the cell controls must be less than 21 foci. The virus control wells were subsequently expanded to between 200 and 1600 spots.

Pymol Designs

Three dimensional (3D) models of COBRA HA sequences were developed by uploading the HA amino acid sequence into the SWISS-MODEL system [19, 20], 2wr1.1.A was used as a template. A 3D rendering of the H5 HA molecules were designed with MacPyMOL version 1.7.4.5 [21] downloaded for educational use. Addition of glycosylation on PBD molecule was created through Glycosciences.de online software (www.glycoscience.de) complex sugar was added to show hinderance of epitope binding sites.

Phylogenetic Tree Design

The unrooted phylogenetic tree was inferred from HA amino acids sequences derived from 42 representative H5Nx isolates and also the COBRA HA using the Jukes Cantor method. Sequences were aligned with the MUSCLE 3.8.425 software [22, 23]. Phylogeny was determined using the Jukes Cantor method with Geneious® software 11.1.2 [24] using MUSCLE alignment, Jukes Cantor, Neighbor Joining tree with No outgroup tree was constructed. Viruses and vaccines of interest are highlighted in colors. Clades and subclades are annotated.

Statistical Analysis Statistical analysis was performed on HAI assays using non-paired, non-parametric test Mann Whitney, where significant differences were annotated with the following key: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

RESULTS

Generation of mutant COBRA HA antigens.

The previously characterized Hu-CO and Hu-Av CO HA gene sequences [4, 6-8] have 6 different amino acids in the HA1 portion of the molecule. Four of these amino acids (140, 141, 155, 156 [mature H5 numbering]) are located near the receptor binding site and part of putative antigenic sites A and B [25]. Pairs of amino acids were exchanged between the two COBRA HA proteins in order to determine which region(s)/epitope(s) of HA are contributing to each antigen phenotype (Figure 3.1A). Amino acids at position positions 281 and 341 were not located near the receptor binding site and were not exchanged. These amino acid changes did not affect HA expression or incorporation of HA on the surface of a virus-like particles (VLP) (Figure 3.1B).

Vaccine elicited antibodies with HAI and neutralizing activity.

In order to determine which amino acids in Hu-CO HA contributed to the elicitation of broadly-protective antibodies against heterologous strains, BALB/c mice (n=11/group) were vaccinated (IM injection at week 0 and 4) with VLP vaccines expressing these HA molecules (Figure 3.3). Two weeks following the second vaccination, sera were analyzed for HAI activity against a panel of 15 H5Nx influenza viruses (Table 1). These viruses were chosen to represent H5Nx viruses isolated between 2005-2014 within clade 2 (Figure 3.2). The Hu-CO and Hu-Av CO HA antigens cluster closely with In/05 HA protein (Clade 2.1.3). HA sequences from newer circulating H5Nx viruses, cluster with viruses from the Clade 2.3.4.4 (Si/14 and gyr/WA/14) (Fig. 2).

Most, but not all, mice vaccinated with the ws/Mo/05 VLP vaccine had antibodies with HAI activity (≥1:40) against ws/Mo/05 (Figure 3.4B) and Vn/04 (Figure 3.4D), however few of these

mice had antibodies with HAI activity against the Eg/10 or Hu/10 viruses (Figure 3.5 C and D). The Hu-CO VLP vaccine elicited antibodies with similar HAI activity against these 4 viruses as the antisera from ws/Mo/05 VLP vaccinated mice. Mice vaccinated with the Hu-Av CO VLP also had antibodies with similar patterns of HAI activity as mice vaccinated with Hu-CO VLPs, albeit fewer mice seroconverted and the overall geometric mean titer (GMT) was lower than mice vaccinated with Hu-CO VLPs. Mice vaccinated with Hu/10 and Si/14 VLPs displayed low HAI activity against the homologous comparator Hu/10 and Si/14 virus (Figure 3.4D and Figure 3.5C) and had no antibody titers against other viruses run in the panel. Mice vaccinated with Si/14 VLP showed antibody titers against both Si/14 and gyr/WA/14 viruses (Figure 3.5).

When the Ser and Pro at positions 140-141 in Hu-CO HA were changed to the associated amino acids (Lys and Ser) in Hu-Av CO HA, all mice vaccinated with VLPs expressing this mutant Hu-CO HA antigen had a significant rise in antibodies with HAI activity against all 4 viruses (average GMT between 1:128-1:512) compared to Hu-CO (Figure 3.4). However, mice vaccinated with the Hu-CO with the NT155-156SA mutation had higher HAI activity against the clade 2.2 viruses, ws/Mo/05 and Eg/10 (Figure 3.4A and C), but not the clade 2.3.2.1 Hu/10 or clade 1 Vn/04 viruses (Figure 3.4B and D). Interestingly, exchanging the amino acids from Ser-Ala at positions 155-156 in the Hu-Av CO HA to the amino acids Asn-Thr found in Hu-CO HA elicited antibodies with high HAI activity against all 4 of these viruses, but only mice vaccinated with the KS140-141SP Hu-Av CO HA VLP vaccine elicited higher titers against only ws/Mo/05 (Figure 3.4A), but not the other 3 viruses. The antisera elicited by each of these vaccines had similar virus neutralization patterns as HAI activity (Figure 3.4E-H). For example, sera from mice vaccinated with ws/Mo/05 VLPs had a log2 serum dilution neutralization titer of 6.3 (50% inhibition) against ws/Mo/05 virus

and baseline log2 serum dilution titer of 2.3 against Hu/10 (Figure 3.4A and B). Consistent with the HAI activity, mice vaccinated with either of the Hu-CO mutant HA VLP vaccines had sera with statistically higher log2 serum dilution neutralization titers (8.9-10.5 at 50% inhibition and 7.9-8.9 at 80% inhibition) than sera collected from ws/Mo/05 VLP vaccinated mice (Figure 3.4A).

Antisera from these same vaccinated mice were assayed for HAI and neutralization activity against four recent viruses from more distant clades (Figure 3.5). Mice vaccinated with ws/Mo/05 VLPs or Hu-Av CO VLPs did not have antibodies with HAI or neutralization activity against any of the 4 viruses. Substituting the amino acids from Hu-Av CO HA into the Hu-CO HA did not enhance the HAI titers against any of these 4 viruses. However, substituting the amino acids from Hu-CO HA into the Hu-Av CO HA sequence significantly enhanced the HAI titers against the ck/Vn/08 virus and viruses in clade 2.3.4.2, such as Gu/13 (Figure 3.5A and B). There was no HAI activity or neutralization activity in mice vaccinated with any of the vaccines against the H5N6 or H5N8 viruses, both from clade 2.3.4.4 (Fig 3.5C and D).

Overall, site directed amino acid substitutions in Hu-CO and Hu-Av CO VLP vaccines enhanced antibody titer to some Clade 2 and Clade 1 viruses and enhanced protection against Hu/10 challenge. Antibody titers against clade 2.3.2.1 viruses such as Hu/10 were enhanced when the amino acids Ser-Pro were replaced with Lys-Ser in position 140-141, and the effectiveness of the sera elicited by the Hu-Av CO VLP was also enhanced by the replacement of Ser-Ala with Asn-Thr at position 155-156, but the elicited sera was not able to pick up viruses that Hu-CO VLP elicited sera could also not detect. The HAI data was correlated with FRA neutralization data, but did not always match up. This data only provides us with a small insight into the importance of

residues located near the receptor binding site. Although small changes were made to Hu-CO VLP vaccine, it did not overall enhance its efficacy to newly emerging viruses such as H5N6 and H5N8.

Challenge Data and Lung Titers

Female BALB/c mice were challenged 4 weeks after IM boost of VLP HA formulated with oil and water adjuvant. The viruses chosen were Hu/10 and Si/14. As predicted, Hu-CO vaccinated mice were fully protected against the Hu/10 (clade 2.3.2.1) challenge. Lung virus titers also corresponded with this protection. However, when the Hu-CO vaccine was altered to remove the glycosylation site from amino acid residues 155-156, we saw a significant drop in survival (100% vs 40%) in the Hu/10 challenged mice. This phenomenon was also seen in the Hu-AV CO vaccine platforms, parental vaccine Hu-Av CO had a survival rate of 80%, however when the glycosylation site was added into site 155-156, survival in the Hu-Av 155-156 vaccinated mice increased to 100%. Overall, all COBRA derived vaccines elicited protective antibody responses compared to the homologous comparator, which has been previously seen in studies [4, 8, 14]. Mice challenged with Si/14 showed increased viral plaque titers (Figure 3.6 B) and decreased survival rates in both Hu-CO and Hu-AV CO vaccinated mice. Hu-CO 141-142 vaccinated mice had a 100% survival rate, however viral plaque titers were still significantly high. Weight loss from mice vaccinated with Hu-CO 141-142 was less severe compared to Hu-CO vaccinated mice (Figure S3.2). The mechanism of protection for Hu-CO 141-142 here in unknown but could be due to other epitopes located on the HA molecule that are not being investigated in this study. Although mice vaccinated with Hu-CO 141-142 did survive challenge, weight-loss and lung viral titers were significantly high, therefore the protection of this vaccine should be taken lightly. Mice vaccinated with Hu/10

and Si/14 VLPs had significantly lower survival rates (25% and 20% respectively) compared to the COBRA vaccinated mice.

DISCUSSION

The COBRA methodology has been successfully used to generate HA proteins for the H1, H3 and H5 influenza subtypes that can capture antigenic epitopes from multiple strains within multiple viral clades in [4, 6-8, 14, 17, 26]. For H5, two COBRA HA antigens were designed using HA sequences from human and avian H5N1 viruses isolates that were collected between 2004-2007 [4, 8]. Antibodies elicited from these two H5 HA COBRA vaccines had different patterns of HAI activity against H5Nx viruses isolated from 2005-2017. Based upon HAI activity elicited by the Hu CO and Hu-Av CO HA vaccines, these H5Nx viruses could be categorized into three groups: (1) viruses that were neutralized by antibodies elicited from both vaccines, (2) viruses that could be neutralized by antibodies elicited by one of the two COBRA HA vaccines, and (3) viruses that could not be neutralized by antibodies elicited from either of these two HA COBRA HA vaccines. To explore these three phenotypes, strategic point mutations in the amino acid sequence were introduced to better elucidate the regions in HA that elicit the broadly-reactive antibody inducing these phenotypes. The HA amino acid sequences between Hu CO and Hu-Av CO differ by 6 residues in the globular head region (Figure 3.1). In this study, four mutant COBRA HA vaccines were generated to determine which antigenic site(s) were responsible for eliciting antibodies against heterologous clade viruses. Viruses that fell into the first category (1) were older circulating viruses, such as ws/Mo/05 and Vn/04. Both Hu-CO and Hu-Av CO HA based vaccines are able to protect mice, ferrets and non-human primates from lethal challenge with ws/Mo/05 [4, 6, 7] and

in mice against Vn/04 [8]. The HAI and neutralization titers mirrored these results. The mutant vaccines Hu SP140-141KS and Hu-Av SA155-156NT had significantly higher titers than mice vaccinated with Hu-CO vaccine. Hu-CO and Hu-Av CO vaccines were both able to elicit neutralizing antibodies against viruses in this first category. Viruses in our panel that fell into the second category (2) included four different viruses; Hu/10, Eg/10, ck/Vn/08 and Gu/13. The first two viruses, Hu/10 and Eg/10 were neutralized by Hu-CO elicited antibodies, but not by Hu-Av CO (Fig. 4). When sites 140-141 were altered in Hu-CO to represent the amino acids located in Hu-Av CO (Lys-Ser), antibody titers were significantly increased compared to the parental Hu-CO vaccine. However, when sites 155-156 were disturbed on the Hu-CO backbone, antibody titers against Hu/10 were abolished. Furthermore, replacing the amino acids Ser-Ala with an Asn-Thr at position 155-156 in Hu-Av CO backbone significantly increased elicited antibody geometric mean titers (GMT) against both Hu/10 and Eg/10. This data suggests that introduction of the amino acid residues Asn-Thr (N-T) into either Hu-CO or Hu-Av CO vaccines helps elicit neutralizing antibodies against H5Nx viruses from heterologous clades 2.3.2.1 and 2.2.1.

The residues 155-156 on the Hu CO HA contains an N-linked glycosylation motif (Asn-X-Ser/Thr). Recently the crystal structure of the Hu CO HA has been resolved and indeed shows a glycosylation on this epitope [27]. This putative glycosylation site in the Hu-CO HA vaccine may mask residues that are critical for eliciting antibodies that neutralize H5Nx viruses. Glycosylation of influenza HA-based viruses often leads to an ineffective vaccine, due to mismatched HA molecule, and can alter the effectiveness of the antibody binding following infection. Glycosylation sites have been thought to “mask” important antigenic epitopes rendering the vaccine ineffective and creating antibody responses to non-neutralizing epitopes [28]. However, the presence of N-linked glycans on the HA surface can also alter antibody binding

epitopes and work as an immune diversion mechanism, possibly disguising areas near the RBS that are not neutralizing and diverting the immune response towards epitopes on the HA molecule that are more neutralizing [29-32]. It is important to note that the glycosylation motif located on Hu-CO HA antigen is not present in the viruses Hu/10 and Eg/10 and may not be participating in antibody binding and recognition, but rather diverting antibody responses towards more neutralizing epitopes that are not targeted when vaccinated with Hu-Av CO. Glycosylation of the HA protein in H5N1 viruses is variable across clades and the addition or removal of N-linked glycosylation sites can serve as an evolutionary roadmap. Viruses from clades 0, 1 and 7 contain a putative glycosylation in site 154, most viruses from clade 2.2, except viruses from clade 2.2.1.1 lack the putative 154-glycosylation site [33].

The Hu/10, Eg/10, Hu-CO SP140-141KS and Hu-Av CO SA155-156NT sequence alignments revealed that there is a shared Serine residue at residue 141. This shared amino acid may aid in virus neutralization if the epitope is targeted by antibodies. It is important to note that although introduction of Asn-Thr into sites 155-156 in Hu-Av CO increased antibody titers, it did not fully restore the response to the Hu-CO 140-141 GMT levels. Mutation of this same location in Hu-Av CO VLP did not completely rescue the HAI activity from all the mice (n=11) compared to sera elicited by the Hu-CO SP140-141KS HA (GMT 6.1 vs 5.0). This indicates that there may be other sites that are located more distant from the RBS that are important for the induced HAI activity. However, Site 282 on the HA1 protein may be a site that induces neutralizing titers against clade 2.3.2.1 viruses. Sequence alignment of Hu/10 and Hu-Co vaccine revealed that they shared the same amino acid Methionine at site 282. Due to the location of this amino acid on the HA molecule, it is very feasible that antibody binding to this site would impact HA conformational

change and inhibit viral infectivity. This site has been shown to be a neutralizing epitope in monoclonal antibody studies [25, 34].

The second set of viruses that fell into pattern (2) are Gu/13 and ck/Eg/08. Antibody titers against these viruses were increased when a Lys-Ser were replaced with the amino acid's Ser-Pro in sites 140 and 141 in the Hu-Av CO backbone. Antibody titers were significantly higher than those elicited by the parental strain Hu-Av CO. Neutralizing antibody titers elicited by Hu-Av CO vaccination were not induced following vaccination, however Hu-CO vaccination induced 50% and/or 80% neutralization titers. The addition of a proline in this epitope increased shared amino acids between Hu-Av CO, ck/Vn/08 and Gu/13, both viruses contain a Proline in site 141. The addition of a Proline can also affect the structure of the HA protein. The Proline amino acid contains a large cyclic structure and affects secondary structure of proteins adding to conformational rigidity. The addition of a Proline residue in site 141 contributes to antibody responses by increasing shared epitopes between viruses ck/Vn/08 and Gu/13. The presence of a Proline near the receptor binding domain increase the stability of the HA protein.

Mice vaccinated with Hu-CO HA protein do not elicit protective antibodies against the more recent circulating strains of the clade 2.3.4.4 (Si/14, gy/WA/14, dk/Eg/17). Antisera elicited by Hu-Co HA VLP vaccines did have some reactivity to newer circulating viruses, such as A/ck/Eg/17 (H5N1, clade 2.2.1.2), however mice vaccinated with Hu-CO construct failed to protect mice from Si/14 challenge. However, mice vaccinated with Hu-Av CO constructs had higher survival odds compared to the Hu-CO vlp vaccines. Neutralizing antibodies at 50% neutralization was higher from Hu-AV 140-141 and Hu-Av 155-156 compared to the Hu-CO vaccinated mice (Fig 5G). Antigenic epitopes associated with the RBS were evaluated for amino acid mutations between sets of viruses and/or vaccine sequences [35]. When comparing the

gy/WA/14 HA sequence with the Hu-CO and Hu-Av CO HA sequences, there were mutations located in the 192-198 region of the HA1 molecule. This immunogenic region is located on the antigenic site B in H3 and or the Sb region in H1 viruses [36]. In addition, there are multiple mutations between Si/14 and Hu-CO/Hu-Av CO HA proteins near the RBS at positions 116, 121-123, 126-129 (overlaps site Sa in H1) [37, 38], 138-141 (site A in H3 and site Ca2 in H1) [37-39], 152-155 (site B in H3) [37, 39]. There is a total of 30 and 31 amino acids that differ between the HA1 portion of Si/14 HA and the Hu-CO, Hu-Av CO HA proteins, the majority of amino acid mutations are associated with the RBS. Changes to these sites greatly impacted cross protective antibody responses elicited by Hu-CO vaccination. Mutations in multiple antigenic sites in the HA render the Hu-CO HA vaccine ineffective against H5N6 and H5N8 viruses.

The correlate of protection for human infection with H5Nx viruses are currently not known. The standard correlate of protection for seasonal influenza vaccinations is defined as a HAI titer of greater than 1:40. However, since avian influenza viruses do not readily infect the human population, the HAI correlate is unknown from vaccine trials. In animal models, low HAI titers (<1:40) and even the absence of HAI and/or neutralization titers were found to decrease the severity of infection and prevent mortality in viral challenge [40-43]. Vaccine elicited protection from lethal challenge with H5N1 viruses can be providing protection via mechanisms that are not detectable through benchmark assays. Therefore, antibodies with HAI or neutralization activity may not always correlate with true protection against HPAI infection. However, these assays are the best methods we have for determining protection. The NA protein in seasonal trivalent vaccine can elicit protective immune responses against lethal challenge with H5N1 influenza viruses with no detectable HAI titers [44]. Although small animal models are currently the best method for studying influenza viruses, the cellular and humoral responses may not provide us with a full

understanding of the human response to avian influenza challenge. Further studies need to be performed in order to determine the correlate of protection for H5Nx viruses in humans.

In this report, we sought out to determine which two specific epitopes located on Hu-CO HA vaccine are responsible for eliciting broadly protective antibodies against heterologous clades of viruses. Vaccinating mice with chimeric HA VLP vaccines enabled us to break down the two specific epitopes responsible for eliciting immune responses capable of neutralizing heterologous clades of viruses compared to Hu-Av CO. Most likely, the putative N-linked glycosylation motif located in amino acid sites 155-156 in Hu-CO 2 is responsible for eliciting HA-directed antibodies against heterologous clade 2.3.2.1 viruses (Hu/10). We also identified the site 140-141 in Hu-CO HA vaccine is also targeted by the immune response and is of most importance to clade 2.3.4.2 and 7.1 viruses. Therefore, Hu-CO HA vaccine is able to elicit antibodies against a broad range of avian influenza viruses due to its unique combinations of epitopes that capture antigenic variations found throughout multiple clades. The Hu-CO HA vaccine, however did not elicit HAI detectable antibodies or protection against newer reassorted viruses in clade 2.3.4.4. Hu-CO HA vaccine was designed using H5N1 input sequences from viruses isolated between the years 2005-2007 and therefore does not contain newer epitopes from HA antigens in H5N6 and H5N8 influenza viruses. There are 9 specific residues that are associated with the antigenic drift of clade 2.3.4.4 viruses and also correspond to antigenic epitopes found on H3N2 viruses which includes sites 124, 140, 156, 189, 198, and 162 [45]. Mutating these residues in the Hu-CO HA to match these seasonal HA residues, the elicited clade 2.3.4.4 epitopes and induce neutralizing antibodies against these circulating strains of viruses. Pre-pandemic vaccine development is an essential preventative measure against HPAI viruses. Further studies need to be conducted in order to develop an avian influenza vaccine that is safe, efficacious, immunogenic and induces a long-lasting memory

response in the human population. Cross-clade protection and neutralization in an essential element to vaccine development and must be considered for H5Nx viruses.

Figures

Fig. 1

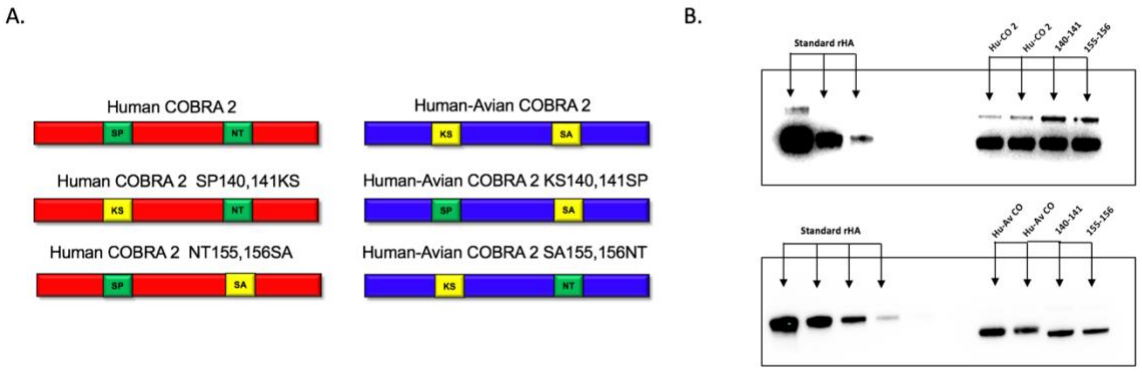


Figure 3.1: Schematic representation of COBRA mutants. Sites 140-141 and 155-156 on Hu-CO were exchanged with the corresponding amino acids located on Hu-Av CO, two Hu-CO mutants are shown (A), Sites 140-141 and 155-156 on Hu-Av CO were exchanged with the corresponding amino acids located on Hu-CO. (B) Western Blot analysis of Hu-CO and Hu-Av CO mutant VLP are shown in the right panel, arrows indicate VLPs on the right side of the blot, protein standards on the left.

Virus	Clade	Type	Stock
A/Vietnam/1203/2004	1	H5N1	Virus PR8
A/whopper swan/Mongolia/244/2005	2.2	H5N1	Virus PR8
A/Indonesia/05/2005	2.1.3	H5N1	Virus PR8
A/Anhui/1/2005	2.3.4	H5N1	Virus PR8
A/Egypt/321/2007	2.2.2	H5N1	Virus PR8
A/chicken/Vietnam/NCVD-016/2008	7.1	H5N1	Virus PR8
A/Egypt/N03072/2010	2.2.1	H5N1	Virus PR8
A/Hubei/1/2010	2.3.2.1	H5N1	Virus PR8
A/Cambodia/X0810301/2013	1.1.2	H5N1	Virus PR8
A/Guizhou/1/2013	2.3.4.2	H5N1	Virus PR8
A/gyrfalcon/Washington/41088-6/2014	2.3.4.4	H5N8	Virus PR8
A/Sichuan/26221/2014	2.3.4.4	H5N6	Virus PR8
A/chicken/Egypt/CAL3-RLQP/2017	2.2.1.2	H5N1	VLP
A/duck/Egypt/S78-RLQP/2017	2.3.4.4	H5N8	VLP

Table 3.1: Table of viruses used in the HAI and FRA panel with subtype, clade and stock type. Most viruses used were from a PR8 backbone with HA and NA from the wild type virus. HA was modified to a low pathogenic cleavage site for use in BSL-2 conditions. Viruses were ordered from International Reagent Resources (IRR). 3 of the viruses were expressed as VLPs in a mammalian cell line and were used for HAI only.

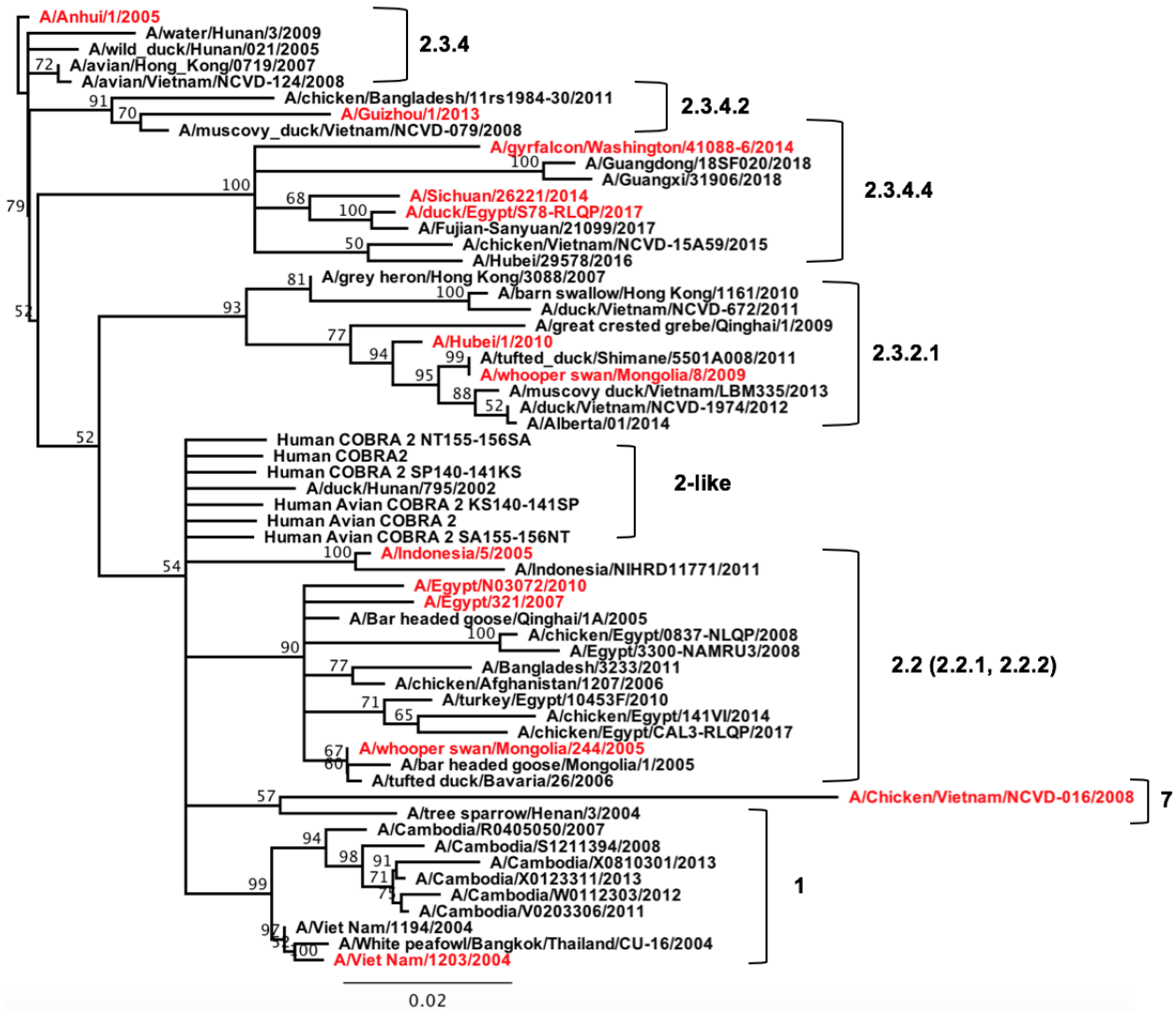


Figure 3.2: Phylogenic tree of H5Nx viruses and vaccine amino acid sequences of the HA molecule. Tree was made using Geneious® version 11.1.2 [24]. Sequences were aligned using MUSCLE 3.7 software, the alignment was refined by Gblocks .91b software. Phylogeny was determined using the maximum-likelihood method with neighbor joining software. Viruses and vaccine used in this study are colored in red, where other strains are black and used for comparison. Clades and subclades are labeled to inform the reader the spread of the viruses used for the HAI panel, and to show antigenic relatedness of the Hu CO and Hu-Av CO vaccine.

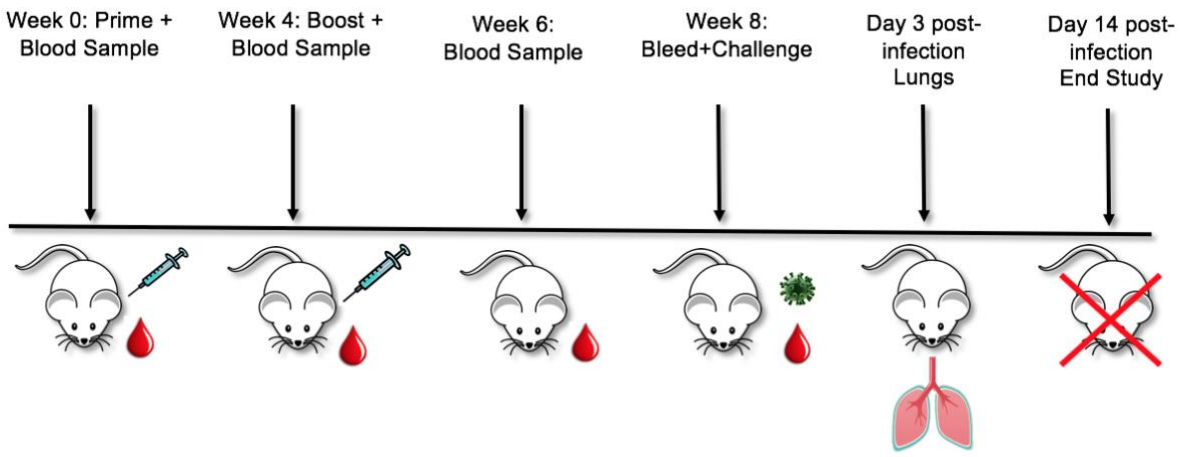
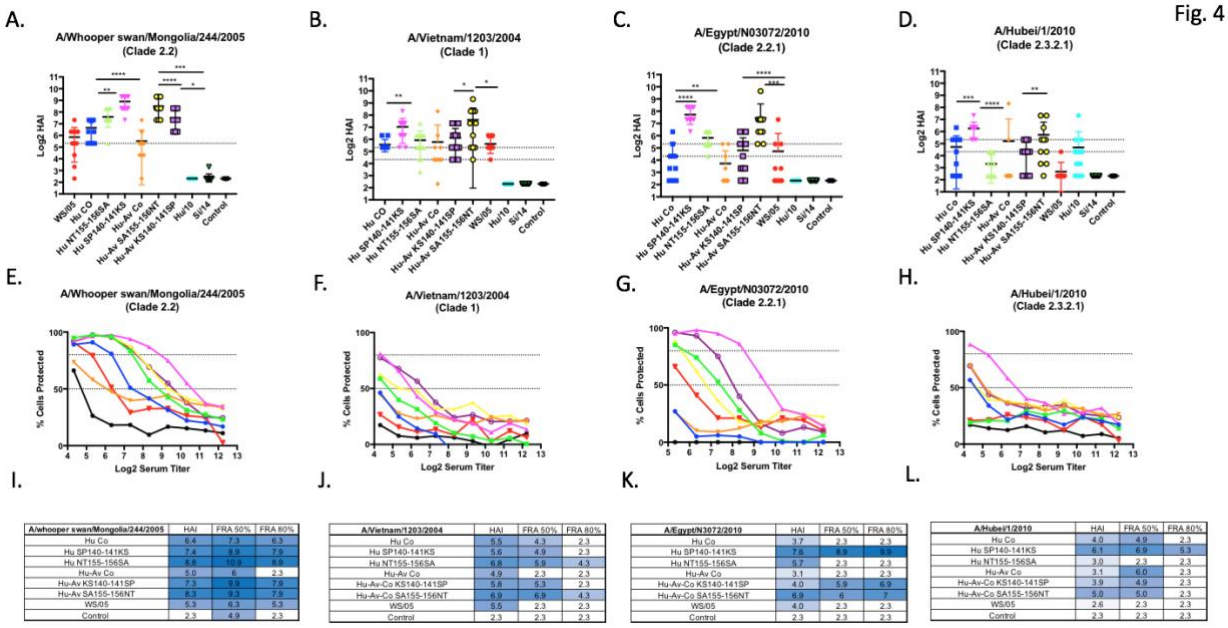


Figure 3.3: Schematic representation of mouse study. Mice were prime and boosted with VLPs expressing Hu-CO and Hu-Av CO HA antigens and their mutants adjuvanted with AFO3. Blood samples were taken intermittently throughout study to confirm antibody titer was sufficient enough to withstand challenge. Mice were challenged with A/Hubei/01/2010 and A/Sichuan/26221/2014 PR8 reassortant viruses and were monitored daily for 14 days post infection (P.I). Lungs were collected on day 3 P.I. of study to test for viral plaques.

Fig. 4



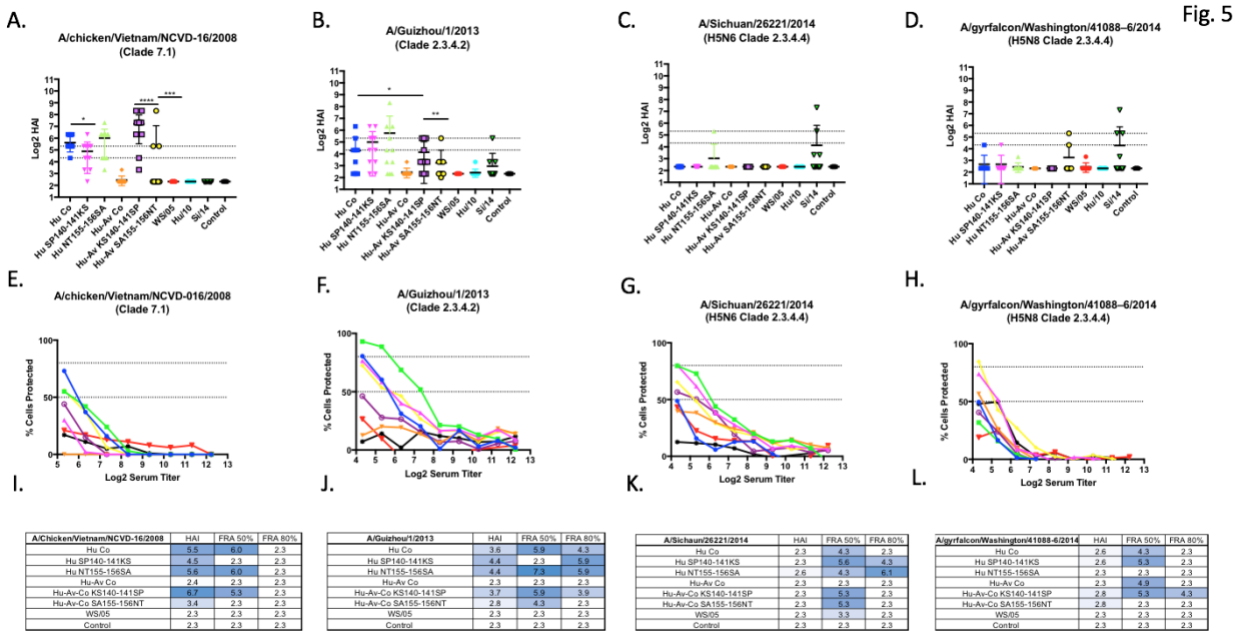
A/whooper swan/Mongolia/244/2005	HAI	FRA 50%	FRA 80%
Hu Co	6.4	7.3	6.3
Hu SP140-141KS	7.4	8.9	7.9
Hu NT155-156SA	8.6	10.8	9
Hu-Av Co	5.0	6	2.3
Hu-Av KS140-141SP	7.3	9.9	7.9
Hu-Av SA155-156NT	8.3	9.3	7.9
WS/05	5.3	6.3	5.3
Control	2.3	4.9	2.3

A/Vietnam/1203/2004	HAI	FRA 50%	FRA 80%
Hu Co	5.5	4.3	2.3
Hu SP140-141KS	5.6	4.9	2.3
Hu NT155-156SA	6.8	5.9	4.3
Hu-Av Co	4.9	2.3	2.3
Hu-Av-Co KS140-141SP	5.8	5.3	2.3
Hu-Av-Co SA155-156NT	6.9	6.9	4.3
WS/05	5.5	2.3	2.3
Control	2.3	2.3	2.3

A/Egypt/N3072/2010	HAI	FRA 50%	FRA 80%
Hu Co	3.7	2.3	2.3
Hu SP140-141KS	7.6	8.9	9.9
Hu NT155-156SA	5.7	2.3	2.3
Hu-Av Co	3.1	2.3	2.3
Hu-Av-Co KS140-141SP	4.0	5.9	6.9
Hu-Av-Co SA155-156NT	5.9	6	7
WS/05	4.0	2.3	2.3
Control	2.3	2.3	2.3

A/Hubei/1/2010	HAI	FRA 50%	FRA 80%
Hu Co	4.0	4.9	2.3
Hu SP140-141KS	6.1	6.9	5.3
Hu NT155-156SA	3.0	2.3	2.3
Hu-Av Co	3.1	6.0	2.3
Hu-Av-Co KS140-141SP	3.9	4.9	2.3
Hu-Av-Co SA155-156NT	5.0	5.0	2.3
WS/05	2.6	2.3	2.3
Control	2.3	2.3	2.3

Figure 3.4: HAI serum antibody titers induced by vaccination of mice with Hu-CO and Hu-AV CO derived vaccines against ws/Mo/05, Hu/10, Eg/10 and Vn/04. HAI titers were determined for each group of mice (n=11) vaccinated twice (days 0 and 28) with 1 of the 7 H5 VLP vaccines expressing surface WT or mutant HA proteins against 4 viruses shown here. Values are the geometric mean titers and errors of the means (SEM) (error bars) from the antisera collected at day 56. The dotted line represents a titer at 1:20 and 1:40 HAI titer range. Focal reduction Assay (FRA) uses pooled sera from the vaccine groups from day 56. For each virus, the virus concentration was standardized to 1.2 x10^4 FFU/ml (corresponding to 600 FFU/50 µl, which is the volume of virus added to each plate). A monolayer of MDCK SIAT cells (2.5x10^5 to 3x10^5 cells/ml) (100 µl/well in a 96-well plate) was added the day before the assay is run. Cells were 95 to 100% confluent at the time of the assay to determine the number of foci detected as percent infected cells normalized to 100%. Pooled sera from each group of mice were tested against ws/Mo/05 (E), Hu/10 (H), Eg/10 (G), Vn/04 (F). The dotted lines represent 50%and 80% protection by sera compared to virus-only control wells. Amino acid sequence alignment performed in Geneious® (I).



A/Chicken/Vietnam/NCVD-16/2008	HAI	FRA 50%	FRA 80%
Hu Co	5.5	6.0	2.3
Hu SP140-141KS	4.5	2.3	2.3
Hu NT155-156SA	5.6	6.0	2.3
Hu-Av Co	2.4	2.3	2.3
Hu-Av-Co KS140-141SP	6.7	5.3	2.3
Hu-Av-Co SA155-156NT	3.4	2.3	2.3
WS/05	2.3	2.3	2.3
Control	2.3	2.3	2.3

A/Guizhou/1/2013	HAI	FRA 50%	FRA 80%
Hu Co	3.6	5.9	4.3
Hu SP140-141KS	4.4	2.3	5.9
Hu NT155-156SA	4.4	7.3	5.9
Hu-Av Co	2.3	2.3	2.3
Hu-Av-Co KS140-141SP	3.7	5.9	3.9
Hu-Av-Co SA155-156NT	2.8	4.3	2.3
WS/05	2.3	2.3	2.3
Control	2.3	2.3	2.3

A/Sichaun/26221/2014	HAI	FRA 50%	FRA 80%
Hu Co	2.3	4.3	2.3
Hu SP140-141KS	2.3	5.6	4.3
Hu NT155-156SA	2.6	4.3	6.1
Hu-Av Co	2.3	2.3	2.3
Hu-Av-Co KS140-141SP	2.3	5.3	2.3
Hu-Av-Co SA155-156NT	2.3	5.3	2.3
WS/05	2.3	3.3	2.3
Control	2.3	2.3	2.3

A/gyrfalcon/Washington/41088-6/2014	HAI	FRA 50%	FRA 80%
Hu Co	2.6	4.3	2.3
Hu SP140-141KS	2.6	5.3	2.3
Hu NT155-156SA	2.3	2.3	2.3
Hu-Av Co	2.3	4.9	2.3
Hu-Av-Co KS140-141SP	2.8	5.3	4.3
Hu-Av-Co SA155-156NT	2.8	2.3	2.3
WS/05	2.3	2.3	2.3
Control	2.3	2.3	2.3

Figure 3.5: HAI serum antibody titers induced by vaccination of mice with Hu-CO and Hu-Av CO derived vaccines against ck/Vn/08 (A), Gu/13 (B), Si/14 (C) and gy/WA/14 (D). HAI titers were determined for each group of mice (n=11) vaccinated twice (days 0 and 28) with 1 of the 7 H5 VLP vaccines expressing surface WT or mutant HA proteins against 4 viruses shown here. Values are the geometric mean titers and errors of the means (SEM) (error bars) from the antisera collected at day 56. The dotted line represents a titer at 1:20 and 1:40 HAI titer range. Focal reduction Assay (FRA) uses pooled sera from the vaccine groups from day 56. For each virus, the virus concentration was standardized to 1.2 x10_4 FFU/ml (corresponding to 600 FFU/50 µl, which is the volume of virus added to each plate). A monolayer of MDCK SIAT cells (2.5x10_5 to 3x10_5 cells/ml) (100 µl/well in a 96-well plate) was added the day before the assay is run. Cells were 95 to 100% confluent at the time of the assay to determine the number of foci detected as percent infected cells normalized to 100%. Pooled sera from each group of mice were tested against ck/Vn/08 (E), Gu/13 (F), Si/14 (G), gy/WA/14 (H). The dotted lines represent 50%and 80% protection by sera compared to virus-only control wells. Amino acid sequence alignment performed in Geneious® (I).

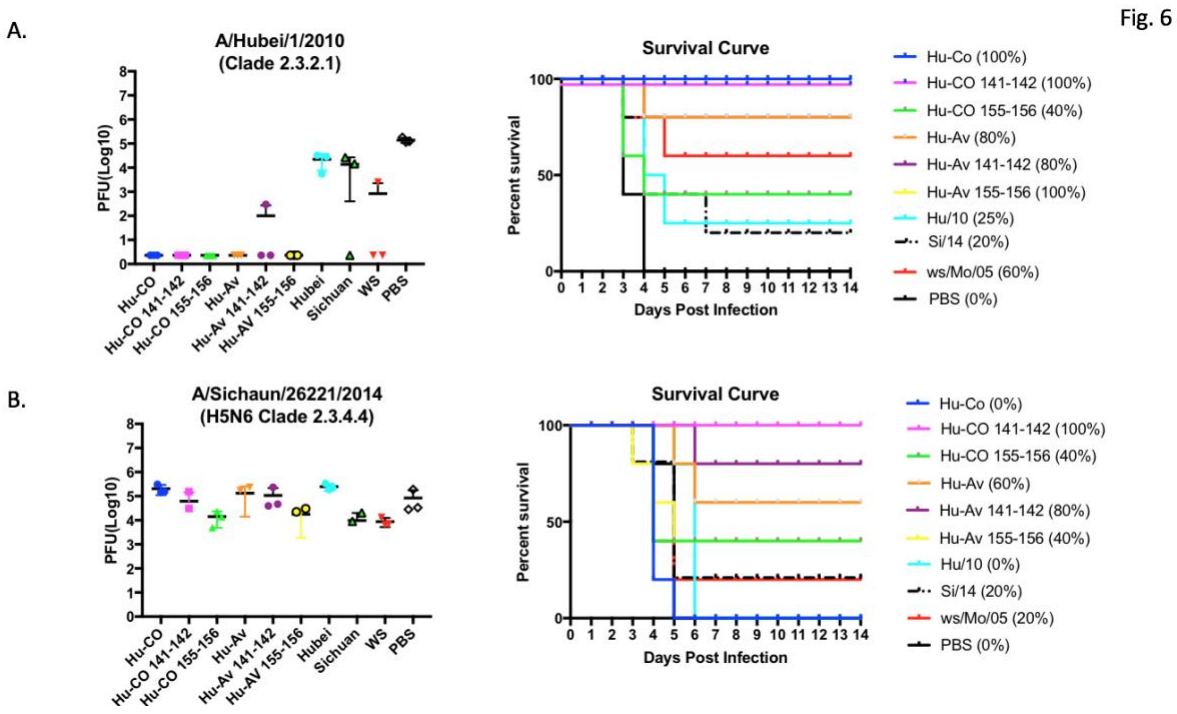


Figure 3.6: Mice were challenged with A/Hubei/01/201 and A/Sichuan/26221/2014 PR8 reassortant viruses 4 weeks following the last VLP+Adjuvanted IM boost. At day 3 post-infection, 3 mice from each vaccine group were sacrificed to collect lungs. Lungs were snapped frozen and were processed to determine viral plaque titers. Survival curves were calculated using the remaining 5 mice.

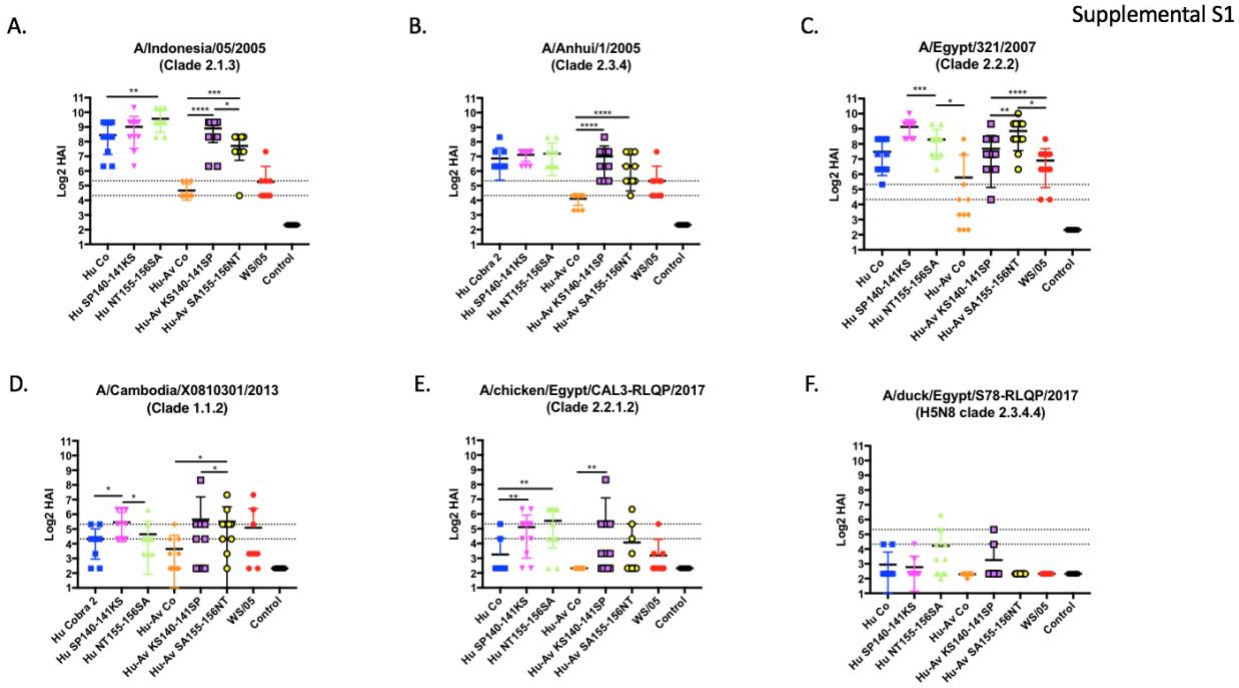


Figure S3.1: HAI serum antibody titers induced by vaccination of mice with Hu-CO and Hu-Av CO HA derived vaccines against In/05 (A), An/05 (B) , Eg/07 (C), Cm/13 (D), ck/Eg/17 (E) and dk/Eg/17 (F). HAI titers were determined for each group of mice (n=11) vaccinated twice (days 0 and 28) with 1 of the 7 H5 VLP vaccines expressing surface WT or mutant HA proteins against 4 viruses shown here. Values are the geometric mean titers and errors of the means (SEM) (error bars) from the antisera collected at day 56. The dotted line represents a titer at 1:20 and 1:40 HAI titer range.

Supplemental S2

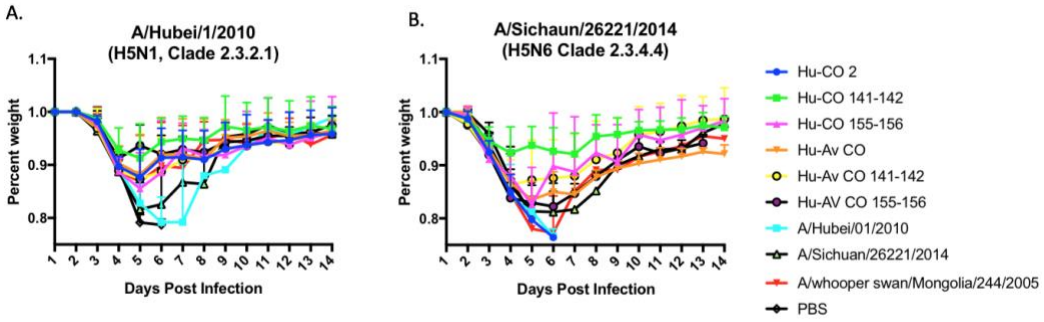


Figure S3.2: Weight loss curves taken 14 days following viral challenge. Days 1-3 include all 8 mice until the day 3 sacrifice. Days post lung collection include 5 mice per-group until mice succumbed to disease. Weight loss of 20% of initial weight was considered as a humane endpoint.

Supplemental S3

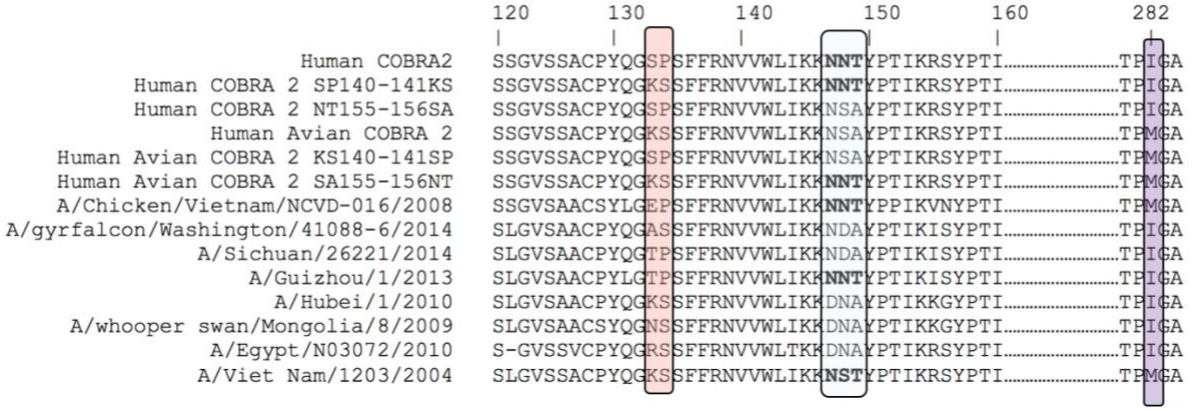


Figure S3.3*:* Amino acid sequence alignment of Hu-CO and Hu-Av CO vaccines and viruses used in challenge and HAI data. Sites 140-141, 155-156 and 282 are highlighted.

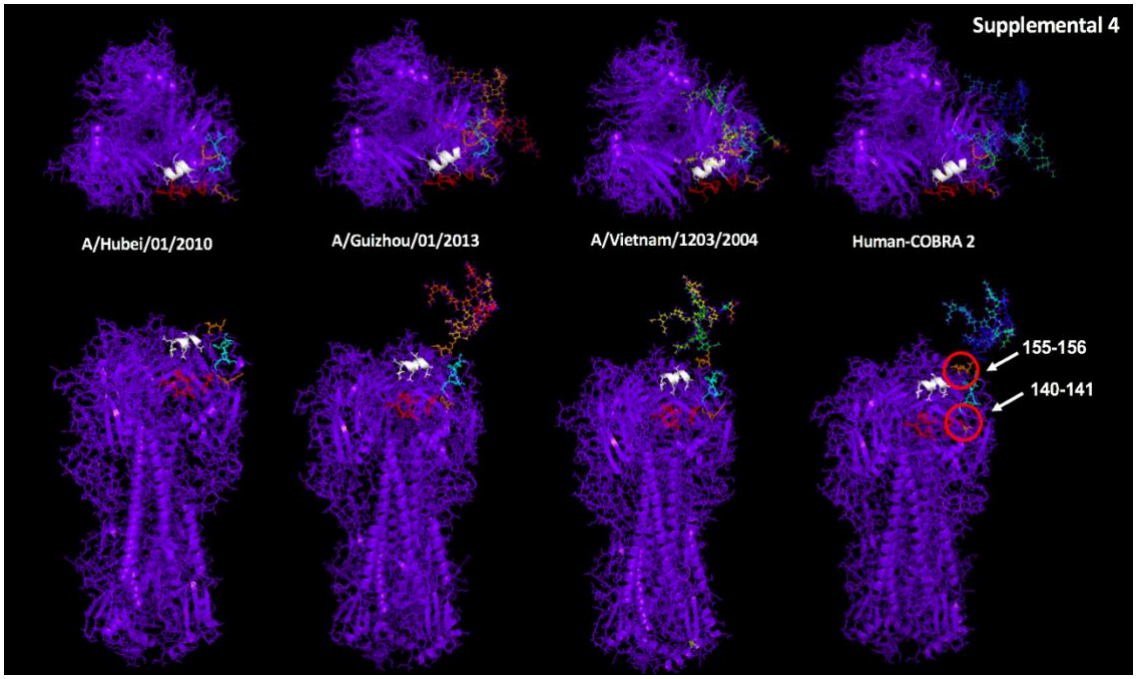


Figure S3.4: Pymol model of Hu-CO 2 HA with complex glycosylation in site 154-155, predicted modeling of masking sub-dominant epitopes. White, blue and red regions mark the amino acid sites predicted to be involved with the receptor binding pocket. White (190 helix) Blue (130 Loop), Red (220 loop). The two orange sites are the sites of interest 140-141 and 155-156.

Virus	Accession Number
A/whooper swan/Mongolia/244/2005	ACD68156.1
A/Viet_Nam/1203/2004	AAW80717.1
A/Egypt/N03072/2010	AEL31632.1
A/Hubei/1/2010	AEO89181.1
A/Chicken/Vietnam/NCVD-016/2008	ACO55047.1
A/Guizhou/1/2013	EPI420386
A/Sichuan/26221/2014	EPI533583
A/gyrfalcon/Washington/41088-6/2014	AJE30333.1
A/Indonesia/5/2005	ABP51969.1
A/Anhui/1/2005	ABD28180.1
A/Egypt/321/2007	ACO55049.1
A/Cambodia/X0810301/2013	KF918470.1

Figure S3.5: Genbank accession numbers

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CHAPTER 4

NEXT GENERATION H5NX COBRA DESIGNS PROVIDE PROTECTION AGAINST MULTIPLE AVIAN DERIVED AVIAN VIRUS CLADES

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ABSTRACT

The original H5N1 COBRA vaccines called Human COBRA 2 and Human-Avian COBRA 2 vaccines were constructed through layering of hemagglutinin (HA) sequences from human isolates collected between 2004-2007 using only Clade 2 isolates. These COBRA HA vaccines in VLP platforms elicit protective immune responses in mouse, ferret and non-human primates. Recent data however shows that these vaccines do not induce neutralizing antibody titers against newly circulating viruses. Therefore, COBRA vaccines were updated in order to elicit protective antibodies against the new dominant circulating clade of H5Nx viruses (clade 2.3.4.4). Next-Generation COBRA vaccines were designed to encompass the newly emerging viruses circulating in wild fowl populations across the globe. Protein HA sequences were downloaded from the GISAID (global initiative on sharing all influenza data) using only complete H5 HA sequences taken from both avian and human isolates. Multiple year filters were used between 2011-2017. Immunological data obtained from vaccinated mice showed that newly generated vaccines were capable of eliciting broad responses detectable through hemagglutinin inhibition assays (HAI) against 5 viral clades and outperformed the wild-type comparators. Three vaccines were further chosen to perform a challenge study to access survival, weight loss, lung viral loads and pathological analysis. Our study shows that one vaccine termed IAN-8 was able to protect mice from viral challenge against A/Sichuan/26621/2014 and VN/04, and also elicited a broader antibody response compared to the wild-type vaccine comparators. Mice vaccinated with IAN-8 rHA vaccine were also able to elicit HAI antibodies against clades 2.2, 2.3.2.1, 2.3.4.2, 2.2.1 and 2.2.2. Histopathological analysis of mouse lungs Day 3 post challenge revealed a decrease of viral load and cellular infiltrates in IAN-8 vaccinated groups compared to controls. We conclude

therefore, a pandemic vaccine should incorporate a mixture of 2 or more COBRA vaccines to elicit antibodies against a variety of viral H5 clades.

INTRODUCTION

Highly pathogenic avian influenza viruses that fall into H5 subtype genetic clade 2.3.4.4 emerged in China in 2010-2011. As of October 2017, clade 2.3.4.4 viruses have been detected in birds in 24 countries in Africa, Asian and Europe [1]. As of February 2020, the WHO has reported 24 cases of human infections [2]. The H5N6 subtype is the only 2.3.4.4 clade virus that has infected people [3]. The two newest cases of H5N6 infection in humans both occurred in China between September 26th 2017 and February 19th 2018, and had HA genes that were phylogenetically distinct from one another [4]. Since its emergence, these H5Nx genetic clade 2.3.4.4 viruses have spread globally and has had devastating effect on the poultry industry. The first 2.3.4.4 virus that emerged was classified as in the H5N6 subtype and it circulated throughout China and traveled to Southeast Asia causing the death of 457 birds in Laos [5]. Over 48 million chickens and turkeys were culled and led to a loss of $1.6 billion USD [6].

In 2014, H5N8 Eurasian subtypes emerged in Canada, Germany, The Netherlands, United Kingdom and East Asia and was concurrently detected in the U.S. state of Washington in captive falcons, wild birds, and poultry [7]. The spread of these intercontinental-like viruses coincided with the bird migration out of Russia and most likely spread by migratory birds [8]. In late 2014, H5N8 viruses were identified in Canada and in Washington State in the United States where it caused infection in captive falcons, wild birds and poultry and spread across mid-Western and North Central States causing devastation to the poultry industry [8]. The clade 2.3.4.4 viruses from

Africa and Europe are primarily of the H5N8 subtype, whereas those viruses isolated in Asia are in the H5N6 subtype, and those strains isolated in in the United States are classified as H5N2 isolates [4]. Clade 2.3.4.4 viruses isolated from Africa and Europe are primarily of the H5N8 subtypes and those found in Asia are H5N6 subtype [1]. The H5N8 viral subtype caused an outbreak in 2014 in South Korea leading to a distinction of two different H5N8 virus subgroups [8-10]. Group A comprises a set of H5N8 isolates and is referred to as the intercontinental group A (icA) group. The icA H5 viruses further evolved into 3 different subgroups, icA1, icA2, icA3. The icA1 subtypes group contains viruses that were isolated from Europe, Russia, and Japan. The icA2 subgroup in composed of H5N8 and HPAIV reassortants H5N2 and H5N1 from North America in 2014. The icA3 subgroup is composed of H5N8 viruses isolated in Japan and in Korea [8].

Avian influenza viruses from clade 2.3.4.4 show different pathogenicity in chicken, duck and mammal species [11, 12]. Ducks infected with H5N6 and H5N8 virus have viral growth in the lung, spleen, kidneys and brain of these animals [12]. However, mortality in the duck species is variable, infection in ducks with H5N6 or H5N8 caused death in one animal per group per virus. Ducks infected with a H5N1 virus resulted in 100% mortality [12]. The pathogenicity of clade 2.3.4.4 viruses in chickens was much more severe, infection with H5N6 and H5N8 viruses resulted in 100% mortality 4-5 days post-infection [12]. H5N6 virus infection in BALB/c mice also has varying pathogenic effects, viruses with increased binding affinities to α-2,3-sialic acids resulted in 100% mortality of mice and viruses that had increased α-2,6-sialic acid binding affinities had no pathogenic effects [11]. Infection of H5N6 viruses in ferrets display different effects of pathogenicity compared to HPAI H5N1 infection. Ferrets infected with H5N6 displayed no signs

of morbidity, except for fever one day post-infection [13]. H5N6 virus is able to grow in the lung and spleen of challenged ferrets but are significantly lower than H5N1 infected tissues [13]. Ferrets infected with H5N6 did however easily transmit the virus to contact naïve cage mates, where H5N1 infected ferrets did not [13]. Although these viruses are able to transmit through direct contact, it does not possess the ability to transmit through airborne droplets in infected ferrets [14].

Viruses from clade 2.3.4.4 have binding affinities to both α-2,3 sialic acids and α-2,6 sialic acid residues [11]. Mutations in the HA domain that are associated with receptor binding alterations include S123P, I151T, T156A and a deletion at position 125. The receptor binding specificity of avian influenza viruses can be altered to bind to human α-2,6-linked sialic acids by introducing a single amino acid mutation into the RBS [15]. Also, sites 182, 222, 223 and 224 have been shown to be important mutations sites for avian viruses to bind to α-2,6 sialic acids. HPAI viruses that are of H5Nx subtype in clade 2.3.4.4 show binding affinities to both sialic acid subtypes, increasing the possibility of these viruses becoming a human transmissible agent.

Viruses in the genetic clade 2.3.4.4 are genetically and antigenically distinct from clade 2 viruses. Clade 2.3.4.4 is composed of viruses that have diversified H5 molecules with different NA subtypes that include N1, N2, N3, N5, N6, and N8 [16]. The H5N6 viruses contain reassortments from multiple viruses, including internal genes that originate from H5N1, H6, H3, and H9N2 [17]. Viruses with internal genes from H9N2 have been responsible for 12 human infections in years 2015-2016 [17]. The H5N6 viruses also have internal genes from HPAI H5N1 viruses from clade 2.3.2.1c [9]. HA genes from clade 2.3.4.4 H5Nx viruses can be divided into four subclades designated I, II, II, and IV [9]. The H5N6 viruses are found in subclades I and II, H5N2 and H5N8

viruses are found in subclade III and subclade IV are mainly H5N8 [9]. These major shifts in the HA, NA and internal gene segments has resulted in H5N6 viruses that are not cross reactive against strains in the H5N1 subtypes [18]. Chickens who are vaccinated with recombinant vaccines protected from the pathogenicity against these H5N6 viruses, but are still able to transmit the virus into other poultry [19]. Poultry vaccinations therefore need to be re-evaluated in order to provide protection against these dominate H5N6 circulating strain.

The prevalence of H5N6 circulating in wild water fowl population has increasingly become an issue for the health of poultry farmers. Viruses from clade 2.3.4.4 have the ability to reassort with NA that are naturally found in avian species and also have an increasing tendency towards binding to sialic acid receptors more commonly found in the upper respiratory tract of humans. These features of the H5Nx viruses have further increased its potential to cross over into the human population. Along with reassortant events, the HA mutational rate has also been problematic, as observed by the phylogenetic branching and lack of HAI titers against reference strains. Between September 2019 and February 2020, 2 major viral clades have been reported to be circulating in the wild water fowl population, clade 2.3.4.4 and 2.3.2.1. [2]

Mandatory vaccination of poultry was established in Guangdong province in China in July 2017 using an inactivated influenza vaccine [20]. This vaccine regimen decreased the prevalence of H7N9 circulation in live poultry markets, however, circulation of H5N6 viruses continued and increased in antigenic diversity compared to the vaccine strain [20]. In 2018, the Chinese Government and the WHO approved of a new A/Guangdong/18F020/2018 candidate vaccine virus [20]. However, vaccine escape mutants are still a risk for the animal and human population.

Reference sera generated by the WHO revealed that reference antigens A/Sichuan/26221/2014, A/Hubei/29578/2016 and A/Fujian-Sanyuan/21099/2017 does not generate antibodies against the A/Guangdong/18SF020/2018 vaccine strain [21].

Although clade 2.3.4.4 viruses are the dominate circulating strain in wild waterfowl and poultry populations, outbreaks of H5N1 viruses from clade 2.3.2.1 have also been reported. In 2015, it was reported that viruses from clade 2.3.2.1 were the cause of the majority of the H5N1 outbreaks since 2011. Similarly, to the 2.3.4.4 viruses, viruses from clade 2.3.2.1 have further diversified into five separate subclades [22]. Since 2010, the HA of clade 2.3.2.1 viruses have spread over provinces in Vietnam and were predominant throughout Vietnam in early 2014. Specifically, viruses from the subclade 2.3.2.1c are predominantly present in southern Vietnam since the first outbreak in 2010 in chickens and ducks [23]. These compounding factors further exacerbate the need for a pandemic vaccine for the at-risk human population that span throughout multiple viral clades.

MATERIALS AND METHODS

Next Generation COBRA design

Next generation COBRA H5 HA antigen were generated through a consensus sequence alignment of H5NX HA sequences from human and avian isolates. Sequences were downloaded through the GISAID database based on area, date of submission and the species of isolation. These sequences were then organized and used to generate multiple consensus sequences in order to capture the repeated and unique H5 epitopes. A rolling COBRA approach was taken to obtain 10-20 primary consensus sequences of a 4-5 year time frames using the sequences taken from: Jan 1st-Dec 31 2011-2012, 2012-2013, 2013-2014, 2014-2015, 2015-2016, and 2016-2017. The time frames used

for the rolling COBRA approach were 4-year long spans (2011-2015, 2012-2016, 2013-2017) and one 5-year long span (2011-2016). The HA sequence was downloaded into Geneious (San Diego, CA, USA) and were aligned using Muscle alignment. The HA1 fragment of each HA sequence was extracted to produce the unique HA sequences. The AAs 17-340 were extracted and were then imported into a new file for re-alignment. The remaining 322 AA are used to create the COBRA HA1 sequence. These sequences were used to generate a phylogenetic tree and are then condensed based upon identity and on the tree. Sequences that are condensed have no more that 2.5% difference and with no ambiguities (X amino acid). Each primary sequence was labeled to represent the original sequences that were used in each primary consensus sequence. These primary consensus sequences were further combined into another phylogenetic tree and were combined to create unique sequences with no ambiguities. Over 50 sequences were generated using this method, but only 8 were chosen due to their unique AA sequence and their placement on the phylogenetic tree. Sequences that were clustered too closely together with wildtype sequences and were not found to be in close associated with the root were ruled out. Each segment was blasted to confirm its uniqueness. The leader sequences (first 17 AA) were taken from a wildtype virus that was closely related to the unique COBRA virus. This was done to ensure the sequence would be properly localized in the cell. The final 8 sequences were generated by Genewiz (South Plainfield, NJ, USA) into out acceptor vector plasmid Zeo+ pcDNA3.1 (Thermo Fisher Scientific, where location).

IAN-1: Traditional COBRA 2013-2017

IAN-2: Rolling COBRA 2011-2015

IAN-3: Rolling COBRA 2011-2015

IAN-4: Rolling COBRA 2012-2016

IAN-5: Rolling COBRA 2011-2015

IAN-6: Rolling COBRA 2012-2016

IAN-7: Rolling COBRA 2014-2018

IAN-8: Rolling COBRA 2011-2016

Recombinant Protein Production

The HA gene cassettes expressing wild type or COBRA HA recombinant protein from the H5NX subtype were cloned into mammalian DNA expression plasmid pcDNA 3.1/Zeo(+)vector (Thermo Fisher Scientific) and were synthesized by Genewiz (South Plainfield, NJ, USA). The plasmid was transformed into Top10 bacterial cell line and was purified using Zympure maxi-prep. The HA1 fragment, which contained a KPNI site was removed from the plasmid and was moved into an acceptor vector containing the Hu-CO2 HA2 domain. The final gene of the HA protein contained an extracellular domains that was terminally fused with the trimeric domain of T4 fibritin, an AviTag sequence and a hexahistidine affinity tag for purification [24]. Each DNA plasmid containing either wild-type or COBRA antigens were transiently transfected into Expi293F HEK suspension cell line (Thermo Fisher Scientific) and was allowed to incubate for 72 hours at 37 degrees C (5% CO2). Supernatants were collected and were tested for protein expression through BCA and Western Blot (His tag antibody). The cells were then pelleted down and the supernatant was purified for protein collection. Soluble HA protein was purified via AKTA Pure System using HisTrap columns following the manufacturers protocol. Eluted fractions were pooled and purified, protein concentration was tested though anti-HIS tag antibody (Biolegend, Sand Diego, CA, USA) using SDS-PAGE and Western blot [25].

Viruses

Viruses were obtained through the Influenza Reagents Resource (IRR) and passaged once in embryonated chicken eggs as per the instructions provided the WHO [26]. Virus lots were tittered with horse erythrocytes and made into aliquots for single-use applications. The H5NX vaccine panel includes the following reassortants viral strains containing internal genes from the mouse adapted strain A/Puerto Rico/8/1934: A/Vietnam/1203/2004 (Vn/04), A/Whooper swan/Mongolia/244/2005 (ws/Mo/05), A/Anhui/1/2005 (An/05), A/Egypt/321/2007 (Eg/07), A/chicken/Vietnam/NCVD-16/2008 (ck/Vn/08), A/Hubei/1/2010 (Hu/10), A/Egypt/N03072/2010 (Eg/10), A/Guizhou/1/2013 (Gu/13), A/Sichuan/26221/2014 (Si/14), A/gyrfalcon/Washington/41088-6/2014 (gyr/WA/14).

Mouse Studies

BALB/c mice (Female, 6-8-week) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in microisolator unites and fed ad libitum. Mice were handled in accordance with UGA and IACUCU protocols and were cared for under the U.S. Department of Agriculture guidelines for laboratory animals. Mice were humanely euthanized in case of weight loss ≥25% of the original weight. After the mice were acclimated for 7 days, they were bled to ensure all were immune naïve prior to vaccination. After naïve mice were confirmed, mice were vaccinated using 5μg of recombinant protein formulated with an oil-in-water nano-emulsion adjuvant AddaVax™ according to manufactures protocols. Mice were vaccinated 3 times on a 4-week interval to obtain appropriate antibody response (n=10). Four weeks following the last vaccination, mice were intranasally infected with 2x107 pfu of recombinant A/Sichuan/26621/2014 virus and 1x107 pfu

of A/Vietnam/1203/2008 PR8 virus. Mice were briefly anesthetized in an isoflurane chamber and were intranasally inoculated with 50μL of virus. The mice were allowed to recover and were monitored 2x daily for weight loss, clinical signs and mortality for up to 14 days.

H&E Staining

To assess the viral replication and pathological effect of infection, mice (n=3) were euthanized 3 days post infection. The right lung lobes were taken for viral plaques and the incision was clamped with a hemostat, a 22 gauge needle was then used to puncture the apex of the heart and sterile PBS was perfused throughout the mouse for 2-3 mins. After the blood was efficiently removed from the lungs, 10% formalin was then perfused to fix the Left lobes. Lungs were removed and placed into formalin for 1-week prior to paraffin embedding. Mouse lungs were embedded into paraffin and were cut using a Lecia microtome. Transverse 5μm sections were placed onto Apex superior adhesive glass slides (Leica biosystem Inc, IL, USA) which were coated for a positive charge. and were processed for H&E staining. Sections were deparaffinized in Xylene and hydrated using different concentrations of ethanol (100%, 95%, 80% and 75%) for 2 mins each. Deparaffinized and hydrated lung sections are stained with Hematoxylin (Millipore sigma, MA,USA) for 8 mins at RT, differentiated in 1% acid alcohol for 10 sec, and then counterstained with Eosin (Millipore sigma, MA,USA) for 30s, slides were dehydrated with 95% and 100% ethanol, cleared by Xylene, and mounted using Permount® mounting media (Thermo Fisher scientific, MA, USA).

Immunohistochemistry staining

The deparaffinizing and hydrating of lung tissues section were subjected to antigen retrieval by sub-boiling in 10nm sodium citrate buffer at PH=6 for 10 mins and then incubated in 3% fresh

made hydrogen peroxide for 10 minutes to inactivate endogenous peroxidase at room temperature. The lung sections were blocked with 5% horse serum in PBS, incubated with mouse Influenza A Nucleoprotein monoclonal antibody at 1:1000 dilution (Bio-Rad, CA, USA) overnight at 4 °C, and then incubated with biotinylated goat-antibody mouse IgG H&L (Abcam, MA, USA) at 1:2000 dilution for 1 hours at RT. The avidin-biotin-peroxidase complex (VectStain Standard ABC kit) (Vector Laboratories, CA, USA) was used to localize the biotinylated antibody, and DAB (Vector Laboratories, CA, USA) was utilized for color development. Sections were then counterstained with hematoxylin, and then mounted using Permount® mounting media (Thermo Fisher scientific, MA, USA). Images were obtained by Aperio digital slide scanner AT2 (Leica biosystem, IL, USA).

Plaque Assays

Viral titers were determined in BALB/c mice using a plaque forming assay as previously described [27-31] using $1x10_6$ Madin-Darby Canine Kidney (MDCK) cells. Mice were euthanized (n=3/group) 3 days post-infection, lungs were taken and snapped frozen and kept at -80°C until processing. Lungs were diluted (10_0 to 10_6) and overlayed onto confluent MDCK cell layers for 1 hour in 200 µL of DMEM supplemented with penicillin-streptomycin. Cells were washed after 1-hour incubation and DMEM was replaced with 4 mL of L15 and 2.4% Avicel (FMC BioPolymer; Philadelphia, PA) (1:1). Cells were incubated for 72 hours at 37°C with 5% CO_2. Avicel and L15 media was removed and washed 2x with sterile PBS, cells were fixed with 10% buffered formalin and stained for 15 mins with 1% crystal Violet. Cells were washed with tap water and allowed to dry. Plaques were counted and the plaque forming units calculated (PFU/mL)

Hemagglutination-Inhibition (HAI) assay

Hemagglutinin inhibition assay (HAI) assay was used to assess receptor-blocking antibodies to the HA protein to inhibit agglutination of horse erythrocytes. The protocol is taken from the CDC laboratory influenza surveillance manual. To inactivate non-specific inhibitors, mouse sera was treated with receptor destroying enzyme (RDE, Denka Seiken, Co., Japan) prior to being tested. Three parts of RDE was added to one-part sera and incubated overnight at 37 degrees C. The RDE was inactivated in 56 degrees C for 30 minutes, when cooled, 6 parts of sterile PBS was added to the sera and was kept at 4 degrees C until use. RDE treated sera was two-fold serially diluted in v-bottom microtiter plates. 25 μL of virus at 8 HAU/50 μL was added to each well (4 HAU per 25 μL). Plates were covered and incubated with virus for 20 minutes at room temperature before adding 1% Horse red blood cells (HRBC) (Lampire Biologicals, Pipersville, PA, USA) in PBS. Red blood cells were washed and stored at 4° C and used within a week of preparation. The plates were mixed by agitation and covered, the RBCs were allowed to settle for 1 hour at room temperature. HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Negative and positive serum controls were included for each plate. All mice were negative (HAI<1:10) for pre-existing antibodies to currently circulating human influenza viruses prior to vaccination.

P-Epitope/P-Sequence Analysis

In order to assess the antigenic distances between the HA sequences used in the vaccines and the HA sequences used in the target strains, a $P_{sequence}$ analysis was performed on the vaccine and virus strain and used to calculated antigenic distances. The epitopic value is calculated by the number of amino acid changes divided by the number of amino acids located in a specific antigenic

epitope. A linear regression analysis was performed in Prism in order to determine a correlation between HAI titer and $P_{epitope}$.

$$P_{sequence} = \frac{\text{Number of substitiutions in the HA1 RBS domain of hemagglutinin}}{\text{Total number of amino acids in the HA1 RBS doamin of hemagglutinin}}$$

RESULTS

All 8-Next generation COBRA vaccines were successfully expressed in mammalian cell lines and generated detectable antibody responses in naïve female 6-8 week old BALB/c mice (Figure 4.1). A preliminary experiment was performed in able to determine which Next Generation COBRA vaccines would be successful in generating a broadly reactive antibody response against multiple virus clades. Along the 8 vaccines used, there were also four wild-type rHA, Hu-CO 2 and a mock control (Figure 4.2). Five viruses were used to test the preliminary panels including WS/05, Gu/13, Hu/10, Si/14 and gy/WA/14. These viruses were used to represent 4 distinct Clade 2 viruses and to spread across timeline (2005-2014) and reassortant status (H5N1 vs H5N6/H5N8) (Figure 4.3 and 4.4). The clades tested in this panel include Si/14 & gy/WA/14 from clade 2.3.4.4, Gu/13 from clade 2.3.4.2, Hu/10 from clade 2.3.2.1 and WS/05 from clade 2.2. Preliminary HAI assays determined that the majority of vaccines behaved similarly to the wild-type rHA comparators, only eliciting antibodies against themselves and against a breath of clades. For example, IAN-3 and IAN-6 behaved very similarly to Si/14 and gy/WA/14, only eliciting antibodies to clade 2.3.4.4 viruses and not to the other 3 viral clades in the panel (Figure 4.3). These antibody responses are very narrow and do not meet the criteria of a broadly protective COBRA vaccines. Other vaccines such as IAN-2 was not immunogenic against any of the viruses on the panel. This could be due to

compromised protein folding or lack of proper glycosylation sites. The failure of this vaccine is unknown, nevertheless, it was removed from animal trials. Overall, only 3 out of the 8 vaccines were successful at eliciting a broad immune response. IAN-4, IAN-7 and IAN-8 all had an average HAI titer of 20 for each 5 viruses used in the panel (Figure 4.3). Wild-type vaccines were also tested to compare the immunogenicity to that of the Next-Generation vaccines. This was used to determine the effectiveness of each vaccine. We showed again that the Hu-CO 2 vaccine was not able to elicit HA specific antibodies against Si/14 or gy/WA/14 [32]. All mock vaccinated mice were serologically negative to the viruses in the panel.

Moving forward, vaccines encoded for IAN-4, IAN-7 and IAN-8 were used for an in-depth study of immunogenicity and survival. Naïve BALB/c mice were vaccinated on a prime-boost-boost regimen and were bleed 2 weeks following the third vaccination to test HAI antibody response (Figure 4.5). Serological analysis reveals that each vaccine elicited a distinct antibody profile against the 10 viruses in the vaccine panel (Figure 4.6). Mice who were vaccinated with the IAN-4 vaccine had high antibody titers against Hu/10 and Gu/13, but did not develop robust antibody titers against the two viral challenge strains VN/04 or Si/14. Mice who were vaccinated with IAN-7 had high HAI antibody titers against viral strains from the clade 2.3.4.4 (Si/14 and gy/WA/14) and moderate titers against An/05 and Eg/07. Mice who were vaccinated with IAN-8 displayed antibody titers against 9 out of the 10 viruses in the HAI panel. High antibody geometric mean titers (GMT) were generated against multiple viruses including VN/04 (GMT: 91), Hu/10 (GMT: 196), Gu/13 (GMT: 80) and Eg/07 (GMT: 211). Mice vaccinated with IAN-8 produced average titers against Si/14 (GMT: 30), gy/WA/14 (GMT: 46), WS/05 (GMT: 56), Eg/10 (GMT: 43) and An/05 (GMT: 65). IAN4, IAN-7 and IAN-8 did not induce titers against the ck/Vn/08 virus.

Mice were intranasally challenged with one of two viruses, either VN/04 ($1x10^7$ pfu/mouse) or Si/14 ($2x10^7$pfu/mouse). Viruses were chosen to represent distinct clade lineages and timelines. Mice who were challenged with the PR8 reassortant strain VN/04 had severe weight loss curves compared to those of the Si/14 challenged mice (Figure 4.8). The survival curves of mice vaccinated with IAN-4, IAN-7 and IAN-8 were closely correlated with the HAI activity to the challenge viruses. For example, whereas IAN-8 vaccinated mice had a HAI GMT of 91.8 and 100% survival, IAN-7 vaccinated mice only elicited a GMT of 5.7 with a 14% survival rate. Therefore, for the vaccine strains in question, the HAI titers elicited from a prime-boost-boost vaccination regimen is directly correlated with the survival of vaccinated mice. Mice that were inoculated with Si/14-PR8 6:2 reassortant virus did not die from infection. Only mice in the control group died following infection (Figure 4.8). However, the weight loss data does show a differential between vaccine groups. In this challenge model, IAN-7 vaccinated mice experienced the least weight loss and clinical symptoms compared to IAN-4 and IAN-8 vaccinated mice (Figure 4.8).

Although mice in this group had a higher survival rate than IAN-4, there were increased viral titers in the lungs of these mice compared to homologous control vaccinated mice (Figure 4.9A). Viral lung loads also showed that all vaccinations protected mice from Si/14 challenge (Figure 4.9B). However, in the VN/04 challenge, mice who were vaccinated with IAN-8 and VN/04 displayed a trend towards lower viral loads.

P-epitope mapping of the COBRA HA constructs and virals HA amino acid sequences revealed a direct correlation between p-epitope value and HAI Cross reactivity. P-epitope analysis was performed on the vaccine and virus strain, as described in Munoz et al [33]. A correlation of p-epitope was performed on the panel of the 2 challenge viruses VN/04 and Si/14 against the 3 vaccine strains IAN-4, IAN-7 and IAN-8 (Figure 4.10).

Lungs were taken on day 3 post vaccination for histopathology analysis using H&E staining (Figures 4.11 & 4.12) and immunohistochemistry (IHC) (Figures 4.13 & 4.14) for specific staining for influenza virus NP nucleoprotein. Mice who were vaccinated with IAN-4, IAN-7, VN/04 and control displayed the highest amounts of cellular infiltrates and inflammation (Figure 4.11 A,B,F&G) when challenged with the Si/14 virus, and were similar to the unvaccinated challenged group (Figure 4.11G). However, mice vaccinated with IAN-8 or the homologous control Si/14 showed more alveolar space and was comparable to the unchallenged mock control lungs (Figure 4.11 C,E&H). The amount of inflammation in IAN-4 vaccinated lungs are correlated with the increased viral titers taken from these lungs (Figure 11A). Lungs taken from mice who were challenged with VN/04 also displayed high inflammation titers in IAN-4 vaccinated mice (Figure 4.12A) and in Si/14 vaccinated mice (Figure 4.12E). IAN-7, IAN-8, Hu-CO 2 and VN/04 vaccines were able to inhibit inflammation compared to the control challenged lungs (Figure 4.12B,C,D,F &G respectively). Notably, the amount of inflammation caused by VN/04 influenza virus challenged was lower than Si/14 challenge. This may be due to the increased cellular trafficking, lowering overall viral pathogenicity.

IHC was also performed on mouse lungs to detect virus within the tissues, which is seen as brown staining. Mice who were challenged with Si/14 virus had the highest amount of virus in epithelial cells in IAN-4, IAN-7, VN/04 vaccinated mice (Figure 4.13 A, B&F) which was comparable to the control unvaccinated mice (Figure 4.13G). Again, we see that the IAN-8, Hu CO 2 and Si/14 vaccinated mice showed the lowest amounts of viral NP staining (Figure 4.13 C, D&E) which was consistent with the amount of inflammation found by H&E staining. Staining performed lungs taken from VN/04 virus challenged mice showed heavy viral staining on epithelial cells on IAN-4, IAN-7, Si/14 and control unvaccinated groups (Figure 4.14 A, B, E&G, respectively). IAN-8 vaccinated groups were comparable to mock unchallenged lungs (Figure 4.13 C&H) with the least viral NP staining. VN/04 vaccinated lungs still contained viral NP staining in epithelial cells, but increased cellular infiltrates were consistent with a lack of NP binding. Suggesting viral clearance in these areas.

DISCUSSION

In this study, COBRA HA designed vaccines were evaluated in a mouse model by comparing their immunogenicity, cross reactivity and protective efficacy. Three vaccines, IAN-4, IAN-7 and IAN-8 were able to protect mice from a lethal challenge of Si/14 with no viral titers detected in lung of mice at day 3 post-infection. When challenged with a lethal dose of VN/04, mice vaccinated with IAN-8 rHA had little weight loss and decreased viral lung titers compared to IAN-4 and IAN-8 vaccinated mice.

It is highly desirable to generate a pandemic vaccine that elicits heterologous protection against multiple viral clades. We assessed the ability of Next Generation COBRA HA vaccines to induce cross reactive immunogenic responses against multiple H5 viral clades. Interestingly, IAN-8 vaccination induced antibody titers against 9 out of the 10 viruses in the HAI panel. However, we found that gy/WA/14 vaccinated mice did not elicit sera with any HAI activity against the VN/04 virus and ~71% of the vaccinated mice survived a lethal challenge. The immune correlates of protection for H5 have not been as well established as the correlates for seasonal influenza viruses, however in this study, the antibody titer elicited by the COBRA HA vaccines against a specific strain, directly correlated with survival. This conflict appears to be systemic within H5 vaccine research. Previous studies have also discovered increased survival rates and decreased pathogenicity without detectable serum-antibody titers present [38, 39]. This non-HAI protection has been attributed to stem-based antibodies [40] and/or anti-NA antibodies [41]. However, for this study, only rHA vaccines without NA were included. The role of cellular immunity cannot be ruled out for this study. The use of Oil-in-water adjuvants such as MF59 enhances T-cell specific influenza vaccine immunity [42, 43]. The role of cellular immunity induced by COBRA vaccination needs to be further analyzed.

Previous data from our laboratory showed that Hu-CO-2 VLP vaccinated mice did not survive challenge and elicited no HAI antibody titers against Si/14 virus. However, neutralizing antibody titers were detectable at high concentrations [32]. However, the Hu-CO 2 vaccinated mice survived challenge. This may be due to increased antibody titers against HA specific epitopes since these vaccines lack of NA and GAG proteins that are included in VLP vaccine formulations. This vaccine decreased the pathogenicity of the H5N6 virus. Future studies to access the efficacy of

IAN-8 should be performed to include highly pathogenic variants of H5N6 or H5N8 to ensure proper viral loads and survival curves. However, obtaining highly pathogenic wild-type strains of viruses in a research facility is difficult and PR8 reassortant strains are more easily obtained. Challenge with a 2.3.2.1 virus should also be performed to ensure that IAN-8 elicited antibodies are sufficient to protect mice from death and lung pathology.

A p-epitope analysis of the three-vaccine strains were performed in order to examine the specific epitopes that were essential for HAI titer elicitation. P-epitope values were plotted against HAI titers for IAN-4, IAN-7 and IAN-7 against VN/04 and Si/14 viruses. Although the specific antigenic sites for H5 is not entirely known, Velkov *et al.* [44] described a broad guideline for antigenic sites. In addition, the amino acid 282 was also included [45, 46]. Antigenic sites for H5 viruses are not as well defined as the seasonal influenza strains such as H1N1 and H3N2. For this study, we defined the antigenic sites according to the review article by Velkov *et al.*, where sites are designated by color and antigenic sites with overlapping monoclonal antibodies [44]. A modification was performed of P-epitope in order to calculate P-epitope, which has been previously discussed [33-36]. The P-epitope was calculated using only the sites associated with receptor binding site (RBS). This is an important tool that can be used when designing vaccines against pandemic strains of viruses.

Future studies using a ferret model of disease will need to be performed to ensure the protective efficacy of IAN-8 vaccination against a highly pathogenic strain of clade 2.3.4.4 virus and 2.3.2.1 virus. When designing the Next Generation COBRA HA vaccines, the goal was to produce a unique HA proteins that encompassed not only the 2.3.4.4 clade, but also other clades that are

circulating in avian species, specifically clade 2.3.2.1 which has been reported to be circulating in Bangladesh, China and India [2]. These results strongly suggest that the IAN-8 rHA vaccine in combination with an oil-in-water adjuvant is a possible candidate for pre-clinical trials against clade 2.3.4.4 and 2.3.2.1 viruses, which are the dominant circulating clades in wild waterfowl populations throughout the world.

Figures

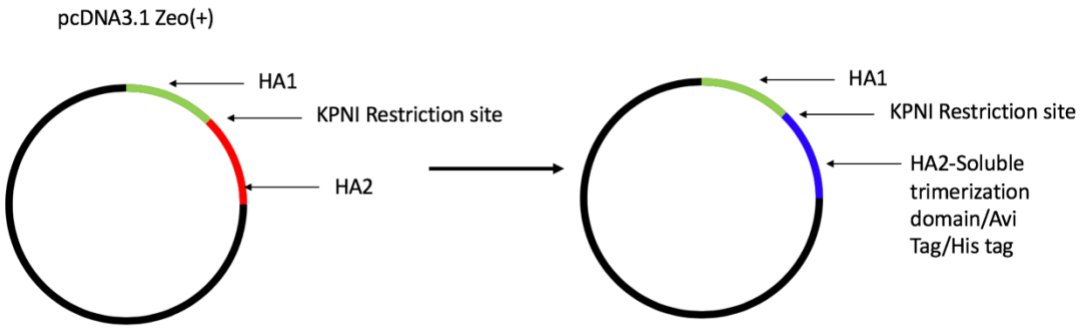

Figure 4.1: Schematic of HA expression plasmids. Unique HA amino acid sequences were placed into pcDNA3.1 Zeo (+) cassettes mammalian expression vectors. The HA1 fragment was swapped into an acceptor vector containing the HA2 domain from Hu-CO 2. The HA2 domain contained T4 fibritin fold on domain and AviTag/His tag markers for purification.

Group	Vaccine	Mice
1	IAN-1	8
2	IAN-2	8
3	IAN-3	8
4	IAN-4	8
5	IAN-5	8
6	IAN-6	8
7	IAN-7	8
8	IAN-8	8
9	A/Sichuan/26221/2014	8
10	A/gyrfalcon/Washington/41088-6/2014	8
11	A/chicken/Egypt/CAL3-RLQP/2017	8
12	Human COBRA	8
13	WS/05	8
14	Mock	8

Figure 4.2: Table of recombinant HA proteins used to vaccinate mice in preliminary serological study. BALB/c female mice were vaccinated on a prime-boost-boost regimen with soluble recombinant HA protein in combination with an oil-in-water emulsion Addavax adjuvant. COBRA vaccines termed IAN-1 – IAN-8 were used along with wild-type comparators to compare the antibodies elicited in a HAI panel.

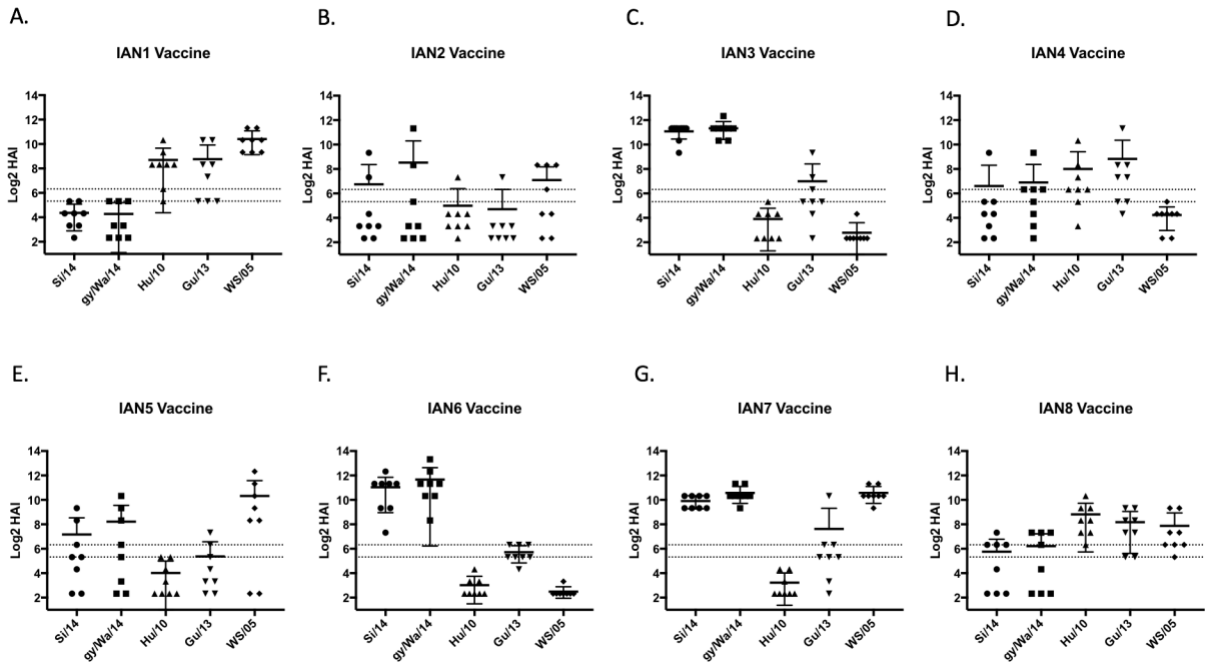

Figure 4.3: Virus HAI antibody panel from next generation COBRA vaccines. Five viruses from 4 viral clades were chosen to represent clades that are currently circulating around the globe in wild waterfowl populations. The virus panel is arranged from newest virus (2014) to the oldest (2005). The viral clades include 2.3.4.4, 2.3.2.1, 2.3.4.2 and 2.2 respectively. IAN-4, IAN-7 and IAN-8 were chosen to continue with further studies. Other vaccines were excluded due to their similar breath to wildtype vaccines or lack of immunogenicity.

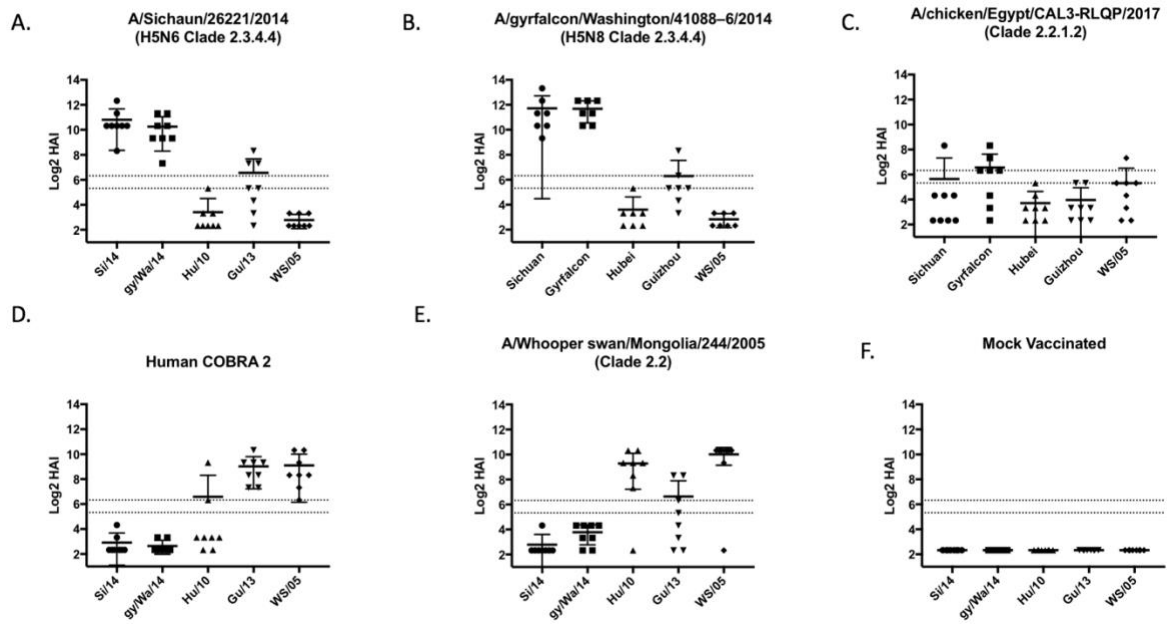

Figure 4.4: Virus HAI antibody panel against antibodies elicited from soluble rHA vaccines encoding wild-type HA sequences and Hu-CO 2 vaccine. Mice vaccinated with rHA encoding wild-type sequences elicited a limited antibody breath against the 5 viruses in the chosen panel. Vaccines encoding HA sequences from clade 2.3.4.4 viruses were only able to elicit antibodies against other 2.3.4.4 viruses and not against the 2.3.2.1 virus Hu/10. The vaccine contained A/ws/Mo/05 elicited antibodies towards Hu/10, Gu/13 and itself, but did no elicit antibodies against 2.3.4.4 clades. Mock vaccinated mice were seronegative to all the viruses in the panel.

Group	Vaccine	Mice
1	IAN-4	16
2	IAN-7	16
3	IAN-8	16
4	HU-CO	16
5	A/VN/04	16
6	A/SI/14	16
7	A/gy/WA/14	16
8	Control	16

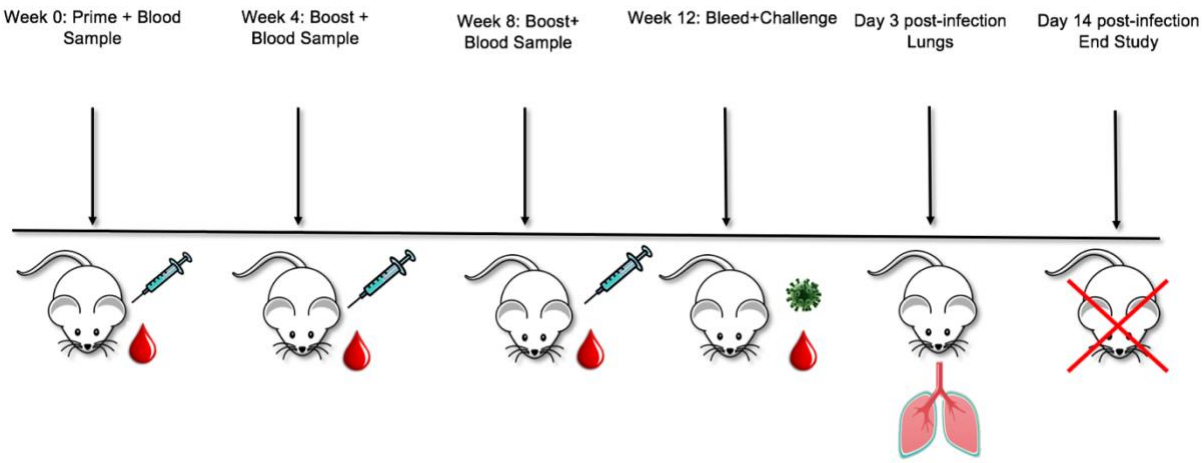

Figure 4.5: Schematic representation of mouse vaccine study. BALB/c female mice 6-8 weeks of age were vaccinated intramuscularly with 5µg of rHA in combination with Addavax. Eight groups of nice (n=10) were vaccinated with one of the 7 vaccines listed in the table above. Blood was taken at weeks 0, 2, 4, 8 and 10 to ensure antibody response was efficient prior to challenge. 4-weeks following the last boost, mice were intranasally challenged with either Vn/04 or Si/14. Three days post challenge, a subset of mice (n=3) were sacrificed to access viral loads and pathology. Mice were monitored 2x a day for the next 14 days to measure weight loss and clinical scores.

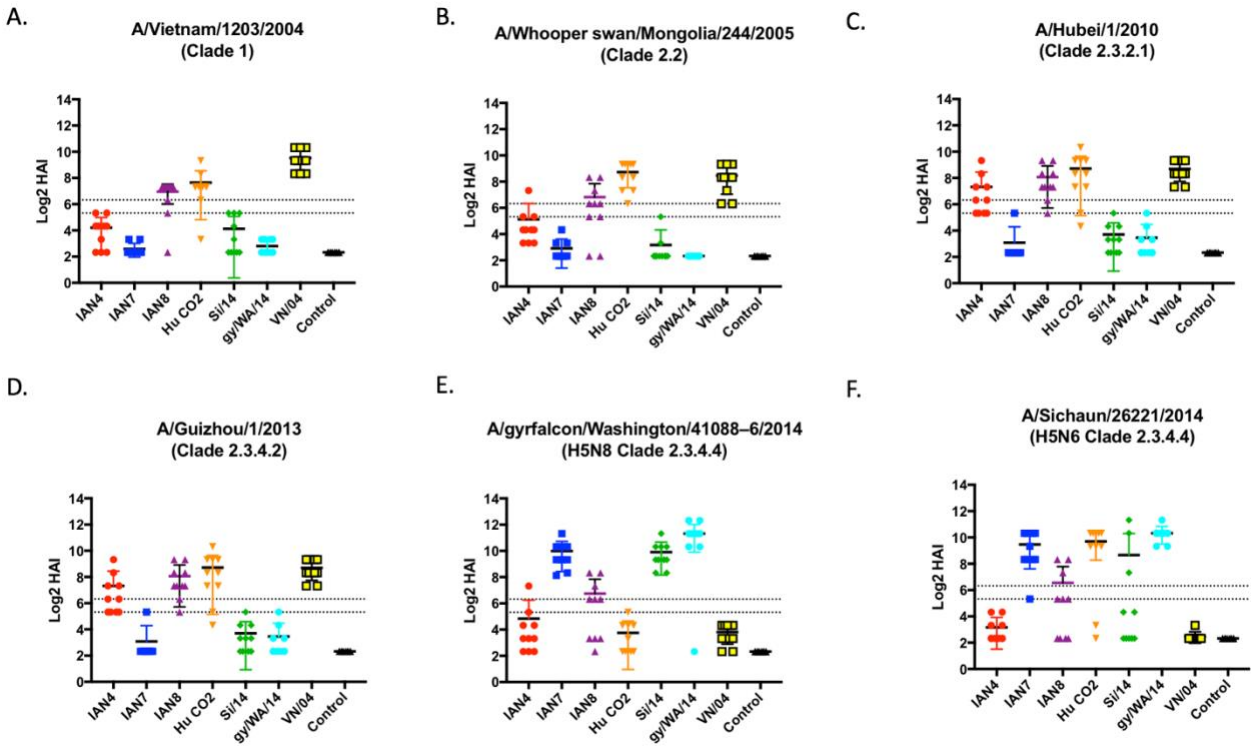

Figure 4.6: Vaccine induced antibodies against six PR8-backbone viruses. Serum from week 10 mice was taken to assess the immunological response against a HAI panel of H5Nx viruses using horse erythrocytes. Antibody responses were calculated according to serum dilution, a value of 5 was given for a negative response. The two dashed lines represent HAI titers of 20 and 40. Each COBRA generated vaccine displayed a unique pattern of antibody response.

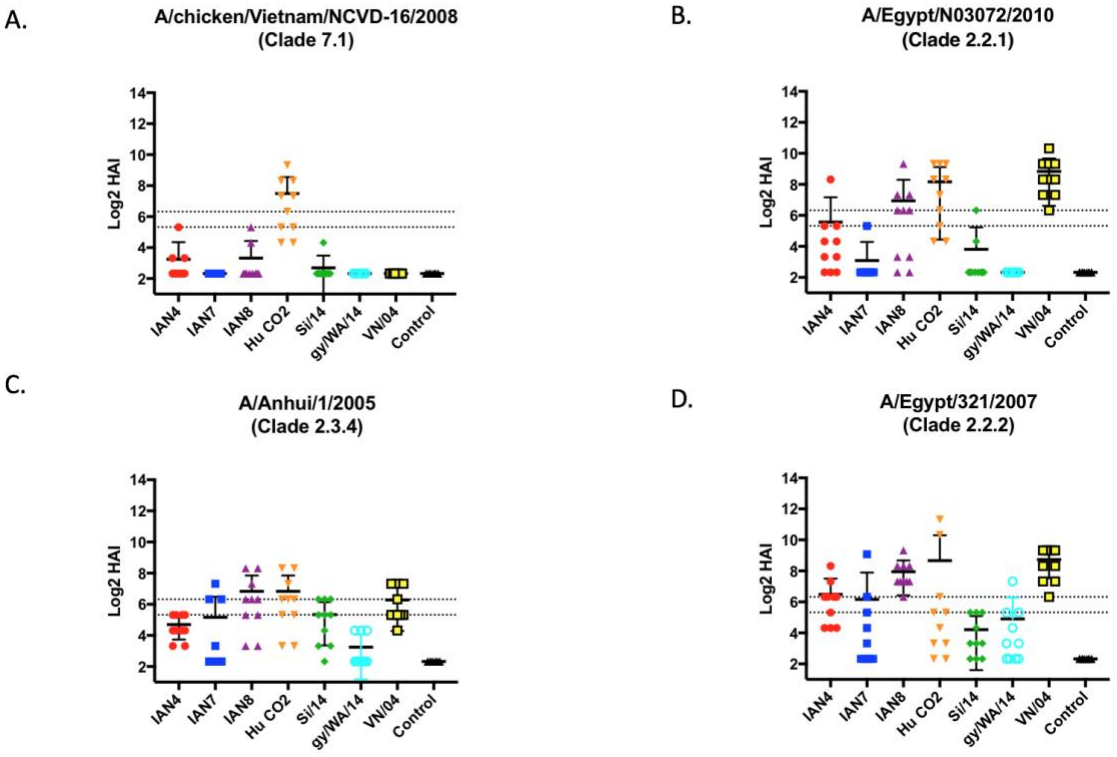

Figure 4.7: Vaccine induced antibodies against four PR8-backbone viruses. Serum from week 10 mice was taken to assess the immunological response against a HAI panel of H5Nx viruses using horse erythrocytes. Antibody responses were calculated according to serum dilution, a value of 5 was given for a negative response. The two dashed lines represent HAI titers of 20 and 40.

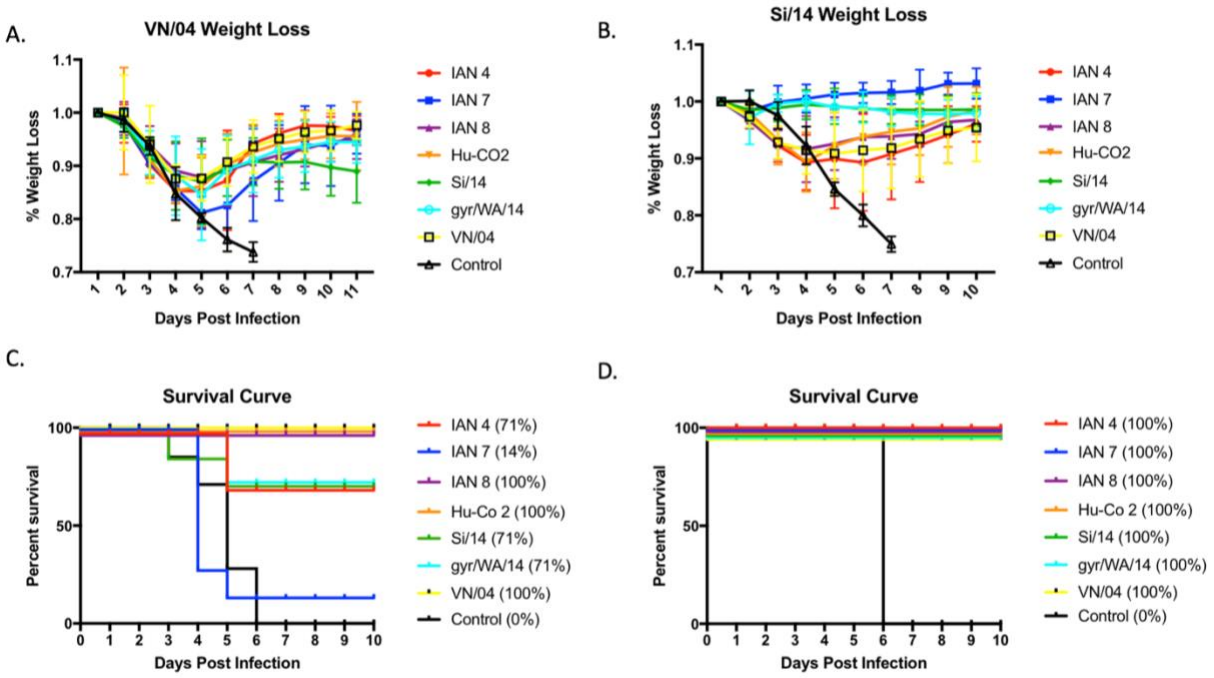

Figure 4.8: Weight loss data and survival curves from Vn/04 and Si/14 intranasal challenge. Four-weeks following the last boost vaccination, mice were intranasally challenged with Vn/04 or Si/14 PR8 virus. Mice challenge with Vn/04 experienced severe weight loss in comparison to those challenged with Si/14. Mice who lost more than 25% of their original weight loss were humanely euthanized. Mice who were vaccinated with IAN-8 had 100% survival rate when challenged with Vn/04 PR8 virus, whereas IAN-4 and IAN-7 were less effective. In Si/14 challenge, all vaccinated mice survived challenge, however, mice vaccinated with IAN-7 experience the lease weight loss when compared to IAN-4 and IAN-8.

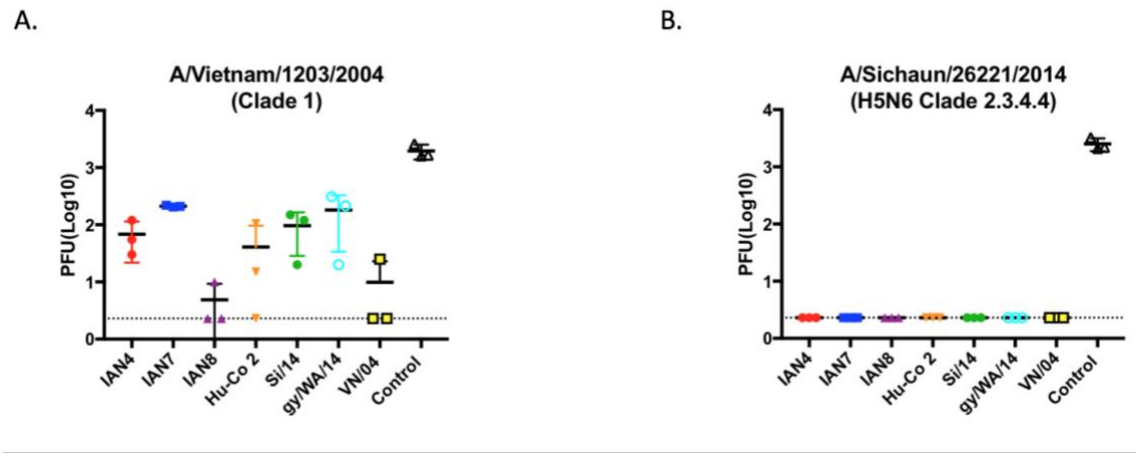

Figure 4.9: Viral lung titers obtained from mice 3-days post challenge. A subset of mice was randomly chosen to be sacrificed 3-days post intranasal infection for lung removal. Lungs were snap-frozen and were stored to process viral lung titers. Lungs were homogenized, remaining supernatants were collected and placed on MDCK monolayers for incubation. Fixation and crystal violet staining revealed viral plaques which were counted and calculated based upon dilution factor. Mice who were vaccinated with IAN-4 and IAN-7 had viral lung titers between 2-3 Log10 PFU. Mice who were vaccinated with IAN-8 has a trend towards lower lung titers but was not statistically significant. As expected, control mice who were not vaccinated had the highest titers compared to vaccinated mice. All vaccinated mice who were challenged with Si/14 had no viral lung titers. This is consisted with the lack of mortality in these groups.

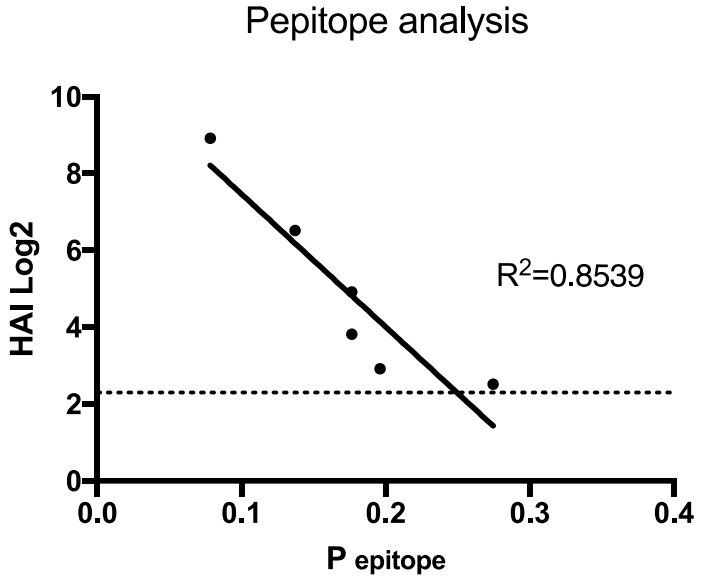

Figure 4.10: P-epitope analysis of HAI data plotted against amino acid sequence similarity. There were 51 amino acids total analyzed in the HA1 portion of the H5 molecule. Amino acid sequences were aligned and analyzed using Geneious® software. the number of amino acid differences was divided by the total amino acid epitopes associated with the RBS. Linear regression analysis was performed on all strains of virus and vaccine HAI titer, there was a significant correlation between HAI titer and Psequence. $P_{sequence} = \frac{\text{Number of substitiutions in the HA1 RBS domain of hemagglutinin}}{\text{Total number of amino acids in the HA1 RBS 113omain of hemagglutinin}}$

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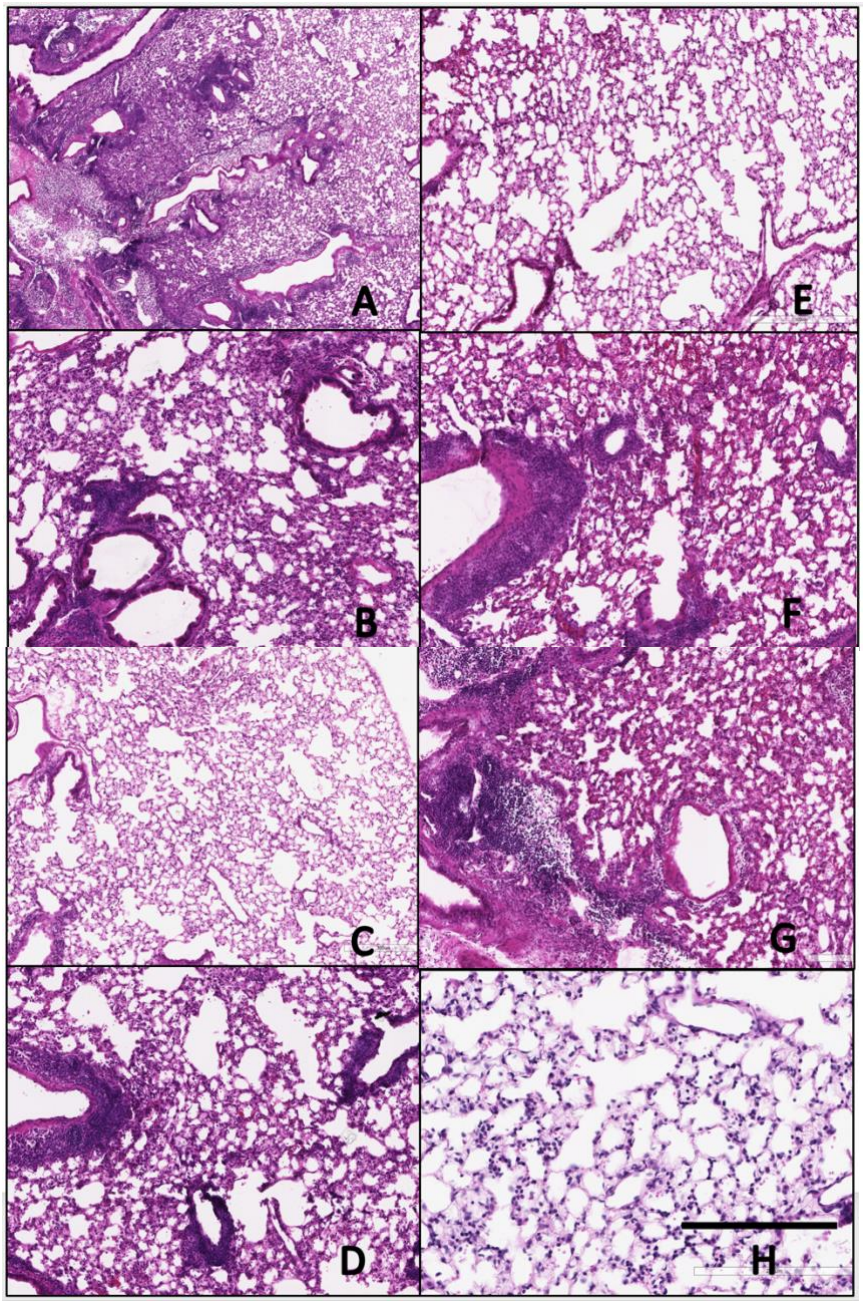

Figure 4.11: Histopathology of lungs taken 3 days following viral infection of Si/14 virus. Lungs were taken from euthanized mice and fixed in formalin for one week prior to examination. Hematoxylin and Eosin staining (H&E) was performed on 5 µm lung slices to determine pathology and cellular infiltrates in vaccinated and non-vaccinated groups. A) IAN-4, B) IAN-7, C) IAN-8, D) Hu-CO 2, E) Si/14, F) VN/04, G) control infected H) control mock (not infected). Bar represents 300µm.

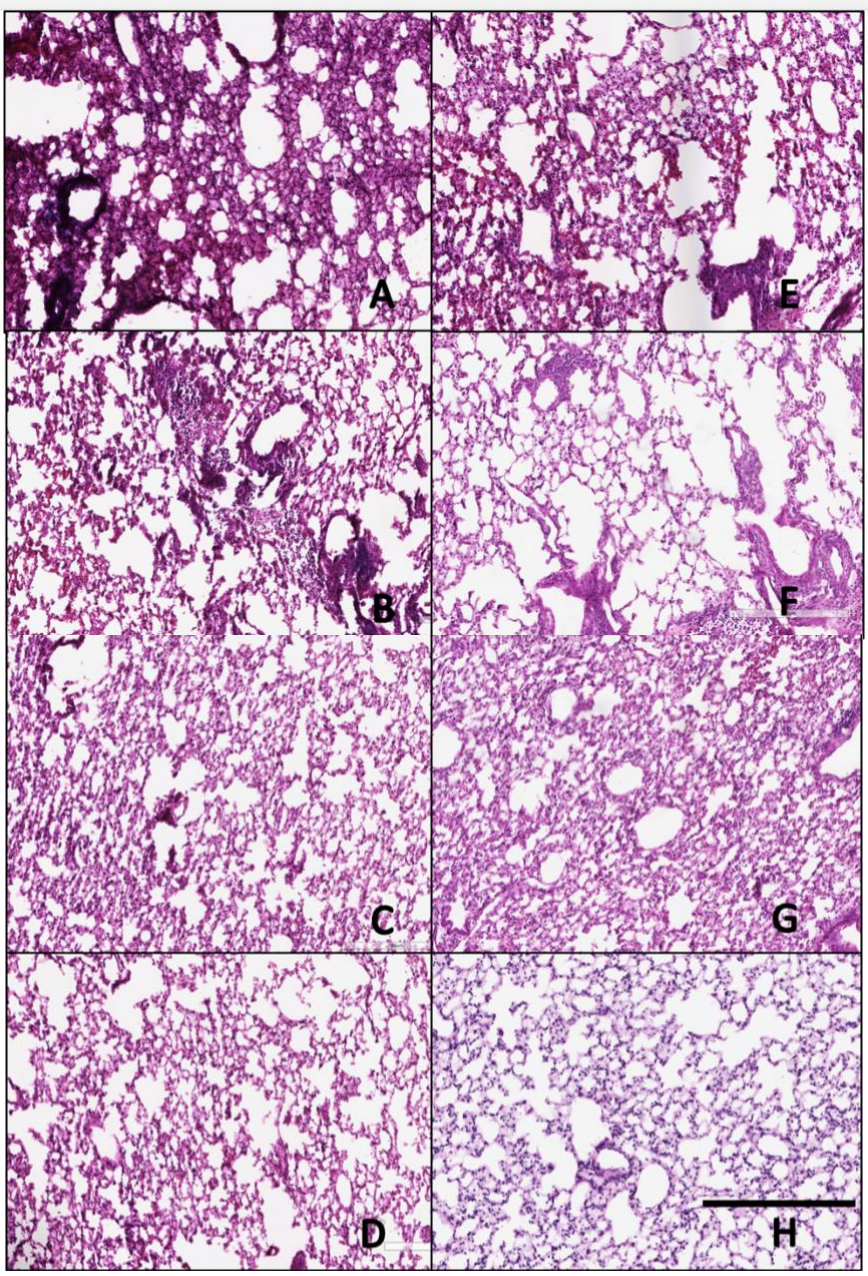

Figure 4.12: Histopathology of lungs taken 3 days following viral infection of VN/04 virus. Lungs were taken from euthanized mice and fixed in formalin for one week prior to examination. Hematoxylin and Eosin staining (H&E) was performed on 5 µm lung slices to determine pathology and cellular infiltrates in vaccinated and non-vaccinated groups. A) IAN-4, B) IAN-7, C) IAN-8, D) Hu-CO 2, E) Si/14, F) VN/04, G) control infected H) control mock (not infected). Bar represents 300µm.

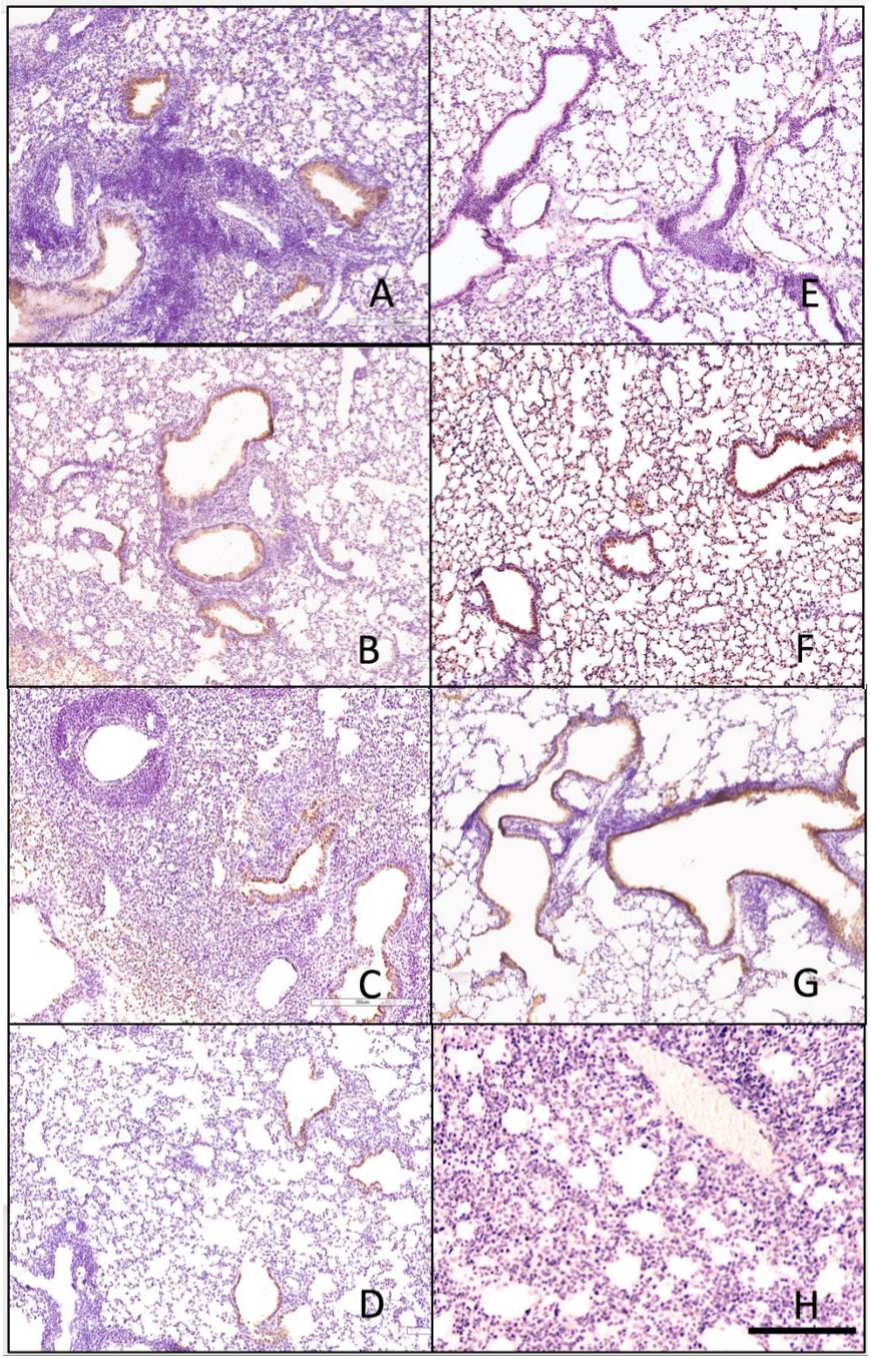

Figure 4.13: Immunohistochemistry of lungs taken 3 days following viral infection of Si/14 virus. Lungs were taken from euthanized mice and fixed in formalin for one week prior to examination. Hematoxylin and Eosin staining (H&E) was performed on 5 µm lung slices to determine pathology and cellular infiltrates in vaccinated and non-vaccinated groups. A) IAN-4, B) IAN-7, C) IAN-8, D) Hu-CO 2, E) Si/14, F) VN/04, G) control infected H) control mock (not infected). Bar represents 300µm.

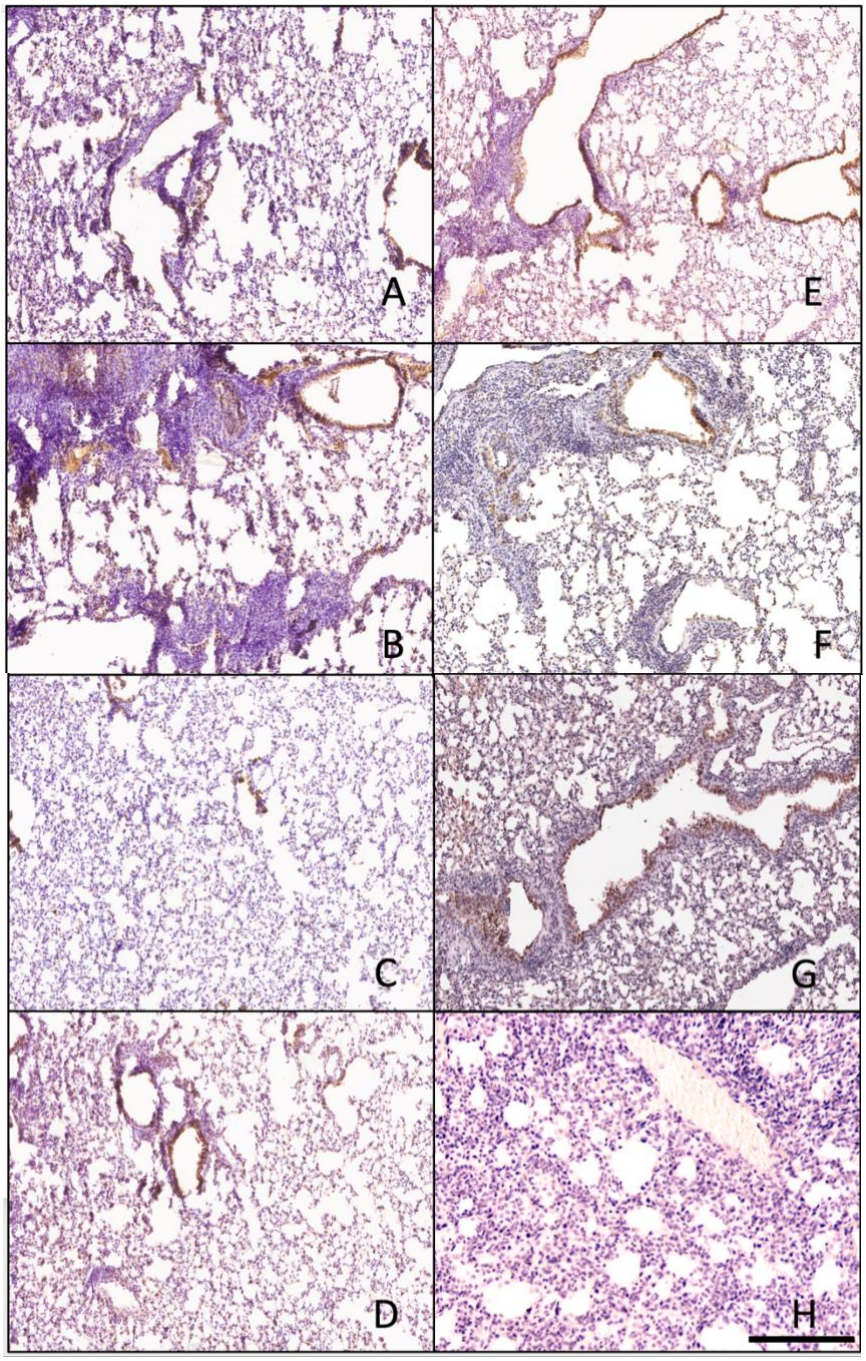

Figure 4.14: Immunohistochemistry of lungs taken 3 days following viral infection of VN/04 virus. Lungs were taken from euthanized mice and fixed in formalin for one week prior to examination. Hematoxylin and Eosin staining (H&E) was performed on 5 µm lung slices to determine pathology and cellular infiltrates in vaccinated and non-vaccinated groups. A) IAN-4, B) IAN-7, C) IAN-8, D) Hu-CO 2, E) Si/14, F) VN/04, G) control infected H) control mock (not infected). Bar represents 300µm.

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CHAPTER 5

H5N1 INFECTION IN THE NAÏVE AND PREIMMUNE FERRET MODEL

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ABSTRACT

The effectiveness of inactivated influenza virus vaccines have been affected by a person's immune imprinting, age, sex and immunocompromised status. An individual's first exposure to influenza virus has a life-long effect that can determine subsequent immune reactions to infection and/or vaccination. Previously, it has been demonstrated that the year a person was born directly correlates with that individual's immune imprinting and therefore antibody repertoire induced by vaccination. The effect of H5N1 virus vaccination on imprinted individuals has not yet been thoroughly assessed. Therefore, a pre-immune ferret model was designed to determine the effect of group 1 or group 2 viral imprinting on pre-pandemic H5N1 virus infections and H5N1-based vaccination. Group 1 imprinting protects ferrets from infection of group 1 pre-pandemic virus infection. In addition, group 2 imprinted ferrets were partially protected from H5N1 virus infection and these animals suffered from a delayed onset of symptoms compared to group 1 imprinted ferrets. Subsequent infection with H1N1 and H3N2 influenza viruses in immunologically naïve ferrets did not interfere with the protection induced by group 1 imprinting. All animals survived challenge with no clinical signs or detectable viral titers. Vaccination of H3N2 influenza viruses (group 2) imprinted ferrets with either seasonal influenza vaccination or human COBRA 2 (Hu-CO 2) virus-like particles ameliorated disease in these ferrets and offered complete protection. Further studies confirmed that soluble recombinant HA protein version of Hu-CO 2 or A/Vietnam/1203/2004 HA protected 100% of the H3N2 imprinted ferrets. Ferrets vaccinated with a soluble, recombinant tetrameric neuraminidase from the H1N1 A/California/04/2009 (CA/09) were also protected against highly pathogenic H5N1 virus challenge, however, the CA/09 HA did not protect all ferrets from disease. Therefore, pre-immunity stems from both HA and NA induced

antibodies and the HA alone from an H1N1 virus was not sufficient to protect ferrets pre-immune to H3N2 influenza viruses.

INTRODUCTION

Infection with seasonal influenza virus early on in life is thought to create an imprint on the immune system [1]. Primary infection with an influenza virus followed by infection with a similar virus elicits antibodies against the first primary virus. This imprinting, also known as "antigenic sin" [2] or "antigenic seniority" [3] is thought to dampen the serological response against subsequent infection with a heterologous strain of virus. Subsequent infection with influenza virus recalls antibody responses against shared antigens, even if the original antigen becomes a secondary or lesser component [4]. The term original antigenic sin (OAS) has been correlated with negative effects such that recall responses are targeted towards antigenic epitopes that have undergone antigenic drift, and recognition of the drift variant is lost [5]. Multiple studies have shown an ineffective cross reactivity to new strains of influenza virus while maintaining protective antibody titers to the primary infection strain [6, 7]. Other studies have shown that repeated vaccination results in the loss of antibody titer completely [8, 9]. These studies however measured antibody protection through HAI assay which only measures the antibody response against the HA head to block sialic acid binding; repeated vaccination can elicit antibody responses against HA-stem regions which is not detectable via HAI. Serological studies of human exposure and seasonal vaccination have shown that pre-immune status often narrows antibody cross-reactivity to influenza viruses, depending on age or date of birth of the individual [3, 10-12]; but does not inhibit the response to unique strains.

Memory B-cells are activated upon infection with similar influenza viruses. Secondary infection with influenza virus will most-likely result in a recall response, even if the virus from primary infection is unique compared to the new virus [13]. Memory B cells have an advantage over naïve B cells, memory B cells initiate responses faster than naive B cells and also divide faster than naive B cells [14, 15]. Memory B cells enter their first division 20-30 hours early than naïve B cells [15]. Naïve B cells can be activated in a secondary infection but the virus either has to be delivered at a high does or administered with immune activators such as Bordetella pertussis toxin or squalene based oil in water emulsion adjuvants [16]. Recalled memory B-cells in response to infection with a heterologous virus do undergo somatic hypermutation in the antibody variable regions and can lead to antibodies that have higher affinities for new antigen [13, 15]. Cross reactive antibodies that have higher binding affinities for the primary viral strain can also provide protection against heterologous viral challenge, albeit binding specificities were much lower in comparison to the primary strain [15].

Although pre-immunity with seasonal influenza viruses have been previously investigated, the effects of pre-pandemic H5Nx vaccination on a seasonal pre-immune background have not been thoroughly explored. Using epidemiological data, there is an age-related response against H5N1 viral infection in the human population. The highest incidence of H5N1 virus infection and mortality occurred in people born between 1957-2009 [17]. Elderly individuals with no record of prior exposure to avian influenza virus had heterotypic binding antibodies against H9N2 and H5N1 influenza viruses isolated in Europe [18]. Individuals who were vaccinated with inactivated seasonal influenza elicited increased cross-protective antibodies following vaccine administration

[18]. Twenty-five of the 174 participants seroconverted to the H5N1 influenza virus, A/Vietnam/1194/2004, after seasonal influenza vaccination. However, ~1% of people who elicited heterotypic antibodies against this H5N1 influenza virus [18]. The age of the individuals (74-79 mean age) in the study may have influenced the results since they had been exposed to viruses similar to the Spanish influenza virus (H1N1 virus) of 1918-1919. This data is in agreement with the epidemiological study performed by Gostic *et al.* [17] that suggested individuals born after 1957 were most susceptible to H5N1 viral (group 1) induced morbidity and were most likely to be imprinted with H3N2 (group 2) viruses [17]. Individuals born before 1957, who were most likely imprinted with a group 1 of influenza viruses (H1N1) had lower case incidences of H5N1 virus infection [17]. Pre-immunity with group 1 or group 2 viruses elicits a long-lasting immunity after exposure to heterologous viruses. Therefore, the pre-immune status of a person may ultimately have an effect on pre-pandemic vaccination strategies and must be investigated in order to produce a successful H5Nx vaccine.

The development of a protective avian influenza virus vaccine has proven to be a difficult task. An avian influenza virus vaccine should be capable of inducing long-lasting memory responses, neutralizing titers, cross-clade protection and cellular and humoral responses. Human clinical trials with avian influenza vaccinations have shown varying results and complications. Avian influenza virus vaccines delivered as inactivated viruses are a safe approach to immunizing naïve populations, but can also have negative set-backs. Inactivated vaccines take 6 months to produce, and can often result in limited immunogenicity and elicit a poor cellular immune response [19]. Inactivated vaccines are also often grown in eggs, which introduces a large amount of egg proteins and can sometimes have vaccine reactogenicity especially in people who are younger that 23 years

of age [20]. Egg viral growth can also introduce glycosylation's that are normally not present in the wild-type strain of virus and can result in a vaccine that is not protective against circulating strains [21]. Growth of HPAI viruses is also highly lethal to embryos and is often difficult to propagate safely in eggs [19]. Most inactivated vaccines for H5N1 are poorly immunogenic and require at least two doses to elicit a long-lasting immune response [19]. Unadjuvanted inactivated split virus and sub-unit H5N1 vaccines elicit neutralizing antibody titers in only 58% of individuals who are vaccinated [22]. Antibody titers in these recipients decreased substantially 6 months after their second dose [22]. The recipients were therefore offered a third does to boost their antibody response, and protective titers were seen in 78% and 67% depending on the dose [23]. Other studies have shown that the combination of inactivated influenza and adjuvant can induce a long-lasting response, however the vaccine doses were very high and neutralizing antibody titers were highest in children aged 6 months-17 years of age [24, 25]. Recombinant protein based vaccines have shown promising results; rHA vaccines are well tolerated but do not easily elicit neutralizing antibodies after two doses [26]. The current pre-pandemic vaccine that is stockpiled is a subvirion vaccine with recombinant H5N1 HA derived from A/Vietnam/1203/2004 virus. Although vaccine was well tolerated it only induced neutralizing titers of 1:40 in 43% of the participants [22]. Heterologous prime-boost performed with this sub-unit vaccine however has been seen to induce cross-clade protection, specifically to clade 2.3.4.4 viruses [27, 28]. However, there are still challenges to providing protection against highly variable and rapid diverging strains of avian influenza viruses and limited number of antigens that can be stockpiled.

In order to test vaccine efficacy and pre-immunity, an influenza virus model must be utilized. Fitch ferrets are commonly used as influenza experimental models. Although mice are most commonly

used for influenza research, they lack clinical symptoms that follow influenza virus infection such as fever, sneezing, nasal discharge and inflammation [29]. The ferret displays clinical symptoms that are similar to that of humans. Ferrets were discovered as influenza virus models in 1933, they can be infected with influenza A viruses that do not require adaptation [30]. Ferrets can also transmit influenza viruses to susceptible cage mates and carry the α-2,6 linked sialic acid receptors in their upper respiratory traction which helps transmit the virus via airborne droplets [31, 32]. Because of the extensive nature of housing ferrets, sample sizes for experimental purposes are often small (n=4-5). The investigation of pandemic viruses such as H5N1 and H7N9 has increased the use of ferrets in influenza studies. Pre-immune status greatly determines antibody production and repertoire and therefore is an area of interest that should be considered when developing a vaccine for pre-pandemic preparedness. This study aimed to determine the protective response of group 1 vs group 2 pre-immunity on H5N1 virus infection and the responses to vaccination depending on pre-immunity.

MATERIALS AND METHODS

Vaccine Preparation

Mammalian 293T cells were transfected with each of three plasmids expressing the influenza neuraminidase (A/Thailand/01/2004,), the HIV p55 Gag sequences and Human COBRA 2 HA expressing plasmids on previously described mammalian expression vectors [33-38]. After 72 h of incubation at 37°C, supernatants from transiently transfected cells were collected, centrifuged to remove cellular debris, and filtered through a 0.22-µm-pore-size membrane. Mammalian derived VLPs were purified and sedimented by ultracentrifugation on a 20% glycerol cushion at

27,000 rpm for 4 hours at 4°C. VLPs were resuspended in phosphate-buffered saline (PBS), and the total protein concentration was assessed by a conventional bicinchoninic acid assay. The hemagglutination activity of each preparation of VLPs was determined by adding equal volume Horse red blood cells (RBCs) to a V-bottom 96-well plate, followed by incubation with serially diluted volumes of VLPs for 60 min at room temperature.

Soluble recombinant HA or NA were also produced for vaccination. The HA/NA gene cassettes expressing wild type or Human COBRA 2 HA recombinant protein were cloned into mammalian DNA expression plasmid pcDNA 3.1/Zeo(+)vector (Thermo Fisher Scientific) and were synthesized by Genewiz (South Plainfield, NJ). The plasmid was transformed into Top10 bacterial cell line and was purified using Zympure maxi-prep. The HA1 fragment, which contained a KPNI site was removed from the plasmid and was moved into an acceptor vector containing the Hu-CO2 HA2 domain. The final gene of the HA protein contained an extracellular domains that was terminally fused with the trimeric domain of T4 fibritin, an AviTag sequence and a hexahistidine affinity tag for purification [39]. Each DNA plasmid containing either wild-type or Hu-CO2 HA antigens were transiently transfected into Expi293F HEK suspension cell line (Thermo Fisher Scientific) and was allowed to incubate for 72 hours at 37 degrees C (5% CO2). Supernatants were collected and were tested for protein expression through BCA and Western Blot (His tag antibody). The cells were then pelleted down and the supernatant was purified for protein collection. Soluble HA protein was purified via AKTA Pure System using HisTrap columns following the manufacturers protocol. Eluted fractions were pooled and purified, protein concentration was tested though anti-HIS tag antibody (Biolegend, Sand Diego, CA, USA) using SDS-PAGE and Western blot [40].

The Quadrivalent inactivated Fluzone® 2018-2019 vaccine (Sanofi Pasteur, Swiftwater, PA, USA) contained the strains A/Michigan/45/2015 (H1N1)pdm09-like virus, A/Singapore/INFIMH-16-0019/2016 A(H3N2)-like virus, B/Colorado/06/2017-like (Victoria lineage) virus (updated), and B/Phuket/3073/2013-like (Yamagata lineage) virus. Ferrets were vaccinated using 500 µL (15 µg) intramuscularly without adjuvant.

Determination of HA content in VLPS

Protein concentration was determined by MicroBCA™ Protein Assay Kit (Thermo Fisher Scientific; Pittsburgh, PA, USA). HA concentration was determined the by western blot and densitometry. Purified VLPs were prepared in standard total protein amounts and were electrophoresed on 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with anti-HA clade 1 influenza A viruses (Immune Technology Corporation; New York, NY, USA) monoclonal antibody. HA-antibody complexes were detected using a goat anti-mouse IgG conjugated to horse radish peroxidase (HRP) (Southern Biotech; Birmingham, AL, USA). HRP was detected by chemiluminescent substrate Clarity™ Western ECL substrate (Bio-Rad Laboratories; Hercules, CA, USA). Density of WT HA bands were used to calculate a standard curve and the density of the purified VLPs was interpolated using the results from the WT HA. Experiments were performed in duplicates. Density of bands was determined using myImageAnalysis™ Software (Thermo Fisher Scientific; Waltham, MA, USA).

Viruses

H1N1, H3N2, and H2N3 viruses were obtained through the Influenza Reagents Resource (IRR), BEI Resources, the CDC or were provided by Sanofi-Pasteur. Viruses were passaged once under the same growth conditions as they were received in embryonated chicken eggs with the instructions provided by the WHO. Titers of virus lots were determines with both guinea pig and turkey erythrocytes and divided into aliquots for single-use applications. Viruses used to infect ferrets were as follows: H1N1: A/Singapore/6/1986 (Sing/86, $1x10^6$ pfu/mL), A/California/07/2009 (Cal/09) and A/AA/Marton/1943 (Mar/43). H3N2: A/Panama/2007/1999 (Pan/99, $1x10^6$ pfu/mL), A/Port Chalmers/1/1973 (PC/73), A/Hong Kong/1/1968 (HK/68). H2N3: A/Swine/Missouri/4296424/2006 (sw/MO/06). H5N1: A/Vietnam/1203/2004 (VN/04, $1x10^5$ pfu/mL).

Animal Studies

Fitch ferrets (*Mustela putorius furo*, female and male, 6 to 12 months of age), negative for antibodies to circulating influenza A (H1N1, H3N2, H5N1, H5N2, H5N6, H5N8) and influenza B viruses were de-scented and purchased from Triple F Farms (Sayre, PA) or sent to us by our collaborator Dr. Alyson Kelvin from Dalhousie University. Ferrets were pair housed in stainless-steel cages (Shor-line Kansas City, KS) containing Sani-Chips laboratory animal bedding (P J. Murphy Forse Products, Monteville, NJ). Ferrets were provided with Teklad Flobal Ferret Diet (Harlan Teklad, Madision WIS) and fresh water ad libitum. The University of Georgia Institutional Animal Care and Use Committee approved all experiments which were conducted in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals*, The Animal Welfare Act, and the CDC/NIH *Biosafety in Microbiological and biomedical laboratories*

guide. Ferrets (n=6-8) were intranasally inoculated with an H1N1 seasonal isolate (A/Singapore/ or a H3N2 isolate. Animals were monitored for weight loss, loss of activity, nasal discharge, sneezing and diarrhea and will be allowed to recover for 90 days. At day 90, ferrets were vaccinated with either VLP, rHA antigens, or inactivated seasonal influenza vaccine intramuscularly. Two weeks following vaccination, ferrets were bled to assess the serological antibody response against homologous and heterologous avian influenza strains. If antibody titers are not elicited after one vaccination, ferrets may have to be boosted for a second time. Vaccines will consist of 15 µg or rHA formulated with AddaVax™ adjuvant (San Diego, CA, USA), State, USA) at a 1:1 ratio. Four weeks after final vaccination, ferrets were challenged intranasally with $1\times10_4$ plaque forming units (PFU) of the highly pathogenic H5N1 virus A/Vietnam/1203/2004 (Clade 1) in a volume of 0.5 ml in each nostril for a total infection volume of 1 ml. After infection, ferrets were monitored daily for weight loss, disease signs and death for 14 days after infection. Individual body weights, sickness scores, and death were recorded for each group on each day after inoculation. Sickness score was determined by evaluating activity (0 = normal, 1 = alert and active with stimulation, 2 = alert but not active after stimulation, 3 = not alert or active after stimulation), nasal discharge (0 = absent, 1 = present), sneezing (0 = absent, 1 = present), decreased food intake (0 = absent, 1 = present), diarrhea (0 = absent, 1 = present), dyspnea (0 = absent, 1 = present) and neurological symptoms (0 = absent, 1 = present). Nasal washes on day 3 after inoculation. Washes were collected and stored at−80 ◦C until use. Experimental endpoint was defined as >25% weight loss, development of neurological symptoms, or an activity score of 3 (not active or alert after stimulation). All H5N1influenza virus studies were performed under high-containment biosafety level 3 enhanced conditions (BSL3+). All procedures were in accordance

with the NRC Guide for the Care and Use of Laboratory Animals and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories

ELISA

Immulon 4HBX plates (Thermo Fisher) were coated overnight at 4°C with of cH6/1 in carbonate buffer (pH 9.4) at 0.5 µg/ml containing 5 µg/ml fraction V bovine serum albumin (BSA [Equitech-Bio, Kerrville, TX]; 50 µl/well) in a humidified chamber. The plates were then blocked with 200 µl/well of ELISA blocking buffer (PBS containing 0.2% BSA plus 0.1% bovine gelatin and 0.05% Tween 20) for 1.5 h at 37°C. Serum samples were serially diluted in blocking buffer, and the plates were incubated overnight at 4°C. The plates were washed three times with PBS-Tween (PBS-T, .05%). Then, 100 µl/well of biotinylated goat anti-ferret IgG H&L HRP (Cambridge, MA) diluted at 1:10000 in blocking buffer was added, and the plates were incubated for 1 h at 37°C. The plates were washed four times with PBS-T. 100 µL of ABTS [2,2=azinobis (3-ethylbenzthiazolinesulfonic acid); Amresco, Solon, OH] substrate with .16% H_2O_2 was added, and the plates were incubated at 37°C for 25 min. Colorimetric conversion was terminated by the addition of 5% SDS (50 µl/well), and the optical density was measured at 414 nm using a spectrophotometer (BioTek, Winooski, VT). After subtraction of the background, End point titers were determined as the reciprocal dilution of the last well which had an OD414 above the mean OD414 plus three times the standard deviations of naïve animal sera.

Determination of viral nasal wash titers

Plaque assays were performed in a high-level biosafety containment facility. Lung samples and nasal wash samples taken at day 3 post-infection were snapped frozen and kept at -80°C until

processing. Lungs were homogenized using a plunger and .2 µm strainer. Madin-Darby canine kidney (MDCK) cells were seeded 24 h prior to use at ($5x10_5$) in each well of a six-well plate. The nasal wash and lung homogenate samples were diluted (final dilution factors of 100 to 10_6) and overlaid onto the cells in 200 µl of Dulbecco modified Eagle medium supplemented with penicillin-streptomycin, followed by incubation for 1 h in 37°C with 5% CO2. Samples were removed, the cells were washed twice, and the medium was replaced with 2 ml of L15 medium plus 0.8% agarose (Cambrex, East Rutherford, NJ), followed by further incubation for 72 h at 37°C with 5% CO2. The agarose was removed and discarded. The cells were fixed with 10% buffered formalin for 10 mins and then stained with 1% crystal violet for 5 min. The plates were then thoroughly washed in distilled water to remove excess crystal violet before being air-dried; the numbers of plaques were then counted, and the numbers of PFU per milliliter were calculated.

H&E Staining

To assess the viral replication and pathological effect of infection, mice (n=3) were euthanized 3 days post infection. The right lung lobes were taken for viral plaques and the incision was clamped with a hemostat, a 22 gauge needle was then used to puncture the apex of the heart and sterile PBS was perfused throughout the mouse for 2-3 mins. After the blood was efficiently removed from the lungs, 10% formalin was then perfused to fix the Left lobes. Lungs were removed and placed into formalin for 1-week prior to paraffin embedding. Mouse lungs were embedded into paraffin and were cut using a Lecia microtome. Transverse 5µm sections were placed onto Apex superior adhesive glass slides (Leica biosystem Inc, IL, USA) which were coated for a positive charge. and were processed for H&E staining. Sections were deparaffinized in Xylene and hydrated using different concentrations of ethanol (100%, 95%, 80% and 75%) for 2 mins each. Deparaffinized

and hydrated lung sections are stained with Hematoxylin (Millipore sigma, MA,USA) for 8 mins at RT, differentiated in 1% acid alcohol for 10 sec, and then counterstained with Eosin (Millipore sigma, MA,USA) for 30s, slides were dehydrated with 95% and 100% ethanol, cleared by Xylene, and mounted using Permount® mounting media (Thermo Fisher scientific, MA, USA).

RESULTS

Influenza group imprinting determines disease and survival in a pre-immune ferret model

In order to determine the effect of influenza A virus imprinting on pandemic H5N1 challenge, naïve female ferrets (n=6) were intranasally inoculated with either H1N1 (Sing/86), H3N2 (Pan/99) or PBS (Figure 5.1). Ferrets were allowed to recover from seasonal virus infection and were tested for seroconversion 14 days following infection. Viruses used to establish pre-immunity did not cause severe disease, but elicited a long-lasting immune response. Following challenge, ferrets were allowed to rest for 84 days. For H5N1 challenge, the ferrets were moved into a high-level biosafety facility and were allowed to acclimate for 3 days prior to challenge. Ferrets were briefly anesthetized and intranasally challenged with $1x10_5$ pfu/ml of VN/04 virus. Weights and clinical scores were taken daily until ferrets reached humane endpoint or until the end of the study. Ferrets who were inoculated with Sing/86 all survived challenge (Figure 5.2A) had little weight loss and had no clinical signs (Figure 5.2A and 5. 2C). However, ferrets who were inoculated with Pan/99 virus experienced clinical symptoms on day 6 post challenge such as lethargy and neurological symptoms (Figure 5.2B and 5.2C). There was significant weight loss in ferrets pre-immune to H1N1 or H3N2 viruses between days 3 and day 8 (Table 5.1). Ferrets, inoculated with the H3N2 influenza virus, began to succumb to disease on day-6 post challenge with high clinical

scores in 4 out of 6 ferrets (66%) (Figure 5.2A). Ferrets who were inoculated with PBS all died from H5N1 virus infection 10-days following challenge and had significant weight loss compared to ferrets pre-immune to either H1N1 or H3N2 influenza viruses (Table 1).

Sera collected from ferrets prior to H5N1 influenza virus challenge was tested for IgG antibodies against the VN/04-HA protein. H1N1 pre-immune ferrets contained cross reactive antibody titers against the VN/04-HA protein (Figure 5.2D). Whereas, only two H3N2 pre-immune ferrets had cross-reactive antibodies against VN/04. Both of these ferrets survived challenge with VN/04. All ferrets sero-converted to the pre-immune challenge strains (Figure 5.3). Sera was also tested against the chimeric HA protein C6/1 to assess the stalk antibody response. The chimeric protein contains an H6 head and a H1 stalk, assuming the ferrets do not have antibodies that are elicited against H6 HA head portion, antibodies that are detected are most likely against the H1 stalk domain [41]. Only two ferrets from the H1N1 pre-immune group had HAI titers against the C6/1 protein. Therefore, influenza group imprinting determines the ability recall cellular immune responses and mount a protective response against pandemic H5N1 challenge and does not include stem antibody responses.

Sequential infection with a group 2 virus does not inhibit group 1 influenza virus imprinting

In a secondary study, we investigated whether group 1 influenza virus imprinting could skew the memory response and interfere with protection against a pre-pandemic H5N1 influenza virus infection. Male naïve ferrets (n=12) were inoculated with an H1N1 strain (Mar/43) and an H3N2 (HK/68), and a sequential infection of H1N1 and H3N2 following 30 days after initial infection. Ferrets were allowed to recover 60 days following the last intranasal infection and were moved

into a high-level biocontainment facility to challenge with VN/04 virus ($1x10^5$ pfu/ml) (Figure 5.4). Similar to the previous study, ferrets who were imprinted on with an H1N1 influenza virus/group 1 all survived H5N1 influenza virus challenge and had no clinical symptoms (Figure 5.5A and 5.5B). H1N1 influenza virus imprinted ferrets had no detectable weight loss, compared to the H3N2 influenza virus imprinted ferrets who had severe weight loss and clinical symptoms (Figure 5.5B and 5.5C). All H3N2 influenza virus infected ferrets were moribund by day 7 post-H5N1 influenza virus challenge. Most noticeably, H3N2 influenza virus imprinted ferrets developed neurological symptoms, extreme lethargy, and hind limb paralysis prior to loosing 75% of their weight.

Nasal washes from all ferrets (n=12) were collected on day 3 post-challenge to determine viral titers in the nasal cavity. Interestingly, only ferrets who were pre-immunized with the H3N2 influenza virus had measurable virus in the nasal wash specimens (Figure 5.5D). Ferrets who were sequentially infected with H1N1 and H3N2 had no weight loss or clinical symptoms following H5N1 influenza virus challenge. Noticeably, ferrets who were sequentially immunized had greater recovery of weight loss compared to ferrets who were solely pre-immune to H1N1 influenza virus. Statistical analysis revealed that days 2-6, ferrets who were sequentially infected with H1N1+H3N2 influenza virus less weight loss compared to H1N1 pre-immunized group (Figure 5.5C, Table 5.1). H3N2 pre-immunized ferrets lost a significant amount of weight compared to either H1N1 or H1N1+H3N2 virus sequentially infected ferrets at days 5, 6 and 7 (Figure 5.5C, Table 5.2). Serological data taken from a subset of ferrets (n=4) was tested for cross-reactive antibodies against VN/04 HA and the C6/1 HA chimeric protein. Ferrets who had been pre-immunized with H1N1 influenza virus developed antibodies against the VN/04 HA protein,

whereas the H3N2 influenza virus pre-immune ferrets were all negative to VN/04 HA (Figure 5.6). Interestingly, ferrets who were sequentially infected with H1N1+H3N2 had significantly lower antibody titers against VN/04 HA compared to H1N1 influenza virus pre-immune ferrets (p=.026). In the H1N1 influenza virus only pre-immune group, 2 out of 4 ferrets elicited antibody titers against the C6/1 HA protein, whereas 3 out of 4 of the sequentially infected ferrets were positive for C6/1 stalk antibody (Figure 5.6B). This data suggests that the stalk directed antibody response may not be a correlate of heterosubtypic protection.

Three days following H5N1 influenza virus challenge, ferrets from each group (n=4) were humanely sacrificed and lung punches were taken from upper and lower quadrants from the right lungs. Plaque assays were performed in order to determine viral titers. Upper and lower lung punches taken from ferrets pre-immunized with H1N1 or H1N1+H3N2 influenza viruses had no detectable viral lung titers (Figure 5.7). However, punches taken from H3N2 influenza virus infected ferrets had high titers in both upper and lower quadrants (Figure 5.7). Therefore, H3N2 influenza virus induced pre-immunity does not provide protection against H5N1 influenza virus challenge, and only ferrets pre-immunized with a group 1 viruses, either Sing/86 or Mar/43, can elicit a protective response against the H5N1 influenza virus pathogenic challenge. Ferrets who were sequentially pre-immunized had increased weight gain compared to H1N1 influenza virus infected ferrets, but had significantly lower antibody IgG titers against VN/04 HA protein. Therefore, antibodies elicited against VN/04 HA protein may not be essential to provide protection against lethal challenge with H5N1 influenza viruses.

Vaccination with either Hu-CO 2 or seasonal Fluzone® ameliorates disease in H3N2 influenza virus pre-immune ferrets

Although H1N1 influenza virus pre-immunity has shown to elicit a protective immune response against H5N1 virus challenge, other group-1 virus induced immunity has not been previously tested. The H2N3 influenza virus also belongs to the group 1 influenza virus clade, and could potentially protect against H5N1 virus infection. Using the H2N3 virus subtype as an immune imprinting group also removes the possible effects of cross-protective neuraminidase interactions between H1N1 and H5N1 influenza virus. Female ferrets were pre-immunized with either an H3N2 (PC/73, n=3), H2N3 (sw/Mo/06, n=4), or sequential vaccination of H1N1+H2N3 (CA/09 and sw/Mo/06, n=4) influenza viruses and were allowed to recover for 84 days prior to challenge with H5N1 influenza virus. Again, the H3N2 influenza virus pre-immunized ferrets experienced significant weight loss compared to group 1 imprinted ferrets (Figure 5.9A, Table 3). In the H3N2 influenza virus pre-immune group, 1 out of 3 ferrets succumbed to disease due to the H5N1 influenza virus infection and had neurological symptoms and hind-limb paralysis (Figure 5.9B). However, H2N3 influenza virus imprinted ferrets experienced no significant weight loss and all survived challenge, along with H1N1+H2N3 influenza virus sequentially infected ferrets (Figure 9A and B). Nasal wash titers taken from ferrets on day 3 post-challenge showed viral titers in only the H3N2 influenza virus pre-immune groups (Figure 5.9C). We therefore conclude that all ferrets who are imprinted on with a group 1 virus, regardless of subtype or group 1 strain and can survive challenge with H5N1 influenza virus.

In a following experiment, vaccination of H3N2 influenza virus pre-immune ferrets was tested using Hu-CO 2 VLP formulated with Addavax™ or quadrivalent Fluzone™ seasonal influenza

vaccine. the seasonal influenza vaccine has previous been reported to protect ferrets against challenge with H5N1 influenza virus challenge, and may help protect the human population from a pandemic outbreak by eliciting temporary non-specific antibody responses [42-44]. Naïve female ferrets (n=10) were pre-immunized with H2N3, H3N2 and H1N1+H2N3 influenza viruses. Ferrets were then allowed to recover from infection for 84 days and were immunized on a prime-boost model with 15μg of VLPs expressing Hu-CO 2 formulated with an oil-in-water adjuvant Addavax™ or quadrivalent Fluzone® (2018-2019) split inactivated vaccine. Four-weeks following the last boost, ferrets were intranasally challenged with H5N1 influenza virus. Vaccination with either Hu-CO 2 VLPs or Fluzone rescued H3N2 influenza virus pre-immune ferrets from clinical symptoms and disease, and these animals did not die from infection (Figure 9D). Therefore, the H3N2 influenza virus group 2 imprinting can be rescued by pre-pandemic vaccination of a chimeric H5 HA VLP protein expressing Hu-CO 2 or through vaccination of seasonal influenza viruses.

Recombinant protein vaccination protects ferrets from lethal challenge with H5N1 influenza virus

The previous studies have showed that group 1 imprinting by either an H1N1 or H2N3 influenza viruses can induce protective immune responses against H5N1 influenza virus challenge. However, the exact mechanism of protection has not been elucidated. Due to the possibility that the neuraminidase from H1N1 CA/09 influenza virus could be aiding in protection, vaccination of solely CA/09 HA or CA/09 NA was performed. In addition, VN/04 HA homologous vaccination and Hu-CO 2 HA vaccination was also tested in a one-shot regimen. A dose sparing technique is of major concern when developing a proper pre-pandemic vaccine, therefore a one vaccination

technique, including adjuvant, should be investigated. Female and male fitch ferrets (n=8) were pre-immunized with an H3N2 (Tex/12) influenza virus by intranasal infection and the animals were allowed to recover for 84 days (Figure 5.10). Following recovery, ferrets were intramuscularly vaccinated with soluble recombinant containing either: VN/04 HA, CA/09 HA, Hu-CO 2 HA, or CA/09 NA formulated with Addavax™ or no vaccination (control). One group (n=8) was intramuscularly vaccinated with seasonal quadrivalent Fluzone® vaccine (500 µl). Four-weeks following vaccination, ferrets were intranasally challenged with the VN/04, H5N1 influenza virus ($1x10_5$ pfu/ml) (Figure 5.10). Ferrets were weighed daily and monitored for clinical signs (Figure 11A and B). Ferrets that were solely vaccinated with CA/09 HA had significant weight loss and clinical symptoms compared to Fluzone® vaccinated ferrets on days 7, 8, 9 and 14 post-infection ($p \leq 0.05$) (Figure 5.10A). At day 14 post-infection, there were no weight loss differences between CA/09 HA vaccinated and the control ferrets. Similar to the previous studies, ferrets displayed neurological signs or were found to be moribund before they reached the 75% weight cut off and experienced high clinical scores (Figure 5.10C). The control ferrets who received only intranasal infection with Tex/12 H3N2 influenza virus with no vaccination had the greatest weight loss with only 28% of ferrets surviving challenge (Figure 5.11B). Interestingly, ferrets who were vaccinated with CA/09 HA had ~40% survival. Ferrets who were vaccinated with either CA/09 NA, VN/04 HA, Hu-CO 2 HA, or Fluzone® all survived challenge (Figure 5.11B). High viral titers were detected in the nasal washes of control ferrets at day 3 post-challenge, however those vaccinated ferrets had no significant viral titers in their nasal washes (Figure 5.11D).

On day-3 post-challenge, three ferrets from each group were euthanized and lung punches from upper and lower quadrants were snap frozen and later process for viral titers. All ferrets had viral titers in both upper and lower quadrants with those in the control group and CA/09 HA vaccinated having the highest viral titers in the upper quadrants (Figure 5.12). Ferrets vaccinated with Fluzone® or VN/04 HA had low viral titers in the upper respiratory tract compared to control vaccinated ferrets and CA/09 HA vaccinated ferrets.

ELISA IgG assays were used to tested collected ferret sera collected prior to challenge to confirm that ferrets had seroconverted to their vaccine strain and against the pre-immune H3N2 influenza strain, Tex/12 (Figure 5.13A and 5.13B). Ferrets who were vaccinated with CA/09HA, VN/04 HA and Fluzone displayed cross reactive antibody titers against the CA/09 HA protein (Figure 5.13A). As expected, ferrets who were vaccinated with CA/09 NA contained high antibody titers against the CA/09 HA protein. Vaccinated ferrets with VN/04 HA or CA/09 HA had anti-HA antibodies against VN/04 HA (Figure 5.13D), however these antibodies did not protect against H5N1 influenza virus challenge. Ferrets vaccinated with Fluzone® developed no detectable antibody titers against the VN/04 HA protein.

Histology

Three days post-challenge, 3 ferrets from each group were humanely euthanized, lungs were collected from left quadrant and the tissue was perfused. All the tissues were stored in 300ml of a 10% formalin solution until tissues were fixed. Two slices from upper and lower lobes were collected for histopathology and were processed for H&E staining (Figure 5.14). All tissues collected from ferrets displayed signs of inflammation (Figure 5.14). Control ferrets displayed the

most inflammation in the upper quadrants of the lungs (Figure 5.14 A&B) with little to no alveolar space. Lower quadrants of control ferrets were also impacted by inflammation, especially in the lowest quadrant taken of the lung (Figure 5.14 C&D). Fluzone® vaccinated ferrets also displayed large amounts of cellular inflammation which correlates with the viral titers found in lung punches (Figure 14 E-H). Ferrets who were vaccinated with CA/09 NA protein had the most cellular infiltrates located in the upper lung (Figure 5.14 I&J) and had less inflammation in the lower lung quadrants (Figure 5.14 F&L). This is consistent with viral titers (Figure 5.12) where a trend of viral load is concentrated in the upper lung. Interestingly, ferrets who were vaccinated with the Hu-CO 2 vaccine had the least amount of inflammation in both upper and lower lung specimens (Figure 5.14 M-P). VN/04 and CA/09 HA vaccinated ferrets showed no differences in inflammation (Figure 5.14 Q-X)

DISCUSSION

In these studies, group 1 imprinting determines clinical outcomes in pre-pandemic H5N1 influenza virus infection. The study involved naïve female fitch ferrets that were H1N1 influenza virus imprinted and resulted in complete protection from H5N1 influenza virus challenge with little weight loss and no clinical signs. Interestingly, ferrets who were pre-immunized with a group 2 influenza virus of the H3N2 subtype were partially protected. These studies have previously been performed, however, one main difference was the timeline in which the H5N1 influenza virus was administered. A previous study showing H1N1 influenza virus/Group 1 imprinting provided protection against H5N1 influenza virus challenge, but was administered 30 days following imprinting. This timeline may not be suitable in detecting a memory response and may allow non-

specific circulating antibodies aid in protection. Allowing the ferrets to recover for 84 days enabled the response of H1N1 influenza virus /group 1 pre-immunity to be elicited from a recall cellular response. Intranasal infection using seasonal influenza viruses or vaccination with TIV/QIV does not elicit HAI or neutralization titers against H5N1 influenza viruses [42, 43, 45]. Therefore, a cellular immune response including the effect of antibody-dependent cellular

Interestingly, no vaccinated ferrets were able to completely inhibit viral replication in the upper or lower quadrants of the lungs, as previously reported [45]. Studies will need to be performed in order to determine if Fluzone® vaccination elicits a memory-cell response sufficient enough to protect group-2 imprinted ferrets from H5N1 influenza virus challenge. Vaccination with trivalent-inactivated influenza vaccine administered three times was not sufficient to protect ferrets from infection 3 months following the last vaccination [45]. However, intranasal infection with live virus from either H1N1 or H3N2 influenza virus was sufficient protect ferrets from disease in H5N1 influenza virus challenge 30 days following pre-immunity [45].

It can be speculated that the protective responses induced by seasonal influenza virus infection are primarily transient [43]. A study using passive transfer of human sera into mice collected from individuals who were vaccinated with a seasonal influenza virus vaccines provided partial protection to naïve mice against viral challenge. However, the ability to provide heterosubtypic immunity diminished 30 days following the initial vaccination [43].

Figures

Figure 5.1: Schematic representation of pre-immune ferret study. Naïve fitch ferrets (*Mustela putorius furo*, female) 6-8 months of age were intranasally infected with a Group 1 influenza virus (H1N1 Sin/86), Group 2 influenza virus (H3N2 Pan/99) or with PBS (control) and were monitored for 14 days following infection. Sera was collected to test for antibody responses on day 14, 28 and 84 post-preimmunization. Ferrets were allowed to recover for 84 days post-challenge. Ferrets were transported into a high-level biocontainment facility for H5N1 challenge with VN/04 ($1x10^5$ pfu/ml) and were monitored daily for weight loss and clinical symptoms. On day 3 post-challenge, ferrets were briefly anesthetized and nasal wash specimens were taken to test for viral titers. The study was ended 14 days post-challenge.

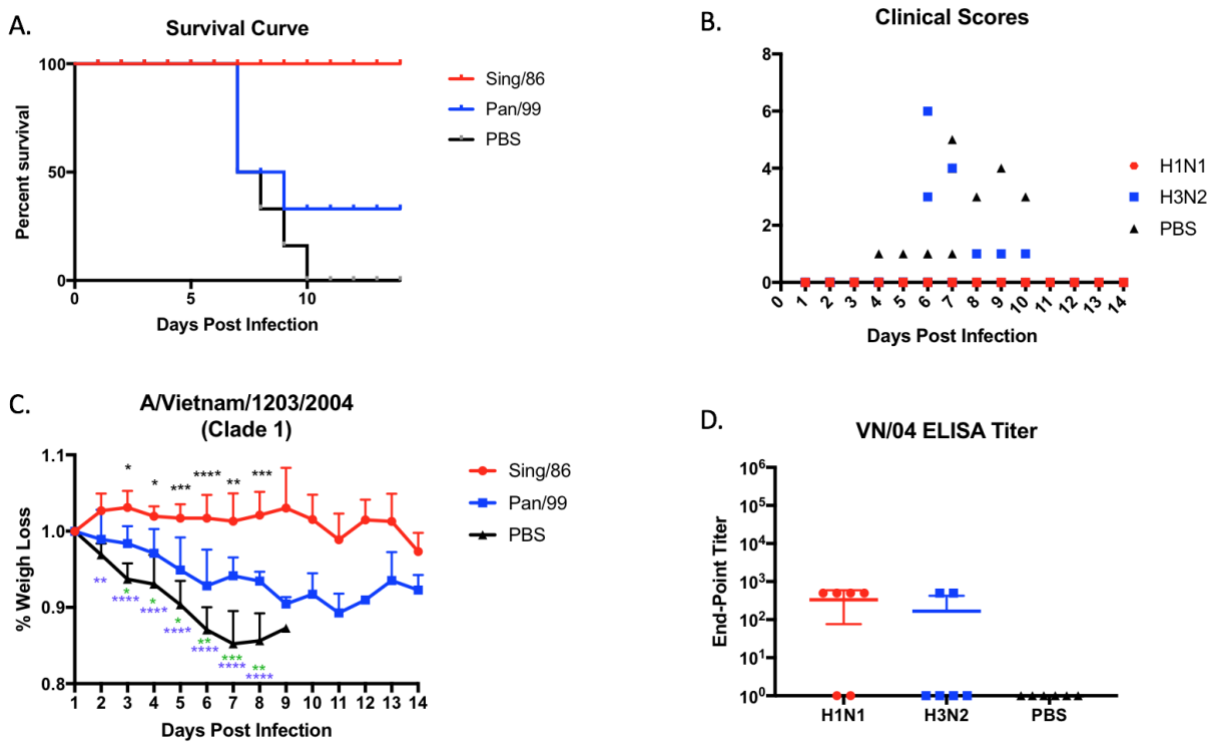

Figure 5.2: H1N1 imprinting provides complete protection against H5N1 challenge in naïve female ferrets. Survival was assessed in each pre-immune group and control (A). Ferrets who were pre-immunized with Sing/86 (red)had a 100% survival rate, whereas Pan/99 vaccinated ferrets (blue) only had 40% (2 out of 6) ferrets survive challenge. All PBS control (Black) ferrets died 10 days post challenge. Clinical scores were recorded for each ferret following challenge (B) PBS and Pan/99 ferrets displayed the highest clinical signs. Weight loss data was calculated for all ferrets (C) and an average loss with error bars are displayed. Statistical analysis was performed using 2-way ANOVA. Black asterisks display differences between H1N1 and H3N2, Purple asterisk display differences between H1N1 and PBS, green asterisks display differences between H3N2 and PBS. Sera taken on day 84 display group-1 imprinted ferrets generating detectable antibodies against VN/04-HA protein using ELISA assay.

Days Post Infection	Groups	Significance	P-value
1	H1N1 vs. H3N2	ns	>0.9999
	H1N1 vs. PBS	ns	>0.9999
	H3N2 vs. PBS	ns	>0.9999
2	H1N1 vs. H3N2	ns	0.0615
	H1N1 vs. PBS	**	0.0016
	H3N2 vs. PBS	ns	0.4183
3	H1N1 vs. H3N2	*	0.013
	H1N1 vs. PBS	****	<0.0001
	H3N2 vs. PBS	*	0.0132
4	H1N1 vs. H3N2	*	0.0104
	H1N1 vs. PBS	****	<0.0001
	H3N2 vs. PBS	*	0.0399
5	H1N1 vs. H3N2	***	0.0002
	H1N1 vs. PBS	****	<0.0001
	H3N2 vs. PBS	*	0.0167
6	H1N1 vs. H3N2	****	<0.0001
	H1N1 vs. PBS	****	<0.0001
	H3N2 vs. PBS	**	0.0017
7	H1N1 vs. H3N2	**	0.0015
	H1N1 vs. PBS	****	<0.0001
	H3N2 vs. PBS	***	0.0006
8	H1N1 vs. H3N2	***	0.0001
	H1N1 vs. PBS	****	<0.0001
	H3N2 vs. PBS	**	0.0089

Table 5.1: Table displaying statistical significance between weight loss in pre-immunized ferret groups on 8 days post-challenge. Data was calculated using 2-way ANOVA, multiple comparisons within each row, compare simple effects within rows (Graphpad Prism, 7). Table correlates with data presented in Figure 2.

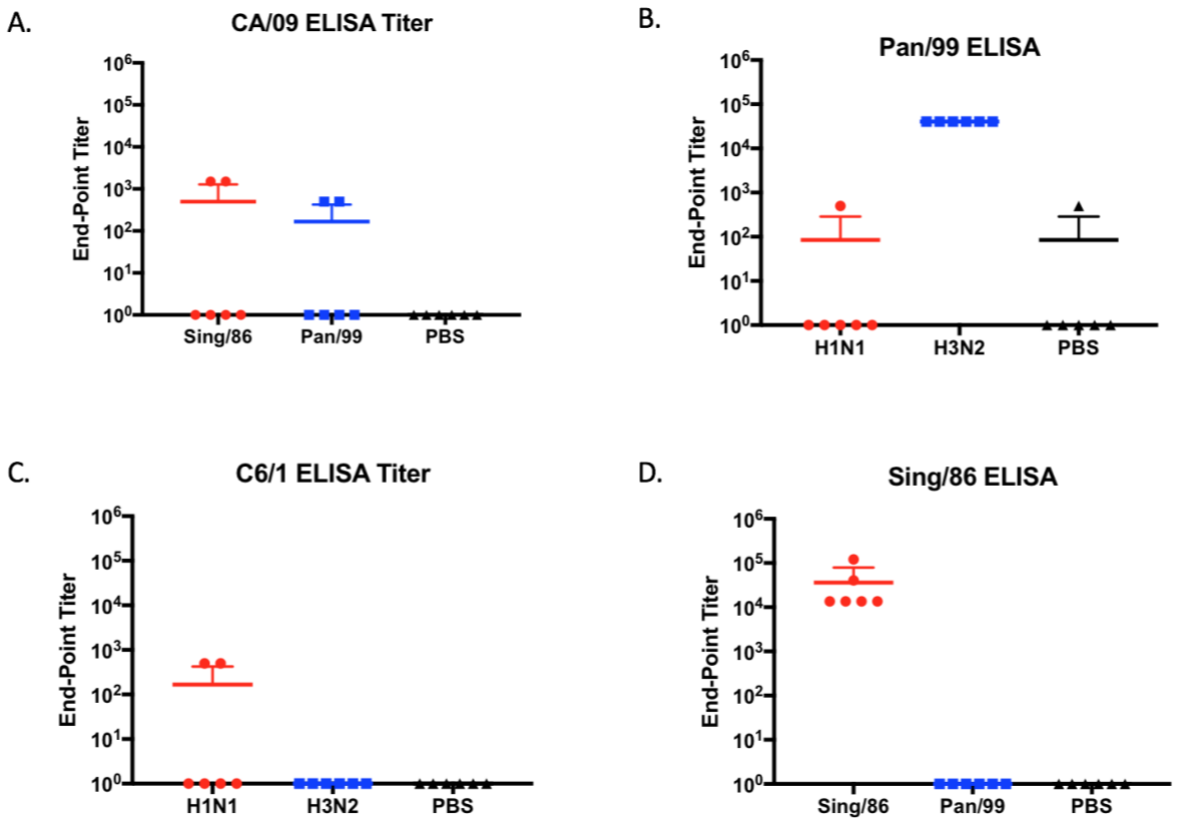

Figure 5.3: Group 1 pre-immunized ferrets do not contain significant antibody titers against C6/1-HA. Sera was taken from ferrets on day 84 prior to infection with H5N1 to test for seroconversion and stalk antibody titers. Sing/86 pre-immunized ferrets contain very little antibody against CA/09-HA (A) All pre-immunized ferrets had detectable antibody titers to their infection strain (Pan/99 B, Sing/86 D). only 2 out of 6 ferrets who were pre-immunized with a group 1 virus displayed low antibody titers against C6/1-HA (C).

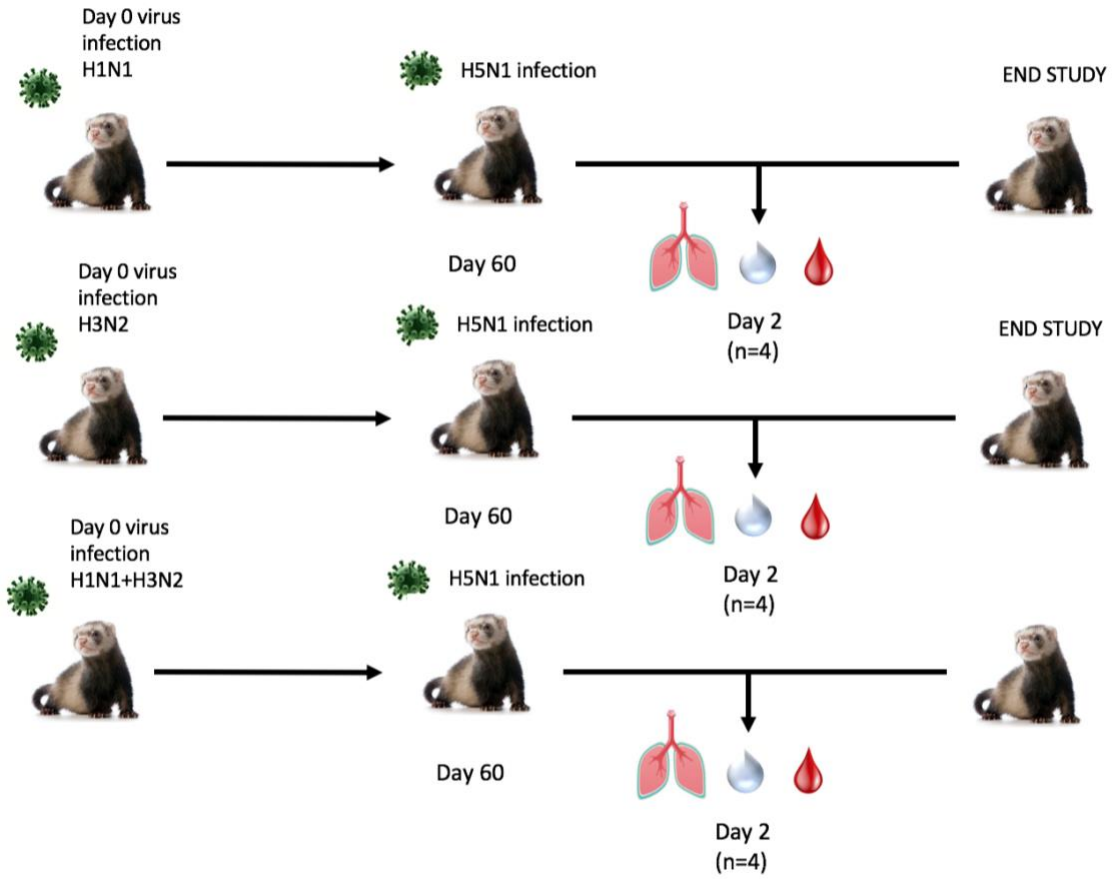

Figure 5.4: Sequential infection with a group 2 virus does not inhibit the imprinting effect of group 1 influenza virus infection. Naïve fitch ferrets (*Mustela putorius furo*, male) aged 4-6 months were preimmunized with either an H1N1/ group 1 virus (Mar/43) an H3N2/group 2 virus (HK/68) or sequential infection of Mar/43 followed by HK/68. Ferrets were allowed to recover for 60 days and were moved into a high-containment facility for challenge with H5N1 VN/04 ($1x10^5$ pfu/mL). On day 2 post-challenge, ferrets were humanely euthanized and nasal wash, lung and sera was taken for analysis. The study ended 14 days following challenge.

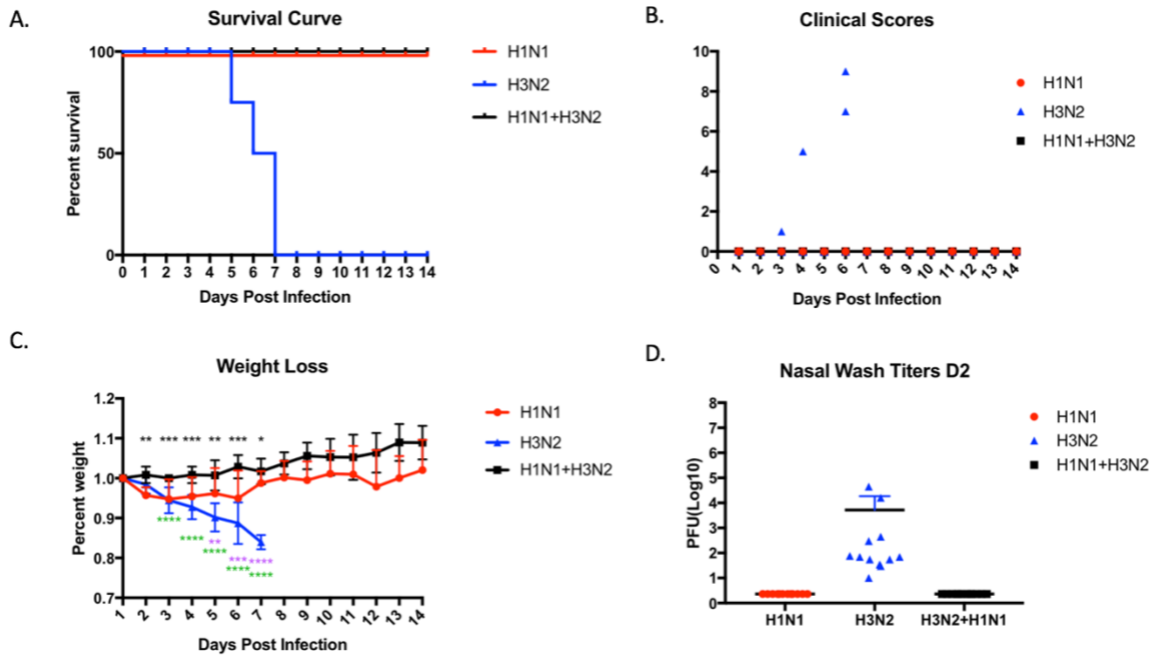

Figure 5.5: Group 1 imprinting provides complete protection against H5N1 challenge in naïve male ferrets. Survival was assessed in each pre-immune group (A). Ferrets who were pre-immunized with the H1N1 strain Mar/43 (red) had a 100% survival rate, whereas H3N2 strain HK/63 infected ferrets (blue) all succumbed to disease 7 days following challenge. Sequentially infected ferrets (Black) ferrets also survived challenge. Clinical scores were recorded for each ferret following challenge (B) H3N2 (HK/63) ferrets displayed the highest clinical signs compared the H1N1 and H1N1+H3N2 sequentially infected ferrets. Weight loss data was calculated for all ferrets (C) and an average loss with error bars are displayed. Statistical analysis was performed using 2-way ANOVA. Black asterisks display differences between H1N1 and H1N1+H3N2, Purple asterisk display differences between H1N1 and H3N2, green asterisks display differences between H1N1 and H3N2. Nasal wash titers showed detectable virus in only H3N2 pre-immunized ferrets (D).

Days Post Infection	Groups	Significance	P-value
1	H1N1 vs. H1N1+H3N2	ns	>0.9999
	H1N1 vs. H3N2	ns	>0.9999
	H1N1+H3N2 vs. H3N2	ns	>0.9999
2	H1N1 vs. H1N1+H3N2	**	0.0015
	H1N1 vs. H3N2	ns	0.1452
	H1N1+H3N2 vs. H3N2	ns	0.2242
3	H1N1 vs. H1N1+H3N2	***	0.0002
	H1N1 vs. H3N2	ns	0.9712
	H1N1+H3N2 vs. H3N2	****	<0.0001
4	H1N1 vs. H1N1+H3N2	***	0.0001
	H1N1 vs. H3N2	ns	0.2033
	H1N1+H3N2 vs. H3N2	****	<0.0001
5	H1N1 vs. H1N1+H3N2	**	0.0055
	H1N1 vs. H3N2	**	0.0021
	H1N1+H3N2 vs. H3N2	****	<0.0001
6	H1N1 vs. H1N1+H3N2	***	0.0002
	H1N1 vs. H3N2	***	0.0005
	H1N1+H3N2 vs. H3N2	****	<0.0001
7	H1N1 vs. H1N1+H3N2	*	0.0197
	H1N1 vs. H3N2	****	<0.0001
	H1N1+H3N2 vs. H3N2	****	<0.0001

Table 5.2: Table displaying statistical significance between weight loss in pre-immunized ferret groups on 7 days post-challenge. Data was calculated using 2-way ANOVA, multiple comparisons within each row, compare simple effects within rows (Graphpad Prism, 7). Table correlates with data presented in Figure 4.

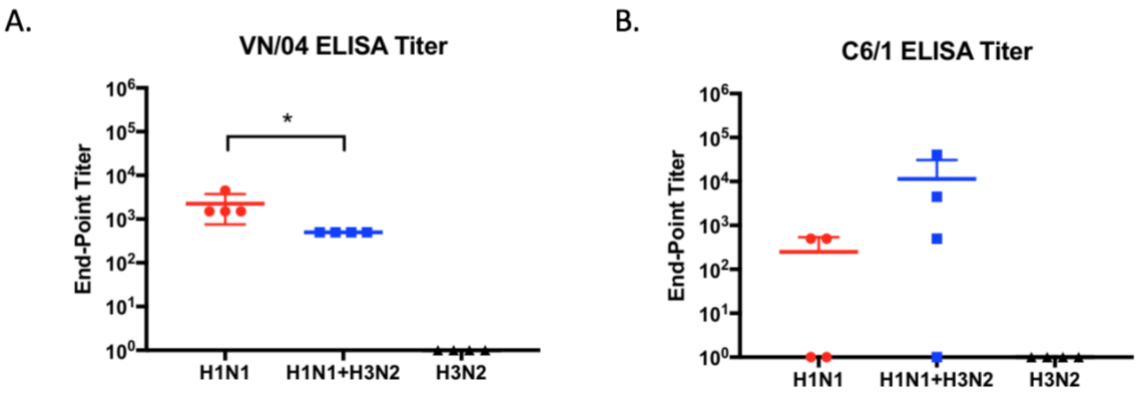

Figure 5.6: Antibodies elicited against VN/04-HA were significantly lower in sequentially infected ferrets. Sera taken from a subset of ferrets (n=4) on day 2 post challenge were tests against VN/04-HA (A) and C6/1-HA (A) in an ELISA assay. Antibody titers against VN/04-HA protein were significantly lower in the sequentially infected ferrets (blue), titers against C6/1-HA used to test stalk antibodies were not significant in either group-1 infected ferrets.

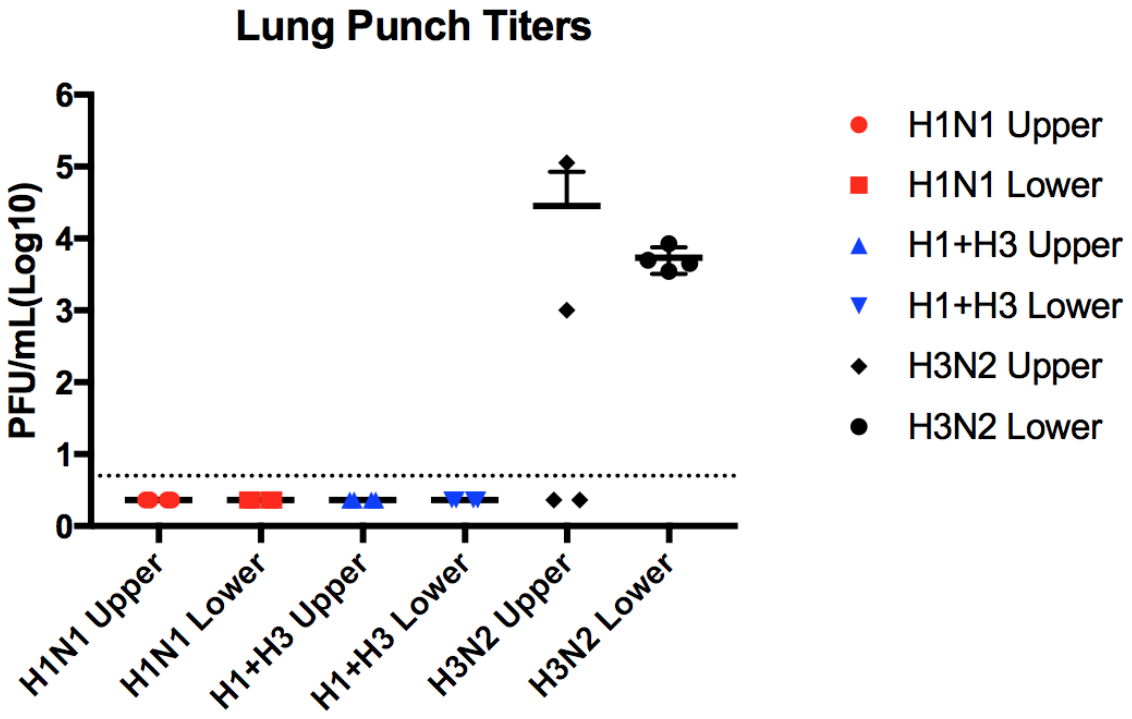

Figure 5.7: Group 1 imprinted ferrets control viral infection in the lungs following challenge. Lungs taken on day 2 post infection were processed for viral titers using a plaque assay. Lung punches were taken from upper and lower quadrants and were processed. Ferrets who were intranasally infected/pre-immunized with H3N2 virus (HK/68) displayed high viral titers in upper and lower lungs. Lower lungs showed a higher trend of viral load. No virus was detected in either H1N1 or H1N1+H3N2 infected ferret groups. Dotted line represents the limit of detection.

Pre-immunity	Vaccination	Challenge
H3N2	Hu-CO 2	H5N1
	Flu-zone	
	Control	
H1/H2	Hu-CO 2	
	Flu-zone	
	Control	
H2N3	Hu-CO 2	
	Flu-zone	
	Control	

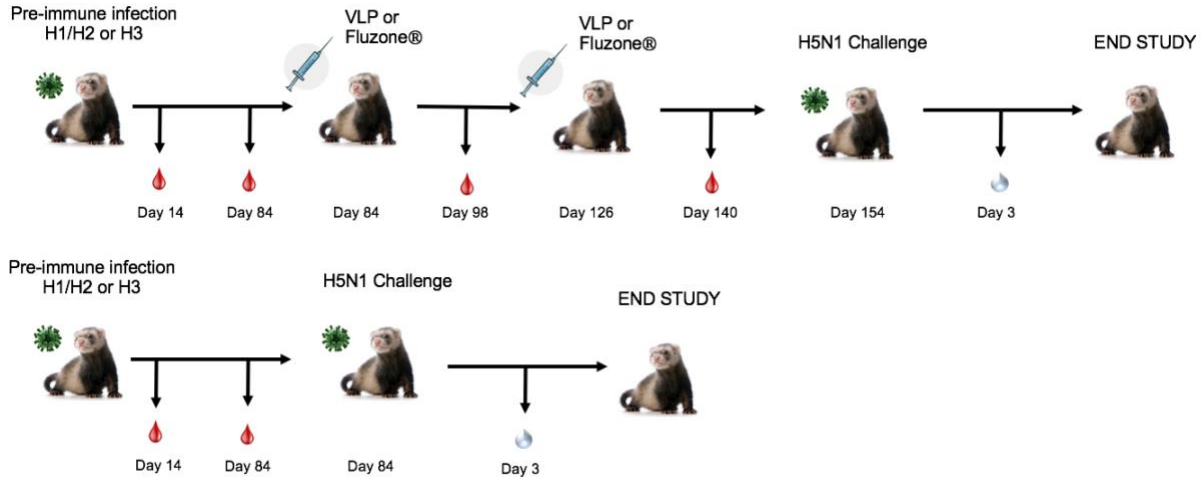

Figure 5.8: Schematic representation of ferret vaccination study. Fitch ferrets (*Mustela putorius furo*, female) aged 9-12 month of age were intranasally infected with either an H3N2 virus (PC/73, n=), H2N3 (sw/MO/06) or a sequential infection of H1N1+H2N3 (CA/09 followed by sw/M0/06). Sera was taken on day 14 and 84 to test for antibody titers. 84 days following pre-immunization, ferrets were either challenged with H5N1 virus (Control) or intramuscularly vaccinated on a prime-boost regimen with either a Hu-CO 2 VLP formulated with Addavax™ adjuvant or with unadjuvanted seasonal Fluzone®, or no vaccine (Control). Ferrets were intranasally challenged 4-weeks following the last vaccination with VN/04 ($1x10_5$ pfu/mL). On day-3 following challenge, nasal washes were taken to test for viral loads. The study was terminated 14 days following challenge.

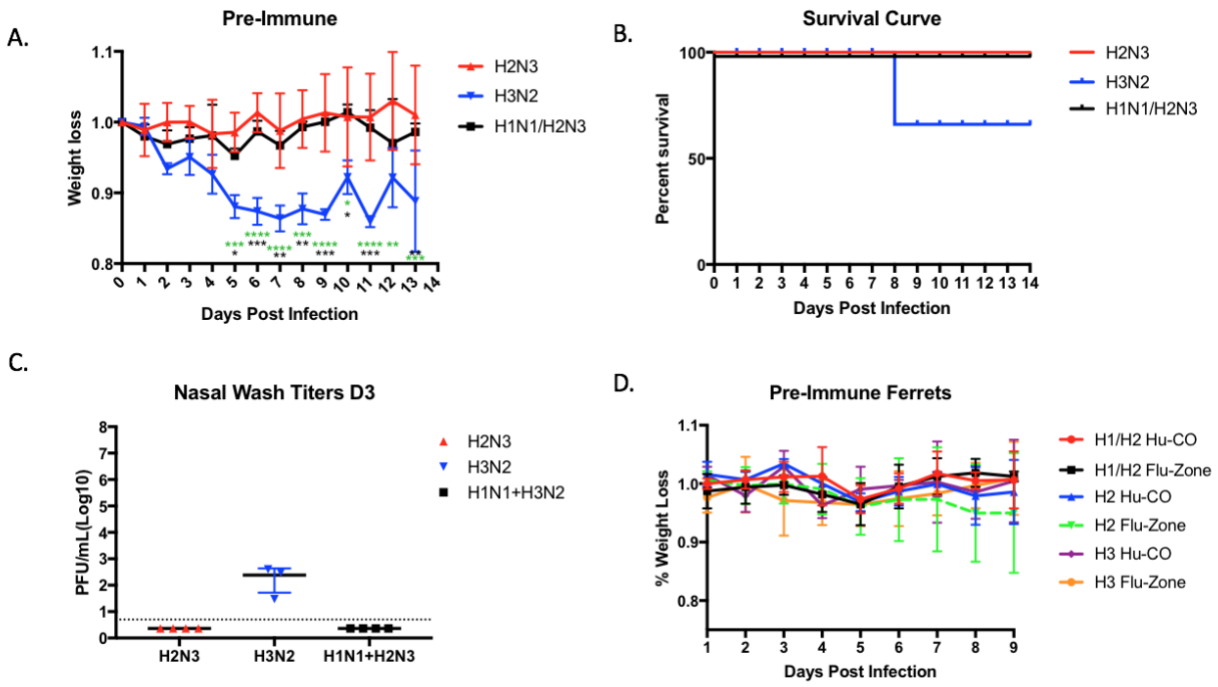

Figure 5.9: All group 1 imprinting protects ferrets from challenge with H5N1 virus, and vaccination with either Hu-CO or Fluzone® can re-educate the immune system of H3N2 imprinted ferrets. Female Fitch ferrets were intranasally infected using H2N3, H3N2 or H1N1+H2N3 strains of viruses. after 84 days, ferrets were intranasally challenged using VN/04 ($1x10_5$ pfu/mL). Weight loss data (A) was graphed according to total % weight loss based on day 0 weights. Ferrets who were H3N2 pre-immune (n=3) experienced a significant amounts of weight loss compared to H2N3 and H3N2 pre-immune ferrets. H2N3 (n=4) and H1N1+H2N3 (n=4) ferrets displayed no significant weight loss compared to one another. Green asterisks display statistical significance between H2N3 and H3N2 groups, black asterisks display statistical significance between H1N1+H2N3 and H3N2 groups. Survival curves were also calculated for pre-immune non-vaccinated groups (B). Nasal wash titers collected from ferrets on day 3 post infection were calculated using plaque assays, dotted represents limit of detection. Ferrets vaccinated with Hu-CO VLP or Fluzone (D) experienced no weight loss or clinical signs.

Days Post Infection	Groups	Significance	P-value
1	H2N3 vs. H1N1+H2N3	ns	0.9324
	H2N3 vs. H3N2	ns	0.9938
	H2N3+H1N1 vs. H3N2	ns	0.9007
2	H2N3 vs. H1N1+H2N3	ns	0.4463
	H2N3 vs. H3N2	ns	0.0513
	H2N3+H1N1 vs. H3N2	ns	0.432
3	H2N3 vs. H1N1+H2N3	ns	0.6219
	H2N3 vs. H3N2	ns	0.2565
	H2N3+H1N1 vs. H3N2	ns	0.7504
4	H2N3 vs. H1N1+H2N3	ns	0.9957
	H2N3 vs. H3N2	ns	0.1673
	H2N3+H1N1 vs. H3N2	ns	0.1951
5	H2N3 vs. H1N1+H2N3	ns	0.3904
	H2N3 vs. H3N2	***	0.0007
	H2N3+H1N1 vs. H3N2	*	0.0297
6	H2N3 vs. H1N1+H2N3	ns	0.5567
	H2N3 vs. H3N2	****	<0.0001
	H2N3+H1N1 vs. H3N2	***	0.0003
7	H2N3 vs. H1N1+H2N3	ns	0.6962
	H2N3 vs. H3N2	****	<0.0001
	H2N3+H1N1 vs. H3N2	**	0.0014
8	H2N3 vs. H1N1+H2N3	ns	0.9012
	H2N3 vs. H3N2	***	0.0005
	H2N3+H1N1 vs. H3N2	**	0.0019
9	H2N3 vs. H1N1+H2N3	ns	0.8665
	H2N3 vs. H3N2	****	<0.0001
	H2N3+H1N1 vs. H3N2	***	0.0002
10	H2N3 vs. H1N1+H2N3	ns	0.9646
	H2N3 vs. H3N2	*	0.02
	H2N3+H1N1 vs. H3N2	*	0.0111
11	H2N3 vs. H1N1+H2N3	ns	0.8238
	H2N3 vs. H3N2	****	<0.0001
	H2N3+H1N1 vs. H3N2	***	0.0001
12	H2N3 vs. H1N1+H2N3	ns	0.0547
	H2N3 vs. H3N2	**	0.0021
	H2N3+H1N1 vs. H3N2	ns	0.2636
13	H2N3 vs. H1N1+H2N3	ns	0.6146
	H2N3 vs. H3N2	***	0.0005
	H2N3+H1N1 vs. H3N2	**	0.0062

Table 5.3: Table displaying statistical significance between weight loss in pre-immunized ferret groups on 13 days post-challenge. Data was calculated using 2-way ANOVA, multiple comparisons within each row, compare simple effects within rows (Graphpad Prism, 7). Table correlates with data presented in Figure 9A.

Pre-immunity	Vaccination	Challenge
H3N2	CA/09	H5N1
	VN/04	
	CA/09 NA	
	Hu-CO 2	
	Flu-zone	
	Control	

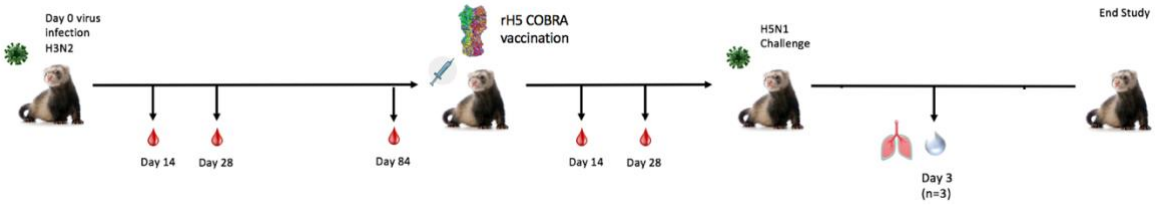

Figure 5.10: Schematic representation of H3N2 pre-immune ferrets vaccine study. Fitch ferrets (*Mustela putorius furo*, female) aged 6-9 months of age were intranasally infected with H3N2 virus (Tex/12). Sera was taken on day 14 and 84 to test for antibody titers. 84 days following pre-immunization, ferrets were vaccinated with recombinant soluble protein formulated with Addavax™ adjuvant or with unadjuvanted seasonal Fluzone®, or no vaccine (Control). Four-weeks following vaccination, ferrets were intranasally challenged with VN/04 ($1x10^5$ pfu/mL). On day-3 following challenge, nasal washes and lungs were taken to test for viral loads and histopathology. The study was terminated 14 days following challenge.

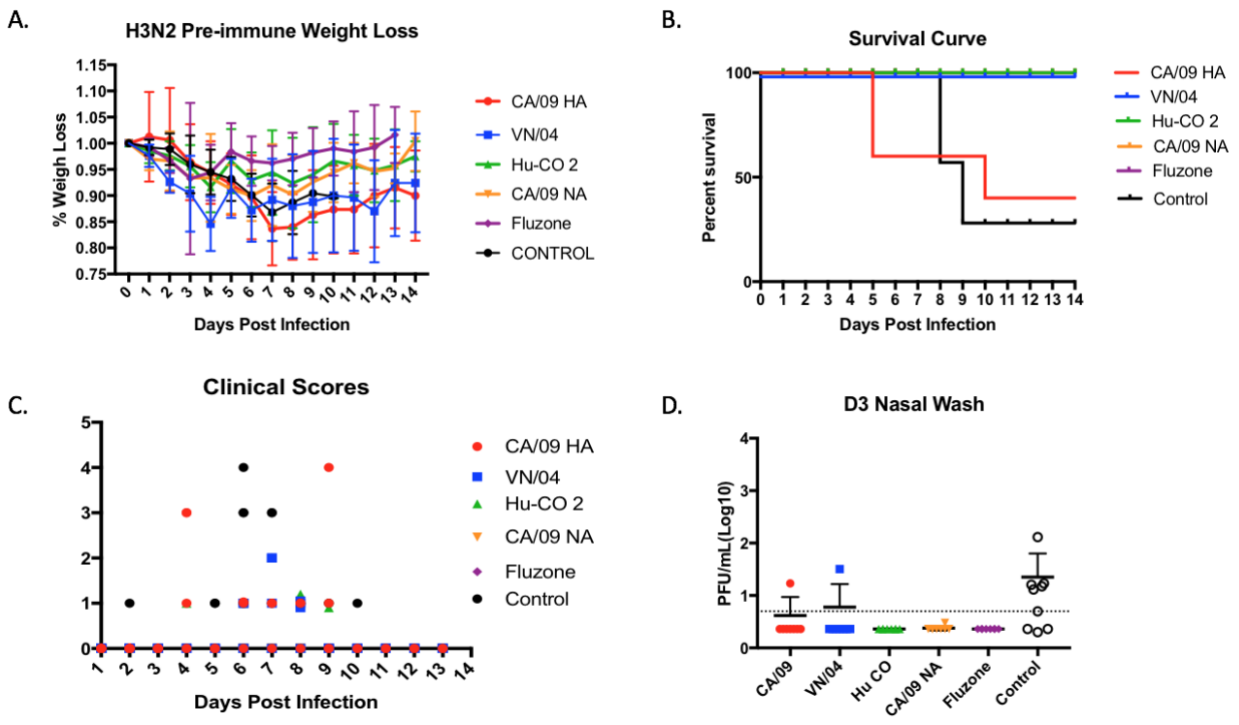

Figure 5.11: Vaccination with inactivated Fluzone vaccine enables protection in H3N2/group 2 imprinted ferrets. Female Fitch ferrets were intranasally infected using H3N2 (Tex/12) virus for imprinting. After 84 days, ferrets were vaccinated with either CA/09-HA, VN/-4-HA, Hu-CO 2-HA, CA/09-NA, Fluzone® or PBS. Ferrets were intranasally challenged using VN/04 ($1x10^5$ pfu/mL). Weight loss data (A) was graphed according to total % weight loss based on day 0 weights. Survival curves were also calculated for pre-immune vaccinated and control groups (B). Clinical scores were calculated for all groups of vaccinated and control ferrets (C) Nasal wash titers collected from ferrets on day 3 post infection were calculated using plaque assays, dotted represents limit of detection. Only H3N2 pre-immunized ferrets who were unvaccinated displayed viral titers in nasal wash specimens.

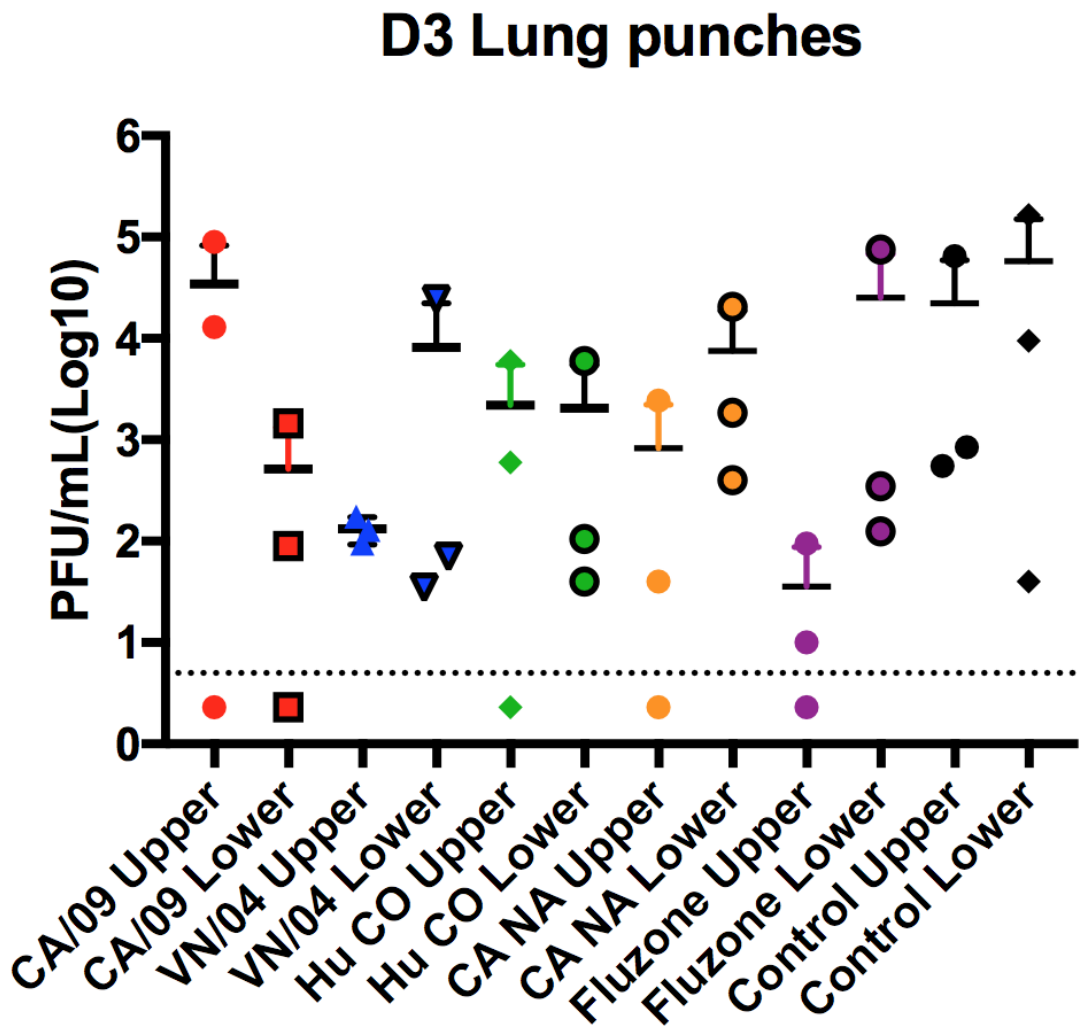

Figure 5.12: Vaccination in H3N2 pre-immune ferrets could not prevent viral infiltration of upper lower lungs. Lung punches were taken from ferrets on day 3 post challenge with VN/04 virus. Lung punches were processed and supernatant from tissue homogenates were plated on a monolayer of MDCK cells in a high-level biocontainment facility. No statistical significance between groups was reported. Dotted line represents the limit of detection.

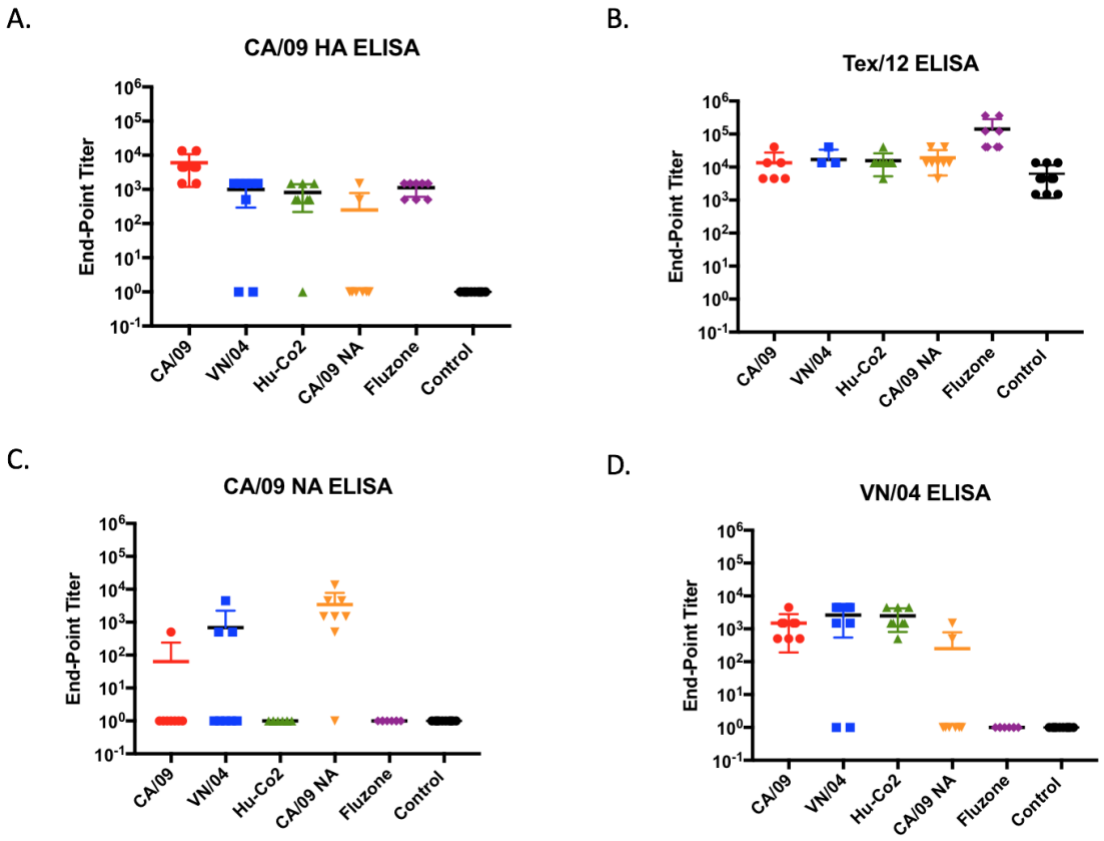

Figure 5.13: antibodies generated against VN/04-HA protein is not a correlate of protection in heterosubtypic immunity. Sera from H3N2 pre-immune vaccinated and control ferrets was taken on day 84 prior to VN/04 challenge. Seroconversion to the vaccine strains were determined (A), (C). ferrets also contained high antibody titers against the Tex/12-HA following infection (B). Antibody titers elicited against the VN/04-HA were detectable in CA/09-HA, VN/04-HA, and Hu-CO 2-HA vaccinated groups. Fluzone® vaccinated ferrets contained no antibody titers against VN/04-HA.

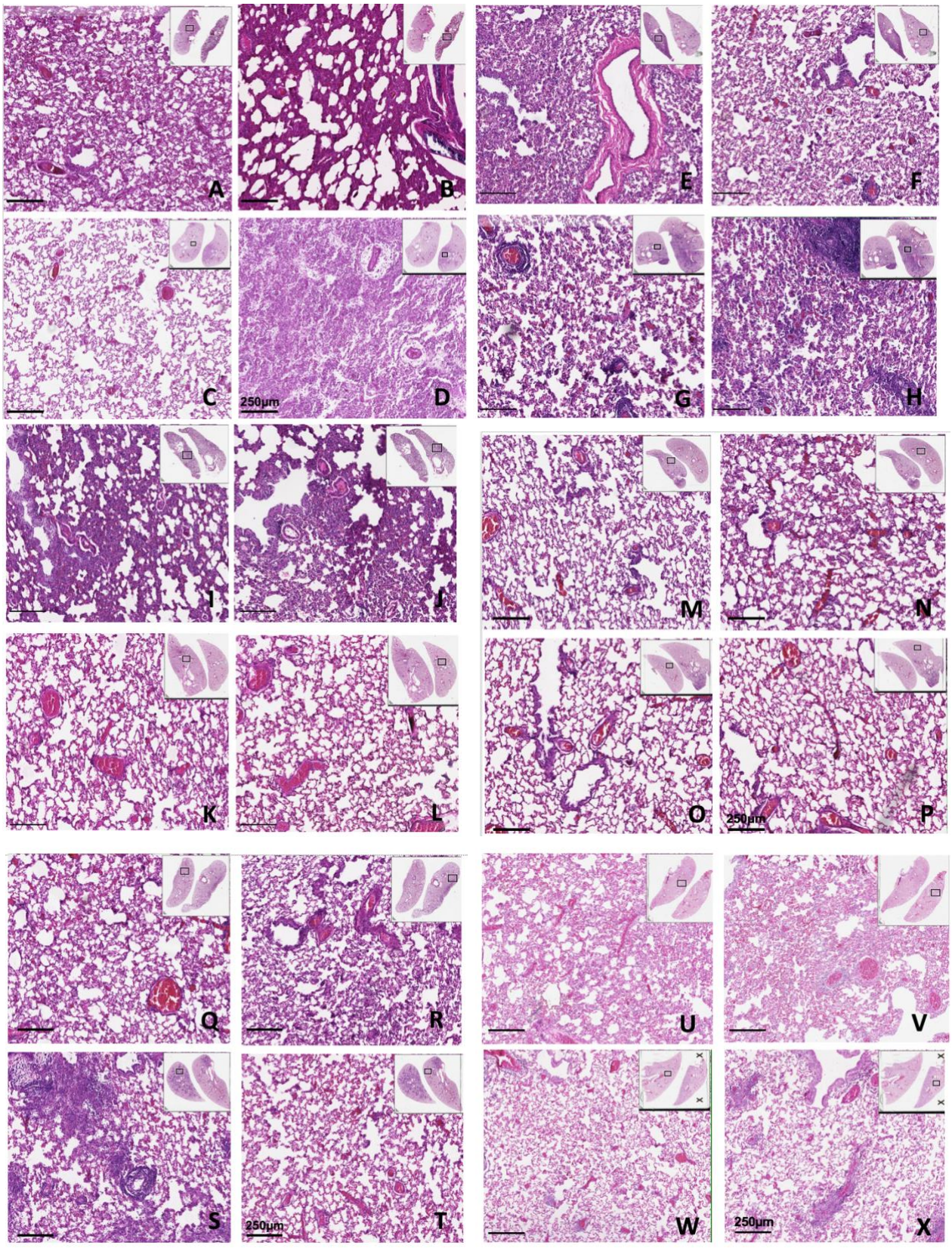
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Figure 5.14: Hematoxylin and Eosin staining of upper and lower lobes of H5N1 infected ferrets. Two samples were taken from each sample of upper and lower lungs. Unvaccinated control ferrets (A-D). A) upper lung, B) upper lung C) lower lung D) lower lung. Fluzone vaccinated ferrets (E-H) E) upper lung, F) upper lung G) lower lung H) lower lung. CA/09 NA vaccinated ferrets (I-L) I) upper lung, J) upper lung K) lower lung L) lower lung. Hu-CO 2 vaccinated ferrets (M-P) M) upper lung, N) upper lung O) lower lung P) lower lung. VN/04 vaccinated ferrets (Q-T) Q) upper lung, R) upper lung S) lower lung T) lower lung. CA/09 vaccinated ferrets (U-X) U) upper lung, V) upper lung W) lower lung X) lower lung.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Avian influenza virus infection has spilled over into the United States poultry population in years 2014 and 2015, resulting in catastrophic effects in the poultry industry and economy. Although the United States Department of Agriculture (USDA) contained the outbreak by culling infected flocks, the spread continues in endemic countries such and Indonesia, Bangladesh, China, India, and Vietnam and remains to be a public health threat [1]. As with current SARS-CoV-2 pandemic, once a virus becomes a human transmissible agent, it is nearly impossible to contain the disease at the site of infection. Fortunately, the ability of H5Nx viruses to spread from one individual to another is very limited. The most common threat of contracting avian influenza remains with individuals who are in close contact with poultry species especially in countries were live animal markets are prevalent. Any contact with poultry, especially in cold dry months, increases the chances of contracting H5Nx influenza viruses.

Avian influenza viruses are constantly undergoing antigenic shift and drift in avian species. Wild bird species are the known reservoir for avian influenza viruses and have been isolated most notably in gulls, terns, shorebirds, ducks, geese and swans [2]. Due to the prevalence of low pathogenic avian influenza in aquatic bird populations, spill over evens in combination with various NA subtypes have led to recombination events in domestic poultry populations [3]. Viral infections that result in little to no disease in wild water fowl can undergo mutations within the

HA gene and result in highly pathogenic viruses in poultry [4], and can also result in the preferential binding to α-2,6-linked sialic acid receptors [5]. Other mutations to the NA or PB2 protein can lead to alternative binding and release patterns of the sialic acid receptor and to change in replication kinetics and temperature kinetics in influenza viruses [6]. Although infection rates of avian to human population are low and are rare in occurrence, there is still a threat of the emergence of an avian influenza pandemic that can infect the human species and spread from one individual to another.

Most concerningly, reference strains that are chosen for vaccine preparedness are not readily available and will take at least 6-months to produce during a pandemic H5Nx outbreak. Therefore, the development of a broadly reactive vaccine that produces antibody responses against the majority of circulating clades is most desirable. Also, a vaccine that produces an immunogenic response after a low dosage of vaccination is also desirable for dose sparing during a pandemic. Our group has previously reported a chimeric computationally derived HA protein that is capable of generating a robust antibody response in mouse, ferret, and non-human primate models of clinical infection. However, the exact mechanism of immunogenicity had not previously been studied.

The first study in this section (Chapter 3) conducted in a mouse model, was aimed to understand the underlying reasons of effectiveness of the Human-COBRA 2 HA vaccine in pre-clinical animal models. Therefore, site-directed mutagenesis was performed at two epitope sites located at amino acids 140-141 and 155-156 (H5 numbering). These studies elucidated the importance of the glycosylation site located near the RBS was important in protecting mice against a heterologous

strain of virus belonging to clade 2.3.2.1. Although the virus in this clade, Hu/10 does not contain this glycosylation site. The sites 140-141 and 155-156 have previously been identified for their immunogenicity and are important epitopes for eliciting immune responses [7-10]. Glycosylation of the HA molecule is associated with epitope masking and immune evasion techniques, especially concerning the H3N2 influenza viruses [11]. Conserved glycosylation sites on the HA protein, most notably at amino acid 47, are essential for ensuring proper protein folding and expression out of the host cell [12].

Most recently, the site located at amino acids 158-160 (H3 numbering) correlates with the 155-156 epitope identified in this study and is important for the pathogenicity of avian influenza viruses. When glycosylated, this site enhanced the pathogenicity of the virus and exacerbated disease in naïve female BALB/c mice [13]. Viruses containing the glycosylation site on amino acid 155-156 also displayed increased viral replication, larger plaque sizes and increased VLP production in a humanized cell line. Mice infected with a virus containing the glycosylation site in 155-156 enhanced transcription of adaptive and innate markers, including the production of mucins in mice lungs compared to a virus that had the glycosylation site removed [13]. In other viruses including Hepatitis C, glycosylation patterns have been directly correlated with increased immunogenicity and higher neutralizing antibody titers [14]. Therefore, this glycosylation site may add to the HA induced immunogenicity by eliciting a powerful immune response that aids in a high titer antibodies being produced and stimulating a memory response strong enough to overcome heterologous virus challenge. As was previously discussed, vaccination using inactivated virus or proteins often results in low antibody titers and a poor memory responses compared to active influenza virus infection. The glycosylation of the Hu-CO 2 HA protein

therefore may be a powerful tool in aiding in the memory response, leading to a higher recruitment of immune cells are memory B cell response.

The second study (Chapter 4) presented included newly generated COBRA HA vaccines that incorporated newer avian isolates from years 2011-2017 termed the Next Generation COBRA Vaccines. Our platform was switched from VLPs to soluble recombinant protein, although we were able to obtain high immunogenicity in mice using VLPs, this platform can be limited due to the cost of production, time of purification and calculation of HA content. Using a soluble recombinant protein as a vaccine platform allowed production of a large amount of antigen without incorporating other viral components. Next Generation COBRA Vaccines were tested in mouse models and one vaccine elicited superior immunity and was selected as the lead pre-pandemic vaccine for future studies. Mice who were vaccinated using a prime-boost-boost model had the most antibody responses to viral isolates in a HAI viral isolate panel. It was also successful in protecting mice from lethal infection from VN/04 viruses. Although this clade of virus is no longer circulating, it is important to test a broad range of viruses against these vaccines in case a once "extinct" strain re-emerges in the avian species.

Mice who were vaccinated and subsequently challenged with the Si/14 virus did not experience any mortality following H5N6 challenge. This may be due to the differing pathogenicity found in reassortant H5N6 viruses, which has previously been reported to show mild to severe clinical symptoms in naïve BALB/c mice, and are 100% lethal in poultry [15]. Differences in pathogenicity may also be altered by using PR8 reassorted H5 viruses, which contain the HA and NA from the wild-type virus, but have the internal genes taken from the A/Puerto Rico/8/1934 H1N1 virus. As

was previously discussed, internal proteins such as the PB1 and PB2 protein may add to the pathogenicity of H5 viruses by altering viral replication kinetics and temperature sensitivities [16, 17]. In addition to this, PR8 reassortant viruses also have the multi-basic cleavage site removed from the HA so the virus can be used in a BSL-2 facility. Although using recombinant PR8 viruses eliminated the need to perform all animal studies and viral assays in a high-containment facility, it may limit the effect of the virus and result in a non-pathogenic strain of virus. All mice were vaccinated using the highest viral PFU/ml that was grown and collected in embryonated chicken eggs, but did not result in viral lung titers or in death in vaccinated mouse groups. Previous studies have shown that using PR8 6:2 or 7:1 reassortments of avian influenza viruses have decreased the pathogenicity of the virus. In a study performed by Hussain et al, viruses that contained internal viral PR8 genes had a significantly lower yield of expression of PA-X proteins, which lead to decreased chicken embryo fatality but ultimately higher yields in viral growth [18]. However, we were able to obtain differences in mouse weight losses which proved to be valuable in assessing vaccine efficacy. In order to obtain information of the immune profiles elicited by IAN-8 vaccination, animals including poultry should be challenged using a wild-type strain of 2.3.4.4 virus that induces lung infiltrates and mortality. Unfortunately, these strains are not available, therefore *in vitro* experiments will be used to determine vaccine efficacy in future studies.

In the next-generation vaccine design, P-epitope mapping was used to determine the correlation between vaccine sequence and HAI titers induced in a mouse model. This is a valuable tool that should be used in future studies when producing a pandemic vaccine for human consumption. P-epitope mapping consolidates amino acids that are specifically know to be targets for antibody

elicited responses and therefore is a powerful tool in predicting vaccine efficacy before animal administration [19].

Lastly, pre-immunity in the context of H5N1 infection was tested in a Fitch Ferret model of influenza virus. Imprinting with a group-1 influenza viruses by intranasal infection from either the H1N1 subtype or the H2N3 subtype enabled ferrets to develop long-lasting immunity that provided protection from infection with H5N1 influenza virus lethal challenge. The first hypothesis was that stalk directed antibodies against group-1 conserved epitopes were responsible for cross reactive heterosubtypic immunity. However, testing serum collected from pre-immunized ferrets, there was no correlation between antibodies elicited to the chimeric C6/1 HA protein or survival (Chapter 5 Figure 3C). Only a subset of ferrets that were pre-immunized with the H1N1 influenza virus Sing/86 had detectable antibody titers against C6/1 HA protein. This was also noted for ferrets pre-immune with the H1N1 influenza virus, Mar/43 (Chapter 5, Figure 6B). This was the first insight into the mechanism of heterosubtypic immunity offered by group-1 virus imprinting that it did not seem to be related to stalk directed group-1 antibody responses.

To investigate further whether HA or NA related antibody titers would protect H3N2 pre-immune groups from H5N1 infection, additional experiments were performed. H3N2 influenza virus heterosubtypic protection was not robust enough to protect 100% of the ferrets, which ranged from 33% protection to 0% mortality, depending on the strain of H3N2 influenza virus used. To determine whether vaccination using Hu-CO 2 HA, Fluzone®, or subunit vaccines containing CA/09 HA or NA would protect H3N2 influenza virus pre-immune ferrets were challenged with H5N1 influenza viruses. Interestingly, vaccination with the CA/09 HA did not afford the same

protection the H3N2 imprinted ferrets as the CA/09 NA vaccination. This was in direct contrast with the initial hypothesis that the group-1 conserved HA stalk domains were directly responsible for eliciting a heterosubtypic response. In order to investigate the amino acid similarity between the first H1N1 influenza virus pre-immune group using Sing/86 and the challenge virus H5N1 influenza virus VN/04, a BLAST search was perform using the NCBI database. The Sing/86 HA protein (accession #: CY020477) has 62.08% identity when compared to the VN/04 HA protein (accession #: AAW80717), whereas the Sing/86 NA protein (accession #SBO383981) had a 79.79% amino acid identity with VN/04 NA protein (accession #ATT73329). The amino acid identity between VN/04 NA and CA/09 NA (accession #: YP_009118627) is 84.22%, whereas the protein identity between VN/04 HA and CA/09 HA (accession #: ACP41105) is 63.43%. Therefore, it is understandable why the CA/09 HA vaccine did not elicit immune responses that provided full protection against H5N1 VN/04 influenza virus challenge. The H1N1 influenza virus imprinting leads to an immunodominant NA effect, where antibodies generated to the NA in H1N1 are cross reactive to N1 proteins that are found the VN/04 viral infection. This hypothesis was strengthened by the fact that ferrets, who were vaccinated with CA/09 NA protein all survived challenge and experienced little to no clinical symptoms compared to ferrets who were vaccinated with the CA/09 HA protein.

Although vaccination in the event of a pandemic outbreak may seem to the best option when there is a lack of a pandemic H5N1 vaccine, negative effects have also been correlated to administering the incorrect antigen as observed in this study using the H3N2 CA/09 HA vaccinated ferrets. Vaccination with H3N2 influenza virus in a H3N2 pre-immune background significantly inhibited the heterosubtypic immunity afforded to animals prior to vaccination [20, 21]. In both mice and

ferret models of infection, animals who were previously imprinted on with a live H3N2 influenza virus, displayed reduced heterosubtypic immunity against H5N1 influenza virus infection following H3N2 influenza virus vaccination [20, 21]. The study also found that ferrets who were infected with H3N2 influenza virus, and were not vaccinated, had fewer clinical symptoms than their vaccinated counterparts [21]. From this data, it can be concluded that vaccination may narrow the once broad immune response and must be properly investigated prior to using a pandemic vaccine into the human population. However, sequential infection of H1N1 influenza virus and H3N2 influenza virus did not dampen the heterosubtypic response against H5N1 influenza virus. I hypothesize that viral infection with H3N2 influenza viruses increased cross-protective responses to internal viral proteins, and therefore did not decrease the heterosubtypic protection against H5N1 influenza virus infection.

The cross protective immunity of antigenically similar NA does not explain, however, the heterosubtypic immune responses induced by H2N3 influenza virus imprinting. This cross protection cannot be afforded to NA similarities, especially in light of the differing NA groups, N1 vs N3. Indeed, sequence analysis between the AA identity between VN/04-NA (accession #: AAT73329.1) and sw/Mo/06-NA (accession #: ABY40440.1) revealed the two proteins only share 42.68% identity. However, comparisons between the HA protein of Sw/Mo/06-HA and VN/04-HA amino acids shared a 75.04% identity. From this information, I predict that pre-immunity elicited protection from H2N3 influenza viruses against VN/04 H5N1 challenge is due to HA identity, whereas H1N1 influenza virus pre-immunity is elicited solely from the NA. In respect to the Fluzone ®vaccinated ferrets, previous studies have reported that seasonal influenza vaccination in ferrets offered protection against challenge of H5N1 virus and was due to

neuraminidase inhibiting antibodies. Further testing of neuraminidase inhibition activity following H1N1 influenza virus imprinting and/or Fluzone® vaccination is required to make an exact conclusion form this data.

None of the pre-immune studies had HAI antibody titers against the challenge virus VN/04 and in some cases, as with the Fluzone® vaccinated ferrets, did not contain ELISA IgG antibody titers against VN/04 HA protein. This has previously been found in other pre-immune heterosubtypic studies. In the absence of neutralizing antibody titers, we can predict that memory T-cell responses are involved in generating a protective response against the lethal H5N1 challenge [22]. Heterosubtypic T-cell responses are the prime effector mechanism for the generation of heterosubtypic response. Cross reactive T cells can recognize highly conserved epitopes located on viral internal proteins and can enhance virus clearance and reduce pathology [22]. Previous studies conducted in a mouse species showed that mice infected with a H3N2 influenza virus strains induced protective immunity against H5N1 influenza virus infection [23]. Mice that were pre-immunized with HK/68 H3N2 influenza virus developed cross reactive CD8+ cells directed towards a NP epitope and that it correlated with protective immunity against A/Indonesia/05/2005 H5N1 influenza virus challenge [23].

Interestingly, in multiple cases, ferrets that were pre-immunized with H3N2 influenza virus were afforded moderate cross-protection against H5N1 influenza virus challenge, but this was not true for all the ferret studies, specifically those involving all male ferrets (Chapter 5, Figure 5A). Cross protective responses to H5N1 influenza virus infection after H3N2 influenza virus pre-immunity have been reported [20, 23]). Specifically, in a mouse model, H3N2 influenza virus infection

produced cellular responses that were directed against a NP epitope that was cross reactive with H5N1 influenza virus [20, 23]. The partial protection elicited by H3N2 influenza virus pre-immunity can be afforded to internal viral proteins such as NP, M1 and PB1.

Therefore, H1N1 pre-immunity fully protects against H5N1 challenge due to either NA inhibiting antibody responses heterosubtypic T-cell responses. Animals that are pre-immune to H3N2 influenza viruses are afforded moderate protection to H5N1 influenza virus infection, most likely due to intraviral proteins and this effect can be negatively affected by vaccinating with a protein that inhibits the heterosubtypic response and narrowing the immune response towards proteins that are not cross-protective. In addition, vaccination with a seasonal influenza virus vaccine can temporarily elicit cross-protection against H5N1 influenza virus infection, but has not yet been tested in a long-term study. Overall, these studies were aimed to investigate the potential of pre-pandemic vaccines and dived into the details of HA-based vaccines containing glycosylation sites, cross protective epitopes, and looked briefly into the effects of influenza virus imprinting and its effects of H5N1 influenza virus infection. Further studies will need to be performed in order to determine the protective responses of Next-generation COBRA HA vaccines in a ferret model. These vaccines will need to be further tested in a pre-immune model to determine the lack of negative narrowing immune effects and seen in CA/09 HA vaccination in ferrets.

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