DEVELOPMENT OF A BROADLY REACTIVE VACCINE FOR HIGHLY PATHOGENIC H5N1 INFLUENZA

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Emerging and re-emerging infectious diseases are increasing throughout the world and highly pathogenic influenza is among those that pose a significant threat to mankind. Pandemic outbreaks of influenza are caused by the emergence of a highly pathogenic and transmissible virus to which the human population is immunologically naïve. Ongoing outbreaks of highly pathogenic avian influenza of the H5N1 subtype are of particular concern because of the high mortality rate (>60%) and novel subtype. Vaccines are considered the most effective way to prevent the morbidity and mortality associated with pandemic influenza and therefore developing an H5N1 vaccine is a public health priority. One of the hurdles facing H5N1 vaccine development is the antigenic diversity of the subtype as evidenced by the identification of ten phylogenetic clades. To overcome the challenge of antigenic diversity, a centralized hemagglutinin was developed and termed computationally optimized broadly reactive antigen (COBRA). The COBRA HA sequence was based upon HA amino acid sequences from clade 2 H5N1 human infections and the expressed protein retained the ability to bind the receptor, as well as mediate particle fusion. COBRA virus-like particle (VLP) vaccines elicited broadly reactive receptor blocking antibodies in multiple animal species: mice, ferrets and non-human primates. The reactivity profile was broader than that elicited by primary isolate-based vaccines given in either monovalent or polyvalent formulations. Although all vaccinated animals were protected from severe disease and death from experimental infection with highly pathogenic H5N1 virus, animals receiving the COBRA vaccine had reduced peak virus replication and cleared the infection more rapidly. COBRA vaccines were shown to be superior to primary isolate-based vaccines with both a broader antibody profile and more efficient protective efficacy. The development of COBRA resulted in a novel antigen generation methodology that is applicable to both pandemic and seasonal influenza.

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PREFACE

I would like to thank my wife Heather Ann and our two daughters, Adelyn Ann and Katherine Margaret, for their patience and encouragement during the course of this research. Through all of the ups and downs that inevitably come with science, I always knew that I could count on your support and love. The thought of your smiling faces warms my heart and gives me strength. You are my inspiration and guiding light. This dissertation is dedicated to you, Heather, Addy, and Katie; for without you, it would not have been possible. I love you all and we finally did it: far enough, fast enough!

1.0 INTRODUCTION

1.1 GENERAL BACKGROUND

Influenza viruses are members of the family *Orthomyxoviridae*, which is comprised of 6 genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Thogotovirus* and *Quarjavirus* [1]. Influenza viruses are pleomorphic, enveloped viruses with negative-stranded, segmented RNA genomes [2]. There are three types of influenza: A, B and C of which only A and B cause significant human disease. Furthermore, only influenza A viruses (IAV) are a significant threat to cause pandemic outbreaks. The distinction among the three types is based upon the antigenic differences in their nucleoprotein (NP) and matrix (M1) proteins that is determined via monoclonal antibody enzymatic immunoassay [3]. Additionally, polymerase chain reaction (PCR) based assays have been developed for more rapid typing of clinical samples [4]. Influenza A and B both have similar virion structures while influenza C is more divergent. Influenza A and B are composed of eight individual gene segments that all encode at least one protein (Table 1). Influenza virions contain three main subviral components: the viral envelope, core and ribonucleoparticle (vRNP) (Figure 1). The viral envelope is derived from the host plasma membrane and contains the viral glycoproteins hemagglutinin (HA) and neuraminidase (NA), as well as the ion channel (M2). The core of the virus is composed of the matrix protein (M1) that packages the vRNP and interacts with cytoplasmic regions of HA and NA at the apical surface of the host cell. The vRNP complex consists of the nucleoprotein (NP), the polymerase

complex (PB1, PB2 and PA), viral RNA and small amounts of nuclear export protein (NEP or NS2). Viral egress usually occurs from the apical membrane of polarized epithelial cells. All viral components (viral envelope, core and vRNP) must be brought together at the assembly site at the apical membrane where viral buds are formed and particles released via the closure of the buds [5].

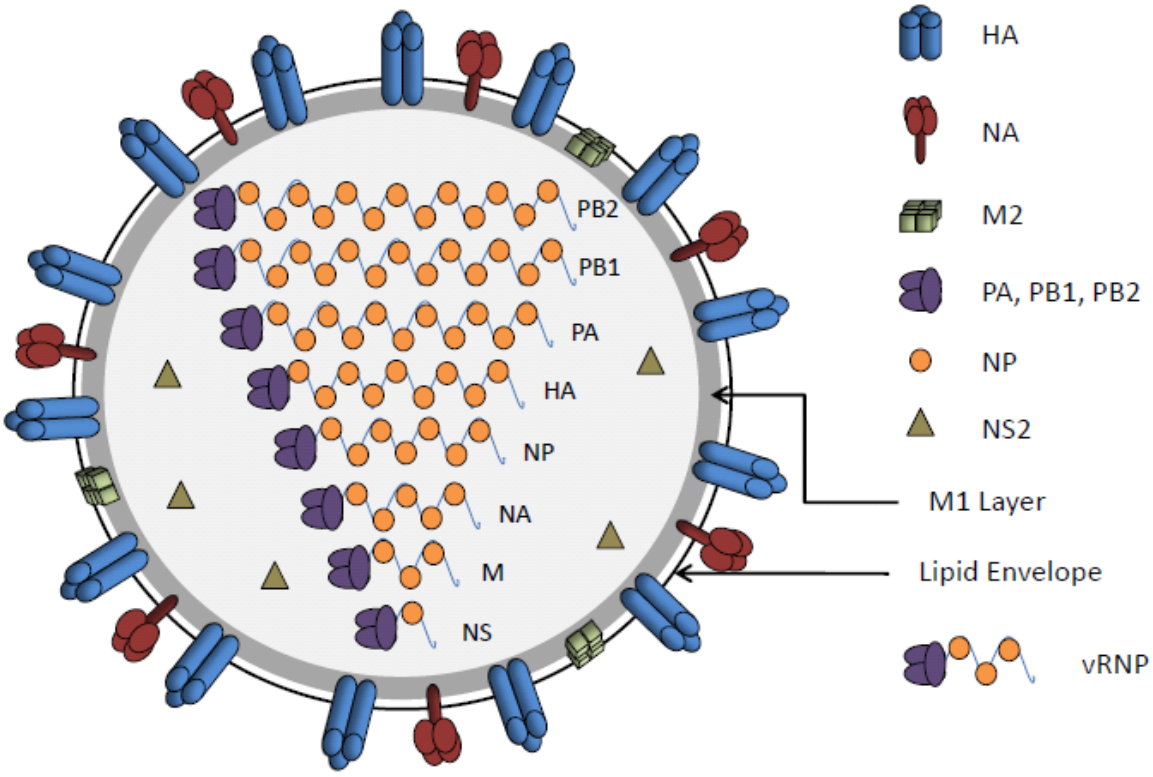

Figure 1: Representation of Influenza A virus. The two viral glycoproteins, HA and NA, and the ion channel, M2, are embedded in the lipid envelope. The M1 protein makes up the core of the particle and interacts with surface proteins and vRNPs. vRNPs consist of the eight gene segments, NP, and the polymerase complex (PA, PB1, PB2). The nuclear export protein (NS2) is also found in the virion.

Table 1: Influenza gene segments.

Segment*	Protein	Function
1	PB2	Polymerase Component
2**	PB1	Polymerase Component
	PB1-F2	Pro-apoptotic
3	PA	Polymerase Component
4	HA	Hemagglutinin Receptor binding Cell Fusion
5	NP	Nucleoprotein Directs RNA synthesis Packages viral RNA
6	NA	Neuraminidase Cleaves sialic acid for viral egress
7**	M1	Matrix Protein vRNP nuclear export Promotes virus assembly
	M2	Ion Channel Facilitates uncoating
8**	NS1	Non-structural protein Type I interferon antagonist Inhibits host protein production
	NS2	Nuclear export protein vRNP nuclear export

*Listed in decreasing order of size

** Encodes two messenger RNAs

1.2 INFLUENZA VIRUS GENOME AND PROTEINS

1.2.1 Hemagglutinin

HA is a glycosylated type I integral membrane protein and functions to both bind the cellular virus receptor and also mediate cell fusion (Figure 2A). The HA protein binds to terminal sialic acids (N-acetyl neuraminic acid) located on sugars found on host glycoproteins. The orientation of the glycosidic bond isomerization between sialic acid and the terminal sugar (galactose or N-Acetylgalactosamine) is a critical factor in HA receptor affinity. The various bond orientations lead to either a “bent” (α2-6) or “flat” (α2-3) conformation that impact how the receptor interacts with the receptor binding domain (RBD) of HA (Figure 2B). Interestingly, HA from IAVs adapted to humans have higher specificity for α2-6 sialic acid while IAVs adapted to avian species have HA proteins that are specific for α2-3 sialic acid [6]. In humans, the upper respiratory tract contains primarily α2-6 sialic acid while the lower respiratory tract has α2-3 sialic acid. The difference in receptor availability restricts replication of human IAVs to the upper respiratory tract and is hypothesized to be one factor involved in limiting human susceptibility to avian IAVs, which will be discussed below (See Section 1.6.2). Once HA has bound its cognate receptor, the virus is internalized into the target cell via receptor mediated endocytosis. The low pH of the endosomal compartment leads to an irreversible conformational change in the HA exposing the fusion peptide that then mediates fusion of the viral envelope and cell membrane. After membrane fusion, the vRNPs are released into the cell and traffic to the nucleus, which will be discussed in detail below (See Section 1.3). Both receptor binding and cell fusion functions of HA require oligomerization and post-translational modifications. The HA protein forms a trimer and each monomer must be proteolytically cleaved to form disulfide-bonded subunits HA1 (containing the receptor binding site) and HA2 (containing the fusion peptide) prior

to activation (Figure 2A). Most IAV require host-derived serine proteases (trypsin-like) for activation. These proteases recognize a conserved Q/E-X-R motif located at the cleavage site between HA1 and HA2. In mammals, the protease is likely the tryptase Clara that is produced by bronchiolar epithelial cells [7]. Addition of insertional mutations into the cleavage site of HA can broaden protease reactivity and lead to systemic spread via intracellular activation. Indeed, presence of this polybasic cleavage site (R-X-R/K-R) is a critical virulence factor in highly pathogenic avian influenza. Although no anti-influenza drugs are currently licensed that target HA, a peptide has been described that specifically binds to HA and prevents sialic acid binding [8]. Furthermore, anti-HA antibodies that prevent receptor binding or cell fusion are neutralizing and are therefore the most important component of anti-influenza immunity because they are able to prevent infection.

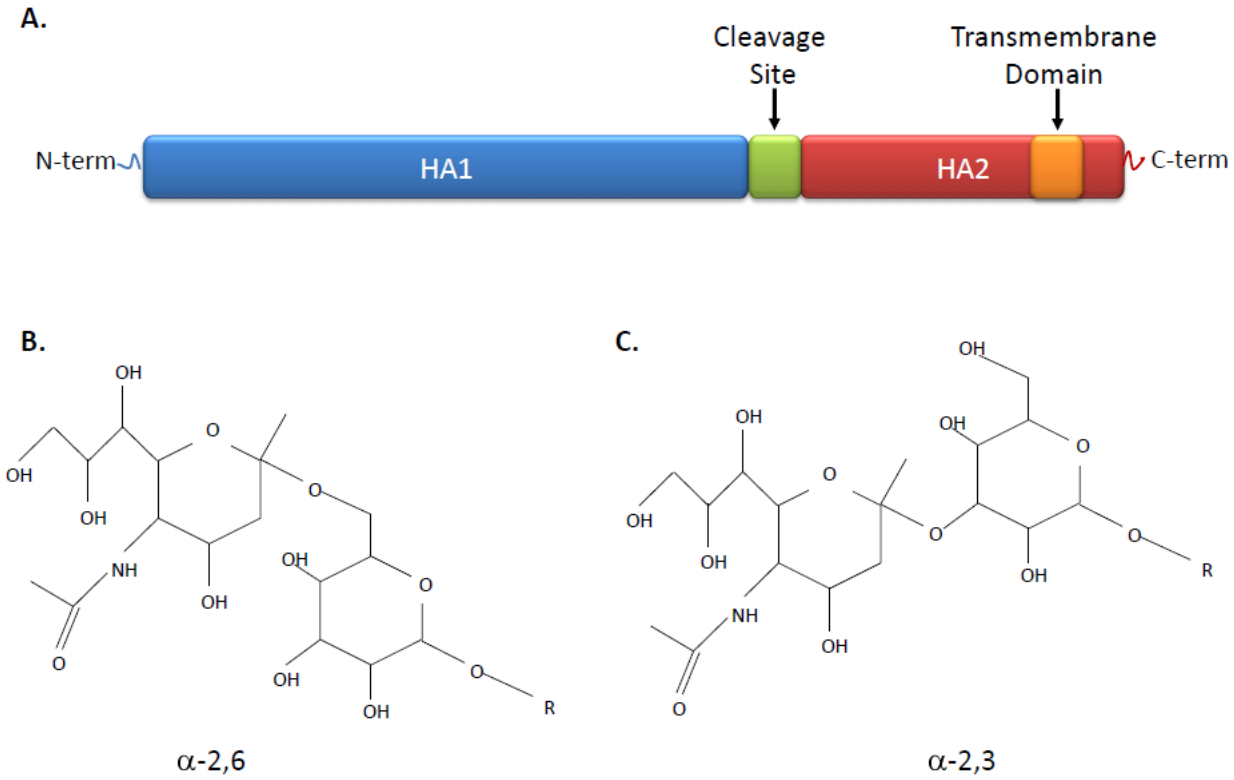

Figure 2: Schematics of HA and sialic acid. HA is translated as a monomer (HA0) and requires protease cleavage to generate the subunits HA1 and HA2. HA1 contains the receptor binding domain and HA2 contains the fusion peptide (A). Sialic acid glycosidic bond isomerization with the terminal sugar impacts conformation leading to either "bent" α2-6 (B) or "flat" α2-3 (C).

1.2.2 Neuraminidase

The NA protein is a type II integral membrane glycoprotein, functions as a tetramer and acts as a sialidase. NA hydrolyzes the glycosidic bond that links the terminal sialic acid to the terminal saccharide (Figure 2). While HA is required for infecting the cell, its presence in the viral buds inhibits particle release by binding to sialic acids on membrane proteins. The enzymatic activity of NA is critical to cleave sialic acid on both host cells to allow particle release during viral egress and also nascent virions to prevent aggregation. The complementary functions of HA binding to sialic acid and NA cleaving sialic acid indicate that these proteins likely require

coadaptation during the course of IAV evolution. The enzymatic activity of NA activates host transforming growth factor beta (TGF-β), which decreases disease severity [9]. Additionally, NA is the target of the anti-influenza drugs oseltamivir and zanamivir which are both sialic acid analogs that bind to the enzymatic pocket of NA and act to inhibit the enzymatic activity. Drug-mediated intervention reduces duration of disease and decreases rates of lower-respiratory tract complications [10]. Despite the effectiveness of the NA-inhibitors, resistance to oseltamivir is present in multiple subtypes of IAV [11-13]. NA is also a target of the humoral immune response and anti-NA antibodies can limit the cell to cell spread of infecting virus, thereby decreasing the severity of illness.

1.2.3 Matrix and Ion Channel

The M1 and M2 proteins are alternative splice products generated from the same gene segment. M1 underlies the viral envelope, interacts with both surface glycoproteins and vRNPs, and promotes assembly and budding [14]. The M2 protein is a small type III integral membrane and functions as a tetramer. M2 is a proton channel and is activated upon exposure to the low pH of the endosome [15]. The influx of protons due to M2 function is crucial during the early phase of the infection cycle to dissociate the association between M1 and vRNPs during viral uncoating [14]. M2 is the target for the adamantane class of influenza antiviral drugs which prevent the low pH activation and therefore inhibit viral uncoating. Additionally, anti-M2 antibodies that prevent activation can limit viral growth by inhibiting M1-vRNP dissociation and have been proposed as a strategy to generate a “universal” influenza vaccine due to the cross-subtype conservation of M2 [16-18].

1.2.4 Nucleoprotein and Polymerase Complex

NP encapsidates all of the RNA gene segments and primarily serves as the structural protein of vRNPs. Additionally, NP is involved in transcription by stabilizing template-polymerase interactions and in intracellular trafficking of vRNPs. NP interacts with several cellular polypeptides: α importin nuclear import receptors, filamentous actin, the nuclear export receptor CRMI and a DEAD-box helicase [19]. The interaction between NP and host proteins is required for proper subcellular localization of viral RNA. In virions, viral RNA is wrapped around NP monomers and, together with the polymerase proteins, are packaged into vRNPs. The polymerase proteins comprise the largest gene products of influenza: polymerase basic proteins 1 and 2 (PB1 and PB2) and polymerase acidic protein (PA). PB1 functions as the RNA-dependent RNA polymerase and PB2 is required for mRNA synthesis by binding and cleaving host 5' mRNA methyl caps [14]. Although PA is essential for all stages of viral replication, including synthesis of both genomic and messenger RNA, its biological roles remain poorly understood. The polymerase complex functions as a heterotrimer and binds to a short hairpin structure derived from a conserved and partially complementary sequence at the terminal 3' and 5' untranslated regions of each gene segment. The roles of the different polymerase proteins during the virus replication cycle will be discussed in detail below (See Section 1.3).

1.2.5 Nonstructural Proteins

The nonstructural proteins (NS1 and NS2) are alternative splice products from the same gene segment. The NS1 protein has multiple functional domains: N-terminal RNA binding domain, a central effector domain, and a C-terminal PDZ binding domain [20]. The RNA binding domain functions via multiple mechanisms: binding and sequestering dsRNA intermediates that are produced during the replication cycle to prevent activation of both 2'-5' oligoadenylate sythetase

(OAS) and retinoic acid inducible gene I (RIG-I) to antagonize the interferon response [21-23] and sequestering small interfering RNA to inhibit induction of RNA interference [24, 25]. The central effector domain interacts directly with distinct cellular proteins: binds and prevents activation of protein kinase R (PKR) to maintain cellular protein synthesis [26], interacts with cleavage and polyadenylation specificity factor (CPSF) and poly (A) binding protein II (PABII) to block cellular mRNA processing and export from the nucleus [27, 28] and functions via a trimeric interaction with eukaryotic translation initiation factor 4 gamma (eIF4GI) and poly (A) binding protein I (PABI) to promote translational initiation of viral mRNAs [29]. The PDZ binding domain interacts with PDZ-containing proteins, many of which are involved in cellular signal transduction pathways [30]. Together, these effects prevent induction of the interferon response by sequestering dsRNA and suppressing cellular gene expression by blocking nuclear mRNA processing. The NS2 protein is also referred to as nuclear export protein (NEP) and is found in small amounts in virions. NS2 facilitates the nuclear export of vRNP complexes and transport to the assembly site at the apical cell membrane. Deletion of the NS1 coding region from the gene segment that encodes for both NS1 and NS2 increases virus susceptibility to the interferon response and results in reduced viral protein production and replication [31]. The NS1 deletion severely attenuates the virus and has been proposed as a mechanism for the generation of new vaccines [32-34].

1.2.6 PB1-F2

An additional small protein that is produced during influenza infection is PB1-F2, which is encoded on the PB1 gene segment in an alternative reading frame. PB1-F2 was initially discovered to be pro-apoptotic and contributes to virulence in animal models by delaying viral clearance [35, 36]. In addition to the pro-apoptotic functions, PB1-F2 down regulates interferon induction by interacting with mitochondrial anti-viral signaling protein (MAVS) and preventing the

activation of interferon regulatory factor 3 (IRF3) [37]. The interferon antagonism acts in a synergistic manner to that of NS1 and together with pro-apoptotic functions make PB1-F2 an important virulence factor and a possible key component in the pathogenicity of pandemic influenza.

1.3 REPLICATION CYCLE

Influenza virus replication cycle (Figure 3) begins with the HA-mediated attachment to cellular surface receptors via engagement of sialic acid. After the attachment step, the virus is internalized via receptor-mediated endocytosis and enters the endosomal pathway in a clathrin dependent manner. Acidification of the endosome to a pH of ~5.0 results in two structural changes in the virion: 1) the M2 ion channel allows protons to enter the virus particle resulting in disruption of M1-vRNP interactions and 2) a conformational change in HA exposes the fusion peptide that inserts into the endosomal membrane and results in envelope fusion. After the fusion event, vRNPs are released into the cytoplasm and imported to the nucleus under the control of nuclear localization signals found on the NP protein.

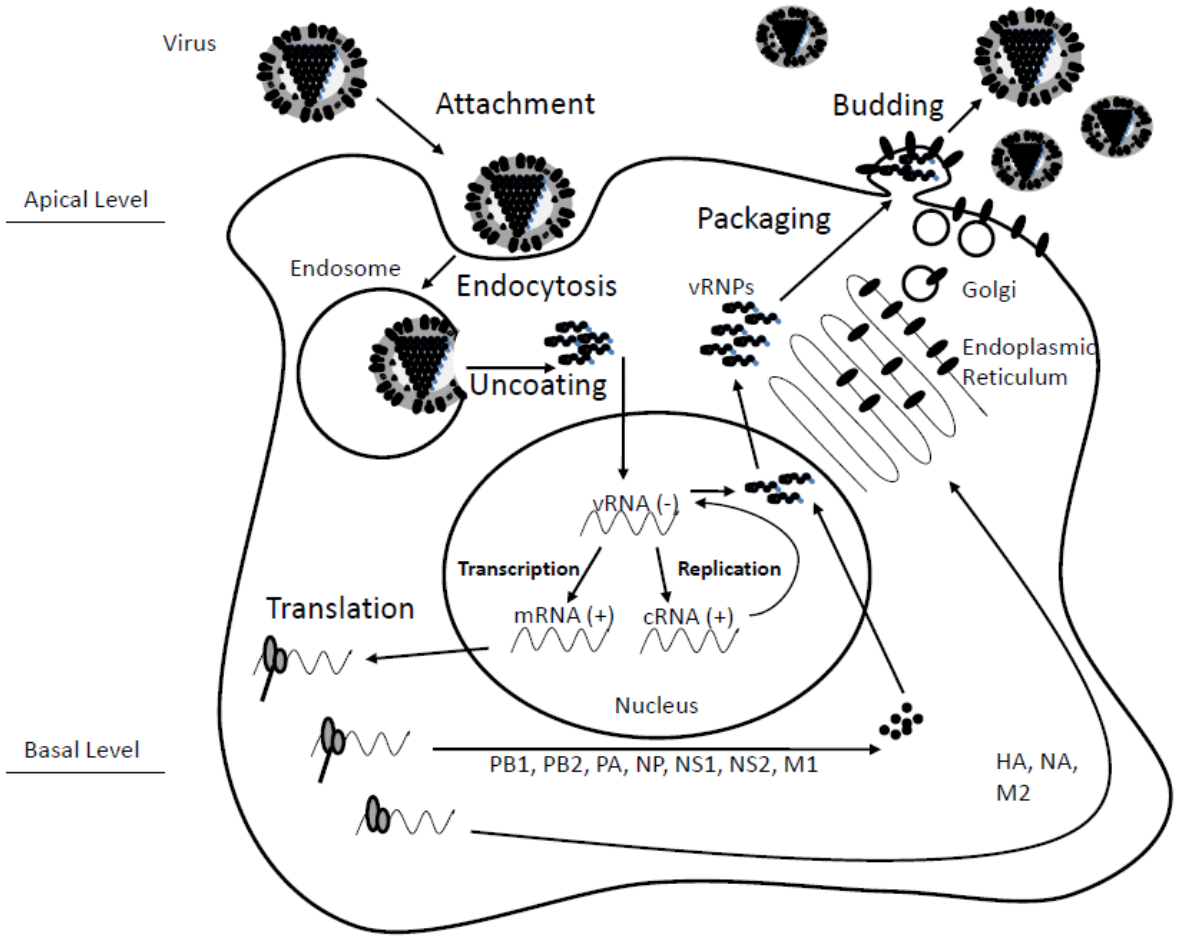

Figure 3: Schematic of influenza virus replication cycle. Infection begins with HA-mediated attachment to cellular receptors. This is followed by endocytosis and transporting through the endosomal pathway. Low pH conditions of the endosome induce membrane fusion and particle uncoating. vRNPs are trafficked to the nucleus where all transcription and replication takes place. Internal proteins are trafficked back into the nucleus where they interact with vRNA to form vRNPs and are eventually trafficked to the apical membrane where they are packaged into nascent virions. Surface proteins enter the endoplasmic reticulum, are trafficked through the golgi apparatus to the cell surface and are incorporated into viral buds.

Once in the nucleus, influenza synthesizes three types of viral RNA: viral messenger RNAs of positive polarity (vmRNA), viral genomic RNAs of negative polarity (vRNA) and viral complementary RNA (cRNA) of positive polarity. Genome replication requires the synthesis of both vRNA for generation of new virus particles and also cRNA as template strands. Synthesis

of vRNA and cRNA is primed by NP, which also binds to elongating strands and blocks the generation of vmRNA (Figure 4A). The vRNA is used in the production of both of the positive polarity RNAs, vmRNA and cRNA. In turn, cRNA is the template from which additional vRNA is synthesized. Transcription of vmRNA utilizes host-derived 5' methyl cap structures to efficiently bind cellular proteins involved in RNA processing, nuclear export and protein production. This process is described as "cap-snatching" and involves PB2 binding to the host mRNA and PB1 endonucleolytically cleaving the host RNA that then serves to prime vmRNA synthesis (Figure 4B). All gene segments are subsequently transcribed into 5' capped, 3' polyadenylated vmRNA for individual proteins. Splicing of vmRNA to generate the coding regions for M2 and NS2 also occurs in the nucleus and is dependent on cellular enzymes. Newly synthesized vmRNA is exported out of the nucleus to the cytoplasm for viral protein production.

After vmRNA is exported into the cytoplasm, viral proteins are translated by cellular protein production machinery. PB1, PB2, PA, NP, NS1, NS2 and M1 are all produced in the cytoplasm by ribosomes that are organized into polysome complexes. Newly synthesized proteins are transported back into the nucleus where they are involved in transcription, genome replication and formation of vRNPs for packaging into nascent viral particles. HA, NA and M2 are synthesized by ribosomes bound to the membrane of the endoplasmic reticulum (ER). The envelope proteins are inserted into the ER and glycosylated prior to transport to the Golgi apparatus. They are then transported to the cell surface via the secretory pathway and are assembled with the vRNP complexes.

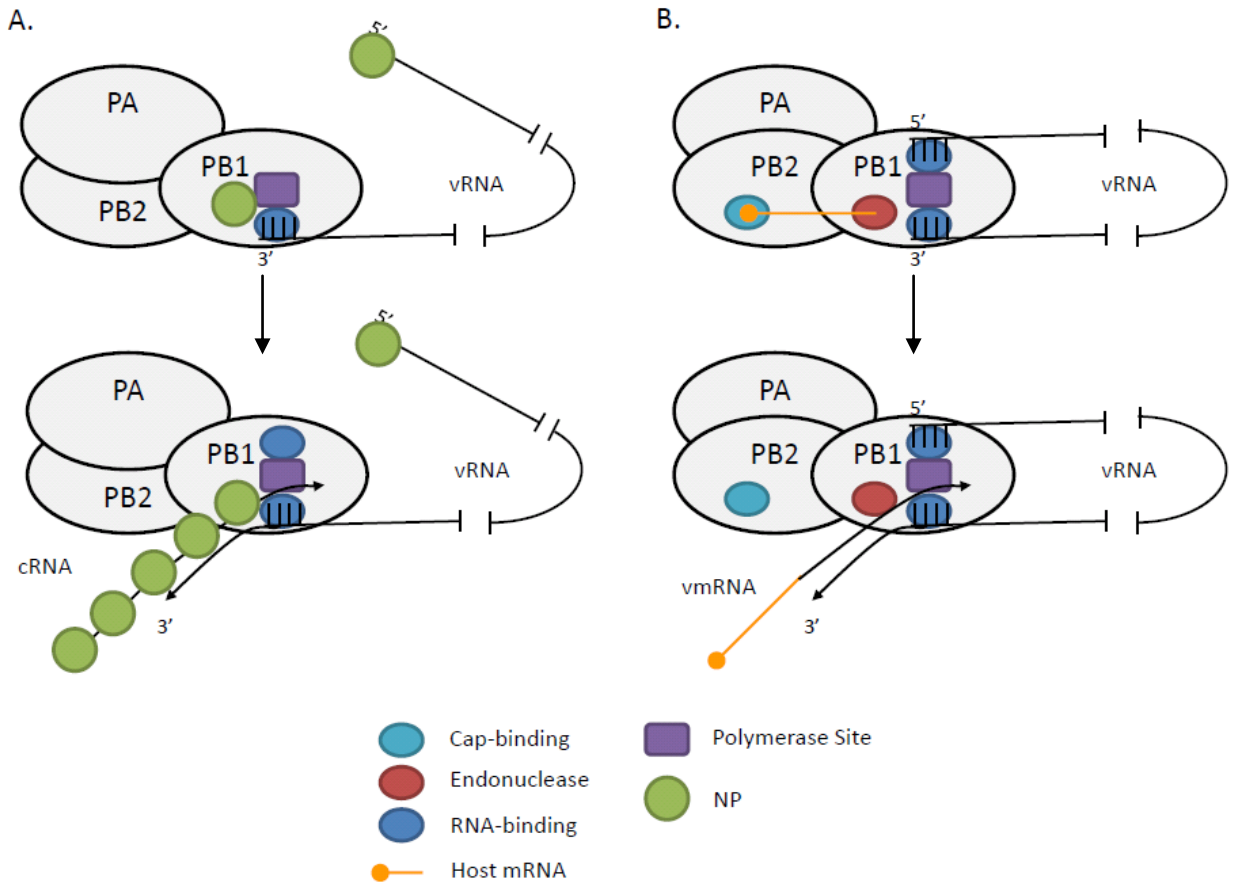

Figure 4: Different priming mechanisms of influenza polymerase complex. The influenza polymerase complex uses NP to prime synthesis of cRNA and vRNA (A). NP also binds to the elongating strand of RNA to promote stability of the polymerase-template interaction. Influenza mRNA is generated using host mRNA to prime synthesis (B). mRNA production is initiated when the 5' untranslated region of the vRNA binds PB1 and activates the cap-binding function of PB2 and endonuclease activity of PB1. After a host mRNA is bound, it is cleaved and vmRNA synthesis is initiated with incorporation of the 5' methyl cap into the vmRNAs.

Formation of vRNP complexes occurs in the nucleus and involves the binding of the viral polymerase complex, NP and NS2 proteins to vRNA. M1 interacts with nuclear vRNPs and, together with NS2, directs the export of M1-vRNP complexes. After nuclear export, M1 inhibits the import of vRNPs back into the nucleus and directs localization to the cell surface. M1-vRNP complexes interact with cytoplasmic regions of HA and NA proteins at the cell membrane and lead to the formation of viral buds. The packaging of vRNPs is a specific event with no more

than 8 gene segments being included in each particle. The exact mechanism by which specific packaging occurs likely involves RNA signals from each gene segment and leads to the vast majority of particles being infectious [38]. Cellular membrane proteins are largely excluded from viral envelopes due to the NA protein. Nascent virions are first released ~8 hours after initial infection [5].

1.4 INFLUENZA DIVERSITY

1.4.1 Antigenic Drift

There is great diversity among type A viruses with 16 HA (H1 to H16) and 9 NA (N1 to N9) subtypes currently identified [39, 40]. Subdivisions of IAV are determined via antigenic characteristics of the HA and NA proteins as measured by double immunodiffusion assays. The different subtypes show up to 30% diversity at the amino acid level, and therefore subtyping IAV is also possible via sequence analysis [41]. Due to the low fidelity RNA-dependent RNA polymerase, IAVs have a high mutation rate ranging from $1x10^{-3}$ to $8x10^{-3}$ substitutions per site per year [42]. Mutations that alter amino acids found in antigenic regions of HA and NA likely confer selective advantages via evasion of pre-existing immunity. Viral diversity generated by the mechanism of mutation accumulation is termed antigenic drift (Figure 5A). Drift variants are critical for IAVs adapted to infect humans because of strong immunological pressures in the host population from both vaccination and prior infection. Antigenic drift mediated diversity results in seasonal epidemics, but is not a cause of pandemic virus emergence.

1.4.2 Antigenic Shift

The segmented nature of the influenza virus genome can lead to another mechanism of diversity in which an entire gene segment(s) is exchanged: a process termed reassortment. Co-infection of one host cell with two different IAVs (*e.g.* H3N2 and H5N1) can lead to the production of progeny virus with genes from both parental strains (*e.g.* H5N2 and H3N1). Reassortment events involving the surface proteins HA and/or NA result in antigenic shift (Figure 5B). From any two parental viruses, there are a possible 256 different combinations of the eight gene segments in nascent virions. Even in the event of co-infection with two viruses of the same subtype, exchange of internal genes such as polymerase proteins or NS can alter the pathogenic phenotypes of viruses that are produced without altering the antigenic classification of the progeny. Indeed, reassortment is an important event that is relatively common in the evolution of IAV [43, 44]. Similarly, drifted viruses of the same subtype can reassort with each other resulting in the selection and spread of the drifted virus. The emergence of a pandemic virus is almost always associated with reassortment events and typically involves both surface and internal proteins [45-49].

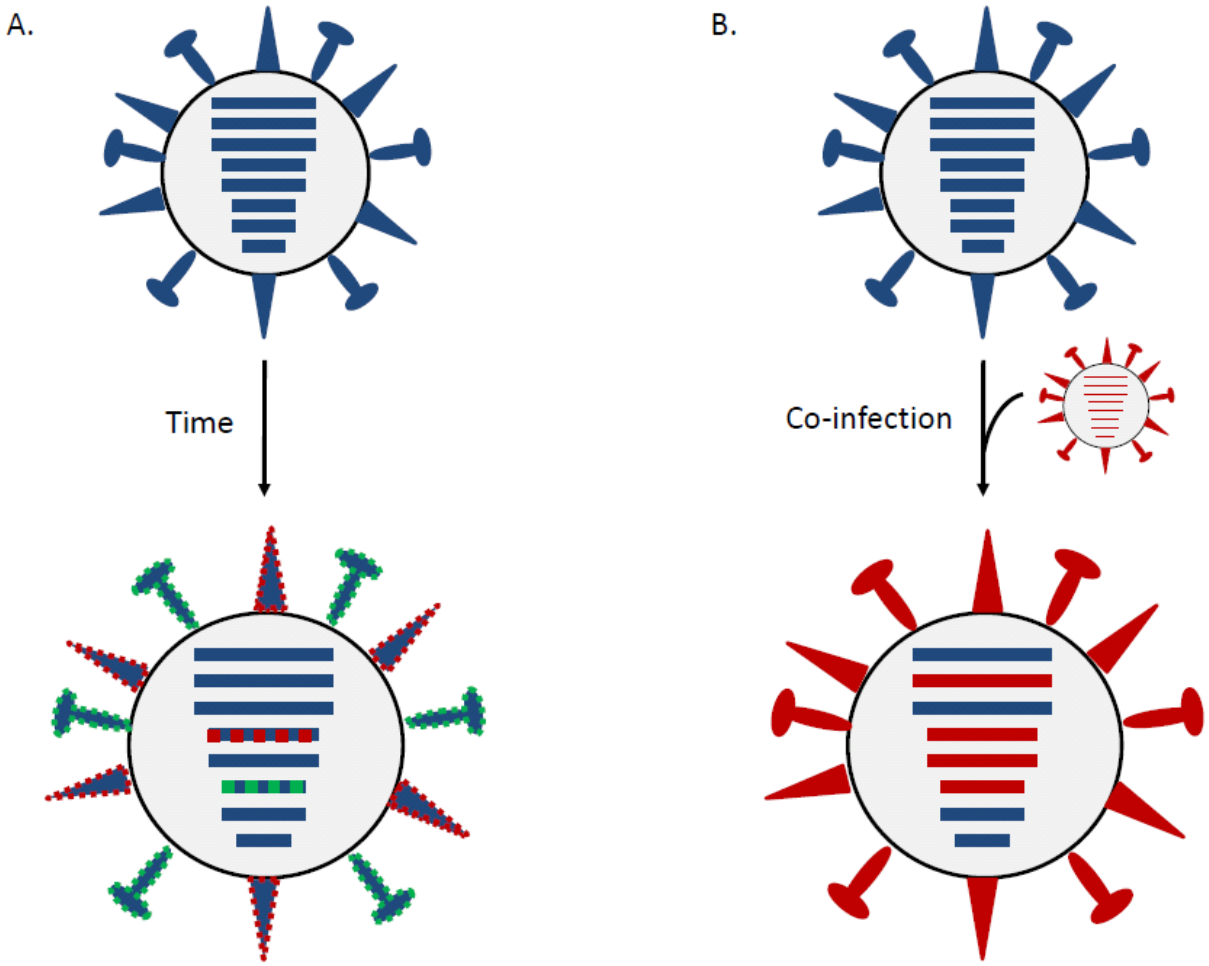

Figure 5: Diversity mechanisms of influenza viruses. Mutations occur over time due to the low fidelity polymerase. When these mutations accumulate in the antigenic regions of the surface proteins and the virus can escape pre-existing immunity the process is termed antigenic drift (A). The segmented nature of the genome allows for rearrangement of gene segments from different viruses when a cell is simultaneously infected. When the gene segment reassortment results in production of progeny virions with novel surface protein(s), the process is termed antigenic shift (B).

1.5 INFLUENZA ANIMAL RESERVOIRS

Wild avian species are reservoir hosts to genetically diverse IAVs across the globe. Most documented infections are asymptomatic and are found in aquatic birds of which over 100 species have been identified as harboring virus [50]. IAVs are usually transmitted via the fecal-oral route where the infection is located in the epithelial cells of the lower intestinal tract. Distribution of HA and NA subtypes is not uniform, with some being more prevalent than others. An example of this disparity is that more than half of the avian isolates in the Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) belong to the H5 or H9 subtypes [51]. It is unknown whether the prevalence of circulating IAV subtypes matches the available data from isolates harvested during routine surveillance. Additionally, the majority of surveillance efforts have focused on aquatic waterfowl and shorebirds and there is not enough data to determine if particular subtypes or strains within a given subtype have evolved to a replicate in a particular species [52]. However, factors such as geographic location, migration patterns and feeding behavior likely are involved in viral adaptation and fitness [53].

Although aquatic birds are the reservoir of IAVs [54], viruses from wild birds are associated with emergence of novel viral lineages that are transmissible in the new host. The most common host switch is to domestic poultry including chicken, turkeys and quails [40]. In addition to avian species, IAVs have also been isolated from multiple mammalian species including humans, pigs, horses, cats, dogs, and even sea mammals [40]. Although IAV strains are isolated consistently from swine and horses, other wild and domestic mammalian species have only sporadic virus isolation which could be due to occasional zoonotic infectious outbreaks that fail to persist. Equine IAV has been recognized for hundreds of years and is often associated with human epidemics and/or pandemics [55]. Both H7N7 and H3N8 IAV subtypes have been isolated from horses, although only the H3N8 subtype persists today. Phylogenetic analysis indicates that equine IAV strains are likely derived directly from avian IAV

and not human or swine influenza virus strains [54]. IAV in swine was first detected in 1918 and persists through today as a stable lineage [56]. Multiple subtypes of IAV have been isolated from swine including H1N1, H2N2 and H3N2 [57]. Swine are susceptible to both avian and human adapted IAV strains and as such are proposed as a “mixing vessel” in which reassortant viruses with pandemic potential can be generated [58]. The susceptibility to diverse strains of IAV could be due to the presence of both α2-3 and α2-6 linked sialic acids, although little α2-3 is found in the upper respiratory tract [59]. Notably, the combination of two unrelated swine H1N1 viruses resulted in the novel H1N1 pandemic of 2009 indicating that swine IAV may be the reservoir from which human pandemics emerge [48, 60].

1.6 HUMAN INFLUENZA INFECTIONS

1.6.1 Seasonal Influenza

Influenza viruses of types A (H1N1 and H3N2) and B are a significant cause of morbidity and mortality in humans and are able to cause repeated disease due to antigenic drift. Human influenza is maintained as a permanent lineage by person to person spread during acute infection. Additionally, there is no evidence of persistent or chronic infection of influenza in humans [40]. Although influenza infection is generally associated with seasonal epidemics, the virus can be detected in a large city throughout the year and influenza is detected year round in various parts of the world [61]. In North America, influenza epidemics generally occur from January through April with occasional outbreaks in December or May [62]. IAV transmits more efficiently in the cold and dry atmospheric conditions that exist in the winter months and this could be one explanation for the pattern of seasonal epidemics [63]. Seasonal influenza

epidemics in the United States result in up to 200,000 hospitalizations and 36,000 excess deaths annually [40, 64].

Infection with influenza occurs in the columnar epithelial cells of the respiratory tract. The virus then spreads from person to person by small-particle respiratory droplets or by direct contact. After infection with influenza virus, the disease presents clinically with fever, headache, sore throat, muscle pain and a dry cough. The illness usually lasts for 3 days with full recovery in 7-10 days. Complications of influenza infection include secondary bacterial infections, myositis, and Reye's syndrome [65]. Secondary bacterial pneumonia usually involves *Streptococcus pneumoniae, Haemophilus influenza* or *Staphylococcus aureus* [66]. Although rare, primary viral pneumonia can also occur and is associated with a high mortality rate [66]. High-risk groups include the young, the elderly, pregnant women, and those with chronic underlying medical conditions, such as chronic obstructive pulmonary disease (COPD), diabetes, or human immunodeficiency virus (HIV) [67]. The most important factor to limiting morbidity post-infection is the presence of pre-existing immunity to influenza, from either vaccination or prior infection with an antigenically similar virus. Indeed, influenza vaccination has been in use for over 50 years and influenza is unique among variable viruses in that it can be prevented by current vaccines but remains a significant cause of morbidity and mortality [68, 69]. An important consideration is that the relative severity of influenza infection is dependent upon the interplay between pre-existing immunity and the pathogenicity of the infecting virus. Furthermore, the relationship of influenza antigenic diversity, pathogenicity, and population immunity is reflected in the size and impact of both seasonal epidemics and also occasional pandemics.

1.6.2 Pandemic Influenza

Pandemic outbreaks of influenza occur sporadically in the human population and appear to have occurred for over 500 years [55, 70]. Up to 50% of the population can be infected during a single pandemic year and this increase can be associated with a dramatic rise in influenza-related mortality [60]. More recently, IAVs have caused four different pandemics in the last 100 years: the 1918 “Spanish” influenza (H1N1), the 1957 “Asian” influenza (H2N2), the 1968 “Hong Kong” influenza (H3N2) and the 2009 novel H1N1 “Swine Flu” (Figure 6) [71, 72]. The three 20th century influenza pandemics are all suspected to have involved antigenic shift resulting from the reassortment of HA and other gene segments from an avian virus with a currently circulating human virus [73]. Although the 2009 pandemic virus included some gene segments from avian IAVs, the novel virus was derived from a reassortment of two swine H1N1 viruses [74]. The “Spanish” influenza of 1918 was the worst pandemic in recorded history and resulted in an estimated 675,000 deaths in the U.S.A. and up to 50 million deaths across the globe [75]. Although the pandemics of 1957, 1968 and 2009 have all been less severe than that of 1918, they have all caused significantly more disease than is observed during seasonal epidemic years. Interestingly, all of the pandemic viruses since 1918 have contained genes derived from the 1918 virus which may indicate the presence of a larger “pandemic era” [76].

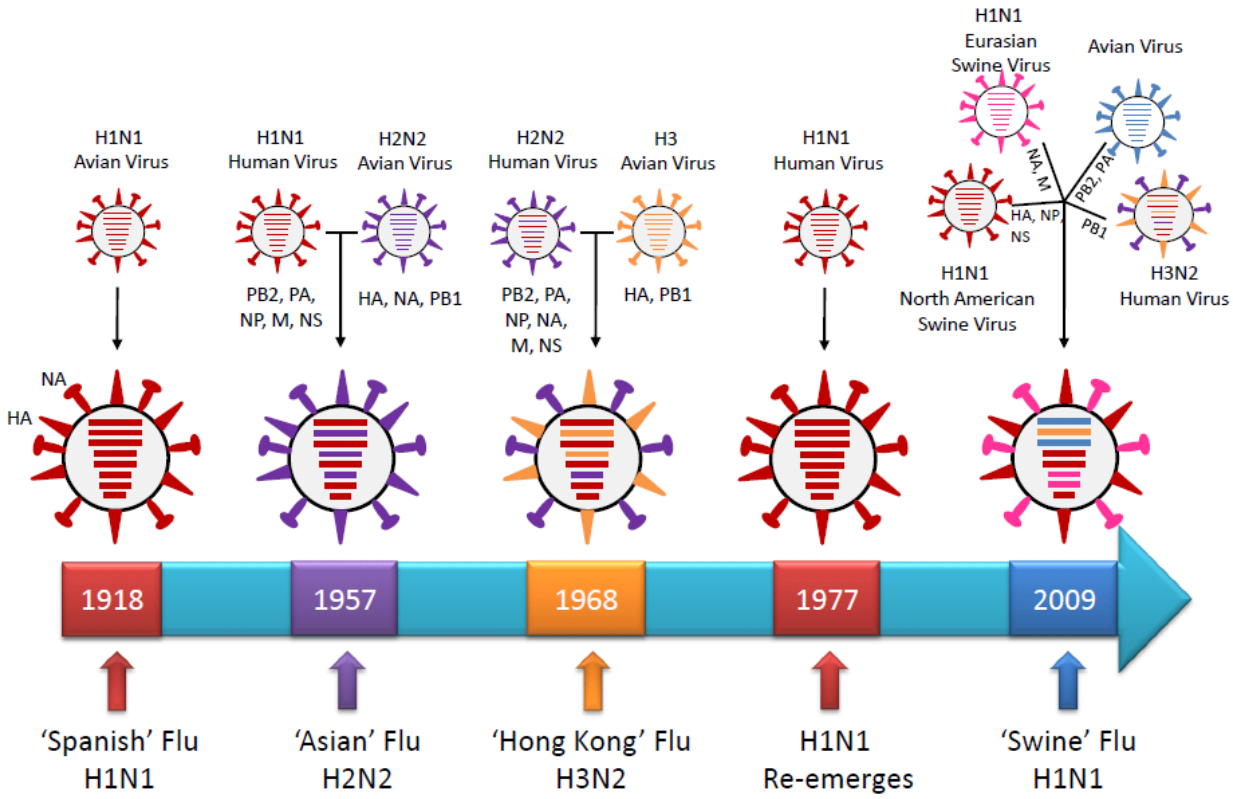

Figure 6: Pandemic viruses from the last 100 years. Pandemic outbreaks of influenza occur sporadically. The 1918 pandemic was caused by the emergence of an avian virus into the human population. The pandemics of 1957 and 1968 were caused by the reassortment of an avian virus with a circulating human virus. In 1977, the human H1N1 virus re-emerged and enough immunity in the population prevented another pandemic. The 2009 pandemic was a reassortant virus combing swine, avian and human viruses. All pandemic viruses have included gene segments from the 1918 virus (red bars).

Emergence of pandemic viruses into the human population from animal reservoirs has indicated that the host switch events are polygenic and involve adaptation of replication and transmission properties [55, 77]. Indeed, recent pandemic viruses have all emerged in different ways, which complicates the understanding of zoonotic IAV adaptation to humans [60]. Direct infection of humans with avian IAVs has occurred in the past 15 years and is predominately associated with the epizootics of H5N1 in Southeast Asia, the Middle East and Africa and

smaller outbreaks of H7N7 in the Netherlands and H7N3 in Canada [78-80]. Prior to these relatively recent zoonotic infections, there was little serological evidence of direct avian to human IAV infection [81]. Genetic mutation signatures of human IAV adaptation have been proposed using comparative analyses to relate contemporary viruses to the host-switching 1918 strain [45, 82]. However, when avian to mammalian switch events are evaluated independently, no parallel evolution has been observed indicating that switch events are polygenic and ongoing evolution of IAV in various hosts is likely changing the requirements for host-switch [49]. Viral properties including virulence, replication competency and transmission may be related, but independent, properties that are associated with different mutations in different gene segments. Indeed, the emergence of the genetically diverse 2009 H1N1 pandemic virus supports the finding that independent and polygenic changes can lead to IAV adaptation in humans [83-85].

Even though identification of specific molecular markers indicating IAV human adaptation has been elusive, several features of host switch events have been identified. IAVs from an avian origin generally have a higher GC content than those viruses adapted to humans [86, 87]. Although the biological significance of the altered nucleotide composition is unclear, possible reasons include higher core temperatures in avian species, host-specific mutations in the cellular components required for RNA replication and avoiding immune detection via host mRNA composition mimicry [86, 87]. Another feature of IAV human adaptation is HA receptor binding specificity. The HA receptor binding domain is conserved in avian IAV, but is mutated in human IAVs. These mutations have been mapped to several key residues and alter the binding preference from α2-3 sialic acid to α2-6 sialic acid [40]. Although receptor usage is clearly important for IAV ability to infect cells, its relative role in the overall phenomenon of host switch is more complicated. Avian IAV of H7N7, H7N3 and H9N2 subtypes have enhanced specificity for α2-6 sialic acid, but have only caused sporadic infections in humans indicating that receptor binding alone is not sufficient for sustained infection in the human population [88-90].

Furthermore, the H5N1 subtype generally has α2-3 receptor preference and introduction of mutations resulting in α2-6 usage did not result in enhanced replication or transmission in a ferret model [91]. Additionally, H5N1 has infected hundreds of humans and fixation of mutations that mediate α2-6 binding has yet to occur [92]. The role of polymerase proteins is also important to host switch as emergence of pandemic strains usually involves the polymerase complex in addition to HA and NA evading prior immunity. The PB1 genes from the 1918, 1957 and 1958 pandemics have all been avian-like indicating that this gene segment retains function without much adaptation to a new host [40, 45, 60]. Additionally, specific residues in the PB2 protein are linked to IAV adaptation to mammalian hosts [93, 94]. Mutations at these residues are linked to pathogenicity, transmission and replication fitness [95-97]. The molecular mechanisms of host switch in IAV are undoubtedly complex and involve the interplay between multiple gene segments. It is clear that no single mutation is associated with replication in mammals, but rather a collection of changes involving HA receptor specificity, polymerase activity and even global nucleic acid composition all are involved in IAV adapting to a new host.

1.7 INFLUENZA IMMUNOLOGY

Neutralizing and receptor-blocking antibodies against HA are the primary means of protection from influenza infection [66]. Antibodies directed against NA play an important, but secondary, role. Although immunity against multiple influenza proteins can mitigate disease, only anti-HA antibodies are able to prevent infection [98]. Five antibody binding regions, A-E, have been determined based upon the H3 subtype and the corresponding structural regions have been mapped onto other subtypes, although the exact position and number of antibody binding

regions remains uncertain for avian subtypes (Figure 7) [99]. The majority of the anti-HA antibody response is focused on the variable epitopes on the surface of the molecule where the virus escapes via antigenic drift. Receptor-blocking antibodies recognize the variable head region of the HA molecule and are subtype or even strain specific [100]. Antibodies that are specific for a more conserved epitope located in the stem region of the HA molecule do not prevent receptor engagement, but neutralize the infectivity of the virus by blocking the low pH conformational change required for fusion [101-103]. These broadly-neutralizing, stem-reactive antibodies are less prevalent than antibodies against the globular head, but are elicited by both infection and vaccination [104, 105]. Several hypotheses have been proposed to explain the sub-dominance of the broadly neutralizing antibody repertoire: the epitope may be inherently poorly immunogenic, the epitope may be not be readily accessible on the intact virion, or the globular head epitopes may be overwhelmingly immunodominant [106]. Antibodies that block the function of HA, via either blocking receptor binding or preventing fusion, are the most important factor in mitigating human disease caused by influenza virus infection.

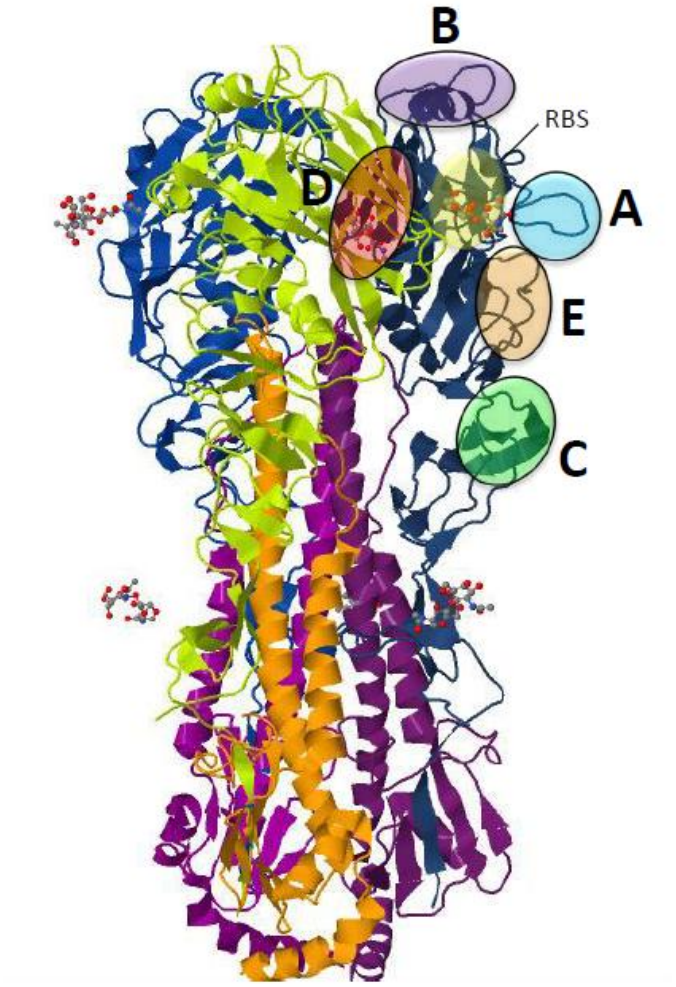

Figure 7: Antigenic regions of HA. The crystal structure of H5 HA is displayed as a trimeric molecule with each subunit (HA1 or HA2) colored per monomer. The antigenic regions for a single monomer that are predicted from H3 residues are circled. The receptor biding site (RBS) is shaded yellow. The image was generated using the 2FKO structure deposited into the Protein Data Bank (www.pdb.org) and visualized with Jmol software [107].

Immune responses to other influenza proteins have also been shown to limit disease, but are not able to prevent infection. NA-inhibiting antibody responses can block the enzymatic activity that is required for particle release. Therefore, anti-NA antibody responses reduce viral spread within the host after initial infection. Preventing NA activity does not prevent infection and appears to play a secondary role in protection, but it can limit disease severity when anti-

HA mediated immunity is not effective [108, 109]. In the context of pandemic influenza, anti-NA antibodies from H2N2 viruses reduced disease severity during the H3N2 pandemic of 1968 but were unable to completely prevent the pandemic [110]. Additionally, anti-NA immunity is increased by vaccination and is believed to contribute to reduced disease in elderly populations during the 2009 H1N1 pandemic [111]. The conserved proteins M2 and NP have also been proposed as antigens against which a universal influenza immune response may be generated [112-115]. Although the efficacy of these antigens has been demonstrated in mice, the findings were not confirmed in a more rigorous ferret challenge [116]. In humans, robust universal protection by conserved antigens is not supported by epidemiological evidence. Repeated infection would not be possible if conserved antigens elicited effective cross-reactive immunity and influenza would resemble one-time childhood illnesses such as measles, mumps or chicken pox. Instead, viruses differing only in their HA and NA are able to spread across the world and re-infect individuals who have previously experienced influenza either through vaccination or primary infection [69].

Even though immunity directed against conserved antigens does not prevent infection and spread of influenza, cell-mediated immunity against these targets does play a role in reducing disease severity. Influenza virus infected cells are eliminated in the absence of antibody responses and animals primed for a cell mediated immune response clear virus more rapidly than naïve animals [117-120]. $CD4^+$ T cells do not efficiently function directly in viral control, but rather are required to promote high quality antibody responses [121]. Additionally, analysis of IFN-γ production has suggested that influenza specific $CD4^+$ T cell populations are smaller, but more diverse than the $CD8^+$ T cell populations [122, 123]. $CD8^+$ effector T cells function to eliminate influenza infected cells via either the Fas-Fas ligand pathway or perforin and granzyme mediated cytotoxicity [124]. Memory CD8+ T cells are generated in large quantities after primary influenza infection, but re-infection even in the presence of large numbers of effector memory $CD8^+$ T cells still results in high viral titers for 48 hours after

infection [120]. Therefore, although cell-mediated immunity to conserved viral antigens is able to restrict viral replication and limit disease severity, influenza can still infect and transmit in the absence of neutralizing antibody regardless of the magnitude of the cell-mediated response.

In addition to adaptive immune responses, innate immunity also plays a role in influenza control and pathogenesis. Type 1 interferons induce a number of anti-viral responses in cells and therefore provide a protective function in the early stages of influenza infection. One of the anti-viral responses that is stimulated is the induction of *Mx* genes that inhibit primary transcription of influenza gene segments [125, 126]. Complement and natural antibody mediate protection in the absence of prior adaptive immunity in mouse models of influenza infection [127, 128]. Additionally, natural killer (NK) cells control influenza infection and have activating receptors that directly recognize HA from multiple subtypes [129, 130]. NK cells kill influenza virus infected cells rather than directly destroying virions. Neutrophils and alveolar macrophages can mediate protection against infection by low pathogenicity influenza viruses [131]. However, these same cell types are associated with excess lung damage during infection with high pathogenicity viruses, such as the reconstructed 1918 H1N1 strain or highly-pathogenic H5N1 influenza isolates [132-134]. Aberrant cytokine and chemokines responses have also been correlated with increased pathogenicity [135]. This “cytokine storm” directly contributes to the pathogenicity of virulent influenza infection and is important factor in severe human infections [136]. Innate immune factors are often able to directly control influenza virus replication, but in the context of highly pathogenic infections, innate immunity can contribute to disease severity and therefore it is of utmost importance for vaccine strategies to utilize the efficient control mechanisms by the adaptive immune response, specifically neutralizing antibodies against HA.

1.8 INFLUENZA VACCINES

Vaccines against influenza have been in use in the human population for over 50 years [68]. Influenza vaccines elicit a strain specific anti-hemagglutinin antibody response and serum hemagglutination inhibition antibody titer is the most commonly measured correlate of protection. Susceptibility to infection is inversely correlated to the reciprocal dilution titers of receptor blocking antibodies with levels of 1:40 preventing infection in about 50% of the population [137]. The majority of influenza-related mortality occurs in the elderly and those with underlying conditions, such as pregnancy, diabetes or HIV infection, and therefore the immunization strategies are focused on protecting these vulnerable populations [138, 139]. Currently licensed vaccines are composed of a trivalent formulation containing a representative strain from each of the circulating strains: IAV (H1N1 and H3N2) and influenza B. Each year, the World Health Organization proposes recommendations to vaccine manufacturers and governmental agencies about which influenza strains to include in the vaccine upcoming influenza season. For the 2011-2012 season, the vaccine strains are A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2) and B/Brisbane/60/2008 (type B). Despite global sequencing efforts, predictions of what will be the dominant circulating strain are not certainties and occasionally drift variants emerge as the most prevalent strain, resulting in vaccine escape [140]. Influenza viruses chosen for vaccine production are propagated in embryonated chicken eggs and although this requires large amounts of eggs and specialized production facilities, this strategy remains the most consistent production system for high titer influenza virus. Cell-based production systems have also been evaluated, but they have not been approved for wide use in humans [141].

Two types of influenza vaccines are approved for use in humans in the United States: inactivated virus (TIV), which includes split and subunit vaccines, and live-attenuated virus (LAIV) vaccines generated from cold-adapted influenza viruses. TIV is approved for use in

individuals greater than 6 months of age, is administered via intramuscular injection and is generally well tolerated [66]. Additionally, TIV is immunogenic and efficacious in children and adults inducing immunity in 60-90% of recipients [67]. Immunogenicity in the elderly is generally reduced and this is due, at least in part, to immunosenescence in the older population [142, 143]. LAIV is administered via an intranasal spray and is approved for use for those individuals between 2 to 49 years of age. Use is not approved for young children due to wheezing concerns, although the relative risk is under debate [144-147]. An advantage of using LAIV is the ability to stimulate $CD8^+$ T cell memory, however, the live-attenuated vaccine does not efficiently elicit serum hemagglutination inhibition antibodies that is the standard measure of immunogenicity. Despite low levels of receptor blocking antibody, LAIV is effective at protecting recipients from infection and this is likely due to mucosal immune responses that are not detected with standard serum antibody assays [148].

New vaccine platforms and adjuvants are also being developed to address the problems of long lead times for traditional egg-based manufacturing and poor immunogenicity in high risk populations. Novel technologies include virus-like particles, recombinant subunits and HA head or stalk specific region vaccines [105, 149-154]. These technologies offer several advantages over traditional egg-based manufacturing including recombinant technology, rapid scale-up and directed immune responses. Adjuvants are vaccine components that enhance the immune response against co-administered antigens. These compounds are used to enhance immunogenicity and/or lower the dose required to achieve protective immunity. Aluminum salts have been used to enhance antibody titers for almost 100 years [155]. These adjuvants function to increase antigen uptake and processing by antigen presenting cells and also activate the NOD-like receptor protein 3 inflammasome pathway [156, 157]. MF59 is an oil-in water emulsion and is another example of an adjuvant licensed for use in humans [158]. This novel adjuvant functions to enhance immune responses via a TLR-independent pathway that involves injection-site activation of a diverse set of immuno-stimulatory genes [159]. Importantly, novel

technologies, such as virus-like particles, and novel adjuvants, such as MF59, have been proposed as methodologies to improve influenza vaccination in high risk populations. The long history of influenza vaccine administration has demonstrated the safety and efficacy of both inactivated and live-attentuated vaccines when matched to the dominant strains in a given year. However, due to the diversity of influenza viruses, the protective capacity of traditional strategies remains limited to that of the individual components and is subject to regular escape by viral evolution. By using new technologies, it may be possible to further improve influenza vaccines via elicitation of immune responses in high risk populations and also increasing the breadth of the antibody repertoire.

1.9 STRATEGIES TO INCREASE VACCINE BREADTH

Simultaneous circulation of diverse IAV (H1N1 and H3N2) and influenza B viruses has been a challenge for seasonal influenza vaccine production for over 30 years. The current seasonal vaccine, both TIV and LAIV, uses a polyvalent formulation to address the issue of distinct viruses circulating simultaneously. Polyvalent influenza vaccine formulations utilize mixtures of the same antigen from different strains. Other polyvalent strategies have included mixing matched antigens from different strains or mixing different antigens. The goal of polyvalency is to increase the breadth of vaccine coverage by combining diverse components into a single vaccination. Polyvalent vaccine strategies increase reactivity for many pathogens including, but not limited to, influenza [160-162], monkey pox [163], HIV [164, 165], HPV [166, 167] and pneumococcal disease [168]. Although polyvalent vaccine formulations clearly expand the breadth of a single vaccine formulation, the reactivity is still limited to the individual components.

An alternative to polyvalent vaccines is the use of centralized sequences to generate vaccine antigens (Figure 8). These vaccines consist of synthetic sequences that represent a population of sequences and are generated by three different methods: center-of-the tree, ancestral, and consensus [169]. Center-of-the tree (COT) sequences are derived by constructing a phylogenetic tree and selecting the sequence that is equidistant from all points [170]. Ancestral sequences represent the most recent common ancestor for the population of sequences selected and are effective influenza vaccine candidates [171, 172]. Consensus sequences encode the most common amino acid found at each position for the selected population [173]. Ancestral and COT sequences are an estimate of an actual sequence that existed in the past, while consensus sequences are more relevant to the current epidemic [173]. The population that is assembled for the generation of a centralized sequence should be an accurate representation of circulating sequences and every effort taken to avoid bias due to sampling or outbreak dominance. Consensus based vaccine strategies elicit broadly reactive immune responses against multiple pathogens including HIV-1 [174-178] and influenza [179-182]. Consensus HA, NA and M1 sequences have been developed for H5N1 DNA vaccination and elicited robust cellular immune responses to each vaccine component, as well as antibodies to the HA [180-182]. Using centralized antigens for influenza vaccines either alone or as components of polyvalent formulations has the potential to overcome the challenge imposed by antigenic diversity and improve the effectiveness of both seasonal and pandemic influenza vaccines.

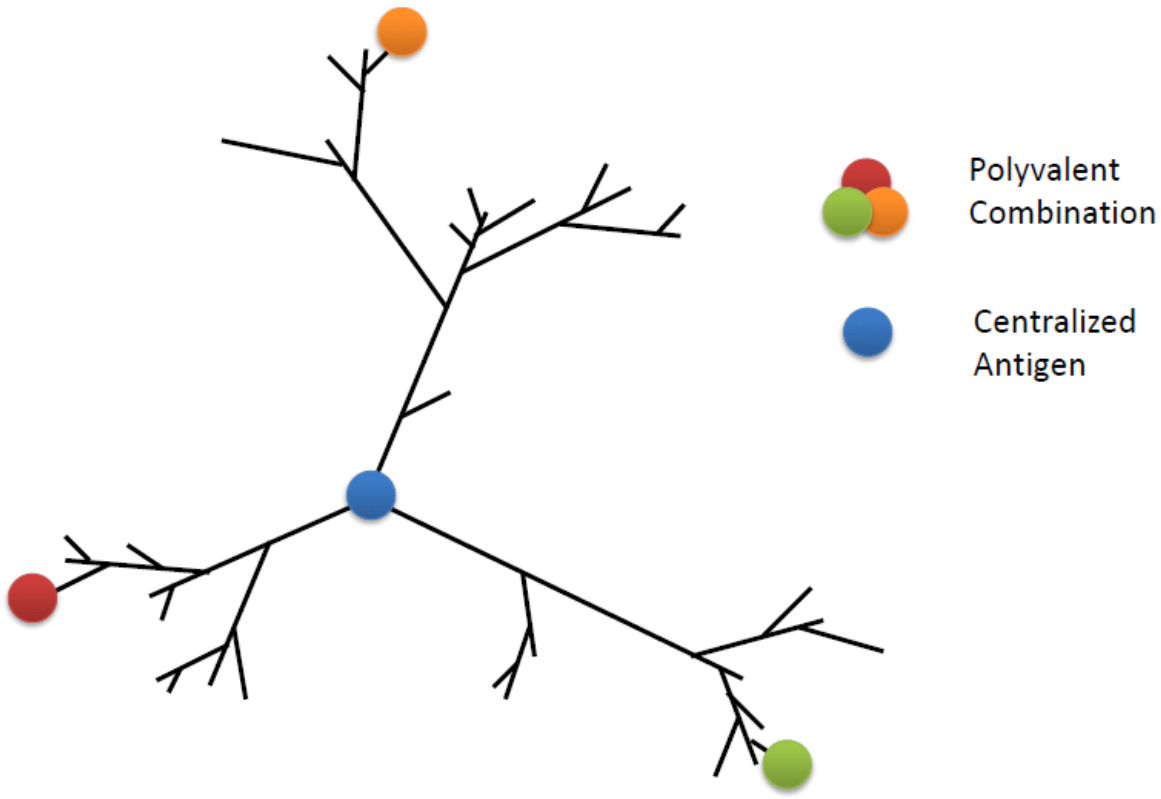

Figure 8: Strategies to elicit broader immune responses. Schematic showing possible vaccine designs for diverse antigens. Red, orange and green circles represent single isolates from different antigenic clusters that are selected for inclusion in a polyvalent mixture. The blue circle represents the hypothetical relationship of a centralized antigen to the various clusters.

1.10 HIGHLY PATHOGENIC H5N1 INFLUENZA

1.10.1 Pandemic Risk

The World Health Organization (WHO) has identified three criteria for defining the start of a pandemic: 1) a virus to which the world's population is mostly naïve must emerge, 2) the new

virus must be able to replicate in and cause severe disease to humans, and 3) the virus must be efficiently transmitted among humans as indicated by chains of human to human spread [183]. Several avian subtypes have caused human infections including H5N1, H7N7, and H9N2. All of these avian strains have a novel HA to which the human population is largely immunologically naïve. In particular, the avian influenza subtype H5N1 has emerged as a highly pathogenic virus in domestic poultry and humans after making its first appearance in 1997. The novel antigenicity of H5N1, as well as its high pathogenicity, fulfills two of the three requirements for pandemic virus set forth by WHO. As revealed by ongoing surveillance, H5N1 is spreading geographically through avian species and represents a significant threat to human health as incidences of human cases involving direct transmission from birds have been reported consistently since 2003 [184]. Through June of 2011, there have been 562 confirmed cases of human infection from H5N1 viruses that have resulted in 329 deaths (Figure 9) [185]. Of obvious concern is the almost 60% case-fatality rate which demonstrates the need to develop effective vaccines to protect against this potentially devastating pandemic.

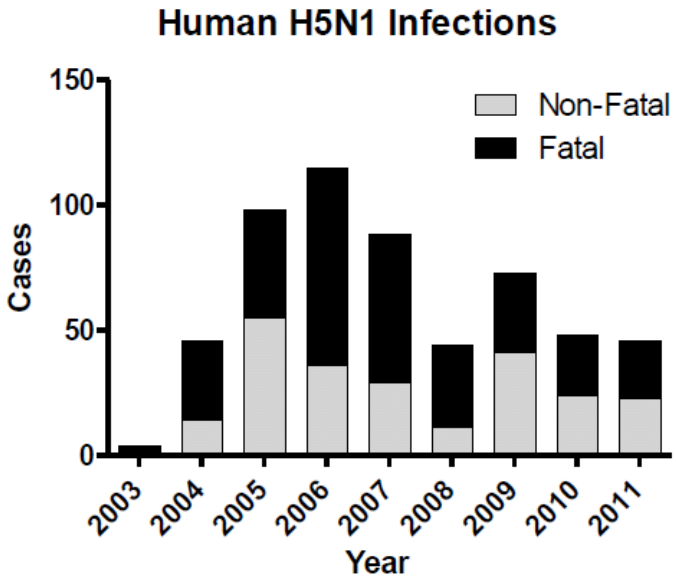

Figure 9: Human infections of H5N1 by year. Human infections continue to occur at a similar frequency since the re-emergence of H5N1 in 2004. The graph shows non-fatal (gray) and fatal (black) human cases of H5N1 infection per year.

1.10.2 Challenges to H5N1 Vaccine Development

Although a clear need exists to develop vaccines against H5N1 influenza, these viruses present unique practical challenges to vaccine development. Many of the vaccine development strategies are being directly applied from the long history with seasonal influenza, but this method may not be directly applicable due to specific challenges presented by H5N1 influenza viruses. Currently licensed influenza vaccines are derived by growing viral stocks in embryonated chicken eggs and the entire process, from strain selection to virus growth to quality assurance, normally takes 6 to 9 months to complete [186]. The high virulence of H5N1 influenza in both poultry and humans makes growing viral stocks for traditional inactivation and generation of split vaccines both difficult and dangerous in the contemporary egg-based systems. The manufacturing process must take place under more rigorous biosafety conditions and these influenza viruses are lethal to the eggs and prevent the harvesting of high titer virus

from the allantoic fluid. A major determinant of the increased virulence involves a multibasic cleavage site in the H5 that is cleaved by ubiquitous cellular proteases. Reverse genetics is a method by which custom influenza viruses and mutations can be engineered by introducing viral DNA into cells with promoters in place to ensure protein production and negative sense RNA synthesis resulting in rescued engineered viruses. These systems can be employed to mutate and eliminate the polybasic cleavage site, thus attenuating the highly pathogenic H5N1 virus and making it less virulent without affecting antigenicity [187]. Another challenge during a new avian influenza pandemic will be the lack of embryonated eggs due to virulence in domesticated poultry and therefore the classical production system would be rendered useless. Cell culture based systems can overcome the practical hurdle of a dwindling egg supply and should be developed for use in a pandemic setting. The HA of H5N1 is poorly immunogenic and requires higher doses of antigen [188] or the use of adjuvants to elicit an effective antibody response [189]. Stockpiling efforts are complicated by the need for higher doses and these dose calculations are insufficient because they are based on the seasonal influenza dose needed to protect a given population. While novel adjuvants have greatly reduced the dose needed to elicit immunity, the long term effects of these new compounds is unknown. Additional difficulties facing vaccine development are insufficient information regarding specific antigenic sites on H5N1 surface proteins and immune correlates of protection are inconclusive, which makes the evaluation of potential vaccine efficacy difficult [190]. Hemagglutination-inhibition (HAI) assays are traditionally the methods employed to determine influenza immunity. These assays take advantage of the viral characteristic of agglutinating chicken or turkey erythrocytes and evaluate immunity via the ability of serum antibodies to inhibit this agglutination. Because H5N1 viruses have different receptor specificity than seasonal influenza viruses, the avian erythrocytes used for seasonal influenza are not useful for the H5N1 hemagglutination inhibition assay. Horse erythrocytes are agglutinated by the H5N1 viruses [191], but the correlation between inhibiting serum antibody titer and protection is unclear. Another method for evaluating serum antibody

efficacy for protection is the neutralization assay. In this assay, serum antibody is evaluated for the ability to inhibit virus from infecting tissue culture cells. Neutralization assays are more time consuming than hemagglutination inhibition assays, but provide more information about the quality of the antibodies and their ability to protect from infection. Hemagglutination inhibition and virus neutralization assays both traditionally require live virus and H5N1 viruses must be handled under high containment conditions adding another problem to evaluating vaccines. To circumvent the need for high containment, non-infectious virus particles expressing H5N1 surface molecules and reporter genes are being developed for use in neutralization and hemagglutination assays [192-194].

1.10.3 H5N1 Diversity

In addition to the practical challenges of H5N1 vaccine development, the antigenic diversity is substantial and poses further challenges. H5N1 viruses are separated into 10 clades based upon phylogenetic relationships among the HA genes (Figure 10) [195]. The vast majority of the viral isolates used to establish these associations are from avian species and have not yet caused human disease. Although limited, human infections have included isolates from clades 0, 1, 2 and 7. Clade 2 H5N1 viruses have caused more reported infections than isolates from the other clades. Antigenic heterogeneity exists between clades and is even beginning to arise within a given clade as demonstrated by subclade and sub-subclade divisions within clade 2. Despite total sequence identity of HA between clades is high (>90%), there is little cross-reactivity in receptor blocking antibodies across clades and even within subclades [196]. An antigenic profile was determined for H5N1 viruses from all clades resulting in the identification of 20 residues that are critical to determining antigenicity [197]. Identity patterns across these 20 amino acids are as low as 30% between clades indicating that total sequence identity likely obscures inter-clade diversity. Each clade and subclade is geographically isolated and evolving

under unique pressures specific to the respective location. As would be expected by the subclade diversity, clade 2 is the most geographically diverse and spans over 60 countries [195]. Independent viral evolution and the subsequent antigenic heterogeneity complicate vaccine preparation and stockpiling efforts because predicting which clade or subclade of H5N1 would be responsible for a pandemic is difficult. The geographic diversity and isolation of H5N1 influenza viruses has led to multiple independently evolving viral lineages posing enormous hurdles for vaccine manufacturers [198].

A goal of H5N1 vaccine development is the elicitation of cross-protective immunity. It is currently impossible to predict which clade may cause a pandemic and therefore an ideal vaccine will elicit immunity to all or multiple clades. Even within clade 2, it is clear that the antigenic difference between subclades is substantial and that a vaccine strain selected from one subclade may not protect against an infection from a virus of a different subclade. The continuing independent evolution of H5N1 virus clades increases the antigenic distance among currently circulating virus strains, as well as the antigenic heterogeneity of strains that will actually be circulating during a potential pandemic. Stockpiling vaccines that are targeted against past viral isolates is a worthwhile strategy only if the virus used to make the vaccine is antigenically similar to the circulating virus during a pandemic. Developing a cross-reactive vaccine that elicits cross-protective immunity to multiple clades may provide more promise than a vaccine that is unable to elicit immunity across current clades. Current vaccine strategies have met this goal with mixed success. In the following sections, studies published before the outset of this work that describe vaccine designs and immunization strategies designed to elicit cross-protective immune responses to H5N1 influenza will be reviewed.

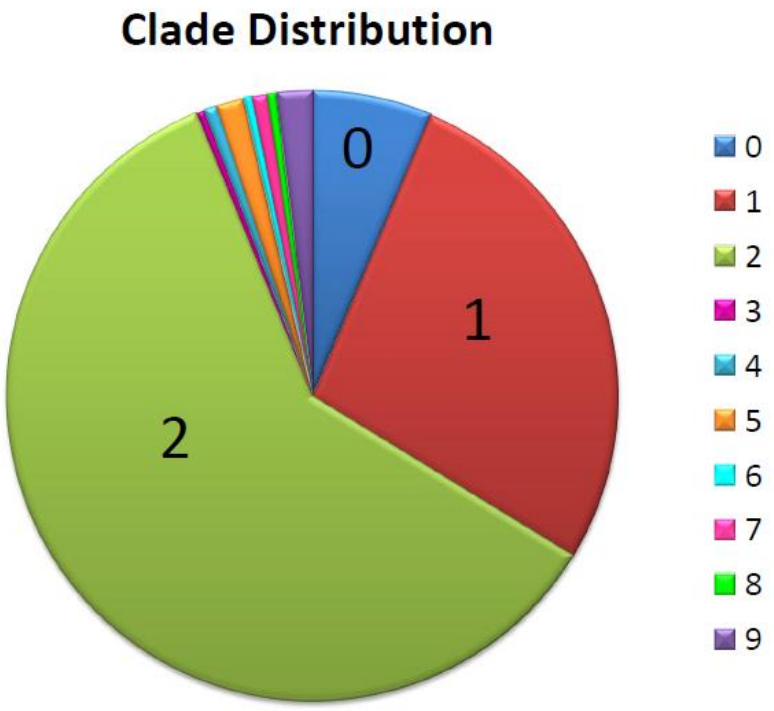

Figure 10: Relative distribution of H5N1 clades. The relative frequency of various clades from both human and environmental infections is plotted. Clade 2 is the most prevalent (olive green) in both human and environmental infections.

1.10.4 Eliciting Immune Responses to H5 Hemagglutinin

1.10.4.1 Inactivated Virus Vaccines. Inactivating virus for use in vaccines is the most common method for generation of current seasonal influenza vaccines. The process entails growing virus in either embryonated chicken eggs or cell culture and then purifying the virus and chemically inactivating it, usually with formalin. Season influenza vaccines are then further processed by splitting the virus particles with detergent and this split vaccine is subsequently used for immunizations. Generating H5N1 inactivated vaccines is challenging in multiple aspects. These highly pathogenic viruses must be handled under greater biosafety conditions than seasonal viruses. More recently, H5N1 viruses have been grown using cell-based methods, but these methods also require increased precautions and safety conditions. One

method of avoiding the need for high containment is to generate reassortant viruses that contain the HA and NA of H5N1 viruses, but have the internal proteins of a high growth virus. Using reverse genetics technology [187, 199], vaccine candidates are grown under less stringent safety conditions and reach a higher titer more easily in eggs. An H5N1 clade 1 reassortant virus generated using the internal proteins from the mouse adapted strain A/Puerto Rico/8/34 (PR8; H1N1) with the HA and NA from A/Hong Kong/213/03 (clade 1) was evaluated as a vaccine candidate [200]. The inactivated whole virion was delivered to ferrets intramuscularly with or without aluminum hydroxide (alum) as an adjuvant. Regardless of adjuvant, the vaccine resulted in antibody responses to homologous virus, but no HAI and neutralization antibody titers were detected to the heterologous virus A/Vietnam/1203/04 (clade 1). Despite the lack of specific antibody, the vaccine protected animals from lethal challenge of A/Vietnam/1203/04 (clade 1), demonstrating heterologous protection despite a lack of immune correlate [200].

Additional reassortant viruses using the HA and NA from either A/Hong Kong/213/03 (clade 1) or A/Vietnam/1203/04 (clade 1) and the internal genes from PR8 were generated and used as inactivated whole virion vaccines delivered subcutaneously with alum [201]. The A/Hong Kong/213/03 (clade 1) vaccine elicited cross-reactive antibodies measured by neutralization to both A/Indonesia/5/05 (clade 2.1) and A/Turkey/12/06 (clade 2.2), but A/Vietnam/1203/04 (clade 1) vaccine did not. Despite relatively low neutralizing titers and no HAI titers, both vaccines were able to protect mice from A/Vietnam/1203/04 (clade 1) lethal challenge at multiple doses with and without adjuvant. Interestingly, the adjuvanted A/Hong Kong/213/03 (clade 1) vaccine protected mice from A/Vietnam/1203/04 (clade 1) virus even better than the A/Vietnam/1203/04 (clade 1) vaccine at lower doses [201]. A proposed mechanism for this phenomenon is that the A/Vietnam/1203/04 (clade 1) virus has a serine at position 224 in HA that may reduce its immunogenicity [202]. Although the A/Hong Kong/213/03 (clade 1) vaccine is efficacious against more contemporary H5N1 viruses such as

A/Indonesia/5/05 (clade 2.1) [201], continued evolution of the virus pushes the antigenic diversity of potential pandemic viruses ever further away from the older isolates.

To meet the challenge of not having a homologous vaccine for contemporary viral isolates, wild type A/Vietnam/1203/04 (clade 1) and A/Indonesia/5/05 (clade 2.1) viruses were prepared by growth in Vero cells under high containment conditions [203]. The wild type viruses were then doubly inactivated with formalin and UV irradiation. The A/Vietnam/1203/04 (clade 1) whole virion vaccine was adjuvanted with alum and elicited neutralizing antibody titers to homologous and heterologous viruses when administered to guinea pigs. Mice immunized subcutaneously with A/Vietnam/1203/04 (clade 1) vaccine produced high titers of antibody and neutralizing antibodies to homologous virus without adjuvant and were subsequently protected from homologous (A/Vietnam/1203/04; clade 1) challenge as well as heterologous (A/Indonesia/5/05; clade 2.1 and A/Hong Kong/156/97; clade 0) challenge [203]. Mice that were vaccinated with A/Indonesia/5/05 (clade 2.1) vaccine were also protected from homologous and heterologous (A/Vietnam/1203/04; clade 1) challenge [203]. The inactivated whole wild-type virion nature of these vaccines is likely responsible for the immunogenicity and effectiveness without adjuvant. Despite double inactivation and rigorous safety testing of the vaccines, the potential for infection with surviving virus is a regulatory hurdle that will be difficult to overcome.

To circumvent the need for whole virus preparations, novel adjuvant technology has been applied to lower the dose of vaccine required to elicit an effective immune response. One adjuvant that has been particularly promising is the MF59 adjuvant from Novartis. As discussed previously, (see Section 1.8) MF59 is an oil-in water emulsion that enhance immune responses via a TLR-independent pathway [158, 159]. A vaccine consisting of reverse genetics engineered A/Vietnam/1194/04 (rgVN1194; clade 1) that had the polybasic cleavage site removed was inactivated and split and then administered to human volunteers with and without the proprietary MF59 adjuvant [204]. Including the adjuvant in the vaccine formulation greatly increased the neutralizing antibody titers to both homologous virus (A/Vietnam/1194/04; clade

1) and heterologous virus (A/Indonesia/5/05; clade 2.1). Importantly, the adjuvant also greatly reduced the amount of antigen administered and allowed for dose sparing. This a critical factor in a pandemic setting as the supply of vaccine is likely to be limited and high dose requirements only exacerbate the problem. Ethical issues obviously preclude the ability to challenge the volunteers who were given the vaccine, so the same vaccine and adjuvant system was analyzed in a ferret model [205]. Ferrets vaccinated with rgVN1194 (clade 1) inactivated split virus with the MF59 adjuvant developed neutralizing antibodies to both A/Vietnam/1194/04 (clade 1) and A/Indonesia/5/05 viruses (clade 2.1) [205]. Upon challenge with both A/Vietnam/1194/04 (clade 1) and A/Indonesia/5/05 (clade 2.1), rgVN1194 (clade 1) with adjuvant vaccinated ferrets had greater survival, lower virus titer in the nose and lungs, and decreased viral shedding than unadjuvanted or mock vaccinated controls [205]. Evidence of safety in humans together with efficacy in ferrets provide promise that an effective pre-pandemic vaccine is possible.

The MF59 adjuvant has also been used in a monovalent HA vaccine from an H5N3 virus, A/Duck/Singapore/97. This vaccine has been tested for safety in human volunteers and elicits heterosubtypic neutralizing antibodies to an antigenically similar virus A/Hong Kong/156/97 (clade 0) and antigenically distinct viruses A/Hong Kong/213/03 (clade 1), A/Vietnam/1203/04 (clade 1), and A/Thailand/16/04 (clade 1) [189]. The A/Duck/Singapore/97 virus was also used as an unadjuvanted, inactivated split vaccine delivered intramuscularly in ferrets to evaluate immunogenicity and effectiveness [206]. Vaccinated animals had low HAI titers to homologous virus and no HAI titers to heterologous viruses A/Hong Kong/156/97 (clade 0) and A/Vietnam/1203/04 (clade 1). Additionally, A/Duck/Singapore/97 elicited neutralizing titers to homologous virus and heterologous A/Hong Kong/156/97 (clade 0) virus but not to heterologous A/Vietnam/1203/04 (clade 1) [206]. Despite the apparently low immunogenicity of the vaccine regimen, the ferrets were protected from severe disease and death upon challenge with A/Vietnam/1203/04 (clade 1) wild type virus [206]. Even without HAI or neutralizing

antibody, the vaccinated animals had lower levels of virus replication in the upper respiratory tract compared to mock vaccinated controls [206]. These data agree with previous studies showing that despite a low or complete lack of HAI or neutralizing antibodies, vaccines can protect animals from infection with heterologous virus [200, 206].

While the heterologous protection shown in response to vaccination with H5 viruses is encouraging and indicates that an effective pre-pandemic vaccine is possible, work with seasonal influenza vaccines has been intriguing. Delivery of influenza vaccines is traditionally administered via intramuscular injection for inactivated vaccines and intranasally for live-attenuated vaccines. By directly targeting mucosal surfaces in the respiratory tract it is possible to elicit secretory antibody that may be more effective at neutralizing virus than serum antibody. Mice were immunized intranasally with inactivated whole virion H3N2 virus X-31 (A/Aichi/2/68 HA and NA with A/Puerto Rico/8/34 internal genes) with mutant heat-labile enterotoxin (LT) from *E. coli* [207]. LT is a toxin produced by enterotoxinogenic strains of *E. coli* and induces chloride flux and fluid loss. The toxin is a potent mucosal adjuvant, but the toxicity of the native toxin precludes its use as an adjuvant. The mutant LT is less toxic than native LT and retains the adjuvant properties of the unaltered toxin. This vaccine strategy elicited serum and mucosal antibodies to both whole H5N1 (A/Hong Kong/483/97; clade 0) virus particles and recombinant H5 HA but these antibodies were unable to neutralize H5N1 virus. Despite a lack of neutralizing antibody, mice vaccinated with X-31 with LT intranasally were protected from lethal challenge with A/Hong Kong/483/97 (clade 0) virus. Mice vaccinated subcutaneously with X-31 plus LT were not protected from lethal challenge and had much lower serum and lung antibody titers to H5N1 virus. Interestingly, mice that were experimentally infected with X-31 prior to challenge with H5N1 were also completely protected from lethality and mice vaccinated intranasally with X-31 without adjuvant were partially protected. Protection correlated with lower virus titers in the lungs and a lack of virus in the nose or brain. This protection was also shown to be dependent on B cells as both CD8 depletion and CD8 deficiency resulted in vaccinated animals

surviving H5N1 challenge while B cell deficiency resulted in death regardless of vaccination. $CD8^+$ cells may not be required for survival, but virus titers were higher in the absence of $CD8^+$ cells indicating an important role in clearance.

The concept of seasonal viruses being used in intranasal vaccines with mucosal adjuvants and prior seasonal influenza infection providing protection to H5N1 infection indicates that an immunologically naïve population may be more protected to novel HA subtypes than suspected. Further evidence of potential protective effects of seasonal influenza vaccines was provided when mice were vaccinated intranasally with trivalent inactivated split vaccine prepared for the 2005-2006 season (A/New Caledonia/20/99 (H1N1), A/New York/55/04 (H3N2), and B/Shanghai/361/02) plus Ampligen, a proprietary TLR-3 agonist [208]. The intranasally delivered, adjuvanted seasonal vaccine did not elicit H5N1 HAI or neutralizing antibodies, but did yield serum and lung antibodies to A/Vietnam/1194/03. Mice that were vaccinated intranasally with adjuvanted trivalent vaccine had increased survival when challenged with multiple H5N1 viruses (A/Vietnam/1194/04; clade 1, A/Indonesia/6/05; clade 2.1 and A/Hong Kong/483/97; clade 0) and had lower virus titer in nasal washes compared to mock vaccinated controls. Intranasal delivery of influenza vaccines has been shown to be an effective way of eliciting heterosubtypic immunity, but an H5N1 vaccine could be even more effective when administered by this route. Inactivated whole virus rgVN1194 (described above, clade 1) was used with Ampligen as an adjuvant to vaccinate mice [209]. Regardless of delivery route or adjuvant, the vaccine elicited neutralizing antibody titers to homologous virus (A/Vietnam/1194; clade 1), but not to heterologous viruses (A/Hong Kong/483/97; clade 0 and A/Indonesia/6/05; clade 2.1). When the vaccine was administered intranasally with the adjuvant, mice were protected from lethal challenge by not only the homologous virus, A/Vietnam/1194/04 (clade 1), but also heterologous viruses: A/Hong Kong/483/97 (clade 0) and A/Indonesia/6 2005 (clade 2.1).

Inactivated vaccines are the main strategy of current seasonal influenza vaccines, but generating inactivated vaccines for H5N1 viruses presents unique challenges because these viruses are highly pathogenic and must be handled under increased biosafety precautions. Furthermore, the viruses are difficult to grow in traditional egg-based systems due to their high pathogenicity and therefore require new methods of virus production such as cell culture. Using whole inactivated virus also causes apprehension among licensing bodies due to the high pathogenicity of the virus and the extremely slight possibility of incomplete inactivation inadvertently infecting patients. Methods, such as reverse genetics, can generate viruses that are less pathogenic than the wild-type strain and that can be handled under lower safety conditions, but these take time and would extend the lead time needed to produce a vaccine after the start of a pandemic. Low immunogenicity and higher dose requirements are additional challenges for inactivated vaccines that further hinder vaccine stockpiling efforts. Novel adjuvants, such as MF59 and Ampligen, are safe not only at lower doses, but also help to elicit cross-protective immunity. Non-traditional delivery methods, such as subcutaneous or intranasal, can also lower the dose that is required and broaden reactivity of the elicited immunity. Inactivated virus vaccines are clearly important and have been progressing through clinical trials, but alternative vaccine strategies can also elicit cross-protective immunity to H5N1 viruses.

1.10.4.2 Live-Attenuated Virus Vaccines. Live-attenuated viruses are potential vaccine candidates for H5N1 influenza. A seasonal influenza live attenuated virus vaccine is currently licensed in the United States for non-pregnant and non-immunocompromised individuals ages 2-49 years of age [210]. Additionally, live-attenuated vaccines have been available in Russia for years [211]. These vaccines are made by adapting an influenza virus to cold temperatures, such that it has limited growth at temperatures encountered in the human body. Surface glycoproteins are then substituted to match the recommended strains for of seasonal influenza

vaccines or H5N1 candidates for pandemic influenza. For H5N1 vaccines, the polybasic cleavage site in HA is removed to return the viruses to a state of trypsin dependence. Live attenuated vaccine strategies have several advantages, including stimulation of an immune response in naïve individuals after a single vaccination, induction of cross-reactive immune responses and poor transmissibility [212, 213]. H5N1 live attenuated vaccines containing the surface glycoproteins of A/Hong Kong/213/03 (clade 1), A/Vietnam/1203/04 (clade 1), and A/Hong Kong/491,486/97 (clade 0; HA from A/Hong Kong/491/97 and NA from A/Hong Kong.486/97) have been generated and evaluated in mice and ferrets [214]. All HA genes were modified to remove the polybasic cleavage site and trypsin dependence was verified. The vaccine strains were all shown to be attenuated by 1) detecting only low titers of virus in the respiratory tract of infected mice or ferrets and 2) the lack of detection of these attenuated strains in the brain. A single vaccination in mice resulted in low neutralizing and HAI antibody responses to both homologous and heterologous viruses, although serum antibodies to recombinant HA were detected by ELISA. Interestingly, a second dose of attenuated virus increased both the titer and cross-reactivity of neutralizing and HAI antibodies even though no replication in the respiratory tract could be detected after the boost. All mice vaccinated with a single dose of attenuated virus survived challenge with both homologous and heterologous virus, including the more recent A/Indonesia/5/05 (clade 2.1) strain despite undetectable neutralizing and HAI antibody responses. Control mice that were immunized with only the attenuated virus without H5N1 surface glycoproteins were partially protected from lethality (40-80% survival) after H5N1 challenge and this protection waned as the challenge dose was escalated. Although complete protection from lethality was achieved in H5N1 vaccinated mice, a single dose of vaccine did not protect from viral replication in the respiratory tract. Viral titers in the lungs were lower in vaccinated mice as compared to controls and virus did not disseminate to the brain, data consistent with low neutralizing antibody titers. Protection that prevented viral replication in the lungs of infected mice was more effective in mice that received

two doses of the vaccines, regardless if the challenge virus was homologous or heterologous to the vaccine strain. Ferrets that received two doses of the vaccine strains were also protected from homologous and heterologous challenge and had lower viral titer in nasal washes. Live attenuated vaccines based upon H5N1 strains that emerged in 1997 (clade 0) are effective at protecting animals from challenge with phylogenetically distinct virus from 2004 (clade 1).

A live attenuated virus has been constructed using the HA from a nonpathogenic, but antigenically related H5N2 avian influenza virus A/Duck/Pottsdam/1042-6/86, with all other gene segments (including NA) from a Russian cold-adapted strain. This vaccine was evaluated for use as both a live attenuated vaccine and as an inactivated vaccine [215]. Mice that were vaccinated intranasally with the live attenuated vaccine did not have cross-reactive neutralizing antibodies while intramuscular administration of the same vaccine inactivated and adjuvanted with alum did yield cross-reactive neutralizing antibodies to an antigenically similar virus, A/Hong Kong/156/97 (clade 0) and an antigenically distinct virus, A/Hong Kong/213/03 (clade 1) but not to a second antigenically distinct virus, A/Vietnam/1203/04 (clade 1). Despite the lack of neutralizing antibodies, antibodies that cross-reacted in an ELISA with HA from A/Vietnam/1203/04 (clade 1) were present in sera from both the live attenuated vaccine and the inactivated vaccine. Mice vaccinated with either the live attenuated virus or the inactivated virus were protected from lethal challenge with A/Vietnam/1203/04 (clade 1) virus despite a lack of neutralizing antibodies. Survival correlated with lower virus titers in vaccinated mice as compared to control animals.

In general, these live attenuated virus vaccines are a safe and effective way to vaccinate for seasonal influenza and are currently licensed for that purpose. Live attenuated viruses have the advantage of replicating to low levels in the host and therefore elicit cellular immunity and are able to generate potent immune responses without the need of a booster vaccination. Applying attenuated virus strategies to H5N1 viruses has been successful in animal models, although multiple doses are needed to increase immunity to the H5N1 antigens. A major hurdle

in order to approve live attenuated viruses as vaccines is, despite the attenuated phenotype, these viruses are still able to transmit from person to person in the case of seasonal vaccines, which is a concern for the development of live-attenuated H5N1 vaccines. Another problem with live attenuated virus vaccines is that if a person is infected with a seasonal strain of influenza at the same time the live attenuated vaccine is administered, a reassortment event could occur that may generate a virus with seasonal influenza internal proteins and H5N1 glycoproteins. Although the HA is mutated to be less pathogenic prior to attenuated vaccine formulation the chance of directly causing reassortment with seasonal influenza is not a favorable possibility. Despite the drawbacks of using a live attenuated vaccine for pre-pandemic immunizations, the live attenuated vaccine's ability to elicit broadly protective immunity with relatively low doses would be a valuable tool at the beginning of a pandemic to limit spread and mortality.

1.10.4.3 Viral Vector Vaccines. Using virus vectors for delivery of H5N1 vaccines is another strategy that is being developed. Viral vectors are low pathogenic viruses that are modified to produce influenza proteins. The vector can also be constructed such that it cannot produce progeny virions and is therefore rendered replication incompetent. Since the vector actually infects cells, viral vector antigen delivery systems have the advantage of producing influenza proteins within the host in a manner similar to live attenuated viruses. This leads to the generation of a more complete immune response that includes cytotoxic T cells in addition to antibody. Several viruses are effective vectors to elicit cross-protection among H5N1 viruses. Adenovirus vectors elicit a potent immune response which is protective against antigenically distinct H5N1 strains [216, 217]. A replication-defective adenovirus vector carrying the full length HA gene of A/Hong Kong/156/97 (clade 0) was used to vaccinate mice both intranasally and intramuscularly and elicited HAI antibody to homologous virus and to heterologous viruses A/Hong Kong/213/03 (clade 1) but not to A/Vietnam/1203/04 (clade 1) regardless of

administration route [217]. Despite the low HAI titers, all vaccinated mice were protected from lethal challenge with both the antigenically similar A//Hong Kong/483/97 (clade 0) and the antigenically distinct A/Vietnam/1203/04 (clade 1). This protection corresponded with low virus titers in the lungs of challenged mice compared to mock vaccinated controls.

Vesicular stomatitis virus vectors that are either replication competent or incompetent and express HA from A/Hong Kong/156/97 (clade 0) elicited neutralizing antibodies to homologous A/Hong Kong/156/97 (clade 0) virus and also to heterologous viruses: A/Vietnam/1203/04 (clade 1) and A/Indonesia/5/05 viruses (clade 2.1) [218]. Vaccinated mice were also protected from challenge with the antigenically similar A/Hong Kong/483/97 (clade 0) virus up to 7.5 months post vaccination. Newcastle disease virus is another vector that has been engineered to express HA from an H5N1 virus, A/Bar-headed goose/Qinghai/3/2005 (clade 2.2) [219]. Highly pathogenic Newcastle disease virus is a threat to poultry across the world and naturally occurring strains are used as vaccines on a regular basis. By combining H5N1 antigens with these vaccine strains, it is proposed that poultry could be protected from two different threats with a single vaccine. The vaccine elicited HAI antibodies to homologous virus and low levels of HAI antibodies to a heterologous virus, A/Duck/Fujian/13/02 (clade 3). Vaccinated mice and chickens were all protected from challenge with both homologous virus and heterologous virus with low levels of virus replication compared to mock vaccinated animals.

Vector delivery of H5N1 vaccines is an effective way to elicit cross-reactive immunity. However, using replication competent viruses for delivery is dangerous due to potential pathogenicity of the vehicle. Pre-existing immunity to the vehicle is a challenge that limits the effectiveness of using viruses as vectors because the vector could be neutralized before it infects and delivers the antigen to the host. Some serotypes of adenovirus have low prevalence in the human population and vesicular stomatitis virus is not prevalent in humans, which makes these two vectors good candidates for vaccine delivery. Using replicating virus as a delivery

system may be ideal for immunizing poultry flocks because the vaccinated birds could spread the vaccine by natural viral replication.

1.10.4.4 Virus-Like Particle Vaccines. Virus-like particles (VLP) are self-assembling, nonpathogenic, genomeless particles that are similar in size and morphology to intact virions [220]. Influenza VLP vaccines have been made that include the influenza proteins matrix, hemagglutinin and neuraminidase. These vaccines are usually produced in a baculovirus expression system and consist of particles that have hemagglutination and neuraminidase activities. VLP vaccines have the advantage that they are particulate in nature and therefore generate a better immune response than recombinant protein vaccines and they are genomeless which renders them completely noninfectious. Additionally, VLP vaccines are produced in an egg-free system and therefore do not require a large supply of embryonated chicken eggs for production and because they are nonpathogenic wild type surface glycoproteins can be used in their formulation. Influenza VLP vaccines have been generated for seasonal influenza viruses and they elicit broad immune responses [221]. VLP vaccines to H5N1 influenza have been developed using the surface glycoproteins from both A/Vietnam/1203/04 (clade 1) and A/Indonesia/5/05 (clade 2.1) viruses [222]. These vaccines elicited strong HAI antibody titers to both homologous virus and heterologous virus. Upon challenge with a lethal dose of reassortant viruses, animals vaccinated with either vaccine were protected from death from both homologous and heterologous virus. VLP vaccines elicit high titer, cross-protective immune responses in the absence of adjuvant. These vaccines have the advantage that they are produced in a cell-based system and do not require high biocontainment for their production. They also have the advantage that they do not have to be inactivated because VLPs are genomeless and noninfectious.

1.10.5 Alternative Targets

1.10.5.1 Neuraminidase. Although immunity against hemagglutinin has been the primary focus of vaccines discussed thus far, other viral proteins are receiving attention as possible antigens on which to base a vaccine. Data indicate that immunity directed at neuraminidase can provide significant cross-protection of antigenically diverse viruses within a given subtype [223]. Although NA antibody does not protect against initial infection, it can reduce severity of disease by limiting viral replication. Current seasonal vaccines contain NA but the concentrations are not standardized like the HA concentrations and HA may dominate the immune response. To target NA specifically, a DNA vaccine expressing A/New Caledonia/20/99 (H1N1) NA was used to vaccinate mice [224]. Vaccinated animals developed high titer antibodies to homologous H1N1 NA but did not develop significant antibodies to heterologous H5N1 NA. Challenges with PR8 reassortant viruses having the NA from A/New Caledonia/20/99 (PR8-huN1) or A/Vietnam/1203/04 (PR8-avN1) and the remaining 7 genes from PR8 resulted in NA vaccinated animals being completely protected from homologous virus (PR8-huN1) and partially protected from heterologous virus (PR8-avN1). Furthermore, wild type A/Vietnam/1203/04 (clade 1) virus was used for challenges and mice were partially protected as would be expected from the reassortant viruses. Because DNA vaccines can elicit potent T cell responses, passive transfer of humoral immunity was also evaluated. Recipient animals were all protected from homologous (PR8-huN1) challenge and partially protected from heterologous (wild type A/Vietnam/1203/04; clade 1) challenge at a rate that is comparable to directly vaccinated mice. Additionally, human serum samples were analyzed for the presence of anti-neuraminidase activity and found high penetrance of high titer NA inhibition to A/New Caledonia/20/99 and low penetrance, low titer NA inhibition to avian isolates (A/Hong Kong/213/03; clade 1 and A/Vietnam/1203/04; clade 1). The results of the animal vaccinations indicate that anti-NA humoral immunity to recent vaccine strains is partially effective at protecting animals

experimentally infected with H5N1. By standardizing the amount of NA currently in vaccine formulations, it is possible that cross-protective immunity could be elicited thereby reducing the morbidity and mortality classically associated with influenza pandemics.

1.10.5.2 M2. Immunity to influenza that is mediated by antibody responses to HA and NA is very effective for antigenically matched strains, but can be ineffective against different subtypes or even within subtypes that have significant drift. Another surface protein in influenza, M2, has been the subject of recent vaccine strategies. M2 is an ion channel that functions in viral entry via facilitating the dissociation of vRNPs from matrix. M2 has a short ectodomain that is exposed to antibodies as well as a long cytoplasmic tail. Anti-M2 monoclonal antibody has been shown to reduce viral replication and identifies M2 as a viable vaccine candidate [17]. A DNA vaccine encoding a full length M2 with a consensus ectodomain was constructed and used to evaluate M2 immunity in mice [179]. Vaccinated mice had specific antibodies to H1N1 M2 that matched the consensus (PR8 and A/FM/1/47) as well as antibodies to H5N1 M2 that diverged from the consensus (A/Thailand/SP-83/2004; clade 1). When the DNA vaccine was followed with an M2-expressing adenoviral boost, animals were completely protected from matched virus (PR8 and A/FM/1/47), as well as partially protected from H5N1 virus (A/Thailand/SP-83/2004; clade 1). M2 as a vaccine antigen is efficacious and is able to elicit broad immunity. The ectodomain is not perfectly conserved with the H5N1 virus A/Hong Kong/213/03 (clade 1) being quite divergent and the consensus vaccine does not elicit antibodies that are specific for M2 of H5N1. Alternative strategies of using M2 in the context of vaccine development are also in progress.

Since M2 plays such a crucial role in viral uncoating following infection, it presents an attractive target for altering to generate live-attenuated viruses. M2 cytoplasmic tail deletion mutants were evaluated in mice for use as live-attenuated vaccines [16]. A panel of M2 deletion mutant viruses was evaluated for their ability to replicate in mice. In contrast to viruses with wild-

type M2, viruses with an 11 cytoplasmic amino acid deletion in M2 (M2del11) replicated to low titers in the lungs and did not spread to the brain. This M2del11 was combined with a mutant HA (HAavir) that was made trypsin dependent by the removal of the polybasic cleavage site and then rescued via reverse genetics with wild type A/Vietnam/1203/04 (M2del11-HAavir; clade 1). The M2del11-HAavir (clade 1) virus was highly attenuated in mice and was restricted to the respiratory tract where it replicated to low titers. Mice that were immunized with M2del11-HAavir (clade 1) had wild type A/Vietnam/1203/04 (clade 1) virus specific antibodies in both the serum and lungs. These antibodies were not neutralizing to either A/Vietnam/1203/04 (clade 1) or A/Indonesia/7/05 (clade 2.1). Despite a lack of neutralization, M2del11-HAavir (clade 1) vaccinated mice were protected from challenge with both homologous A/Vietnam/1203/04 (clade 1) virus and heterologous A/Indonesia/7/05 (clade 2.1) virus. This survival corresponded to no detectable virus titer in A/Vietnam/1203/04 (clade 1) challenged mice and very low virus titer in A/Indonesia/7/06 (clade 2.1) challenged mice. M2 is an attractive vaccine candidate as both a direct target for immunity and also as a target for attenuation. Highly conserved proteins such as M2 are appealing for vaccine design due to the possibility of eliciting broad immunity and this strategy has been used to target other highly conserved proteins in addition to M2

1.10.5.3 Nucleoprotein and Matrix. A major reason for the need to update the seasonal influenza vaccine annually is the poor antigenic match between the vaccine strains. Humoral immunity is generally accepted to be the major correlate of protection, but continuous antigenic drift enables influenza to escape antibodies elicited to prior strains. Because drift within a subtype is enough to lower the efficacy of prior vaccines of the same subtype, emergence of a novel subtype into the population could have devastating effects. In the absence of a matched humoral response, cytotoxic T lymphocytes (CTL) may mediate protection and impact disease severity and mortality. CTL responses are directed towards conserved epitopes of internal proteins like nucleoprotein (NP) and matrix. These internal proteins are well conserved within

subtypes and can even be conserved between subtypes. To elicit effective CTL responses, antigens must be delivered efficiently to the cytosol in order to access the MHC Class I processing pathway and be presented to CTL. Vaccine strategies that ensure this delivery include live attenuated vaccines and DNA vaccines due to their ability to replicate within the cells of the host or virosomes and VLPs that are able to fuse with cellular membranes and deliver contents to the cytosol. To evaluate the role of CTL in eliciting cross-protective immunity, a live-attenuated vaccine was compared to a DNA prime-adenovirus boost strategy [225]. A live-attenuated H3N2 (A/Alaska/6/77) on a cold adapted H2N2 (A/Ann Arbor/6/60) background was compared to DNA prime-adenovirus boost vaccines expressing PR8 nucleoprotein with M2 (A/NP+M2). Both vaccine strategies elicited CTL responses as measured by IFN-γ ELISpot with A/NP+M2 having a better response. Upon challenge with A/Vietnam/1203/04 (clade 1), A/NP+M2 protected much better than live-attenuated (80% and 20% survival, respectively). While CTL immunity to conserved proteins is certainly important in generating cross-protective immune responses, CTL can also be directed towards HA.

To compare the efficacy of NP DNA vaccines to HA DNA vaccines, both were given to chickens to evaluate protective efficacy [226]. The HA from both A/Turkey/Ireland/83 (H5N8) and A/Chick/Victoria/1/85 (H7N7) was used to generate single vaccines as well as the NP from A/Turkey/Ireland/83. Chickens that received the NP vaccine were partially protected (~50%) from both A/Turkey/Ireland/83 and A/Chick/Victoria/1/85. The HA vaccines did not elicit HAI antibodies to either virus, but when administered alone they were protective when challenged with the homologous virus. Interestingly, when the HA DNA vaccines were administered together the protection was to both A/Turkey/Ireland/83 and A/Chick/Victoria/1/85. Consensus DNA sequences is another way of immunizing for CTL while attempting to broaden the antigenic range of the immunogen. A consensus sequence is a version of a gene not found in nature but rather assembled *in silico* to contain the most common amino acid in a given population at each residue. Consensus DNA immunogens have been shown in HIV research to induce strong T

cell responses that are more cross-reactive than their components [178]. Consensus HA, NA, and matrix were all used as DNA vaccines to determine if cellular immunity could be elicited [180]. All constructs tested elicited CTL immunity as measured by IFN-γ ELISpot to custom peptide pools of the respective immunogen. Additionally, the HA consensus vaccine elicited low titer antibodies to the recombinant consensus protein. When this recombinant protein was used as a vaccine with complete Freund's adjuvant, the consensus HA was able to protect mice from A/Hanoi/30408/05 challenge [180]. CTL immunity is important to generating cross-protective immunity to influenza in the absence of an antigenically matched humoral response. Although CTL cannot prevent infection, cell-mediated immune responses can reduce the morbidity and mortality associated lethal influenza infection. Additionally, CTL immunity can reduce transmission of the virus by decreasing the level of replication and the duration of infection.

1.11 CONCLUDING REMARKS

Influenza pandemics occur sporadically and are characterized by the introduction of novel surface glycoproteins into circulating strains. This event results in viruses to which the population has little or no humoral immunity and the viruses are therefore able to spread unchecked. H5N1 influenza has emerged as a potential pandemic virus because it is highly pathogenic and there is little pre-existing immunity in the human population. Geographic spread of H5N1 in avian species is continuing and, subsequent human infections are affecting more of the globe. Several challenges exist in the development of vaccines to H5N1 influenza, such as the high pathogenicity of the virus, antigenic variation within the subtype, poor immunogenicity, and no established immune correlates of protection [188, 190]. Despite these obstacles,

progress is being made toward developing vaccines that protect against both the homologous virus and also heterologous viruses of the same and even different subtypes.

Vaccine strategies that have successfully elicited broad immunity include inactivated virus, live-attenuated virus, viral vectors, virus-like particles, and DNA vaccines [180, 205, 214, 217, 222]. These strategies can be further enhanced by the use of adjuvants and alternative routes of inoculation. The Novartis adjuvant MF59 is particularly effective at eliciting cross-reactive immunity. The vaccine formulation is safe in human volunteers and this same formulation protects ferrets from lethal challenge with antigenically distinct viruses [204, 205]. The intranasal route of vaccination also improves cross-reactivity. When combined with a mucosal adjuvant, Ampligen, seasonal subtype whole inactivated virions were able to protect mice from lethal challenge with H5N1 viruses [207, 208]. Use of adjuvants and intranasal administration of H5N1 vaccines is proving to be a successful way of eliciting broad immunity. Targeting internal proteins of influenza is also an effective way of eliciting cross-reactive immunity and although this immunity does not prevent infection, it may be effective at reducing the duration and severity of illness [227].

Data on long term safety of new adjuvants is lacking and as patient cohorts age more information will become available. The future of broadly reactive pandemic vaccines is open to new discoveries. Lessons learned from seasonal vaccine development should be applicable to pandemic vaccine development, such as the important concept of polyvalency. Seasonal influenza vaccines are able to elicit protective immunity to three strains of influenza each year and it is reasonable to explore the idea of generating polyvalent pandemic vaccines. Because avian isolates other than H5N1 have infected humans (H7N7 and H9N2), a polyvalent vaccine that includes all three subtypes could be the future of pandemic vaccines. Of course, the antigenic diversity of H5N1 alone could warrant the development of a polyvalent vaccine to multiple clades within a single subtype. When developing a pandemic vaccine, it is prudent to

consider subtypes that have previously infected humans and but are no longer circulating in humans, such as H2N2. Pre-existing immunity to influenza of any subtype also appears to provide some protection to H5N1 infection. Infection or prior vaccination with seasonal influenza induces CTL responses and could generate NA antibodies that may reduce disease severity in the event of a pandemic. The roles of pre-existing immunity to a novel subtype are ongoing questions with great relevance to public health. Establishing correlates of protection is a crucial step in comparing vaccines. Protection in the absence of hemagglutination inhibition or virus neutralization complicates evaluation of vaccine candidates and evaluating immunity in human trials must have an interpretable result other than protection. While H5N1 has not caused wide spread infections in humans to date, information learned about pandemic preparedness and vaccine cross-protection will certainly benefit in the event of a pandemic caused by any subtype of influenza.

2.0 MATERIALS AND METHODS

2.1 ANTIGEN CONSTRUCTION AND SYNTHESIS

Influenza A HA nucleotide sequences from viruses isolated during human infections were downloaded from the NCBI Influenza Virus Resource database [51]. 129 full length sequences from H5N1 clade 2 human infections collected from 2004-2006 were acquired and used for subsequent consensus generations (Table 2). Nucleotide sequences were translated into protein sequences using the standard genetic code and alignments were performed using protein sequences. For each round of consensus generation, multiple alignment analysis was applied and the consensus sequence was generated using AlignX software (Vector NTI). The sequences were first grouped into subclades and then further divided into outbreak groups based upon time and location of isolation. Primary consensus sequences were generated based upon outbreak groups within each subclade. Next, primary consensus sequences within each subclade were aligned and used to generate secondary consensus sequences for the respective subclades. Finally, the secondary, subclade-specific, consensus sequences were aligned and the resulting consensus sequence was termed computationally optimized broadly reactive antigen (COBRA; Figure 11). The COBRA protein sequence was reverse translated and optimized for expression in mammalian cells, including codon usage and RNA optimization (GeneArt; Regensburg, Germany). The resulting construct was then synthesized and inserted into the pTR600 expression vector.

Table 2: COBRA input sequences

Strain	Clade	Accession	Host	Country	Year
A/Indonesia/534H/2006	2.1.2	EU146737	Human	Indonesia	2006
A/Indonesia/535H/2006	2.1.2	EU146753	Human	Indonesia	2006
A/Indonesia/536H/2006	2.1.2	EU146754	Human	Indonesia	2006
A/Indonesia/538H/2006	2.1.2	EU146745	Human	Indonesia	2006
A/Indonesia/546bH/2006	2.1.2	EU146793	Human	Indonesia	2006
A/Indonesia/546H/2006	2.1.2	EU146755	Human	Indonesia	2006
A/Indonesia/560H/2006	2.1.2	EU146785	Human	Indonesia	2006
A/Indonesia/CDC594/2006	2.1.2	CY014272	Human	Indonesia	2006
A/Indonesia/CDC595/2006	2.1.2	CY014280	Human	Indonesia	2006
A/Indonesia/CDC596/2006	2.1.2	CY014288	Human	Indonesia	2006
A/Indonesia/CDC597/2006	2.1.2	CY014296	Human	Indonesia	2006
A/Indonesia/CDC599/2006	2.1.2	CY014303	Human	Indonesia	2006
A/Indonesia/CDC599N/2006	2.1.2	CY014477	Human	Indonesia	2006
A/Indonesia/CDC625/2006	2.1.2	CY014433	Human	Indonesia	2006
A/Indonesia/CDC625L/2006	2.1.2	CY014465	Human	Indonesia	2006
A/Indonesia/160H/2005	2.1.3	EU146648	Human	Indonesia	2005
A/Indonesia/175H/2005	2.1.3	EU146640	Human	Indonesia	2005
A/Indonesia/177H/2005	2.1.3	EU146680	Human	Indonesia	2005
A/Indonesia/195H/2005	2.1.3	EU146656	Human	Indonesia	2005
A/Indonesia/239H/2005	2.1.3	EU146664	Human	Indonesia	2005
A/Indonesia/245H/2005	2.1.3	EU146672	Human	Indonesia	2005
A/Indonesia/283H/2006	2.1.3	EU146681	Human	Indonesia	2006
A/Indonesia/286H/2006	2.1.3	EU146688	Human	Indonesia	2006
A/Indonesia/292H/2006	2.1.3	EU146713	Human	Indonesia	2006
A/Indonesia/298H/2006	2.1.3	EU146697	Human	Indonesia	2006
A/Indonesia/304H/2006	2.1.3	EU146705	Human	Indonesia	2006
A/Indonesia/321H/2006	2.1.3	EU146721	Human	Indonesia	2006
A/Indonesia/341H/2006	2.1.3	EU146729	Human	Indonesia	2006
A/Indonesia/5/2005	2.1.3	EF541394	Human	Indonesia	2005
A/Indonesia/542H/2006	2.1.3	EU146777	Human	Indonesia	2006
A/Indonesia/567H/2006	2.1.3	EU146801	Human	Indonesia	2006
A/Indonesia/569H/2006	2.1.3	EU146809	Human	Indonesia	2006
A/Indonesia/583H/2006	2.1.3	EU146817	Human	Indonesia	2006
A/Indonesia/604H/2006	2.1.3	EU146825	Human	Indonesia	2006
A/Indonesia/7/2005	2.1.3	EU146632	Human	Indonesia	2005
A/Indonesia/CDC184/2005	2.1.3	CY014197	Human	Indonesia	2005
A/Indonesia/CDC194P/2005	2.1.3	CY014168	Human	Indonesia	2005
A/Indonesia/CDC287E/2005	2.1.3	CY014198	Human	Indonesia	2005

Table 2 (continued)

A/Indonesia/CDC287T/2005	2.1.3	CY014199	Human	Indonesia	2005
A/Indonesia/CDC292N/2005	2.1.3	CY014200	Human	Indonesia	2005
A/Indonesia/CDC292T/2005	2.1.3	CY014201	Human	Indonesia	2005
A/Indonesia/CDC326/2006	2.1.3	CY014204	Human	Indonesia	2006
A/Indonesia/CDC326N/2006	2.1.3	CY014202	Human	Indonesia	2006
A/Indonesia/CDC326N2/2006	2.1.3	CY014203	Human	Indonesia	2006
A/Indonesia/CDC326T/2006	2.1.3	CY014205	Human	Indonesia	2006
A/Indonesia/CDC329/2006	2.1.3	CY014206	Human	Indonesia	2006
A/Indonesia/CDC357/2006	2.1.3	CY014207	Human	Indonesia	2006
A/Indonesia/CDC370/2006	2.1.3	CY014209	Human	Indonesia	2006
A/Indonesia/CDC370E/2006	2.1.3	CY014210	Human	Indonesia	2006
A/Indonesia/CDC370P/2006	2.1.3	CY014211	Human	Indonesia	2006
A/Indonesia/CDC370T/2006	2.1.3	CY014212	Human	Indonesia	2006
A/Indonesia/CDC390/2006	2.1.3	CY014213	Human	Indonesia	2006
A/Indonesia/CDC523/2006	2.1.3	CY014311	Human	Indonesia	2006
A/Indonesia/CDC523E/2006	2.1.3	CY014368	Human	Indonesia	2006
A/Indonesia/CDC523T/2006	2.1.3	CY014376	Human	Indonesia	2006
A/Indonesia/CDC582/2006	2.1.3	CY014384	Human	Indonesia	2006
A/Indonesia/CDC610/2006	2.1.3	CY014393	Human	Indonesia	2006
A/Indonesia/CDC623/2006	2.1.3	CY014401	Human	Indonesia	2006
A/Indonesia/CDC623E/2006	2.1.3	CY014409	Human	Indonesia	2006
A/Indonesia/CDC624/2006	2.1.3	CY014417	Human	Indonesia	2006
A/Indonesia/CDC624E/2006	2.1.3	CY014425	Human	Indonesia	2006
A/Indonesia/CDC634/2006	2.1.3	CY014441	Human	Indonesia	2006
A/Indonesia/CDC634P/2006	2.1.3	CY014449	Human	Indonesia	2006
A/Indonesia/CDC634T/2006	2.1.3	CY014457	Human	Indonesia	2006
A/Indonesia/CDC644/2006	2.1.3	CY014518	Human	Indonesia	2006
A/Indonesia/CDC644T/2006	2.1.3	CY014510	Human	Indonesia	2006
A/Indonesia/CDC669/2006	2.1.3	CY014481	Human	Indonesia	2006
A/Indonesia/CDC669P/2006	2.1.3	CY014489	Human	Indonesia	2006
A/Indonesia/CDC699/2006	2.1.3	CY014497	Human	Indonesia	2006
A/Indonesia/CDC7/2005	2.1.3	CY014177	Human	Indonesia	2005
A/Indonesia/CDC739/2006	2.1.3	CY014529	Human	Indonesia	2006
A/Indonesia/CDC759/2006	2.1.3	CY014543	Human	Indonesia	2006
A/Indonesia/CDC835/2006	2.1.3	CY017662	Human	Indonesia	2006
A/Indonesia/CDC836/2006	2.1.3	CY017670	Human	Indonesia	2006
A/Indonesia/CDC836T/2006	2.1.3	CY017678	Human	Indonesia	2006
A/Indonesia/CDC887/2006	2.1.3	CY017688	Human	Indonesia	2006
A/Indonesia/CDC938/2006	2.1.3	CY017638	Human	Indonesia	2006
A/Indonesia/CDC938E/2006	2.1.3	CY017646	Human	Indonesia	2006

Table 2 (continued)

A/Indonesia/CDC940/2006	2.1.3	CY017654	Human	Indonesia	2006
A/Indonesia/TLL001/2006	2.1.3	EU015403	Human	Indonesia	2006
A/Indonesia/TLL002/2006	2.1.3	EU015404	Human	Indonesia	2006
A/Indonesia/TLL003/2006	2.1.3	EU015405	Human	Indonesia	2006
A/Indonesia/TLL004/2006	2.1.3	EU015406	Human	Indonesia	2006
A/Indonesia/TLL005/2006	2.1.3	EU015407	Human	Indonesia	2006
A/Indonesia/TLL006/2006	2.1.3	EU015408	Human	Indonesia	2006
A/Indonesia/TLL007/2006	2.1.3	EU015409	Human	Indonesia	2006
A/Indonesia/TLL008/2006	2.1.3	EU015410	Human	Indonesia	2006
A/Indonesia/TLL009/2006	2.1.3	EU015411	Human	Indonesia	2006
A/Indonesia/TLL010/2006	2.1.3	EU015412	Human	Indonesia	2006
A/Indonesia/TLL011/2006	2.1.3	EU015413	Human	Indonesia	2006
A/Indonesia/TLL012/2006	2.1.3	EU015414	Human	Indonesia	2006
A/Indonesia/TLL013/2006	2.1.3	EU015415	Human	Indonesia	2006
A/Indonesia/TLL014/2006	2.1.3	EU015416	Human	Indonesia	2006
A/Djibouti/5691NAMRU3/2006	2.2	DQ666146	Human	Djibouti	2006
A/Egypt/7021-NAMRU3/2006	2.2	CY062439	Human	Egypt	2006
A/human/Iraq/207-NAMRU3/2006	2.2	DQ435202	Human	Iraq	2006
A/Iraq/1/2006	2.2	EU146870	Human	Iraq	2006
A/Iraq/659/2006	2.2	EU146876	Human	Iraq	2006
A/Iraq/754/2006	2.2	EU146877	Human	Iraq	2006
A/Iraq/755/2006	2.2	EU146869	Human	Iraq	2006
A/Iraq/756/2006	2.2	EU146878	Human	Iraq	2006
A/Turkey/12/2006	2.2	EF619982	Human	Turkey	2006
A/Turkey/15/2006	2.2	EF619989	Human	Turkey	2006
A/Turkey/651242/2006	2.2	EF619990	Human	Turkey	2006
A/Turkey/65596/2006	2.2	EF619998	Human	Turkey	2006
A/Xinjiang/1/2006	2.2	FJ492886	Human	China	2006
A/Egypt/14724-NAMRU3/2006	2.2.1	EF200512	Human	Egypt	2006
A/Egypt/14725-NAMRU3/2006	2.2.1	EF200513	Human	Egypt	2006
A/Egypt/2782-NAMRU3/2006	2.2.1	DQ464377	Human	Egypt	2006
A/Egypt/2991-NAMRU3/2006	2.2.1	EU095023	Human	Egypt	2006
A/Egypt/2992-NAMRU3/2006	2.2.1	EU095024	Human	Egypt	2006
A/Egypt/902782/2006	2.2.1	EU146867	Human	Egypt	2006
A/Egypt/902786/2006	2.2.1	EU146868	Human	Egypt	2006
A/Anhui/1/2005	2.3.4	DQ371928	Human	China	2005
A/Anhui/2/2005	2.3.4	DQ371929	Human	China	2005
A/China/2006	2.3.4	EF624256	Human	China	2006
A/China/GD01/2006	2.3.4	DQ835313	Human	China	2006
A/Fujian/1/2005	2.3.4	FJ492882	Human	China	2005

Table 2 (continued)

A/Guangdong/1/2006	2.3.4	FJ492884	Human	China	2006
A/Guangxi/1/2005	2.3.4	DQ371930	Human	China	2005
A/human/China/GD02/2006	2.3.4	EU263981	Human	China	2006
A/Hunan/1/2006	2.3.4	FJ492879	Human	China	2006
A/Jiangxi/1/2005	2.3.4	FJ492885	Human	China	2005
A/Shanghai/1/2006	2.3.4	AB462295	Human	China	2006
A/Shenzhen/406H/2006	2.3.4	EF137706	Human	China	2006
A/Sichuan/1/2006	2.3.4	FJ492881	Human	China	2006
A/Vietnam/UT30850/2005	2.3.4	HM114537	Human	Viet Nam	2005
A/Zhejiang/1/2006	2.3.4	FJ492880	Human	China	2006
A/Zhejiang/16/2006	2.3.4	DQ643809	Human	China	2006

2.2 COBRA FUNCTIONAL CHARACTERIZATION

2.2.1 Protein Expression

To evaluate protein expression, $1x10^6$ Human embryonic kidney (HEK) 293T cells were transiently transfected with 3 μg DNA expressing COBRA. Cells were incubated for 72 h at 37^{o}C, supernatants were removed, the cells were lysed with 1% Triton-X 100 and cell lysates were collected. Cell lysates were electrophoresed on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with mouse polyclonal antisera pooled from mice infected with 6:2 reassortant H5N1 viruses with the surface glycoproteins derived from either A/Vietnam/1203/2004 or A/Whooper Swan/244/2005 and the 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 (Pierce Biotechnology; Rockford IL, USA) and exposed to X-ray film (ThermoFisher; Pittsburgh, PA, USA).

```
   1 - ATGGAAAAGATCGTGCTGCTGCTGGCTATCGTGAGCCTGGTGAAGAGCGACCAGATTTGC - 60
   1 -  M  E  K  I  V  L  L  L  A  I  V  S  L  V  K  S  D  Q  I  C  - 20

  61 - ATCGGCTACCACGCCAACAACAGCACCGAGCAGGTGGACACCATCATGGAAAAGAACGTC - 120
  21 -  I  G  Y  H  A  N  N  S  T  E  Q  V  D  T  I  M  E  K  N  V  - 40

 121 - ACCGTGACCCACGCCCAGGACATCCTGGAAAAGACCCACAACGGCAAGCTGTGCGACCTG - 180
  41 -  T  V  T  H  A  Q  D  I  L  E  K  T  H  N  G  K  L  C  D  L  - 60

 181 - GACGGCGTGAAGCCCCTGATCCTGAGGGACTGCAGCGTGGCCGGCTGGCTGCTGGGCAAC - 240
  61 -  D  G  V  K  P  L  I  L  R  D  C  S  V  A  G  W  L  L  G  N  - 80

 241 - CCCATGTGCGACGAGTTCATCAACGTGCCCGAGTGGAGCTACATCGTGGAGAAGGCCAAC - 300
  81 -  P  M  C  D  E  F  I  N  V  P  E  W  S  Y  I  V  E  K  A  N  - 100

 301 - CCCGCCAACGACCTGTGCTACCCCGGCAACTTCAACGACTACGAGGAACTGAAGCACCTG - 360
 101 -  P  A  N  D  L  C  Y  P  G  N  F  N  D  Y  E  E  L  K  H  L  - 120

 361 - CTGTCCAGGATCAACCACTTCGAGAAGATCCAGATCATCCCCAAGAGCAGCTGGTCCGAC - 420
 121 -  L  S  R  I  N  H  F  E  K  I  Q  I  I  P  K  S  S  W  S  D  - 140

 421 - CACGAGGCCAGCAGCGGCGTGAGCAGCGCCTGCCCATACCAGGGCAGCCCCAGCTTCTTC - 480
 141 -  H  E  A  S  S  G  V  S  S  A  C  P  Y  Q  G  S  P  S  F  F  - 160

 481 - AGAAACGTGGTGTGGCTGATCAAGAAGAACAACACCTACCCCACCATCAAGAGGTCCTAC - 540
 161 -  R  N  V  V  W  L  I  K  K  N  N  T  Y  P  T  I  K  R  S  Y  - 180

 541 - AACAACACCAACCAGGAAGATCTGCTGGTCCTGTGGGGCATCCACCACCCTAATGACGCC - 600
 181 -  N  N  T  N  Q  E  D  L  L  V  L  W  G  I  H  H  P  N  D  A  - 200

 601 - GCCGAACAGACCAGGCTGTACCAGAACCCCACCACCTACATCAGCGTGGGCACAAGCACC - 660
 201 -  A  E  Q  T  R  L  Y  Q  N  P  T  T  Y  I  S  V  G  T  S  T  - 220

 661 - CTGAACCAGAGGCTGGTGCCCAAGATCGCCACCAGGTCCAAGGTGAACGGACAGTCCGGC - 720
 221 -  L  N  Q  R  L  V  P  K  I  A  T  R  S  K  V  N  G  Q  S  G  - 240

 721 - AGGATGGAATTCTTCTGGACCATCCTGAAGCCTAACGACGCCATCAACTTCGAGAGCAAC - 780
 241 -  R  M  E  F  F  W  T  I  L  K  P  N  D  A  I  N  F  E  S  N  - 260

 781 - GGCAACTTTATCGCCCCCGAGTACGCCTACAAGATCGTGAAGAAGGGCGACAGCGCCATC - 840
 261 -  G  N  F  I  A  P  E  Y  A  Y  K  I  V  K  K  G  D  S  A  I  - 280

 841 - ATGAAGAGCGAGCTGGAATACGGCAACTGCAACACCAAGTGCCAGACCCCCATCGGCGCC - 900
 281 -  M  K  S  E  L  E  Y  G  N  C  N  T  K  C  Q  T  P  I  G  A  - 300

 901 - ATCAACAGCAGCATGCCCTTCCACAACATCCACCCCCTGACCATCGGCGAGTGCCCCAAG - 960
 301 -  I  N  S  S  M  P  F  H  N  I  H  P  L  T  I  G  E  C  P  K  - 320

 961 - TACGTGAAGAGCAACAGGCTGGTGCTGGCCACCGGCCTGAGGAACAGCCCCCAGAGAGAG - 1020
 321 -  Y  V  K  S  N  R  L  V  L  A  T  G  L  R  N  S  P  Q  R  E  - 340

1021 - AGCAGAAGAAAGAAGAGGGGCCTGTTCGGCGCTATCGCCGGCTTCATCGAGGGCGGCTGG - 1080
 341 -  S  R  R  K  K  R  G  L  F  G  A  I  A  G  F  I  E  G  G  W  - 360

1081 - CAGGGCATGGTGGACGGGTGGTACGGCTACCACCACTCTAACGAGCAGGGCAGCGGCTAC - 1140
 361 -  Q  G  M  V  D  G  W  Y  G  Y  H  H  S  N  E  Q  G  S  G  Y  - 380

1141 - GCCGCCGACAAAGAGAGCACCCAGAAGGCCATCGACGGCGTCACCAACAAGGTGAACAGC - 1200
 381 -  A  A  D  K  E  S  T  Q  K  A  I  D  G  V  T  N  K  V  N  S  - 400
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1201 - ATCATCGACAAGATGAACACCCAGTTCGAGGCCGTGGGCAGAGAGTTCAACAACCTGGAA - 1260
 401 -  I  I  D  K  M  N  T  Q  F  E  A  V  G  R  E  F  N  N  L  E  - 420

1261 - AGGCGGATCGAGAACCTGAACAAGAAAATGGAAGATGGCTTCCTGGACGTGTGGACCTAC - 1320
 421 -  R  R  I  E  N  L  N  K  K  M  E  D  G  F  L  D  V  W  T  Y  - 440

1321 - AACGCCGAGCTGCTGGTGCTGATGGAAAACGAGAGGACCCTGGACTTCCACGACAGCAAC - 1380
 441 -  N  A  E  L  L  V  L  M  E  N  E  R  T  L  D  F  H  D  S  N  - 460

1381 - GTGAAGAACCTGTACGACAAAGTGCGGCTGCAGCTGAGGGACAACGCCAAAGAGCTGGGC - 1440
 461 -  V  K  N  L  Y  D  K  V  R  L  Q  L  R  D  N  A  K  E  L  G  - 480

1441 - AACGGCTGCTTCGAGTTCTACCACAAGTGCGACAACGAGTGCATGGAAAGCGTGAGGAAC - 1500
 481 -  N  G  C  F  E  F  Y  H  K  C  D  N  E  C  M  E  S  V  R  N  - 500

1501 - GGCACCTACGACTACCCCCAGTACAGCGAGGAAGCCAGGCTGAAGAGGGAAGAGATCAGC - 1560
 501 -  G  T  Y  D  Y  P  Q  Y  S  E  E  A  R  L  K  R  E  E  I  S  - 520

1561 - GGAGTGAAGCTGGAAAGCATCGGCACCTACCAGATCCTGAGCATCTACAGCACCGTCGCC - 1620
 521 -  G  V  K  L  E  S  I  G  T  Y  Q  I  L  S  I  Y  S  T  V  A  - 540

1621 - AGCAGCCTGGCCCTGGCTATCATGGTGGCCGGACTGAGCCTGTGGATGTGCAGCAACGGC - 1680
 541 -  S  S  L  A  L  A  I  M  V  A  G  L  S  L  W  M  C  S  N  G  - 560

1681 - AGCCTGCAGTGCAGGATCTGCATCGGATCCGAGCTC - 1716
 561 -  S  L  Q  C  R  I  C  I  G  S  E  L  - 580
```

Figure 11: COBRA nucleotide and corresponding amino acid sequences

2.2.2 Receptor Binding

To determine receptor binding characteristics, virus-like particles (VLPs) containing COBRA HA proteins were purified from the supernatants of mammalian cell lines. HEK 293T cells were transiently transfected with plasmids expressing HIV Gag, COBRA HA and neuraminidase (NA, A/Thailand/1(KAN-1)/2004) and incubated for 72 h at 37^{o}C. Supernatants were collected and VLPs were purified via ultracentrifugation (100,000 X g through 20% glycerol, weight per volume) for 4 h at 4^{o}C. The pellets were subsequently resuspended in phosphate buffered saline PBS, pH 7.2 and stored at -80^{o}C until use. Protein concentration was determined by Micro BCA^{TM} Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). COBRA HA VLPs were prepared in various amounts as measured by total protein and each individual preparation was two-fold serially diluted in v-bottom microtiter plates. An equal volume of either

1% turkey or 1% horse erythrocytes (RBC) (Lampire; Pipersville, PA, USA) in PBS was added to the diluted VLPs and incubated for 30-60 minutes at room temperature. The HA titer was determined by the reciprocal dilution of the last well which contained agglutinated RBC.

2.2.3 Cell Fusion

To determine endosomal fusion characteristics, COBRA-pseudotyped lentiviral vectors expressing a luciferase reporter gene were produced as described [228]. Briefly, 293T cells were co-transfected by using the following plasmids: 7μg of pCMVdeltaR8.2, 7μg of pHR CMV-Luc, 3μg pCMV/R N1(Kan-1) (all kindly provided by Dr. G. Nabel) and 3μg pTR600 COBRA. Cells were transiently transfected and incubated for 72h at 37^{o}C. Supernatants were harvested, centrifuged to clear cell debris, filtered through a 0.22 μm syringe filter, aliquotted, and used immediately or frozen at -80°C. For fusion assays, 100 μl of pseudoviruses were added to confluent MDCK cells in 48-well plates (~30,000 cells per well). Plates were incubated at room temperature for 30 min, washed, and fresh medium added. Forty-eight hours after infection, cells were lysed in mammalian cell lysis buffer (Promega; Madison, WI, USA). A standard quantity of cell lysate was used in a luciferase assay with luciferase assay reagent (Promega; Madison, WI, USA) according to the manufacturer's protocol.

2.3 VACCINE PREPARATION

2.3.1 Virus-like Particle Purification

HEK 293T cells were transiently transfected with plasmids expressing M1 (A/Puerto Rico/8/1934, optimized for expression in mammalian cells), NA (A/Thailand/1(KAN-1)/2004, optimized for expression in mammalian cells) and various HA and incubated for 72h at 37^{o}C. Supernatants were collected and cell debris removed by low speed centrifugation followed by vacuum filtration through a 0.22 μm sterile filter. VLPs were purified via ultracentrifugation (100,000 X g through 20% glycerol, weight per volume) for 4 h at 4C. The pellets were subsequently resuspended in PBS pH 7.2 and stored in single use aliquots at -80C until use. Total protein concentration was determined by Micro BCA$_{TM}$ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

2.3.2 HA Quantification

HA specific content was determined by western blot and densitometry. Purified recombinant HA matched to the respective VLP and purified VLPs were prepared in standard total protein amounts (2μg to 0.15μg for purified protein and 5μg to 10μmg for VLPs) and were electrophoresed on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with mouse polyclonal antisera pooled from mice infected with 6:2 reassortant H5N1 viruses with the surface glycoproteins derived from either A/Vietnam/1203/2004 or A/Whooper Swan/244/2005 and the 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 (Pierce Biotechnology; Rockford IL, USA) and

exposed to X-ray film (ThermoFisher; Pittsburgh, PA, USA). Density of bands was determined using ImageJ software (NIH) [229]. Density of recombinant HA bands were used to calculate a standard curve and the density of the purified VLPs was interpolated using the results from the recombinant HA. Experiments were performed in triplicate and multiple exposure times were analyzed for all iterations.

2.4 ANIMAL EXPERIMENTS

2.4.1 Mouse Studies

BALB/c mice (*Mus musculis*, females, 6–8 weeks) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA) and housed in microisolator units and allowed free access to food and water and were cared for under USDA guidelines for laboratory animals. For dosing studies, mice (5 mice per group) were vaccinated with one of two doses of purified VLPs (1.5μg or 0.3μg), based upon HA content from the densitometry assay, via intramuscular injection at week 0 and then boosted with the same dose at week 3. For comparison studies, mice (20 mice per group) were vaccinated with purified VLPs (3μg), based upon HA content from the densitometry assay, via intramuscular injection at week 0 and then boosted with the same dose at week 3. Vaccines at each dose were formulated with Imject® alum adjuvant (Imject® Alum, Pierce Biotechnology; Rockford, IL, USA) according to the manufacturer's protocol or vehicle alone. Fourteen to twenty-one days after each vaccination, blood was collected from anesthetized mice via the retro-orbital plexus and transferred to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at -20 ± 5°C.

Three weeks after final vaccination, mice were challenged intranasally with $5x10^3$ plaque forming units (PFU) of either highly pathogenic wildtype H5N1 virus A/Whooper Swan/Mongolia/244/2005 (n=20/group) or 6:2 reassortant virus with internal genes from the mouse adapted virus A/Puerto Rico/8/1934 and the surface proteins HA and NA from A/Vietnam/1203/2004 (n=10/group) in a total volume of 50μl. Challenge doses for both viruses were established independently and represent approximately $50LD_{50}$ (data not shown). After infection, mice 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=reduced, 2=severely reduced), hunched back (0=absent, 1=present) and ruffled fur (0=absent, 1=present) [230]. Experimental endpoint was determined by >20% weight loss or display of neurological disease such as hind limb paralysis. All highly pathogenic wild type H5N1 influenza 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, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

2.4.2 Ferret Studies

Fitch ferrets (*Mustela putorius furo*, female, 6-12-months of age), influenza naïve and descented, were purchased from Marshall Farms (Sayre, PA, USA). Ferrets were pair housed in stainless steel cages (Shor-line, Kansas City, KS, USA) containing Sani-chips Laboratory Animal Bedding (P.J. Murphy Forest Products, Montville, NJ, USA). Ferrets were provided with Teklad Global Ferret Diet (Harlan Teklad, Madison, WI, USA) and fresh water ad libitum. The VLPs were diluted in PBS, pH 7.2 to achieve final concentration. Ferrets (n=9) were vaccinated with 15μg of purified VLPs, based upon HA content as determined by densitometry assay, via

intramuscular injection in the quadriceps muscle in a volume of 0.50 ml at week 0 and then boosted with the same dose at week 3. Vaccines were stored at -80°C prior to use and formulated with Imject® alum adjuvant (Imject® Alum; Pierce Biotechnology, Rockford, IL, USA) immediately prior to use according to manufacturer's protocol. Animals were monitored for adverse events including weight loss, temperature, loss of activity, nasal discharge, sneezing and diarrhea weekly during the vaccination regimen. Prior to vaccination, animals were confirmed by HAI assay to be seronegative for circulating influenza A (H1N1 and H3N2) and influenza B viruses. Fourteen to twenty-one days after each vaccination, blood was collected from anesthetized ferrets via the anterior vena cava and transferred to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at -20 ± 5°C.

Three weeks after final vaccination, ferrets were challenged intranasally with $1x10^6$ plaque forming units (PFU) of the highly pathogenic H5N1 virus A/Whooper Swan/Mongolia/244/2005 (clade 2.2) 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 were performed by instilling 3 ml of PBS into the nares of anesthetized ferrets each day for 14 days after inoculation. Washes were collected and stored at -80°C until use. Experimental endpoint was defined as >20% weight loss, development of neurological symptoms, or an activity score of 3 (not active or alert after stimulation). All H5N1 influenza 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, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

2.4.3 Non-Human Primate Studies

Cynomolgus macaques (*Macaca fascicularis,* male, 3-5 years old) were vaccinated with 15 μg (based upon HA content) of purified VLPs (n=7 per group), via intramuscular injection at weeks 0, 3 and 6. Vaccines at each dose were formulated with alum adjuvant (Imject Alum, Pierce Biotechnology; Rockford, IL, USA) immediately prior to use. Twenty-one days after each vaccination, blood was collected from anesthetized macaques via the femoral vein and transferred to a serum separator tube. After clotting, tubes were centrifuged and sera removed and frozen at -80 ± 5°C.

Three weeks after final vaccination, macaques were placed into BSL3+ isolator units (Bioqual, Inc., Rockville, MD) and then challenged by a multi-route of infection (ocular, nasal, tracheal) as previously described [132, 231, 232] using $1x10^6$ plaque forming units (PFU) of the highly pathogenic H5N1 virus, A/Whooper Swan/Mongolia/244/2005 (clade 2.2), in a volume of 1 ml at each location for a total infection dose of $1x10^6$ PFU in a total volume of 3 ml. Macaques were monitored daily for weight loss, signs of disease, and mortality until 7 days after infection. Individual body weights, sickness scores (based upon lethargy, temperature change, nasal discharge, lack of appetite, dehydration, lack of responsiveness), and death were recorded for each group. Nasal and tracheal washes were performed at day 0, 1, 3, 5, and 7 post-infection. In addition, subsets of monkeys were sacrificed following administration of anesthesia and necropsies were performed according to standard procedures.

2.5 SEROLOGICAL ANALYSIS

2.5.1 ELISA Assay

The ELISA assay was used to assess total antibody titer and IgG isotype titer to the HA. High binding, 96-well polystyrene plates (Costar; Lowell, MA, USA) were coated overnight with 50ng/well of recombinant HA. Coating antigens were derived from the following representative viral isolates: A/Vietnam/1203/2004 (clade 1), A/Indonesia/5/2005 (clade 2.1), A/Whooper Swan/244/2005 (clade 2.2) and A/Anhui/1/2005 (clade 2.3). Plates were blocked with 5% milk diluted in PBS with 0.05% Tween 20. Serum samples were diluted in blocking buffer and added to plates. Serum was two-fold serially diluted and allowed to incubate for 1 hour at room temperature. Plates were washed and species specific antibody against IgG, IgG1, IgG2a, IgG2b or IgG3 and linked to horseradish peroxidase (HRP) (Southern Biotech; Birmingham, AL, USA) were diluted in blocking buffer and added to plates. Plates were incubated for 1 hour at room temperature. Plates were washed and HRP was developed with TMB substrate (Sigma-Aldrich; St. Louis, MO, USA). Plates were incubated in the dark for 15 minutes and then the reaction was stopped with 2N H_2SO_4. Optical densities at a wavelength of 450nm (OD_{450}) were read by a spectrophotometer (BioTek; Winooski, VT, USA) and end point dilution titers were determined. End point titers were determined as the reciprocal dilution of the last well which had an OD_{450} above the mean OD_{450} plus two standard deviations of naïve animal sera.

2.5.2 Hemagglutination Inhibition (HAI) Assay

The HAI assay was used to assess functional antibodies to the HA able to inhibit agglutination of horse erythrocytes. The protocol was adapted from the CDC laboratory-based influenza surveillance manual [233]. To inactivate non-specific inhibitors, sera were treated with receptor

destroying enzyme (RDE; Denka Seiken, Co., Japan) prior to being tested [234-238]. Briefly, three parts RDE was added to one part sera and incubated overnight at 37°C. RDE was inactivated by incubation at 56°C for ~30 min. RDE treated sera was two-fold serially diluted in v-bottom microtiter plates. An equal volume of wild type or reassortant virus, adjusted to approximately 8 HAU/50µl, was added to each well. The plates were covered and incubated at room temperature for 20 min followed by the addition of 1% horse erythrocytes (HRBC) (Lampire Biologicals, Pipersville, PA, USA) in PBS. Red blood cells were stored at 4°C and used within 72 h of preparation. The plates were mixed by agitation, covered, and the RBCs were allowed to settle for 1 h at room temperature [239]. The HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Positive and negative 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.

2.5.3 Virus Microneutralization Assay

Serum neutralizing antibody titers were determined by microneutralization (mVN) assays performed on Madin Darby Canine Kidney (MDCK) cells following an adaptation of the procedure of Rowe et al [240]. Briefly, individual heat inactivated serum samples were serially diluted 2-fold (starting at a 1:10 dilution) in MDCK diluent buffer in a cell culture plate followed by the addition of a pre-determined amount (100 $TCID_{50}$) of each virus. Sera and viruses were mixed and incubated at 37 °C for 2 hours. After the incubation the virus antibody mixtures were added to plate containing MDCK cells and were incubated for 2 hours at 37 °C in a 5% CO_2 cell culture incubator. The virus antibody was then removed and cells were incubated until CPE was observed. Cells were then fixed in 10% formalin and stained with 1% crystal violet to quantify CPE. The neutralizing antibody titers are expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 $TCID_{50}$ of virus in MDCK cells. Positive serum control

and negative cell controls with no serum were included on each plate. Geometric mean neutralizing antibody titers were calculated for each group.

2.6 VIROLOGICAL ASSAYS

For mouse infections, lung virus titers were evaluated. For ferret infections, nasal wash virus titers were used to assess viral burden. Both lungs and nasal wash virus titers were determined using a plaque assay [241, 242]. Briefly, lungs from mice infected with virus were harvested post infection, snap-frozen and stored at -80°C until use. Samples were thawed, weighed and single cell suspensions were prepared via passage through a 70 μm mesh (BD Falcon, Bedford, MA, USA) in an appropriate volume of DMEM supplemented with penicillin-streptomycin (iDEME) as to achieve 100mg/ml final concentration. Cell suspensions were centrifuged at 2000 rpm for 5 min and the supernatants were collected.

Madin-Darby Canine Kidney (MDCK) cells were plated (5×10^5) in each well of a 6 well plate. Samples (lung supernatants for mice and nasal washes for ferrets) were diluted (dilution factors of 1×10^1 to 10^6) and overlayed onto the cells in 100 μl of iDMEM and incubated for 1hr. Virus-containing medium was removed and replaced with 2 mls of L15 medium plus 0.8% agarose (Cambrex, East Rutherford, NJ, USA) and incubated for 96 hrs at 37°C with 5% CO_2. Agarose was removed and discarded. Cells were fixed with 10% buffered formalin, and then stained with 1% crystal violet for 15 min. Following thorough washing in dH_2O to remove excess crystal violet, plates were allowed to dry, plaques counted, and the plaque forming units (PFU)/g for or PFU/ml for nasal washes were calculated.

2.7 CELLULAR ASSAYS

2.7.1 Interferon Gamma Enzyme-Linked Immunospot

The number of anti-influenza specific cells secreting interferon gamma (IFN-γ) was determined in mice by enzyme-linked immunospot (ELISpot) assay (R&D systems, Minneapolis, MN, USA) following the manufacturer's protocol. Mice were sacrificed at 6 days post infection (DPI) and spleens and lungs were harvested and prepared in single cell suspensions. Briefly, pre-coated anti-IFNγ plates were blocked with RPMI plus 10% FCS and antibiotics (cRPMI) for 30 minutes at room temperature. Media was removed from wells and 10^5 cells were added to each well. Cells were stimulated with purified recombinant HA from A/Vietnam/1203/2004 (truncated at residue 530; 1μg/well), inactivated 6:2 reassortant virus A/Vietnam/1203/2004 (1:100 dilution of inactivated stock; 100μl/well) or the immunodominant H2-K^d $CD8^+$ T cell epitope in H5 HA: HA_{533} (IYSTVASSL; 1μg/well) (Pepscan Presto, Leystad, Netherlands). Additional wells were stimulated with PMA (50ng/well) and ionomycin (500ng/well) as positive controls or Ova_{257} (SIINFEKL; 1μg/well) (Pepscan Presto, Leystad, Netherlands) as negative controls. Additionally, IL-2 (10U/ml) was added to each well. Plates were incubated at 37°C for 48 hours. After incubation, plates were washed four times with R&D wash buffer and were incubated at 4°C overnight with biotinylated anti-mouse IFNγ. Plates were washed as before and incubated at room temperature for 2 hours with streptavidin conjugated to alkaline phosphatase. Plates were washed as before and spots were developed by incubating at room temperature for 1 hour in the dark with BCIP/NBT chromogen substrate. The plates were washed extensively with DI H_2O and allowed to dry overnight prior to spots being counted using an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

2.7.2 Antibody Secreting Cell Assay

The number of mouse anti-HA and anti-NA specific antibody secreting cells was determined by B cell ELISpot assay as previously described [243-245]. Mice were sacrificed at 6 DPI and spleens and lungs were harvested and prepared in single cell suspensions. Briefly, 0.45μm PVDF membrane plates (Millipore, Billerica, MA, USA) were coated with either purified recombinant HA from A/Vietnam/1203/2004 or purified recombinant NA from A/Thailand/1(KAN-1)/2004 (250ng/well) and incubated at 4°C overnight. Plates were washed three times with PBS and blocked with cRPMI for at 37°C for 3-4 hours. Media was removed from wells and 10^5 cells were added to each well. Plates were incubated at 37°C for 48 hours. After incubation, plates were washed as before and incubated at room temperature for 2 hours with horse radish peroxidase conjugated anti-mouse IgG or IgA (Southern Biotech, Birmingham, AL, USA). Plates were washed as before and spots were developed at room temperature for 1 hour in the dark with detection substrate (NovaRED™; Vector Labs, Burlingame, CA, USA). The plates were washed extensively with DI H_2O and allowed to dry overnight prior to spots being counted using an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

2.8 PASSIVE TRANSFER OF SERA

Serum from vaccinated mice was pooled and passively transferred into 9 week old recipient BALB/c mice (n=5/group). Equal amounts of serum from each mouse in a particular vaccine group were pooled and heat inactivated for 30 minutes at 56°C. 200 μl of pooled and inactivated serum was transferred to recipient mice via IP injection. 24 hours post transfer, mice

were infected with 6:2 reassortant virus with internal genes from the mouse adapted virus A/Puerto Rico/8/1934 and surface antigens from A/Vietnam/1203/2004 as described above.

3.0 SPECIFIC AIMS

3.1 OVERALL OBJECTIVE AND RATIONALE

The overall objective of this project was to develop a broadly reactive vaccine for highly pathogenic H5N1 influenza. The antigenic diversity of circulating H5N1 viruses makes it highly unlikely that a vaccine derived from a single primary isolate would be able to elicit cross-reactive immune responses in the event of an emerging pandemic. Antibodies capable of blocking the functions of the viral hemagglutinin (HA) are the only immune response capable of preventing influenza infection and represent the standard measure for predicting influenza vaccine efficacy. At the beginning of this dissertation research, there had been no reports of an H5N1 vaccine eliciting broadly reactive receptor blocking antibodies. The focus of this work was the development of a centralized HA vaccine that will elicit broad antibody responses to antigenically diverse H5N1 influenza viruses. Increasing the breadth of influenza vaccines represents a significant advancement for both pandemic and seasonal influenza vaccine development. Broadly reactive pre-pandemic vaccines would enable effective stock-piling in anticipation of the next pandemic. Additionally, increasing the breadth of seasonal vaccines would improve the vaccine efficacy and decrease influenza-related morbidity and mortality during inter-pandemic periods.

3.1.1 H5N1 Clade 2 Vaccines

Although H5N1 is separated into 10 phylogenetic clades, clade 2 is the most prevalent, diverse and widespread of all clades (see Section 1.10.3). Clade 2 is separated into antigenically distinct subclades, of which clades 2.1, 2.2 and 2.3 have caused human disease. The centralized antigen for this work was designed to specifically address the diversity within clade 2. The molecular determinants of host-switch and emergence of avian influenza virus into the human population are not fully elucidated, but HA properties, such as receptor specificity and cleavage site, are proposed to be involved (see Section 1.6.2). Only HA sequences from human isolates were used in centralized antigen generation. Unidentified characteristics in the HA molecule that enable human infection are expected to be conserved. Increasing the breadth of influenza vaccines is traditionally achieved via polyvalency. Therefore, a clade 2-focused polyvalent vaccine was used as the standard strategy against which the centralized antigen was compared (see Section 1.9). The polyvalent vaccine components were chosen from primary isolates in clades 2.1, 2.2 and 2.3. The selected strains were primarily isolated from human infections. Furthermore, all selected polyvalent vaccine components were individually recommended by WHO for vaccine development and therefore are expected to be antigenically matched to their respective subclade. Until this dissertation, the breadth of centralized and polyvalent influenza vaccines had never been directly compared.

3.1.2 Test Antigens

The primary test antigens used to evaluate antibody responses were representative isolates from subclades 2.1, 2.2 and 2.3. The selected test antigens represent unique viral strains that do not elicit cross-reactive antibodies between subclades. Furthermore, the primary test

antigens were matched to the components of the polyvalent vaccine. The panel of primary antigens used to initially evaluate antibody breadth meets or exceeds the standard measures established in prior reports. In some of the studies described here, additional antigens were used to more thoroughly evaluate receptor blocking antibody breadth. These antigens were not included in either the centralized or polyvalent vaccine designs, and were therefore useful as a measure of additional antibody breadth. The panels of test antigens used to evaluate antibody breadth are the most diverse and comprehensive analysis of H5N1 vaccine breadth to date.

3.1.3 Challenge Viruses

The vaccines evaluated in this project were all designed to address the diversity within clade 2 of H5N1. Therefore, the primary challenge virus used to evaluate vaccine efficacy was a clade 2.2 isolate: A/Whooper Swan/Mongolia/244/2005. The virus was a wild type, highly pathogenic avian influenza virus isolated from an avian host. Wild type clade 2 viruses isolated from human hosts were not available when these studies were conducted. Additionally, the challenge virus was matched to the clade 2.2 component of the polyvalent vaccine. The pathogenicity of the challenge virus used in most of these studies was compared to a clade 1 virus (A/Vietnam/1203/2004) isolated from a human infection. The viruses resulted in equivalent disease in mice and ferrets. Additionally, cross-clade protection from centralized and polyvalent vaccines was evaluated using the A/Vietnam/1203/2004 (clade 1) virus.

3.1.4 Animal Models

Mice and ferrets are the most commonly used animal models in influenza virus vaccine and infection studies. Although infection of mice with influenza virus usually requires extensive species-specific adaptation of the virus, highly pathogenic viruses such as 1918 or H5N1 cause

severe disease without prior adaptation. Mice provide several advantages for the analysis of vaccine induced immune responses due to the limited genetic variation and extensive, multi-disciplinary use. These advantages include, but are not limited to, the availability of reagents for determining the antibody isotype profile and evaluating cellular immune responses in an antigen specific manner.

Ferrets are the traditional animal model for influenza infection and demonstrate a disease course that is similar to human infection. Additionally, ferrets are susceptible to infection with human influenza viruses without prior adaptation and transmit the virus. The analysis of immune responses in ferrets is restricted due to the genetic variability and relatively limited scientific use. Although assays to evaluate cellular immunity are being developed, they are not widely available and therefore analysis of vaccine immune response is limited to antibody breadth.

Non-human primates (NHP) were also used as models to evaluate the centralized vaccine antigen. Similar to ferrets, NHP have high levels of genetic variability, but generally have more reagents available for immune response analysis. Importantly, pre-clinical findings in NHPs are more likely to translate to humans than those from either mice or ferrets. NHP were utilized in this work to further evaluate the breadth of antibody responses elicited by the centralized antigen.

3.2 OVERALL HYPOTHESIS AND AIMS

Overall Hypothesis: A synthetic consensus clade 2 H5N1 HA vaccine will elicit broad immunity and protective efficacy against diverse H5N1 viral isolates.

SPECIFIC AIM I: Design and characterize a consensus HA H5N1 vaccine.

Hypothesis: The consensus HA (COBRA) sequence will be a fully functional protein that is antigenically matched to a diverse panel of H5N1 viruses.

SPECIFIC AIM II: Evaluate the breadth and intensity of immune responses elicited by a consensus HA H5N1 vaccine.

Hypothesis: A virus-like particle containing the COBRA HA will elicit broad immune responses to a diverse panel of H5N1 viral isolates.

SPECIFIC AIM III: Determine the protective efficacy of a consensus HA H5N1 vaccine against H5N1 influenza challenge.

*Hypothesis***:** The COBRA HA vaccine will protect animals following virus challenge with H5N1 viruses.

3.3 SPECIFIC AIM I

Goal: Design and functionally characterize a consensus HA H5N1 vaccine.

Hypothesis: The consensus HA sequence will be a fully functional protein that is antigenically matched to a diverse panel of H5N1 viruses.

Summary: A consensus HA was generated using all available, full length sequences isolated from H5N1 clade 2 human infections from 2004-2006. To avoid sampling bias, a layered consensus building approach was developed that used multiple rounds of consensus generation. Sequences were first grouped into phylogenetic subclades and then further divided into outbreak groups within each subclade based on time and location of isolation. A primary consensus sequence was generated for each individual outbreak group. Primary consensus sequences within each subclade were then aligned and secondary consensus sequences were generated representing each subclade. Secondary consensus sequences were then aligned and used to generate a final consensus sequence representing all of the input sequences. This final consensus sequence was termed Computationally Optimized Broadly Reactive Antigen (COBRA) and was used for all subsequent studies. The expression of COBRA HA was confirmed by transient transfection of HEK 293T cells. To verify the functional activity of COBRA, lentiviral pseudoparticles were generated and used in receptor binding and cell fusion assays. COBRA receptor binding was demonstrated by hemagglutination assay. COBRA-mediated cell fusion was independent of trypsin and was as efficient as that observed with a primary isolate-derived HA. Taken together, COBRA was demonstrated to retain all of the functions of a natural hemagglutinin protein. In addition to the functional characterization of COBRA, the antigenicity was also determined using ferret antisera. Antisera elicited against both clade 1 and clade 2 viruses had hemagglutination inhibition (HAI) activity against COBRA pseudoparticles indicating conservation of binding epitopes. After the COBRA HA protein was

characterized, the virus-like particle (VLP) vaccines were produced and further characterized. To determine the HA content of VLP vaccines, a novel quantification assay was developed. Multiple dilutions of purified HA protein matched to an individual VLP were analyzed by SDS-PAGE followed by an immunoblot for the HA. The relative density of each dilution was calculated and used to generate a standard curve. The curve was then used to interpolate the amount of HA in the VLPs using the density of the HA band in VLP lanes.

Conclusion: The consensus HA (termed COBRA) was a functional protein that was antigenically matched to multiple H5N1 viruses. H5N1 vaccines were purified and a novel method of antigen quantification was developed.

3.4 SPECIFIC AIM II

Goal: Evaluate the breadth and intensity of immune responses elicited by a consensus HA H5N1 vaccine.

Hypothesis: A virus-like particle containing the COBRA HA will elicit broad immune responses to a diverse panel of H5N1 viral isolates.

Summary: The immunogenicity of HA from H5N1 is lower than that of seasonal influenza and vaccines require higher doses or adjuvant inclusion to elicit HAI responses. Serum HAI antibodies are the standard measure for influenza vaccine immunogenicity. For H5N1 vaccines, vaccinated mice and ferrets that fail to develop detectable HAI titer are often protected from severe disease and death upon H5N1 challenge. However, the human correlate for protection in H5N1 remains unknown and HAI antibodies are considered the most consistent predictor of protection. Therefore, the HAI assay was the standard measure used to determine antibody breadth. Various doses of COBRA VLPs were evaluated for the elicitation of immune

responses alone or co-administered with adjuvants in BALB/c mice. Although lower doses of COBRA VLPs elicited broad antibody responses when formulated with alum adjuvant, the greatest breadth of antibody response was elicited when using a high dose (1.5μg HA) together with alum adjuvant. Therefore, the high dose and alum adjuvant was used in subsequent vaccinations. Dosing studies in ferrets had similar results: the highest dose (15μg HA) together with alum elicited the greatest antibody breadth and this formulation was used for additional ferret experiments. Next, the breadth of the antibody response elicited by the COBRA HA was compared to a vaccine derived from a primary clade 2.2 isolate (A/Whooper Swan/Mongolia/244/2005). In mice, both vaccines elicited similar high titers of total IgG to diverse HA coating antigens. However, the COBRA VLP vaccinated mice had both higher titers and increased breadth of HAI antibodies compared to those vaccinated with the clade 2.2 VLPs. Similar results were found in ferrets: both COBRA and clade 2.2 VLPs elicited similar total IgG levels, but only those animals that received the COBRA vaccine had broadly reactive HAI antibody responses. NHP were also vaccinated to evaluate the breadth of antibody responses of COBRA VLPs. For these studies, a larger panel was utilized with test antigens within and outside of clade 2. COBRA vaccinated NHP had antibody responses that were more broadly reactive than clade 2.2 vaccinated animals and included HAI activity against viruses from clades 1, 2.1, 2.2.1, 2.2.2, 2.3.4, and 7.

Having established the increased breadth of COBRA-based vaccines in comparison to a clade 2.2 vaccine, COBRA was then compared to a polyvalent formulation. BALB/c mice vaccinated with either COBRA or polyvalent vaccines developed similar high titer total IgG against multiple HA antigens. Additionally, the polyvalent vaccine elicited HAI antibodies to all of the clade 2 test antigens (all were homologous to the vaccine components), but the COBRA vaccine elicited higher titer HAI antibodies and extended the reactivity to clade 1. Similar results were found in ferrets: equivalent levels of total IgG titers between vaccine groups, but increased

breadth and titer of HAI antibodies in the COBRA vaccinated animals. Although humoral immunity is the most potent immune response in protecting against influenza infection, cellular mediated immune responses can limit the severity and duration of infection (see Section 1.7). Cellular immune responses were compared for COBRA and polyvalent vaccinated mice. Both COBRA and polyvalent vaccines elicited cellular immune responses that were detected by IFN-γ ELISpot when stimulated with the BALB/c immunodominant H2-K^d $CD8^+$ T cell epitope in H5 HA. Additionally, stimulation with overlapping peptides spanning the HA molecule did not reveal any novel epitopes in the COBRA HA. This finding confirmed the results from epitope prediction software indicating that COBRA did not contain any novel T cell epitopes.

Conclusion: COBRA VLPs elicit broadly reactive antibody responses of increased breadth and intensity compared to the immune responses elicited by both clade 2.2 and polyvalent VLPs. Additionally, cellular mediated immune responses were equivalent between COBRA and polyvalent vaccines.

3.5 SPECIFIC AIM III

Goal: Determine the protective efficacy of a consensus HA H5N1 vaccine against H5N1 influenza challenge.

Hypothesis: The COBRA HA will protect animals following challenge with H5N1 viruses.

Summary: The most important factor for evaluating influenza vaccines is the protective efficacy against virus challenge. H5N1 vaccine efficacy is primarily evaluated in the context of protection from severe disease and death. These are undoubtedly crucial factors in determining the success of a vaccine, but fail to account for a vaccine's ability to control viral burden. Higher levels of viral replication increase the opportunities for virus evolution and transmission.

Morbidity, mortality and viral burden were all factors used to evaluate vaccine efficacy. Vaccines were designed to specifically address the diversity within clade 2 and as such the primary challenge virus was a clade 2.2 isolate: A/Whooper Swan/Mongolia/244/2005 (WS/05). Prior to its use in vaccine studies, the virus was first titered to determine relative lethality and disease course compared to a well-characterized clade 1 virus: A/Vietnam/1203/2004 (VN/04). At the same infectious dose, 5000 plaque forming units (PFU), weight loss and viral replication in the lung were similar for the two viruses indicating that the WS/05 virus is indeed highly lethal to mice. This dose was calculated as approximately 50 LD_{50} and was used in all subsequent studies. Having established the challenge parameters, vaccine efficacy was then evaluated. Mice receiving various doses of COBRA VLPs with and without adjuvant were challenged with WS/05 and all vaccinated animals were completely protected from weight loss and death. Additionally, when COBRA and clade 2.2 vaccinated animals were challenged, they also were protected from disease. Similar to the mice, all vaccinated ferrets were protected from sickness and death. However, when NHP were challenged, the infection did not result in visible illness in unvaccinated control animals. This was not because of the isolate (WS/05) used in the infection because additional animals infected with VN/04 also failed to develop visible signs of disease. Although no visible disease was observed in challenged animals, histopathological analysis of the lungs revealed focal inflammation and detection of viral antigen in unvaccinated animals that was greatly reduced in COBRA vaccinated animals. Mice and ferrets vaccinated with the polyvalent formulation were then compared to COBRA vaccinated animals using a WS/05 challenge. Once again, all vaccinated animals were protected from sickness and death. When lungs (mice) and nasal washes (ferrets) were assayed for virus, COBRA vaccinated animals had decreased viral burdens compared to polyvalent vaccinated animals. The decrease in viral replication was observed in both magnitude of virus titer and duration of replication. To further evaluate the breadth of protection elicited by COBRA and polyvalent vaccines, mice were challenged with the clade 1 virus VN/04. All vaccinated animals were protected from sickness

and death with viral burdens again being decreased in the COBRA vaccinated animals. These results indicated that although both COBRA and polyvalent vaccines protect animals from severe disease, the COBRA vaccine more efficiently restricted viral replication. Finally, to determine whether serum factors were sufficient to protect animals from severe disease, immune sera from COBRA and polyvalent vaccinated animals were transferred to naïve recipient mice. All mice receiving sera from VLP vaccinated animals were protected from death during VN/04 challenge. All mice lost weight and displayed signs of illness, however, these symptoms were decreased in the cohort receiving COBRA-immune serum.

Conclusion: COBRA VLPs effectively protected vaccinated animals from multiple H5N1 virus challenges. Polyvalent vaccines also protected animals from overt disease and death. However, the COBRA vaccine more efficiently restricted viral replication compared to the polyvalent vaccine. Additionally, COBRA-immune serum recipient animals displayed reduced signs and duration of illness compared to polyvalent-immune serum recipients.

4.0

A COMPUTATIONALLY OPTIMIZED BROADLY REACTIVE ANTIGEN (COBRA) BASED H5N1 VLP VACCINE ELICITS BROADLY REACTIVE ANTIBODIES IN MICE AND FERRETS

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Brendan M. Giles and Ted M. Ross

A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets

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4.1 FOREWORD

The work described in this chapter addresses features of Specific Aims I, II and III (see Sections 3.3, 3.4 and 3.5). The design and functional characterization of the consensus hemagglutinin outlined in Specific Aim I is detailed in this chapter and the work presented here encompasses all of Specific Aim I. Additionally, the vaccine dosing and clade 2.2 comparison studies evaluating vaccine immunogenicity that are outlined in Specific Aim II are included in this chapter. The challenge studies evaluating the protective efficacy of the various vaccine formulations that are outlined in Specific Aim III are also contained in this chapter. Therefore, the work presented here is the entirety of Specific Aim I with additional studies involving features of both Specific Aims II and III.

4.2 ABSTRACT

Pandemic outbreaks of influenza are caused by the emergence of a pathogenic and transmissible virus to which the human population is immunologically naïve. Recent outbreaks of highly pathogenic avian influenza (HPAI) of the H5N1 subtype are of particular concern because of the high mortality rate (60% case fatality rate) and novel subtype. In order to develop a vaccine that elicits broadly reactive antibody responses against emerging H5N1 isolates, we utilized a novel antigen design technique termed computationally optimized broadly reactive antigen (COBRA). The COBRA HA sequence was based upon HA amino acid sequences from clade 2 H5N1 human infections and the expressed protein retained the ability to bind the receptor, as well as mediate particle fusion. Non-infectious recombinant VLP vaccines using the COBRA HA were purified from a mammalian expression system. Mice and

ferrets vaccinated with COBRA HA H5N1 VLPs had protective levels of HAI antibodies to a representative isolates from each subclade of clade 2. Furthermore, VLP vaccinated animals were completely protected from a lethal challenge of the clade 2.2 H5N1 virus A/Whooper Swan/Mongolia/244/2005. This is the first report describing the use of COBRA-based antigen design. The COBRA HA H5N1 VLP vaccine elicited broadly reactive antibodies and is an effective influenza vaccine against HPAI virus.

4.3 INTRODUCTION

The swine-origin H1N1 pandemic of 2009 reminded the worldwide community of the ever-present threat of pandemic influenza. Pandemic outbreaks of influenza are caused by the emergence and spread of a pathogenic and transmissible virus to which the human population is immunologically naïve [183]. Although predicting an emerging pandemic subtype of influenza is difficult, outbreaks of highly pathogenic avian influenza of the H5N1 subtype are of particular concern because of the high mortality rate (60% case fatality rate) and novel subtype [246]. To date, H5N1 influenza has not transmitted efficiently from person to person, but accumulation of mutations or reassortment with a human transmissible virus could result in a highly transmissible H5N1 virus [247]. H5N1 and contemporary H3N2 seasonal influenza viruses are able to generate stable reassortant viruses although the pathogenic potential of the reassortant viruses is less than that of the highly pathogenic H5N1 virus [248, 249]. Additionally, a recent report has demonstrated that not only can H1N1 pandemic viruses and highly pathogenic H5N1 viruses efficiently reassort, but these reassortants can replicate to higher titers than the source

H5N1 virus [250]. The genetic compatibility between the influenza viruses, combined with the continued spread of both novel H1N1 in humans and highly pathogenic H5N1 in wild birds, highlights the potential for a new emerging H5N1 influenza infecting the human population and therefore, the need to develop effective vaccines against H5N1 isolates.

One of the challenges to developing effective H5N1 vaccines is the antigenic diversity within the subtype. H5N1 viruses are separated into distinct clades based upon phylogenetic distance among the hemagglutinin (HA) genes [195]. The clades are geographically diverse and are evolving under unique pressures specific to each respective location [251]. The majority of human infections were identified within the antigenically distinct clades 1 and 2, with clade 2 infections spanning over 60 countries and moving westward from Asia into Africa and the Middle East [252]. Genetic diversity within clade 2 has resulted in distinct subclades including 2.1, 2.2, 2.3, 2.4 and 2.5 with some subclades being further divided into additional sub-subclades [195]. Despite high levels of HA protein sequence homology between clades (>90%), there is little receptor blocking antibody cross-reactivity across clades and even within subclades [195]. Developing vaccines that are able to overcome the challenge of H5N1 antigenic diversity is a crucial step in pandemic preparedness.

The antigenic diversity of all subtypes of influenza is a challenge to influenza vaccine development in general. The current seasonal influenza vaccine uses a polyvalent formulation to address the issue of multiple subtypes simultaneously circulating in the human population. Even though a representative vaccine strain is selected and is expected to represent the most common strain for each subtype in a given season, vaccine escape occurs and yearly epidemics continue to happen. A recent report has demonstrated that a polyvalent H5N1 vaccine with components derived from various clades can elicit cross-clade antibody cross-reactivity and protective efficacy [161]. Alternative strategies that have been investigated for addressing the challenge of antigenic diversity include targeting conserved viral proteins, such as the M2 ion channel or nucleoprotein (NP) and targeting conserved domains of HA [105, 179,

226, 253]. Additionally, engineering synthetic antigens that capture common immune epitopes from a population of primary viruses has the potential to overcome antigenic diversity.

Consensus-based H5N1 antigens have been generated for several influenza proteins including HA, neuraminidase (NA) and matrix (M1) and each has elicited cross-reactive immune responses [180-182, 254]. Consensus sequences are traditionally generated by aligning a population of sequences and selecting the most common residue at each position. These sequences are expected to effectively capture conserved linear epitopes and elicit cross-reactive cellular immune responses [180, 255-257]. Furthermore, consensus-based H5N1 HA immunogens expressed from DNA plasmids elicit broad antibody responses [181, 254]. However, consensus-based antigen design is inherently influenced by the input sequences used to generate the synthetic molecule and as such is subject to sampling bias. Using the NCBI Influenza Virus Resource database, the vast majority of HA sequences from both human and environmental isolates are from clades 1 and 2 [51]. Furthermore, within clade 2 the majority of human isolates are from infections in Indonesia and the subclade 2.1. The predominance of certain isolates, *i.e.* a majority of clade 2.1 isolates from Indonesia, can bias the output consensus sequence generated and may not accurately reflect the genetic diversity of H5N1 influenza viruses. In order to overcome these limitations, we report a new methodology of antigen design using multiple rounds of consensus generation termed computationally optimized broadly reactive antigen (COBRA). This method was designed to address the diversity specifically within clade 2 and utilized global surveillance efforts to generate a vaccine with the potential to elicit increased breadth of antibody responses within this antigenically diverse clade.

In this study, COBRA HA antigens are expressed on virus-like particles (VLPs) and purified as vaccine immunogens. VLPs are self-assembling, nonpathogenic, genomeless particles that are similar in size and morphology to intact virions [258]. VLPs can be produced in a variety of eukaryotic expression systems including yeast, insect and mammalian cells.

Importantly, A VLP-based vaccine which protects against human papillomavirus has been approved for human use [259]. Influenza virus VLP vaccines are attractive alternatives to traditional split vaccines because they do not require the use of any live virus at any step of the vaccine production process [222]. Additionally, VLPs present surface antigens in their native oligomeric structures which are important for maintaining conformational epitopes..

We hypothesized that the COBRA HA delivered in the context of a VLP will elicit an antibody response that demonstrates increased breadth when compared to that of a VLP containing an HA molecule derived from a primary isolate. Here, we report the COBRA HA is a functional molecule, elicits broadly reactive antibody responses, and completely protects mice and ferrets from a heterologous, highly pathogenic H5N1 virus challenge.

4.4 MATERIALS AND METHODS

4.4.1 Antigen construction and synthesis

Influenza A HA nucleotide sequences isolated from human H5N1 infections were downloaded from the NCBI Influenza Virus Resource database (see Section 2.1) [51]. Nucleotide sequences were translated into protein sequences using the standard genetic code. Full length sequences from H5N1 clade 2 human infections from 2004 to 2006 were acquired and used for subsequent consensus generations. For each round of consensus generation, multiple alignment analysis was applied and the consensus sequence was generated using AlignX (Vector NTI). The final amino acid sequence, termed computationally optimized broadly reactive antigen (COBRA), was reverse translated and optimized for expression in mammalian

cells, including codon usage and RNA optimization (GeneArt; Regensburg, Germany). This construct was then synthesized and inserted into the pTR600 expression vector [238].

4.4.2 *In vitro* expression

Human embryonic kidney (HEK) 293T cells ($1x10^6$) were transiently transfected with 3 μg DNA expressing COBRA. Cells were incubated for 72 h at 37°C, supernatants were removed, the cells were lysed with 1% Triton-X 100 and cell lysates were collected. Cell lysates were electrophoresed on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with mouse polyclonal antisera pooled from mice infected with 6:2 reassortant H5N1 viruses with the surface glycoproteins derived from either A/Vietnam/1203/2004 (clade 1) or A/Whooper Swan/244/2005 (clade 2.2) and the 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 (Pierce Biotechnology; Rockford IL, USA) and exposed to X-ray film (ThermoFisher; Pittsburgh, PA, USA).

4.4.3 Functional characterization

To determine receptor binding characteristics, virus-like particles (VLPs) containing COBRA HA proteins were purified from the supernatants of mammalian cell lines. HEK 293T cells were transiently transfected with plasmids expressing HIV Gag, COBRA HA and neuraminidase (NA, A/Thailand/1(KAN-1)/2004) and incubated for 72 h at 37°C. Supernatants were collected and VLPs were purified via ultracentrifugation (100,000 X g through 20% glycerol, weight per volume) for 4 h at 4°C. The pellets were subsequently resuspended in phosphate buffered saline PBS, pH 7.2 and stored at -80°C until use. Protein concentration was determined by

Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). COBRA HA VLPs were prepared in various amounts as measured by total protein and each individual preparation was two-fold serially diluted in v-bottom microtiter plates. An equal volume of either 1% turkey or 1% horse erythrocytes (RBC) (Lampire; Pipersville, PA, USA) in PBS was added to the diluted VLPs and incubated for 30-60 minutes at room temperature. The HA titer was determined by the reciprocal dilution of the last well which contained agglutinated RBC.

To determine endosomal fusion characteristics, COBRA-pseudotyped lentiviral vectors expressing a luciferase reporter gene were produced as described [228]. Briefly, 293T cells were co-transfected by using the following plasmids: 7μg of pCMVΔR8.2, 7μg of pHR CMV-Luc, 3μg pCMV/R N1(Kan-1) (all kindly provided by Dr. G. Nabel) and 3μg pTR600 COBRA. Cells were transiently transfected and incubated for 72h at 37°C. Supernatants were harvested, centrifuged to clear cell debris, filtered through a 0.22μm syringe filter, aliquotted, and used immediately or frozen at -80°C. For fusion assays, 100μl of pseudoviruses were added to confluent MDCK cells in 48-well plates (~30,000 cells per well). Plates were incubated at room temperature for 30 min, washed, and fresh medium added. Forty-eight hours after infection, cells were lysed in mammalian cell lysis buffer (Promega; Madison, WI, USA). A standard quantity of cell lysate was used in a luciferase assay with luciferase assay reagent (Promega; Madison, WI, USA) according to the manufacturer's protocol.

4.4.4 Vaccine preparation

HEK 293T cells were transiently transfected with plasmids expressing M1 (A/Puerto Rico/8/1934, optimized for expression in mammalian cells), NA (A/Thailand/1(KAN-1)/2004, optimized for expression in mammalian cells) and COBRA HA and incubated for 72h at 37°C. Supernatants were collected and cell debris removed by low speed centrifugation followed by

vacuum filtration through a 0.22μm sterile filter. VLPs were purified via ultracentrifugation (100,000 X *g* through 20% glycerol, weight per volume) for 4 h at 4C. The pellets were subsequently resuspended in PBS pH 7.2 and stored in single use aliquots at -80C until use. Total protein concentration was determined by Micro BCA_{TM} Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

HA specific content was determined by western blot and densitometry. Purified recombinant COBRA HA and purified VLPs were prepared in standard total protein amounts and were electrophoresed on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with mouse polyclonal antisera pooled from mice infected with 6:2 reassortant H5N1 viruses with the surface glycoproteins derived from either A/Vietnam/1203/2004 or A/Whooper Swan/244/2005 and the 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 (Pierce Biotechnology; Rockford IL, USA) and exposed to X-ray film (ThermoFisher; Pittsburgh, PA, USA). Density of bands was determined using ImageJ software (NIH) [229]. Density of recombinant HA bands were used to calculate a standard curve and the density of the purified VLPs was interpolated using the results from the recombinant HA. Experiments were performed in triplicate and multiple exposure times were analyzed for all iterations.

4.4.5 Mouse studies

BALB/c mice (*Mus musculis*, females, 6–8 weeks) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA) and housed in microisolator units and allowed free access to food and water and were cared for under USDA guidelines for laboratory animals. For dosing studies, mice (5 mice per group) were vaccinated with one of two doses of purified VLPs (1.5μg

or 0.3μg), based upon HA content from the densitometry assay, via intramuscular injection at week 0 and then boosted with the same dose at week 3. For comparison studies, mice (20 mice per group) were vaccinated with purified VLPs (3μg), based upon HA content from the densitometry assay, via intramuscular injection at week 0 and then boosted with the same dose at week 3. Vaccines at each dose were formulated with Imject® alum adjuvant (Imject® Alum, Pierce Biotechnology; Rockford, IL, USA) according to the manufacturer's protocol or vehicle alone. Fourteen to twenty-one days after each vaccination, blood was collected from anesthetized mice via the retro-orbital plexus and transferred to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at -20 ± 5°C.

Three weeks after final vaccination, mice were challenged intranasally with $5x10^3$ plaque forming units (PFU) of the highly pathogenic H5N1 virus A/Whooper Swan/Mongolia/244/2005 (Clade 2.2) in a volume of 50μl. The challenge dose represents approximately $50LD_{50}$ in mice. After infection, mice 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=reduced, 2=severely reduced), hunched back (0=absent, 1=present) and ruffled fur (0=absent, 1=present) [230]. Experimental endpoint was defined as >20% weight loss or display of neurological disease such as hind limb paralysis. All H5N1 influenza 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, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

4.4.6 Ferret studies

Fitch ferrets (*Mustela putorius furo*, female, 6-12-months of age), influenza naïve and descented, were purchased from Marshall Farms (Sayre, PA, USA). Ferrets were pair housed in stainless steel cages (Shor-line, Kansas City, KS, USA) containing Sani-chips Laboratory Animal Bedding (P.J. Murphy Forest Products, Montville, NJ, USA). Ferrets were provided with Teklad Global Ferret Diet (Harlan Teklad, Madison, WI, USA) and fresh water ad libitum. The VLPs were diluted in PBS, pH 7.2 to achieve final concentration. Ferrets (n=9) were vaccinated with 15μg of purified VLPs, based upon HA content as determined by densitometry assay, via intramuscular injection in the quadriceps muscle in a volume of 0.50ml at week 0 and then boosted with the same dose at week 3. Vaccines were stored at -80°C prior to use and formulated with Imject® alum adjuvant (Imject® Alum; Pierce Biotechnology, Rockford, IL, USA) immediately prior to use according to manufacturer's protocol. Animals were monitored for adverse events including weight loss, temperature, loss of activity, nasal discharge, sneezing and diarrhea weekly during the vaccination regimen. Prior to vaccination, animals were confirmed by HAI assay to be seronegative for circulating influenza A (H1N1 and H3N2) and influenza B viruses. Fourteen to twenty-one days after each vaccination, blood was collected from anesthetized ferrets via the anterior vena cava and transferred to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at -20 ± 5°C.

Three weeks after final vaccination, ferrets were challenged intranasally with $1x10^6$ plaque forming units (PFU) of the highly pathogenic H5N1 virus A/Whooper Swan/Mongolia/244/2005 (Clade 2.2) in a volume of 0.5ml 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 were performed by instilling 3ml of PBS into the nares of anesthetized ferrets each day for 14 days after inoculation. Washes were collected and stored at -80°C until use. Experimental endpoint was defined as >20% weight loss, development of neurological symptoms, or an activity score of 3 (not active or alert after stimulation). All H5N1 influenza 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, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

4.4.7 ELISA assay

The ELISA assay was used to assess total antibody titer and IgG isotype titer to the HA. High binding, 96-well polystyrene plates (Costar; Lowell, MA, USA) were coated overnight with 50ng/well of recombinant HA. Coating antigens were derived from the following representative viral isolates: A/Vietnam/1203/2004 (clade 1), A/Indonesia/5/2005 (clade 2.1), A/Whooper Swan/244/2005 (clade 2.2) and A/Anhui/1/2005 (clade 2.3). Plates were blocked with 5% milk diluted in PBS with 0.05% Tween 20. Serum samples were diluted in blocking buffer and added to plates. Serum was two-fold serially diluted and allowed to incubate for 1 hour at room temperature. Plates were washed and species specific antibody against IgG, IgG1, IgG2a, IgG2b or IgG3 and linked to horseradish peroxidase (HRP) (Southern Biotech; Birmingham, AL, USA) were diluted in blocking buffer and added to plates. Plates were incubated for 1 hour at room temperature. Plates were washed and HRP was developed with TMB substrate (Sigma-Aldrich; St. Louis, MO, USA). Plates were incubated in the dark for 15 minutes and then the

reaction was stopped with 2N H_2SO_4. Optical densities at a wavelength of 450nm (OD_{450}) were read by a spectrophotometer (BioTek; Winooski, VT, USA) and end point dilution titers were determined. End point titers were determined as the reciprocal dilution of the last well which had an OD_{450} above the mean OD_{450} plus two standard deviations of naïve animal sera.

4.4.8 Hemagglutination inhibition (HAI) assay

The HAI assay was used to assess functional antibodies to the HA able to inhibit agglutination of horse erythrocytes. The protocol was adapted from the CDC laboratory-based influenza surveillance manual [233]. To inactivate non-specific inhibitors, sera were treated with receptor destroying enzyme (RDE; Denka Seiken, Co., Japan) prior to being tested [234-238]. Briefly, three parts RDE was added to one part sera and incubated overnight at 37°C. RDE was inactivated by incubation at 56°C for ~30 min. RDE treated sera was two-fold serially diluted in v-bottom microtiter plates. An equal volume of reassortant virus, adjusted to approximately 8 HAU/50µl, was added to each well. The reassortant viruses contained the internal genes from the mouse adapted strain A/Puerto Rico/8/1934 and the surface proteins HA and NA from the following representative viral isolates: A/Vietnam/1203/2004 (clade 1), A/Indonesia/5/2005 (clade 2.1), A/Whooper Swan/244/2005 (clade 2.2) and A/Anhui/1/2005 (clade 2.3). The plates were covered and incubated at room temperature for 20 min followed by the addition of 1% horse erythrocytes (HRBC) (Lampire Biologicals, Pipersville, PA, USA) in PBS. Red blood cells were stored at 4°C and used within 72 h of preparation. The plates were mixed by agitation, covered, and the RBCs were allowed to settle for 1 h at room temperature [239]. The HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Positive and negative 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.

4.4.9 Plaque assay

Madin-Darby Canine Kidney (MDCK) cells were plated (5×10^5) in each well of a 6-well plate. Samples were diluted (final dilution factors of 10^0 to 10^{-6}) and overlayed onto the cells in 100µl of DMEM supplemented with penicillin-streptomycin and incubated for 1hr. Samples were removed, cells were washed twice and media was replaced with 2 ml of L15 medium plus 0.8% agarose (Cambrex; East Rutherford, NJ, USA) and incubated for 72 h at 37°C with 5% CO_2. Agarose was removed and discarded. Cells were fixed with 10% buffered formalin, and then stained with 1% crystal violet for 15 min. Following thorough washing in dH_2O to remove excess crystal violet, plates were allowed to dry, plaques counted, and the plaque forming units (PFU)/ml were calculated.

4.4.10 Statistical analysis

Statistical significance of the antibody data was determined using a two-way analysis of variance (ANOVA) with Bonferroni's post-test to analyze differences between each vaccine group for the different test antigens (multiparametric). Differences in weight loss, sickness score, and viral titers were analyzed by two-way ANOVA, followed by Bonferroni's post-test for each vaccine group at multiple time points. Significance was defined as $p < 0.05$. Statistical analyses were done using GraphPad Prism software.

4.5 RESULTS

4.5.1 Computationally optimized broadly reactive antigen design

To address the challenge of antigenic diversity present in H5N1 influenza, we designed a computationally optimized broadly reactive antigen (COBRA). For the first step of antigen generation, 129 unique hemagglutinin (HA) sequences were downloaded from the NCBI Influenza Virus Resource (IVR) sequence database [51] representing clade 2 H5N1 viruses isolated from human infections between 2004 and 2006. The sequences were first grouped into phylogenetic subclades and then further divided into individual outbreak groups within each subclade based upon geographic location and time of isolation. HA amino acid sequences for each individual outbreak group were aligned and the most common amino acid at each position was determined resulting in primary consensus sequences representing each outbreak group within each subclade (Figure 12A). Primary consensus sequences within each subclade were then aligned and the most common amino acid was chosen resulting in secondary consensus sequences representing each subclade (Figure 12A). The secondary consensus sequences were aligned and the most common amino acid at each position was selected resulting in the final consensus sequence referred to as clade 2 COBRA HA (Figure 12A). Phylogenetic analysis of the COBRA HA with all human isolates of H5N1 HA proteins indicated that COBRA retained a clade 2-like sequence without being grouped specifically within any clade 2 subclade cluster (Figure 12B). Furthermore, a BLAST search using the COBRA HA sequence revealed that it is a unique sequence that has not been isolated from the environment (data not shown).

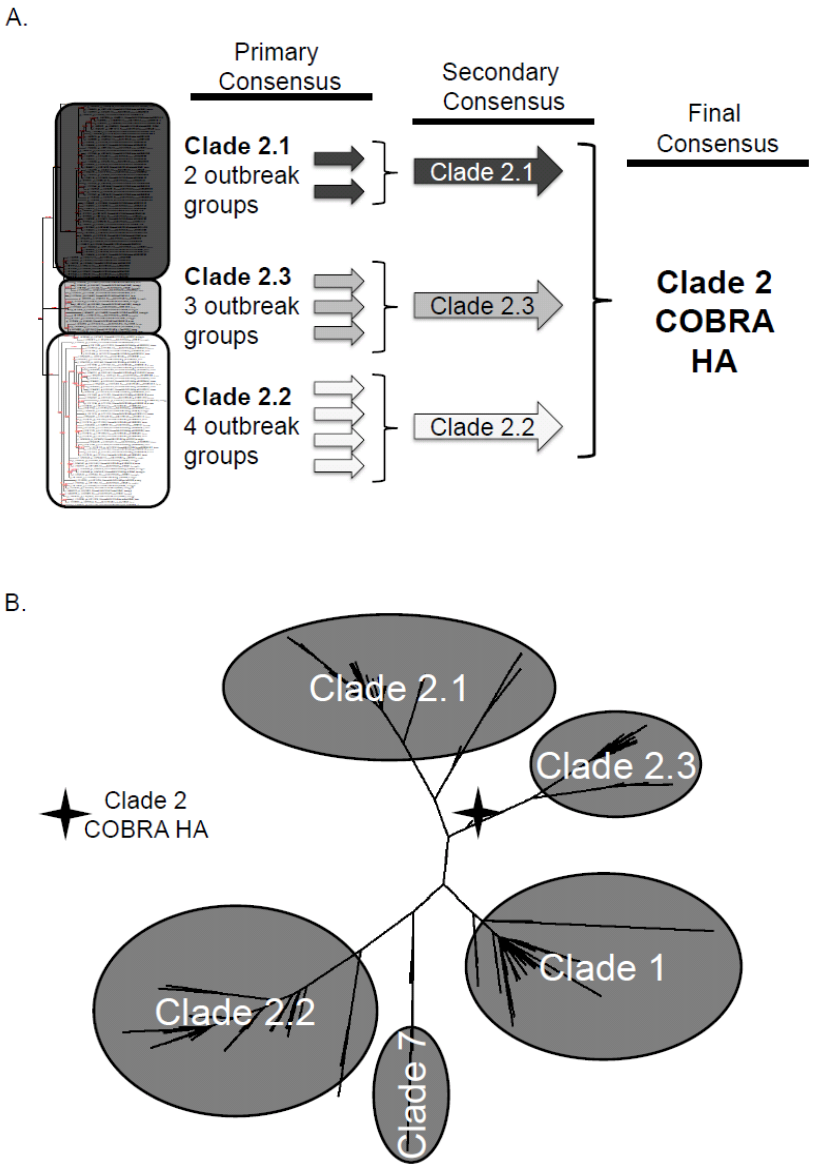

Figure 12: COBRA HA Design. Schematic to describe how the COBRA HA molecule was designed (A). The phylogenetic tree was inferred from hemagglutinin amino acid sequences using the maximum likelihood method and clade/sub-clade groupings were identified. Primary consensus sequences were generated for each outbreak group. Secondary consensus sequences were then generated for each subclade using the primary sequences as input. The secondary consensus sequences were then aligned and the resulting consensus, designated COBRA, was generated. Phylogenetic analysis of the COBRA HA (B). The unrooted phylogenetic tree was inferred from hemagglutinin amino acid sequences from human H5N1 infections isolated from 2004 to 2009 and the clade/sub-clade groupings are indicated. The star represents the COBRA HA sequence relative to human H5N1 infections.

4.5.2 Characterization of COBRA

Since COBRA is a fully synthetic protein, the retention of natural hemagglutinin function was confirmed. Initially, COBRA expression was verified by transient transfection of mammalian cells. Analysis of the total cell lysate demonstrated that the COBRA HA migrates at its predicted molecular weight of approximately 73kDa (Figure 13A). Because the poly-basic cleavage site was retained in the COBRA HA sequence, both HA0 and the HA1 subunits were detected by immunoblot at similar molecular weights as recombinant HA and the HA on the H5N1 virion (Figure 13A). Virus-like particles (VLPs) with COBRA HA on the surface bound sialic acid in a dose-dependent manner and this binding was specific to COBRA, since empty lentiviral core alone did not bind to the receptor (Figure 13B).

To determine if the COBRA HA was functional, the protein was pseudotyped onto lentiviral Gag_{p24} to generate pseudoparticles [192, 260]. COBRA HA containing pseudoparticles mediated cell fusion as efficiently as H5N1 control pseudoparticles without the requirement for trypsin. In contrast, H1N1 pseudoparticles did require trypsin and pseudoparticles without surface HA produced luciferace at similar levels as the cell only controls (Figure 13C). Taken together, these results demonstrate that although the COBRA HA is a synthetic protein not found in nature, it retains all of the functions of a natural hemagglutinin protein.

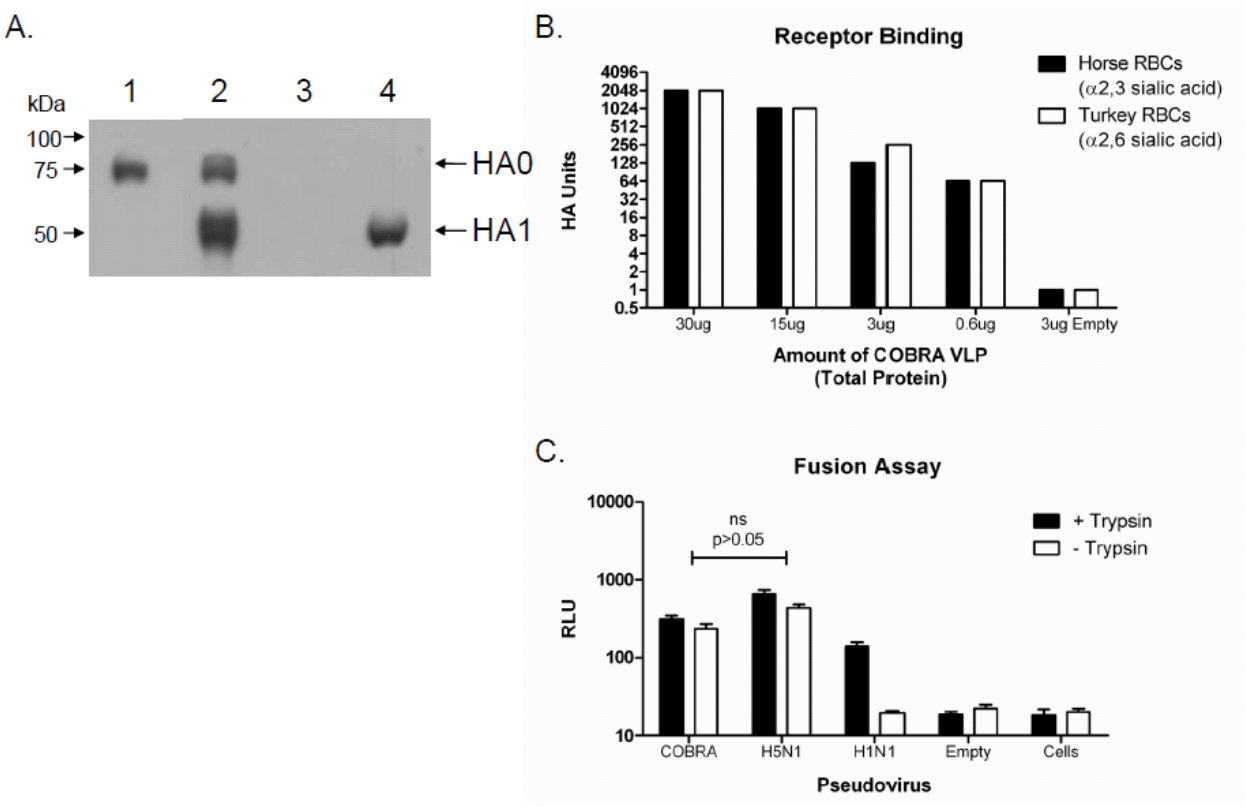

Figure 13: COBRA HA Functional Characterization. COBRA HA was translated *in vitro* and the cell culture lysates were analyzed by SDS-PAGE (A). Lane designations: 1) H5N1 recombinant HA; 2) COBRA HA; 3) Expression vector; 4) H5N1 reassortant virus. The COBRA HA (lane 2) migrates at its expected molecular weight confirming expression of the synthetic protein. COBRA HA VLPs were prepared in various amounts, serially diluted, and incubated with 1% erythrocytes to evaluate receptor binding (B). HA titer was determined as the last well in which the RBCs remained suspended in a lattice structure. COBRA HA and control lentiviral pseudoparticles packaging a CMV-Luc gene were generated in HEK 293T cells and used to infect MDCK cells with or without trypsin (C). Particle fusion was determined by luciferace production by infected cells.

4.5.3 Mouse Dosing Immunizations

Mice (BALB/c; n=5) were vaccinated (week 0 and 3) via intramuscular injection with purified COBRA VLPs at either a high dose (1.5ug HA) or low dose (0.3ug HA) with and without Imject® alum adjuvant. At week 5, all COBRA VLP-vaccinated animals had anti-HA antibodies that recognized heterologous recombinant HA derived from both clade 1 and also subclades of clade 2 (Figure 14A and 14B). Imject® alum significantly increased anti-HA antibody titers in both low and high dose groups as compared to the non-adjuvanted groups ($p<0.01$). The IgG isotype subclasses elicited by the VLP vaccines against a clade 2.1 coating antigen consisted mainly of IgG1 and IgG2a, indicating a mixed T helper response (Figure 14C and 14D). Similar results were found for additional coating antigens representing clade 1, clade 2.2 and clade 2.3 (data not shown). In addition to recognizing HA, antibodies were also evaluated for the ability to block virus from binding its receptor via inhibition of viral-induced agglutination of horse erythrocytes (HAI). All mice administered Imject® alum adjuvanted vaccines, regardless of dose, had HAI titers $\geq$1:40 to viruses expressing HA from clades 2.1 and 2.2 and 90% of the mice had titers $\geq$1:40 to a clade 2.3 representative virus (Figure 14E and 14F). Non-adjuvanted vaccines elicited generally lower HAI antibody titers with 100% of high dose animals achieving titers $\geq$1:40 only against clade 2.1 viruses. Imject® alum adjuvanted vaccines elicited significantly higher HAI antibody titers to clade 2.2 and clade 2.3 viruses regardless of dose as compared to non-adjuvanted vaccines ($p<0.05$ for high dose and $p<0.001$ for low dose, respectively). Subsequent vaccine formulations utilized a high dose and included Imject® alum as adjuvant because of the increased antibody breadth observed in these experiments. None of the vaccines elicited high HAI titer antibodies to a clade 1 virus.

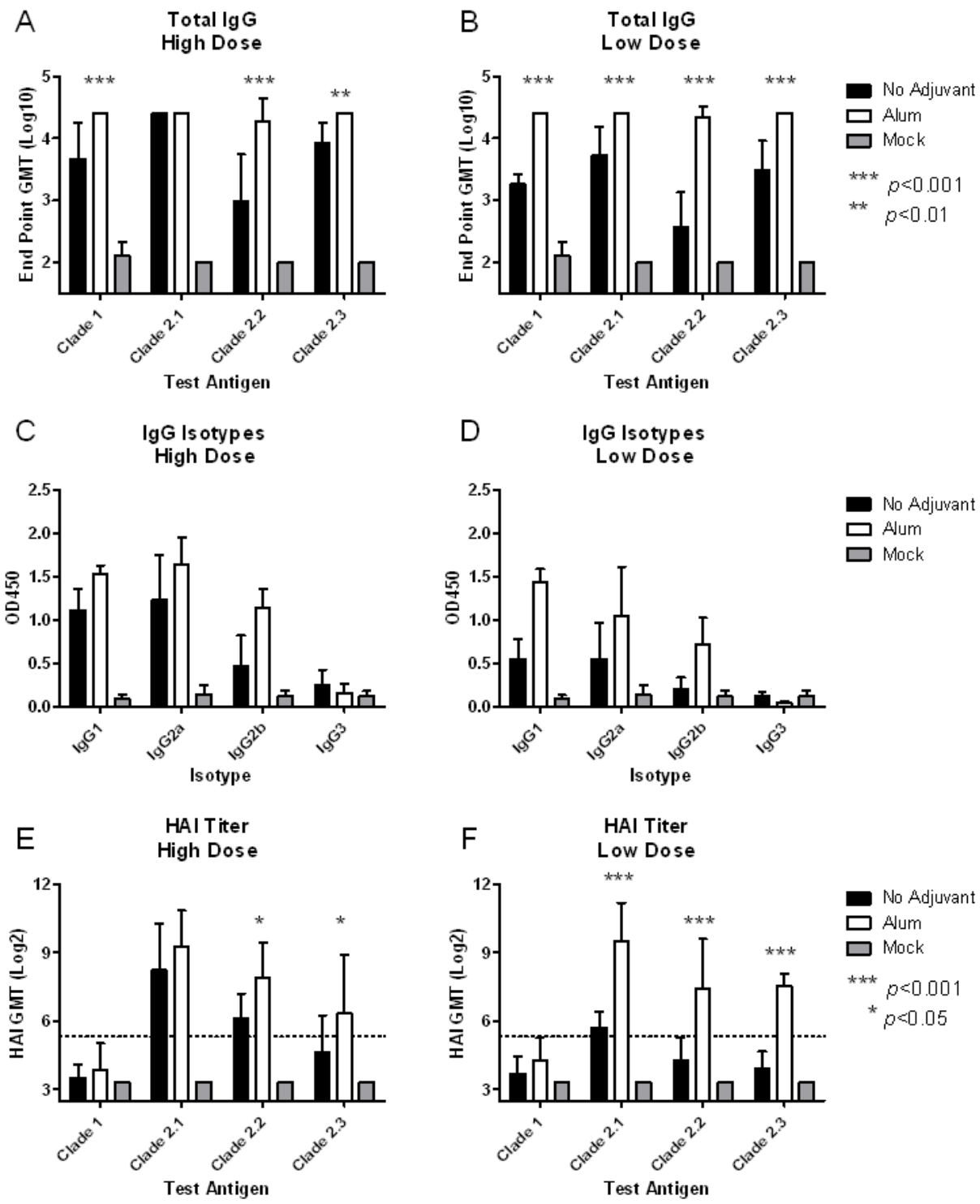
A
Total IgG
High Dose
End Point GMT (Log10)
Clade 1
Clade 2.1
Clade 2.2
Clade 2.3
Test Antigen
B
Total IgG
Low Dose
C
IgG Isotypes
High Dose
OD450
IgG1
IgG2a
IgG2b
IgG3
Isotype
D
IgG Isotypes
Low Dose
E
HAI Titer
High Dose
HAI GMT (Log2)
F
HAI Titer
Low Dose
No Adjuvant
Alum
Mock
*** p<0.001
** p<0.01
* p<0.05

Figure 14: COBRA HA Mouse Dosing Immunogenicity. BALB/c mice (n=5/group) were vaccinated at 0 and 3 weeks with blood collected at 14 to 21 days after each vaccination. Vaccines were formulated at high (1.5ug HA), and low (0.03ug HA) doses; with and without Imject® alum and delivered intramuscularly. Total IgG at week 5 was determined via ELISA for each vaccine group (A and B). Values represent the geometric mean titer (±95% confidence interval) of log_{10} transformed endpoint titers. IgG isotypes were evaluated via ELISA for each vaccine group (C and D). Values represent the mean OD_{450} of a 1:200 dilution of serum. Hemagglutination inhibition (HAI) serum antibody titer for each vaccine group was determined at week 5 using representative reassortant viruses (E and F). Values represent the geometric mean titer (±95% confidence interval) of log_2 transformed titers. The dotted line represents the 1:40 titer. Significant differences were determined by two-way ANOVA with Bonferroni's post-test to evaluate differences between the vaccine formulations for each test antigen. A *p* value of less than 0.05 was considered significant.

4.5.4 Mouse Dosing Challenge

Mice that received the COBRA VLP vaccines or mock vaccinated control mice were challenged intranasally with a lethal dose of clade 2.2 H5N1 highly pathogenic avian influenza (A/Whooper Swan/Mongolia/244/2005) to evaluate the protective efficacy of the different COBRA vaccine formulations. All COBRA vaccinated mice, regardless of dose or the presence of adjuvant, were protected from weight loss and death following lethal challenge, while all mock vaccinated animals rapidly lost weight and required euthanasia by day 6 post infection (Figure 15A and 15B). Additionally, COBRA VLP vaccinated mice had no signs of disease, while mock vaccinated animals developed such symptoms as ruffled fur, hunched back, and lethargy (Figure 15C and 15D).

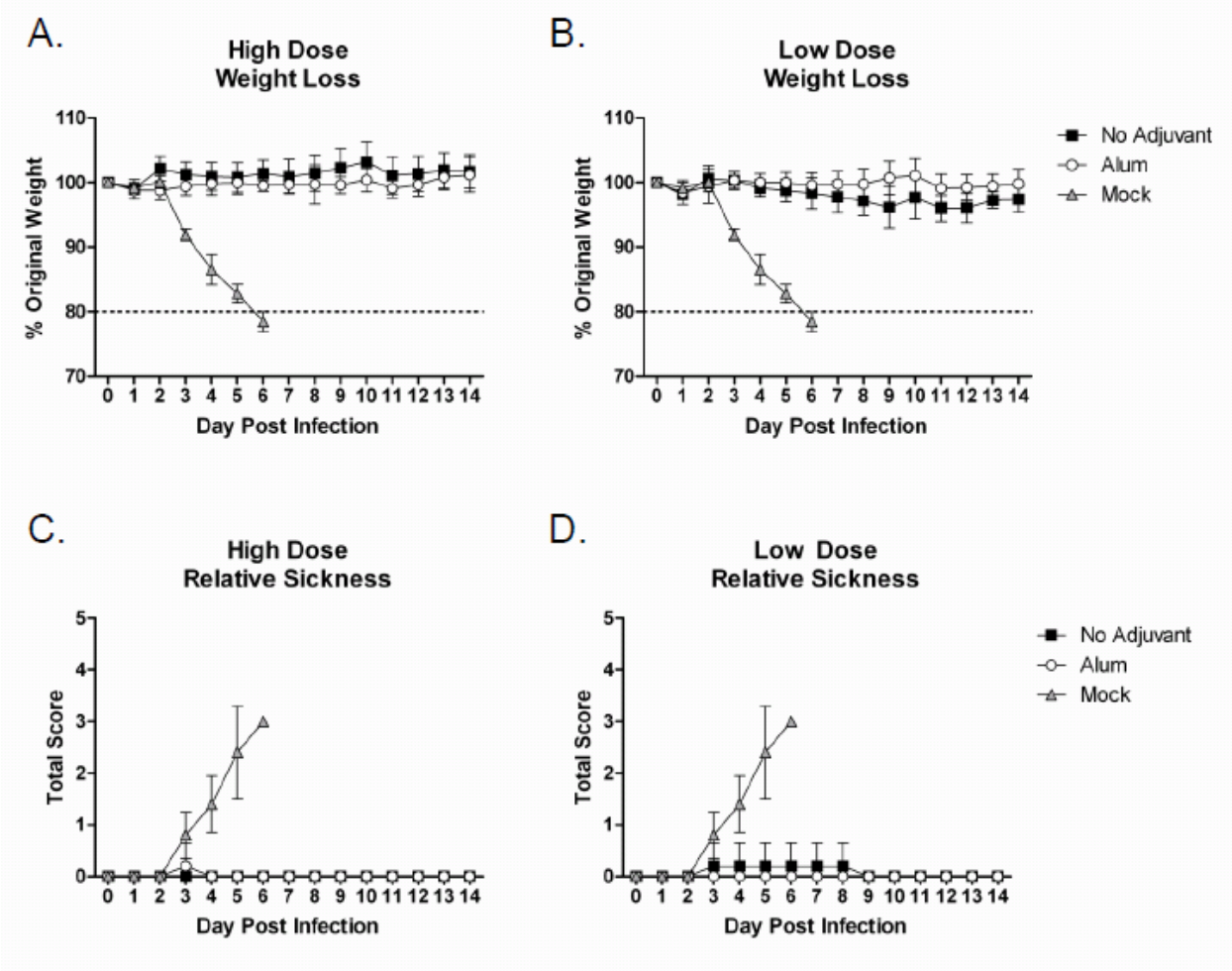

Figure 15: COBRA HA Mouse Dosing Efficacy. BALB/c mice (n=5/group) were vaccinated with COBRA HA VLPs with or without adjuvant. Mice were infected with $5x10^3$ PFU of the highly pathogenic clade 2.2 H5N1 virus A/Whooper Swan/Mongolia/244/2005. Mice were followed to monitor weight loss (A and B) and sickness (C and D). Sickness score was determined by evaluating activity (0=normal, 1=reduced, 2=severely reduced), hunched back (0=absent, 1=present) and ruffled fur (0=absent, 1=present). All mock vaccinated mice reached the experimental endpoint and required euthanasia by 6 days post infection.

4.5.5 Mouse Comparison Immunizations

To determine if the COBRA HA vaccine elicits a broader antibody response compared to a vaccine derived from a primary isolate, an additional set of mice were vaccinated with either COBRA VLPs or clade 2.2 (A/Whooper Swan/Mongolia/244/2005) VLPs. Mice (BALB/c; n=20) were vaccinated (week 0 and 3) via intramuscular injection with either COBRA VLPs or clade 2.2 VLPs at a high dose (3ug HA) with Imject® alum adjuvant. At week 5, all COBRA VLP-vaccinated mice and all clade 2.2 VLP-vaccinated mice had anti-HA antibodies that recognized heterologous recombinant HA derived from both clade 1 and various subclades of clade 2 (Figure 16A). Although no significant differences were found in total IgG titers between vaccine groups, COBRA VLP-vaccinated animals had higher HAI antibody titers against all viruses tested as compared to clade 2.2 VLP-vaccinated animals ($p<0.01$; Figure 16B). Furthermore, COBRA VLP-vaccinated animals had an increased frequency of HAI titers of $\geq$1:40 compared to clade 2.2 VLP-vaccinated animals (Table 3).

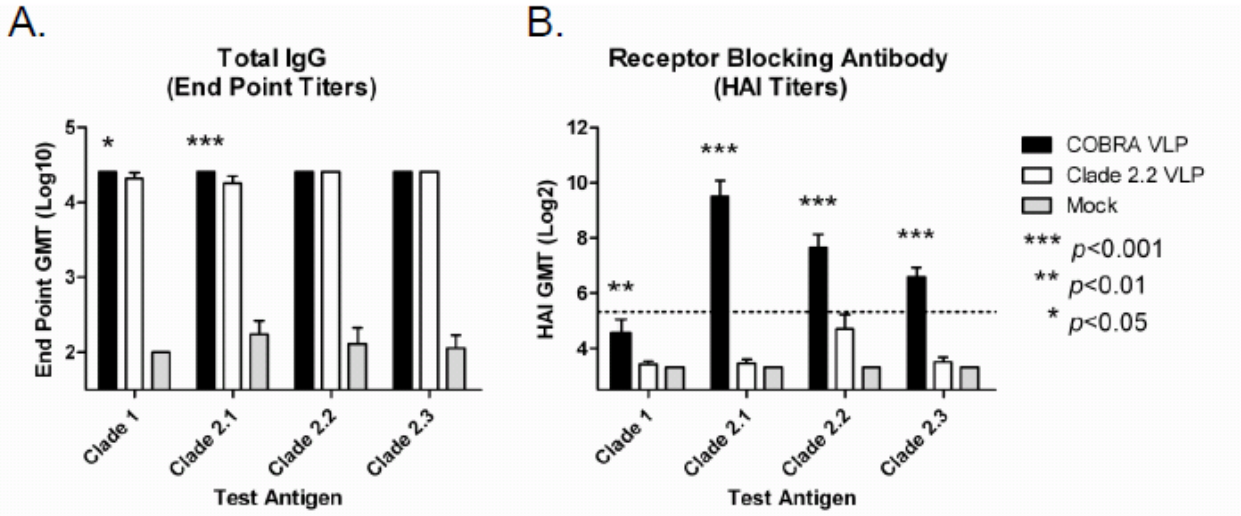

Figure 16: Mouse Comparison Immunogenicity. BALB/c mice (n=20/group) were vaccinated at 0 and 3 weeks with blood collected at 14 to 21 days after each vaccination. Vaccines were formulated at a high dose (3μg HA) with Imject® alum and delivered intramuscularly. Total IgG at week 5 was determined via ELISA for each vaccine group (A). Values represent the geometric mean titer (±95% confidence interval) of $\log_{10}$ transformed endpoint titers. Hemagglutination inhibition (HAI) serum antibody titer for each vaccine group was determined at week 5 using representative reassortant viruses (B). Values represent the geometric mean titer (±95% confidence interval) of $\log_2$ transformed titers. The dotted line represents the 1:40 titer. Significant differences were determined by two-way ANOVA with Bonferroni's post-test to evaluate differences between the vaccine formulations for each test antigen. A *p* value of less than 0.05 was considered significant.

Table 3: Mouse seroconversion frequency.

Vaccine Antigen	Clade 1[a]	Clade 2.1[b]	Clade 2.2[c]	Clade 2.3[d]
COBRA	45% (9/20)	100% (20/20)	100% (20/20)	100% (20/20)
Clade 2.2[c]	0% (0/20)	0% (0/20)	50% (10/20)	0% (0/20)

Percentage of VLP-vaccinated mice achieving an HAI titer of ≥1:40 to each test antigen

[a] A/Vietnam/1203/2004

[b] A/Indonesia/5/2005

[c] A/Whooper Swan/Mongolia/244/2005

[d] A/Anhui/1/2005

4.5.6 Mouse Comparison Challenge

Mice that received the COBRA VLP vaccine, clade 2.2 VLP vaccine or mock vaccinated control mice were challenged intranasally with a lethal dose of clade 2.2 H5N1 highly pathogenic avian influenza (A/Whooper Swan/Mongolia/244/2005) to evaluate the protective efficacy of the VLP vaccines. All VLP-vaccinated mice were protected from weight loss and death following lethal challenge while all mock vaccinated animals rapidly lost weight and required euthanasia by day 6 post infection (Figure 17A). Additionally, VLP vaccinated mice did not show signs of disease, while mock vaccinated animals developed ruffled fur, hunched back, and lethargy (Figure 17B). Even though the clade 2.2 VLP was matched to the challenge virus, no significant differences were found between COBRA VLP and clade 2.2 VLP vaccinated mice in any of the parameters analyzed indicating that the COBRA VLP vaccine protected animals as efficiently as the homologous vaccine.

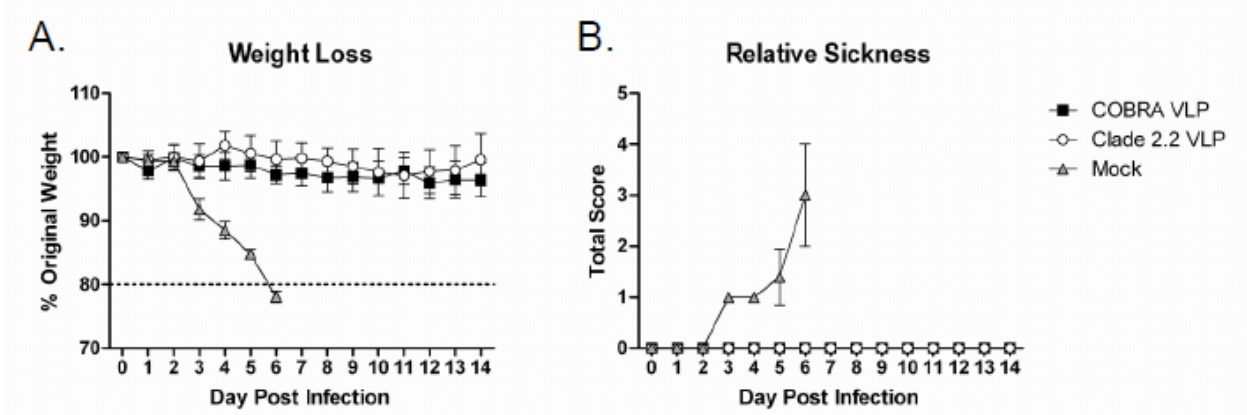

Figure 17: Mouse Comparison Efficacy. BALB/c mice (n=20/group) were vaccinated with VLPs with adjuvant. Mice were infected with $5x10^3$ PFU of the highly pathogenic clade 2.2 H5N1 virus A/Whooper Swan/Mongolia/244/2005. Mice were followed to monitor weight loss (A) and sickness (B). Sickness score was determined by evaluating activity (0=normal, 1=reduced, 2=severely reduced), hunched back (0=absent, 1=present) and ruffled fur (0=absent, 1=present). All mock (adjuvant-only) vaccinated mice reached the experimental endpoint and required euthanasia by 6 days post infection.

4.5.7 Ferret Comparison Immunizations

Ferrets are the most relevant model for influenza disease and as such the COBRA vaccine was tested in this more rigorous animal model. Ferrets (Fitch; n=9) were vaccinated (week 0 and 3) via intramuscular injection with COBRA VLPs or clade 2.2 VLPs at a high dose (15ug HA) with Imject® alum adjuvant. Serum was collected from ferrets at week 5 and antibody responses to the COBRA vaccines were evaluated. All vaccinated ferrets had anti-HA antibodies that recognized heterologous recombinant HA derived from both clade 1 and also subclades of clade 2 (Figure 18A). No significant difference in anti-HA antibody was found between the COBRA VLP vaccine and the clade 2.2 VLP vaccine for any of the antigens tested ($p>0.05$). In addition to recognizing HA, antibodies were also evaluated for HAI activity. COBRA VLP-vaccinated animals had higher HAI antibody titers against clade 2.1 and clade 2.3 viruses as compared to clade 2.2 VLP-vaccinated animals ($p<0.01$ Figure 18B). Similar to the mice, COBRA VLP-vaccinated ferrets displayed an increased rate of achieving HAI titers $\geq$1:40 when compared to clade 2.2 VLP-vaccinated ferrets (Table 4). Although both COBRA and clade 2.2 VLP vaccines elicited anti-HA binding antibody to a clade 1 virus that was of equivalent titer to that of the clade 2 viruses, neither of the vaccines elicited high HAI titer antibodies to a clade 1 reassortant virus.

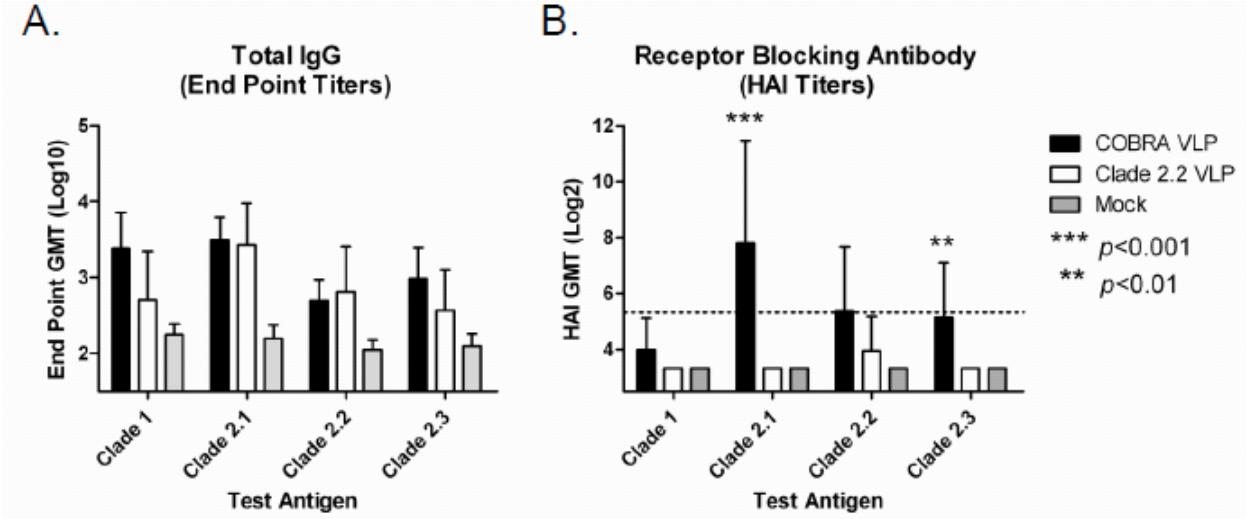

Figure 18: Ferret Immunogenicity. Ferrets (n=9/group) were vaccinated with VLPs (15ug HA) with Imject® alum at weeks 0 and 3 and serum collected at week 5. Total IgG at week 5 was determined via ELISA for each vaccine group (A). Values represent the geometric mean titer (±95% confidence interval) of $\log_{10}$ transformed endpoint titers. Hemagglutination inhibition (HAI) serum antibody titer for each vaccine group was determined at week 5 using representative reassortant viruses (B). Values represent the geometric mean titer (±95% confidence interval) of $\log_2$ transformed titers. The dotted line represents the 1:40 titer. Significant differences were determined by two-way ANOVA with Bonferroni's post-test to evaluate differences between the vaccine formulations for each test antigen. A *p* value of less than 0.05 was considered significant.

Table 4: Ferret seroconversion frequency

Vaccine Antigen	Clade 1[a]	Clade 2.1[b]	Clade 2.2[c]	Clade 2.3[d]
COBRA	0% (0/9)	78% (7/9)	56% (5/9)	56% (5/9)
Clade 2.2[c]	0% (0/9)	0% (0/9)	22% (2/9)	0% (0/9)

Percentage of VLP-vaccinated ferrets achieving an HAI titer of ≥1:40 to each test antigen

[a] A/Vietnam/1203/2004

[b] A/Indonesia/5/2005

[c] A/Whooper Swan/Mongolia/244/2005

[d] A/Anhui/1/2005

4.5.8 Ferret Comparison Challenge

Ferrets that received the COBRA VLP vaccines, clade 2.2 VLP vaccines or mock vaccinated control animals were challenged intranasally with clade 2.2 H5N1 highly pathogenic avian influenza (A/Whooper Swan/Mongolia/244/2005) to evaluate the protective efficacy of the COBRA vaccine in the ferret model of influenza infection. All VLP vaccinated ferrets were protected from weight loss and death following viral challenge, while all mock vaccinated animals rapidly lost weight and 78% (7/9) of mock vaccinated animals required euthanasia by day 7 post-infection (Figure 19A and 19B). Additionally, both COBRA VLP-vaccinated and clade 2.2-vaccinated ferrets were protected from acute fever and failed to develop significant signs of disease while mock vaccinated animals had an elevated body temperature and developed such symptoms as lethargy, diarrhea and decreased food and water intake (Figure 19C and 19D). In addition to monitoring outward signs of disease progression, nasal washes were collected for determination of viral replication in the upper respiratory tract. Ferrets vaccinated with either COBRA VLPs or clade 2.2 VLPs did not have detectable virus at any point after infection, while mock vaccinated animals had high levels of viral replication for the first five days of the infection (Figure 19E). Importantly, no significant differences were found between COBRA VLP and clade 2.2 VLP vaccinated ferrets in any of the challenge parameters analyzed confirming the findings in mice that the COBRA VLP vaccine protected animals as efficiently as the homologous vaccine.

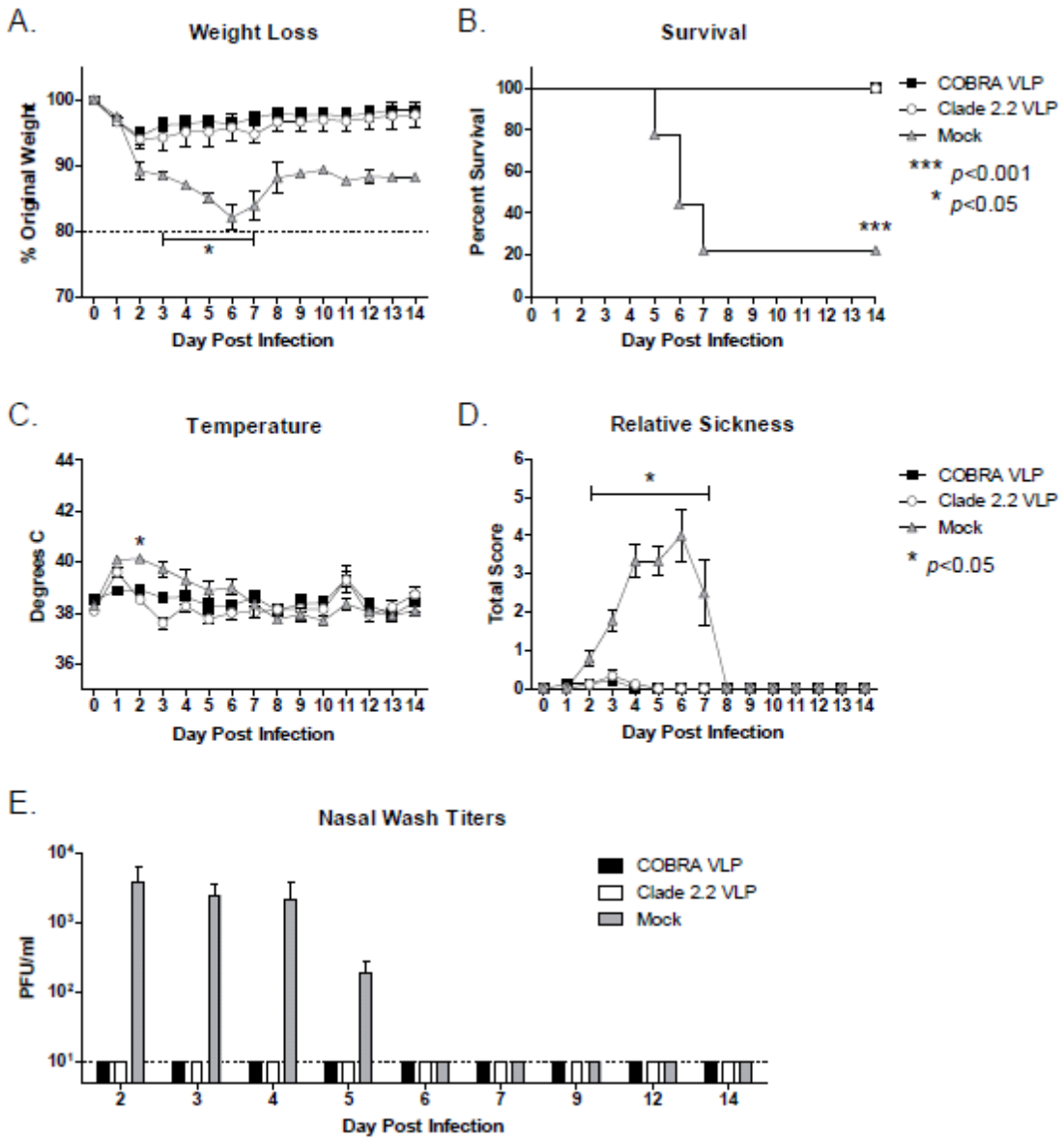

Figure 19: Ferret Efficacy. Ferrets (n=9/group) were vaccinated with VLPs formulated with adjuvant. Ferrets were challenged with $1x10^6$ PFU of the highly pathogenic clade 2.2 H5N1 virus A/Whooper Swan/Mongolia/244/2005. Animals were monitored daily for weight loss (A), survival (B), temperature (C) and clinical symptoms (D). Relative sickness scores were determined by measuring lethargy (0-3), runny nose (0-1), sneezing (0-1), loss of appetite (0-1) and diarrhea (0-1). Animals reaching experimental endpoint were euthanized according to institutional guidelines. Nasal washes were collected serially post infection and virus titers determined via plaque assay (E). Statistical significance was determined using a two-way ANOVA with Bonferroni's post test. A *p* value of less than 0.05 was considered significant.

4.6 DISCUSSION

In this study, we investigated the efficacy of a computationally optimized broadly reactive antigen (COBRA) approach designed for influenza HA as a H5N1 vaccine candidate. Centralized vaccines are a potential strategy for inducing broadly reactive immune responses and are comprised of synthetic antigens that represent a population of sequences. These vaccine antigens are generated by three different methods: center-of-the tree, ancestral, and consensus [169]. Center-of-the tree (COT) sequences are derived by constructing a phylogenetic tree and selecting the sequence that is equidistant from all points [170]. Ancestral sequences represent the most recent common ancestor for the population of sequences selected [171]. Consensus sequences encode the most common amino acid found at each position for the selected population [173]. Ancestral and COT sequences are expected to be an estimate of an actual sequence that existed in the past while consensus sequences are expected to be more relevant to the current epidemic [173]. A recent report has demonstrated the efficacy of using ancestral HA and NA sequences to generate infectious virus capable of eliciting broadly-reactive antibodies post infection in ferrets [172]. Consensus-based vaccines have been previously investigated as strategies for eliciting broadly reactive immune responses for multiple pathogens including HIV-1 [174-178] and influenza [179-182]. Although the COBRA HA investigated in this report utilized a consensus-based approach, important design features differentiate COBRA from prior strategies. COBRA represents a novel method of sequence construction that features a layered building approach that is intended to capture the most common antigenic characteristics, while avoiding the complication of differential sequence availability that can bias a consensus sequence to the most prevalent antigenic cluster. Avoiding this sampling bias is essential to generating a centralized vaccine that accurately represents the population of input sequences. COBRA HA phylogenetic analysis in the context of H5N1 human infections demonstrates that using a layered consensus building approach can

indeed overcome the challenges of sequence availability and results in a sequence that retains similarity to the input without localizing into any one antigenic cluster (Figure 12B).

Previously, consensus HA, NA and Matrix sequences developed for H5N1 DNA vaccination elicited robust cellular immune responses to each vaccine component, as well as antibodies to the HA [180-182]. This consensus HA consisted of 16 input HA sequences, primarily clade 1 viruses and derived from fatal human infections [180]. For this study, the COBRA antigen utilized over 100 input sequences from human clade 2 infections, which represents the more rapidly emerging clade of H5N1 influenza. Importantly, the work presented here confirms the earlier reports of the efficacy of a centralized H5N1 vaccine and extends those findings using DNA based vaccinations to a virus-like particle (VLP) platform. With any whole protein derived from a centralized sequence, proper folding and retention of function cannot be assumed. Although COBRA HA is a synthetic sequence, we demonstrated that the COBRA HA retained the binding and fusion functions of wild-type HA proteins (Figure 13).

Influenza vaccines delivered as VLPs represent a promising new generation of cell-based candidate vaccines [221, 222, 261]. The high virulence of H5N1 makes growing viral stocks for traditional inactivation and generation of split vaccines both difficult and dangerous [186]. VLPs are self assembling, nonpathogenic, genomeless particles that are similar in size and morphology to intact virions [220, 258]. The influenza VLPs described in these studies include the viral proteins HA, NA and matrix and are flexible enough to incorporate a synthetic HA without a decrease in stability or function. The COBRA HA vaccine was evaluated for immunogenicity in mice with and without adjuvant at multiple doses (Figure 14). The COBRA vaccine elicited anti-HA total IgG regardless of dose or adjuvant with an isotype profile indicative of a mixed T helper response. IgG1 and IgG2a have been shown to have non-redundant actions in influenza immunity with IgG1 associated with virus neutralization and IgG2a associated with viral clearance [262]. Inclusion of Imject® alum as an adjuvant did not impact

the total IgG titers or isotype profile but resulted in increased HAI antibody responses at all doses tested. Aluminum salts have been used to enhance antibody responses to vaccines for almost 100 years [155]. Imject® alum was selected for use in these studies because it is an aluminum salt-based adjuvant and its commercial analogs are the most widely used adjuvants that have been used in Diphteria, Tetanus, Pertussis and Hepatitis A and B vaccines [263]. Aluminum salts enhance antigen uptake by antigen presenting cells and stimulate a pro-inflammatory response via activation of the NOD-like receptor protein 3 inflammasome pathway [156]. Inclusion of an adjuvant increased the breadth and intensity of HAI antibody responses in mice vaccinated with the COBRA HA compared to non-adjuvanted vaccine recipients. Additionally, the relative affinity of the anti-HA antibodies was increased in the adjuvanted groups compared to the non-adjuvanted groups (data not shown). Importantly, the increase in breadth of the HAI antibody response was not a non-specific effect of the adjuvant. An adjuvanted vaccine based on the HA of the clade 2.2 virus A/Whooper Swan/Mongolia/244/2005 failed to elicit significant HAI antibody titers to heterologous test antigens (Figures 16 and 18).

One of the challenges in developing a pre-pandemic vaccine for H5N1 is the diversity within the subtype. An important lesson from the novel H1N1 pandemic of 2009 is that predicting the origin of a pandemic virus is currently not impossible. If the next influenza pandemic is caused by H5N1, the clade or subclade of the pandemic virus may not be identified until the outbreak is well underway. An effective pre-pandemic vaccine should be immunogenic and broadly reactive so as to give the best chance of preventing disease caused by drift variants. Identifying vaccine candidate strains based on HA sequence may not effectively select an isolate that will elicit a broad antibody response. The COBRA HA sequence is similar to the clade 2.2 HA used as a vaccine in these studies (95% identity) and the two different HA molecules have similar identity profiles to the other test antigens investigated here (Table 5).

Despite the high levels of identity between immunogens, structural modeling predicted the COBRA sequence to retain the most common structural features found in representative sequences while the clade 2.2 HA was divergent in potentially antigenic regions surrounding the receptor binding domain (data not shown). Consistent with these predictions, the COBRA based H5N1 vaccine elicited a broader antibody response than the clade 2.2 primary isolate based H5N1 vaccine. Although the breadth of this immunity was limited to clade 2, the antigen was designed specifically to address the wide diversity specifically within clade 2. Further work and antigen design is currently underway to include input sequences from more diverse clades to address the cross-clade diversity of H5N1. In addition to the broadened antibody responses, the COBRA vaccine protected experimentally infected animals as efficiently as a homologous vaccine.

Table 5: Percent Identity of Test Antigens.

Vaccine Antigen	Clade 1[a]	Clade 2.1[b]	Clade 2.2[c]	Clade 2.3[d]
COBRA	97	97	95	97
Clade 2.2[c]	94	97	*100*	94

HA amino acid sequences were aligned and percent identity across the entire protein was determined for the vaccine immunogens compared to the representative test antigens.

[a] A/Vietnam/1203/2004

[b] A/Indonesia/5/2005

[c] A/Whooper Swan/Mongolia/244/2005

[d] A/Anhui/1/2005

Although the HAI assay has historically been the most commonly used serological assay for monitoring influenza immunity, no correlation between HAI titer and protective efficacy against H5N1 infection has been reported [200, 201, 206, 214, 215, 217]. For seasonal influenza, an HAI titer $\geq$1:40 against a seasonal virus protects ~50% of the vaccinated

population [264]. Despite the lack of correlation of protection for H5N1 infection, the 1:40 HAI titer remains the standard for evaluating vaccine immunogenicity. Using the 1:40 titer as an indicator of positive responses, the adjuvanted COBRA vaccines consistently elicited a high rate of seroconversion to diverse clade 2 viruses (Table 3 and 4). Consistent with prior findings, even in the absence of positive HAI titers, vaccinated animals were protected from experimental infection (Figure 15, 17 and 19). Protection could be due to non-neutralizing anti-HA antibodies. All COBRA HA immunized animals had total IgG titers to a diverse number of H5N1 HA antigens. Additionally, both anti-NA antibodies and cellular responses elicited by these vaccines against influenza proteins could contribute to protection against H5N1 infection [223, 224, 265, 266]. The COBRA HA sequence does not contain unique epitopes, as predicted by immune epitope software (www.immuneepitope.org), compared to representative primary clade 2 isolates [267]. However, VLPs elicit cellular immune responses [222] and while these responses are predicted to be equivalent between the COBRA and primary isolate vaccine groups, the protective capacity of the cell-mediated immune responses cannot be ruled out.

This is the first report of a consensus-based H5N1 vaccine delivered in the context of a virus-like particle. The COBRA HA represents a new generation of centralized vaccine design and elicited a broadly reactive antibody response that protected experimentally infected animals from a heterologous H5N1 challenge. Increasing the breadth of antibody responses is a challenge not only for H5N1, but all influenza viruses. Utilizing COBRA-based vaccine design may enable the development of influenza vaccines more resistant to antigenic drift variants and warrants further evaluation.

4.7 ACKNOWLEDGMENTS

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5.0

A COMPUTATIONALLY-OPTIMIZED HEMAGGLUTININ VLP VACCINE ELICITS BROADLY-REACTIVE ANTIBODIES THAT PROTECT MONKEYS FROM H5N1 INFECTION.

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5.1 FOREWORD

The work presented in this chapter addresses features of Specific Aims II and III (see Sections 3.4 and 3.5). Specifically, the non-human primate immunogenicity studies described in Specific Aim II are detailed in this chapter. This work includes consensus and clade 2.2 vaccine comparisons and features the more detailed analysis of antibody breadth within and outside of clade 2. Additionally, non-human primate efficacy studies outlined in Specific Aim III are described in this chapter. Therefore, the work presented here describes studies encompassed by both Specific Aims II and III.

5.2 ABSTRACT

Highly pathogenic H5N1 avian influenza viruses continue to spread via waterfowl across Asia, Europe, and Africa causing lethal infections in humans. Vaccines can prevent the morbidity and mortality associated with pandemic influenza isolates, but predicting the specific isolate that may emerge from one of the 10 H5N1 clades is a tremendous challenge for vaccine design. In this study, we generated a synthetic hemagglutinin (HA) molecule based upon a new methodology, computationally optimized broadly reactive antigen (COBRA), which utilizes worldwide sequencing and surveillance efforts and is specifically focused on sequences from H5N1 clade 2 human isolates. Cynomolgus macaques vaccinated with COBRA clade 2 HA H5N1 VLPs had hemagglutination-inhibition antibody titers that recognized a broader number of representative isolates from each subclade of clade 2, as well as other divergent clades, compared to monkeys vaccinated with a clade 2.2 HA VLP based upon A/Whooper Swan/Mongolia/244/2005 (WS/05). Furthermore, all vaccinated animals were protected from

WS/05 virus challenge with no virus detected in the nasal or tracheal washes, however, COBRA VLP vaccinated monkeys had greatly reduced lung inflammation and gross pathology compared to WS/05 VLP vaccines. These results demonstrate that the COBRA clade 2 HA H5N1 VLP elicits broad humoral immunity against multiple H5N1 isolates from different clades, which was more effective against a HPAI virus challenge than a homologous vaccine.

5.3 INTRODUCTION

Despite the attention devoted to the 2009 outbreak, highly pathogenic avian influenza virus still represents a considerable threat to global public health. Since the first identified cases of H5N1 influenza in Hong Kong [268], the World Health Organization (WHO) influenza surveillance network increased its intensive monitoring of both human and avian populations for influenza infections [269]. Following the resurgence of H5N1 influenza in 2004 and subsequent spread across Asia, the Middle East, Africa, and Europe, there has been an intense need for vaccine strategies that elicit broadly reactive immunity against H5N1 isolates in light of its pandemic potential. Currently, efficient human to human transmission of H5N1 is limited, however, reassortment with a human seasonal influenza strain or adaptation to human cells could easily result in a virus that can transmit efficiently from human to human. Vaccination is a potent and cost-effective counter-measure to the threat of seasonal or pandemic outbreaks of influenza [233]. However, there are several challenges for designing an effective vaccine against H5N1 influenza, including the phylogenetic characterization and sequence homology in HA that groups H5N1 viruses into 10 clades as defined by the WHO/OIE/FAO H5N1 Evolution Working Group [270] with additional subclades and sub-subclades having been identified [271] (Figure 20). Human infections have been limited to clades 0, 1, 2 and 7. Clade 2 has several

subclades that continue to circulate and evolve independently [269]. Antibodies that inhibit virus receptor binding or neutralize infection are often clade-specific and do not always recognize isolates within even subclades of the same clade [269]. The clades are geographically diverse and are evolving under unique pressures specific to each respective location. The viruses isolated during the 1997 outbreak in Hong Kong represent clade 0. Clade 1 viruses were isolated between 2003–2005 in Vietnam and Thailand, and clade 2.1 viruses are attributed to human outbreaks in Indonesia. Viruses in clade 2.3 and clade 7 are predominantly isolated in southern China [272]. In this study, we chose to focus on a clade 2.2 virus, A/Whooper Swan/Mongolia/244/2005, as a challenge virus to analyze the protective responses to H5N1 challenge because the clade 2.2 viruses are the most geographically widespread of the H5N1 isolates having spread from Qinghai Lake in China throughout Asia, Europe, the Middle East, and Africa [273-276].

For vaccine manufacturers, it is unclear which clade(s) or subclade(s) of H5N1 may eventually emerge and cause pandemic, therefore designing an effective H5N1 vaccine in advance of an outbreak is challenging. To address the sequence diversity inherent in H5N1 isolates circulating in avian populations, consensus strategies have been designed for vaccine development [180, 277]. Our research group has expanded upon the consensus strategy and developed computationally-optimized broadly reactive antigen (COBRA) strategies to overcome pathogen sequence diversity. Traditional consensus sequences are generated by aligning a population of sequences and selecting the most common residue at each position. These sequences are expected to effectively capture conserved epitopes and elicit cross-reactive immune responses [180, 255-257]. The COBRA methodology of antigen design uses multiple rounds of consensus generation to address antigenic diversity, specifically within clade 2 H5N1 hemagglutinin (HA) sequences.

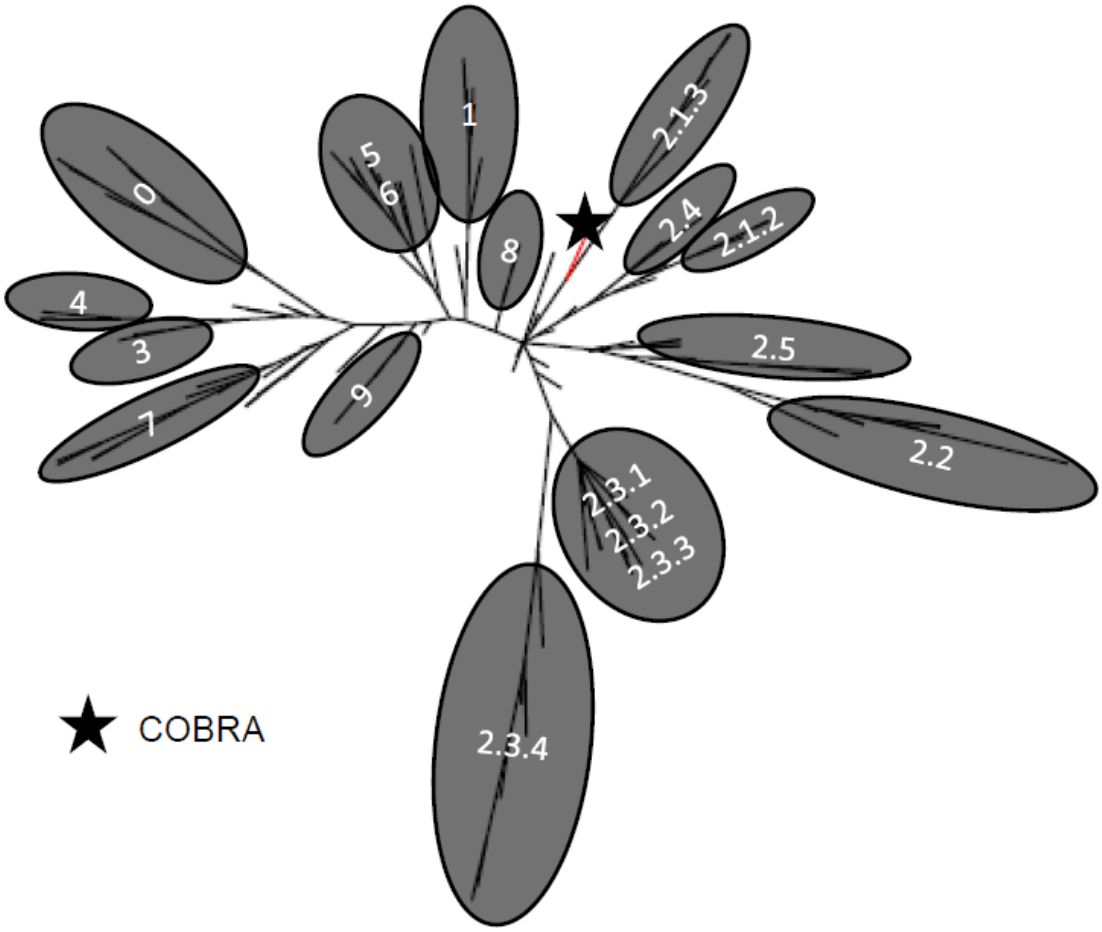

Figure 20: Phylogenetic diversity of H5N1 influenza. The unrooted phylogenetic tree was inferred from HA amino acid sequences derived from 8 to 10 representative isolates in all clades and subclades and also the COBRA HA using the maximum likelihood method. Clade/subclade clusters were identified and are indicated in the shaded ovals. The star identifies where the COBRA antigen is located relative to the various representative isolates. Sequences were aligned with MUSCLE 3.7 software and the alignment was refined by Gblocks 0.91b software. Phylogeny was determined using the maximum likelihood method with PhyML software. Trees were rendered using TreeDyn 198.3 software [278]. The NCBI accession numbers for the HA sequences used in phylogeny inference were obtained through the Influenza Virus Resource [51].

Candidate H5N1 vaccines have been previously tested in mice and ferret models. While these models are effective for seasonal influenza vaccine studies [206, 221, 237, 279-281], they

are not as informative for determining protective efficacy by H5N1 vaccines. There is often a lack of correlation between HAI activity and protection against H5N1 challenge [200, 201, 206, 221, 222]. Unvaccinated mice or ferrets that are challenged with H5N1 influenza viruses quickly succumb to disease, however, when these animals are vaccinated with an H5N1 hemagglutinin-bearing HA, they are protected against challenge that does not always correlate with HAI titers. Therefore, we chose to use a non-human primate model to determine the breadth of HAI activity and the ability to protect against H5N1 infection and pathology. Monkey models have been effectively used for H5N1 (clades 0 and 1) [282], as well as the reconstructed 1918 H1N1 influenza virus [132], since they have similar pathology to humans when infected with these highly pathogenic influenza viruses. However, there is currently no published non-human primate model based upon an avian clade 2 H5N1 isolate demonstrating the pathology or immune responses induced following infection of vaccinated or unvaccinated macaques.

In this report, COBRA HA proteins were displayed on the surface of a virus-like particle (VLP) and used to vaccinate cynomolgus macaques. The elicited immune responses were directly compared to a VLP with the HA from a clade 2.2 isolate, A/Whooper swan/Mongolia/244/2005 (WS/05). The COBRA HA VLP not only elicited broader antibody responses against H5N1, but also protected monkeys from clade 2.2 H5N1 challenge more efficiently than the homologous vaccine.

5.4 MATERIAL AND METHODS

5.4.1 Expression and purification of virus-like particles

The COBRA HA sequence has been described [283]. Briefly, the sequence was generated by aligning amino acid sequences of all available HA proteins isolated from clade 2 H5N1 human infections (2004-2007) and deposited in the Influenza Virus Resource (IVR) database available through NCBI. 293T cells were transiently transfected with plasmids expressing HA, M1, and NA using Lipofectamine 2000 in low serum media, incubated for 72h at 37^0C, and purified by ultracentrifugation through a 20% glycerol cushion as previously described [283]. All VLP vaccines were engineered using the same NA from A/Thailand/1(KAN-1)/2004. HA content was quantified as previously described [283]. Two different VLP preparations were purified, each containing one of the HA influenza gene products: WS/05 or the COBRA HA.

5.4.2 Primate immunizations and H5N1 challenges

Cynomolgus macaques (*Macaca fascicularis,* male, 3-5 years old) were vaccinated with 15 μg (based upon HA content) of purified COBRA HA VLPs (n=7), WS/05 VLPs (n=7) or vehicle alone (n=6), via intramuscular injection at weeks 0, 3 and 6. Vaccines were prepared in PBS and formulated with alum adjuvant (Imject Alum, Pierce Biotechnology; Rockford, IL, USA) immediately prior to use. Twenty-one days after each vaccination, blood was collected from anesthetized macaques via the femoral vein and transferred to a serum separator tube. After clotting, tubes were centrifuged and sera removed and frozen at -80 ± 5°C.

Three weeks after final vaccination, macaques were placed into BSL3+ isolator units (Bioqual, Inc., Rockville, MD) and then challenged by a multi-route of infection (ocular, nasal, tracheal) as previously described [132, 231, 232, 283] using $1x10^6$ plaque forming units (PFU)

of the highly pathogenic H5N1 virus, A/Whooper Swan/Mongolia/244/2005 (clade 2.2), in a volume of 1 ml at each location for a total infection dose of $1x10^6$ PFU in a total volume of 3 ml. Macaques were monitored daily for weight loss, signs of disease, and mortality until 7 days after infection. Individual body weights, sickness scores (based upon lethargy, temperature change, nasal discharge, lack of appetite, dehydration, lack of responsiveness), and death were recorded for each group.

Nasal and tracheal washes were performed at day 0, 1, 3, 5, and 7 post-infection. In addition, subsets of monkeys were sacrificed following administration of anesthesia and necropsies were performed according to standard procedures. These time points for euthanasia were chosen because it allowed the assessment of gross pathologic and histopathologic changes, as well as the extent of virus replication.

5.4.3 Serological assays

A quantitative ELISA was performed to assess anti-HA specific IgG in immune serum as previously described [222, 283]. The hemagglutination inhibition (HAI) assay was used to assess functional antibodies to the HA able to inhibit agglutination of horse red blood cells [239]. The protocol was adapted from the CDC laboratory-based influenza surveillance manual and performed as previously described [222, 233]. To inactivate non-specific inhibitors, sera were treated with receptor destroying enzyme (RDE; Denka Seiken, Co., Japan) prior to being tested [234-238]. The HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Positive and negative serum controls were included for each plate. All monkeys were negative (HAI ≤1:10) for pre-existing antibodies to currently circulating human influenza viruses prior to vaccination.

Serum neutralizing antibody titers were determined by microneutralization (mVN) assays performed on Madin Darby Canine Kidney (MDCK) cells following the procedure [240]. Briefly,

individual heat inactivated serum samples were serially diluted 2-fold (starting at a 1:10 dilution) in MDCK diluent buffer in a cell culture plate followed by the addition of a pre-determined amount (100 $TCID_{50}$) of each virus. Sera and viruses were mixed and incubated at 37°C for 2 hours. After the incubation the virus antibody mixtures were added to plate containing MDCK cells and were incubated for 2 hours at 37°C in a 5% CO_2 cell culture incubator. The virus antibody was then removed and cells were incubated until CPE was observed. Cells were then fixed in 10% formalin and stained with 1% crystal violet to quantify CPE. The neutralizing antibody titers are expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 $TCID_{50}$ of virus in MDCK cells. Positive serum control and negative cell controls with no serum were included on each plate. Geometric mean neutralizing antibody titers were calculated for each group.

5.4.4 Histopathologic evaluation and immunohistochemical analysis.

Formalin-inflated lungs and trachea were fixed in 10% neutral buffered formalin. Cross-sections of upper and lower left and right lung lobes and trachea were made, concentrating on gross-lesions. Tissue was paraffin embedded and 6-μm sections were stained with hematoxylin and eosin for histologic evaluation. Sequential sections were processed for immunohistochemistry or *in situ* hybridization (ISH). Immunohistochemistry was performed as described before [284] using an immunoperoxidase method with a polyclonal antibody (Maine Biotechnology Services, Portland, ME) directed against influenza A virus. A biotinylated donkey anti–goat IgG (Rockland Immunochemicals, Gilbertsville, PA) was used as the secondary antibody. ISH was performed as described previously [285] using a ^{35}S-labeled riboprobe synthesized using templates derived from 760 bp of influenza A/California/04/2009 (H1N1) matrix protein.

5.4.5 Statistical analysis

Statistical significance of antibody data was determined using a two-way analysis of variance (ANOVA) with Bonferroni's post-test to analyze differences between each vaccine group for the different test antigens (multiparametric). To determine antibody breadth to more divergent test antigens, analysis was done with log2 transformed titers. Significance was defined as $p<0.05$. All statistical analysis were performed using GraphPad Prism software.

5.5 RESULTS

5.5.1 Vaccine induced antibody responses

To address the challenge of antigenic diversity present in H5N1 influenza, we designed a computationally optimized broadly reactive antigen (COBRA) for hemagglutinin. This HA molecule was engineered using 129 unique hemagglutinin sequences representing clade 2 H5N1 viruses isolated from human infections as previously described [283]. The fully functional COBRA HA was pseudotyped, along with neuraminidase, onto influenza M1 to generate pseudoparticles (VLPs) to be used as vaccine immunogens.

Cynomolgus macaques (3-5 years of age; n=7) were vaccinated intramuscularly with 15μg purified COBRA VLPs (dose based upon HA content) or WS/05 VLPs formulated with Imject® alum at 0, 3 and 6 weeks. At week 3 post-vaccination, all COBRA VLP-vaccinated animals had anti-HA antibodies that recognized recombinant HA derived from three subclades of clade 2, which were boosted at week 6 (Figure 21). There was no statistical difference in the anti-HA titers elicited against any of the HA proteins, except monkeys vaccinated with COBRA

VLPs had a statistically higher titer against the Indo/05 HA (clade 2.1) compared with monkeys vaccinated with the WS/05 VLP (derived from clade 2.2) on week 6.

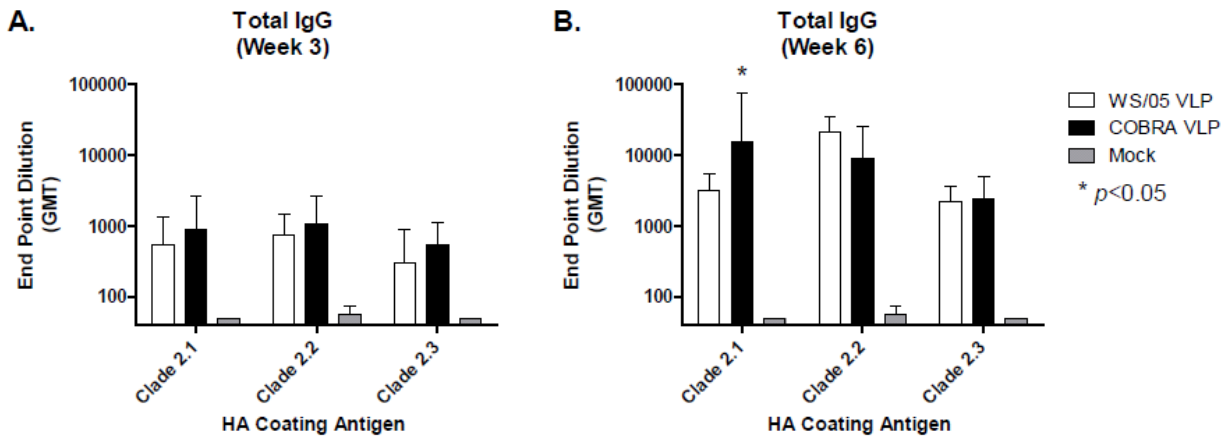

Figure 21: Anti-HA IgG ELISA antibodies. Total IgG at week 3 (A) and week 6 (B) was determined via ELISA for each vaccine group. Each collected antiserum was assayed for antibody binding to representative HA molecules from clade 2.1 (A/Indonesia/5/2005), clade 2.2 (A/Whooper Swan/Mongolia/244/2005), and clade 2.3 (A/Anhui/1/2005). Values represent the geometric mean titer (±95% confidence interval) of endpoint titers.

5.5.2 A single COBRA VLP vaccination induced high titer HAI antibodies to clade 2 H5N1 viruses.

Monkeys vaccinated with COBRA VLPs (but not with WS/05 VLPs) had hemagglutination-inhibiting antibodies (HAI) against viruses representing all three clade 2 subclades after a single vaccination (Figure 22A). Four to six monkeys responded to the COBRA VLP vaccine with an HAI titer ≥1:40 for the all of the various test antigens. In contrast, 4 of 7 monkeys vaccinated with the WS/05 VLP responded to the homologous clade 2.2 virus, but none of these vaccinated monkeys responded to the clade 2.1 or 2.3 virus. Following a second vaccination, almost all the

monkeys vaccinated with either vaccine responded to all three viruses (Figure 22B). These results were confirmed by microneutralization assay (Figure 22C and 22D).

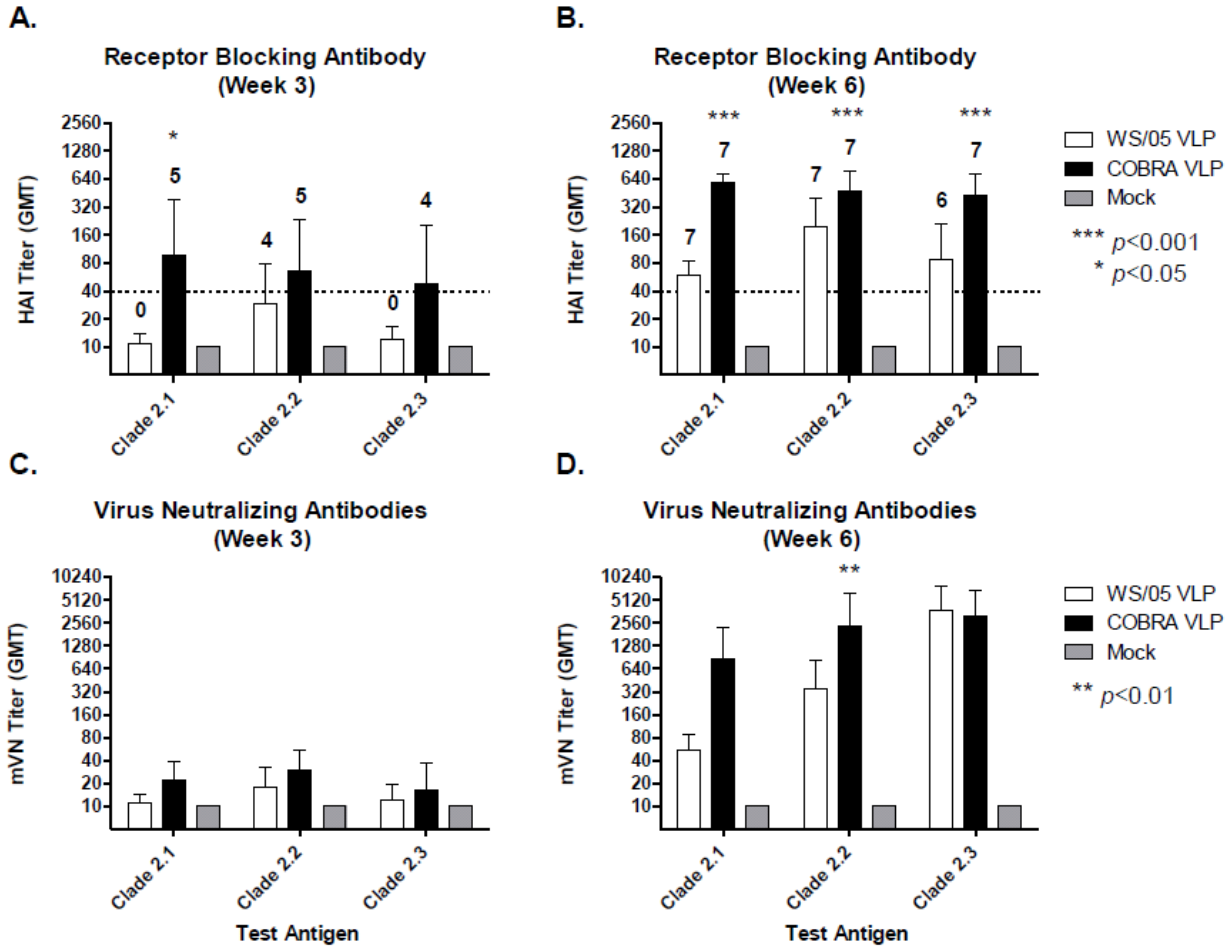

Figure 22 : Hemagglutination inhibition (HAI) and microneutralization (mVN) serum antibody titers for representative clade 2 isolates. Receptor blocking antibody titer at week 3 (A) and week 6 (B) was determined via HAI for each vaccine group. Values represent the geometric mean titer (±95% confidence interval). The dotted line represents the 1:40 titer. The number of monkeys that responded with a titer greater than 1:40 is listed above each bar. None of the mock vaccinated monkeys responded and no value is listed. Neutralizing antibody at week 3 (C) and week 6 (D) was determined via mVN for each vaccine group. Values represent the geometric mean titer (±95% confidence interval).

5.5.3 COBRA VLPs induced HAI antibodies that recognize broader numbers of H5N1 viruses.

In order to determine if the COBRA HA elicited antibodies that recognized a broader number of H5N1 isolates, serum was collected and tested for the ability to inhibit influenza virus induced hemagglutination of red blood cells *in vitro*. Antisera collected from both vaccinated and unvaccinated monkeys were then tested against a broad panel of H5N1 viruses representing not only subclades of clade 2, but also non-clade 2 H5N1 virus strains (0, 1, 4, and 7) by HAI. Monkeys vaccinated with the COBRA VLP had high average HAI titers against all clade 2 isolates, regardless of subclade (Figure 23). In general, all 7 monkeys responded to the COBRA VLP vaccine and seroconverted with an HAI titer $\geq$1:40 against all the clade 2 viruses. In contrast, monkeys vaccinated with the WS/05 VLP vaccine had lower HAI titers against clade 2 viruses (Figure 23) and fewer number of monkeys responded to the vaccine. Of the 10 clade 2 viruses tested in the HAI assay, WS/05 VLP vaccinated monkeys responded more poorly (fewer than 4 of 7 monkeys) to 4 of the isolates and none of these monkeys had antibodies that responded to the Dk/HU/02 (clade 2.1.1) or Eg/3300/08 (clade 2.2.1) isolates. The COBRA VLPs elicited significantly higher HAI titers against almost all of the clade 2 viruses than the WS/05 VLPs (Figure 23).

In addition to clade 2 isolates, a minimum of five COBRA VLP vaccinated monkeys had HAI antibodies against both clade 1 and 7 virus isolates (Figure 23). In comparison, almost none of the WS/05 VLP vaccinated monkeys had HAI antibodies against clade 1 and clade 7 viruses. None of the monkeys, regardless of the vaccine, had antibodies that responded to the clade 0 or 4 isolates. None of the mock vaccinated monkeys recognized any of the H5N1 isolates.

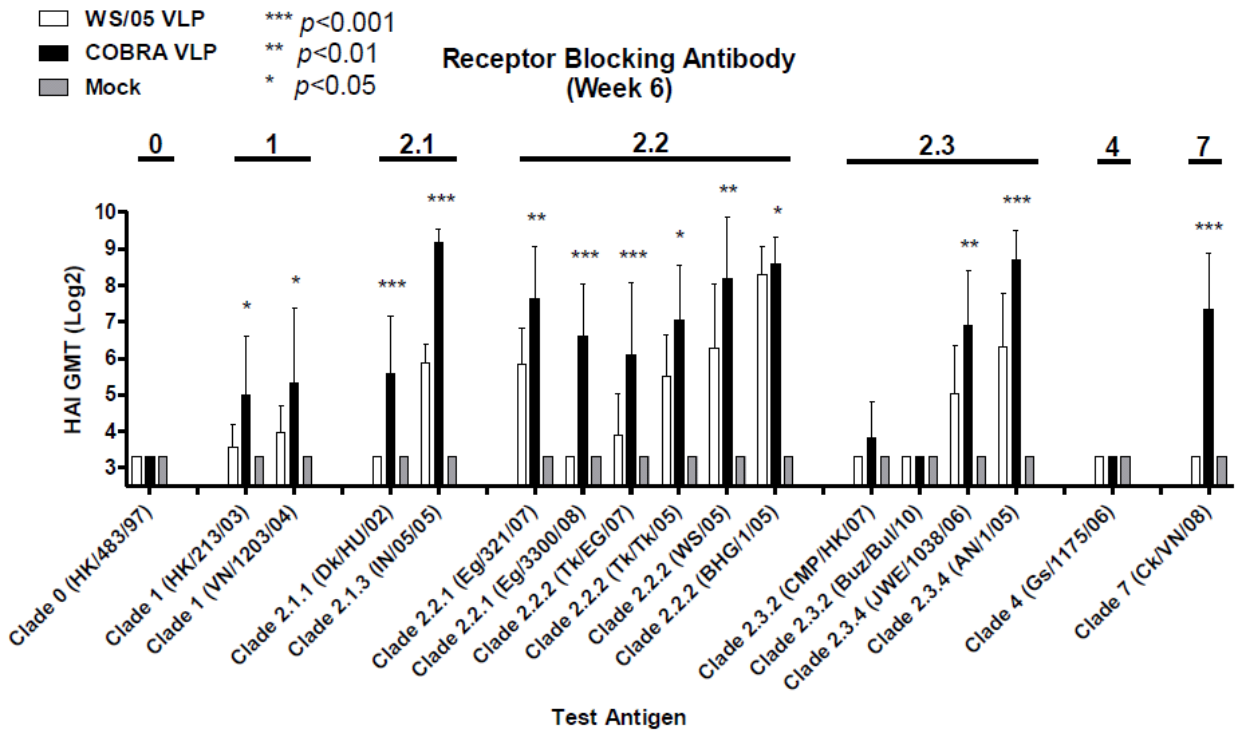

Figure 23: HAI serum antibody titers from vaccinated monkeys against a panel of clade 0, 1, 2, 4, and 7 isolates. HAI titer for each vaccine group (n=7) was determined at week 6 using diverse H5N1 influenza viruses. Values represent the geometric mean titer (±95% confidence interval) of $\log_2$ transformed titers.

5.5.4 Challenge of primates with H5N1 clade 2.2 virus.

Three weeks after final vaccination, both VLP vaccinated and mock-vaccinated monkeys were transferred to ABSL3+ isolator units and then challenged with highly pathogenic H5N1 virus, A/Whooper Swan/Mongolia/244/2005 (clade 2.2) ($1x10^6$ pfu), by a multi-route (ocular, nasal, tracheal, oral) of infection [132, 231, 232, 283]. There was no significant weight loss or mortality in any of the monkeys over the 7 day period of observation. Unvaccinated monkeys had an elevated temperature of ~2^0 C that was sustained for 5 days post-infection and higher gross pathology scores by day 3 post-infection (Table 6). The lungs of unvaccinated monkeys had

mild to moderate acute pneumonia with alveolar pulmonary exudate by day 3 post-infection by H&E staining (Figure 24A). ISH showed focal collections of H5N1 infected cells present at day 3 post-infection in alveolar spaces and to a lesser extent in bronchial epithelium (Figure 24E). These results were similar to unvaccinated monkeys infected with the clade 1 H5N1 virus, A/Vietnam/1203/2004. In contrast, monkeys vaccinated with either the COBRA VLP or the WS/05 VLP vaccine had a reduced, but not statistically significant, gross pathology scores of 2.1-3.3 at day 3 post-infection with a milder increase in body temperature (1.1-1.3^{0}C) that spiked between days 2-3 post-infection and then returned to pre-infection temperatures (Table 6). Vaccinated animals had fewer H5N1 infected cells that were detected primarily on day 1 post-infection (Figure 24F & G and Table 7). However, monkeys vaccinated with the COBRA VLP had little to no signs of lung inflammation by H&E staining (Figure 24C), while animals vaccinated with the WS/05 VLP vaccine had similar signs of inflammation as non-vaccinated monkeys (Figure 24B and Table 8). In addition, unvaccinated monkeys had high titers of virus in both the nasal and tracheal washes between days 3 and 5 post-infection. In contrast, no virus was detected in either vaccinated groups (Table 6).

Table 6: Monkey H5N1 Infections

Vaccine	Lung Pathology Score [a]	Elevated Temperature [b]	Peak Viral Titer [c]
Mock	5.3	1.9ºC(1-5 DPI)	Nasal Wash: 2.2 – 2.5 (5DPI) Tracheal Wash: 2.0 – 4.4 (3DPI)
WS/05 VLP	3.3	1.1ºC – 1.3ºC (1-5 DPI)	Nasal Wash: <2 Tracheal Wash: <2
COBRA VLP	2.1	1.3ºC(2 DPI)	Nasal Wash: <2 Tracheal Wash: <2

[a] Average gross lung pathology score (3 DPI; n=3/group)

[b] Elevated temperature above baseline (days elevated)

[c] Log 10 of average viral load at peak PFU/ml (peak day)

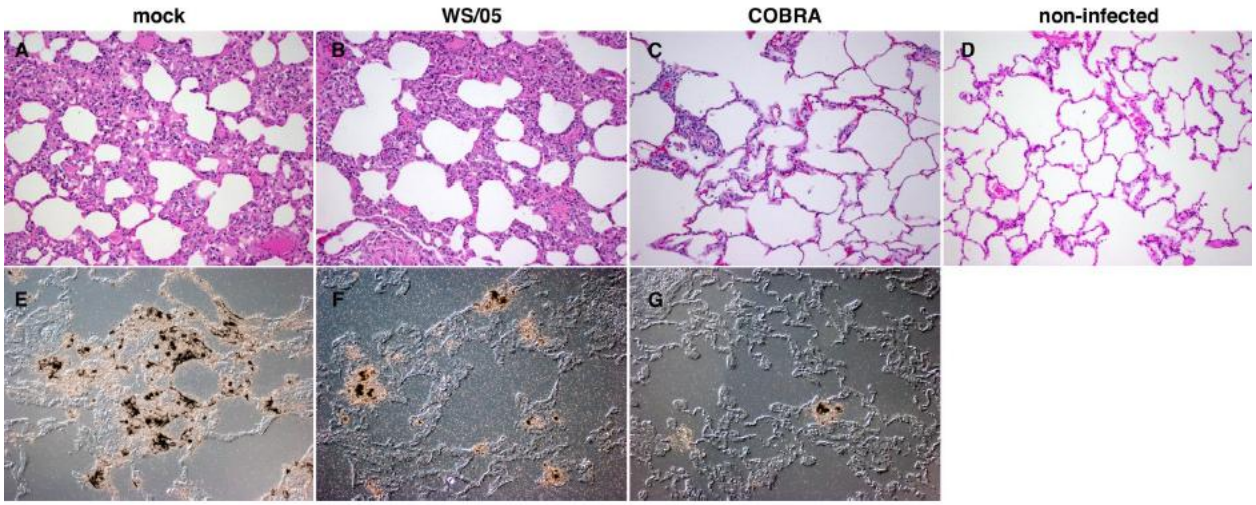

Figure 24: Histopathology of H5N1 infected lung. H&E stained sections of lungs from day 3 post-infection: (A) unvaccinated, (B) WS/05 VLP, (C) COBRA VLP, (D) unvaccinated, non-infected. Influenza ISH from day 1 post-infection: (E) unvaccinated, (F) WS/05 VLP, (G) COBRA VLP.

Table 7: H5N1 lung infection scores.

Vaccine	Bronchial infection score			Alveolar infection score			Submucosal infection score		
DPI	1	3	5	1	3	5	1	3	5
Mock	1.00	0.05	0	1.10	0.48	0.25	0	0	0
WS/05 VLP	0.05	0	0	0.55	0.10	0	0	0	0
COBRA VLP	0	0	0	0.60	0.03	0.05	0	0	0

ISH for influenza was performed on tissue sections of from upper and lower left and right lung. A semi-quantitative scoring system was developed to evaluate the presence of influenza infected cells. Scores were then averaged: 0.2 = rare or occasional cells but <5% of fields, 1 = >1/2 to 1/4 low power fields, 2 = >1/4 low power fields, 3 = essentially all low power fields.

Table 8: Lung involvement and inflammation scores.

Vaccine	% lung involvement [a]			Bronchial inflammation [b]			Alveolar inflammation [b]		
DPI	1	3	5	1	3	5	1	3	5
Mock	0.38 (0-1)	1.13 (0-2)	1.25 (0-2)	0.63 (0-1)	0.75 (0-2)	1.25 (0-2)	0.63 (0-1)	1.00 (0-2)	1.25 (0-2)
WS/05 VLP	0.75 (0-2)	1.50 (0-3)	0.88 (0-3)	1.00 (1)	1.42 (1-2)	0.63 (0-2)	1.00 (0-2)	1.25 (0-2)	1.00 (0-2)
COBRA VLP	0.88 (0-2)	0.50 (0-2)	0.38 (0-2)	1.13 (1-2)	0.75 (0-2)	0.88 (0-2)	1.13 (0-2)	0.67 (0-2)	0.25 (0-1)

[a] % lung involvement. Tissue sections from upper and lower left and right lung were evaluated for percent area demonstrating pneumonia. Scores were then averaged. Range in parentheses. 0 = <10%, 1 = 10-24%, 2 = 25-50%, 3 = >50%.

[b] bronchial and alveolar inflammation scores. Tissue sections from upper and lower left and right lung were evaluated for presence of bronchial inflammation and denudation and alveolar immune cell infiltration. Scores were then averages. 0 = absent, 1 = present, 2 = abundant

5.6 DISCUSSION

Highly pathogenic avian influenza virus represents a considerable threat to global public health. Outbreaks of highly pathogenic avian influenza of the H5N1 subtype are of particular concern because of the high mortality rate (60% case fatality rate) and novel subtype [286]. This report describes the immunogenicity and efficacy of a computationally optimized broadly reactive antigen (COBRA) approach designed for H5N1 influenza HA in a non-human primate model. The recent spread of the virulent avian H5N1 viruses throughout Asia to North Africa and Europe has raised serious concerns about the possibility of a novel human influenza pandemic efficiently transmitting to humans [287, 288]. These highly pathogenic H5N1 viruses represent isolates from clade 2, which contain distinct subclades, with some subclades being further

divided into additional sub-subclades [289]. Several current H5N1 vaccine candidates elicit immune responses that are able to neutralize viruses within a homologous clade, but lack the ability to neutralize cross-clade. Developing vaccines that are able to overcome these challenges of H5N1 antigenic diversity is a crucial step in pandemic preparedness. In this report, we describe for the first time, a non-human primate model for a clade 2 H5N1 influenza virus and use this model to test the protective efficacy induced by the clade 2 COBRA VLP vaccine.

The COBRA vaccine strategy represents a novel method of sequence construction that features a layered building approach that is intended to capture the most common antigenic characteristics, while avoiding the complication of differential sequence availability that can bias a consensus sequence to the most prevalent antigenic cluster. Avoiding this sampling bias is essential to generating a centralized vaccine that accurately represents the population of input sequences. A COBRA designed HA phylogenetic analysis in the context of H5N1 human infections demonstrated that using a layered consensus building approach can indeed overcome the challenges of sequence availability and results in a sequence that retains similarity to the input without localizing into any one antigenic cluster .

Why do COBRA induced antibodies recognize a broader number of H5N1 isolates? Several possibilities have been explored. First, COBRA HA antigens may have a combination of antibody epitopes that are not all present in any given wild-type sequence and therefore elicit a unique antibody profile. However, only one low titer linear peptide was identified as having differential reactivity (aa. 136-152) by ELISA (data not shown) and therefore, any putative epitopes are most likely conformational. Second, COBRA HA elicited antisera did not readily recognize viruses from clade 0 and 4 by HAI assay (Figure 23). Viruses in these clades have conserved HA residues between them, but these residues are divergent from the other HA antigens in our panel at several positions reported to be localized in antigenic regions: 100, 140 (region B), 154 (region A), 171 (region B) and 228 (region D) (Figure 7). It is possible that these

particular residues could play key roles in evading neutralizing antibody responses. The COBRA HA used in this study was based upon HA sequences from clade 2 viruses isolated from human infections [283]. The addition of HA sequences from divergent clades may expand the breadth of elicited antibodies to recognize viruses in other clades by HAI or mVN assays, however, it is not known if this expansion will reduce the reactivity to isolates within clade 2. In addition, antibodies elicited by COBRA HA immunogens may elicit higher affinity antibodies due to exposure of unique epitopes on the COBRA molecule that result in neutralization of a broad panel of primary virus isolates. COBRA-based immunogen design could also improve T helper epitopes, but high levels of sequence identity between HA molecules (>90%) indicates this is likely not occurring. Furthermore, epitope prediction software has failed to identify the presence of any unique linear immune epitopes in the COBRA HA. T cell responses were not evaluated in the course of these studies and cannot be ruled out at this time. How any of these results correlate with human H5N1 vaccination and subsequent protection from challenge is not known and the COBRA HA immunogen will need to be tested in future clinical trials.

Following vaccination of non-human primates, the H5N1 COBRA HA expressed on the surface of a virus-like particle elicited broadly reactive antibody (HAI) titers against not only clade 2 (Figure 22), but also across multiple clades of H5N1 (Figure 23). In general, experimental H5N1 vaccines elicit clade-specific HAI titers [161, 290]. Emergence of the novel H1N1 isolates in 2009, once again demonstrated that influenza viruses can recombine with avian and swine influenza viruses resulting in human transmissible viruses. Since the clade of an emerging, human-transmissible H5N1 isolate cannot be known in advance, a number of approaches have been employed to generate vaccines targeting a broad number of H5N1 isolates/clades, including targeting the highly conserved ion channel protein (M2) and the nucleoprotein (NP) [291, 292]. However, these influenza proteins are not highly immunogenic. For seasonal influenza viruses, T cells directed against NP and antibodies against NA can be effective at preventing viral budding and spread within a host, and thereby reducing symptoms

of disease, but only antibodies to HA can prevent infection. More recently, a vaccine approach based upon the stalk region of the influenza virus conferred protection in mice against multiple lethal influenza viral challenges representing different subtypes [105]. This approach may have merit, since antibodies elicited during the recent 2009 H1N1 outbreak had broadly reactive neutralizing activity and cross-reacted against epitopes in the HA stalk and head domain of multiple influenza strains [293]. Furthermore, it is possible that vaccines targeting the HA stalk region could be improved by using a COBRA based immunogen design. We demonstrated that antibodies elicited by the clade 2 COBRA HA recognized viruses from clades 1, 7, as well as isolates representing various subclades of clades 2.1, 2.2, and 2.3 with a larger phylogenetic footprint than antibodies elicited by VLPs expressing the clade 2.2-specific HA from A/Whooper swan/Mongolia/244/2005, which had a more restricted footprint (Figure 25). These antibodies recognized a subset of isolates within clade 2, and no viruses outside clade 2 (Figure 24). It was recently reported that vaccination with three HA molecules representing clades 1, 2.1, and 2.3 elicited broadly reactive antibody responses to divergent H5N1 clades [161]. However, the COBRA HA used in this study elicited similar antibody breadth using a single HA immunogen. The addition of HA gene sequences from other clades may expand the breadth of elicited antibodies to more clades of H5N1.

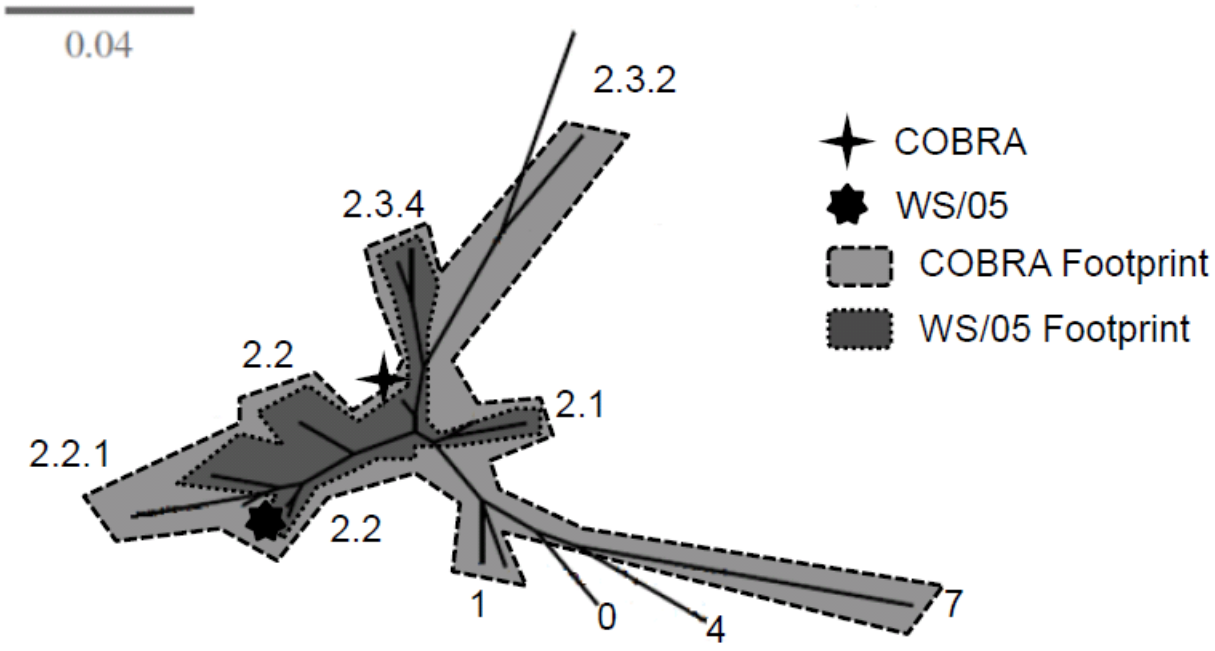

Figure 25: Footprint of HAI reactivity. The unrooted phylogenetic tree was inferred from HA amino acid sequences derived from both vaccines and test antigens using the maximum likelihood method. Phylogenetic analysis is the same as described for Figure 20. Numbers above each branch identify the clade/sub-clade of the respective cluster. The stars identify where the two vaccine antigens (COBRA and WS/05) are located relative to the test antigens. The shading indicates the footprint of HAI reactivity for the COBRA vaccine (light gray) and WS/05 vaccine (dark gray).

Non-human primates offer some advantages for studying vaccine induced immune responses, including the identification of immunological parameters outside of antibodies that may correlate with protection induced by experimental influenza vaccines [182, 294-296]. We developed this cynomolgus macaque clade 2 infection model to study both H5N1 viral-induced pathology, as well as testing vaccine efficacy in a primate immune model. Grossly, unvaccinated monkeys had lung pathology as soon as day 3 post-infection that continued until day 7. Similar results were detected in clade 1 infected monkeys [294]. Hemorrhage and lung consolidation were associated with clade 2.2 (WS/05) H5N1 infection. Monkeys vaccinated with either COBRA VLPs or WS/05 VLPs had few signs of disease and greatly reduced viral titers in

the lungs upon challenge (Table 5), but COBRA VLP-vaccinated monkeys had little lung inflammation and fewer virally infected cells than WS/05 VLP vaccinated animals (Figure 24). The COBRA HA was as effective in protecting monkeys from challenge as the homologous WS/05 HA, indicating that the COBRA HA can protect against a heterologous clade 2 viral challenge. However, there were few clinical signs associated with infection with this strain of clade 2 H5N1 and therefore, differences in vaccine efficacy could only be determined by lower viral titers and reduced pathology following necropsy and histopathological staining, which may limit the use of this model for vaccine studies.

In this report, we hypothesized that the COBRA HA delivered in the context of a VLP will elicit an antibody response that would increase the breadth of elicited antibody when compared to that of a VLP containing an HA molecule derived from a primary isolate. This is the first report of a COBRA HA eliciting broadly reactive antibody responses across multiple clades of H5N1 and also reduces inflammation and pathology in non-human primates from a heterologous, highly pathogenic H5N1 virus challenge.

5.7 ACKNOWLEDGMENTS

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Swan/Mongolia/244/2005 influenza virus. In addition, we thank Richard Webby for providing additional H5N1 viruses for assays and Mark Lewis and Wendeline Wagner at Bioqual for veterinary assistance for ABSL3+ H5N1 challenges.

6.0

ANTIBODY BREADTH AND PROTECTIVE EFFICACY IS INCREASED BY VACCINATION WITH COMPUTATIONALLY OPTIMIZED HEMAGGLUTININ BUT NOT WITH POLYVALENT HEMAGGLUTININ BASED H5N1 VLP VACCINES

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6.1 FOREWORD

The work presented in this chapter addresses features of Specific Aims II and III (see Sections 3.4 and 3.5, respectively). Specifically, the comparison of the consensus and polyvalent vaccines is described here. Evaluations of both antibody and cellular immune responses outlined in Specific Aim II are detailed in this chapter. For Specific Aim III, the challenge studies evaluating vaccine efficacy against both clade 2.2 and clade 1 viral challenges are included here. Additionally, the finding that vaccine-induced serum factors provide protection from severe disease is contained in this chapter. Therefore, the work presented here completes the studies outlined in Specific Aims II and III.

6.2 ABSTRACT

Influenza pandemics occur sporadically and are caused by the emergence of a pathogenic and transmissible virus to which the human population is immunologically naïve. Human infections with highly pathogenic avian influenza of the H5N1 subtype were first identified in 1997 and have continued to occur through today. The H5N1 subtype is of particular concern due to the novel subtype and high pathogenicity. One of the challenges for developing an H5N1 vaccine is the diversity within the subtype. H5N1 is separated into 10 phylogenetic clades that are co-circulating and antigenically distinct. Vaccines that are able to elicit cross-reactive antibodies to multiple strains are needed for effective pre-pandemic stockpiling. We have previously described a novel hemagglutinin using a methodology termed computationally optimized broadly reactive antigen (COBRA) that elicited broad antibody responses. In this report, COBRA HA vaccine is compared to a polyvalent H5N1 vaccine. Polyvalent vaccines are a

traditional method for increasing the breadth of antibody responses by influenza vaccines and still remain the strategy employed for generating seasonal influenza vaccine formulations. COBRA HA elicited higher titer antibody responses to antigens homologous and heterologous within the polyvalent formulation. Both COBRA and polyvalent vaccines protected vaccinated animals from experimental infection with highly lethal H5N1 influenza viruses, but COBRA vaccinated animals had decreased viral replication and histological involvement in the lungs of mice and reduced virus recovery in ferret nasal washes. Both vaccines had similar cellular responses post-challenge indicating that higher titer serum antibody likely restricts the duration of viral replication. Furthermore, passively transferred immune serum from COBRA vaccinated mice protected recipient animals more efficiently than immune serum from polyvalent vaccinated mice. This is the first report comparing two strategies for increasing breadth of influenza vaccines: centralized and polyvalent. We show that a single COBRA antigen elicits broader antibody responses and is more effective than a polyvalent mixture of primary antigens.

6.3 INTRODUCTION

In addition to yearly epidemics, pandemic outbreaks of influenza have occurred sporadically throughout human history [55, 71]. Pandemics occur when a novel pathogenic and transmissible virus emerges into the human population. A critical factor in the emergence of a pandemic virus is that it must be antigenically divergent from circulating strains to evade prior immunity in the human population. Potentially pandemic viruses can therefore emerge from novel subtypes, such as H5N1 or H7N7, or divergent strains of currently circulating subtypes, such as H1N1. Indeed, the influenza pandemic of 2009 was caused by the emergence of a novel swine-origin H1N1 virus into the human population [72]. Avian viruses of the subtypes

H5N1, H7N7 and H9N2 have all demonstrated the ability to directly infect humans [297]. H5N1 is of particular concern because of the continued cross-species infection and the high pathogenicity of the virus (60% mortality) [185]. Although H5N1 has not displayed efficient human to human transmission, *in vitro* studies have established that stable reassortant viruses that retain the pathogenic phenotype of H5N1 can be created with both H3N2 and novel H1N1 viruses [248-250]. Reassortment with transmissible viruses and/or accumulation of mutations could result in the emergence of a highly transmissible H5N1 virus. The genetic compatibility of H5N1 with currently circulating human and swine viruses highlights the need for the development of effective vaccines against H5N1.

Development of pre-pandemic H5N1 vaccines is complicated by the antigenic diversity within the subtype. Phylogenetic distances of the hemagglutinin (HA) genes of H5N1 viruses distinguishes the ten distinct clades [196]. HA-based diversity within clade 2 alone has led to characterization of distinct subclades and sub-subclades. Most human infections were by isolates belonging to clades 1 or 2 with isolates from clade 2 being detected in over 60 countries and moving westward into the Middle East and Africa [298]. Although H5N1 HA proteins display a high degree of similarity (>90% identity), there is little receptor blocking antibody cross-reactivity between clades. Furthermore, the subclades of clade 2 are also antigenically distinct as determined by cross-reactivity of receptor blocking antibodies [196]. Despite the risk imposed by highly pathogenic H5N1 influenza, the magnitude of diversity within the subtype complicates vaccine antigen selection for either pre-pandemic usage or stockpiling. Vaccines that are able to overcome the challenge of antigenic diversity are therefore crucial to effective pandemic preparedness.

Influenza antigenic diversity is not a unique problem for H5N1 vaccine development. Rather, simultaneous circulation of diverse influenza A (H1N1 and H3N2) and influenza B viruses has been a challenge for seasonal influenza vaccine production for over 30 years. The current seasonal vaccine uses a polyvalent formulation to address the issue of distinct viruses

circulating at the same time and therefore is a standard strategy to elicit increased antibody breadth by influenza vaccination. Indeed, multivalent H5N1 vaccines increase the breadth of receptor blocking antibody responses [160, 161]. Another strategy for expanding antibody breadth involves engineering synthetic centralized antigens that are predicted to capture common immune epitopes. Consensus and ancestral sequences have been used for H5N1 vaccine antigens and these centralized vaccines are a feasible mechanism by which antibody breadth can be expanded [172, 180-182, 254]. Our research group has used a consensus-based approach to develop a novel strategy termed computationally optimized broadly reactive antigen (COBRA) [283]. Traditional consensus sequences are inherently biased by the input sequences used in the alignment and subsequent sequence generation. Because sequencing efforts for H5N1 are reactionary to outbreaks rather than systematic, the available input sequences to generate any centralized sequence are subject to outbreak dominance and bias. COBRA methodology seeks to overcome this limitation by layering together multiple rounds of consensus sequence generation. This method was specifically applied to the diversity of HA within clade 2 because this clade is the most antigenically diverse and has been implicated in the majority of human infections and has the widest geographic spread.

Here, we demonstrate that our previously described COBRA antigen elicits a broader receptor blocking antibody response than a clade 2 polyvalent formulation in mice and ferrets. Both vaccines were able to protect mice and ferrets from highly pathogenic H5N1 challenge with COBRA vaccinated animals showing more efficient viral clearance. Additionally, both vaccines protected mice from a divergent clade 1 challenge and immune serum from COBRA vaccinated animals protected animals more efficiently than serum from polyvalent vaccine recipients.

6.4 MATERIALS AND METHODS

6.4.1 Vaccine antigens and preparation

The design and characterization of the computationally optimized broadly reactive antigen (COBRA) has been described previously [283]. Briefly, the COBRA HA antigen was generated by multiple rounds of consensus generation using HA sequences from H5N1 clade 2 human infections collected from 2004 to 2006. Polyvalent vaccine HA antigens were derived via reverse transcription from the following 6:2 reassortant H5N1 viruses: A/Indonesia/5/2005 (clade 2.1; IN/05), A/Whooper Swan/Mongolia/244/2005 (clade 2.2; WS/05) and A/Anhui/1/2005 (clade 2.3; AN/05). All HA antigens were cloned into the pTR600 expression vector [238].

Virus-like particles (VLPs) were generated by transiently transfecting HEK 293T cells with plasmids expressing M1 (A/Puerto Rico/8/1934), NA (A/Thailand/1(KAN-1)/2004), and a single H5N1 HA for each preparation. Cells were incubated for 72h at 37^{o}C after which supernatants were harvested. Cell debris was cleared by low speed centrifugation followed by vacuum filtration through a 0.22μm sterile filter. VLPs were purified by ultracentrifugation (100,000 x g through 20% glycerol, weight to volume) for 4h at 4^{o}C. Pellets were then resuspended in PBS (pH 7.2) and stored in single use aliquots at -80^{o}C until use. Total protein concentration was determined by MicroBCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). HA specific content of each VLP was determined by scanning densitometry as described previously [283]. Briefly, purified HA matched to each VLP was electrophoresed with purified VLPs, transferred to a PVDF membrane and probed by western blot with H5-specific antisera. The relative density of the HA band in the purified protein lanes was used to calculate a standard curve and the density of the HA in the VLP lanes was interpolated. In total, four different VLP preparations were purified and HA content

quantified independently, each containing one of the three wild-type influenza gene products (IN/05, WS/05, AN/05) or the COBRA HA.

6.4.2 Mouse studies

BALB/c mice (*Mus musculis*, females, 6–8 weeks) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA) and housed in microisolator units and allowed free access to food and water and were cared for under USDA guidelines for laboratory animals. Mice were vaccinated with purified COBRA VLPs (3μg HA) or a polyvalent formulation of VLPs consisting of 1μg HA each IN/05, WS/05 and AN/05 (3μg HA total) via intramuscular injection at week 0 and then boosted at week 3. Vaccines were formulated with Imject® alum adjuvant (Imject® Alum, Pierce Biotechnology; Rockford, IL, USA) according to the manufacturer's protocol. Fourteen to twenty-one days after each vaccination, blood was collected from anesthetized mice via the retro-orbital plexus and transferred to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at -20 ± 5°C.

Three weeks after final vaccination, mice were challenged intranasally with $5x10^3$ plaque forming units (PFU) of either highly pathogenic wild type H5N1 virus A/Whooper Swan/Mongolia/244/2005 (n=20/group) or 6:2 reassortant virus with internal genes from the mouse adapted virus A/Puerto Rico/8/1934 and the surface proteins HA and NA from A/Vietnam/1203/2004 (n=10/group) in a total volume of 50μl. Challenge doses for both viruses were established independently and represent approximately $50LD_{50}$ (data not shown). After infection, mice 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 scores were determined by evaluating activity (0=normal, 1=reduced, 2=severely reduced), hunched back (0=absent, 1=present) and ruffled fur

(0=absent, 1=present) [230]. Experimental endpoints were determined by >20% weight loss or display of neurological disease such as hind limb paralysis. All highly pathogenic wild type H5N1 influenza 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, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

6.4.3 Ferret studies

Fitch ferrets (*Mustela putorius furo*, female, 6-12-months of age), influenza naïve and descented, were purchased from Marshall Farms (Sayre, PA, USA). Ferrets were pair housed in stainless steel cages (Shor-line, Kansas City, KS, USA) containing Sani-chips Laboratory Animal Bedding (P.J. Murphy Forest Products, Montville, NJ, USA). Ferrets were provided with Teklad Global Ferret Diet (Harlan Teklad, Madison, WI, USA) and fresh water ad libitum. The VLPs were diluted in PBS (pH 7.2) to achieve final concentration. Ferrets (n=6) were vaccinated with purified COBRA VLPs (15μg HA) or a polyvalent formulation of VLPs consisting of 5μg HA each IN/05, WS/05 and AN/05 (15μg HA total) via intramuscular injection at week 0 and then boosted at week 3. Vaccines were formulated with Imject® alum adjuvant (Imject® Alum, Pierce Biotechnology; Rockford, IL, USA) immediately prior to use according to the manufacturer's protocol. Animals were monitored weekly during the vaccination regimen for adverse events including weight loss, temperature, loss of activity, nasal discharge, sneezing and diarrhea. Prior to vaccination, animals were confirmed by HAI assay to be seronegative for circulating influenza A (H1N1 and H3N2) and influenza B viruses. Fourteen to twenty-one days after each vaccination, blood was collected from anesthetized ferrets via the anterior vena cava

and transferred to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at -20 ± 5°C.

Three weeks after final vaccination, ferrets were challenged intranasally with $1x10^6$ plaque forming units (PFU) of the highly pathogenic H5N1 virus A/Whooper Swan/Mongolia/244/2005 (clade 2.2) in a volume of 0.5ml 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 scores were determined by evaluating activity (0=normal, 1=alert and active after stimulation, 2=alert but not active after stimulation, 3=neither active nor alert 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) as previously described [283]. Experimental endpoints were defined as >20% weight loss, development of neurological disease or an activity score of 3 (neither active nor alert after stimulation). Nasal washes were performed by instilling 3 ml of PBS into the nares of anesthetized ferrets each day for 14 days after inoculation. Washes were collected and stored at -80°C until use. All highly pathogenic wild type H5N1 influenza 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, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

6.4.4 ELISA assay

The ELISA assay was used to assess total antibody titer to the HA. High binding, 96-well polystyrene plates (Costar; Lowell, MA, USA) were coated overnight with 50ng/well of recombinant HA. Coating antigens were derived from the following representative viral isolates:

A/Vietnam/1203/2004 (clade 1), A/Indonesia/5/2005 (clade 2.1), A/Whooper Swan/Mongolia/244/2005 (clade 2.2) and A/Anhui/1/2005 (clade 2.3). Plates were blocked with 5% milk diluted in PBS with 0.05% Tween 20. Serum samples were diluted in blocking buffer and added to plates. Serum was two-fold serially diluted and allowed to incubate for 1 hour at room temperature. Plates were washed and horseradish peroxidase (HRP)-linked species specific antibody against IgG was diluted in blocking buffer and added to plates. Plates were incubated for 1 hour at room temperature. Plates were washed and HRP was developed with TMB substrate (Sigma-Aldrich; St. Louis, MO, USA). Plates were incubated in the dark for 15 minutes and then the reaction was stopped with 2N H_2SO_4. Optical densities at a wavelength of 450nm (OD_{450}) were read by a spectrophotometer (BioTek; Winooski, VT, USA) and end point dilution titers were determined as the reciprocal dilution of the last well which had an OD_{450} above the mean OD_{450} plus two standard deviations of naïve animal sera.

6.4.5 Hemagglutination inhibition (HAI) assay

The HAI assay was used to assess functional antibodies to the HA able to inhibit agglutination of horse erythrocytes. The protocol was adapted from the CDC laboratory-based influenza surveillance manual [299]. To inactivate non-specific inhibitors, sera were treated with receptor destroying enzyme (RDE; Denka Seiken, Co., Japan) prior to being tested [234-238]. Briefly, three parts RDE was added to one part sera and incubated overnight at 37°C. RDE was inactivated by incubation at 56°C for ~30 min. RDE treated sera was two-fold serially diluted in v-bottom microtiter plates. An equal volume of reassortant virus, adjusted to approximately 8 HAU/50µl, was added to each well. The reassortant viruses contained the internal genes from the mouse adapted strain A/Puerto Rico/8/1934 and the surface proteins HA and NA from the following representative viral isolates: A/Vietnam/1203/2004 (clade 1), A/Indonesia/5/2005 (clade 2.1), A/Whooper Swan/Mongolia/244/2005 (clade 2.2) and A/Anhui/1/2005 (clade 2.3).

The plates were covered and incubated at room temperature for 20 min followed by the addition of 1% horse erythrocytes (HRBC) (Lampire Biologicals, Pipersville, PA, USA) in PBS. Red blood cells were stored at 4°C and used within 72 h of preparation. The plates were mixed by agitation, covered, and the RBCs were allowed to settle for 1 h at room temperature [239]. The HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Positive and negative serum controls were included for each plate. All mice and ferrets were negative (HAI ≤1:10) for pre-existing antibodies to currently circulating human influenza viruses prior to vaccination.

6.4.6 Plaque assay

For mouse infections, lung virus titers were evaluated. For ferret infections, nasal wash virus titers were used to assess viral burden. Both lungs and nasal wash virus titers were determined using a plaque assay [241, 242]. Briefly, lungs from infected mice were harvested post infection, snap-frozen and stored at -80°C until use. Samples were thawed, weighed and single cell suspensions were prepared via passage through a 70 μm mesh (BD Falcon, Bedford, MA, USA) in an appropriate volume of DMEM supplemented with penicillin-streptomycin (iDMEM) as to achieve 100mg/ml final concentration. Cell suspensions were centrifuged at 2000 rpm for 5 min and the supernatants were collected.

Madin-Darby Canine Kidney (MDCK) cells were plated (5×10^5) in each well of a 6 well plate. Samples (lung supernatants for mice and nasal washes for ferrets) were diluted (dilution factors of 1×10^1 to 10^6) and overlayed onto the cells in 100 μl of iDMEM and incubated for 1hr. Virus-containing medium was removed and replaced with 2 mls of L15 medium plus 0.8% agarose (Cambrex, East Rutherford, NJ, USA) and incubated for 96 hrs at 37°C with 5% CO_2. Agarose was removed and discarded. Cells were fixed with 10% buffered formalin, and then

stained with 1% crystal violet for 15 min. Following thorough washing in dH_2O to remove excess crystal violet, plates were allowed to dry, plaques counted, and the plaque forming units (PFU)/g for lung supernatants or PFU/ml for nasal washes were calculated.

6.4.7 Histopathological analysis

Left lobes of lungs from infected mice were collected 1, 3 and 5 days post-infection and placed into 10% buffered formalin. After fixation, lungs were paraffin embedded and 6 μm sections were prepared for histopathological analysis. Tissue sections were stained with hematoxylin and eosin. For *in situ* hybridization (ISH), vectors containing 760 bp of Influenza/California/04/2009 matrix protein were linearized to create antisense and sense templates. ^{35}S-labeled riboprobes were generated using MAXIscript *in vitro* transcription kit (Ambion, Austin, TX). ISH was performed as described before [300]. Control riboprobes did not hybridize to lung tissue at any time point post-infection and non-infected tissue did not show hybridization with viral probes. Hybridized slides were assessed and scored for abundance of foci.

6.4.8 Cellular assays

The number of anti-influenza specific cells secreting interferon gamma (IFN-γ) was determined by enzyme-linked immunospot (ELISpot) assay (R&D systems, Minneapolis, MN, USA) following the manufacturer's protocol. Mice were sacrificed at 6 days post infection (DPI) and spleens and lungs were harvested and prepared in single cell suspensions. Briefly, pre-coated anti-IFNγ plates were blocked with RPMI plus 10% FCS and antibiotics (cRPMI) for 30 minutes at room temperature. Media was removed from wells and 10^5 cells were added to each well.

Cells were stimulated with purified recombinant HA from A/Vietnam/1203/2004 (truncated at residue 530; 1μg/well), inactivated 6:2 reassortant virus A/Vietnam/1203/2004 (1:100 dilution of inactivated stock; 100μl/well) or the immunodominant H2-K^d $CD8^+$ T cell epitope in H5 HA: HA_{533} (IYSTVASSL; 1μg/well) (Pepscan Presto, Leystad, Netherlands). Additional wells were stimulated with PMA (50ng/well) and ionomycin (500ng/well) as positive controls or Ova_{257} (SIINFEKL; 1μg/well) (Pepscan Presto, Leystad, Netherlands) as negative controls. Additionally, IL-2 (10U/ml) was added to each well. Plates were incubated at 37°C for 48 hours. After incubation, plates were washed four times with R&D wash buffer and were incubated at 4°C overnight with biotinylated anti-mouse IFNγ. Plates were washed as before and incubated at room temperature for 2 hours with streptavidin conjugated to alkaline phosphatase. Plates were washed as before and spots were developed by incubating at room temperature for 1 hour in the dark with BCIP/NBT chromogen substrate. The plates were washed extensively with DI H_2O and allowed to dry overnight prior to spots being counted using an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

The number of anti-HA and anti-NA specific antibody secreting cells was determined by B cell ELISpot assay as previously described [243-245]. Mice were sacrificed at 6 DPI and spleens and lungs were harvested and prepared in single cell suspensions. Briefly, 0.45μm PVDF membrane plates (Millipore, Billerica, MA, USA) were coated with either purified recombinant HA from A/Vietnam/1203/2004 or purified recombinant NA from A/Thailand/1(KAN-1)/2004 (250ng/well) and incubated at 4°C overnight. Plates were washed three times with PBS and blocked with cRPMI at 37°C for 3-4 hours. Media was removed from wells and 10^5 cells were added to each well. Plates were incubated at 37°C for 48 hours. After incubation, plates were washed as before and incubated at room temperature for 2 hours with HRP-conjugated anti-mouse IgG or IgA (Southern Biotech, Birmingham, AL, USA). Plates were washed as before and spots were developed at room temperature for 1 hour in the dark with detection

substrate (NovaRED™; Vector Labs, Burlingame, CA, USA). The plates were washed extensively with DI H_2O and allowed to dry overnight prior to spots being counted using an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

6.4.9 Passive transfer of sera

Serum from vaccinated mice was pooled and passively transferred into 9 week old recipient BALB/c mice (n=5/group). Equal amounts of serum from each mouse in a particular vaccine group were pooled and heat inactivated for 30 minutes at 56^{o}C. 200 μl of pooled and inactivated serum was transferred to recipient mice via intraperitoneal (IP) injection. 24 hours post transfer, mice were infected with 6:2 reassortant virus with internal genes from the mouse adapted virus A/Puerto Rico/8/1934 and surface antigens from A/Vietnam/1203/2004 as described above.

6.4.10 Statistical analysis

Statistical significance of the antibody and cellular immunology data was determined using a two-tailed Student's T test to analyze differences between COBRA and polyvalent vaccine groups for each of the different test antigens. Differences in weight loss and sickness scores were analyzed by two-way ANOVA, followed by Bonferroni's post test for each vaccine group at multiple time points (multiparametric). Statistical siginificance of viral titer data was evaluated using a two-tailed Student's T test on Log_{10} transformed values. Significance was defined as $p < 0.05$. Statistical analyses were done using GraphPad Prism software.

6.5 RESULTS

6.5.1 Immunogenicity in mice and ferrets

To expand our previous findings comparing COBRA to clade 2.2 VLPs [283], BALB/c mice were vaccinated twice via intramuscular injection with either purified COBRA or polyvalent VLPs and two weeks after the second vaccination serum was analyzed for antibody responses. All vaccinated mice had high titer anti-HA antibodies that bound to recombinant HA derived from both clade 1 and various subclades of clade 2 (Figure 26A). Although both COBRA and polyvalent vaccines elicited similar total IgG, COBRA vaccinated animals had higher HAI antibody titers for all viruses tested ($p<0.001$; Figure 26B). In addition to higher HAI titer, COBRA vaccinated mice had an increased frequency of HAI titers $\geq$1:40 for all viruses tested, including those which were components of the polyvalent formulation (TABLE 9).

These results were confirmed in ferrets, which are a more rigorous animal model than mice. Similar to the mouse results, all vaccinated ferrets had anti-HA antibodies that bound to diverse recombinant HA and the relative total IgG titers were equivalent for both COBRA and polyvalent vaccines (Figure 26C). COBRA vaccinated ferrets demonstrated increased HAI antibody titers compared to polyvalent vaccinated animals against all viruses tested, however only the antibodies to the clade 2.1 virus were significantly different ($p<0.05$; Figure 26D). Furthermore, the frequency of COBRA vaccinated animals with an HAI titer of $\geq$1:40 was increased compared to the polyvalent vaccinated ferrets for all test antigens (TABLE 9).

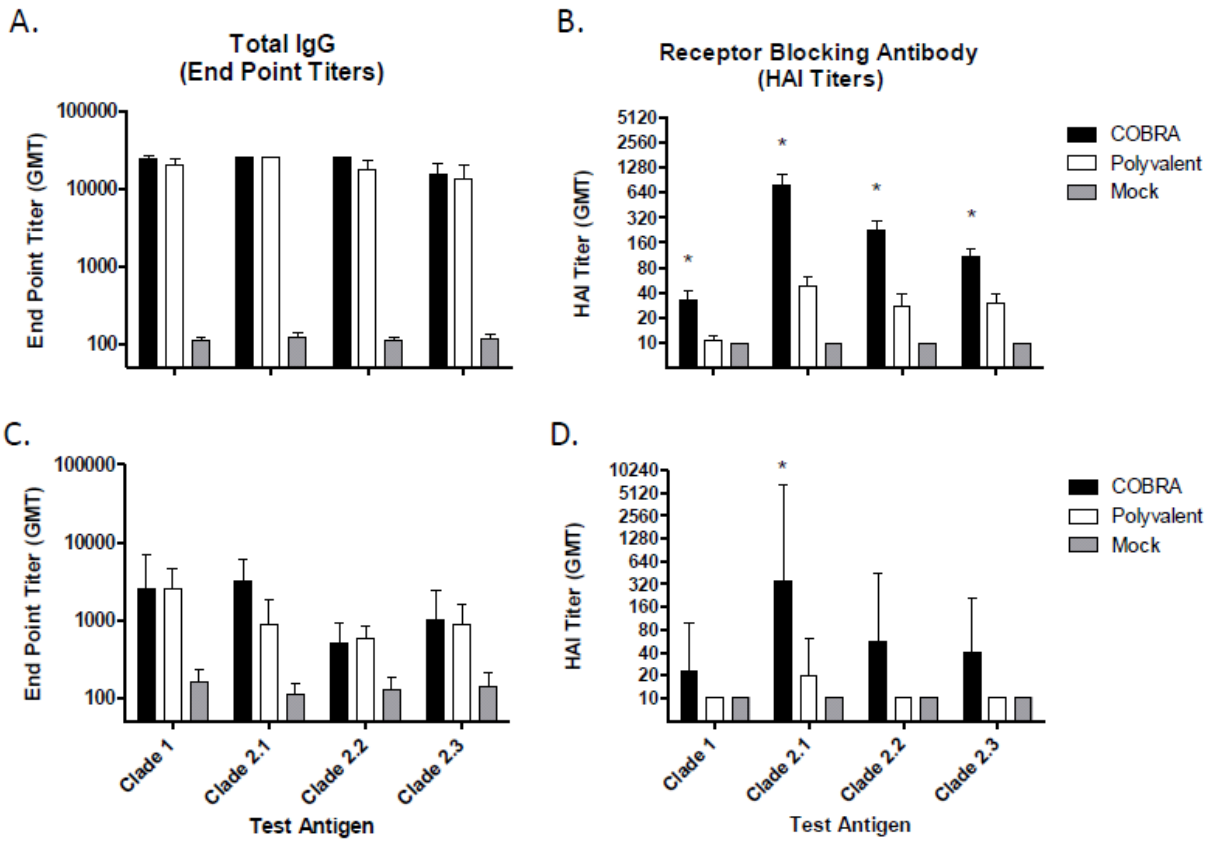

Figure 26: Vaccine induced serum antibody responses. BALB/c mice (n=30/group) (A and B) or Fitch ferrets (n=6/group) (C and D) were vaccinated at 0 and 3 weeks with blood collected 14 days after each vaccination. Total IgG after the second vaccination was determined via ELISA for each vaccine group (A and C). Receptor blocking antibody titers after the second vaccination were determined via hemagglutination inhibition (HAI) for each vaccine group (B and D). Values represent the geometric mean of the reciprocal dilution (+/- 95% confidence interval) of the last positive well. Significant differences between COBRA and polyvalent vaccines were determined by a two-tailed Student's T test and a *p* value of less than 0.05 was considered significant (*).

Table 9: Seroconversion frequency. HAI titers ≥ 1:40.

Species	Vaccine antigen	Clade 1	Clade 2.1	Clade 2.2	Clade 2.3
Mouse	COBRA	60% (18/30)	100% (30/30)	100% (30/30)	100% (30/30)
	Polyvalent	3.3% (1/30)	70% (21/30)	50% (15/30)	53% (16/30)
Ferret	COBRA	33% (2/6)	67% (4/6)	50% (3/6)	50% (3/6)
	Polyvalent	0% (0/6)	33% (2/6)	0% (0/6)	0% (0/6)

6.5.2 Wild type clade 2.2 challenge

To confirm protective efficacy against highly pathogenic H5N1 infection, vaccinated animals were challenged with a lethal dose of the wild-type clade 2.2 isolate A/Whooper Swan/Mongolia/244/2005. All VLP vaccinated mice were protected from weight loss and death, while mock vaccinated animals rapidly lost weight and reached experimental end-points by 6 days post infection (DPI; Figure 27A). COBRA and polyvalent vaccinated mice both had a mean maximum weight loss of 4% by 12 and 13 DPI, respectively. Additionally, all VLP vaccinated mice failed to develop any overt signs of disease, while mock vaccinated mice developed visible illness (Figure 27B).

Similar to the mice, all VLP vaccinated ferrets were protected from death following a lethal challenge. Vaccinated ferrets demonstrated mild weight loss in response to the infection, with COBRA vaccinated animals having mean maximum weight loss of 5.5% at 2 DPI and polyvalent vaccinated animals losing 6.8% by 3 DPI (Figure 27C). Both groups rapidly recovered weight and failed to develop any significant signs of disease (Figure 27D). Furthermore, VLP vaccinated animals had no temperature spikes, while mock vaccinated animals had an elevated temperature of ~3^{o}C for 1-3 DPI (data not shown).

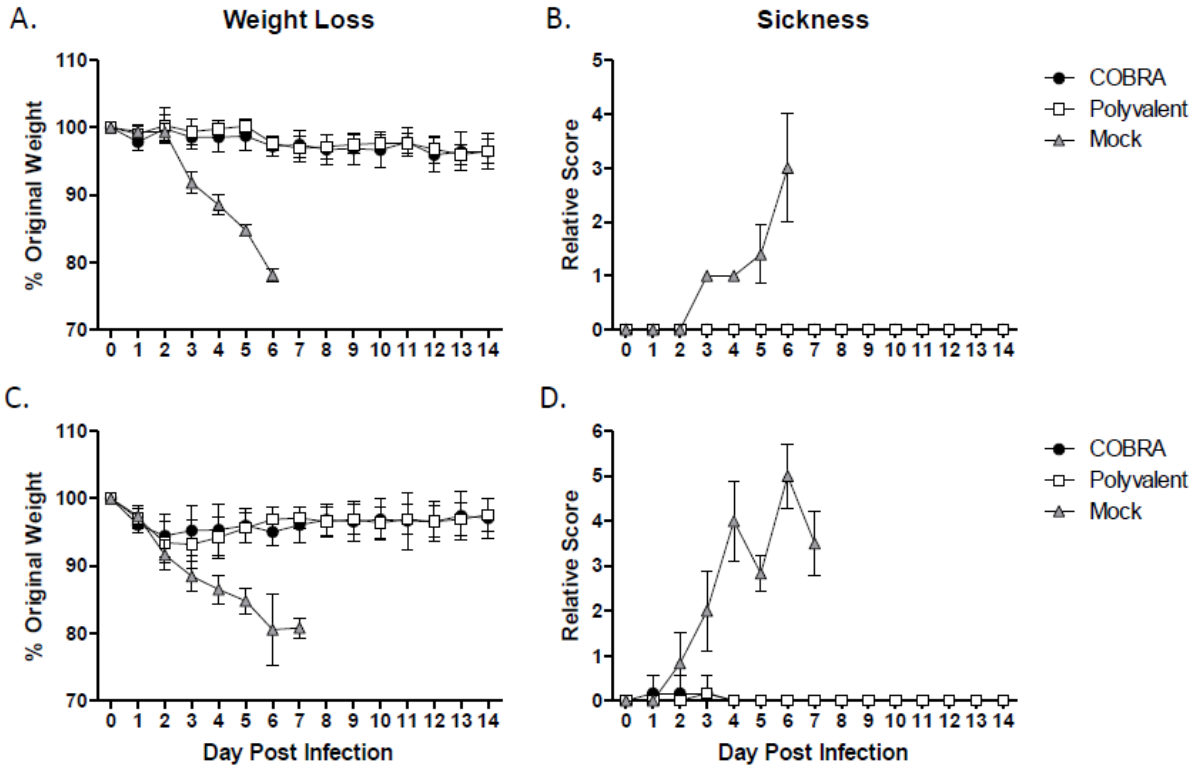

Figure 27: Highly pathogenic clade 2.2 challenge. Vaccinated BALB/c mice (n=5/group) were infected with $5x10^3$ PFU of the highly pathogenic clade 2.2 H5N1 virus A/Whooper Swan/Mongolia/244/2005 (WS/05). Mice were monitored daily for weight loss (A) and sickness (B). Vaccinated Fitch ferrets (n=6/group) were infected with $1x10^6$ PFU of the highly pathogenic clade 2.2 WS/05 virus. Ferrets were monitored daily for weight loss (C) and sickness (D). Values represent mean (+/- SEM) for each group.

To evaluate vaccine efficacy with a more sensitive output than morbidity and mortality, we also determined the viral burden of infected animals. Both COBRA and polyvalent vaccinated mice had reduced lung viral titers as soon as 1 DPI when compared to mock vaccinated animals. Furthermore, COBRA vaccinated mice did not have detectable virus by 3 DPI, while polyvalent vaccinated mice demonstrated prolonged viral replication with 1.8×10^3 PFU/g detected at 3DPI ($p<0.05$; Figure 28A). Additionally, both VLP vaccines prevented extra-pulmonary spread of the virus, while mock vaccinated animals had detectable virus in both

kidney and liver by 3 DPI (data not shown). Control of virus replication in ferrets was similar to that observed in mice, although complete clearance of the virus was delayed (Figure 28B). All VLP vaccinated animals had decreased recovery of virus in nasal washes compared to mock vaccinated ferrets at every time point tested (p<0.05). COBRA vaccinated animals did not have detectable virus by 5 DPI. In contrast, virus replication did not reach undetectable levels until 9DPI in polyvalent vaccinated ferrets (data not shown).

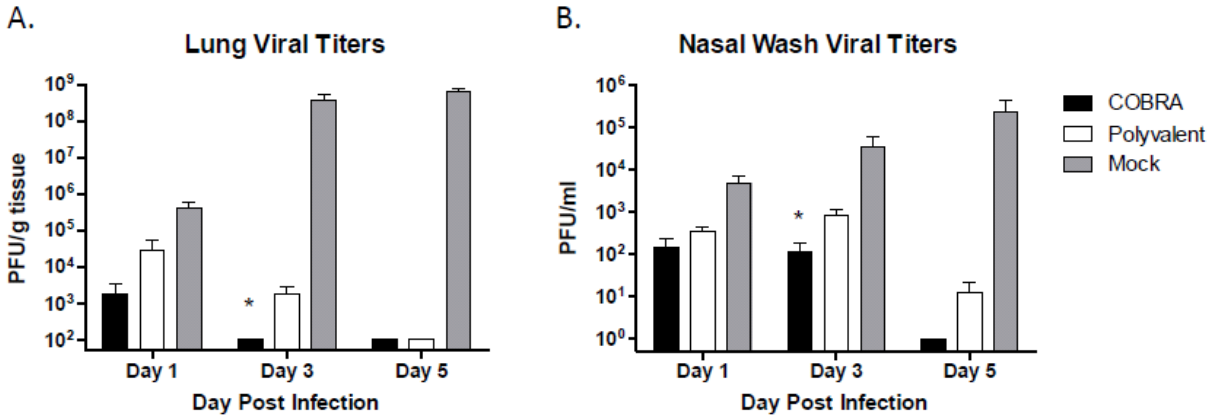

Figure 28: Clade 2.2 viral loads. Vaccinated BALB/c mice (n=15/group) were infected with $5x10^3$ PFU of the highly pathogenic clade 2.2 H5N1 virus A/Whooper Swan/Mongolia/244/2005 (WS/05). Cohorts of mice (n=5/group) were sacrificed at 1, 3 and 5 days post infection, lungs harvested, and viral load determined by plaque assay (A). Vaccinated Fitch ferrets (n=6/group) were infected with $1x10^6$ PFU of the highly pathogenic WS/05 virus. Nasal washes were collected and viral load determined by plaque assay (B). Values represent mean (+/- SEM) viral titer for each group. Significant differences between COBRA and polyvalent vaccines were determined by a two-tailed Student's T test and a p value of less than 0.05 was considered significant (*).

6.5.3 Histopathology of infected lungs.

To evaluate the location and severity of influenza viral replication, ISH for influenza A MP was scored on 1, 3 and 5 DPI lung sections. COBRA vaccinated animals had rare bronchial epithelium infection on 1 DPI (Figure 29C), and animals receiving polyvalent vaccines had occasional bronchial epithelium infection that was comparable to the COBRA vaccinated animals (Figure 29C). On 3 DPI COBRA vaccinated animals had no detectable viral replication, while animals receiving polyvalent vaccines showed some bronchial epithelium infection (Figure 29A and 29C). This was in contrast to significant bronchial epithelium infection and replication observed in mock animals (Figure 29A and 29C).

To determine if vaccination protected animals from lung inflammation, hematoxylin and eosin stained sections were evaluated for histopathological changes at 1, 3 and 5 DPI. COBRA vaccinated animals had less lung involvement than polyvalent vaccinated animals at all time points examined (Figure 29B and 29D). COBRA vaccinated animals showed minor bronchial inflammation while polyvalent vaccinated animals exhibited slight thickening of the bronchial epithelium (Figure 29B). Both vaccinated groups showed little alveolar involvement compared to mock vaccinated animals. Mock vaccinated animals demonstrated greater lung involvement and severe alveolar infiltration (Figure 29B and 29D).

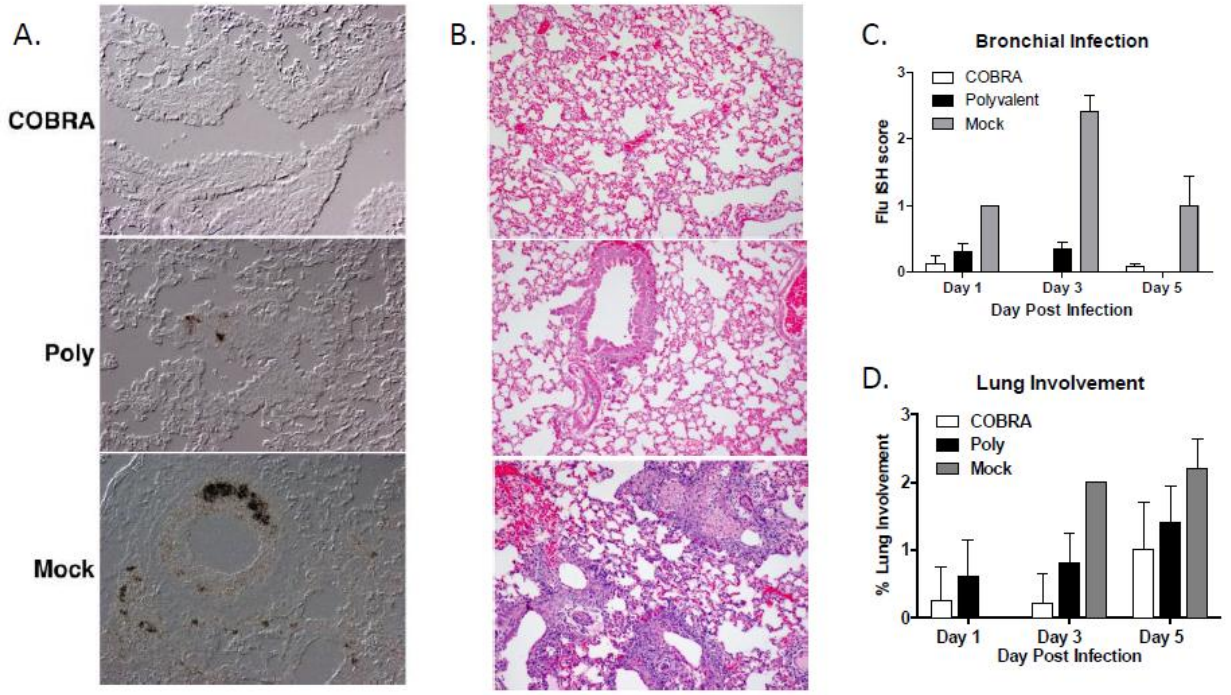

Figure 29: Histopathology of infected lungs. Vaccinated BALB/c mice (n=15/group) were infected with $5x10^3$ PFU of the highly pathogenic clade 2.2 H5N1 virus A/Whooper Swan/Mongolia/244/2005 (WS/05). Cohorts of mice (n=5/group) were sacrificed at 1, 3 and 5 days post infection. ISH for influenza MP (A) and hematoxylin and eosin staining (B) was performed on sections from paraffin embedded lung tissue. Representative ISH images are shown from 3 days post infection. Severity of influenza ISH foci was accessed in the bronchi at 1, 3 and 5 DPI (C). Scoring: 0 = no definitive signal; 1 = occasional focus; 2 = focus in most fields; 3 = more than one focus per field. Percentage of lung involvement was assessed in lung sections (D). Scoring: 0 = <10%; 1 = 10-24%; 2 = 25-50%; 3 = >50%.

6.5.4 Clade 1 challenge

Having established the clade 2.2 protective profile of both the COBRA and polyvalent vaccines, we next evaluated the efficacy of these vaccines against a more divergent, clade 1 challenge in mice. COBRA and polyvalent vaccinated mice were challenged with 6:2 reassortant virus containing the HA and NA proteins from the clade 1 virus A/Vietnam/1203/2004. All VLP

vaccinated animals were protected from weight loss and death, while mock vaccinated animals rapidly lost weight and reached experimental endpoint by 7 DPI (Figure 30A). Furthermore, vaccinated mice also did not develop any signs of disease throughout the course of the study (Figure 30B). Lungs were harvested at 3 DPI for determination of viral burden (Figure 30C). COBRA vaccinated animals did not have detectable virus while polyvalent animals had 1.1 x 10^3 PFU/g virus (p=0.12). Importantly, both vaccines had significantly less recoverable virus than mock vaccinated animals at 3 DPI (p<0.01).

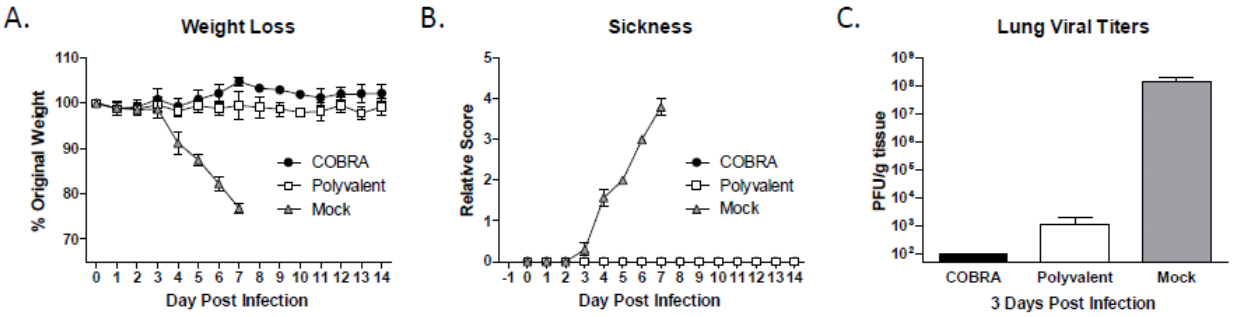

Figure 30: Clade 1 challenge. Vaccinated BALB/c mice (n=4/group) were infected with 5x10^3 PFU of reassortant virus containing the HA and NA genes from the clade 1 H5N1 virus A/Vietnam/1203/2004 (VN/04). Mice were monitored daily for weight loss (A) and sickness (B). Values represent mean (+/- SD) for each group. An additional cohort of vaccinated mice (n=3/group) were infected and lungs were harvested 3 days post infection for analysis of viral burden (C). Values represent mean (+/- SEM) viral titer for each group.

6.5.5 Post-challenge cellular immune responses

The magnitude of influenza specific cellular immune responses in the lungs post-infection was evaluated via ELISpot assay for both antibody secreting cells (ASC) and IFN-γ producing cells. Vaccinated mice were infected with reassortant A/Vietnam/1203/2004 virus as described above and lungs were harvested at 6 DPI. COBRA and polyvalent vaccinated animals had statistically equivalent numbers of both IgG and IgA ASC specific for the A/Vietnam/1203/2004 challenge virus HA (p>0.05; Figure 31A). No ASC were detected in mock vaccinated animals indicating

that the 6 DPI time point is likely representative of a recall response. Additionally, the majority of the ASC response to infection was specific for HA as lower numbers of cells were detected for the NA component of the vaccines.

VLP vaccine primed IFN-γ secreting cells were also evaluated after infection. IFN-γ responses were equivalent between VLP vaccine groups regardless of stimulating antigen ($p>0.05$; Figure 31B). As expected, recombinant HA and inactivated virus were inefficient stimulators of IFN-γ production compared to the HA_{533} peptide. HA_{533} is the immunodominant, HA-derived $CD8^+$ T cell epitope in BALB/c mice and is conserved in all HA vaccine antigens used in this study [301]. Overlapping peptide pools spanning the entire HA molecule were also used to stimulate cells and no differences were observed between COBRA and polyvalent vaccines for any of the pools (data not shown). Similar to the ASC data, no influenza specific IFN-γ responses were detectable above background in mock vaccinated animals at 6 DPI.

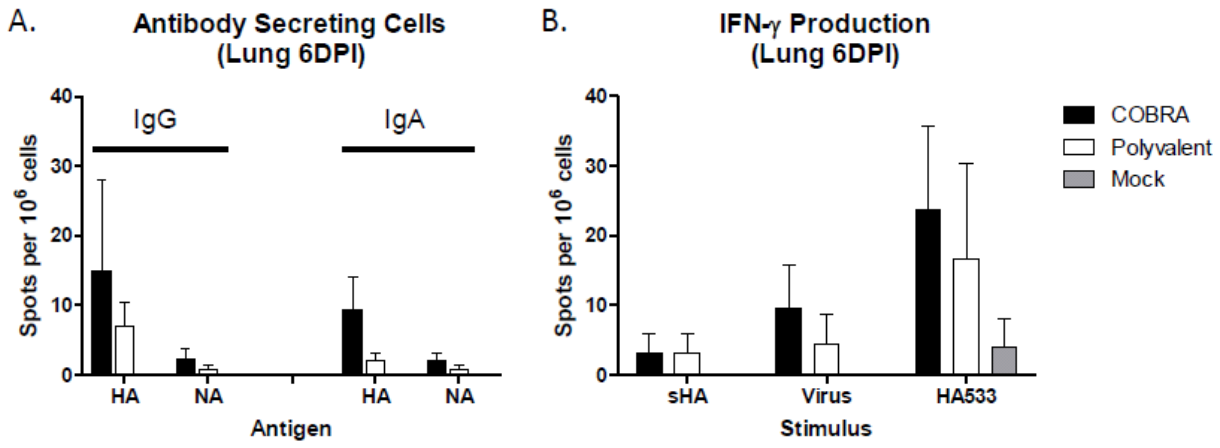

Figure 31: Post-challenge cellular immune responses. Vaccinated BALB/c mice (n=3/group) were infected with $5x10^3$ PFU of reassortant virus containing the HA and NA genes from the clade 1 H5N1 virus A/Vietnam/1203/2004 (VN/04). Mice were sacrificed 6 days post infection, lungs were harvested and the numbers of antibody secreting cells (A) and IFN-γ producing cells (B) were determined by ELISpot assay. Cells were stimulated with soluble HA (sHA), inactivated virus, or class I immunodominant peptide (HA533). Values represent the mean (+/- SEM) spots for each group.

6.5.6 Passive transfer of immune sera

The contribution of serum factors to protection from clade 1 challenge was evaluated using a passive transfer model. Nine week old recipient mice were administered pooled sera via IP injection from COBRA, polyvalent and mock vaccinated mice. The next day, recipient mice were challenged with the clade 1 reassortant A/Vietnam/1203/2004 virus. All recipient mice lost weight and became visibly ill (Figure 32A and 32B). Interestingly, COBRA serum recipient mice lost less weight than polyvalent recipient mice with maximum losses of 5.2% (6 DPI) and 11.8% (7 DPI), respectively ($p<0.05$ at 7 DPI). COBRA serum recipient mice also began to resolve the clinical symptoms more rapidly than polyvalent recipient mice ($p<0.05$ at 7 DPI). Although COBRA serum prevented recipient mice from developing illness more efficiently than polyvalent serum, both COBRA and polyvalent serum protected all recipient mice from death. Conversely, all mice receiving serum from mock vaccinated mice rapidly lost weight, became visibly ill and reached experimental endpoint by 7 DPI.

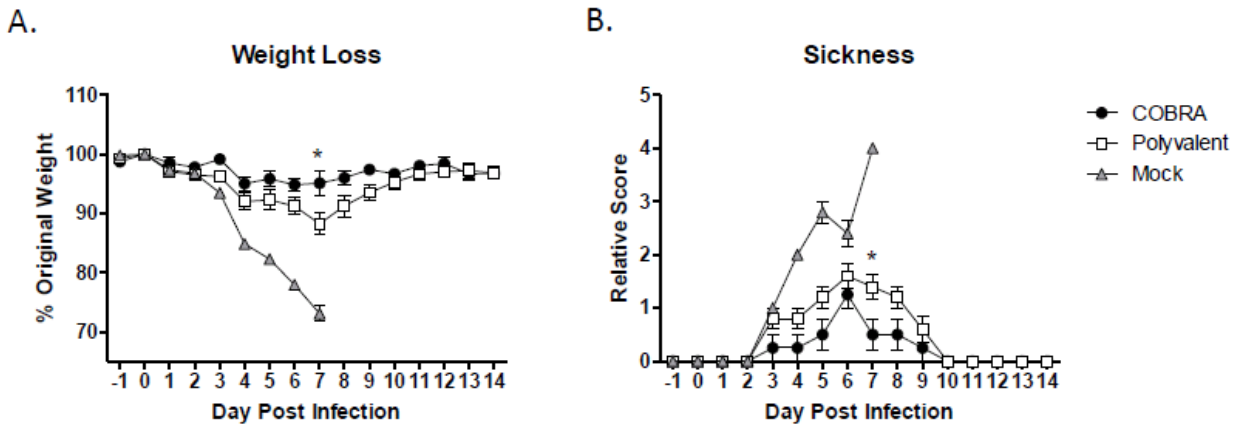

Figure 32: Passive transfer clade 1 challenge. BALB/c mice (n=10/group) were vaccinated at 0 and 3 weeks with blood collected 14 to 21 days after each vaccination. Serum collected after the second vaccination was pooled for each vaccine group and administered to naïve recipient mice (n=5/group). 1 day after passive transfer, recipient mice were infected with $5x10^3$ PFU of reassortant virus containing the HA and NA genes from the clade 1 H5N1 virus A/Vietnam/1203/2004 (VN/04). Mice were monitored daily for weight loss (A) and sickness (B). Values represent mean (+/- SD) for each group. Significant differences were determined by two-way ANOVA with Bonferroni's post-test to evaluate differences between vaccines at each day. A *p* value of less than 0.05 was considered significant (*).

6.6 DISCUSSION

In this study, we compared the immunogenicity and efficacy of two strategies proposed to increase breadth: computationally optimized broadly reactive antigen (COBRA) and a polyvalent mixture of primary antigens. Polyvalent vaccines have been used as a vaccine strategy to increase reactivity for many pathogens including, but not limited to, influenza [160-162], monkeypox [163], HIV [164, 165], HPV [166, 167] and pneumococcal disease [168]. Polyvalent vaccines consist of a mixture of several antigens and are designed to elicit an immune response that is broader than that elicited by any single component. Seasonal influenza vaccines have

traditionally been delivered as polyvalent formulations to address the diversity of currently circulating strains of influenza A (H1N1 and H3N2) and influenza B. Although polyvalent vaccine strategies are undoubtedly effective at expanding breadth, several limitations exist. First, with any polyvalent vaccine, production must be increased as to include several different antigens. In the context of H5N1 vaccination, doses required for seroconversion are higher than seasonal vaccines [188, 204] and to produce multiple vaccines at higher doses could become a difficult hurdle for vaccine manufacturers. Second, strain selection remains critical to polyvalent vaccine efficacy as best evidenced by seasonal influenza vaccine escape and continual yearly epidemics. Despite these limitations, the polyvalency strategy remains the standard approach for influenza vaccine design. Therefore, in this study, the breadth of immune responses elicited by COBRA HA antigens was compared to a polyvalent mixture of primary H5N1 HA antigens. Previous polyvalent vaccines for H5N1 have focused on inclusion of antigens from different clades [160, 161]. The COBRA HA antigen used in these studies is designed specifically to address the diversity present within clade 2 [283] and therefore selected clade 2 antigens from primary H5N1 isolates were generated as the polyvalent vaccine. Clade 2 is genetically diverse and is divided into distinct sub-clades including 2.1, 2.2, 2.3, 2.4 and 2.5 with some subclades being further divided into sub-subclades [196]. Furthermore, within clade 2, humans have been infected with isolates representing clades 2.1, 2.2 and 2.3 with the most recent human infections in Egypt identified as clade 2.2 [196]. To generate a polyvalent mixture covering the most prevalent subclades of clade 2, representative isolate were selected from clade 2.1 (A/Indonesia/5/2005), clade 2.2 (A/Whooper Swan/Mongolia//244/2005) and clade 2.3 (A/Anhui/1/2005).

Both COBRA and polyvalent vaccines efficiently elicited broad binding antibodies in mice and ferrets (Figure 26A and 26C). Even though both vaccines had equivalent levels of binding antibodies, the COBRA vaccine elicited a broader profile of receptor blocking antibodies (Figure 26B and 26D). Although the COBRA vaccine elicited higher titer receptor blocking antibodies,

the polyvalent vaccine did elicit antibodies to each of the components confirming the validity of using a polyvalent strategy to increase vaccine breadth (Figure 26B). Importantly, when any of the polyvalent components were used as a monovalent formulation, the receptor blocking antibody profile was limited to the homologous test antigen (data not shown). One reason for the decreased receptor blocking antibody titers in the polyvalent VLP vaccinated groups could be that each of the components was administered at one third of the total dose. While this is certainly a possible explanation, when individual components are given at a full dose, the titers are equivalent to the polyvalent vaccine for the homologous test antigen and remain decreased compared to COBRA (data not shown). Furthermore, prior studies indicated equivalent antibody responses and protection profiles for the different HA doses used in these studies, both total HA dose and individual component HA doses ([283] and unpublished observations).

Both COBRA and polyvalent vaccines protected mice and ferrets from highly pathogenic clade 2 H5N1 viral challenge (Figure 27). All vaccinated animals were protected from significant weight loss and did not develop overt signs of disease. Although protection from severe illness and death is certainly critical for pandemic vaccines, decreasing viral replication is also important to reduce complications from secondary infections and limit the potential for transmission. Efficient human-to-human transmission is an essential factor in pandemic influenza emergence and to date, H5N1 does not easily spread between people [302-304]. However, if H5N1 were to acquire an efficient human transmission phenotype, the ability of a vaccine to reduce viral titers and thereby limit potential spread in addition to preventing severe disease is an important factor. Animals receiving the COBRA vaccine had decreased levels of replicating virus and returned to baseline more rapidly than polyvalent vaccinated animals (Figure 28 and 29). Receptor blocking antibodies are not required to protect experimental animals against severe disease and death induced by highly pathogenic H5N1 influenza infection [200, 201, 206, 214, 215]. The findings reported here are consistent with those reports, but higher receptor blocking antibody titers against the challenge virus did correlate with

a reduction in duration of viral replication (Figures 26 and 28). Therefore, although high titer receptor blocking antibodies are not required for protection from severe disease, the presence of these antibodies may be predictive of reduced viral burden. Receptor blocking antibodies may be more effective at preventing nascent virions from infecting new cells and thereby limiting viral replication within the host. Viral replication could provide a more sensitive output for evaluating H5N1 vaccine efficacy.

The goal of these studies was to compare the breadth of two independent broadening strategies: COBRA and polyvalent. Although clade 2 is the most diverse and is spreading westward into the Middle East and Africa, clade 1 is still circulating and causing human disease in south-east Asia [196]. Clade 1 is not represented as a component of our polyvalent mixture nor was it part of the COBRA design and as such it represents a stringent test to evaluate vaccine efficacy for both broadening strategies. Vaccinated animals had anti-clade 1 binding antibodies at levels equivalent to that of clade 2 test antigens (Figure 26A and 26C). Despite these high titer binding antibodies, both vaccines elicited low to undetectable levels of anti-clade 1 receptor blocking antibodies (Figure 26B and 26D). Consistent with the findings that receptor blocking antibodies are not predictive of protection from severe disease, all vaccinated animals were protected from weight loss and development of visible illness after challenge with a reassortant virus containing the HA and NA antigens derived from a clade 1 virus (Figure 30). Furthermore, COBRA vaccinated animals did not have detectable virus after 3 days post infection, while polyvalent had low levels of virus present. Although the differences were robust as predicted, the viral replication in COBRA vaccinated animals was below the limit of detection of the assay and animals receiving the polyvalent vaccine had recoverable virus 3 days after clade 1 challenge. These results indicate that even though clade 1 sequences were not included in the design of COBRA, the synthetic antigen serves as an effective vaccine against a divergent virus, even one that is antigenically distinct from the original input sequences.

In the absence of receptor blocking antibody, it is possible that cellular immune responses contribute to the protection from severe disease and death [305-307]. Both COBRA and polyvalent vaccines elicited similar cellular recall responses at 6 days post infection (Figure 31). Both IgG and IgA antibody secreting cells (ASC) specific for the clade 1 HA were recruited to the lungs of vaccinated animals and this is likely a recall response as unvaccinated controls did not have any ASC above background at 6 days post infection (Figure 31A). ASC in either the spleen or bone marrow were not detectable above the background of the assay in any group before or after challenge (data not shown). Interestingly, the majority of ASC were specific for HA rather than NA. Although the NA content of the vaccines was not standardized, all vaccinated animals had high anti-NA serum IgG prior to challenge (data not shown). It is possible that NA-mediated immunity contributes to protection from challenge in the absence of receptor blocking antibodies, but the preferential recruitment of anti-HA ASC implies a more critical role for anti-HA binding antibodies. An important limitation of the ASC assay used in this report is there was no stimulation of cells *ex vivo*, so it is possible that equivalent numbers of anti-HA and anti-NA memory cells are present, but mainly anti-HA memory cells are activated and recruited to the lung in response to infection. An additional implication for the equivalent numbers of ASC for both vaccine groups is that antibody titer is not directly correlated with responsible receptor blocking (Figure 26B). This is not a surprising finding since the titer of binding antibody were equivalent between the two vaccine groups (Figure 26A). Importantly, both strategies resulted in recruitment of equivalent numbers of ASC in response to a completely heterologous infection.

One of the proposed advantages for utilizing centralized antigens is expanding the breadth of T cell epitopes [180, 255, 257]. Indeed, a consensus-based H5N1 elicited IFN-γ producing cells in response to multiple peptide pools of HA [181]. Both the COBRA and polyvalent vaccines elicited IFN-γ responses in the lungs of infected mice by 6 days post

infection and the numbers of responding cells between the vaccine groups were equivalent regardless of stimulating antigen (Figure 31B). The immunodominant epitope in HA for BALB/c mice, HA_{533}, is conserved in all of the vaccine antigens and may be responsible for the equivalent responses between the two vaccine groups. Additionally, the COBRA vaccine did not result in expansion of breadth of T cell reactivity across different regions of HA, as measured by stimulation with overlapping peptide pools (data not shown). COBRA failed to expand the breadth of T cell responses in these experiments, which may be a result of the high levels of homology between HA antigens. H5N1 HA antigens are >90% identical amino acids between strains and the majority of the diversity is focused on altering antibody binding sites that are usually non-linear, conformational epitopes. Therefore, any potential T cell epitopes are not changing due to immune pressure and likely remain highly conserved between strains. Indeed, the HA_{533} BALB/c immunodominant epitope is conserved not only throughout H5N1 strains but also H1N1 and H9N2.

The presence of B and T cell recall responses in the lungs of vaccinated mice after challenge could be a potential mechanism for protection rather than serum antibody. To evaluate the role of serum factors in protecting mice from a heterologous challenge, we passively transferred immune serum to naïve recipients and challenged with a clade 1 reassortant virus. Both COBRA and polyvalent immune serum protected mice from severe disease and death (Figure 32). Therefore, serum antibody was able to protect from lethal challenge in the absence of cellular recall responses. COBRA immune sera recipient animals lost less weight and recovered from illness more rapidly than polyvalent recipient animals, most likely as a result of moderate levels of anti-clade 1 receptor blocking antibodies in the COBRA serum (Figure 26B). In comparison to directly vaccinated and challenged mice, passive transfer recipient mice lost more weight and developed more clinical signs. One possibility for this disparity is that for the transferred antibodies to gain access to the site of infection in the airway,

there must first be damage to lung. Transferred antibodies could then function in several ways: direct neutralization of virus, opsonization of viral particles, complement fixation and/or impairment of viral egress from infected cells. The initial damage would then be associated with the observed mild weight loss and development of visible sickness and the speed of recovery related to the quality and function of the transferred antibody. Importantly, this is the first description of passive transfer-mediated protection by a centralized antigen for H5N1 indicating that serum antibody is indeed an important factor in heterologous protection.

This is the first report comparing the breadth of a centralized H5N1 antigen, COBRA, with the more traditional strategy of a polyvalent mixture. The COBRA vaccine elicited a more robust antibody profile than the polyvalent vaccine or any of its individual components. This immunity was completely protective against both clade 2 and clade 1 virus challenges and COBRA vaccinated animals had lower viral burdens and lung involvement compared to polyvalent vaccinated animals. The cellular immune components (ASC and IFN-γ) were equivalent between the two VLP vaccinated mouse groups, indicating that the enhanced control of viral replication observed in the COBRA vaccinated animals is likely antibody mediated. Indeed, a passive transfer model demonstrated that animals receiving COBRA immune serum were more efficiently protected from disease than those receiving polyvalent immune serum. The data presented in this report indicate that although binding antibodies are sufficient for protection from severe disease and death, receptor blocking antibodies are essential for reducing viral replication. This observation suggests that in the context of pandemic preparedness, vaccines that elicit receptor blocking antibodies to a diverse set of viruses would be more effective at limiting viral replication, transmission, and disease impact. Antigenic diversity is a challenge for not only H5N1 influenza, but all influenza subtypes. Polyvalent vaccines are currently utilized to address multiple types and subtypes of influenza simultaneously circulating in the human population. It remains to be determined if COBRA-

based vaccine design will be able to overcome diversity between subtypes, as greater levels of diversity and structural limitations are challenges for antigen design. Both COBRA and polyvalent strategies are effective at expanding antibody breadth in the context of influenza vaccination. These two strategies are not mutually exclusive and an intriguing possibility is a combination thereof: a polyvalent mixture of COBRA antigens. Combining the intrasubtype broadening ability of COBRA with the intersubtype advantages of polyvalent mixtures represents an interesting strategy for expanding breadth within and between subtypes. COBRA-based vaccines are effective at broadening the antibody repertoire against H5N1 and applying this design strategy to other subtypes of influenza warrants further investigation.

7.0

ELICITATION OF ANTI-1918 INFLUENZA IMMUNITY EARLY IN LIFE PREVENTS MORBIDITY AND LOWER-LUNG INFECTION BY 2009 PANDEMIC H1N1 INFLUENZA IN AGED MICE.

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7.1 FOREWORD

The work presented in this chapter is in addition to the studies outlined in the Specific Aims (see Chapter 3). The studies detailed previously (Chapters 4, 5 and 6) focused on broadly reactive vaccine development in anticipation of what may be the next influenza pandemic: H5N1. However, during the course of the work, the 2009 H1N1 "swine flu" influenza pandemic occurred. The emergence of a novel strain of human influenza is a rare event and provided an opportunity to evaluate pandemic influenza vaccines in "real-time." Thus, the work presented in this chapter describes H1N1 pandemic vaccine studies. In particular, the longevity and mechanism of protective immune responses was evaluated. Although the work described here specifically addresses H1N1, the findings of long lasting immunity can be applied to vaccine responses in general and demonstrate that pandemic influenza vaccines have the potential to elicit long-lasting immune responses that can protect from pandemic influenza infection years after original vaccination.

7.2 ABSTRACT

The rapid emergence and spread of swine origin H1N1 in 2009 resulted in the first pandemic of the 21st century. It is estimated that the "Spanish" influenza pandemic of 1918 was responsible for 40 to 50 million deaths worldwide and is antigenically similar to the swine lineage of influenza that is represented by the 2009 pandemic. Emergence of the 2009 pandemic from swine into humans has raised the possibility that low-levels of cross protective immunity to past shared epitopes could confer some protection. In this study, we have engineered an influenza virus-like particle (VLP) expressing the HA and NA genes from the 1918 influenza virus to

evaluate the duration of cross-protective efficacy to the 2009 H1N1 pandemic strain by vaccinating young mice (8-12 weeks) and then allowing the animals to age to 20 months. 1918 VLPs were found to elicit protective immunity in adult mice when lethally challenged with the reconstructed 1918 influenza virus. This immunity was long-lasting with homologous receptor blocking antibodies detected throughout the lifespan of vaccinated mice. Furthermore, the 1918 VLP fully protected aged mice from 2009 pandemic H1N1 virus challenge 16 months after vaccination. Histopathological assessment showed that aged vaccinated mice had significant protection from alveolar infection, but less protection of the bronchial tissue than adult vaccinated mice. Additionally, passive transfer of immune serum from aged vaccinated mice resulted in protection from death, but not morbidity. This is the first report describing the lifelong duration of cross-reactive immune responses elicited by a 1918 VLP vaccine in a murine model. The results show that antibody responses elicited early in life are sufficient for protection from morbidity and mortality when challenged with an antigenically similar virus 16 months post-vaccination. Importantly, these life-long immune responses did not result in decreased total viral replication, but did prevent infection of the lower respiratory tract. These findings demonstrate that immunity acquired early in life can restrict the anatomical location of influenza viral replication, rather than preventing infection, in the aged.

7.3 INTRODUCTION

Infections with influenza virus result in ~200,000 hospitalizations and 36,000 deaths for a typical endemic season in the United States [64]. In 2009, a novel strain of H1N1 influenza emerged from swine and quickly spread among humans resulting in the World Health Organization declaring the first pandemic of the 21st century [72]. The 1918 “Spanish” influenza pandemic

was the worst pandemic in recorded history and caused severe disease and mortality (675,000 total deaths) in the United States [308] and killed up to 50 million people worldwide [75]. In comparison to 1918, the 2009 pandemic virus is much more moderate with the majority of cases being uncomplicated [309]. The most common feature of fatal disease is varying degrees of alveolar infection and damage [310-312]. This differs from seasonal influenza, as fatal cases rarely involve alveolar cells, with virus located primarily in the major airways such as the trachea and bronchioles [313, 314]. Interestingly, the majority of severe cases from the 2009 H1N1 pandemic were reported in children and young adults, while the elderly population was relatively protected from infection and severe disease [309]. This pattern of susceptibility to severe disease is in direct contrast to what is normally observed during seasonal influenza epidemics, but is similar to what was reported for the 1918 pandemic [315]. Although the 1918 pandemic is believed to have emerged from avian species into both swine and humans nearly simultaneously, the human and swine lineages quickly diverged. Sequence analysis indicated that the 2009 H1N1 pandemic virus is related to the 1918 H1N1 virus and it has been proposed that the swine population has maintained an “antigenically frozen” H1N1 lineage [316]. Structural analysis demonstrated conservation within antigenic regions of 1918 and 2009 pandemic hemagglutinin (HA) proteins that is not present in contemporary seasonal H1N1 viruses [317, 318].

Antigenic similarities together with the abnormal protection from severe disease in the elderly population have led to the hypothesis that exposure to 1918-like viruses confers cross-protective immune responses [319, 320]. Several studies have indicated cross-reactive antibodies to the 2009 pandemic H1N1 viruses in elderly human populations [321, 322] with antibodies derived from survivors of the 1918 pandemic cross-neutralizing the 2009 pandemic viruses [323]. Additionally, direct evidence of the cross-protective efficacy elicited by exposure to 1918-like viruses has been demonstrated in small animal models [316, 324], but the duration of this cross-protective immune response(s) has not been evaluated.

Aging is associated with decreasing ability of the immune system to respond to new antigens [325]. Although elderly individuals are impaired in their immune responses, immunological memory responses to antigens experienced prior to the onset of decreased immune function can be retained and offers protection against re-exposure to similar pathogens. Indeed, successful vaccines are usually antibody-based and memory B cell responses, at both the cellular and antibody level, can persist for a lifetime [326]. After exposure to antigen, B cells can develop into two main types of long lived cells: memory B cells (MBC) and long-lived plasma cells (LLPC) [327]. Both subsets generally develop in the germinal center and go through differential degrees of affinity maturation: MBC differentiate earlier and have fewer somatic mutations while LLPC emerge later with higher affinity [327-329]. Both subsets play important roles in preventing reinfection with LLPC producing high affinity antibody in the absence of antigen stimulation and MBC being able to respond to a second infection that escapes circulating antibody [330]. The evidence of cross-reactive antibodies and decreased disease severity in the older human populations indicates that memory B cell responses to previously experienced antigens are cross-reactive to the 2009 pandemic virus. Protection of the elderly population has largely been attributed to circulating antibody derived from the LLPC arm of the B cell memory response. Interestingly, infection with 2009 pandemic virus in humans led to development of broadly-neutralizing antibody producing cells and it has been suggested that these cells were derived from pre-existing MBC [331]. Animal models have confirmed the hypothesis that prior exposure to various H1N1 virus strains can indeed protect from 2009 pandemic challenge, but mechanistic inquiries into the reason for the cross-protection have been largely antibody-focused and have thus far failed to evaluate the involvement of the cellular component [316, 324].

Our research group has developed virus-like particle (VLP) vaccines against a variety of viral pathogens [221, 222, 283, 332-334]. These vaccines are composed of non-infectious, non-replicating VLPs that present functional HA and NA on the surface of a viral particle. VLPs

efficiently elicit high titer immune responses that protect small animals against lethal viral challenge [222, 283]. In this study, HA and NA from the 1918 influenza virus, A/South Carolina/1/1918, were expressed on the surface of a VLP that was expressed and purified from mammalian cells. Mice were vaccinated at a young age (8-12 weeks) and allowed to age to elderly status (20 months). Antibody titer to the 1918 antigens persisted for the lifetime of the animals indicating that the non-replicating VLP vaccine elicits enduring antibody titers. The 1918 VLPs efficiently protected the aged mice from 2009 pandemic challenge and prevented alveolar infection. Additionally, the protection involved not only the previously described cross-reactive antibodies but also a robust cellular recall response.

7.4 MATERIALS AND METHODS

7.4.1 Purification of virus-like particles

Human embryonic kidney (HEK) 293T cells (1×10^6) were transiently transfected with plasmids expressing Gag_{p24} alone or together with HA and NA and incubated for 72h at 37°C. Plasmids expressing the HIV-1_{NL4-3} Gag gene products only, $pGag_{p24}$, was derived from codon-optimized sequences (phGag), as previously described [335]. $pGag_{p24}$ encodes for an immature, unprocessed HIV-1 Gag particle. Plasmids expressing HA (A/South Carolina/1/1918) and NA (A/Brevig Mission/1/1918) genes were kindly provided by Dr. A. Garcia-Sastre. Supernatants were collected and cell debris removed by low speed centrifugation followed by vacuum filtration through a 0.22 μm sterile filter. VLPs were purified via ultracentrifugation (100,000 X g through 20% glycerol, weight per volume) for 4 h at 4°C. The pellets were subsequently resuspended in phosphate buffered saline (PBS) and stored at -80°C until use. Protein concentration was

determined by Micro BCA™ Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA).

7.4.2 Animals and vaccinations

BALB/c mice (*Mus musculis*, females, 6–8 weeks) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA), housed in microisolator units, allowed free access to food and water and cared for under USDA guidelines for laboratory animals. Mice were vaccinated with 3μg of purified VLPs based upon total protein via intramuscular injection at study weeks 0 and 3. Vaccination experiments were initially prepared with and without 10μg CpG oligonucleotides (Sigma-Aldrich, St. Louis, MO, USA). Due to no observed effect of the adjuvant, subsequent vaccinations investigating longevity and cross-reactivity were performed without inclusion of adjuvant. 14 to 21 days after each vaccination, blood was collected from anesthetized mice via the retro-orbital plexus and transferred to a microfuge tube. Tubes were centrifuged and sera was removed and frozen at -20°C. A subset of vaccinated mice was allowed to age to a final age of 20 months (~17 months post final vaccination) with blood collected at 10 months and 20 months of age. All procedures were in accordance with the NRC Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

7.4.3 ELISA assay

The ELISA assay was used to assess total antibody titer and IgG isotype titer to the 1918 HA. High binding, 96-well polystyrene plates (Costar; Lowell, MA, USA) were coated overnight with 50ng/well of recombinant 1918 HA. Plates were blocked with 5% milk diluted in PBS with

0.05% Tween 20. Serum samples were diluted in blocking buffer and added to plates. Serum was two-fold serially diluted and allowed to incubate for 1 hour at room temperature. Plates were washed and species specific antibody against IgG, IgG1, IgG2a, IgG2b or IgG3 and linked to horseradish peroxidase (HRP) (Southern Biotech; Birmingham, AL, USA) were diluted in blocking buffer and added to plates. Plates were incubated for 1 hour at room temperature. Plates were washed and HRP was developed with TMB substrate (Sigma-Aldrich; St. Louis, MO, USA). Plates were incubated in the dark for 30 minutes and then the reaction was stopped with 2N H_2SO_4. Optical densities at a wavelength of 450nm (OD_{450}) were read by a spectrophotometer (BioTek; Winooski, VT, USA) and end point dilution titers were determined. End point titers were determined as the reciprocal dilution of the last well which had an OD_{450} above the mean OD_{450} plus two standard deviations of naïve animal sera.

7.4.4 Hemagglutination inhibition assay

The hemagglutination inhibition (HAI) assay was used to assess functional antibodies to the HA able to inhibit agglutination of turkey erythrocytes. The protocol was adapted from the CDC laboratory-based influenza surveillance manual [233]. To inactivate non-specific inhibitors, sera were treated with receptor destroying enzyme (RDE) prior to being tested [234-238]. Briefly, three parts RDE was added to one part sera and incubated overnight at 37°C. RDE was inactivated by incubation at 56°C for ~30 min. RDE treated sera was two-fold serially diluted in v-bottom microtiter plates. An equal volume of either 1918 VLP or wild type pandemic H1N1 virus, adjusted to approximately 8 HAU/50µl, was added to each well. 1918 VLPs were produced as described above. Wild type viruses were propagated in eggs and included the strains A/California/07/2009 and A/Mexico/4108/2009. The plates were covered and incubated at room temperature for 20 min followed by the addition of 1% turkey erythrocytes (RBC) (Lampire Biologicals, Pipersville, PA, USA) in PBS. Red blood cells were stored at 4°C and

used within 72 hours of preparation. The plates were mixed by agitation, covered, and the RBCs were allowed to settle for 30 min at room temperature [239]. The HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Positive and negative serum controls were included for each plate. All mice were negative (HAI ≤ 10) for pre-existing antibodies to currently circulating human influenza viruses prior to vaccination.

7.4.5 Virus microneutralization

To assess the ability of mouse immune antisera to inhibit replication of live 1918 virus, the virus microneutralization assay was used as previously described [336]. Briefly, sera were two-fold serially diluted and then incubated with 100 $TCID_{50}$ of 1918 virus for 60 minutes at room temperature. The serum-virus mixture was then added to MDCK cells and allowed to incubate for 2 days at 37°C. Specific neutralizing activity was calculated as the lowest concentration of serum that displayed neutralizing activity.

7.4.6 Viral challenge

To determine the homologous efficacy of the 1918 vaccine, mice were challenged with the reconstructed 1918 virus [133, 337]. Briefly, two weeks after the final vaccination, adult animals were challenged intranasally with 50 LD_{50} of 1918 virus in a volume of 50μl. Mice were monitored daily for disease signs and death for 16 days post-infection. Body weights were recorded for individual mice at various days post-inoculation. All virus challenge experiments were performed under the guidance of the U.S. National Select Agent Program in negative-pressure HEPA-filtered biosafety level 3+ (BSL-3+) enhanced laboratories with the use of a battery-powered Racal HEPA filter respirator and according to Biomedical Microbiological and Biomedical Laboratory procedures [338].

To determine the cross-protective efficacy of the 1918 vaccine, mice were infected with a 2009 pandemic H1N1 isolate: A/Mexico/4108/2009. Although this virus is not highly lethal in adult BALB/c mice, it does cause 10-20% weight loss and development of clinical illness (data not shown). Briefly, animals were challenged intranasally with 1 x 10^6 plaque forming units (PFU) of A/Mexico/4108/2009 virus in a volume of 50μl. Mice were monitored daily for disease signs and death for 14 days post-infection. Body weights and sickness scores were recorded for individual mice at various days post-infection. Sickness scores were determined by evaluating activity (0=normal, 1=reduced, 2=severely reduced), hunched back (0=absent, 1=present) and ruffled fur (0=absent, 1=present) [230]. Any animal reaching >20% weight loss was humanely euthanized. All experiments using 2009 pandemic H1N1 virus were performed under biosafety level 2 (BSL2) conditions.

7.4.7 Virus titrations

On day 4 post 1918 virus challenge, four mice per group were exsanguinated, euthanized and their lungs removed for virus titration. Lungs were homogenized in 1 ml of sterile PBS, and clarified homogenate virus titers were determined using a 50% egg infectious dose (EID_{50}) method. Homogenates were titrated for virus infectivity in eggs from initial dilutions of 1:10, and EID_{50} was calculated using the method of Reed and Muench [339]. The limit of virus detection was $10^{1.5}$ EID_{50}/ml.

For 2009 pandemic H1N1 virus infections, lung virus titers were determined using a plaque assay [241, 242]. Briefly, lungs from infected mice were harvested 4 days post-infection (DPI), snap-frozen and stored at -80°C until use. Samples were thawed, weighed and single cell suspensions were prepared via passage through a 70 μm mesh (BD Falcon, Bedford, MA, USA) in an appropriate volume of PBS as to achieve 100mg/ml final concentration. Cell

suspensions were centrifuged at 2000 rpm for 5 min and the supernatants were collected. Madin-Darby Canine Kidney (MDCK) cells were plated (5×10^5) in each well of a 6 well plate. Lung supernatants were diluted (dilution factors of 1×10^1 to 10^6) and overlayed onto the cells in 100 μl of DMEM supplemented with penicillin-streptomycin and incubated for 1hr. Virus-containing medium was removed and replaced with 2 mls of L12 medium plus 0.8% agarose (Cambrex, East Rutherford, NJ, USA) and incubated for 96 hrs at 37°C with 5% CO_2. Agarose was removed and discarded. Cells were fixed with 10% buffered formalin, and then stained with 1% crystal violet for 15 min. Following thorough washing in dH_2O to remove excess crystal violet, plates were allowed to dry, plaques counted, and the plaque forming units (PFU)/g were calculated.

7.4.8 Histopathological analysis

Left lobes of lungs from infected mice were collected 4 days post-infection and placed into 10% buffered formalin. After fixation, lungs were paraffin embedded and 6 μm sections were prepared for histopathological analysis. Tissue sections were stained with hematoxylin and eosin and examined for bronchial inflammation and denudation and alveolar infiltration.

Immunohistochemistry was performed as described before [340]. Sections containing lung were stained using antibodies against Influenza A virus (1:500; Maine Biotechnology Services, Portland, ME), Iba1 (1:500; Wako Pure Chemical Industries, Osaka, Japan), CD3 (1:500; Dako, Carpinteria, CA), myeloperoxidase (1:250; Abcam, Cambridge, MA), IgA (1:500; Sigma), IgG (1:500; Sigma), and IgM (1:1000; Sigma) followed by species appropriate secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA and Rockland Immunochemicals, Gilbertsville, PA) conjugated to a fluorophore for fluorescent stains or biotin for peroxidase-based stains. Stains were assessed and scored for frequency of influenza-

infected cells and abundance of CD3+ T cells, Iba1+ macrophages, myeloperoxidase-labeled neutrophils, and IgA expressing cells.

For *in situ* hybridization (ISH), vectors containing 760 bp of Influenza/California/04/2009 matrix protein and full-length murine interferon-β (Open Biosystems, Huntsville, AL) were linearized to create antisense and sense templates. ^{35}S-labeled riboprobes were generated using MAXIscript *in vitro* transcription kit (Ambion, Austin, TX). ISH was performed as described before (Bissel et al 2011 "Accepted Article" doi: 10.1111/j.1750-3639.2010.00514.x). Control riboprobes did not hybridize to lung tissue at any time point post-infection and non-infected tissue did not show hybridization with viral probes. Hybridized slides were assessed and scored for abundance of foci.

7.4.9 Cellular assays

The number of anti-influenza specific cells secreting interferon gamma (IFNγ) was determined by enzyme-linked immunospot (ELISpot) assay (R&D systems, Minneapolis, MN, USA) following the manufacturer's protocol. Mice were sacrificed at 6 DPI and spleens and lungs were harvested and prepared in single cell suspensions. Briefly, pre-coated anti-IFNγ plates were blocked with RPMI plus 10% FCS and antibiotics (cRPMI) for 30 minutes at room temperature. Media was removed from wells and 10^5 cells were added to each well. Cells were stimulated with 1918 recombinant HA (truncated at residue 530; 1μg/well), inactivated A/Mexico/4108/2009 virus (1:100 dilution of inactivated stock; 100μl/well) or the immunodominant H2-K^d CD8$^+$ T cell epitope in H1 HA: HA_{533} (IYSTVASSL; 1μg/well) (Pepscan Presto, Leystad, Netherlands). Additional wells were stimulated with PMA (50ng/well) and ionomycin (500ng/well) as positive controls or Ova_{257} (SIINFEKL; 1μg/well) (Pepscan Presto, Leystad, Netherlands) as negative controls. Additionally, IL-2 (10U/ml) was added to each well.

Plates were incubated at 37°C for 48 hours. After incubation, plates were washed four times with R&D wash buffer and incubated at 4°C overnight with biotinylated anti-mouse IFNγ. Plates were washed as before and incubated at room temperature for 2 hours with streptavidin conjugated to alkaline phosphatase. Plates were washed as before and spots were developed by incubating at room temperature for 1 hour in the dark with BCIP/NBT chromogen substrate. The plates were washed extensively with DI H_2O and allowed to dry overnight prior to spots being counted using an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

The number of anti-1918 HA specific antibody secreting cells was determined by B cell ELISpot assay as previously described [243-245]. Mice were sacrificed at 6 DPI and spleens and lungs were harvested and prepared in single cell suspensions. Briefly, 0.45μm PVDF membrane plates (Millipore, Billerica, MA, USA) were coated with 1918 recombinant HA (250ng/well) and incubated at 4^oC overnight. Plates were washed three times with PBS and blocked with cRPMI for at 37°C for 3-4 hours. Media was removed from wells and 10^5 cells were added to each well. Plates were incubated at 37^oC for 48 hours. After incubation, plates were washed as before and incubated at room temperature for 2 hours with horse radish peroxidase-conjugated anti-mouse IgG (Southern Biotech, Birmingham, AL, USA). Plates were washed as before and spots were developed at room temperature for 1 hour in the dark with detection substrate (NovaRED™; Vector Labs, Burlingame, CA, USA). The plates were washed extensively with DI H_2O and allowed to dry overnight prior to spots being counted using an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

7.4.10 Passive transfer of sera

Sera from adult and aged vaccinated mice were pooled within the respective age group and passively transferred into 9 week old recipient BALB/c mice (n=5/group). Equal amounts of serum from each mouse in a particular vaccine/age group were pooled and heat inactivated for 30 minutes at 56^{o}C. 200 μl of pooled and inactivated serum was transferred to recipient mice via IP injection. 24 hours post-transfer, mice were infected with 2009 pandemic H1N1 virus as described above.

7.4.11 Statistical analysis

Statistical analyses of immune responses were performed using a One-way analysis of variance (ANOVA) with Dunn's post-test to compare each group. A *p*-value of <0.05 was considered significant. For challenge experiments, statistical analysis was performed using a two-way ANOVA with Bonferroni's post-test to compare each group at multiple time points. A *p*-value of <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism software.

7.5 RESULTS

7.5.1 Homologous immunogenicity of 1918 VLP vaccines in mice

BALB/c mice (6-8 weeks) were vaccinated twice at weeks 0 and 3 via intramuscular injection with purified influenza VLP vaccines plus CpG adjuvant. Two weeks after the final vaccination

serum was analyzed for antibody responses. All mice vaccinated with 1918 VLPs had HAI antibodies to the homologous test antigen with a geometric mean titer (GMT) of 1:113 (Figure 33A). In contrast, mice receiving Gag VLPs or CpG adjuvant alone failed to generate any HAI antibodies to the 1918 VLP (Figure 33A). To evaluate the ability of the elicited antibody response to block live virus infection, serum was tested in a live virus neutralization assay. Similar to the 1918 VLP HAI results, all mice vaccinated with 1918 VLPs had neutralizing antibodies with a GMT of 1:208, while mice receiving Gag VLPs or CpG alone failed to generate any detectable neutralizing antibodies (Figure 33B). Additional vaccines administered without the CpG adjuvant elicited similar HAI titers and therefore CpG adjuvant was removed from subsequent vaccine preparations (data not shown).

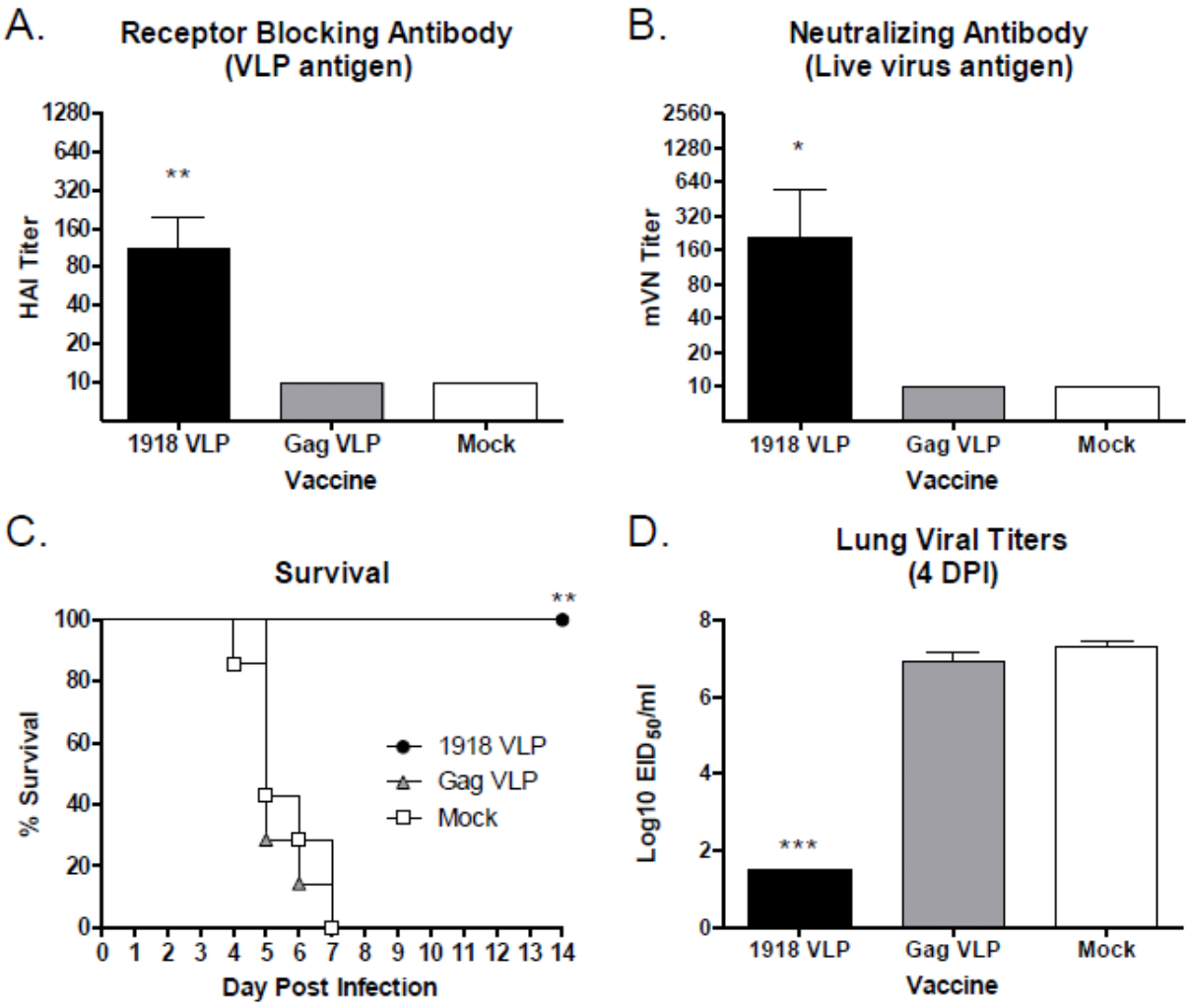

Figure 33: 1918 immunogenicity and protection. BALB/c mice (n=10/group) were vaccinated at 0 and 3 weeks with blood collected 2 weeks after final vaccination. Hemagglutinin inhibition (HAI) serum antibody titers were determined for each vaccine group using 1918 VLPs as the test antigen (A). Bars represent the geometric mean titer (±95% confidence interval). Virus neutralizing (mVN) antibody titer was measured using reconstructed 1918 virus (B). Bars represent the geometric mean titer (±95% confidence interval). 2 weeks after final vaccination, mice were infected with a lethal dose of reconstructed 1918 virus (C). 4 days post infection, lung virus titers (n=3/group) were determined (D). Bars represent the log10 transformed mean virus titer (± standard deviation). A p value of less than 0.05 was considered significant (*p<0.05, **p<0.01, ***p<0.001).

7.5.2 Protection against 1918 influenza virus challenge

Two weeks after the final vaccination, mice were challenged intranasally with a lethal dose of live 1918 virus to evaluate the protective efficacy of the vaccines. 1918 VLP-vaccinated mice were protected from weight loss (maximum 5.2% at 2 DPI) while Gag VLP and CpG alone vaccinated animals rapidly lost weight (maximum weight loss of 14.2% at 5 DPI and 13.7% at 5 DPI, respectively) (data not shown). Furthermore, mice receiving the 1918 VLP vaccine were completely protected from death while Gag VLP and CpG alone vaccinated animals completely succumbed to infection by 7 DPI (Figure 33C). To determine the ability of the vaccines to control viral replication in the respiratory tract, lungs of infected mice (n=3) were collected at 4 DPI and analyzed for viral titers (Figure 33D). Mice vaccinated with 1918 VLPs did not have detectable virus while mice receiving the Gag VLPs or CpG alone had significantly higher viral loads (p<0.001).

7.5.3 Longevity of the antibody response

To determine the duration of the antibody response elicited by the 1918 VLP, mice (n=15) were vaccinated twice with 3μg of purified 1918 VLP without adjuvant and allowed to age. Serum was collected at 3 months (two weeks post-final vaccination), 10 months and 20 months of age and analyzed for antibody responses to the 1918 VLP (Figure 34A). Homologous antibody responses were detected in vaccinated mice over the lifetime of the animals. Although the HAI GMT decreased from 1:127 to 1:88 to 1:54 at 3, 10 and 20 months, respectively, the observed differences were not significant (p>0.05) indicating that the 1918 VLPs elicited lifelong homologous antibody responses.

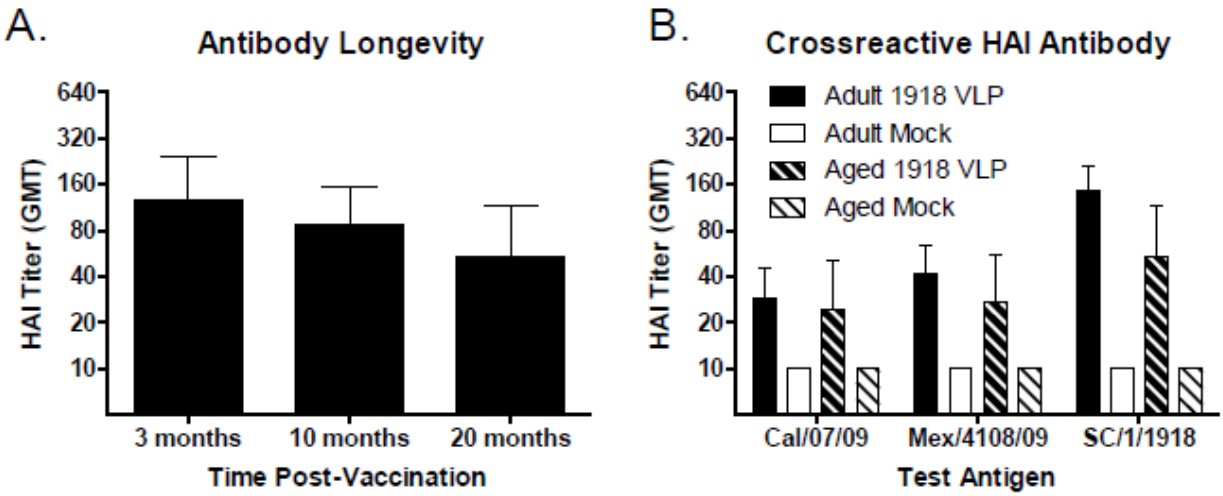

Figure 34: Duration of cross-reactive antibody response. BALB/c mice were vaccinated in the first 12 weeks of life with blood collected at 3 months, 10 months and 20 months. Hemagglutination inhibition (HAI) serum antibody titers were evaluated at each time point using 1918 VLPs as the test antigen (A). Sera from adult vaccinated mice (2 weeks post final vaccination) and aged vaccinated mice (16 months post final vaccination) were evaluated for HAI activity against 2009 pandemic viruses (Cal/07/09 and mex/4108/09) and 1918 VLPs (SC/1/1918) (B). Bars represent geometric mean titer (±95% confidence interval). A p value of less than 0.05 was considered significant.

7.5.4 Heterologous antibody responses in aged mice

To evaluate if antibodies elicited by the 1918 VLPs cross-reacted with 2009 pandemic viruses, sera from 1918 VLP vaccinated adult (3-4 months; n=13) and aged (20 months; n=14) animals were analyzed for heterologous antibody responses. Sera from adult mice was collected 2 weeks after the final vaccination, while sera from aged mice was collected 16 months (~64 weeks) after the final vaccination. Both adult and aged animals had 2009 pandemic cross-reactive antibodies and no significant differences between the age groups was detected (p>0.05; Figure 34B). Although the 2009 pandemic HAI titers in the 1918 VLP vaccinated animals failed to achieve significance compared to the titer of the mock vaccinated animals, 100% of adult animals and 50% of the aged animals receiving the 1918 VLP vaccine had detectable HAI titers (range of 1:20-1:160 and 1:10-1:320 for adult and aged, respectively), while the mock vaccinated animals were all negative (1:10). Multiple 2009 pandemic viral isolates were tested to ensure that the observed cross-reactivity was not unique to a single virus and similar titers were found between strains. Additionally, adult and aged 1918 VLP vaccinated animals had equivalent titers to the homologous 1918 test antigen (p>0.05) and these titers were significantly increased compared to age-matched mock vaccinated controls (p<0.05).

7.5.5 Protection against 2009 pandemic virus challenge

Adult (2 weeks post final vaccination) and aged (16 months post final vaccination) vaccinated mice were then challenged intranasally with 1 x 10^6 PFU of the 2009 pandemic virus A/Mexico/4108/2009. This isolate is not mouse adapted and does not cause a lethal infection in adult or aged BALB/c mice, but the dose used resulted in significant weight loss and development of clinical disease. Both adult and aged animals receiving the 1918 VLP were

protected from development of clinical sickness and weight loss, while mock vaccinated animals became ill and rapidly lost weight (Figure 35A and 35B). Furthermore, 1918 VLP vaccinated animals, regardless of age, were significantly protected from sickness ($p<0.05$; 4-9 DPI and 3-13 DPI for adults and aged, respectively) and weight loss ($p<0.05$; 3-12 DPI and 3-14 DPI for adults and aged, respectively) compared to the age-matched mock vaccinated control animals. No significant differences were found between adult and aged animals within the 1918 VLP or mock vaccine groups. To evaluate viral burden, lungs were harvested from infected mice at 4 DPI (n=3/group) and viral replication was determined (Figure 35C). Adult 1918 VLP vaccinated mice had significantly decreased lung viral titers compared to the adult mock vaccinated animals. In contrast, aged 1918 VLP and mock vaccinated mice had equivalent viral titers despite the differences observed for disease signs and weight loss.

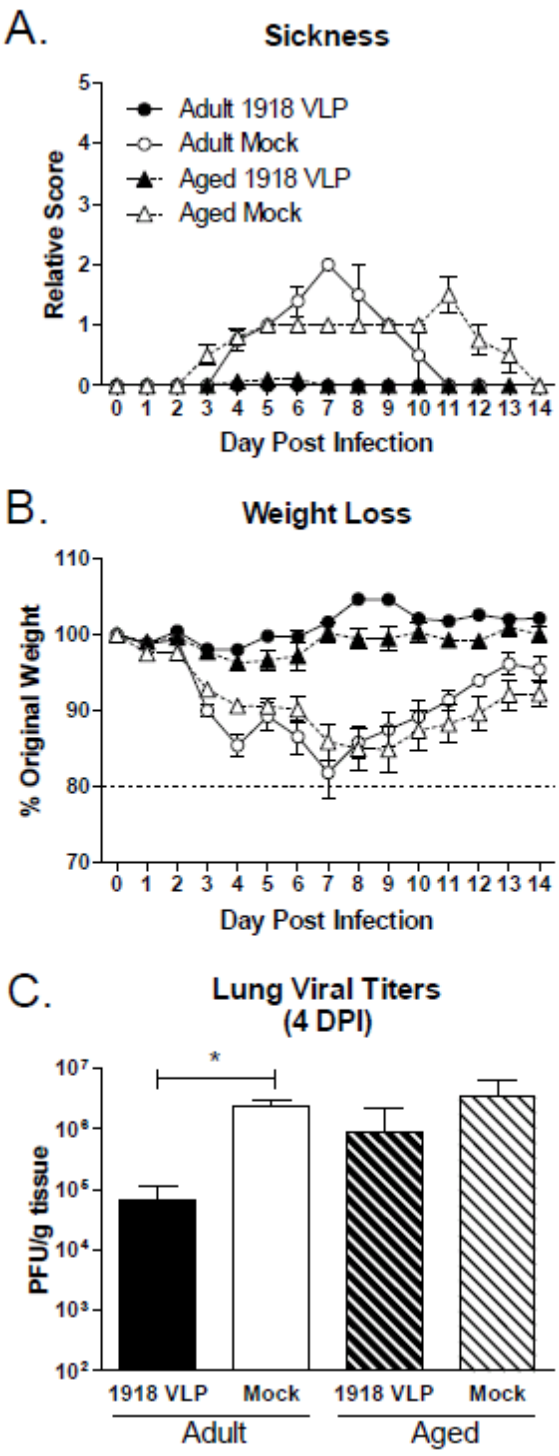

Figure 35: Protection from 2009 pandemic virus challenge. BALB/c mice were vaccinated with 1918 VLPs and infected with 2009 pandemic virus (A/Mexico/4108/2009) 2 weeks (adult; n=5) or 16 months (aged; n=4) after final vaccination. Mice were evaluated daily to monitor sickness (A) and weight loss (B). Sickness score was determined by evaluating activity (0=normal, 1=reduced, 2=severely reduced) and hunched appearance (0=absent, 1=present). Lung virus titers were determined 4 days post infection. Bars represent mean virus titer (± standard deviation). A *p* value of less than 0.05 was considered significant (*$p<0.05$).

7.5.6 Histopathology of infected lungs

To determine if histopathological features after the A/Mexico/4108/2009 challenge could explain the dichotomy observed in aged 1918 VLP vaccinated mice protection from disease but increased viral titers, lung sections were assessed for histopathological changes, presence of influenza and differences in immune response. On 4 DPI, the lungs of adult 1918 VLP vaccinated mice had minimal bronchial and alveolar inflammation compared to adult mock vaccinated mice that showed bronchial epithelial intracellular edema and necrosis with moderate inflammatory infiltration and areas of intra-alveolar exudate and cellular consolidation (Figure 36A and 36B). Similar to adult 1918 VLP vaccinated mice, aged 1918 VLP vaccinated mice showed minor alveolar involvement, however bronchial epithelium had moderate intracellular edema with necrotic epithelium sloughing into airway spaces (Figure 36C). Aged mock vaccinated mice showed severe bronchial inflammation and epithelial necrosis, but alveolar spaces were less involved than adult mock vaccinated mice (Figure 36D).

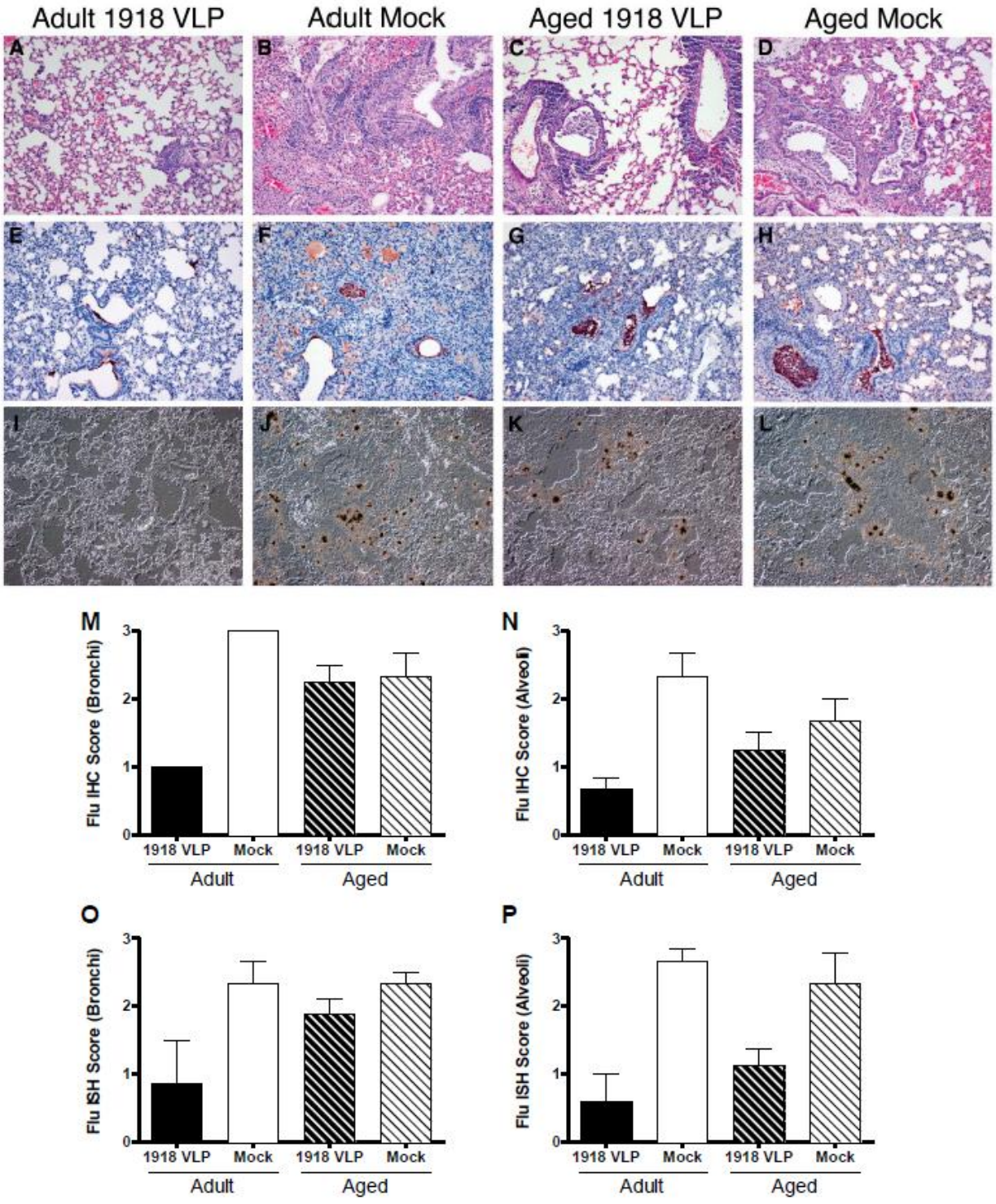

Figure 36: Histopathology and influenza antigen detection of lungs after 2009 pandemic challenge. BALB/c mice were vaccinated with 1918 VLPs and infected with 2009 pandemic virus (A/Mexico/4108/2009) 2 weeks (adult) or 16 months (aged) after final vaccination. Lungs were collected 4 days post infection, formalin fixed and paraffin embedded. Representative

images from hematoxylin and eosin stained sections: adult 1918 VLP vaccinated mice (A), adult mock vaccinated mice (B), aged 1918 VLP vaccinated mice (C), and aged mock vaccinated mice (D). Immunohistochemistry for influenza antigen (E-H) and ISH for influenza MP (I-L) was performed on sections from paraffin embedded lung tissue at 4 DPI. Severity of viral antigen or influenza ISH foci at 4 DPI was accessed in the bronchi (M and O) and alveolar spaces (N and P). Scoring: 0 = no definitive signal; 1 = occasional focus; 2 = focus in most fields; 3 = more than one focus per field.

To evaluate the location and severity of influenza viral antigen and viral replication, immunohistochemical staining using an antibody against influenza A and ISH for influenza A MP was scored on 4 DPI lung sections. Adult 1918 VLP vaccinated animals had occasional bronchial epithelium infection and viral replication and even less infection in alveolar spaces (Figure 36E, 36I and 36M-P). This was in contrast to significant bronchial epithelium infection and replication observed in adult mock and aged animals, regardless of vaccination (Figure 36F-H, 36J-L, 36M and 36O). Alveolar spaces in adult and aged mock animals had pronounced influenza antigen and RNA, but aged VLP vaccinated mice showed less alveolar infection than mock animals (Figure 36F-H, 36J-L, 36N and 36P).

To assess differences in immune responses in situ, lung sections from challenged mice were scored for presence of macrophages, T cells, neutrophils, IgA secreting cells. To evaluate the antiviral response, lung sections were scored for IFNβ transcription. Macrophage and T cell infiltrates were less abundant in adult 1918 VLP vaccinated mice compared to adult mock and aged animals, regardless of vaccination (Figure 37A-J). Similar results were observed with neutrophils (data not shown). Total numbers of IgA positive cells (not influenza specific) were higher in aged animals than adult mice, regardless of vaccination status (Figure 37K-O). Immunohistochemistry for IgG and IgM was attempted but the level of background staining precluded analysis. Adult 1918 VLP vaccinated mice showed few IFNβ RNA foci compared to adult and aged mock and aged 1918 VLP vaccinated mice (Figure 37P-T).

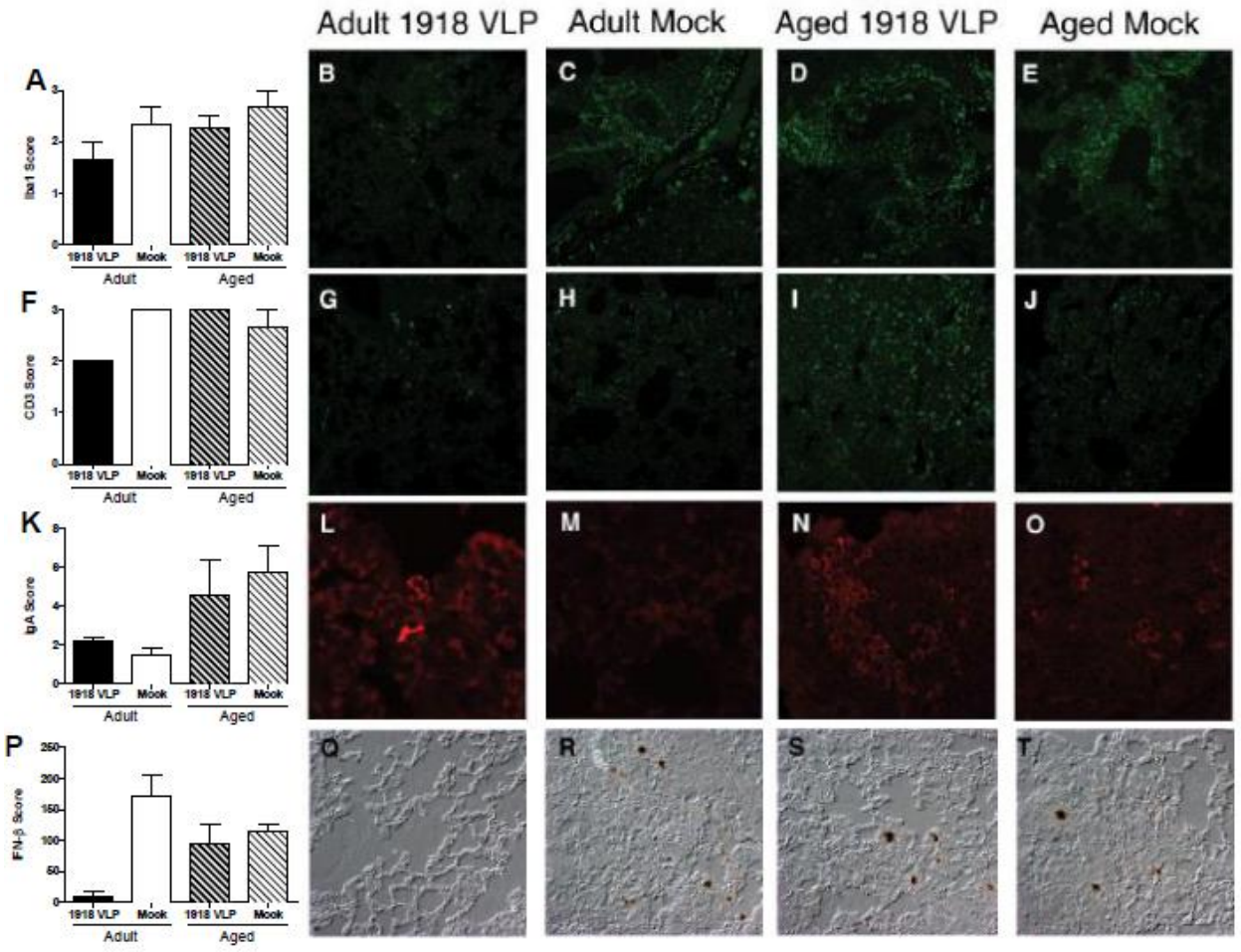

Figure 37: Analysis of immune response by immunohistochemistry and ISH after 2009 pandemic challenge. BALB/c mice were vaccinated with 1918 VLPs and infected with 2009 pandemic virus (A/Mexico/4108/2009) 2 weeks (adult) or 16 months (aged) after final vaccination. Lungs were collected 4 days post infection, formalin fixed and paraffin embedded. Immunohistochemistry for Iba1+ macrophages (B-E), CD3+ T cells (G-J) and IgA+ cells (L-O) and ISH for IFNβ (Q-T) was performed on sections from paraffin embedded lung tissue. Sections were scored for severity of infiltrate: Iba1+ macrophages (A), CD3+ T cells (F), IgA+ cells (K) and IFNβ foci (P). Iba1 scoring: 0 = scattered throughout section; 1 = collected around bronchi; 2 = forming cuff around bronchi; 3 = severe cuffing of bronchi, extending into alveolar spaces. CD3 scoring: 0 = rare; 1 = scattered throughout section; 2 = abundant throughout section; 3 = abundant with collections surrounding bronchi. IgA scoring: average of the number of positive cells in 25 microscopic fields (40X). IFNβ scoring: number of foci in section.

7.5.7 Post-infection cellular responses

To determine the magnitude of influenza-specific cellular responses post-infection, spleens and lungs from vaccinated animals (n=3/group) were harvested 6 DPI and both antibody secreting cells (ASC) and IFN-γ producing cells were analyzed by ELISpot assay. Although the vaccines were administered via intramuscular injection, anti-1918 HA IgG secreting cells were not detected in significant quantity in the spleens of any animals, but 1918 specific ASC were detected in the lungs of 1918 VLP vaccinated mice regardless of age (Figure 38A). Importantly, both adult and aged 1918 VLP vaccinated animals had equivalent numbers of lung ASC which were significantly increased compared to the age matched controls ($p<0.05$).

Additionally, 1918 VLP vaccine primed influenza-specific IFN-γ producing cells were analyzed (Figure 38B). IFN-γ production in the spleen was low to undetectable and no significant differences were found between any of the groups regardless of stimulating antigen. Adult mice receiving the 1918 VLP had significantly more lung IFN-γ producing cells responding to the immunodominant peptide HA_{533} compared to adult mock animals ($p<0.05$). Aged mice vaccinated with 1918 VLPs and stimulated with HA_{533} peptide had increased numbers of IFN-γ producing cells compared to aged mock animals ($p<0.05$). Similar to the ASC numbers, both adult and aged 1918 VLP vaccinated animals had equivalent numbers of IFN-γ producing cells after infection. Although IFN-γ producing cells were detected in the lung using truncated 1918 HA protein or intact virus as stimulating antigens, no significant differences between groups were observed.

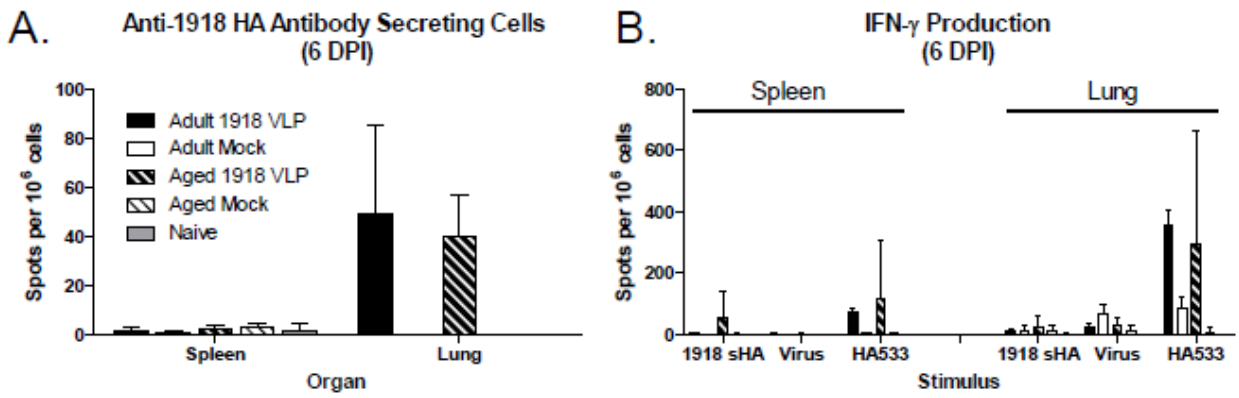

Figure 38: Post-infection antigen specific cellular immune response. BALB/c mice were vaccinated with 1918 VLPs and infected with 2009 pandemic virus (A/Mexico/4108/2009) 2 weeks (adult) or 16 months (aged) after final vaccination. Lungs were collected 6 days post infection, single cell suspensions were prepared and the numbers of anti-1918 HA antibody secreting cells (A) and influenza-specific IFN-γ producing cells (B) were determined by ELISpot assay. Cells were stimulated with 1918 soluble HA (1918 sHA), inactivated virus, and class I immunodominant peptide (HA533). Values represent the mean spots (± standard deviation) for each group.

7.5.8 Passive transfer of immune sera

To evaluate the contribution of serum factors to protection, 9 week old recipient animals (n=5/group) were administered pooled sera via IP injection from adult (2 weeks post vaccination) and aged (16 months post vaccination) 1918 vaccinated animals and aged mock animals. At 24 h after serum transfer, mice were challenged intranasally with the 2009 pandemic virus A/Mexico/4108/2009 (1 X 10^6 PFU). Although this isolate does not cause a lethal infection in adult or aged BALB/c mice, the disease course in 9 week old mice is more severe and, as such, the young mice are a more sensitive model for evaluating protective efficacy (unpublished observations). Pooled serum from adult and aged 1918 VLP vaccinated animals was confirmed to have equivalent levels of anti-1918 antibody titers prior to transfer

(total IgG end point dilution of 1:3200 and HAI of 1:80 for each VLP group). Mice receiving either adult or aged 1918 VLP vaccinated serum developed mild clinical illness, while mice receiving mock vaccinated serum developed more severe disease ($p<0.05$; 4-8 DPI) (Figure 39A). Interestingly, adult 1918 VLP serum recipients resolved the clinical symptoms more rapidly than the aged 1918 VLP serum recipients ($p<0.05$; 7-8 DPI). Mice receiving adult 1918 VLP serum had significantly less weight loss compared to both mock serum recipients ($p<0.05$; 4-8 DPI) and aged 1918 VLP serum recipients ($p<0.05$; 6-8 DPI) (Figure 39B). Aged 1918 VLP serum recipients had equivalent weight loss compared to the mock serum recipients at every time point except for 8 DPI. Although the aged 1918 VLP serum recipient mice developed longer-lasting disease and lost more weight than the adult 1918 VLP serum recipients, both adult and aged 1918 VLP recipients were completely protected from death while 80% of the mock recipients had reached experimental endpoint by 8 DPI ($p<0.01$; Figure 39C). Interestingly, both adult and aged 1918 VLP transferred serum had similar levels of IgG_1, IgG_{2a} and IgG_{2b}, but only the adult 1918 VLP serum had detectable IgG_3 (Figure 39D).

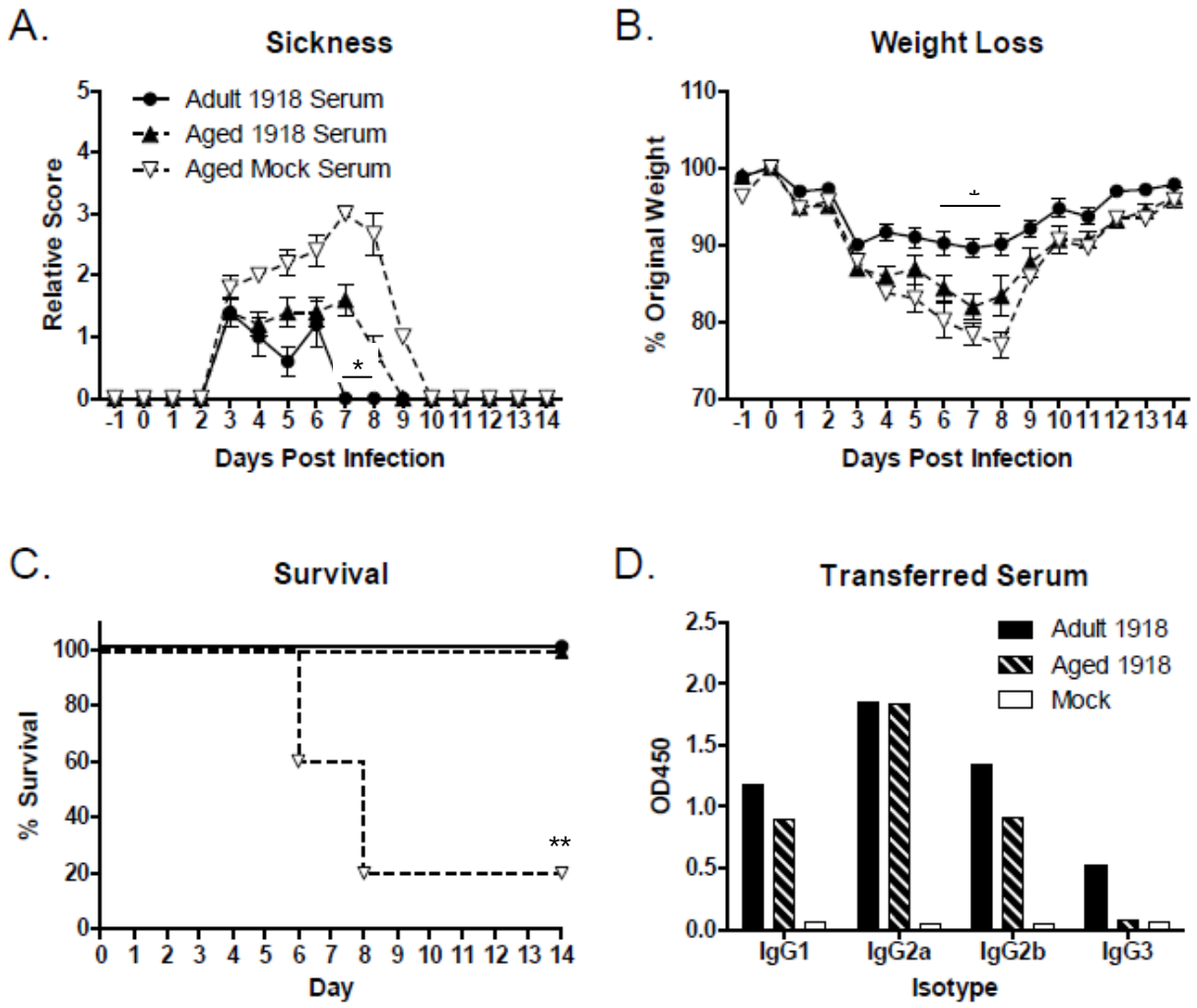

Figure 39: Passive transfer protection from 2009 pandemic challenge. BALB/c mice were vaccinated with 1918 VLPs and blood collected 2 weeks (adult) or 16 months (aged) after final vaccination. Serum was pooled for each age group, heat inactivated and transferred via IP injection to naïve recipient mice. 1 day after transfer, recipient mice were infected with 2009 pandemic virus (A/Mexico/4108/2009). Mice were monitored daily for sickness (A) and weight loss (B). Mice reaching the experimental end point of >20% weight loss were humanely euthanized (C). Sickness score was determined by evaluating activity (0=normal, 1=reduced, 2=severely reduced), hunched appearance (0=absent, 1=present) and ruffled fur (0=absent, 1=present). Prevalence of IgG isotypes in pooled transferred serum was determined via ELISA (D). A *p* value of less than 0.05 was considered significant (**p*<0.05; ***p*<0.01).

7.6 DISCUSSION

In this study, we evaluated the efficacy of pre-immunity to 1918 derived antigens in protecting against 2009 pandemic H1N1 virus in aged mice. Sequence analysis indicated that the HA protein from the 2009 H1N1 pandemic virus is more closely related to the 1918 virus than the H1N1 strains that re-emerged in humans in 1977 [316]. Indeed, structural similarities between 1918 HA and 2009 pandemic HA have been elegantly described and indicate conservation within antigenic sites that is not present in contemporary seasonal HA molecules [317]. Additionally, neutralizing antibodies derived from human survivors of the 1918 pandemic were able to cross-neutralize 2009 pandemic virus, confirming the antigenic predictions [323, 341]. Although severe seasonal influenza infections usually occur in the very young and older age groups, epidemiological evidence has indicated that elderly populations were unusually protected from severe infections during the 2009 H1N1 pandemic. Our group and others have found high levels of cross-reactive antibodies in the older populations and it is hypothesized that this is due to prior exposure to antigenically similar influenza virus [319, 320, 322]. Furthermore, there is direct evidence of the protective efficacy in mice with prior exposure to 1918-like or classical swine H1N1 influenza virus to 2009 pandemic infection [316, 324]. Cross-protective efficacy of older viruses to 2009 pandemic infection in animal models has provided mechanistic evidence for the observed phenomenon of decreased disease severity in the older human population, but has not directly evaluated the duration of the cross-reactive immune responses. We sought to confirm and expand these findings to aged animals in order to more closely mimic the findings in humans. Our results indicate that the anti-1918 influenza immunity acquired early in life can indeed retain its cross-protective efficacy against a 2009 pandemic challenge in later stages of life.

Age-associated defects in immune responses generally lead to increased susceptibility to infectious disease and decreased responsiveness to vaccines [325]. Furthermore, intrinsic

defects within the B cell response directly cause decreased responses to influenza vaccine in elderly humans [342]. Although several studies have established a defect in immune responses to vaccination in the elderly [342-345], we sought to evaluate the durability and efficacy of immune responses initially elicited in young animals. We found that vaccine responses were produced efficiently in adult mice (Figure 33A and 33B) and were robust enough to protect against the highly lethal reconstructed 1918 virus challenge (Figure 33C and 33D). The duration of homologous receptor blocking antibody was evaluated and titers elicited as adults were maintained throughout the lifespan of the mouse (Figure 34A). Although the antibody titers tended to decrease with age, these differences were not statistically significant (p>0.05). Structural analysis and neutralizing antibody binding has indicated cross-reactivity between 1918 and 2009 pandemic HA proteins and this has been confirmed in adult mice [316, 317, 323]. Shortly after vaccination, the antibody response is dominated by low-affinity responses produced by short-lived plasma cells that may have the benefit of being more cross-reactive due to reduced somatic mutation in response to a specific antigen [328]. Prior studies have only evaluated antibody responses at 2-4 weeks after antigen exposure and as such the observed cross-reactivity may be due to the kinetics of the antibody response and not completely indicative of the long-lasting antibody repertoire. We found that similar to adult mice, aged animals maintained equivalent levels of cross-reactive antibody titers (Figure 34B). These results indicate that the cross-reactive antibodies observed in adult animals are long lasting and therefore probably produced not only by short-lived plasma cells generated immediately in response to vaccination, but rather by long-lived plasma cells (LLPC) that continue to produce antibody for an entire lifetime. An important caveat of this study is that we evaluated a single antigen and its role in eliciting cross-protective immunity. A more realistic scenario is one that includes multiple exposures, via infection or vaccination, of antigenically distinct viruses over a lifetime. Although LLPC would be unable to respond to the new antigens, an accumulation of diverse LLPC and resulting serum antibodies could lead to even more robust cross-reactivity. In

support of this idea, serologic data from humans suggests that those individuals who have anti-2009 pandemic cross-reactive antibodies are more likely to be positive for other historic viruses [322]. A second, but not mutually exclusive, possibility is that memory B cells (MBC) may respond to the new antigens and a cross-reactive epitope(s) could be specifically boosted by sequential exposure. Indeed, the increased numbers of broadly-neutralizing antibody secreting cells in response to 2009 pandemic infection in humans supports the notion that the MBC specific for a cross-reactive epitope can be activated during heterologous infection [331].

Most human influenza viruses require adaptation to cause disease in mice, except for highly pathogenic viruses, including 1918 and H5N1 isolates [346]. The 2009 pandemic virus also readily infects mice, although lethality differences have been reported when comparing multiple virus isolates [316]. The strain used for challenge infections in these studies (A/Mexico/4108/2009) is not lethal to adult mice, but does cause significant morbidity (unpublished observations). Aged mice have an impaired immune response following influenza infection and display increased morbidity and delayed innate immune activation [230]. Interestingly, 2009 pandemic virus infections in older adult (1 year) mice have decreased severity compared to young (4 week) mice [347]. We found that naïve aged and adult animals had similar morbidity profiles with aged animals displaying prolonged signs of disease (Figure 35A). This could be due to a delayed immune response in the aged animals at both the initiation and contraction stages leading to prolonged inflammation in the lungs [230]. Vaccinated animals from both age groups did not develop any signs of morbidity in response to the infection even though high levels of virus were recovered from lungs of aged vaccinated animals 4 days post-infection (Figure 35C). Although the differences were not significant between the vaccinated groups, the observation was confirmed by immunohistochemistry for influenza antigen and *in situ* hybridization for influenza RNA. *In situ* analysis of pathological responses and viral replication revealed that adult vaccinated animals were protected from infection of both bronchial and alveolar spaces, while aged vaccinated animals were only

protected from alveolar infection (Figure 36). This suggests that alveolar inflammation and/or infection has a greater contribution to the development of morbidity than bronchial infection which is consistent with post-mortem analysis of fatal human cases [310-312]. One possible explanation for why virus was detected in the bronchial epithelium in aged vaccinated animals, but not in adult vaccinated animals, is that the initial immune response is delayed in the aged cohort [230]. A delay in the kinetics of antigen presentation could allow for additional spread within the lung that results in the observed bronchial disease. Alternatively, the time from vaccination could also contribute to the differences in viral replication between adult and aged vaccinated mice. Adult vaccinated mice were challenged only 2 weeks after the final vaccination, while aged mice were challenged 16 months after final vaccination. Because of the shorter timeframe, larger numbers of effector cells in adult mice could be available at the time of infection and therefore more efficiently control virus replication. However, it was surprising that the aged animals showed greater numbers of IgA-expressing cells than adult mice. Since this was not dependent on vaccination status, it could be a reflection of the lifespan difference between mice and the number of pathogen exposures. Overall in the histopathological analysis of the immune response, we observed greater infiltrates of macrophages, T cells, neutrophils and IFNβ RNA expressing cells in the aged and adult mock mice than the adult 1918 VLP vaccinated mice. This suggests that the intensity of the observed immune response is a reflection of the level of viral replication in the lungs. Aged-vaccinated mice had similar viral burden and immune infiltrate compared to unvaccinated mice, but they did not lose weight or display any signs of disease. Therefore, restriction of viral replication to the bronchial spaces in aged vaccinated mice likely contributed to the less severe disease than that observed in the naïve adult or aged mice. These findings confirm prior observations that a 1918 influenza vaccine protects adult mice from 2009 pandemic influenza challenge and extend this finding to aged animals that originally experienced the 1918 antigens early in life; a finding that is analogous to humans.

Influenza-specific CD8+ T cells are primarily responsible for the clearance of virus infected cells after influenza infection and are detectable after primary infection by day 5 [348]. One defect that is associated with the aging immune response is the reduction in CD8+ T cell function [349]. Our results indicate that IFN-γ producing T cells specific to a Class I immunodominant peptide are recruited to the site of infection as efficiently in vaccinated aged animals as in adult animals (Figure 38). Consistent with aging-associated T cell defects, a reduced, albeit not significant, number of IFN-γ producing cells were found in naïve aged animals compared to the adult controls. Interestingly, when inactivated whole virus was used as the stimulating antigen, IFN-γ producing cells were not significantly detected above background. This is likely due to the vaccine containing only HA and NA antigens derived from influenza virus and a HIV-Gag core. In addition to T cell related age defects, B cells are also impaired [350, 351]. Vaccinated animals in both age groups had equivalent levels of serum antibody pre-challenge and similar numbers of antigen-specific antibody secreting cells detectable in the lungs 6 days post-infection while unvaccinated animals did not have any detectable antigen-specific antibody secreting cells (Figure 38). Interestingly, antigen-specific antibody secreting cells were not detectable in any group prior to challenge (data not shown). The rapid recall of both T and B cells in the lungs of vaccinated animals indicates that the adaptive immune response that was primed in young animals is maintained late in life, even though the ability to respond to new antigens might be impaired. This observation is consistent with the finding that accumulation of memory B cells in aged animals negatively regulates lymphpoeisis of B cells, which are required to respond to new antigens [352]. Although the adaptive response to vaccines is known to be impaired in aged mice, the immunological memory elicited by vaccination as a young adult is maintained and aged animals respond to challenge as efficiently as adults.

Serum surveillance of humans has indicated that the elderly population has an increased frequency of 2009 pandemic cross-reactive antibodies [319, 320, 322]. To determine if cross-reactive systemic antibody is sufficient to protect from 2009 pandemic challenge, we passively transferred immune serum from both adult and aged mice into naïve recipient mice prior to challenge. Consistent with published results, young mice (<10 weeks of age) are highly susceptible to 2009 pandemic challenge and therefore provide a sensitive model for evaluating protective efficacy [347]. Serum from vaccinated mice, regardless of age-group, protected mice from death while naïve animal serum did not (Figure 39). Interestingly, adult serum recipients lost less weight and had reduced morbidity compared to aged serum recipients. This was not due to differences in administered serum antibody titer as adult and aged serum had equivalent levels of both total anti-HA antibody and receptor blocking antibody (data not shown). Additionally, the IgG subclass profile indicated that although the adult and aged animals had equivalent levels of the dominant isotypes (IgG_1, IgG_{2a} and IgG_{2b}), the adult mice had low but increased amounts of IgG_3 compared to the aged mice (Figure 39D). IgG_3 is a minor fraction of antibody to T cell dependent antigens and is the major isotype to T cell independent antigens [353]. Additionally, IgG_3 is a potent activator of the classical complement pathway, likely due the properties of cooperative binding [354, 355]. Enhanced complement fixation mediated by the HA-specific IgG_3 in adult sera could be a mechanism responsible for the decreased morbidity observed in the recipient mice. It is interesting to speculate that the loss of IgG_3 in aged mice is due to LLPC being more efficiently generated in the presence of T cell help, whereas it is retained in the adult mice because of the shorter kinetics of the vaccine regimen. In addition to IgG_3 variability, differences in affinity profiles or non-HA antibodies could also explain the observed morbidity differences. Antibodies to the NA protein of 2009 pandemic virus are increased in elderly humans [111] and the protective role of anti-NA immunity cannot be excluded in this study. Another possibility for the differences in morbidity is that the antibody milieu in the adult serum is more diverse than the aged animals. Although the anti-HA specific

antibody titer is equivalent between the age groups, the LLPC producing the antibody in the aged animals are likely less diverse than those cells producing the antibody in the adult animals due to the kinetics of the vaccination. Additionally, the finding that the vaccinated animals did not display any signs of morbidity implies a critical protective role for the cellular component of the immune response in addition to the cross-reactive receptor blocking antibodies. Cellular immunity in presence of protective antibodies is also more predictive of protection in the context of highly pathogenic 1918 influenza virus challenge [356]. Therefore, although serum antibody is sufficient to protect from severe disease and death, cellular immune responses in both the T and B cell compartments likely contribute to protection from the morbidity associated with 2009 pandemic infection in both aged and adult mice.

Virus-like particles are an intriguing platform for developing new influenza vaccines [150, 221, 260, 357]. The VLPs are self-assembling and completely non-pathogenic particles similar in morphology to intact virions [258]. For the vaccines used in this study, the influenza proteins HA and NA were psuedotyped onto the surface of HIV Gag particles. This strategy has been used for multiple applications and takes advantage of the robust budding properties of the Gag protein [192, 260]. Lifelong antibody responses are more efficiently produced by virus infection than by non-replicating antigens [358]. We found that vaccination with 1918 influenza VLPs elicited robust lifelong immunity that was effective at protecting against heterologous 2009 pandemic virus challenge. Importantly, the cohort of vaccinated animals allowed to age was not vaccinated in the presence of adjuvant indicating that the duration of the elicited immune response was not a function of an adjuvant. Although antibody producing cells were not detectable in the bone marrow or spleens of vaccinated animals prior to challenge (data not shown), presence of specific antibody in the serum could be due to one of at least two possibilities: the cell frequency was below the detection limit of the assay but sufficient to maintain serum IgG titer or secreting cells were in a lymphoid compartment that was not analyzed. These studies were performed in mice and only evaluated a single HA antigen,

however, the findings reported here indicate VLP-based vaccinations are capable of eliciting lifelong immunity as measured by both serum antibody (Figure 34) and cellular recall to infection (Figure 38).

This is the first evaluation of cross-reactive immunity to 2009 pandemic influenza in an aged animal model. Antigenic similarities between the pandemic influenza strains of 1918 and 2009 have been demonstrated at the structural level, indicated by human data and confirmed in adult animal models [316, 317, 319]. Here, we show animals that experience 1918 influenza antigens during adulthood maintain the cross-protective immunity to 2009 pandemic H1N1 influenza late into life. The aged animals were not protected from viral replication, but restricted the virus to larger airways and did not show signs of alveolar infection, which is the most common feature of fatal human disease. The life-long immunity evaluated in these studies was established by vaccination with a non-replicating VLP rather than by infection, and included B and T cell cross-reactive responses in addition to serum antibody. The studies reported here confirm prior work by others that 1918 influenza can elicit cross-reactive antibody responses to 2009 pandemic influenza and expands those findings to aged animals, further validating the hypothesis that decreased disease severity in the elderly human population observed during the 2009 H1N1 pandemic may be due to prior exposure to antigenically similar viruses.

7.7 ACKNOWLEDGEMENTS

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8.0 SUMMARY AND DISCUSSION

Pandemic outbreaks of influenza have occurred four times in the past 100 years and were responsible for tens of millions of deaths worldwide [60, 71]. The World Health Organization (WHO) has identified three criteria for defining the start of a pandemic: 1) the emergence of a virus to which the world's population is mostly naïve, 2) the new virus must be able to replicate and cause severe disease in humans and 3) the virus must be efficiently transmitted among humans, as evidenced by chains of human to human spread [183]. Highly pathogenic avian influenza of the H5N1 subtype has fulfilled two of these criteria: it is a novel subtype and results in an approximately 60% fatality rate [55, 185]. Neutralizing antibody to the viral glycoprotein hemagglutinin (HA) is the most important aspect of immunity to influenza [359]. Targeting HA via vaccination is the traditional method for reducing the impact of influenza-related illness. H5N1 diversity complicates pre-pandemic vaccine development because of antigenic differences in the HA. Phylogenetic distance in the HA gene separates H5N1 into 10 distinct clades and the majority of human infections are from clades 1 and 2. Furthermore, diversity within clade 2 alone has resulted in the identification of distinct subclades and sub-subclades [360]. There is little receptor blocking antibody cross reactivity between clades and subclades despite high levels of HA protein identity (>90%). Developing vaccines that are able to overcome the antigenic diversity of H5N1 is a crucial step in pandemic preparedness.

The overall objective of this research was to develop a broadly reactive vaccine for highly pathogenic H5N1 influenza. The HA antigen was the primary target for vaccine

development because neutralizing antibody is the most important factor in preventing influenza-related disease. The first aim of this work was to design and characterize a consensus-based H5N1 HA. The second aim was to evaluate the breadth and intensity of the immune responses elicited by the consensus HA designed in aim 1. The third aim was to determine the protective efficacy of the consensus HA vaccine against H5N1 influenza challenges. This work demonstrated for the first time that a consensus-based HA virus-like particle vaccine elicits a broad antibody profile. Additionally, the centralized antigen generation methodology developed for increasing vaccine breadth is applicable to both seasonal and pandemic influenza vaccine design.

The work presented in this dissertation resulted in the development of a consensus-based HA antigen that was termed computationally optimized broadly reactive antigen (COBRA). The COBRA sequence was unique and has not been isolated from either human or environmental infections. The fully synthetic COBRA protein retained the natural functions of HA: receptor binding and cell fusion. COBRA virus-like particle (VLP) vaccines elicited a broad antibody response that had receptor blocking activity against a greater number of virus isolates compared to a polyvalent vaccine. All H5N1 VLP vaccines investigated during the course of this work protected animals from severe disease and death. Consistent with prior reports, protection from severe disease and death did not correlate with receptor blocking antibodies to the challenge virus. Protection in the absence of receptor blocking antibodies could be mediated by several mechanisms such as non-receptor blocking antibodies, cellular immunity, or anti-neuraminidase (NA) antibodies. Antibodies to the HA protein can neutralize virus infectivity without blocking receptor binding by inhibiting cell fusion. For the vaccines tested, neutralizing antibodies correlated with receptor blocking antibodies. Non-neutralizing anti-HA antibodies can also mediate protection via complement activation and opsonization of infected cells. Indeed, transferred serum that had non-neutralizing anti-HA antibodies protected recipient animals from severe disease and death indicating a potential role for binding antibody in protection. All

vaccines tested also elicited anti-HA cellular recall responses to infection. However, the major targets of the cellular immune response, NP and PB2 [117, 118], are not contained in the VLPs tested in this work and it is unknown if the anti-HA cellular response alone would be sufficient for protection from severe disease. Additionally, all of the vaccines contained NA and elicited anti-NA antibodies. It is therefore possible that the NA component in all of the VLP vaccines contributes, at least in part, to the observed protection from severe disease. Although the mechanism underlying protection from severe disease and death remains unclear, the COBRA vaccine more efficiently limited virus replication compared to a polyvalent vaccine. The reduced viral burden in COBRA vaccinated animals suggests a correlation of increased receptor blocking antibody titer with decreased viral replication.

Preventing clinical symptoms without reducing viral shedding allows for transmission and viral evolution. Vaccines that prevent clinical illness but permit viral replication and transmission can hasten virus escape, and are proposed to have led to the selection of the H5N1 variants currently circulating in Asia [77]. For the first time, this work compared a centralized H5N1 vaccine to both monovalent and polyvalent primary isolate-based H5N1 vaccines. Even though all of the tested vaccines protected animals from severe disease, the reduced viral titers in the COBRA vaccinated animals represent a novel finding. Surprisingly, the COBRA vaccine was more effective at reducing viral titers than a comparator vaccine homologous to the challenge virus. One explanation for this observation is that COBRA elicited higher titer receptor blocking antibodies to the challenge virus than the primary isolate-derived vaccine. This is consistent with prior reports showing the poor immunogenicity of naturally occurring H5N1 HA [188, 189, 361]. If a different challenge virus was used and the matched vaccine elicited similar antibody titers as COBRA, then the differential control of virus would likely be ameliorated. This work therefore suggests that the synthetic design of COBRA may have altered the structure of the protein to make it more immunogenic. One feature of COBRA that may enhance its relative immunogenicity compared to primary isolates is the retention of the polybasic cleavage site in

COBRA, but not in the comparator vaccines. The cleavage site mutations are essential for attenuating H5N1 viruses and the primary isolate vaccines used in these studies were generated from such mutated viruses. Removal of the polybasic cleavage site has been associated with decreased immunogenicity of live-attenuated vaccines [214], but this is likely due to decreased levels of replication. Retaining the cleavage site could also result in structural features that more accurately reflect that which are found in the challenge virus. Other features known to impact immunogenicity, such as glycosylation [235] or a serine at position 223 [202], are not different between COBRA and the comparator vaccines (data not shown). Although the mechanism by which COBRA vaccinated animals controlled virus replication more efficiently than those receiving matched vaccine remains to be elucidated, the result is a novel and interesting finding. Centralized vaccines developed by other research groups have either failed to detect a difference in viral replication [172] or did not compare the centralized antigen to primary vaccine candidates [180-182, 254]. Reducing viral burden is a critical aspect in evaluating pandemic vaccines because limiting viral replication will directly impact the transmissibility of the emerging virus. This work has established COBRA as a superior vaccine compared to prior H5N1 vaccines due to the decreased levels of virus detected in COBRA vaccinated animals. Additionally, the comparison of COBRA to other antigens using the same delivery system is critical in demonstrating that COBRA itself results in a broader antibody profile and more efficient protection. VLPs have been shown to broaden antibody responses alone [221], but non-COBRA antigens in the context of the VLP did not have as broad of a reactivity profile as COBRA. The studies described in this dissertation have, for the first time, compared a centralized antigen to primary isolate-based vaccines using a VLP delivery platform. This work demonstrated COBRA to elicit increased breadth of receptor blocking antibodies and more efficiently control viral replication compared to primary isolate-based antigens.

COBRA vaccines elicited a broader profile of receptor blocking antibodies than either monovalent or polyvalent vaccines. This difference cannot be explained by total sequence identity as all vaccines studied were >93% identical. HA proteins in general are highly similar and total protein identity scores can often obscure the more subtle differences in antigenic positions. For H5N1, a panel of 20 antigenic residues has been identified [197]. Restricting analysis of amino acid identity of the vaccine antigens to these 20 antigenic positions is a better representation of the diversity between the proteins (Table 10). Furthermore, structure modeling of the HA proteins has indicated that the COBRA protein retains the most common structural features at proposed antigenic sites (Figure 40). An interesting finding in the structural models is that surface residues are not the only ones that alter antigenic regions. Several of the 20 antigenic residues did not map to the surface of a particular antigenic site, but remained variable and antigenically important. When modeled onto a molecular structure, many of these “internal” residues altered the position or orientation of surface antigenic structures. These observations suggest that predicting antigenic divergence based on surface antigen identity fails to capture all of the possible changes, and that differences outside of accepted antigenic sites can still impact antibody binding. A limitation of the antigenic structure analysis is that the models are hypothetical and based on solved structures of similar molecules. An important next step for this work is to determine the structure of the COBRA HA protein. A solved structure would enable more definitive analysis of antigenic regions and could indicate which epitopes are contributing to the increased breadth of elicited antibodies. It is unlikely that linear epitopes are responsible for the differences in antibody breadth because peptide ELISAs using diverse antisera failed to detect any differences between vaccines (data not shown). Non-linear epitopes are the most common structural features at the various antigenic sites and it is likely that these conformational epitopes are the critical determinants of antibody breadth. Rationally designed vaccine antigens based on epitope structure could represent the future of

vaccine design and the work presented in this dissertation indicates that structural features are important determinants of vaccine breadth.

Table 10: Sequence Identity

	Clade 1	Clade 2.1	Clade 2.2	Clade 2.3	COBRA
Clade 1	100	96	94	96	94
Clade 2.1	55	100	94	96	97
Clade 2.2	50	60	100	94	95
Clade 2.3	25	35	50	100	97
COBRA	50	75	85	60	100

Antigenic Residues | Total Protein

The COBRA-based HA described in these studies addressed the objective of developing a broadly reactive vaccine for H5N1 influenza. Although COBRA did elicit broadly reactive antibodies, the mechanism behind the expanded breadth remains unclear. At least two explanations are plausible: 1) the COBRA antigen contains a unique, single epitope against which a broadly neutralizing antibody is elicited, or 2) the COBRA antigen captures a collection of epitopes against which multiple antibodies are elicited. One way to address this lingering question is through the development of monoclonal antibodies. Unique monoclonal antibodies could each be evaluated for their receptor-blocking profile and then mapped onto the original antigen. If monoclonal antibody mapping indicates that expanded breadth is due to a single epitope, future antigen design could focus on the given epitope and expand its reactive profile. Alternatively, if multiple antibodies that each react within a cluster of antigenically related viruses are identified, future antigen design could use an individual epitope to specifically address a single antigenic family. For example, a chimeric HA could be designed that captures a clade specific neutralizing epitope in each antigenic site. COBRA methodologies could be applied to developing clade-specific antigenic sites and then the different sites engineered into a single molecule. In addition to investigating the underlying mechanism of broad reactivity, COBRA-

derived monoclonal antibodies would also be useful in the development of therapeutics to be used in the event of a pandemic. Indeed, neutralizing antibodies are effective at ameliorating disease from highly pathogenic influenza even when administered after infection [103].

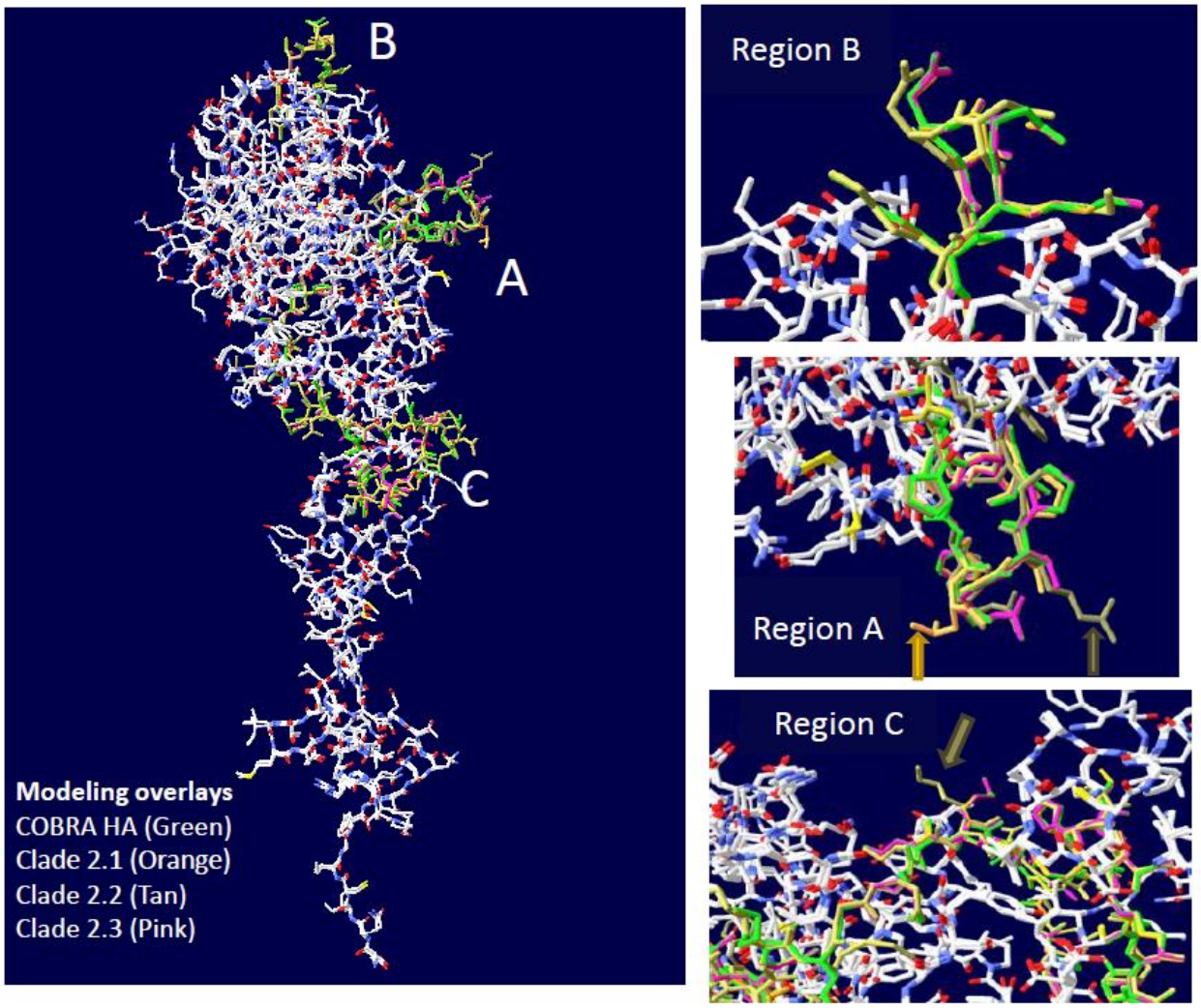

Figure 40: Structural modeling of antigenic regions. HA1 structures were predicted using 3D Jigsaw comparative modeling (http://bmm.cancerresearchuk.org/~3djigsaw/) [362]. Structures were overlayed and antigenic regions analyzed for changes. The left panel shows the whole HA1 and right panels show close ups of three antigenic regions: A, B and C. Arrows indicate divergent structures.

Another possible outcome of broad reactivity is more rapidly evolving strains of influenza. COBRA vaccinated animals had decreased viral replication compared to polyvalent

vaccinated animals, but the immunity was not sterilizing. Even the low levels of viral replication found in animals receiving COBRA vaccines provide the virus an opportunity to mutate and evade the broad antibody response. This possibility could be evaluated by serially passaging influenza virus in the presence of various antisera (or monoclonal antibodies) and monitoring the pathogenicity and growth characteristics of the escaped viruses. The new viruses could then be sequenced and evasion mutations in the HA protein identified. It is possible that viruses selected under the pressure of COBRA antisera would more rapidly accumulate antigenic variability and result in highly divergent strains. Alternatively, the functional constraints of the HA molecule combined with the broadly reactive COBRA antibodies could fully restrict the virus and prevent further divergence. Either outcome would provide insights into the virologic outcomes of broadly reactive immune responses, which are important considerations as broadly reactive vaccines are being developed.

Table 11: Resistance associated residues.

Region:	A (154)	A (157)	B (140)	B (171)	C (100)	D (228)
COBRA	Q	P	D	N	N	K
Clade 0	L	S	N	S	S	E
Clade 4	L	S	N	S	S	T
Clade 2.1	L	P	D	S	N	K
Clade 2.2	Q	S	D	N	N	K
Clade 2.3	Q	P	D	N	N	K
Clade 7	L	P	N	N	S	K

Resistant isolates Susceptible isolates

The COBRA vaccines investigated in this work were focused on the diversity specifically within clade 2 of H5N1. The results of the studies presented here demonstrate that COBRA is effective at increasing breadth within clade 2, but is limited when the test antigens are from divergent clades. Furthermore, the COBRA antigen evaluated in these studies included only sequences from human isolates and could be failing to capture avian epitopes. Although clade 2 is the most widespread and is responsible for the majority of human infections, an ideal H5N1

vaccine would not be restricted to any single clade. The COBRA vaccine failed to elicit receptor-blocking antibodies to representative isolates from clades 0 and 4. Interestingly, the resistant viruses shared antigenic residues that were not found in susceptible viruses (Table 11). For example, in region A, resistant viruses were divergent from COBRA at two positions (154 Q→L and 157 P→S) and although susceptible viruses may have one of the changes, none had both (Figure 41A). Additionally, divergence at two amino acids in region B (140 D→N and 171 N→S) was associated with resistance (Figure 41B). None of the divergent residues were associated with the specific host from which the isolate was derived: the clade 4 virus is an avian isolate and the clade 0 virus is a human isolate. Due to the antigenic location of the divergent amino acids, it is possible that they play key roles in evading receptor blocking antibody responses. Furthermore, the criteria for clade designation only consider total HA sequence identity and it is possible that the current classification system fails to efficiently group antigenically related viruses. Because the layered design of COBRA utilized pre-determined clades and sub-clades, subtle antigenic differences that drive resistance may fail to cluster within any specific clade and would have been lost in the antigen design. To overcome these limitations, new COBRA antigens could be designed using different input sequences or different layering techniques. For example, clade 2 vaccines could be improved by adding avian sequences and including additional subclades (2.4, 2.5 and 2.6) that only have avian isolates. To expand the vaccine to cover all of H5N1, COBRA technology could again be utilized by layering together all of the clades. Additionally, changing the layering process to ignore total HA-based clade designations and instead utilize antigenic residue-based clustering could alter the breadth of any new COBRA vaccine. Altering either the input sequence population used to generate the antigen or the layering technique by which the antigen is constructed are both predicted to impact the reactivity profile of the new vaccines. Indeed, phylogenetic analysis of newly designed antigens indicates that either inclusion of clade 2 avian sequences or other clades results in a sequence that is more central to all of H5N1 (Figure 42). One possible

outcome of making an antigen more central to all clades of H5N1 is the loss of reactivity within the more divergent clade 2. If a single COBRA antigen is unable to expand antibody breadth to all clades and subclades of H5N1, a polyvalent approach could be used. This work and others have demonstrated the validity of using polyvalent vaccinations to increase the breadth of H5N1 vaccines (see Chapter 6 and [161]). Using COBRA antigens as polyvalent components would enable the inclusion of multiple COBRA antigens, each addressing a specific antigenic cluster. Avian influenza of the H7 and H9 subtypes have also caused human disease and are additional reservoirs from which pandemic influenza could emerge. COBRA antigens could be developed for each subtype, H5, H7 and H9, and then combined for pre-pandemic vaccinations. Pandemic influenza emerges suddenly, and the vast diversity of influenza A viruses renders impossible the accurate prediction of the exact strain that will cause the next pandemic. To overcome this challenge facing pandemic preparedness, development of broadly reactive vaccines (*e.g.* COBRA) and strategies (*e.g.* polyvalency) are critical.

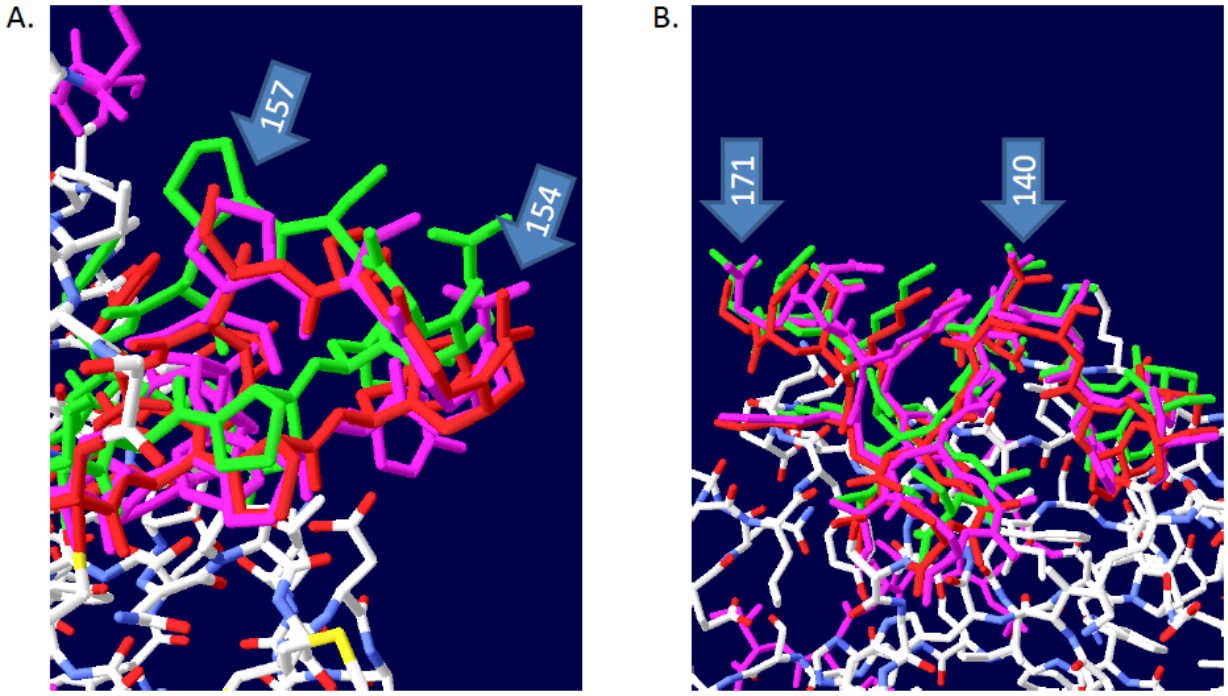

Figure 41: Modeling of resistant residues. HA1 structures representing COBRA (green), clade 2.3 (pink; susceptible) and clade 0 (red; resistant) were predicted and overlayed as before (Figure 40). Predicted location of resistance associated residues is indicated by arrows for antigenic region A (A) and antigenic region B (B).

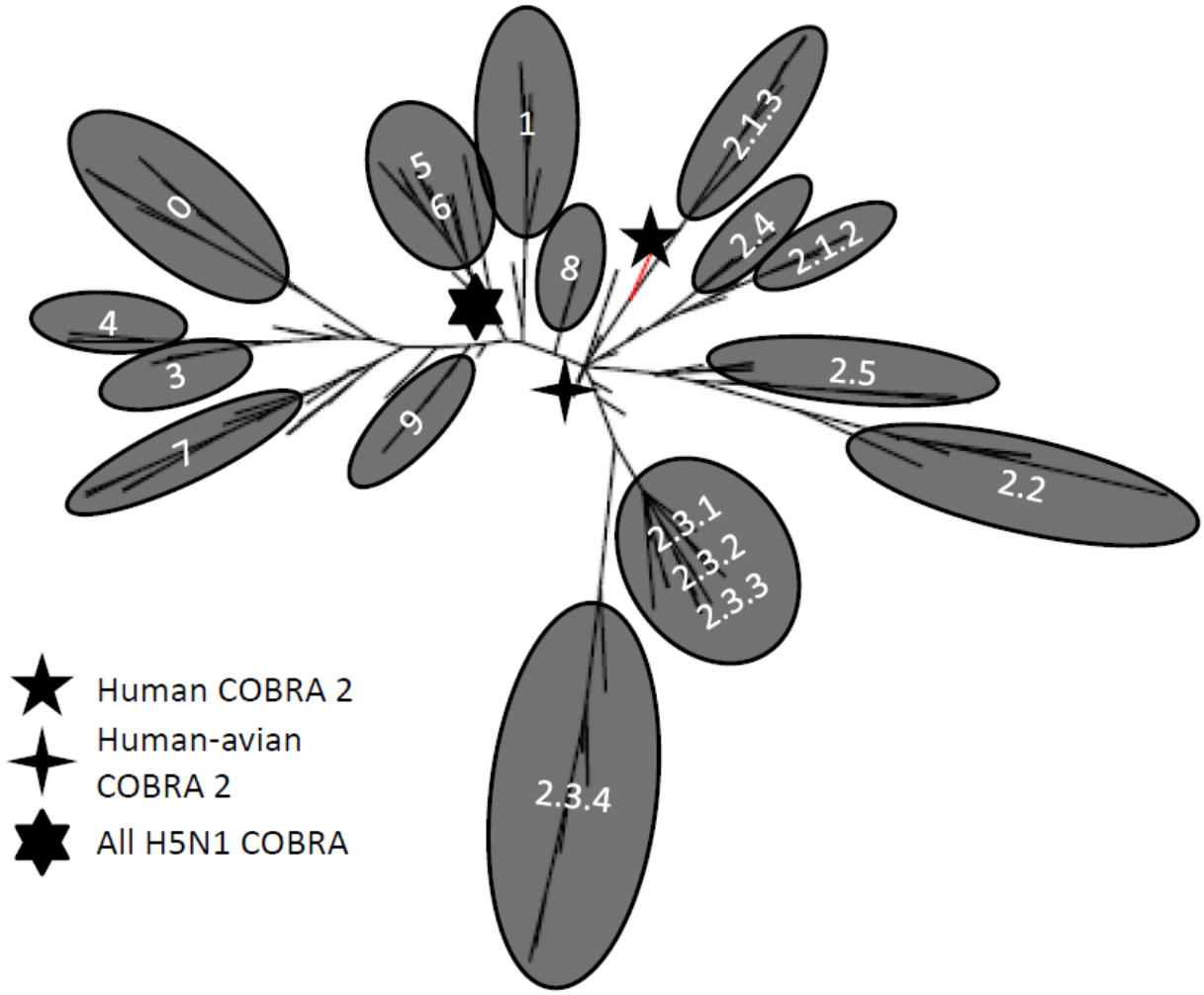

Figure 42: Phylogenetic analysis of additional H5N1 COBRA genes. The unrooted phylogenetic tree was inferred from HA amino acid sequences derived from 8 to 10 representative isolates in all clades and subclades and also the COBRA HA using the maximum likelihood method. Clade/subclade clusters were identified and are indicated in the shaded ovals. The stars identify where the COBRA antigens are located relative to the various representative isolates.

A limitation of the work presented in this dissertation is that the vaccines were all delivered using a virus-like particle (VLP) platform. VLPs are a promising new generation of influenza vaccines [150, 221, 222, 363], but are not approved for human use as influenza vaccines. Importantly, VLPs expand vaccine breadth, regardless of antigen, compared to recombinant protein vaccines [221]. The expanded antibody breadth elicited by COBRA VLPs was not due to the VLP itself as vaccines containing only a different HA failed to achieve the same broad responses. It remains to be determined if a COBRA antigen can continue to elicit broadly reactive antibodies when used in a non-VLP platform. Because currently licensed influenza vaccines are virus-based (either split and inactivated or live-attenuated; see Section 1.8), the COBRA antigen should be evaluated in these platforms. Reverse genetics technology (see Section 1.10.2) could be used to generate novel viruses expressing the COBRA HA. These would be H5N1 viruses that do not circulate in nature and therefore non-wild type internal genes (*e.g.* cold adapted virus) would be required. The COBRA-virus could then be evaluated as a vaccine candidate. The broadly reactive antibody profile elicited by COBRA is likely due to unique antigenic features of the synthetic HA rather than the VLP delivery system. However, the trimeric conformation on the surface of the VLP is probably an important feature of the vaccines evaluated in this work and splitting the COBRA-virus could affect the oligomerization. Investigating COBRA vaccine antigens in multiple vaccine platforms is an important step forward to demonstrate that the broad antibody profile is not an artifact of the VLP. Furthermore, the most direct path for licensure of a COBRA-based influenza vaccine is to utilize established and standardized influenza production and purification methods. For COBRA-based influenza vaccines to have the most significant impact on improving human health, changing the vaccine platform from experimental VLPs to licensed methodologies is essential.

In addition to directly vaccinating humans, a vaccine utilizing the COBRA antigen described in this work could make an impact via use in poultry. Poultry vaccines can successfully limit the spread of H5N1 through farms [77]. Additionally, agricultural vaccines

have less rigorous standards to which they must adhere. VLPs and other experimental delivery platforms would be more easily transitioned into animal use than for human use. Vaccinating poultry with COBRA could positively impact the world in at least two different ways. First, decreasing the amount of circulating virus reduces the chances for transmission to humans. Sporadic human cases continue to cause severe disease and each transmission event provides a new opportunity for the virus to acquire additional mutations and/or gene segments that will enable a stable host-switch. Vaccinating poultry would further reduce the current human disease burden as well as decrease the opportunities for avian viruses to evolve to mammalian hosts. Second, large-scale poultry vaccinations would prevent the culling of flocks in response to outbreaks. Eradication of an emerging virus is the optimal strategy of control, and for domestic poultry this means destroying infected flocks. This large scale loss of agricultural resources can have devastating impacts on local economies and resulted in the loss of 1.5 million birds in Hong Kong in late 1997 [364]. The COBRA vaccines described here were immunogenic and effective in three animal models: mice, ferrets and non-human primates. Although the relative immunogenicity in avian species is unknown, it is reasonable to expect that COBRA will retain its immunogenic properties because of the past success with multiple animal models.

The work presented here focused on developing pre-pandemic vaccines. Influenza pandemics occur sporadically and can result in severe human disease. Despite the attention devoted to pandemic influenza, seasonal epidemics continue to cause human disease and result in up to 36,000 deaths each year. Antigenic diversity in circulating human influenza remains a challenge for influenza vaccines and is currently addressed by re-formulating the vaccine when an escaped drift variant emerges. Applying COBRA antigen-design methods to seasonal influenza could reduce the frequency of replacing vaccine strains. A large amount of data is available for the antigenic properties of human influenza viruses. The exact antigen design approach would be different for seasonal influenza than H5N1 influenza due to the

different evolutionary trajectories. However, COBRA-based methods could be used to capture the most common antigenic features of each subtype. Antigenic clusters, for instance, have been identified and could be utilized for the COBRA-based layering approach [48, 365, 366]. Applying COBRA methodology to seasonal influenza could provide a more “real world” impact on human health than pre-pandemic vaccine development. Therefore, this dissertation has provided the framework within which broadly reactive influenza vaccines, both seasonal and pandemic, may be developed.

The COBRA vaccines evaluated during the course of these studies demonstrated that a broadly reactive influenza vaccine is possible. Although the antibody reactivity profile was broad, the mechanism underlying this phenomenon is unclear. Future work should be focused on elucidating the reasons for increased breadth. First, determining the structure of the molecule should be investigated. Modeling has indicated a structural basis of breadth, but these observations should be confirmed. Next, monoclonal mapping of the broadly reactive profile should be completed. Information gained from determining the epitope(s) that elicits the broad antibody response could then be applied to future COBRA antigens. Finally, viral escape should be driven with COBRA-derived antibodies to investigate the virologic outcomes of broadly reactive antibodies. Together, these studies would be expected to determine the mechanism underlying COBRA-mediated antibody breadth. Additionally, development of novel COBRA antigens incorporating additional clades could continue to expand the antibody breadth to eventually cover all of H5N1. It will be important to determine if a single antigen can retain reactivity with the diverse clades or if multiple, clade-specific COBRA antigens will need to be developed.

The work presented here demonstrated that a centralized HA (COBRA) based VLP vaccine elicited a broadly reactive antibody response. The reactivity profile of COBRA receptor blocking antibodies was broader than that of a polyvalent comparator. Importantly, the immune responses of all vaccines evaluated protected animals from severe disease and death, but

COBRA vaccines more effectively reduced viral replication. It remains to be elucidated what aspect of the vaccine protected animals from severe disease, but for the first time a centralized vaccine was shown to reduce virus burden more efficiently than primary isolate-based vaccines. Further work is needed to understand the mechanism of increased antibody breadth from the COBRA vaccines to improve future iterations of antigen design. The data presented in this dissertation indicate that broadly reactive influenza vaccines are possible, and that COBRA-based antigens should be developed as both seasonal and pandemic influenza vaccines.

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APPENDIX B

LIST OF PUBLICATIONS

Peer-reviewed Publications

1. **Giles, B.M.,** S.J. Bissel, D.R. DeAlmeida, C.A. Wiley, and T.M. Ross. 2011. Antibody breadth and protective efficacy is increased by vaccination with computationally optimized hemagglutinin but not with polyvalent hemagglutinin based H5N1 VLP vaccines. *Clinical and Vaccine Immunology.* Under review.
2. **Giles, B.M.**, C.J. Crevar, D.M. Carter, S.J. Bissel, S. Schultz-Cherry, C.A. Wiley, and T.M. Ross. 2011. A Computationally-Optimized Hemagglutinin VLP Vaccine Elicits Broadly-Reactive Antibodies that Protect Monkeys from H5N1 Infection. *Journal of Infectious Disease*. In Press.
3. **Giles, B.M.,** S.J. Bissel, J.K. Craigo, D.R. DeAlmeida, C.A. Wiley, T.M. Tumpey, and T.M. Ross. 2011. Elicitation of anti-1918 influenza immunity early in life prevents morbidity and lower lung infection by 2009 pandemic H1N1 influenza in aged mice. *Journal of Virology.* In revision.
4. **Giles, B.M.** and T.M. Ross. 2011. A Computationally Optimized Broadly Reactive Antigen (COBRA) Based H5N1 VLP Vaccine Elicits Broadly Reactive Antibodies in Mice and Ferrets. *Vaccine* 29(16):3043-54
5. Bissel, S.J., **B.M. Giles**, G. Wang, D.C. Olevian, T.M. Ross, and C.A. Wiley. 2011. Acute Murine H5N1 Influenza A Encephalitis. *Brain Pathology.* In press.
6. Schneider-Ohrum, K., **B.M. Giles**, H.K. Weierback, B.L. Williams, D.R. DeAlmeida, and T.M. Ross. 2011. Adjuvants that Stimulate TLR3 or NLPR3 Pathways Enhance the Efficiency of Influenza Virus-like Particle Vaccines in Aged Mice. *Vaccine.* In press.
7. Carter, D.M., H.R. Lu, C.E. Bloom, **B.M. Giles**, C.J. Crevar, J.L. Cherry, D.J. Lipman, and T.M. Ross. 2011. Complex patterns of human antisera reactivity to novel 2009 H1N1 and historical H1N1 influenza strains. In preparation.
8. Zimmer, S.M., C.J. Crevar, D.M. Carter, J.H. Stark, **B.M. Giles,** R.K. Zimmerman, S. Ostroff, B.Y. Lee, D.S. Burke, and T.M. Ross. 2010. Seroprevalence Following the Second Wave of Pandemic 2009 H1N1 Influenza in Pittsburgh, PA, USA. *PLoS One* 5(7):e11601.
9. Rosenkranz, M.E., D.C. Wilson, A.D. Marinov, A. Preuss, D. Kirchner, **B. Giles,** P. Reynolds, K. Kolli, S. Thompson, and R. Hirsch. 2010. Synovial Fluid Proteins Differentiate Between the Subtypes of Juvenile Idiopathic Arthritis. *Arthritis & Rheumatism* 62(6):1813-1823.
10. **Giles, B.M.**, S.N. Tchepeleva, J.J. Kachinski, K. Ruff, B.P. Croker, L. Morel, and S.A. Boackle. 2007. Augmentation of NZB Autoimmune Phenotypes by the Sle1c Murine Lupus Susceptibility Interval. *The Journal of Immunology* 178:4667-4675.

Book Chapters

1. Ross, T.M., N. Bhardwaj, H.S. Eugene, **B.M. Giles,** S.P. McBurney, S.L. Rossi, K. Schneider-Ohrum, and X. Tang. "Virus Like-Particle Vaccines: Advantages and Challenges." Development of Vaccines: From Discovery to Clinical Testing. 2009. Wiley and Sons (USA).

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