# COMPARATIVE EVALUATION OF HUMAN AND PORCINE ADENOVIRUS VECTORS FOR VACCINE APPLICATION AGAINST AVIAN INFLUENZA (H5N1)

BY

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Patel, A., S. Tikoo, and G. Kobinger. 2010. A porcine adenovirus with low human seroprevalence is a promising alternative vaccine vector to human adenovirus 5 in an H5N1 virus disease model. *PLoS One* 5:e15301.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| °C | degrees Celsius |
| Ad | adenovirus |
| AdHu5 | human adenovirus serotype 5 |
| APC | antigen presenting cells |
| Arg | arginine |
| BAV3 | bovine adenovirus 3 |
| BGH | bovine growth hormone |
| BSA | bovine serum albumin |
| CAG | chicken β-actin |
| CAR | cocksackie B-adenovirus receptor |
| CCHF | Crimean Congo Hemorrhagic Fever virus |
| CMV | cytomegalovirus |
| CPE | cytopathic effects |
| CsCl | cesium chloride |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DPT | diphtheria, polio, tetanus, combined vaccine |
| ELISPOT | enzyme-linked immunosorbant spot assay |
| FBS | fetal bovine serum |
| FITC | fluorescein isothiocynate |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| H05 | A/Hanoi/30408/2005 |
| H1N1-1918 | A/South Carolina/1/1918, *Spanish Influenza/Flu* |
| H1N1-PR8 | A/Puerto Rico/8/1934 |
| H5N1 | avian influenza A virus, subtype H5N1 |
| HA | hemagglutinin |
| HEK 293 | human embryonic kidney 293 cells |
| HI | hemagglutination inhibition |
| HIV | human immunodeficiency virus |
| HK97 | A/Hong Kong/483/1997 |
| HPAI | highly pathogenic avian influenza |
| HRP | horseradish peroxidise |
| i.m. | intramuscular |
| i.n. | intranasal |
| i.v. | intravenous |
| IFN | interferon |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| IL | interleukin |
| In05 | A/Indonesia/05/2005 |

| | |
|---|---|
| kDa | kiloDalton ($10^3$ daltons) |
| kg | kilograms ($10^3$ grams) |
| L-15 | Lebovitz-15 medium |
| LAIV | live-attenuated influenza virus |
| $LD_{50}$ | lethal dose of virus resulting in 50% survival |
| LPAI | low pathogenic avian influenza |
| Lys | lysine |
| MCK | muscle-specific creatine kinase |
| MDCK | Mardin-Darby canine kidney cells |
| MEM | minimum essential medium |
| MEM alpha | minimum essential medium containing alpha salts |
| µg | microgram ($10^{-6}$ gram) |
| µl | microlitre ($10^{-6}$ litre) |
| mg | milligrams ($10^{-3}$ grams) |
| MHC | major histocompatibility complex |
| ml | millilitre ($10^{-3}$ litre) |
| mM | millimolar ($10^{-3}$ molar) |
| MMR | measles, mumps, and rubella combined vaccine |
| NA | neuraminidase |
| NAB | neutralizing antibody |
| NaCl | sodium chloride |
| NaPyr | sodium pyruvate |
| neaa | non-essential amino acids |
| NEP | nuclear export protein |
| NK | natural killer cells |
| NML | National Microbiology Laboratory |
| NP | nucleoprotein |
| NS | non-structural protein |
| PAGE | polyacrylamide gel electrophoresis |
| PAV3 | porcine adenovirus 3 |
| PBS | phosphate buffered saline |
| PE | phycoerythrin |
| PEG | polyethylene glycol |
| PerCPCy5.5 | peridinin–chlorophyll–protein complex |
| pfu | plaque forming units |
| PHAC | Public Health Agency of Canada |
| polyA | polyadenylation signal |
| PRR | patter recognition receptor |
| PVDF | polyvinylidene fluoride |
| RBC | red blood cells |
| RDE | receptor destroying enzyme |
| RIPA buffer | radioimmunoprecipitation assay buffer |
| RLR | retinoic acid inducible-like receptor |
| RNA | ribonucleic acid |
| RNP complex | ribonucleoprotein complex |
| rpm | revolutions per minute |

| | |
|---|---|
| SDS | sodium dodecyl sulphate |
| sfc | spot forming cells |
| sIgA | secretory immunoglobulin A |
| spp | species |
| SV | split-virion inactivated influenza vaccine |
| SV40 | simian virus 50 |
| $TCID_{50}$ | infectious dose in 50% of tissue culture cells |
| TLR | toll-like receptors |
| VIDO | Vaccine and Infectious Disease Organization |
| VLP | virus-like particles |
| Vn04 | A/Vietnam/1203/2004 |
| vp | virus particles |
| VSV | vesicular stomatitis virus |
| WHO | World Health Organization |
| WV | whole-virion inactivated influenza vaccine |
| ZGP | *Zaire Ebolavirus* glycoprotein |
| ΔE1/E3 | adenovirus containing deletions in the E1 and E3 genes |

## ABSTRACT

First in 1997, and later re-emerging in 2003, highly pathogenic avian influenza A virus, subtype H5N1, has spread from wild bird reservoirs to domestic bird flocks. As a result, cross-transmission has been confirmed in people living or working in close contact with infected birds. H5N1 virus infection is associated with a high mortality rate (>60%) in humans and the rapid evolution of the virus suggests that it could potentially develop into a new, and possibly severe, pandemic influenza virus. To-date, conventional inactivated and live-attenuated vaccine strategies offers the best protection against influenza virus infection; however, poor immunogenicity and weaker efficacy have been observed against H5N1 viruses. It was hypothesized that experimental adenovirus-based vaccines based on human adenovirus serotype 5 (AdHu5) or porcine adenovirus serotype 3 (PAV3) can offer protection against a broad range of avian influenza, subtype H5N1, viruses. Ad vaccine vectors are highly immunogenic and have demonstrated protective efficacy against several disease models. However, natural immunity against AdHu5 can interfere with vector efficacy. The nonhuman PAV3 was not neutralized by pooled human serum from 10,000-60,000 individuals and offers a promising alternative to AdHu5-based vectors. Systematic antigen screening using DNA vaccines identified the hemagglutinin (HA) glycoprotein as the most immunogenic H5N1 antigen. HA was then inserted directly into PAV3 or AdHu5. Comparable immune responses were observed between both vectors but, interestingly, the PAV3-based vaccine generated stronger T-cell responses and better rapid protection 8 days following immunization. Additionally, better long-term protection 1 year following vaccination was observed with the PAV3-

HA vaccine. The co-administration of multiple H5N1 antigens was also screened to improve protection against divergent H5N1 challenge. Combinations of DNA vaccines expressing (HA+NA) and (HA+NP) offered the best promise for enhancing protection against homologous and heterologous H5N1 challenges, respectively. However, addition of three or more antigens reduced overall protection possibly by antigen dilution, competition, or interference. Co-administration of PAV3 or AdHu5 vectors expressing both the HA and NP antigens reduced protection against homologous and heterologous H5N1 virus challenges. For all combination vaccines, T-cell responses were strong against HA but significantly decreased against additional antigens in each combination vaccine. Overall, the experimental porcine-based Ad-based vaccine offered better protection than the H5N1 conventional vaccine against a broad range of different H5N1 viruses. Understanding of the relationship between immune parameters and protection will be critical in future improvement of adenovirus-based and other vaccines against avian influenza H5N1.

# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Literature Review

### *1.1.1 Vaccination*

The first historical evidence of vaccination describes the treatment of smallpox using the technique of variolation. Several sources describe the use of variolation in ancient China, India, and Egypt, where the scabs from infected patients were dried and inhaled in order to prevent infection (Kennedy et al., 2009; Riedel, 2005). Variolation reduced the mortality rate of smallpox from around 30% down to less than 2%. However, variolation was associated with adverse side effects including the possible development of natural smallpox infection. In 1796, Edward Jenner successfully tested his hypothesis that inoculation with cowpox could prevent smallpox infection, resulting to first published report describing vaccination. This was a considerable success since smallpox was considered one of the most infectious and dangerous pathogens at that time (Riedel, 2005). Later, a live-attenuated vaccine derived from vaccinia virus was developed and distributed as part of government directed immunization programmes. In December 1979, the World Health Organization (WHO) officially declared the eradication of smallpox (WHO). To date, smallpox is the only pathogen that has been completely eradicated. The elimination of poliovirus has come close, however, lack of compliance to vaccine programmes have lead to re-emergence in several countries which had formally eradicated the virus (Kelly et al., 2009; Kennedy et al., 2009).

Several vaccines have been developed to protect against a range of infectious pathogens (Table 1.1). Many of these vaccines are administered as part of early childhood immunization programmes and have been successful at reducing morbidity and mortality worldwide. Approximately 2-3 million lives are saved yearly due to effective vaccination programmes (WHO, 2010b).

### *1.1.2 Types of Vaccines*

Several vaccine platforms are being developed in order to provide protection against various infectious pathogens. The selection of an appropriate vaccine platform is generally pathogen specific and the development of inactivated (killed), live-attenuated, and subunit vaccines offer different approaches to establish protective immunity. In addition to overall vaccine efficacy, other design considerations include: stability, safety, economic feasibility, dosage, and route of immunization.

#### *1.1.2.1 Inactivated (killed) Vaccines*

Inactivated "killed" vaccines are generated through the inactivation of bacteria or live virus particles by heat, chemical (e.g. formalin), or irradiation. These vaccines contain entire microbial antigens but are non-infectious, non-pathogenic, and cannot re-acquire virulence following inactivation. Inactivated vaccines are frequently administered by intramuscular injection (Amorij et al., 2010) but may also be given through subcutaneous (Kitchener et al., 2006) or intradermal routes (PHAC, 2008). They can generate strong antibody-mediated immune response but have limited ability to activate cell-mediated immune responses. Additionally, multiple doses may be necessary in order to generate sufficiently protective immune responses (Fiore et al., 2009).

**Table 1.1:** List of currently approved vaccines in Canada (2010) (PHAC, 2008)

| Vaccine | Type |
|---|---|
| ***Early Childhood and Adult Vaccines*** | |
| Cholera | live-attenuated |
| Diphtheria | subunit |
| *Haemophilus influenzae* b | subunit |
| Hepatitis A | inactivated |
| Hepatitis B | subunit |
| Human papillomavirus | subunit |
| Influenza A virus | inactivated, live-attenuated |
| Influenza B virus | Inactivated, live-attenuated |
| Japanese encephalitis | inactivated |
| Measles | live-attenuated |
| Meningococcus | subunit |
| Mumps | live-attenuated |
| Pertussis | subunit |
| Pneumococcus | subunit |
| Polio | inactivated |
| Rabies | inactivated |
| Rotavirus | live-attenuated |
| Rubella | live-attenuated |
| Tetanus | subunit |
| Tick-borne encephalitis | inactivated |
| Tuberculosis | live-attenuated |
| Typhoid | subunit/live-attenuated |
| Varicella | live-attenuated |
| Yellow fever | live-attenuated |
| ***Approved but not widely-distributed*** | |
| *Anthrax* | *subunit* |
| *Smallpox* | *live-attenuated* |

*1.1.2.2 Live-attenuated Vaccines*

Live-attenuated vaccines are derived using either non-pathogenic or attenuated pathogens in order to reduce the virulence. This can be achieved through numerous passages of a highly pathogenic virus in tissue culture and in animal models to accumulate mutations, resulting in a less pathogenic or attenuated phenotype. Alternatively, the antigens from a more pathogenic isolate may be incorporated onto the backbone of a less pathogenic strain by reverse genetics (Cinatl et al., 2007b). Live virus vaccines can be administered intramuscularly or by mucosal routes (intranasal, oral) and as a result generate both systemic and mucosal immunity. In addition to B cell responses, live-attenuated vaccines can also stimulate cell-mediated immune responses (Cinatl et al., 2007b).

However, there are safety concerns associated with live-attenuated vaccines. An attenuated virus may undergo reassortment with more virulent strains or acquire mutations that may result in possible loss of attenuation. Codon-deoptimization has also been suggested as an alternative method for achieving attenuation and has been successful against poliovirus (Mueller et al., 2006), but potential reversion to a wild type phenotype may occur through silent mutations.

*1.1.2.3 Subunit vaccines*

Subunit vaccines contain only one or more selected antigens from a pathogen. Bacterial toxins or capsular antigens are often delivered as part of a subunit vaccine. Additionally, experimental subunit vaccines containing purified recombinant or synthetic peptides, virus-like particles, DNA vaccines, or viral-based vectors are also being

considered as ways to efficiently delivery antigen and stimulate strong immune responses. Immune responses vary depending on the type of subunit vaccine. Many subunit vaccine candidates can generate strong humoral and cell-mediated immune responses.

### *1.1.3 Influenza and Vaccination*

To date, prophylactic vaccination is the best method of preventing influenza virus infection and the use of vaccines, coupled with appropriate immunization programmes, has considerably reduced the severity and mortality associated with seasonal and episodic influenza outbreaks. However, achieving protection against emerging zoonotic influenza viruses is challenging and may require alternative vaccine strategies. The next section is a review of research literature on avian influenza A, subtype H5N1 and the development of protective vaccines.

### *1.1.4 Avian Influenza A virus, subtype H5N1*

Most influenza A viruses are maintained in wild bird reservoirs but a few subtypes are pathogenic in humans. Low pathogenic avian influenza (LPAI) A viruses persist in the gastrointestinal and respiratory system of aquatic birds as an asymptomatic or mild infection and are occasionally transmitted to urban flocks and domestic poultry. In 1997, a highly pathogenic avian influenza (HPAI) A virus subtype H5N1 was identified from chicken in Hong Kong and several human clinical cases were identified in people who lived or worked in close contact with infected birds. Six human fatalities were reported (Neumann et al., 2009). Although no cases of human-to-human transmission were reported, this raised concerns that H5N1 may emerge as a potential

pandemic influenza virus. As a direct result, domestic poultry flocks were immediately culled to prevent further cross-transmission of the HPAI H5N1 virus to humans. LPAI H5N1 viruses continued to persist in wild birds and in 2003 a new HPAI H5N1 variant emerged in wild and domestic bird populations in South East Asia. Since 2003, over 500 human cases (WHO, 2010a) have been reported in Asia, the Middle East, and Africa and isolated cases of human-to-human transmission have been confirmed in Indonesia (Yang et al., 2007), Pakistan (WHO, 2008), and China (Wang et al., 2008a). The spread of H5N1 viruses between humans is limited however the high mortality rate (>60%) suggests that the virus could be extremely virulent should it establish more efficient transmission in humans. Therefore, improvement of current vaccines and the development of alternative preventative and therapeutic strategies will be necessary to prepare for the emergence of potential pandemic avian influenza viruses like H5N1 or other HPAI viruses such as H3N7, H7N7, and H9N2. Additionally, similar vaccine strategies may be applicable to improve seasonal vaccines against H1 and H3 influenza viruses or against other emerging and re-emerging influenza viruses.

#### *1.1.4.1 Pandemic influenza viruses*

Influenza pandemics occur when the currently circulating seasonal viruses are replaced by a different strain or subtype through reassortment or replacement with a virus of zoonotic origin. The classification of an influenza virus as *pandemic* does not mean that it will be associated with high mortality; it is an index to assess the global spread of the virus.

The 1918 *Spanish Influenza* (Spanish Flu, H1N1-1918) is the most familiar influenza pandemic virus in recent history. The H1N1-1918 virus spread globally

between 1918 and 1919 and was characterized by unusually high mortality and morbidity in young adults (aged 20-40) (Ligon, 2005). Reports suggest that close to 50 million deaths were directly or indirectly associated with infection, including the development of secondary bacterial pneumonia (Johnson and Mueller, 2002; Morens et al., 2008). Older adults experienced a milder infection and may have been protected by pre-existing antibodies to previously circulating H1N1 viruses. The high virulence of H1N1-1918 is suggested to have been characterized by massive up-regulation of immune responses, including cytokines and increased infiltration by neutrophils and macrophages into lung tissues and the generation of strong pro-inflammatory responses (Ahmed et al., 2007). War, sanitation conditions, and the absence of antibiotics are also likely contributors to the high mortality observed during the Spanish Flu (Kuszewski and Brydak, 2000).

The *Asian Flu* (H2N2-1957) originated in China and spread throughout South East Asia during the 1957-1958 influenza season, resulting in approximately 1 million deaths. Another pandemic virus, the *Hong Kong Flu* (H3N2-1968), spread in Hong Kong between 1968 and 1969, with a mortality of around 500 000 (Potter, 2001). The H1N1-1918 virus is believed to have originated in a swine intermediate host where it acquired a highly virulent PB2 gene (Reid and Taubenberger, 2003). The zoonotic origins of the H2N2-1957 and H3N2-1968 viruses are still uncertain.

More recently, a swine-like H1N1 influenza A virus originating in Mexico spread worldwide in 2009 (H1N1-2009). The H1N1-2009 pandemic virus was the result of genome reassortment between swine and human influenza H1N1 viruses resulting in a novel virus including previously not encountered gene segments. The establishment of public health and government guidelines in the past decade contributed to the swift

identification and control of the pandemic. However, the recent H1N1-2009 influenza pandemic also highlighted the need to improve and develop better cross-protective influenza vaccines and address rapid vaccination during a pandemic.

*1.1.4.2 Genome structure, antigens, and life cycle*

Influenza A viruses, family *Orthomyxoviridae,* consist of an enveloped, negative-sense single-stranded RNA genome with 8 segments encoding 10 genes: hemagglutinin (HA), neuraminidase (NA), matrix proteins (M1 and M2), polymerase proteins (PB1, PB2, PA), non-structural proteins (NS1 and NS2), and the nucleoprotein (NP) (Skeik and Jabr, 2008; Webster et al., 1992; Wong and Yuen, 2006; Wright PF, 2007). Sixteen different HA genes (H1-16) and 9 NAs (N1-9) have been isolated from wild birds but only a few subtypes are pathogenic to humans (Wong and Yuen, 2006). Standard influenza virus nomenclature refers to combinations of the external glycoproteins to identify virus subtypes, regardless of the origin of the internal genes. Additionally, each strain is tagged with the location and year of isolation for further reference.

The influenza envelope contains the HA, NA, and M2 proteins. The HA glycoprotein mediates entry, fusion, and attachment of the virus through binding to sialic acid receptors on the surface of epithelial cells (Kodihalli et al., 1999; Skeik and Jabr, 2008). HA is expressed in the envelope as the HA0 precursor protein, comprising the HA1 globular domain and the HA2 stalk domain. HPAI H5N1 viruses contain a multi-basic amino acid site between HA1 and HA2 that can be cleaved by cellular proteases such as furin or PC6 (Hatta et al., 2001; Subbarao and Joseph, 2007). This initiates cleavage of the HA0 precursor independent of tissue specificity and increases the potential for systemic dissemination of the virus. Following cleavage, HA2 mediates

fusion of the virus to the endosome membrane (Steel et al., 2010). The multi-basic cleavage site is not frequently found in LPAI viruses, perhaps explaining the more limited tissue range and localised infection (Suguitan et al., 2009; Vandegrift et al., 2010).

The NA glycoprotein interacts with sialic acid receptors on the surface of epithelial cells, cleaving terminal sialic (neuraminic) acid residues allowing mature influenza virus to be released from infected cells (Cinatl et al., 2007a; Fischer II and Frederick). Additionally, NA also cleaves sialic acid residues on the HA glycoprotein to prevent self-aggregation of mature influenza viruses (Fischer II and Frederick). Matrix proteins M1 and M2 are important for the structural integrity of the influenza virion. M2 is a small, transmembrane protein that contains a short ectodomain that serves as an ion channel. Following endosomal fusion, the M2 ion channel regulates entry of hydrogen ions into the virus. The decrease in virus pH triggers uncoating of M1 and release of the ribonucleoprotein (RNP) complex into the cytoplasm (Tisdale, 2009).

The influenza A virus PB1, PB2, and PA proteins form the polymerase complex, encoding a viral RNA-dependent RNA polymerase. The NS1 protein acts as an interferon antagonist, interacting with RIG-I and inhibiting interferon β (IFNβ) and has been associated with induction of cytokines and chemokines (Laddy et al., 2007; Neumann et al., 2009). NS2 encodes the nuclear export protein (NEP) that transports the uncoated virus RNP-complex into the nucleus. Lastly, NP comprises the majority of the total protein content of the influenza virion. NP forms the RNP complex with the individual RNA genome segments.

In H5N1 viruses, HA, PB2, and NS1 have been associated with increases in virulence (Hatta et al., 2001; Neumann et al., 2009). Virulence may also be associated with the temperature in the lung environment (Hatta et al., 2007).

*1.1.4.3 H5N1 Clades*

The WHO has defined 10 different H5N1 clades in birds and humans, each further dividing into multiple sub-lineages and different isolates based on sequence homology and antigenic relationship (Martin et al., 2006; WHO, 2009). The HPAI A/Goose/Guangdong/1/1996 H5N1 virus lineage ("Z" lineage) is believed to be the precursor for the HPAI Hong Kong H5N1 viruses (Clade 0) (Martin et al., 2006). Further phylogenetic analysis has identified several HPAI H5N1 human clinical isolates in Clades 1, 2.1, 2.2, 2.3 and 7 (Dong et al., 2009; WHO, 2009). A Clade 2.1 virus was isolated from a case of human-to-human transmission in Indonesia (Yang et al., 2007).

*1.1.4.4 Host Specificity*

Influenza viruses mediate virus entry into epithelial cells through interaction of sialic acid receptors linked to different configurations of galactose. Avian influenza H5N1 viruses bind preferentially to α2,3-linked sialic acid receptors present on respiratory epithelial cells in birds. In contrast, human influenza viruses bind α2,6-linked sialic acid receptors that are present on human upper respiratory epithelia (Stephenson et al., 2004). Although the human upper respiratory epithelia express a small percentage of α2,3-linked receptors, the majority are found on lower respiratory epithelial cells. *In vitro* studies demonstrated the presence of α2,3-linked sialic acid receptors on the surface of ciliated epithelial cells in the lower respiratory tract (Neumann et al., 2009), partially

explaining the inability for H5N1 viruses to establish more efficient human-to-human transmission. A Lys193Arg mutation has been implicated in the transition from bird to human receptor specificity (Stevens et al., 2008).

Swine epithelial cells have been shown to express both α2,3-linked and α2,6-linked sialic acid receptors (Reid and Taubenberger, 2003), allowing fusion and entry of both avian and human influenza A viruses. Co-infection of cells with different influenza viruses can lead to genetic reassortment between influenza viruses, resulting in the generation of viable progeny which may contain human, pig, and/or bird genes.

*1.1.4.5 Antigenic Drift and Shift*

Influenza virus evolution is driven by two mechanisms: a*ntigenic drift* and *antigenic shift*. The accumulation of point mutations in influenza genes by the error-prone RNA-dependent RNA polymerase and/or selective pressure is called *antigenic drift* (Peiris et al., 2007). Point mutations in hypervariable regions of the glycoproteins can lead to immune evasion by altering antibody-binding and T-cell epitopes (Peiris et al., 2007). *Antigenic drift* can impair vaccine efficacy between antigenically related viruses.

*Antigenic shift* is the result of virus genome reassortment occurring through gene transfer between different influenza viruses. The successful exchange of external antigens can potentially generate a new influenza subtype that may not be effectively neutralized by pre-existing antibodies against either parent virus (Johansson and Brett, 2007). *Antigenic shift* is frequently described for the HA or NA genes, however, reassortment can potentially occur with each of the 8 RNA segments. Studies suggest that the high virulence of the H1N1-1918 virus was the result of gene transfer of the PB2 gene from a swine intermediate host (Ahmed et al., 2007; Reid and Taubenberger, 2003).

The evolution of the recent H1N1-2009 pandemic virus resulted from reassortment of multiple gene segments, over several decades, from avian H1N1 (N1, M, PB2, PA), swine H1N1 (H1, NP, NS) and human H3N2 (PB1) viruses (Smith et al., 2009).

### *1.1.5 Influenza Pathogenesis and Treatment*

Influenza A viruses cause an acute, upper respiratory tract infection in humans and are transmitted primarily through aerosol contact. The clinical presentation of influenza infection includes muscle aches, fever, headache, cough, sore throat, and may be related to secondary infections like bacterial pneumonia (caused by opportunistic *Staphylococcus spp.*) (Brundage and Shanks, 2008; Morens et al., 2008). Most people recover successfully from seasonal influenza infections but each year over 250 000-500 000 people die worldwide (Vandegrift et al., 2010). Young children, the elderly, and immunocompromised individuals are at most risk for influenza infection and mortality.

Typical lung pathology in humans following infection by H5N1 viruses includes alveolar damage, inflammation of the bronchioles, thrombocytopenia, lymphocytopenia, increased liver enzymes, increased infiltration of intra-alveolar macrophages, and hyperplasia and desquamation of type II pneumocytes (Guarner and Falcon-Escobedo, 2009). Similar pathology has been observed in animal models of infection including mice (Perrone et al., 2008), ferrets (Zitzow et al., 2002), and non-human primates (Laddy et al., 2007). Cell death due to apoptosis in the upper airway epithelia has also been reported following H5N1 infection (Daidoji et al., 2008).

*1.1.5.1 Immune responses*

Clearance of primary influenza infection is mediated predominantly by cell-mediated immune responses before a strong humoral immune response can be generated (Wiley et al., 2001; Woodland et al., 2001). Infected epithelial cells attract antigen presenting cells (APCs) including dendritic cells and macrophages which will process the viral antigens and become activated. Mature APCs migrate to the regional draining lymph nodes and present antigen on the surface of MHC molecules to circulating T-cells. Predominantly $CD8^+$ T-cells have been shown to be activated following antigen presentation (Wiley et al., 2001) and more specifically, cytotoxic T lymphocytes increase leading to clearance of influenza virus by Fas- or perforin activated pathways (Wiley et al., 2001).

Antigen-specific memory T-cells are resident in the respiratory tissues following primary infection, however, the re-activation efficiency decreases rapidly, as soon as three months following infection (Hogan et al., 2001). Therefore, the generation of long-term antibody-mediated protection is necessary to prevent against re-infection with homologous virus.

In the case of natural secondary infection, both mucosal (IgA) and systemic (IgG) antibody responses play a major role in protection (Wong and Yuen, 2006). A humoral neutralizing antibody response is generated against the HA glycoprotein and prevents secondary infection with homologous virus. Reactivation of the memory T-cell response may influence viral clearance and recovery. Cytotoxic T lymphocyte responses generated against conserved influenza epitopes may improve cross-protection against mismatched viruses (Doherty OC, 2001; Fleischer et al., 1985; Yewdell JW, 1989). Cell-

mediated immune responses may also play a role in protection against infection with a heterologous influenza virus.

Influenza virus infection can be treated using antiviral drugs and supportive therapies however prophylactic vaccination is the best method of protection.

*1.1.5.2 Antiviral drugs*

Antiviral drugs offer therapeutic treatment post-infection by blocking different stages of the virus replication and release cycle. Amantadine and rimantadine are M2 ion channel blockers that prevent uncoating of the M1 protein and release of the RNP complex early during replication (Fischer II and Frederick). However, resistance to both drugs has been identified across many influenza A subtypes, including all H3N2, some H1N1, some Clade 1 H5N1, and all Clade 2 H5N1 viruses (Fischer II and Frederick). Alternatively, the NA inhibitors oseltamivir (Tamiflu®) and zanamivir (Relenza®) interfere with the final stage of virus release by blocking cleavage of the terminal sialic acid residues and preventing release of mature virus (Cinatl et al., 2007a; Fischer II and Frederick). Similar to M2 inhibitors, oseltamivir resistance has been identified in several seasonal and H5N1 influenza isolates. Zanamivir has a lower bioavailability than oseltamivir, but resistance is less prevalent and the few resistant influenza strains are lab-generated (Fischer II and Frederick). Alternatively, the drug ribavirin has not been associated with any drug resistant influenza phenotypes. Ribavirin inhibits virus replication however it is cytotoxic and has been associated with many adverse side effects, including anaemia, electrolyte disturbance, and potential teratogenesis (Fischer II and Frederick; Rowe et al., 2010). Additionally, clinical data regarding the efficacy of ribavirin treatment is inconsistent. Nevertheless, ribavirin treatment in conjunction with

NA inhibitors may be a consideration for severe influenza infections (Chan-Tack et al., 2009; Ray et al., 1989; Rowe et al., 2010).

Following influenza infection, antiviral drugs can prevent influenza replication and release and are most effective for immediate response in cases of suspected contact or recent exposure. Alternatively, the passive transfer of serum antibodies from convalescent serum can prevent active influenza virus infection (Fischer II and Frederick; Tao et al., 2009). Passive immunotherapy is, however, limited by the cost of production of serum, the quantity (in mg/kg), and the antigenic variability between influenza virus strains. Prophylactic vaccination offers the best method to actively prevent influenza A virus infection and generate more robust immune responses leading to protection and recovery. Conventional inactivated and live-attenuated vaccines are commercially available and several experimental vaccine platforms are currently being evaluated to improve protection and cross-protection against various influenza subtypes including H5N1.

### *1.1.6 Conventional Influenza Vaccines*

The first conventional influenza vaccine was developed in the 1940s as a whole virus inactivated vaccine and was successfully evaluated in U.S. military personnel before receiving approval for public administration in 1945 (Kitchen and Vaughn, 2007). Current conventional influenza vaccines are generally composed of three inactivated or live-attenuated virus strains: one H1 influenza A, one H3 influenza A, and one influenza B virus. The influenza strains included in each seasonal vaccine are predicted based on the predominantly circulating strain from the previous year (Ellebedy and Webby, 2009; Subbarao and Joseph, 2007). Each vaccine contains 15µg of the HA antigen from each

virus, an unspecified amount of NA, and may be administered with or without an adjuvant (Hampson, 2008).

*1.1.6.1 Inactivated Vaccines*

Formalin-inactivated vaccines are the most commonly administered influenza vaccines. Two types of influenza vaccines have been generated: whole-virion (WV) and split-virion (SV), although most vaccines today are SV. The HA and NA proteins from the vaccine strain are added onto a non-pathogenic A/H1N1/Puerto Rico/8/1934 (H1N1-PR8) influenza virus backbone and amplified in the allantoic cavity of chicken eggs (Cox et al., 2004). Mature virus particles are then chemically inactivated using aqueous formaldehyde or β-propriolactone solutions (Cinatl et al., 2007b; Wong and Yuen, 2005). WV influenza vaccines are highly immunogenic and can generate protective antibody responses. WV vaccines have been associated with undesirable clinical side effects including increased local and systemic symptoms, therefore SV vaccines are generated by disrupting the inactivated virus particle with a solvent like sodium deoxycholate (Hampson, 2008) or Triton X-100 (Gross et al., 1981). SV vaccines are less immunogenic than WV vaccines (Cox et al., 2004), but they may be improved when combined with an adjuvant. One study suggests that the immunogenicity of the WV vaccine is mediated by enhanced activation of TLR-7 signalling pathways recognizing the viral single-stranded viral RNA (Geeraedts et al., 2008). This is impaired in SV vaccines due to disruption of the influenza particle leading to potential degradation of viral proteins.

The inactivated influenza vaccines generate predominantly systemic immune responses, producing IgG antibodies against the influenza virus, and are good for

presenting conformational antibody epitopes (Welsh and Fujinami, 2007). However, they do not generate significant cell-mediated immune responses. Currently, three H5N1 SV vaccines have been approved for human use based on the A/Vietnam/1194/2004 H5N1 virus: an aluminum hydroxide-adjuvanted Influenza Virus Vaccine (30µg HA, Sanofi Pasteur, U.S.A.) (Bresson et al., 2006), Panvax (30µg, Australia) (Nolan et al., 2008), and AS03-adjuvanted Pandemrix (3.75µg HA, GlaxoSmithKline, Europe) (Leroux-Roels et al., 2007).

Several clinical trials are underway to evaluate various WV and SV inactivated and live-attenuated H5N1 vaccines (reviewed in (Subbarao and Luke, 2007) and (Prieto-Lara and Llanos-Mendez)). Many vaccines are based on the A/Vietnam/1203/2004 (H5N1-Vn04) isolate. One WV inactivated vaccine candidate, currently in Phase III clinical trials, observed successful protection with low doses (7.5µg HA) of the vaccine without adjuvant, and a detectable antibody response (NIH, 2010). The vaccine has demonstrated a comparable safety profile to season influenza SV vaccine and may be recommended for young children.

*1.1.6.2 Live-attenuated Vaccines*

A live-attenuated influenza vaccine (LAIV) has also been approved for commercial use and is administered by an intranasal aerosol mist. The HA and NA proteins from the vaccine strain are added onto the non-pathogenic A/Ann Arbor/6/60 or B/Ann Arbor/1/66 influenza backbones (Cox et al., 2004). The LAIV virus has a cold-adapted phenotype and the backbone is first attenuated by serial passage on tissue culture cells at 25°C (Beyer et al., 2002; Cinatl et al., 2007b). The virus is passaged in chicken eggs to achieve further attenuation. The attenuated phenotype is attributed to five

mutations in the PB1, PB2, and NP gene segments and does not replicate in the lungs of mice and ferrets (Jin et al., 2003). LAIV vaccines induce innate and adaptive immune responses at the site of administration and have been associated with increased systemic IgG antibodies and mucosal IgA antibodies (Beyer et al., 2002; Cheng et al., 2009). Additionally, they can generate T-cell responses and several studies have reported protection against heterologous virus strains (Suguitan et al., 2006; Veits et al., 2008).

FluMist® (MedImmune) is a commercially available LAIV that has been approved in the U.S. since 2007 and more recently in Canada (2010). LAIV vaccines are effective for administration in children older than 6 (Wareing and Tannock, 2001). The vaccine has been associated with increased risk of adverse side effects in young children with asthma (18-35 months), however, in older children it appears to be more stable (Redding et al., 2002). There are no approved LAIV vaccines against H5N1, however ,several studies have shown protection in mice and ferrets (Lu et al., 2006; Suguitan et al., 2006). Current H5N1 LAIV vaccines in clinical trials include two candidates, one based on the H5N1-Vn04 isolate and another based on A/duck/Potsdam H5N2 (Keitel and Atmar, 2009; Rudenko et al., 2008).

### *1.1.7 Immune Responses to Conventional Vaccines*

Although the inactivated and LAIV vaccines activate different adaptive immune responses, both vaccines offer similar protection against homologous secondary infection (Beyer et al., 2002). Inactivated vaccines can stimulate both local and systemic humoral responses, leading to the generation of IgG antibodies against the HA and NA glycoproteins. Weak IgA responses can also be detected (Brokstad et al., 1995; Cox et al., 1994). Protective neutralizing antibodies against the HA are generated within the first

2 weeks following vaccination in 90% of individuals (Cox et al., 2004; Cox et al., 1994) and cross-protection against antigenically similar influenza isolates has also been reported with inactivated vaccines. LAIV vaccines can generate systemic IgG antibodies, however, lower hemagglutination inhibition (HI) antibody titres are frequently reported following administration compared with inactivated vaccines. Additionally, serum IgG antibodies following LAIV immunization do not always correlate with protection (Clover et al., 1991), suggesting that other innate and adaptive immune responses may also be involved in protection (Cox et al., 2004). Rather, LAIV vaccines can induce strong secretory IgA (sIgA) immune responses. The generation of cell-mediated immunity in response to internal influenza antigens in the live vaccine may also influence recovery following protection. In older adults, the strength of the cell-mediated immune response may be a better indication of protection than neutralizing antibody titres (McElhaney et al., 2006; Rimmelzwaan et al., 2007).

In addition, the route of immunization can play significant role in vaccine immunogenicity and protection against influenza viruses. Inactivated and live-attenuated influenza vaccines can be delivered by intramuscular, intranasal, oral, or intradermal routes of immunization. Vaccines can also be combined with an adjuvant to induce stronger immunogenicity and protection. Different cytokine profiles have also been observed following immunization at different locations.

*1.1.7.1 Intramuscular (i.m.)*

Intramuscular immunization of inactivated influenza vaccines offers a fast rate of absorption into muscle tissue (Treanor, 2004) and can generate strong serum IgG antibody titres. Intramuscular inactivated vaccine administration demonstrated better

induction of HI antibody titres than LAIV intranasal or dermal administration (Fiore et al., 2009) and could be correlated with survival (Amorij et al., 2010). As well, some evidence suggests that weak cell-mediated immune responses can also be generated following i.m. administration. Unfortunately, i.m. vaccination is associated with some discomfort and pain and the generation of immune responses in immunocompromised individuals may not be optimal. Needle stick injuries are also a potential occupational risk for health care workers. Therefore, other alternative immunization routes are being considered for influenza vaccine delivery.

*1.1.7.2 Intranasal*

Influenza viruses naturally infect humans through the nasopharyngeal route, suggesting that vaccines administered by intranasal aerosol or dry powder can also induce protective mucosal immune responses at the primary site of influenza infection (Amorij et al., 2010; Dean, 2006). This can lead to the generation of mucosal sIgA as well as systemic IgG antibodies. Intranasal inactivated and LAIV vaccines offer similar protection against homologous influenza virus challenge. LAIV vaccines offer better protection against heterologous challenges possibly through the generation of mucosal cell-mediated immune responses (Suguitan et al., 2006; Wareing and Tannock, 2001). The immunogenicity of intranasal influenza vaccines can be improved when combined with an adjuvant. There is some possibility of CNS side effects and patients with respiratory disorders may have additional implications. However, intranasal vaccination has shown similar efficacy to intramuscular administration (Beyer et al., 2002).

*1.1.7.3 Oral*

There are currently no available oral vaccines against influenza viruses. One of the challenges with oral administration is the pH of the gastrointestinal tract leading to potential degradation of the vaccine. Additionally, cleavage or dissociate of the vaccine must be considered in order to facilitate interactions with the M cells of the intestines to stimulate mucosal immune responses (Dean, 2006). For this reason, oral administration is very inefficient and often requires considerably more vaccine than other routes of immunization. The delivery of vaccine by sublingual or gingival routes has shown promise in one clinical trial (Amorij et al., 2010). A WV influenza vaccine was delivered to the oral cavity by using an aerosolized spray. Although IgA antibodies were not induced, serum IgG and hemagglutination inhibition antibodies against influenza were detected (Bakke et al., 2006). Cell-mediated immune responses were not detectable.

*1.1.7.4 Intradermal and Subcutaneous*

More recent studies are evaluating the use of intradermal or subcutaneous delivery for inactivated influenza vaccines. Full protection was observed in mice following microneedle delivery of liquid or powder influenza vaccines (Amorij et al., 2010). Additionally, dose-sparing was also observed with intradermal administration compared to intramuscular immunization (Kenney et al., 2004; La Montagne and Fauci, 2004). Intradermal immunization has been associated with an increase in antigen presentation through recruitment of Langerhans cells in the skin dermis (Amorij et al., 2010). This can induce the generation of both systemic and mucosal immune responses, leading to antibody production by B cells (Belshe et al., 2004) and the potential activation of cellular immune responses.

### *1.1.8 Influenza Vaccine Adjuvants*

Adjuvants are often administered in conjunction with H5N1 vaccines to improve immunogenicity. Aluminum-based salts, aluminum hydroxide and aluminum phosphate, are often combined with the conventional H5N1 vaccines (Bernstein et al., 2008; Miyaki et al., 2010). GlaxoSmithKline has also developed a line of adjuvants: AS03 (squalene-based), AS04 (aluminum hydroxide-based) that have been combined with their seasonal influenza vaccines. Alternatively, MF59 (Fluad, Chiron Vaccine) is an oil-in-water emulsion that has also demonstrated immunogenicity when combined with seasonal inactivated vaccines (Forrest et al., 2009). Immune stimulating complexes (ISCOMs) are derived from the bark of the *Quillaia saponaria* Molina tree and have also shown to stimulate strong humoral and cellular immune responses (Sjolander et al., 1998).

Other experimental adjuvants include the addition of cytokines and other pattern recognition molecules to stimulate innate immune responses such as interleukins 12 and 15, TLR adjuvants, or GM-CSF (Fang et al., 2010; Kutzler et al., 2005; Loudon et al.; Morrow et al., 2009; Riedl et al., 2008).

#### *1.1.8.1 Safety Concerns*

Although the safety of conventional inactivated and LAIV vaccines is well-documented, several adverse side effects have been reported following immunization including allergic reactions or the development of undesirable conditions. Allergic reactions are rare, however, they have been reported against vaccine components such as formaldehyde, Tween-80, or sodium deoxycholate (Hampson, 2008). As well, commercially available influenza vaccines are produced in chicken eggs, therefore,

administration is not advisable for individuals who are allergic to eggs or chicken protein (Cox et al., 2004).

Thiomersal (thimerosal) is an ethylmercury preservative that is frequently included as part of the inactivated vaccine to prevent bacterial and fungal contamination (Bigham and Copes, 2005). The use of thiomersal has been contraindicated by reports of potential toxicity and the development of allergic reactions. Mercury poisoning has been reported in animal studies (Hornig et al., 2004; Magos et al., 1985), as well as anecdotal cases of human toxicity, however, little conclusive data has been presented to support these claims. The WHO still recommends the inclusion of thiomersal in several vaccine candidates (WHO, 2010b). Allergies have been associated with the subcutaneous administration of thiomersal-containing vaccine, however, no significant reactions have been identified with intramuscular immunization (Harry et al., 2004). Nevertheless, the manufacturers of commercial influenza vaccines are reducing or eliminating the content of thiomersal as a preventative measure (WHO, 2010b).

Occulorespiratory syndrome (ORS) has been associated with the inactivated influenza vaccine. It is often associated with red eyes, respiratory distress, pain, and mild discomfort (Boulianne et al., 2001), typically resolving within 48 hours. Systemic symptoms may include a high fever and headache (Boulianne et al., 2001), however, ORS usually resolves without any additional treatment. ORS was first reported in Canada following vaccination with the 2000-2001 seasonal influenza vaccine. An abnormally large proportion of unsplit virions in the SV vaccine lead to the formation of large aggregates of virus particles (Skowronski et al., 2003), resulting in adverse side

effects. ORS may occur in subsequent vaccinations, however, the symptoms have been reported to be mild and do not interfere with future vaccination (Grenier et al., 2004).

The rare development of Guillan-Barré Syndrome (GBS) may also occur following vaccination. Although it is most frequently associated with complications following *Campylobacter jejuni* infection, GBS may develop following vaccination against respiratory illnesses including influenza and parainfluenza (Kett and Loharikar, 2009). GBS is commonly associated with peripheral nerve cell damage, the development of muscle weakness, and may occasionally present with paralysis and may last from weeks to months. Several cases of GBS were reported following the 1976 swine influenza vaccination due to components in the vaccines. Overall, approximately one in every million people develops GBS. It is believed that the development of GBS may be related to egg components in conventional vaccines (Wong and Yuen, 2005).

With LAIV vaccines, there are also concerns that the vaccine may revert from attenuation to a more virulent phenotype or reassort with other influenza viruses. As a safety feature, LAIV vaccines are temperature sensitive so that they will only replicate in the upper respiratory tract and reduce the likelihood of virus reassortment (Gillim-Ross and Subbarao, 2006; Wong and Yuen, 2005). Immunization with the LAIV Flumist® vaccine was also associated with increased early upregulation of anti-inflammatory cytokine interleukin 10 (IL-10) (Kobinger et al., 2010). Higher mRNA levels of IL-10 were detected in the upper and lower airways of ferrets possibly contributing to increased virus replication through suppression of the pro-inflammatory cytokine interleukin 6 (IL-6).

*1.1.8.2 Conventional Vaccines and H5N1*

Conventional influenza vaccines offer considerable protection and can reduce the illness associated with seasonal and episodic influenza outbreaks. The vaccines are reformulated yearly in order to ensure optimal protection, however, the co-circulation of multiple influenza lineages may lead to antigenic mismatch between the circulating and vaccine strain resulting in inadequate coverage. Such a mismatch was observed during the 2005-2006 influenza season where the A/California/7/2004 H3N2 vaccine strain varied antigenically from the A/Wisconsin/67/2005 circulating strain (Poland et al., 2008). This presents a challenge for the development of vaccines against emerging influenza viruses like H5N1 viruses as most populations are immunologically naive. The development of conventional vaccines against H5N1 viruses has also been hampered by the low immunogenicity of the H5 HA protein (Jin et al., 2008; Keitel and Atmar, 2009; Subbarao and Luke, 2007) resulting in reduced vaccine efficacy and limited protection. While conventional vaccines are available against H5N1 viruses, higher or multiple doses of vaccine are required in order to achieve comparable protection levels as with seasonal viruses. An H5N1 SV vaccine required 12 times the dose of HA as the seasonal influenza vaccine to achieve the same protection (Nolan et al., 2008).

HPAI H5N1 viruses are also lethal in chicken, presenting a challenge for mass-production of influenza vaccines in fertilized chicken eggs (Subbarao and Joseph, 2007). Additional concerns include potential mutation and/or reassortment of the vaccine strain in chicken eggs prior to inactivation. Vaccine production requires several months in order to produce, inactivate, and distribute the approved vaccines. As an alternative,

tissue-culture can be used to amplify vaccine strains rapidly, with few of the side effects associated with egg-based methods (Barrett et al., 2010).

The major challenge with H5N1 vaccines has been the establishment of cross-clade protective immunity (Baras et al., 2008; Cinatl et al., 2007b). Therefore, several experimental subunit vaccines are being considered as potential options to conventional vaccination for achieving better protection against avian influenza H5N1 viruses.

### *1.1.9 Experimental Subunit Vaccines*

The limitations of conventional H5N1 vaccines have encouraged the development of several experimental approaches to improve protective efficacy against matched and divergent viruses. Experimental subunit vaccines can express one or more H5N1 antigens without administration of whole influenza virus particles. Expression of H5N1-HA, -NA, -NP, and matrix proteins (M1, M2) has been evaluated in various single or combination vaccines candidates (Cinatl et al., 2007b; Johansson and Brett, 2007). The generation of protective neutralizing antibody responses against the HA protein has been the focus of the majority of experimental platforms. The generation of antibodies against NA and cell-mediated immune responses against the well-conserved matrix proteins and NP are also being considered as part of experimental vaccine design (Laddy et al., 2007; Rimmelzwaan et al., 2007; Tompkins et al., 2007). Several experimental subunit vaccines have been evaluated including purified viral proteins, virus-like particles (VLP), DNA expression vectors (DNA vaccines), and viral-based vectors.

Purified viral protein (native and recombinant) antigens were first considered as potential vaccine candidates (Johansson and Brett, 2007). However, the protein vaccines could not generate strong cell-mediated immune responses and poor vaccine efficacy was

reported *in vivo* (Treanor et al., 2001). A purified H5N1-HA protein vaccine offered only partial protection against lethal challenge in ferrets, compared to full protection achieved using an experimental VLP vaccine expressing the same antigen (Mahmood et al., 2008). The *de novo* synthesis of antigens based on consensus amino acid sequences from diverging H5N1 viruses was evaluated in several experiments. In one study, a consensus-based HA gene was constructed from the sequences of over 400 H5N1-HA antigens belonging to different clades and expressed in an experimental DNA platform (Chen et al., 2008). Another study evaluated a consensus-based DNA vaccine expressing well-conserved H5N1 antigens (Laddy et al., 2007). However, ensuring that the consensus-based protein retains functionality is one weakness of this approach. Additionally, the generation of low affinity antibodies against epitopes in the consensus-based antigen may lead to reduced immune responses and possibly lower protection. Overall, experimental subunit vaccines based on virus-like particles, DNA-based, and recombinant viral vectors have shown the most promise as potential vaccine platforms against avian influenza H5N1 viruses.

#### *1.1.9.1 Vaccines in Development*

##### 1.1.9.1.1 Virus-like particles (VLPs)

Virus-like particles are non-infectious vaccine experimental platforms that can express one or more structural proteins but contain no viral nucleic acids (RNA or DNA), and are, therefore, non-replicating. The development of VLP vaccines against enveloped viruses has been challenging (Noad and Roy, 2003) but several successful candidates have been generated against H5N1 viruses. The H5N1-HA, NA, and matrix proteins can be expressed in a helper-dependent baculovirus system that allows VLP production by

self-assembly (Jin et al., 2008; Wu et al., 2009). H5N1-VLP vaccines preserve the native structure of influenza proteins, maintaining conformational B and T-cell epitopes. Mass-production of VLPs can be achieved using insect tissue culture cell lines, thereby avoiding traditional egg-based vaccine production methods (Bright et al., 2008). Steel et al (2010) evaluated an influenza VLP expressing the more conserved HA2 stalk domain and achieved successful protection (Steel et al., 2010). Alternatively, another study has developed H5N1-VLPs derived from plants (D'Aoust et al., 2010).

VLP-based vaccines have been shown to be safe and immunogenic in mice (Song et al., 2010), ferrets (Mahmood et al., 2008), NHPs, and humans (Akahata et al., 2010; Evans et al., 2001; Harro et al., 2001). The first successful VLP vaccine was generated using hepatitis B surface antigen (HBsAg), forming a virus-like particle without any internal nucleic acids (McAleer et al., 1992; Noad and Roy, 2003). The commercial human papilloma virus (HPV, Gardasil) vaccine is also a VLP-based vaccine and has demonstrated both systemic and mucosal immune responses (Evans et al., 2001). An H5N1-VLP vaccine expressing HA and M1 proteins offered full protection in mice against homologous challenge and generated strong humoral and cell-mediated immune responses (Song et al., 2010). Protective IgG1 and IgG2a antibodies were detected along with the induction of Th1 immune responses and increased IFNγ secretion. Additionally, heterosubtypic protection between an H1-VLP and H5N1 challenge was reported in ferrets (Perrone et al., 2009).

However, long-term immune responses have not been evaluated for H5N1-VLPs. Traditional VLPs are non-infectious and, therefore, cannot prolong continuous expression of antigens (Spohn and Bachmann, 2008). Additionally, immune responses are limited to

the antigens presented in the vaccine and may not generate as broad an immune response as an infectious virus. Small VLPs have also been shown to have trouble inducing immune responses (Noad and Roy, 2003).

1.1.9.1.2 DNA Vaccines

The delivery of naked DNA expressing viral antigens is another experimental strategy. DNA vaccines were first considered in the context of gene transfer. Mouse muscle was injected with naked DNA expressing reporter genes luciferase or β-galactosidase (Wolff et al., 1990). It was suggested that DNA could deliver the corrected copy of a defective gene directly to muscle cells or potentially an antigen as an alternative vaccination approach (Ulmer et al., 1993; Wolff et al., 1990). DNA vaccines are non-infectious double-stranded DNA expression plasmids that express a gene of interest under the control of a eukaryotic promoter. The first vectors were promising vaccine candidates in small rodents however it was discovered that naked DNA was poorly immunogenic in larger animal models and humans (Garmory et al., 2003; Luxembourg et al., 2007). One of the first considerations was to improve the DNA backbone by including tissue-specific promoters and other enhancing elements to increase antigen expression and vector immunogenicity (Garmory et al., 2003). Selection of certain mammalian (creatine kinase, Ubiquitin c, chicken β-actin) or viral promoters (SV40, RSV, CMV) can improve tissue targeting and increase gene expression. Other enhancer elements can be included such as a Kozak sequence (GCC(R)CCATGG) to improve ribosome binding and initiation of transcription, and a polyadenylation signal (BGH, SV40, rabbit β-globin) to stabilize the mRNA transcript (Garmory et al., 2003). Additionally, gene optimization has been considered in several

DNA vaccine candidates to improve expression through consideration of codon-bias and selection of optimal amino acids for expression in mammalian cells (Gurunathan et al., 2000). Together, these elements have greatly improved the immunogenicity of DNA expression vectors and several candidates are being evaluated against bacterial (Lowrie, 1999), fungal (Deepe, 1997), parasitic (Carvalho et al., 2010), and viral pathogens (Gurunathan et al., 2000; Kim and Jacob, 2009) in animals and humans [reviewed in (Mor and Eliza, 2001) (Faurez et al., 2010). Three DNA vaccines are currently licensed for administration in animals to prevent infectious hematopoietic necrosis in salmon, West Nile virus in horses, and canine oral melanoma (Faurez et al., 2010).

The exact mechanism of naked DNA uptake into cells is unclear, however, it is hypothesized to enter through either receptor- or pinocytosis-mediated endocytic uptake (Kim and Jacob, 2009). How DNA plasmids are eliminated from the cell over time is also uncertain. Following intramuscular administration, DNA vaccines enter keratinocytes and dendritic cells, in particular Langerhans' cells (Kim and Jacob, 2009). Activated dendritic cells migrate to the regional draining lymph nodes where they activate circulating T-cells by presentation and cross-presentation on MHC Class I and II molecules. DNA vaccines can induce the generation of humoral and cell-mediated adaptive immune responses leading to activation of IFNγ and the activation of cytotoxic T lymphocytes. The generation of specific Th1 or Th2 immune responses depends on the route of immunization and antigen. DNA vaccines can also activate innate immune pathways, including intracellular TLR-9. The bacterial origin of replication and antibiotic selection marker frequently contain, or are flanked by, immunogenic CpG motifs that can act as a natural adjuvant (Kendirgi et al., 2008).

1.1.9.1.3 DNA vaccines against Influenza and H5N1

In 1993, Ulmer et al described the first DNA vaccine against an influenza A virus. A naked DNA vector expressing an H1N1-PR8 NP antigen offered promising levels of protection against homologous and heterologous H3N2-1968 challenges (Montgomery et al., 1993; Ulmer et al., 1993). Since then, influenza DNA vaccine candidates have been developed mostly in the context of the HA antigen, however, several vaccines have also evaluated more conserved antigens. Chen et al (2009) evaluated DNA vaccines expressing H1N1-PR8 HA, NA, M2 and NP antigens in mice, achieving successful protection against homologous but only partial protection against diverging H1 and H3 influenza viruses (Chen et al., 2009) .

HA-based H5N1 DNA vaccines have offered mixed success at achieving protection against matched and divergent challenges. In one experiment, an HA DNA vaccine offered full protection in mice against homologous virus but did not prevent heterologous infection. Other strategies have included the evaluation of multivalent vaccines containing a mix of DNA plasmids expressing the HA genes from different H5N1 isolates and subtypes. Kodihalli et al (1999) described protection in mice and chicken following immunization with a DNA vaccine combining H5 and H7 HA antigens (Kodihalli et al., 1999). The study was able to achieve cross-protection against both viruses. Protection was also evaluated in mice (Chen et al., 2009) and chicken (Jiang et al., 2007) using the pCAGGS expression plasmid, containing a chicken β-actin promoter.

Other studies have considered the generation of strong T-cell responses to improve DNA vaccine efficacy. Although a DNA vaccine delivering H5N1 NP did not offer full protection in mice, it was able to achieve 50% protection against lethal homologous

challenge (Epstein et al., 2002). The same study combined H5 NP and M2 plasmids but only observed protection at low challenge doses. In another study, combining H5 HA and NP antigen was able to offer 100% protection from homologous challenge and significant partial protection against a divergent H5N1 virus (Price et al., 2009). Recently, Rao et al (2009) generated cross-protective immunity following administration of a DNA vaccine combining the H5N1 HA, NP, and M2 antigens (Rao et al., 2009).

1.1.9.1.4 Improving immunogenicity

Although promising, the majority of successful DNA vaccines require multiple immunizations, often combined with other pharmaceutical or mechanical aids to improve bioavailability. Cationic lipid delivery molecules such as liposomes have been considered as one way to coat the DNA for better delivery (Thueng-in et al., 2010). Electroporation is another strategy to improve delivery of naked DNA to the cells by disrupting the cell membrane and creating transient pores, allowing DNA to enter the cell; the electroporation is performed following intramuscular injection of the vaccine (Luxembourg et al., 2007; Wang et al., 2008b). Also, particle-mediated gene delivery (gene gun) is another option (Sharpe et al., 2007; Wang et al., 2008b). Gold-particles are coated with DNA and used to physically bombard the cell with the vaccine. All strategies have considerably improved DNA vaccine delivery and therefore also have had a great impact on vector immunogenicity.

1.1.9.1.5 Limitations of DNA Vaccines

Although naked DNA vaccines are an attractive vaccine candidate, there are several concerns including possible integration of bacterial genes into the human genome leading

to insertional mutagenesis and potential oncogenesis (Kendirgi et al., 2008). However, there is little evidence of DNA plasmid insertion into the cellular genome, to-date. Alternatively, bacterial genes may also stimulate undesirable immune responses. To avoid this problem, linear expression cassettes (LECs) and other linear DNA vectors that lack a bacterial origin of replication, bacterial promoter, and selective resistance marker are also being developed (Kendirgi et al., 2008). Cell-free synthesis method can be used to amplify the linear DNA using a mesophilic DNA polymerase process similar to PCR. One study observed high levels of protection (93%) in mice vaccinated with an H5N1 HA expressing LEC.

Recently, an H5N1 DNA vaccine (VGX-3400X, Inovio), delivered in conjunction with electroporation, has entered phase I clinical trials. Alternatively, the use of DNA vaccines has also been considered for initial priming of the immune response before or following immunization with a conventional or another experimental vaccine. Another current clinical trial is evaluating the VRC-AVIDNA036-00-VP DNA vaccine (NIAID) for administration following the Sanofi Pasteur H5N1 inactivated virus vaccine.

DNA vaccines can be combined with viral based vectors to boost protective immunity and induce long-term immune responses as part of a prime boost strategy. Viral-based vectors can also generate robust immune responses in larger animals and may improve vaccine efficacy and expand cross-protection against multiple H5N1 lineages.

*1.1.9.2 Viral Vectors*

As another alternative to conventional vaccines, experimental H5N1 vaccines based on recombinant viral vectors are being considered to generate strong immune responses and protection (Kopecky-Bromberg and Palese, 2009; Palese et al., 1997).

Several properties of viral vaccines can be exploited including activation of pattern recognition receptors (PRR) like Toll-like receptors (TLR) and RIG-like receptors (RLR), leading to activation innate and adaptive immune responses (Spohn and Bachmann, 2008). Early genes involved in virus replication are frequently deleted to increase the carrying capacity of a viral vector and as a safety feature. Deletion of essential genes can prevent unregulated replication of a vector, thereby avoiding undesirable side effects against the virus. Several replication-competent and deficient viruses have been developed for human and animal H5N1 vaccines including single-stranded RNA vectors: alphavirus replicons (Perri et al., 2003), vesicular stomatitis virus (Schwartz et al., 2007), Newcastle disease virus (DiNapoli et al., 2009) and double-stranded DNA vectors: poxviruses (canarypox, vaccinia)(Kreijtz et al., 2009), and adenovirus (Ad) [reviewed in (Kopecky-Bromberg and Palese, 2009). In particular, Ad-based viral vectors can generate robust, protective immune responses and have shown promise against H5N1 viruses in animal models.

1.1.9.2.1 Adenovirus vectors

Adenoviruses are non-enveloped, linear, double-stranded DNA viruses, belonging to the family *Adenoviridae*. The genome size of wild type adenoviruses is typically between 30-40kb (Volpers and Kochanek, 2004). Infection generally causes a mild respiratory or gastrointestinal illness and has also been associated with conjunctivitis. A live-oral vaccine against human adenovirus serotypes 4 (Ad4) and 7 (Ad7) has been administered to U.S. military personnel since the 1960s and is generally well-tolerated with few side effects (Kitchen and Vaughn, 2007; Roy-Chowdhury J, 2002).

The use of adenovirus as a vaccine vector was first considered for gene therapy and cancer applications. The initial discovery came from simian virus 40 (SV40) contaminated primary monkey tissue culture cells that were being used to propagate the live adenovirus vaccines (Roy-Chowdhury J, 2002). Experiments identified the insertion of SV40 T-antigen into the E1 region of Ad. This was the first indication that an adenovirus could be used to carry and express foreign DNA (Roy-Chowdhury J, 2002). First-generation Ad vectors contain deletions in the early immediate E1 genes, rendering the virus incapable of replication without help from a stable cell line expressing the missing genes. Ad vectors were further developed to accommodate larger fragments (up to 8kb) of foreign DNA through additional deletions in the E3 and E4 genes. A replication-deficient vector can transiently express heterologous genes without integration into the host chromosome; the vector is maintained as an episome following infection.

Adenoviruses can infect various human cell types and tissues *in vitro* and *in vivo*. The well-characterized human adenovirus serotype 5 (AdHu5) gains cell entry through interactions between the cocksackie B-adenovirus receptor (CAR) and β-integrins ($\alpha_v\beta_3/\alpha_v\beta_5$, and occasionally $\alpha_v\beta_1$) (Bangari and Mittal, 2005; Varnavski et al., 2003) leading to receptor-mediated endocytosis. AdHu5 vectors can act as a natural adjuvant through the induction of innate immune responses including activation of transmembrane TLR2 and intracellular TLR9 pathways leading to upregulation of type I interferons (IFNα and IFNβ), type II interferon (IFNγ) and proinflammatory cytokines (Hartman et al., 2008; Nayak and Herzog; Price et al., 2009). Combined with influenza antigens, AdHu5 can also potentially activate intracellular TLRs 3 and 7 (Price et al., 2009).

Specifically, activation of the MYD88/TRAIL pathways can lead to activation of IL-6 and IL-12 and there is possible evidence of NALP3 inflammasome involvement (Nayak and Herzog). Activation of type I interferon pathways by AdHu5 can activate natural killer (NK) cells, leading to the upregulation of IL-1 and TNFα. AdHu5 can also induce TLR-independent pathways that can also lead to maturation of dendritic cells, initiate complement cascades, and induce endothelial cell activation (Hartman et al., 2008; Nayak and Herzog).

Ad vectors can induce adaptive immune responses including both systemic and mucosal antibody responses, depending on the route of immunization (Croyle et al., 2008). Activation of cell-mediated immune pathways can lead to further upregulation of IFNγ and generation of Th1 immune responses similar to influenza virus infection (Nayak and Herzog). When administered i.v. the virus is rapidly cleared (90% in 24hours), however, it can persist in tissues for a much longer period. The generation of long-term memory responses has also been reported in several animal models (Lasaro and Ertl, 2009; Tatsis et al., 2007).

1.1.9.2.2 Ad-based Vaccine Development

Vaccine research has focused on the development and evaluation of Group C (genera *Mastadenovirus*) adenoviruses which includes the well-characterized human serotype 2 (AdHu2) and human serotype 5 (AdHu5) vectors. The generation of diverse humoral and cell-mediated immune response may not be advantageous for certain gene therapy applications (Hartman et al., 2008; Jooss and Chirmule, 2003), however, the strong immunogenicity of Ad-based vectors suggests that they may be a promising platform against endemic parasitic and viral infections, as well as emerging zoonotic

viruses. Several vaccine candidates have been evaluated against malaria (*Plasmodium flaciparum*) (Todryk and Hill, 2007), dengue virus (Raviprakash et al., 2008), Hepatitis C (Fattori et al., 2006), Ebola (Richardson et al., 2009), SARS (Zakhartchouk et al., 2005), and influenza virus [reviewed in (Kopecky-Bromberg and Palese, 2009). Additionally, the protective efficacy of several promising AdHu5-based candidates is being evaluated in mouse and non-human primate animal models and in human clinical trials (NIH, 2010; Seregin and Amalfitano, 2009). AdH5-based vaccines may provide alternative vaccine platforms to conventional influenza vaccines for generation of protective immune responses against avian influenza H5N1 viruses. Although adenovirus vectors contain virus particles, they can be lyophilized for easy storage and transport (Souza et al., 2005).

Several AdHu5-based vectors have been developed expressing the H5N1-HA antigen. An AdHu5 vector expressing the full HA0 or HA1 fragment offered better protection and higher antibody responses were detected compared to HA2 (Gao et al., 2006). Another study combined multiple AdHu5-HA vectors expressing HAs from multiple H5N1 clades and observed partial protection. The combination of AdHu5-NP along with two divergent AdHu5-HAs improved protection (Hoelscher et al., 2008). Holman et al (2007) evaluated an AdHu5-based vaccine platform (CAdVax) that contains multiple promoters, allowing expression of more than one gene in trans (Holman et al., 2008). Partial protection against clade 1 and 2 H5N1 viruses was observed by an AdHu5 vaccine expressing HA, NA, M1. An AdHu5-M2 vaccine was also developed and offered some protection but was more efficient with DNA priming (Tompkins et al., 2007).

However, the development of AdHu5-based vaccines has met with a couple setbacks. In 1999, a gene therapy clinical trial to correct ornithine transcarbamylase (OTC) deficiency was halted following the death of one of the patients (Jooss and Chirmule, 2003). The clinical disease, high toxicity, and uncontrollable upregulation of cytokines were contributing factors leading to death. More recently, Merck evaluated a trivalent AdHu5-based vaccine expressing the *gag*, *nef*, and *pol* genes in its HIV-STEP clinical trial. Although initially promising, it was determined during a follow-up that patients who received the vaccine had an increased risk of HIV infection. Further studies implicated the possibility of pre-existing neutralizing antibodies against the AdHu5 vector as well as pre-existing memory T-cells against AdHu5 that may have also interfered with vector efficacy (Watkins et al., 2008). However, despite the setbacks, AdHu5 vectors are being re-developed and other adenovirus serotypes are also being considered.

1.1.9.2.3 Pre-existing immunity

Although AdHu5-based H5N1 vaccines have demonstrated protective efficacy against matched and distant virus challenges, the future development of the vector is hindered by the presence of pre-existing natural immunity in a large percentage of the human population (30-50% in North America (Lasaro and Ertl, 2009)). Naturally-acquired neutralizing antibodies against AdHu5 can interfere with vaccine efficacy by reducing transgene expression and potentially leading to undesirable clinical outcomes following immunization (Brunetti-Pierri et al., 2004; Hartman et al., 2008; Watkins et al., 2008). Pre-existing immunity to AdHu5 can be overcome with high doses of vaccine

($10^{13}$ vp/kg), however, this has been associated with lymphocytopenia, elevated liver enzymes, and other signs of toxicity (Hartman et al., 2008; Varnavski et al., 2005).

This has had implications for sequential immunization regimens that may require re-administration of the same vaccine and for administration of different vaccines using the same vector backbone. Therefore, several strategies are being considered to circumvent pre-existing immunity to AdHu5, including the development of modified and chimeric AdHu5 vectors (Campos and Barry, 2007), alternative sites of administrations such as intranasal immunization (Croyle et al., 2008; Park et al., 2009), and the development and evaluation of rare human and mammalian adenovirus vectors with low human seroprevalence [reviewed in (Bangari and Mittal, 2006a)].

1.1.9.2.4 Chimeric AdHu5 vectors

The adenovirus spike consists of the major capsid proteins: hexon, fibre, and penton. Immunodominant epitopes encoded by the AdHu5 hexon protein can be replaced by the desired antigen sequence however better immune responses against the transgene have been observed when it is incorporated into the Ad vector (Lasaro and Ertl, 2009). Swapping of the fibre knob with other Ad vectors may circumvent pre-existing immunity. Chimeric Ad vectors have been developed by replacing the knob fibre on the AdHu5 hexon protein with one from another Ad serotype such as Ad6, Ad12, or Ad35 (Campos and Barry, 2007; Lasaro and Ertl, 2009; Seregin and Amalfitano, 2009). A chimeric simian adenovirus 21 vector provided protection in mice against *Ebolavirus* challenge and T-cell responses were generated against the glycoprotein in nonhuman primates (Kobinger et al., 2006). AdHu5-based vaccines can also be PEGylated to non-covalently shield the virus from neutralizing antibodies (Croyle et al., 2005).

Modification of AdHu5 vectors can also lead to tissue de-targeting which may be potentially harmful if not taken into consideration during vaccine development (Seregin and Amalfitano, 2009). Additionally, modification may also alter biological characteristics of the AdHu5 vector and lead to changes in toxicity and immune epitope cross-reactivity.

1.1.9.2.5 Development of alternative Ad vectors against H5N1 viruses

Rare human and mammalian adenovirus vectors which have a low prevalence of neutralizing antibodies in the general human population are attractive candidates to avoid the limitations associated with AdHu5 vectors. Rare human Group B and D adenoviruses are being considered as potential alternative vectors (Lasaro and Ertl, 2009). However, a recent article reported that certain populations had a high percentage of pre-existing neutralizing antibodies against Ad26, which was previously believed to have low seroprevalence in humans (Chen et al., 2010). Therefore, the evaluation of adenoviruses from other mammals holds better promise such as simian adenoviruses (AdC6, AdC7, AdC68), bovine adenovirus 3, and porcine adenovirus 3 ((Barouch, 2008; Kobinger et al., 2006; Roy et al., 2006), reviewed in (Bangari and Mittal, 2006b)). A chimpanzee adenovirus 7 (AdCh7) vector expressing an H5N1-NP antigen demonstrated similar protection and immunogenicity when compared in parallel with AdHu5-NP (Roy et al., 2007). Both AdCh7-NP and AdHu5-NP afforded similar levels of partial protection against lethal H5N1 challenge. In another study, a replication-deficient bovine adenovirus 3 (BAV3) vector expressing an H5N1-HA antigen offered comparable efficacy to AdHu5. Two doses of the BAV3 vector offered similar levels of protection with strong antibody responses (Singh et al., 2008). Additionally, the vector evaded both

AdHu5 and BAV3 pre-existing neutralizing antibodies. Interestingly, sequential re-administration of the BAV3 vector was successful (Singh et al., 2008).

1.1.9.2.6 Porcine Adenovirus 3 (PAV3)

PAV3 is a mild gastrointestinal pathogen in pigs and does not cause any known illness in humans (Kleiboeker, 2006). PAV3-based vectors share a similar genome arrangement and structure to AdHu5 and are attractive vaccine candidates for potential human use. The vector has demonstrated *in vivo* efficacy in swine models against classical swine fever and pseudorabies viruses (Hammond et al., 2001a; Hammond et al., 2001b; Hammond and Johnson, 2005; Hammond et al., 2000). PAV3-based vaccines provided long-term protection and were able to evade pre-existing anti-vector immunity *in vitro* (Bangari and Mittal, 2004; Sharma et al., 2010) and *in vivo* (Hammond et al., 2003; Hammond and Johnson, 2005), suggesting a potential application for vector re-administration.

Unlike AdHu5, the cellular receptors are uncertain for PAV3. PAV3 does not mediate attachment by the CAR receptor and has been shown to be β-integrin independent (Bangari and Mittal, 2005). The virus lacks the RGD binding motif on its hexon protein which is necessary for integrin binding (Sharma et al., 2009). This suggests that PAV3 may initiate activation of immune responses by different mechanisms to AdHu5 and could potentially offer an alternative strategy to avoid pre-existing AdHu5 immunity.

1.1.9.2.7 Limitations of Ad vectors

In addition to pre-existing immunity one of the major concerns with Ad vectors is the development of oncogenesis. For this reason, most adenovirus vectors contain deletions in the E1 genes to prevent virus-driven replication (Volpers and Kochanek, 2004). Additionally, replication-deficient adenovirus vectors may not be efficient in immunocompromised individuals. As an alternative, third generation helper-dependent, or gutless, Ad vectors (3$^{rd}$ generation) contain only the virus long terminal repeats (LTRs) and can hold up to 35kb of foreign DNA (Nayak and Herzog). However, gutless Ad vectors have been associated with lower immunogenicity. Nevertheless, adenovirus-based vaccine platforms offer a promising alternative to conventional vaccination. Immunogenicity can also be enhanced when the virus is administered through different routes of immunization.

## 1.2 Future Prospects

The emergence of HPAI H5N1 viruses in birds and cross-transmission to humans underlines concerns that the next pandemic influenza virus may be of zoonotic origin. Current conventional vaccines can provide reasonable protection against H5N1 viruses however they are not efficient and may not be protective against divergent isolates. The prediction of future seasonal influenza viruses is not always accurate and could present a difficulty when forecasting potential pandemic viruses. It will therefore be necessary to develop alternative vaccine candidates that can offer broad spectrum protection against a range of influenza viruses. Experimental vaccines may address several weaknesses associated with conventional H5N1 vaccines by providing better immunogenicity and long-term protection. However, the development of experimental vaccines will require

rapid formulation and extensive assessment in animal models, followed by clinical studies before approval for human administration. Combined with an appropriate immunization schedule and vaccination programmes, it should be possible to develop a vaccine against avian influenza that can successfully offer cross-protection against multiple H5N1 clades and potentially other influenza A virus subtypes including circulating seasonal isolates.

## 1.3 Background

Vaccination is the best available strategy to prevent infectious disease (Kitchen and Vaughn, 2007). Approved vaccines are commercially available against several pathogens and administration has considerably improved public health over the past century by reducing both morbidity and mortality following infection. While the ultimate goal of vaccination is complete eradication of an infectious pathogen, the reality of vaccine development is more practical. Vaccination aims to prevent and reduce infectious disease pathogenesis in endemic regions and effectively control potential epidemic and pandemic outbreaks. The field of vaccine research has contributed tremendously to the basic understanding of pathogenesis, infection, and immunology of many infections pathogens (Kitchen and Vaughn, 2007) (Ellebedy and Webby, 2009).

The emergence and re-emergence of zoonotic pathogens like avian influenza A virus, subtype H5N1, highlights the need to develop better vaccines that can protect against a wide range of influenza viruses. Although conventional vaccines are available against H5N1 they are not as efficient as vaccines against seasonal influenza viruses and, therefore, may not offer sufficient protection against heterologous virus strains. New vaccines are being evaluated to improve traditional strategies as well as develop experimental platforms that will improve protection against antigenically variable and emerging pathogens including influenza viruses.

The field of experimental subunit vaccines has benefited enormously from the advances and failures of gene therapy vectors. Early experiments observed that DNA could be delivered to cells using different carriers such as naked DNA or viral-based vectors (Roy-Chowdhury J, 2002; Wolff et al., 1990). Low vector immunogenicity and

transgene tolerance are beneficial characteristics for gene therapy and the induction of strong immune responses against vaccine vectors is often a disadvantage. On the other hand, this characteristic may be advantageous for infectious disease vaccines that require the generation of robust humoral and cell-mediated immune responses to establish protective immunity. Several experimental platforms including DNA and adenovirus-based vaccine vectors offer promises for developing strong protective immunity and importantly the generation of cross-protective immune responses against diverging H5N1 viruses.

### *1.3.1 Hypothesis*

The hypothesis for this thesis is that adenovirus-based viral vectors expressing optimized H5N1 antigens can generate cross-protective immune responses against a broad range of avian influenza A, subtype H5N1, viruses.

Specifically, the evaluation of nonhuman adenovirus platforms such as porcine adenovirus 3 (PAV3) can offer an alternative to the commonly used human adenovirus 5 (AdHu5) platform. Additionally, it was hypothesized that H5N1 DNA vaccines can also offer protection against lethal challenge and be a useful tool for preliminary vaccine assessment before evaluation in a more complex Ad-based platform.

### *1.3.2 Thesis Organization*

The following objectives were undertaken to test this hypothesis:

1) Screen potential H5N1 antigens using DNA vaccines
2) Using the best antigen, develop and evaluate PAV3 and AdHu5-based viral vaccines
3) Evaluate immune responses generated by the DNA and Ad-based vaccine candidates and how this correlates with survival
4) Evaluate the combination of different H5N1 antigens through co-administration of DNA and Ad-based vaccine candidates

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Cell lines and Viruses

Madin-Darby canine kidney cells (MDCK) were maintained in MEM (Sigma) supplemented with 10% FBS (Wisent) and 1% penicillin/streptomycin (Gibco). Human embryonic kidney (HEK) 293 cells were maintained in DMEM (Sigma), supplemented with 10% FBS, L-glutamate (Gibco), NaPyr (Gibco), and antibiotics. Fetal porcine retina cells (VR1BL E1), expressing AdHu5 E1a genes and PAV3 E1b large genes, were maintained in MEM containing alpha salts (Gibco), supplemented with 10% FBS, L-glutamate, sodium pyruvate (NaPyr), non-essential amino acids (neaa) Gibco), HEPES buffered saline, penicillin/streptomycin, and 50μg/ml Hygromycin B (BD Biosciences). Syngenic mouse AB12 cells (C57BL6) were maintained in culture in DMEM with 5% FBS and antibiotics.

Avian influenza H5N1 strain A/Hanoi/30408/2005 (Clade 1 H5N1-H05, $LD_{50}$=1.05 pfu/mouse) was generously provided by Q. Mai Le and T. Hien Nguyen, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. The A/Hong Kong/483/1997 (Clade 0 H5N1-HK97, $LD_{50}$=4.5 pfu/mouse), and A/Indonesia/05/2005 (Clade 2.1 H5N1-In05 $LD_{50}$=1.00 pfu/mouse) H5N1 influenza viruses were generated at the National Microbiology Laboratory, Public Health Agency of Canada by reverse engineering (Darwyn Kobasa, Julie Boutot). Viruses were propagated on MDCK cells cultured with virus diluent (MEM, 0.3% BSA, and antibiotics) containing 1.0μg/ml TPCK-trypsin and titred by plaque assay.

All infectious recombinant adenovirus constructs were propagated on HEK 293 or VR1BL E1 cells and purified by a cesium chloride density gradient.

## 2.2 Vector and Antigen selection

As highlighted in the introduction, the expression vector selection can influence vaccine immunogenicity. Therefore careful selection of regulatory elements through tissue-specific targeting can greatly enhance transgene expression and immunogenicity. Various studies have evaluated different eukaryotic (muscle-specific creatine kinase, human ubiquitin C, chicken β-actin (CAG)) and viral promoters (SV40, CMV) (reviewed in (Garmory et al., 2003). A Hepatitis B DNA vaccine expressing HBsAg was poorly immunogenic under the control of an MCK promoter however generated strong responses when combined with the viral CMV promoter (Garmory et al., 2003). Early H5N1 studies observed similar expression levels between CAG and CMV expressing the HA protein. Alternatively, a hybrid-CAG promoter fused to the CMV early immediate enhancer generated greater luciferase expression compared to each individual promoter. An Ad vector expressing Ebola glycoprotein offered higher expression with the hybrid-CAG promoter than CMV for Ebola GP and induced more robust immune responses and better overall protection (Richardson et al., 2009). Gene therapy and cancer treatment may benefit from low transgene expression and low reactogenicity leading to potential tolerance of the vector. However, it is advantageous for an infectious disease vaccine to generate robust immune responses following over-expression of an immunogenic transgene. The identification of tissue-specific promoters is necessary to ensure that the correct cell types are transduced following immunization. While an MCK promoter may

target muscle tissues other viral promoters may enhance transduction of additional cell types such as antigen-presenting cells and lymphocytes (Garmory et al., 2003).

Additionally, appropriate antigen selection is also important. Four avian influenza H5N1 antigens, HA, NA, NP, and M2, were selected for vaccine development based on the potential to generate humoral or cell-mediated immune responses. The antigenic variability of the envelope glycoproteins (HA and NA) and conservation (M2 and NP) were also an additional consideration. The glycoproteins HA and NA are exposed at the virion surface and are targets for the humoral antibody immune response. The presence of strong anti-HA antibody responses are virus neutralizing and are frequently correlated with protection against influenza A viruses (Corti et al.; Ellebedy and Webby, 2009; Hannoun et al., 2004). Antibodies against NA do not result in sterile immunity but they can prevent enzymatic cleavage of residues by neuraminidase (Qiu et al., 2006) and prevent the release of mature virus from infected cells. However, the antigenic diversity of H5N1 viruses and selective pressure on the HA and NA proteins can contribute to inefficient antibody binding and may reduce cross-protection against divergent isolates. Therefore, the generation of cell-mediated immune responses against less antigenically variable regions in H5N1 proteins and against more conserved antigens like NP and M2 may be an alternative strategy (Ellebedy and Webby, 2009; Kodihalli et al., 2000). The M2 ion channel can potentially generate a weak antibody response but more importantly the high conservation between diverging H5N1 isolates make it a better candidate for generating T-cell immune responses (Epstein et al., 2002; Tompkins et al., 2007). Alternatively, NP can also generate cell-mediated immune responses and has

been shown to lead to clearance of influenza viruses from the respiratory mucosa and inhibit viral replication, leading to recovery and improved survival (Laddy et al., 2008).

## 2.3 Vaccine Development

The pCAGα DNA vector containing a CAG promoter was selected as the backbone for expressing the selected H5N1 antigens. The vector was designed based on the pCAGGS expression plasmid (Miyazaki et al., 1989; Niwa et al., 1991), modified to remove 829 base pairs from the intron between the *Eco47III/XbaI* sites (Ao et al., 2008; Patel et al., 2009). The pCAGα vector controls gene expression through a hybrid-CAG promoter coupled to a cytomegalovirus early enhancer and encodes a rabbit β-globin polyadenylation (polyA) signal to stabilize the mRNA transcript. The CAG promoter allows efficient transcription of viral genes in muscle tissues and has been shown to generate higher expression of gene products than other muscle promoters like CMV or creatine kinase (Garmory et al., 2003).

The pCAGα expression cassette was inserted into Ad-based vaccine vectors. The evaluation of nonhuman adenovirus-based vaccine vectors with low seroprevalence of neutralizing antibodies in humans can offer another option to human adenovirus serotypes with higher pre-existing immunity like AdHu5. Bovine, canine, simian, and porcine adenovirus vectors have been all been suggested as possible candidates for further vaccine development [reviewed in (Bangari and Mittal, 2006a) and (Bangari and Mittal, 2006b)]. Five porcine adenovirus serotypes have been identified however PAV3 is the best characterized (Reddy et al., 1999). A replication-deficient PAV3 vector was originally developed as a veterinary vaccine vector (Reddy et al., 1999) and afforded successful protection in pigs against different disease models (Hammond and Johnson,

2005). The porcine immune system shares many similarities with that of humans (Butler et al., 2009). The success of PAV3 in pigs suggests that the vector may have potential applications for human vaccines. Although human adenoviruses can infect pigs, there have been no confirmed reports of PAV3 infection in humans (Kleiboeker, 2006).

The PAV3 virus is not cross-neutralized by neutralizing antibodies against AdHu5 or other nonhuman adenoviruses *in vitro* (Adair and McFerran, 1976; Bangari and Mittal, 2004). Preliminary studies in pigs also suggest that anti-vector immunity can be surpassed using higher doses of vector without the toxicity associated observed with high doses of AdHu5 vectors (Hammond and Johnson, 2005). Recent studies indicate that PAV3 can stimulate both innate and adaptive immune responses through pathways that are independent of the CAR/β-integrins (Sharma et al., 2009).

### *2.3.1 Construction of DNA Vaccines*

The cDNA sequences were obtained from Genbank/NCBI for the A/Hanoi/30404/2005 H5N1-H05 HA, NA, M2, and NP genes and codon-optimized to enhance gene expression, followed by synthesis using overlapping oligonucleotide primers (Patel et al., 2009). The genes were inserted into the pCAGα vector and four DNA vaccine constructs were generated: pCAGα-HA, NA, NP, and M2. Additionally, *Zaire ebolavirus* glycoprotein (GP, strain Mayinga 76) (Richardson et al., 2009) and the Crimean Congo Hemorrhagic Fever virus glycoprotein precursor (CCHF-M, strain *IbAr10-200*), were also constructed to generate pCAGα-GP (Richardson et al., 2009) and pCAGα-CCHF-M (Mickey Sahib). The sequence and integrity of each vaccine was confirmed by restriction digest and DNA sequencing (NML, DNACore). DNA vaccines were scaled for vaccine production using endotoxin-free Qiagen mega- and gigapreps.

Vaccines were re-suspended in endotoxin-free buffer and stored at -20°C ready for immunization.

### *2.3.2 Transfection and Detection of DNA vaccine proteins*

To confirm expression of each antigen, HEK 293T cells were cultured to an 80-90% confluent monolayer in 60mm tissue culture dishes. The cells were then transfected with 8-10μg of pCAGα-HA, -NA, -NP, or -M2 DNA vaccines using calcium phosphate precipitation (BD Biosciences). After 48-72 hours, the cells were harvested in RIPA buffer (Triton X-100, 1M Tris-HCl pH 7.7., 5M NaCl, 2% sodium deoxycholate, 0.1% SDS, 0.5M EDTA pH 8.0, water) buffer to preserve protein from degradation. Samples were passed through a 21 1/2-guage needle to lyse the cells, incubated on ice for 20 minutes, and then centrifuged at 14 000 rpm to remove the cellular debris. Protein expression of each DNA vaccine was evaluated by western blotting. The protein samples were separated on a 10% SDS-PAGE gel and wet transferred to a PVDF membrane (GE Healthcare) overnight at 32V. The HA protein blot was incubated with sera obtained from mice infected with a sub-lethal dose of the H5N1-H05 influenza virus (Figure 2.1). The NA, NP, and M2 blots were incubated with anti-mouse antibodies against each protein. All samples were then probed using a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (KPL). The expected protein bands were visualized using the ECL Detection kit (GE Healthcare).

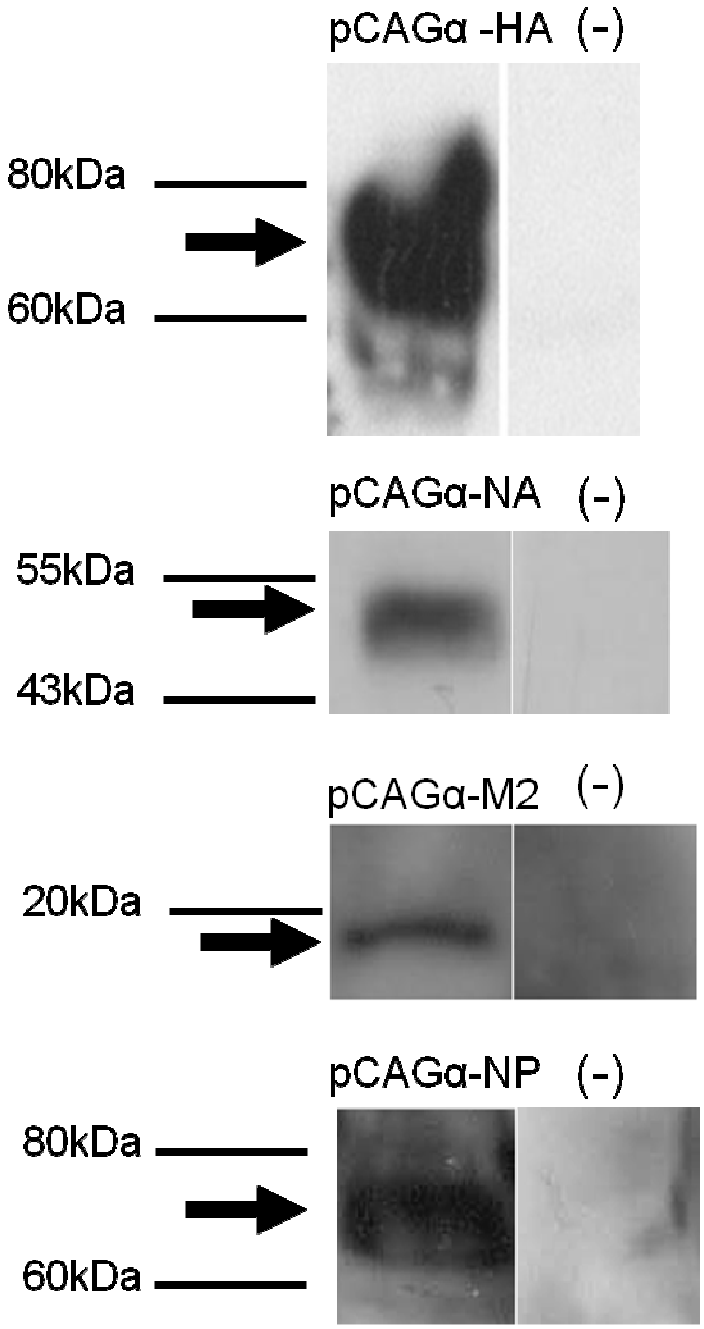



**Figure 2.1 Detection of H5N1-H05 HA, NA, M2, and NP expression by Western blot**. HEK 293T cells were transfected with the H5N1 pCAG-HA, -NA, -M2, or –NP DNA vaccines. HA, NA, M2 and NP proteins were detected by Western blot and probed with polyclonal anti-H5N1 serum, anti-NA, anti-M2 or anti-NP antibody followed by a goat anti-mouse- HRP. Control well (−) contained protein derived from 293T cells transfected with the empty plasmid.

### *2.3.3 Construction of transfer plasmids and AdHu5-based vaccines*

The entire gene expression cassette from pCAGα-HA or pCAGα-NP was excised and cloned into the pShuttle2 transfer vector (Clontech). Insertion of the expression cassette replaced the existing pShuttle2 CMV promoter with the CAG promoter, resulting in the pShuttle2-HA and pShuttle2-NP constructs. The transgene cassettes were then excised from pShuttle2 and inserted into a replication-deficient (ΔE1/E3) pAdenoX (AdHu5, Clontech) using the unique restriction homing endonucleases *I-CeuI/PI-SceI* and ligation of complementary cohesive ends using T4 DNA ligase (Invitrogen). Two AdHu5 constructs were generated: pAdenoX-HA and pAdenoX-NP. The integrity of the H05-HA or H05-NP cassettes in each vaccine were confirmed through *EcoRI* restriction digests.

### *2.3.4 Construction of transfer plasmids and PAV3-based vaccines*

The pPAV227 transfer plasmid (VIDO, University of Saskatchewan), was modified to include an SV40 polyA signal for comparison between the AdHu5 vaccines and PAV3-based vaccines, generating pPAV227-PolyA. The SV40 polyA signal was amplified from the pShuttle2 plasmid using forward and reverse primers and the inserted into the *SpeI* restriction site in pPAV227. Each expression cassette from pCAGα-HA or pCAGα-NP was cloned into pPAV227-PolyA (*SpeI*) and then transferred by homologous recombination into the replication-deficient (ΔE1/E3 described in (Reddy et al., 1999)) pFPAV227 vector (PAV3, VIDO, University of Saskatchewan). pFPAV227-HA and pFPAV227-NP were generated through homologous recombination using the chemically competent *Escherichia coli* strain BJ5183 (*recBC, sbcBC)* (Reddy et al., 1999) and linearized pPAV227-HA or NP (Eco47III/Tth1III and Eco47III/KflI, respectively) and

pFPAV227 (*PacI*). Two PAV3-based vaccines were generated: PAV3-HA and PAV3-NP. The integrity of the H05-HA or H05-NP cassettes in each vaccine were confirmed through *EcoR*I or *Hind*III restriction digests.

### *2.3.5 Transfection and Detection of Ad vector proteins*

All four Ad vector constructs were linearized with the *PacI* restriction enzyme. To obtain the AdHu5-HA and AdHu5-NP vaccines, HEK 293 cells were transfected with 10µg of the linearized pAdenoX-HA or pAdenoX-NP DNA using calcium phosphate precipitation and cells were cultured until the appearance of CPE (approximately 20 days). Similarly, the PAV3-HA and PAV3-NP vaccines were obtained by transfecting VR1BL E1 cells with 10µg of linearized pFPAV227-HA or pFPAV227-NP DNA combined with Lipofectamine2000 transfection reagent (Invitrogen). Cells were cultured until CPE was visible (approximately 18 days). The cell lysates from each transfection were harvested, freeze-thawed three times, and used to amplify each adenovirus for vaccine production. Samples of each cell lysate were amplified by PCR using multiple primer sets corresponding to the H05-HA or H05-NP genes. All vaccine genes were confirmed by sequencing (DNA Core, NML). Expression of all four vaccines was confirmed in human, porcine or mouse cells by western blot using anti-HA mouse serum (Figure 2.2a) or commercial anti-NP antibodies (Figure 2.2b). Additionally, expression was evaluated in syngenic mouse AB12 cells to confirm a previous report that PAV3 and AdHu5 can transduce in mouse cells (Bangari et al., 2005).

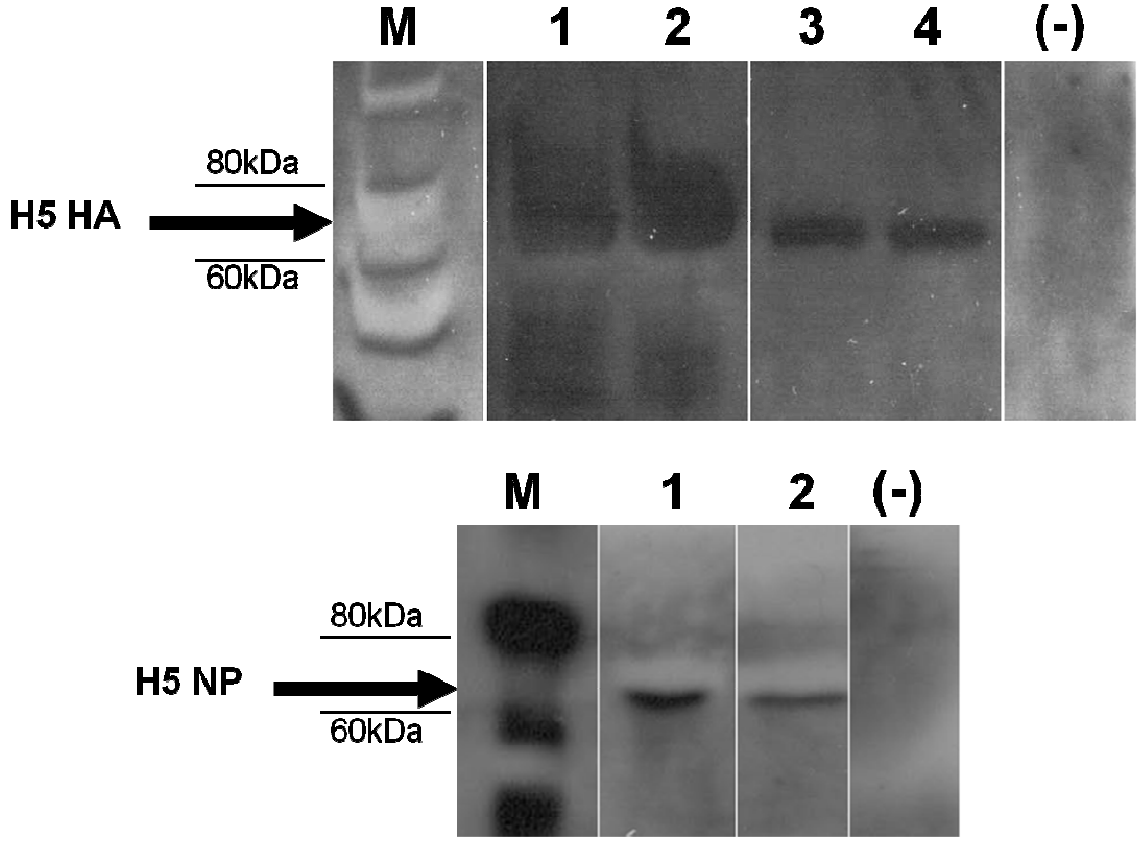



**Figure 2.2 Comparative detection of H5N1-H05 HA and NP expressed by AdHu5 and PAV3 vaccines.** Transgene expression was detected by Western blot using polyclonal anti-H5N1 mouse sera or a mouse anti-NP antibody, followed by a goat anti-mouse-HRP secondary antibody. (A) M=Marker, Lane 1 = expression of AdHu5-HA in HEK293 cells, Lane 2 = expression of PAV3-HA in VRIBL E1 cells, Lanes 3 and 4 = expression of AdHu5-HA and PAV3-HA, respectively, in mouse syngenic AB12 cells, (-) negative control (untransfected cell lysate). (B) M= Marker, Lane 1 = expression of AdHu5-NP in HEK293 cells, Lane 2 = expression of PAV3-NP in VRIBL E1 cells, (-) negative control.

Each adenovirus vaccine was amplified for vaccine production by infected fifty 140mm tissue culture dishes (NUNC) and virus was harvested following the appearance of wide-spread CPE (approximately 40-48 hours). Cells were harvested, centrifuged at 4000rpm for 5 minutes and the cell pellet was re-suspended in a 10mM Tris-HCl solution. The cell supernatant also contains infectious virus and this was also saved for additional vaccine production. Cell lysates were free-thawed three times and then purified using low and high CsCl density gradients, first at 20 000rpm for 3 hours, no brake, and secondly at 20 000rpm overnight. The virus band was removed using a 21 ½-guage needle and placed directly into a 0.5-3ml dialysis cassette (Pierce). The virus was dialyzed in PBS for 2 hours, PBS 1 hour, and finally in 10mM Tris-HCl for 1 hour. The final vaccine was removed from the dialysis cassette and combined with a glycerol vaccine solution for storage at -80C.

Total virus particles (vp) were determined by OD260 and total infectious particles was determined using anti-hexon antibodies against AdHu5 (AdenoX Rapid Titer kit, Clontech) or against PAV3 (VIDO, University of Saskatchewan). Four independent vector preparations were used for each HA vaccine. Total infectious particles and total viral particles were determined for both adenovirus vaccines, with ratios of 1:285, 1:333, 1:250, and 1:300 for PAV3-HA and 1:150, 1:250, 1:220, and 1:250 for AdHu5-HA. One virus preparation was used for each NP vaccine: PAV3-NP 1:300 and Ad-NP 1:311.

### *2.3.6 Combination Vaccines*

Co-administration of different H5N1 antigens was achieved by mixing the desired concentration of each pCAGα DNA vaccine together in solution and diluting in PBS (pH 7.4) and then administering the vaccine at a single site of vaccination. Alternatively,

individual pCAGα DNA vaccines were also administered at multiple immunization sites. Adenovirus combined vaccines were generated by determining the desired dose in total virus particles and then combining the necessary volumes together in solution. The combined Ad vaccines consisted of $10^{10}$vp of each vaccine vector, total $2x10^{10}$vp/mouse.

## 2.4 Immunization schedule

### *2.4.1 DNA Vaccines*

*2.4.1.1 Protection studies*

Groups of 8-10 BALB/c mice (Charles River Canada) were immunized with a range of doses of each DNA vaccine individual or in selected combinations by intramuscular injection of 50µl into each hind limb. For vaccines administered at multiple sites of immunization, 50µl of each individual vector was administered into each hind and forelimb. Mice were anaesthetized with isoflurane (Aerrane, Baxter) and challenged by intranasal inoculation with $100LD_{50}$ of H5N1 virus in 50µl of virus diluent (MEM, 0.3%BSA, antibiotics).

### *2.4.2 Adenovirus Vaccines*

Groups of 10 BALB/c were vaccinated with $10^8$, $10^9$, or $10^{10}$ virus particles (vp) of recombinant adenovirus PAV3-HA or AdHu5-HA by intramuscular administration. Each vaccine was diluted in 100µl and 50µl was administered in each of the right and left hind limbs. All mice were anesthetized and challenged after 28 days through intranasal inoculation with $100LD_{50}$ in 50 µl virus diluent.

*2.4.2.1 Infectious work and Containment*

All animal procedures were approved by the Institutional Animal Care Committee at the National Microbiology Laboratory (NML) at the Public Health Agency of Canada (PHAC), according to the guidelines of the Canadian Council on Animal Care. All infectious work was performed in the biocontainment BSL4 laboratory at NML/PHAC.

### *2.4.3 Animal Scoring*

All animal work was first approved by the inhouse animal care committee, according to guideline set by the Canadian Council on Animal Care. Mice were monitored for weight loss and signs of disease over a period of 15-20 days following infection. The mice were scored on a scale of 0-3: 0 = no symptoms, 1=ruffled fur, slowing activity, loss of body conditions, 2 =hind limb paralysis, laboured breathing, hunched posture, 3= death).

## 2.5 Immune Assays

### *2.5.1 Antibody Assays*

Sera collected from immunized mice were evaluated by hemagglutination inhibition and neutralization assays (Kodihalli et al., 1999; Rowe et al., 1999). Blood was collected via the saphenous vein for all samples. For all antibody assays, sera were treated overnight at 37°C with the receptor destroying enzyme (RDE) and then inactivated at 56°C for 45 min the following day.

### *2.5.2 Hemagglutination Inhibition*

RDE-treated sera were serially diluted in 2-fold steps starting with a 1:10 dilution and 50ul/well was added to a V-bottom 96-well microtitre plate. Four hemeagglutinating

doses of H5N1-H05 virus were added to each well and the plate was incubated at room temperature for 1 h. Following incubation, 50ul of 0.5% guinea pig, 0.5% horse, or 0.5% turkey red blood cells were added to each well and the assay was incubated at room temperature for 1 h, 1 h, or 45 min, respectively. The hemagglutination inhibition titre was scored as the highest dilution where red blood cell agglutination did not occur and the data were reported as the reciprocal of this dilution. Each assay was performed in triplicate.

### *2.5.3 Neutralizing Antibody*

(also called microneutraliziation or virus neutralization)

Twofold serial dilutions of each sample, starting with a 1:10 dilution, were prepared in virus diluent and mixed with equal volume of H5N1 virus (100 plaque forming units (pfu) per well) and incubated at 37°C for 60 min. The mixture was then transferred onto subconfluent MDCK cells in 96-well flat-bottomed plates and incubated for 5–10min at room temperature. 100µl of virus diluent supplemented with 2.0µg/ml TPCK-trypsin was then added to each well and plates were incubated at 37°C, 5% CO2 for 48 h. Cells were subsequently scored for the presence or the absence of cytopathic effects (CPE) under a light microscope. The highest serum dilution not exhibiting CPE was scored positive for neutralizing antibody and neutralization titres were reported as the reciprocal of this dilution.

All infectious *in vitro* work was performed in the biocontainment level 4 laboratory of the NML, PHAC.

### *2.5.4 T-cell Assays*

Groups of 4 BALB/c mice were vaccinated with the pCAGα DNA vaccines (50μg /mouse) or PAV3- and AdHu5-based vaccines ($10^{10}$ vp/mouse). Spleens were harvested on days 10 post-immunization for each DNA vaccine and days 8, 10, 14, and 21 following vaccination with each adenovirus vaccine.

#### *2.5.4.1 Suspension of lyophilized peptide libraries*

Peptide libraries containing 15amino acids long sequences with 10 amino acid long overlaps covering the entire H5N1 HA, NA, NP, and M2 proteins were obtained lyophilized (Mimitopes, Australia). The lyophilized peptides were resuspended in dimethyl sulfoxide (DMSO) to a final concentration of 100μg/μl, aliquoted, and stored at -80°C for long term storage. A matrix of peptide pools was created for efficient screening of the entire peptide library. Pools of 28, 20, 19, and 10 peptides were made for HA, NA, NP, and M2, respectively.

#### *2.5.4.2 Harvesting of Splenocytes*

T-cell responses were evaluated following vaccination using ELISPOT assays to detect the production of IFNγ. ELISPOT assays were performed using the Mouse IFN-γ ELISPOT Set (BD Biosciences) according to the manufacturer's instructions. Ninety-six well Immobilon®-P flat bottom microtitre plates (PVDF membrane, Millipore) were coated overnight at 4°C with purified anti-mouse IFNγ antibody (BD Biosciences), diluted in PBS. On the day of the experiment, each plate was washed with RPMI 1640 medium, supplemented with 10% FBS and antibiotics, and then blocked in the same medium for a minimum of three hours at room temperature. Spleens were harvested

from vaccinated mice at different time points post-vaccination and ground against a fine mesh filter in L-15 medium (GIBCO). The cells were filtered through a 0.45µm filter and centrifuged at 485g for 6-7minutes. Mononuclear cells were re-suspended in 10ml of L-15 and counted using a haemocytometer.

*2.5.4.3 ELISPOT*

For each assay, the peptide pools were diluted in RMPI 1640 and added to the 96-well microtitre plate to give a final concentration of 2.5µg/ml per peptide (total final DMSO less than 0.1%). Spleens were re-suspended in RMPI 1640 (complex supplemented with 10% FBS, penicillin/streptomycin, l-glutamine, neaa, NaPyr, HEPES buffer, 5×10−3 2-ME) and $5x10^5$cells were added per well in 100µl. An additional 100µl of 5µg/ml per peptide was added to each well to give a final peptide concentration of 2.5µg/ml. ELISPOT plates were incubated overnight at 37°C, 5% $CO_2$. After 18-20 hours, the ELISPOT plates were washed twice with water, soaking for 3-5 minutes, followed by 3 washes of Solution I (PBS+tween-20 0.1%), and incubated with biotinylated anti-mouse IFNγ for 2-3 hours at room temperature. After incubation, the plates were washed 3 times in Solution I soaking in between for 1-2 minutes, and then incubated with streptavidin-HRP. Cells positive for IFNγ production were detected using the AEC Substrate Reagent (BD Biosciences). Positive cells were visualized as spots on the PVDF membrane and counted using an AID ELISPOT reader (Cell Technology, Colombia, MD). All experiments were repeated independently in triplicate

*2.5.4.4 Flow Cytometry*

To further characterize the T-cell response, flow cytometry was performed. Spleens were harvested at different time points post-vaccination and plated at $2x10^6$ cells/well. Cells were re-stimulated for 5 hours with the individual 9 amino acid long peptides corresponding to the H5N1-H05. A immunodominant epitope (IYSTVASSL), or control peptides from H1N1-PR8 NP (TYQRTRALV) and EbolaGP (TELRTFSI) in complex DMEM (supplemented with 10% FBS, penicillin/streptomycin, l-glutamine, non-essential amino acids, NaPyr, HEPES buffer, 5×10−3 2-ME) along with brefeldin A (GolgiStop, BD Biosciences) and IL2.

Following incubation, cells were stained with either anti-mouse CD8-FITC (fluorescein isothiocynate) or anti-mouse CD4-PerCPCy5.5 (peridinin–chlorophyll–protein complex) at 4°C for 30 min. Splenocytes were fixed and permeabilized using the BD Cytofix and permwash protocols (BD Biosciences). The next day, cells were stained for interferon gamma using IFNγ-PE (phycoerythrin). All samples were read on the LSRII Flow cytometer (BD Biosciences). Data was analyzed using BD FACSDiva 6.0.1 software (BD Biosciences).

## 2.6 Lung virus titre ($TCID_{50}$)

Nine animals were monitored for survival and lung tissue and blood were harvested from the remaining 6 mice three days post-infection. Virus load was determined by the tissue culture infectious dose which will result in CPE of fifty percent of cell culture ($TCID_{50}$). $TCID_{50}$ was performed by added serial dilutions of homogenized lung tissue onto MDCK cells and monitoring the presence of CPE after 48 hours. The $TCID_{50}$ titre

was calculated using the Reed & Muench method (Reed and Muench, 1938) and normalized per gram of lung tissue.

## 2.7 Statistical analysis

Data were analyzed for statistical difference by performing unpaired t-test, one-way analysis of variance (ANOVA) when appropriate. The differences in the mean or raw values among treatment groups were considered significant when $p < 0.05$.

# CHAPTER III

# SYSTEMATIC EVALUATION OF VARIABLE AND CONSERVED H5N1 ANTIGENS USING DNA VACCINES

## 3.1 Introduction

In 2003, avian influenza A virus subtype H5N1 re-merged in South East Asia in wild aquatic bird and domestic poultry populations. Confirmation of bird-to-human cross-transmission, high mortality (>60%), and isolated reports of human-to-human transmission highlights current concerns that potential pandemic viruses may originate from an H5N1 virus. The efficacy of conventional inactivated and live-attenuated influenza vaccines is dependent on the accurate prediction of future circulating strains and, therefore, may offer limited protection against an emerging influenza viruses like H5N1. The H5N1 virus lineage is diverse and the co-circulation of multiple strains could impact vaccine efficacy and the development of cross-protective immunity (Dong et al., 2009; Heiny et al., 2007). The generation of strong, robust immune responses will likely be important to ensure optimal protection against the antigenically divergent H5N1 virus and other potential pandemic influenza isolates.

The process of vaccine development is often empirical however rational design can be used to facilitate the selection and evaluation of potential vaccine candidates. Experimental DNA subunit vaccines expressing viral antigens can be delivered directly to the cell and candidates have demonstrated the induction of protective immune responses leading to better survival against several pathogens including Hepatitis B (Mancini-Bourgine et al., 2006), Ebola (Martin et al., 2006), HPV (Kim et al., 2004), and

influenza viruses (Kim and Jacob, 2009). DNA vaccines against alphaviruses (Chen et al., 1999a), dengue virus (Raviprakash et al., 2006), and HIV have been used to screen potential antigens and identify optimal candidates for further vaccine development (Bazhan et al.). Potential H5N1 antigens can be carefully selected and systematically evaluated in parallel for protein expression, immune responses, protection, and survival.

The current thesis chapter describes the systematic evaluation of DNA vaccine candidates in parallel, individually expressing four potential H5N1 influenza A antigens: HA, NA, NP, and M2. An optimized DNA vaccine was selected and all antigens were codon-optimized to increase gene expression in mammalian cells. Together, the data from systematically evaluating H5N1 antigens can be used as an efficient method to effectively evaluate immune responses prior to challenge and support the use of DNA vaccines for protection studies and other vaccine platforms.

The experiments described in this chapter were designed to test the following hypotheses:

1) A single DNA vaccine expressing an optimized avian influenza H5N1 antigen can provide protection against homologous and heterologous challenges.
2) A single DNA vaccine can generate detectable immune responses which can correlate with observed survival and protection.

## 3.2 Results

### *3.2.1 Evaluation of single DNA vaccines expressing H5N1-H05 HA, NA, NP, or M2 antigens against lethal homologous challenge*

A DNA vaccine expressing an H5N1 antigen may potentially offer protection against a matched, homologous challenge. In the first set of experiments, H5N1 antigens were selected based on potential for generating either antibody (HA, NA, M2) or cell-mediated (M2, NP) immune responses. Each antigen was first codon-optimized to improve protein expression in mammalian cells and then cloned into the pCAGα expression vector to produce each DNA vaccine. Four vaccines were developed: pCAGα-HA, -NA, -NP, and –M2.

Each vaccine was administered in parallel to groups of 10 BALB/c mice by i.m. injection and challenged 28 days following immunization with $100LD_{50}$ of homologous H5N1-H05 virus by i.n. inoculation. Mice were monitored daily for weight loss and signs of disease were scored according to a 0-3 scale (0=no symptoms, 3=severe signs of disease/death). Full protection against both weight loss and signs of disease was observed following immunization with the pCAGα-HA DNA vaccine (Table 3.1). Although the pCAGα-NA, -NP, and -M2 vaccines afforded 100% survival, signs of disease and weight loss of 12%, 24%, and >25% were observed, respectively. Therefore, the pCAGα-HA DNA vaccine was selected as the best candidate against homologous challenge. Decreasing doses of the pCAGα-HA were administered to identify a dose response curve and establish the minimum dose required for full protection in mice. A single dose of 10μg /mouse was sufficient to afford 100% survival with no signs of

**Table 3.1:** Efficacy of different influenza antigen-based DNA vaccines following homologous challenge (H5N1-H05)

| Treatment | Protection (%) | Weight loss (%)† | Signs of disease‡ |
|---|---|---|---|
| **Vehicle** | **0** | **>25** | **Death** |
| **HA (100 μg)** | **100** | **0** | **None** |
| **NA (100 μg)** | **100** | **12** | **Mild** |
| **NP (100 μg)** | **100** | **24** | **Severe** |
| **M2 (100 μg)** | **90** | **>25** | **Severe** |

**† Weight loss: Percentage difference from pre-challenge weight to the lowest weight observed.**
**‡ Signs of Disease: Change in observed clinical signs. None: no change from pre-challenge signs.**

disease. Fifty percent survival and partial protection from signs of disease and weight loss was observed with the 5μg /mouse dose (Figure 3.1).

### *3.2.2 Evaluation of single DNA vaccines expressing HA, NA, NP, or M2 against lethal heterologous challenge*

Each vaccine was evaluated to identify which vaccine could extend the best protection against a more divergent H5N1 challenge. The H5N1-HK97 virus (Clade 0) shares 95.5% amino acid identity to the H5N1-HA (Clade 1) gene expressed by pCAGα-HA. 100μg of the HA, NA, NP, or NP vaccines were administered to groups of 10 BALB/c mice, in parallel. An additional group of BALB/c mice was primed with a 100μg dose of the HA DNA vaccine and then boosted 28 days following immunization with another 100μg dose. All groups of mice were challenged with $100LD_{50}$ of the heterologous H5N1-HK97 virus. A single dose of the HA DNA vaccine at 50μg and 100μg offered 70% partial protection, with significant weight loss and signs of disease (Table 3.2). A dose of 200μg offered full survival, but significant signs of weight loss and disease were observed. The NA, NP, and M2 vaccines at 100μg did not offer any protection against lethal challenge. A prime-boost regimen with the HA DNA vaccine was fully protective with no signs of disease and weight loss HK97 challenge.

### *3.2.3 Immune responses in BALB/c mice*

#### *3.2.3.1 Antibody Responses*

Antibody responses were measured to evaluated B-cell responses following vaccination. HI and NAB levels were detected against H5N1-H05 and –HK97 viruses

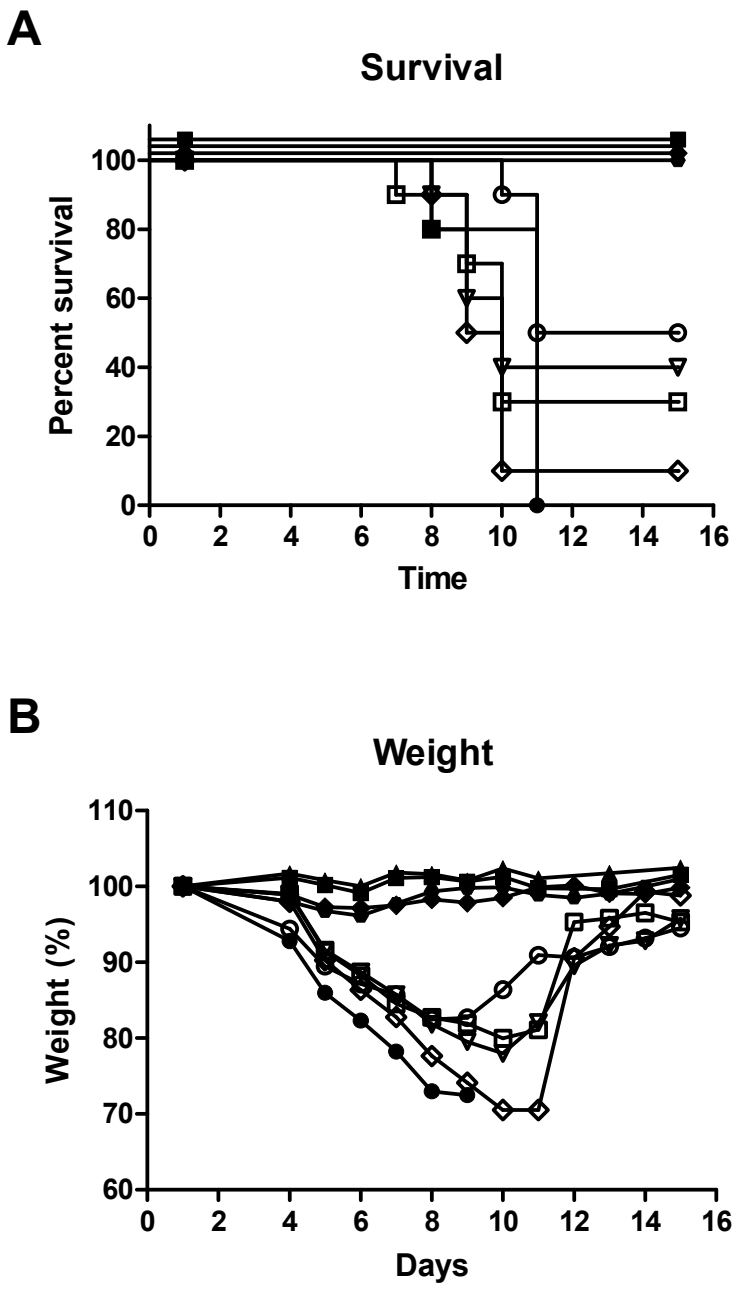



**Figure 3.1 Dose titration of H5N1-H05 HA DNA vaccine.** Groups of 10 BALB/c mice were administer decreasing dose of the pCAGα-HA: 100 (■), 50 (▲), 25 (◆), 10 (•), 5(○), 1(□), 0,5 (▽), or 0.1 (◇) µg/mouse. Control mice were administered an equal volume of PBS (●). (A) Survival and (B) Weight (%) were monitored following homologous H5N1-H05 challenge. The data represent average values from 2 experiments performed with at least two different preparations of the pCAGα-HA DNA vaccine.

Table 3.2: Efficacy of different influenza antigen-based DNA vaccines following heterologous challenge (H5N1-HK97)

| Treatment | Protection (%) | Weight loss (%)[†] | Signs of Disease[‡] |
|---|---|---|---|
| **Vehicle** | **0** | **>25** | **Death** |
| **HA (100µg)** | **70** | **21** | **Mild to Severe** |
| **NA (100µg)** | **0** | **>25** | **Death** |
| **NP (100µg)** | **0** | **>25** | **Death** |
| **M2 (100µg)** | **0** | **>25** | **Death** |
| **HA-HA (100µg- 100µg)** | **100** | **0** | **None** |
| **HA (200µg)** | **100** | **20** | **Mild to Severe** |

[†] Weight loss: Percentage difference from pre-challenge weight to the lowest weight observed.
[‡] Signs of Disease: Change in observed clinical signs. None: no change from pre challenge signs.

before challenge for each dose of the HA DNA vaccine 1-100μg (Figure 3.2a). HI antibodies were detected in three different red blood cells: guinea pig, horse, and turkey. Guinea pigs cells and turkey RBCs are frequently used for evaluating HI titres for seasonal influenza strains. However, several studies report horse RBCs as being more sensitive for the evaluation of H5N1 HI antibodies. Therefore, HI titres were detected in all three red blood cells for comparison. Reciprocal titres with guinea pig, horse, and turkey RBCs ranged from 10±9 to 32±20, 0 to 60±35, and 0 to 66±23, respectively, in mice. HI titres against the H5N1-HK97 virus were not detectable with guinea pig or turkey RBCs (Figure 3.2b). At the 100μg dose of pCAGα-HA HI titres of 33±10 were detected against H5N1-HK97 with horse RBCs. Average HI titres of 7±6 were observed with the 50μg dose, although this was below the limit of detection of the assay.

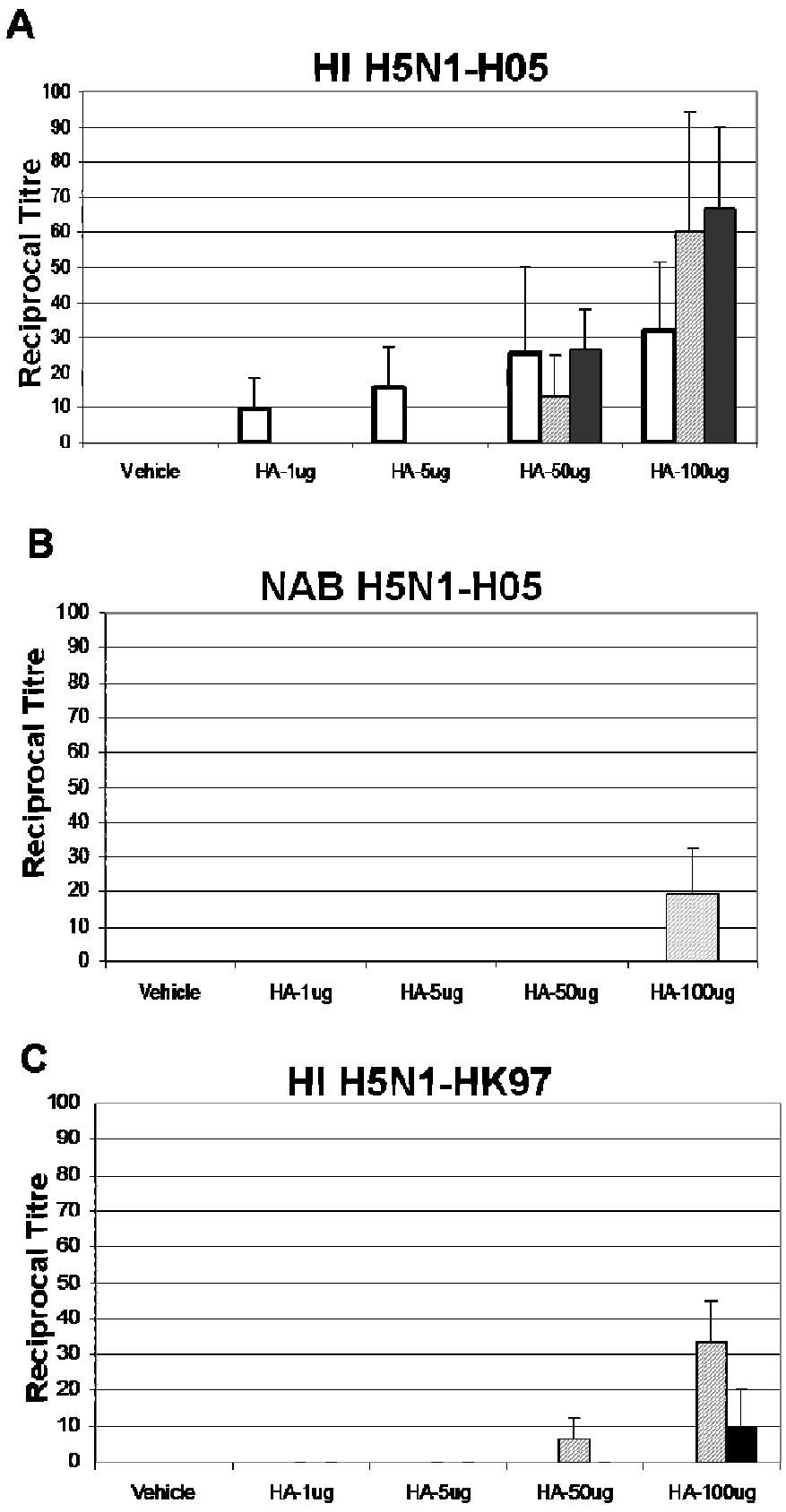
Neutralizing antibodies were evaluated on MDCK cells to detect that ability of antibodies to prevent active influenza infection. The lowest dose of the HA DNA vaccine that was able to generate a detectable NAB titre was 100μg, with an average NAB titre of 20 (Figure 2c). NAB responses could not be detected against H5N1-HK97.

HI and NAB titres were not detectable in the NA, NP, or M2 vaccinated mice against homologous H5N1-H05 or heterologous H5N1-HK97 viruses.

*3.2.3.2 T-cell Responses*

The cell-mediated T-cell response was assessed in two ways, first by an ELISPOT assay and secondly by flow cytometry. Parallel groups of 4 BALB/c mice were immunized with 50μg of each DNA vaccine and T-cell responses were detected 10 days

**Figure 3.2 Pre-challenge HI and NAB titres against H5N1-H05 and HK97.** Serum samples from BALB/c mice vaccinated with the pCAGα-HA DNA vaccine (1µg , 5µg , 50µg , 100µg ) were pre-treated overnight with RDE and inactivated the following morning. Serial dilutions were performed and 4 hemagglutinating doses of H5N1-H05 or –HK97 virus were added to each well. The serum-virus mixture was incubated with 0.5% guinea pig (□), horse (▩), or turkey (▨) RBCs and HI titres were reported as the reciprocal of the highest dilution of serum which prevented agglutination. (A) HI titres against the H5N1-H05 virus. (B) NAB titres against H5N1-H05 were determined as the reciprocal of the highest dilution which did not exhibit cytopathic effects on MDCK cells. (C) HI tires against H5N1-HK97. Groups of 5-10 mice were analyzed for each assay. Error bars represent the standard deviation of the data from 3 different experiments.


A
HI H5N1-H05
Reciprocal Titre
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50
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HA-100ug

following vaccination. Doses of less than 50μg did not result in any detectable T-cell responses. Spleens were harvested and re-stimulated with pools of overlapping peptides representing the HA, NA, NP, or M2 proteins from H5N1-H05. Spleens were then evaluated for the production of IFNγ using an ELISPOT assay to detect IFNγ secretion. The number of IFNγ secreting cells varied for each peptide (Figure 3.3). Spleens from mice immunized with the pCAGα-HA vaccine had the highest number of IFN-producing cells, followed by NA>NP>M2 with 10,096±179, 3551±132, 2775±53 and 245±3.42 spot-forming cells (sfc)/million mononuclear cells for HA, NA, NP and M2, respectively. The individual immunodominant T-cell epitopes were identified for H5N1-H05 HA and ranked using the SYFPEITHI algorithm (Appendix A) (Rammensee et al., 1999; Schuler et al., 2007). H5N1-H05 and –HK97 HA were aligned and showed high conservation between shared immunodominant epitopes with only a single AA change between the two; however this variance was observed in the highest ranked immunodominant T-cell epitope. Interestingly, the T-cell immunodominant epitope against HA is highly conserved across other H5 and H1 virus isolates.

Flow cytometry was used to further characterize the T-cell response and evaluate the percentage of CD8+ or CD4+ T-cells secreting IFNγ. Spleens were re-stimulated with the individual 9aa peptide corresponding to the H5N1-HA immunodominant epitope (IYSTVASSL). CD8+ and CD4+ responses following re-stimulation were 1.8±0.3% and 0.3±0.1%, respectively and 0.1% and 0.1%, respectively, with control NP peptide (Fig. 3.4, $p<0.001$).

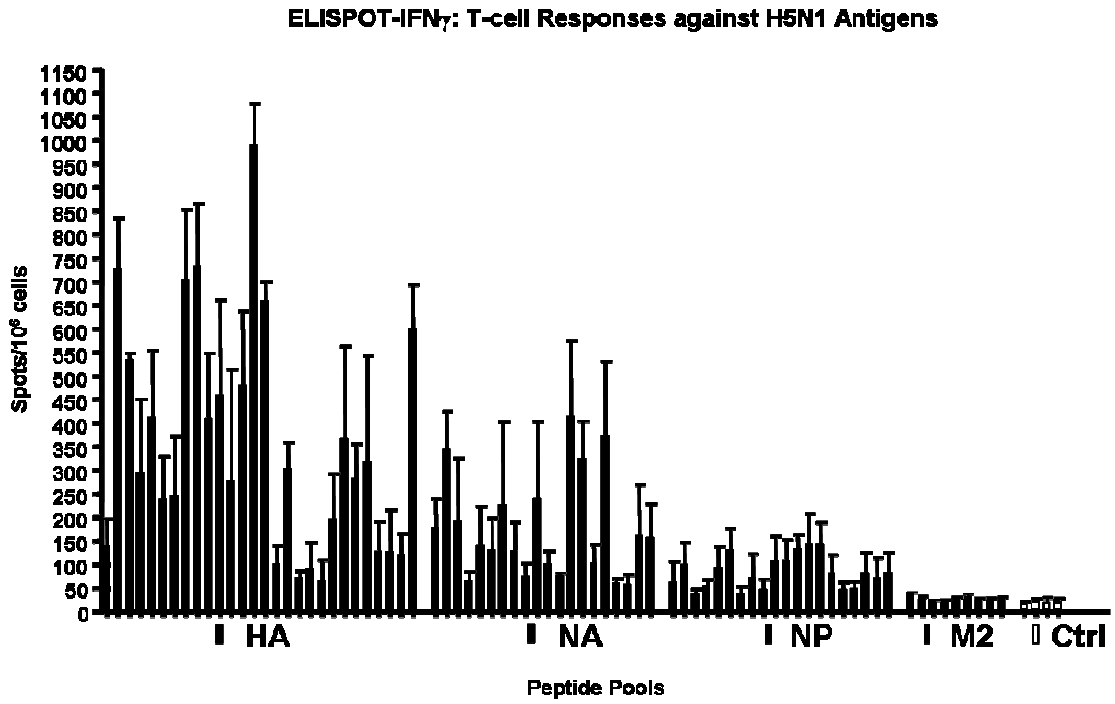



**Figure 3.3 ELISPOT-IFNγ T-cell responses against H5N1-H05 HA, NA, NP, M2 following immunization.** BALB/c mice were vaccinated with 50μg of the pCAGα-HA, -NA, -NP, or -M2 DNA vaccines. Splenocytes were harvested 10 days post-immunization and $5\times10^5$ cells were stimulated with pools of 15aa peptides covering the entire H5N1-H05 HA, NA, NP and M2 proteins Responses were visualized as spots representing T-cell IFNγ production. Four mice were analyzed per group and the experiment was repeated at least three times. Error bars represent the standard deviation of the data.

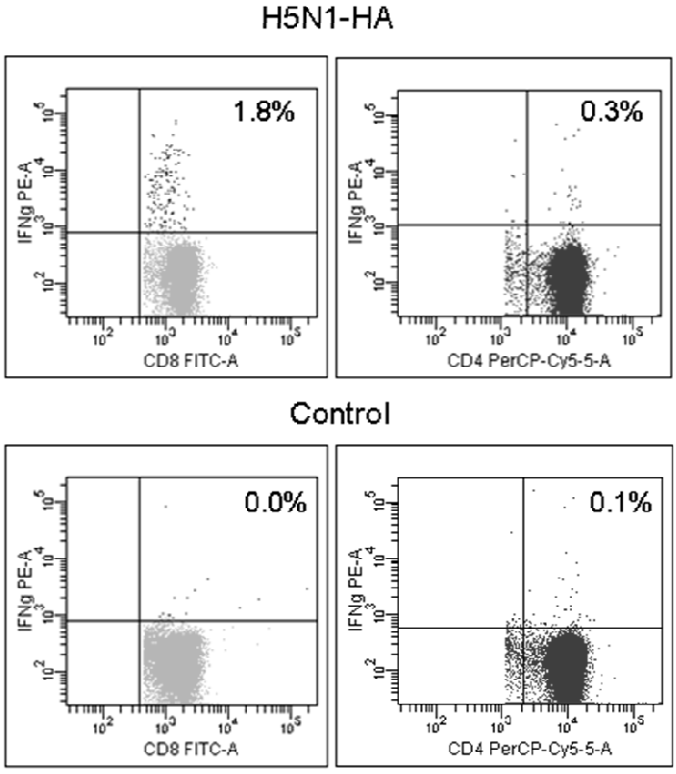



**Figure 3.4 Detection of T-cell responses against H5N1-H05 HA by flow cytometry.** BALB/c mice were vaccinated with 50µg of the pCAGα-HA vaccine and spleens were harvest on day 10 following vaccination. $2x10^6$cells were stimulated with the 9aa peptide corresponding to the H5N1-H05 HA immunodominant epitope (IYSTVASSL, H-$2K^d$)) or NP peptide as a control (TYQRTRALV H-$2K^d$)**.** The percentage of $CD8^+$ and $CD4^+$ T cells secreting IFNγ was detected by flow cytometry. The data represent average values and standard deviations from 3 experiments performed separately.

## 3.3 Discussion

The systematic evaluation of influenza antigens can facilitate the development of optimized experimental subunit vaccines against emerging avian influenza viruses like H5N1. Establishing protection against diverging H5N1 viruses will likely require the generation of broad adaptive immune responses. Extensive research has evaluated different HA-based conventional and subunit vaccines to generate strong neutralizing antibodies and protection. Individual antigens have been assessed in the context of overall protection, however, few studies have evaluated the exact relationship between humoral and cell-mediated immune parameters and overall clinical outcome. The current chapter describes four experimental DNA subunit vaccine candidates expressing H5N1-H05 HA, NA, NP, and M2 antigens and the parallel assessment of protection, humoral and cell-mediated immune responses against homologous and heterologous challenges H5N1.

Early influenza DNA vaccines were poorly immunogenic and required high doses in order to achieve full protection (Kim and Jacob, 2009). Therefore, the appropriate selection of promoters and enhancer elements, as well as vector and gene optimization was necessary in order improve *in vivo* efficacy. Previous studies reported a minimum single dose of 50μg HA DNA achieved full protection against lethal homologous challenge and lower doses were observed when coupled with adjuvants (Lalor et al., 2008) or various delivery techniques such as electroporation (Wang et al., 2008; Zheng et al., 2009). Similar studies evaluated pCAGGS-based DNA vaccines against H1 and H5 influenza viruses in the context of mouse models (Chen et al., 1999b) and as a vaccine for chicken (Jiang et al., 2007) and were able to achieve full protection with two doses of

10μg of naked HA DNA. The experiments described in this thesis employed a modified pCAGGS expression vector containing an additional deletion in the intron to improve gene expression of a codon-optimized H5N1-H05 HA gene. Using the optimized platform, a single dose of 10μg provided full protection against H5N1-H05 infection; a dose that was much lower than previously reported in other studies. Additionally, a dose of 200μg afforded full survival against heterologous challenge with signs of disease, while a prime-boost approach with two doses of vaccine prevented clinical signs of disease.

Although strong antibody responses are regarded as the best indicator for protection against influenza viruses, cell-mediated immune responses are also likely to contribute to the establishment of protective immunity following DNA vaccination. Full protection was observed with low doses of the pCAGα-HA DNA vaccine in the absence of antibodies against lethal homologous and heterologous H5N1 viruses as detected by HI or NAB assays. Similar observations have also been reported following DNA vaccination in several animal models. Kodhalli et al. (1999) were unable to detect antibodies from the serum of mice immunized with DNA vaccines expressing H5 and H7 HA antigens but still observed full protection against lethal H5N1 challenge (Kodihalli et al., 1999). This was also described by Rao et al. (2008) in mice and chicken and Lipatov et al (2004) in ferrets using similar HA DNA vaccine constructs. In this thesis, variable levels of protection were also observed with the pCAGα-NA, -NP, and –M2 vaccines against H5N1-H05 and with pCAGα-NA and –NP against H5N1-HK97 challenge. This has also been previously observed with several –NP or –M2 H5N1 DNA vaccine candidates (Chen et al., 2009; Chen et al., 1999b; Epstein et al., 2005; Epstein et al.,

2002). Antibody responses targeting NP are generally inefficient since it is an internal protein, therefore, other immune pathways are likely activated in order to achieve protection in the absence of effective antibodies. Laddy et al (2008) observed that conserved influenza components could reduce mortality and morbidity following infection (Laddy et al., 2008).

One limitation may be the sensitivity of current HI and NAB assays. Several studies recommend the evaluation of HI antibody titres using horse RBCs for detection rather than standard guinea pig or turkey RBCs (Stephenson et al., 2004). The HI antibody assay detects the ability for serum anti-HA antibodies to prevent interaction between influenza virus and sialic acid receptors on the surface of erythrocytes. Horse RBCs express mostly α2,3-linked sialic acid receptors, the preferred receptor for avian influenza viruses like H5N1, and this may explain better sensitivity. Turkey RBCs express both α2,3 and α2,6-linked receptors while guinea pig cells express mostly α2,6-linked sialic acid receptors (Stephenson et al., 2004). Turkey RBCs are preferred because they are nucleated and sediment rapidly (30-45min), and are easy to read using V-bottom plates. In contrast, horse and guinea pig RBC take longer to settle (1h-1hr15min and 1hour, respectively) and are more difficult to accurately analyse. To evaluate the sensitivity of horse red blood cells, the experiments in this chapter evaluated all three RBCs in parallel. Horse and turkey RBCs were more sensitive than guinea pig red bloods cell in the assays. Reports suggest that detection of neutralizing antibodies is more sensitive for H5N1 viruses and a better measure of prevention of infection by influenza virus (Uhl et al.). The WHO current recommends suspected avian influenza H5N1 cases to be confirmed using horse RBCs with confirmation at a titre >1:160 or

neutralizing antibody titre >1:80 (WHO, 2010). As a guideline, recommended vaccines should have HI titres >1:40 and NAB titres >1:80 (WHO, 2010). However, this does not provide a full explanation since the titre of detected antibodies was at or below the limit of detection for several protective vaccines. It suggests that either current antibody detection assays must be further improved in order to better characterize protective immune responses and/or that other adaptive immune responses like cell-mediated immunity may play an important role in protection.

A strong cell-mediated immune response may complement an inadequate or absent antibody response and potentially play a supportive role in virus clearance and a reduction in viral replication. T-cell responses against H5N1-H05 peptide pools, as detected by secretion of IFNγ, were highest against following vaccination with the pCAGα-HA DNA vaccine, followed by –NA, –NP, and –M2, respectively. Further characterization identified the activation of predominantly $CD8^{+}IFN\gamma^{+}$ cytotoxic T-cells following re-stimulation with a peptide corresponding to the 9aa H05-HA immunodominant epitope (IYSTVASSL). As an addition to the experiments presented in this chapter, cynomolgus macaques were infected with the H5N1-Vn04 virus and T-cell immune responses were evaluated during the course of infection (Patel et al., 2009). In all surviving animals, T-cell responses were strongest against re-stimulation with peptide pools for HA, followed by NA, NP, and M2, similar to the observations following DNA vaccination in BALB/c mice. This may suggest that in addition to evaluating the antibody response, the strength of the T-cell response may be used to predict clinical outcome following immunization, although more studies will need to be evaluated in order to assess this conclusion. Based on previous studies (Epstein et al., 2005; Epstein

et al., 2002; Heiny et al., 2007) it was hypothesized that a DNA vaccine expressing a well-conserved antigens such NP can generate strong cell-mediated immune responses and this can contribute towards protective immunity. Both the data from DNA vaccination and natural infection suggest that the T-cell response against HA is the strongest, contrary to the previous reports suggesting that cell-mediated immune responses are strongest against NP. The exact contribution of cell-mediated immune responses during influenza infection still remains unclear and will need to be further evaluated. However, protection in the absence of T-cell responses has been observed in mice depleted of $CD4^+$ and $CD8^+$ cells in the presence of strong HI and neutralizing antibodies against influenza (Woodland et al., 2001), suggesting that it is not essential for protection against secondary infection.

The experiments described in this chapter observed full protection with the pCAGα-HA DNA vaccine against homologous and heterologous challenges. Better protection was achieved against heterologous HK97 using a DNA-DNA prime-boost vaccination regimen. Survival has been shown to be a function of the strength of the antibody response however a strong T-cell response may also play an important role in protection (Rimmelzwaan et al., 2007). The generation of T-cell responses against the HA antigen and other H5N1 proteins is likely to also contribute towards protective immunity in the absence of adequate humoral immune responses. Several advances to DNA vaccine technology including optimization, lipid-mediated delivery, electroporation, and particle-mediated delivery (Garmory et al., 2003; Lalor et al., 2008; Luxembourg et al., 2007; Sharpe et al., 2007) have improved vaccine immunogenicity and long-term protection in larger animal models and several groups are revisiting the

possibility of using DNA vaccines for human applications. Still, DNA vaccine platforms provide an efficient method to evaluate antigens in small animal models like mice. From the experiments in this chapter, expression cassettes from a promising DNA vaccine can be excised and inserted into other platforms like adenovirus-based viral vectors.

# CHAPTER IV

# EVALUATION OF HUMAN AND PORCINE ADENOVIRUS-BASED VACCINE VECTORS EXPRESSING AN H5N1 ANTIGEN

## 4.1 Introduction

As another option to conventional vaccines, adenovirus vectors can distinctively promote the generation of more comprehensive humoral and cell-mediated immune responses and improve protection against emerging and re-emerging pathogens like avian influenza H5N1. Ad-based vectors are more challenging to produce, however, they offer specific advantages over DNA vaccination including more potent gene expression, unique activation of innate immunity, and the stimulation of stronger adaptive immune responses in small and large animal models and in humans (Lasaro and Ertl, 2009; McCoy et al., 2007; Sharma et al., 2009). Several AdHu5-based vaccine candidates have demonstrated strong protective efficacy against H5N1 viruses (Singh et al., 2008; Tompkins et al., 2007) and can generate cross-protection against heterologous and homologous challenges (Boon and Webby, 2009; Hoelscher et al., 2006; Hoelscher et al., 2008).

Unfortunately, the presence of naturally occurring anti-AdHu5 neutralizing antibodies in a large percentage of the human population can interfere with the vector and limit vaccine efficacy. In North America, 30-50% of people have pre-existing immunity to AdHu5. This percentage is >90% in developing countries (Lasaro and Ertl, 2009). Therefore, one strategy to evade pre-existing immunity against AdHu5 is the development and evaluation of rare mammalian Ad vectors with low human

seroprevalence of neutralizing antibodies. A bovine adenovirus 3 (BAV3)-based vaccine expressing an H5N1-HA antigen previously demonstrated successful protection in mice against homologous H5N1 challenge (Singh et al., 2008). BAV3 was not neutralized by AdHu5 neutralizing antibodies *in vitro* (Bangari et al., 2005) and the BAV3-HA vaccine also escaped pre-existing antibodies against AdHu5 *in vivo* (Singh et al., 2008). In another study, a chimpanzee Ad vector (AdC7) with low human seroprevalence expressing an H1-NP antigen offered partial protection against lethal H5N1 challenge in mice, also supporting the idea that Ad-based vectors can generate cross-protection against divergent influenza A viruses from the same lineage or closely-related subtypes (Roy et al., 2007).

Porcine adenovirus 3 (PAV3) is another potential vector with low human seroprevalence that is able to evade pre-existing immunity to AdHu5 *in vitro* (Bangari et al., 2005) and anti-vector immunity *in vivo* in pigs (Hammond and Johnson, 2005). The PAV3 backbone can be developed to generate vaccines against H5N1 viruses and additionally the humoral and cell-mediate immune responses generated by the influenza model of infection can be used to further characterize the efficacy of PAV3 as a vaccine vector.

As a logical extension of the experiments detailed in Chapter 3, the expression cassette from the best single DNA vaccine, pCAGα-HA (expressing the H5N1-H05 HA antigen), was excised and inserted into replication-deficient PAV3 or AdHu5 vectors. The PAV3-HA vaccine was compared in parallel with AdHu5-HA for the induction of humoral and cell-mediated immune responses and the rapid generation of protection following lethal challenge with homologous and heterologous H5N1 viruses. Overall,

the data from the experiments can be applied towards the further development PAV3 vectors as vaccine platforms and provide an alternative strategy for the more efficient generation of protective immunity against emerging zoonotic influenza A viruses such as H5N1.

The experiments in this chapter were designed to evaluate the following hypotheses:

1) A single Adenovirus based vector expressing an optimized expression cassette from a promising DNA vaccine can offer protection against avian influenza H5N1
2) A PAV3 vector can offer comparable or better immune responses and protective efficacy to a similar AdHu5 vector

## 4.2 Results

### *4.2.1 Seroprevalence of PAV3 and AdHu5 in pooled human Immunoglobulin*

The presence of pre-existing antibodies against a vector can interfere with vaccine efficacy and is an important consideration during the evaluation of potential Ad vectors. PAV3 shares a similar genome arrangement and structure to AdHu5; however, the actual sequence is distinct. Previous studies demonstrated that PAV3 and AdHu5 antibodies do not cross-react *in vitro* (Bangari and Mittal, 2004; Bangari et al., 2005). Bangari et al. assessed serum from 50 randomly selected individuals for the presence of antibodies against PAV3. PAV3 was not neutralized by the serum samples even at the lowest dilution of 1:4 (Bangari and Mittal, 2004).

To further address PAV3 neutralization in an extended population, the current set of experiments evaluated a commercial pool of human immunoglobulin (Ig) from 10,000-60,000 for the presence of anti-PAV3 antibodies. AdHu5, evaluated as a control, was neutralized at a dilution of 1:160 ($6.25x10^{-3}$ mg/ml human Ig). PAV3 was not neutralized at 1:20, the lowest dilution tested (5.0x10-2 mg/ml). Dilutions lower than 1:20 are below the limit of detection for this assay and not considered accurate.

### *4.2.2 Vaccine development*

The optimized expression cassette from the single HA DNA vaccine (described in Chapter 3) was excised and inserted into a replication-deficient (ΔE1/E3) PAV3 or AdHu5 vaccine vector. The immune responses and protective efficacy for PAV3 and AdHu5 were compared in parallel for all experiments.

### *4.2.3 Immune responses in BALB/c mice*

#### *4.2.3.1 Antibody Responses*

##### 4.2.3.1.1 Development of the antibody response

Robust humoral responses are important for survival against influenza virus infection and strong neutralizing antibody responses against the HA protein are protective. The development of antibody responses was evaluated through detection of both HI and NAB titres at different time points following immunization. Groups of 10 BALB/c mice were vaccinated with $10^{10}$ vp/mouse of PAV3-HA or AdHu5-HA and serum was collected from the animals at 8, 10, 14, and 21 days post-vaccination. All serum samples were treated with RDE and complement was inactivated after 18-20 hours.

HI antibody titres were assayed in horse red blood cells and scored as the reciprocal of the highest dilution which did not agglutinate red blood cells. Reciprocal titres of 20±0, 120±69, 213±92, 533±184 were detected for PAV3-HA and 27±11, 67±23, 213±92, 426±184 for AdHu5-HA against the H5N1-H05 virus at each time point, respectively. No statistical differences were observed between the two vaccines (Figure 4.1a).

Neutralizing antibodies were evaluated to detect active inhibition of H5N1-H05 virus infection. The reciprocal of the highest dilution which did not display signs of CPE was scored as positive for neutralizing antibodies. Reciprocal titres of 0±0, 7±12, 7±12 and 90±50 or 0±0, 7±12, 10±10 and 40±0 reciprocal dilutions were detected with the PAV3-HA or AdHu5-HA vaccines at 8, 10, 14, and 21 days, respectively (Figure 4.1b)

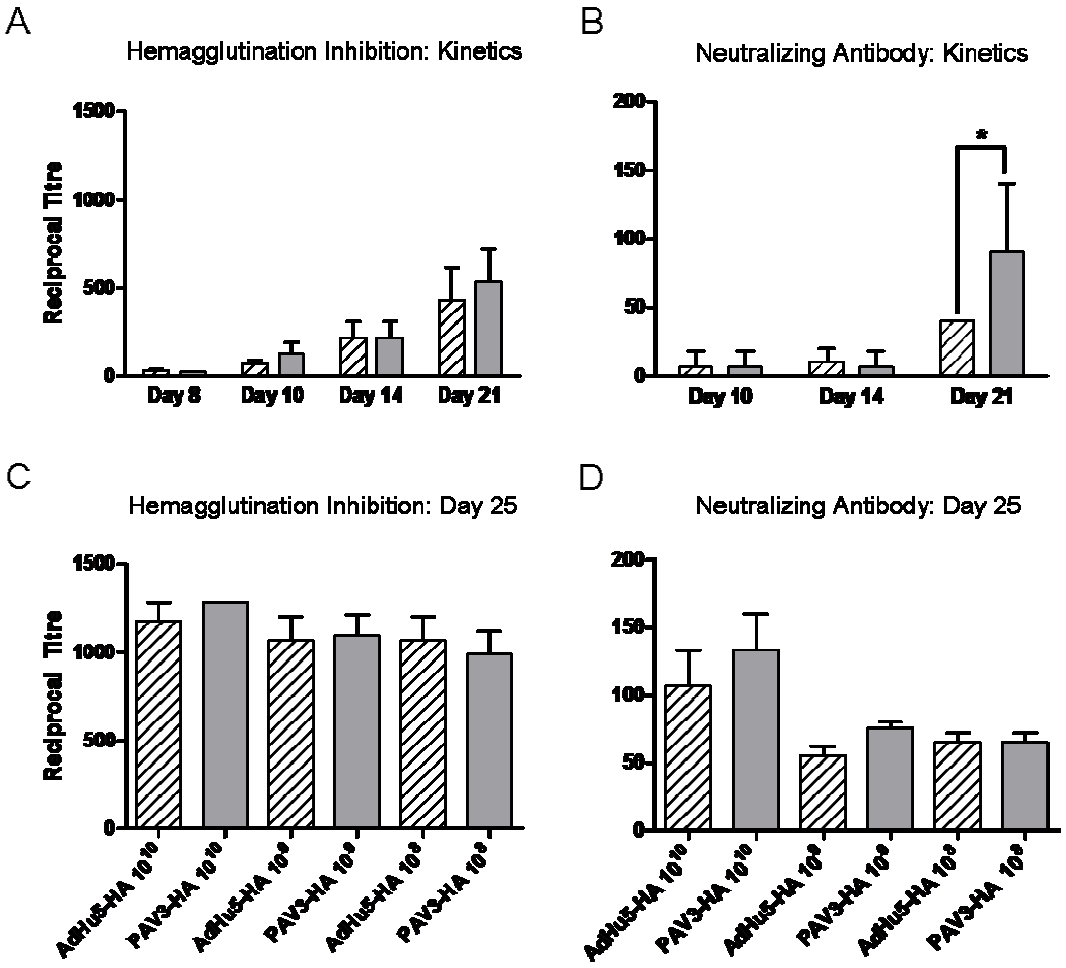



**Figure 4.1 Detection of HI antibody and NAB titres following vaccination with PAV3-HA and AdHu5-HA.** Serum was obtained from groups of PAV3-HA (▭) or AdHu5-HA (▨) vaccinated BALB/c mice and treated overnight with RDE followed by complement inactivation the following day. (A) Kinetics of the HI antibody response. Serum was obtained from groups of 4 BALB/c mice on days 8, 10, 14, and 21 post-vaccination with $10^{10}$ vp/mouse of PAV3-HA or AdHu5-HA. Four hemeagglutinating doses of H5N1-H05 virus were added to serial dilutions of serum and samples were incubated with horse RBCs. (B) Kinetics of the NAB response. Serum from obtained on 8, 10, 14, and 21 days post-vaccination with $10^{10}$ vp/mouse of PAV3-HA or AdHu5-HA. Serial dilutions of was also incubated with 100pfu of H5N1-H05 virus, added to MDCK cells and monitored for CPE. (C) HI titre against H5N1-H05. Serum was obtained 25 days post-immunization from at least 10 BALB/c mice vaccinated with $10^8$, $10^9$, and $10^{10}$ vp/mouse of AdHu5-HA or PAV3-HA. (D) NAB titres against H5N1-H05. Serum from 25 days post-immunization was also monitored for the presence of NAB. The data represent average values and standard deviations from 3 to 5 experiments performed with 2 independent vector preparations of each vaccine (* represents $p<0.05$).

*4.2.3.2 Antibody responses before challenge*

Groups of 10 BALB/c mice were vaccinated with $10^8$, $10^9$, or $10^{10}$ vp/mouse of PAV3-HA or AdHu5-HA and serum was collected at day 25 post-immunisation to assess antibody titres before challenge. HI titres of 986±326, 1093±293, and 1280 were detected for the PAV3-HA vaccine and 1066±330, 1066±330, and 1173±261 for AdHu5-HA were detected for each dose, respectively. Both vaccines had similar HI antibody titres at all doses, with no significant differences detected between the PAV3-HA and AdHu5-HA ($p=0.57$, Figure 4.1c).

Average NAB reciprocal titres were 65±20, 75±14, or 106±46 reciprocal dilution at $10^8$, $10^9$ or $10^{10}$ PAV3-HA vp/mouse respectively (Figure 4.1d). The AdHu5-HA vaccine generated NAB titres of 65±20, 55±20 or 133±46 at $10^8$, $10^9$ or $10^{10}$ vp/mouse, respectively. No significant differences were detected between different doses of the two vaccines.

*4.2.3.3 Antibody Responses against diverging H5N1 Viruses*

To assess cross-reactive antibody responses against diverging H5N1 viruses, HI and NAB titres were also evaluated against H5N1-HK97 and -In05. The PAV3-HA vaccine generated HI reciprocal titres of 54±23 and 80±48 against the HK97 and In05, respectively (Figure 4.2a). NAB titres were 50±8 and 25±3 against each virus, respectively (Figure 4.2b). Reciprocal titres for the AdHu5-HA vaccine were 36±8 and 53±20 against HK97 and In05 and NAB titres were 44±9 and 13±4.

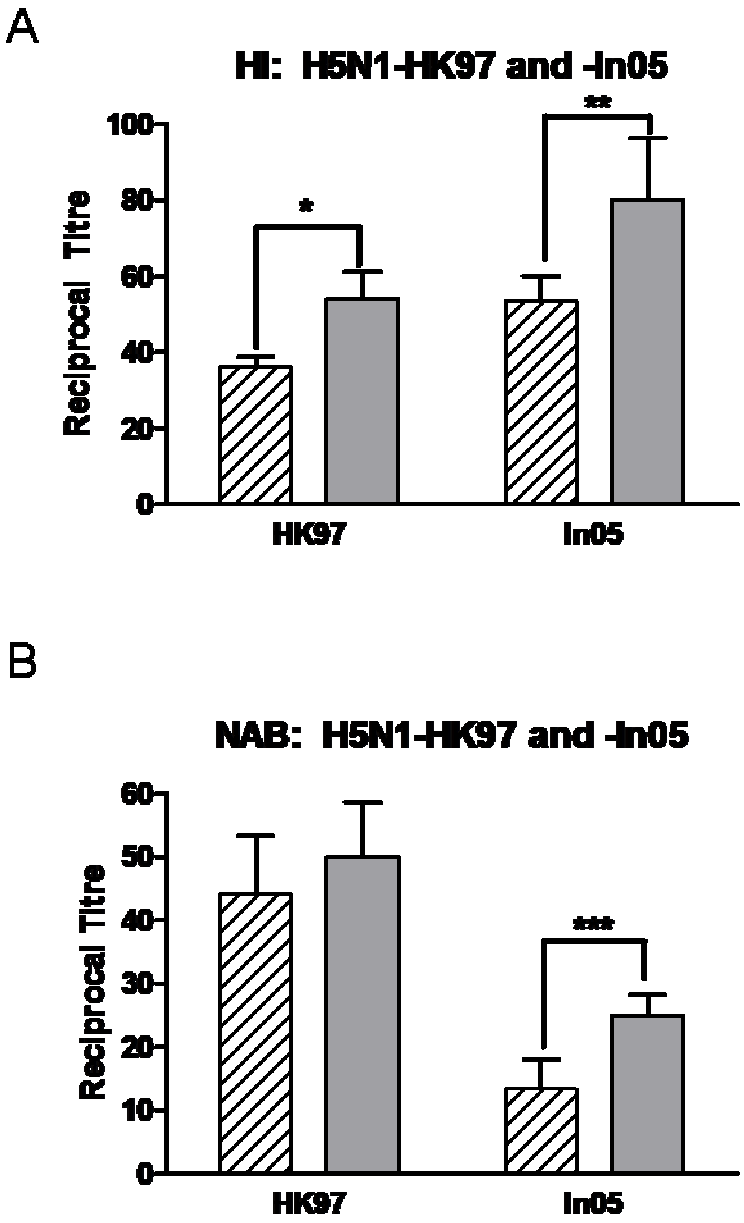



**Figure 4.2 Detection of HI antibody and NAB titres against H5N1-HK97 and –In05 viruses.** Serum was obtained from groups of 10 BALB/c mice vaccinated with PAV3-HA (■) and AdHu5-HA (▨). RDE-treated sera were assayed for (A) HI titres against H5N1-HK97 and –In05 and (B) NAB titres against H5N1-HK97 and –In05. Error bars represent the standard deviation of the experiments from one experiment using a single vector preparation of each vaccine. (*represents p=0.025, ** p=0.011, *** p=0.024).

*4.2.3.4 T-cell response kinetics*

The presence of strong HI and neutralizing antibody responses are important for immediate and long-term protection against influenza viruses. Early cell-mediated immune responses following vaccination may supplement developing antibody responses and contribute to better clearance of virus-infected cells following H5N1 influenza virus infection. The development of the T-cell response was evaluated by ELISPOT-IFNγ assay at different time points post-immunization. Groups of 4 BALB/c mice were vaccinated with $10^{10}$ vp/mouse of either PAV3-HA or AdHu5-HA and spleens were harvested on days 8, 10, 14, and 21 days. Spleen cells were re-stimulated with pools of overlapping peptides corresponding to the H5N1-HA protein. T-cell responses were visualized through detection of spot forming cells secreting IFNγ. T-cell responses from mice vaccinated with the PAV3-HA vaccine were: 13,598±2066, 14,442±2541, 6954±392, or 2056±633 sfc/million splenocytes at each time point, respectively (Figure 4.3a). AdHu5-HA generated 1,965±341, 12,858±1749, 7332±93, and 1902±372 sfc/million, respectively. Statistically significant higher sfc/million splenocytes were detected at day 8 following vaccination with the PAV3-HA compared to AdHu5-HA ($p<0.005$).

The T-cell response was further evaluated following stimulation by the individual 9 amino acid peptide corresponding to the H5N1-H05 immunodominant epitope (IYSTVASSL). A peptide representing the NP immunodominant epitope (TYQRTRALV) was evaluated as a negative control. Similar to the pooled T-cell responses, the H5N1-H05 HA immunodominant epitope stimulated the strongest T-cell responses on day 8 post-immunisation with PAV3-HA. T-cell responses at day 8

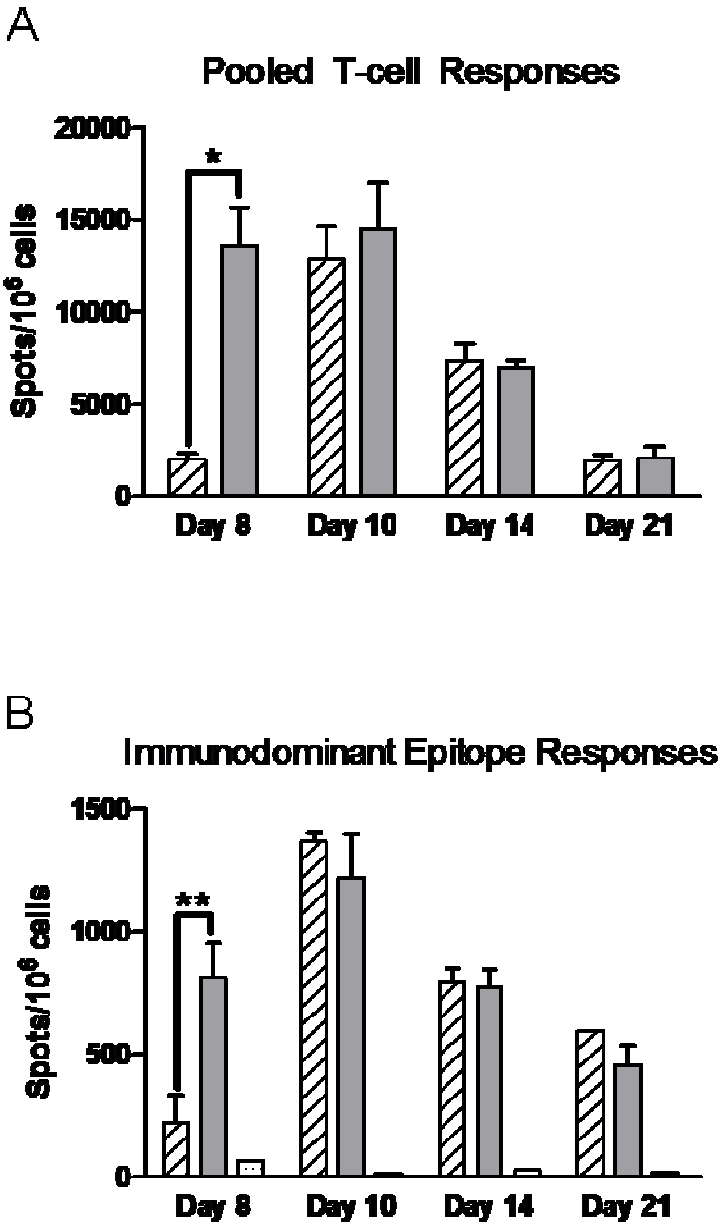



**Figure 4.3 T-cell responses detected by ELISPOT-IFNγ assay.** Groups of 4 BALB/c mice were vaccinated with $10^{10}$ vp/mouse of either PAV3-HA (▭) or AdHu5-HA (▨). Mouse spleens were harvested 8, 10, 14, and 21 days post-immunization and restimulated with H5N1-HA peptides. (A) Pooled T-cell responses. Splenocytes were restimulated with 15mer overlapping peptides from an H5N1-H05 HA peptide library. Bars represent the total number of sfc/million cells for all peptide pools combined. (B) T-cell responses following stimulation with the H5N1-H05 HA immunodominant peptide. Splenocytes were restimulated with the 9mer peptide representing the HA immunodominant epitope (IYSTVASSL, H-2K$^d$) or control peptide H1N1-PR8 NP (TYQRTRALV, H-2K$^d$). Bars represent the total number of sfc/$10^6$ sfc/million cells. The data represent average values and standard deviations from 4 experiments performed with 3 independent vector preparations of each vaccine (* represents $p<0.005$, ** $p<0.05$).

averaged 812±138 sfc/million were detected for PAV3HA and 220±133 sfc/million were detected from AdHu5-HA mice at the same time point (Figure 4.3b, p=0.048). Both vaccines shared similar T-cell responses on day 10: 1,216±178 and 1,364±36 sfc/million for PAV3-HA and AdHu5-HA, respectively (p=0.258). Responses at days 14 and 21 post-vaccination were: 770±74 and 453±84 for PAV3-HA and AdHu5-HA and 791±58 and 590±84, respectively. Splenocytes for the control NP peptide repeatedly generated spots less than 65 sfc/million.

A further experiment characterizing the percentage of $CD8^+$T cells expressing IFNγ was evaluated by flow cytometry. Spleens harvested ten days post-immunization were re-stimulated with IYSTVASSL or unrelated peptides NP or ZGP (Zaire Ebolavirus immunodominant epitope) as negative control peptides. Splenocytes were probed with anti-mouse FITC-CD8 and anti-mouse PE-IFNγ. The frequency of IFNγ positive $CD8^+$ T-cells was 3.4±0.3 and 2.8±0.3 percent for PAV3-HA or AdHu5-HA, respectively (Figure 4.4). Cells stimulated with control peptide NP or ZGP showed frequencies of $CD8^+$ IFNγ positive T-cells equal to 0.3±0.05 or 0.6±0.05 percent, respectively.

### *4.2.4 Protective efficacy of PAV3-HA and AdHu5-HA following lethal H5N1-H05 challenge*

During an influenza epidemic or pandemic, a rapid response following potential exposure will be essential to prevent further spread of the virus. The early induction of strong T-cell responses by the PAV3-HA vaccine suggested that it may be a good candidate for immediate administration during a suspected H5N1 exposure. The efficacy of PAV3-HA and AdHu5-HA vaccines was evaluated at different time points immediately after infection with homologous H5N1-H05 virus. Rapid vaccination was

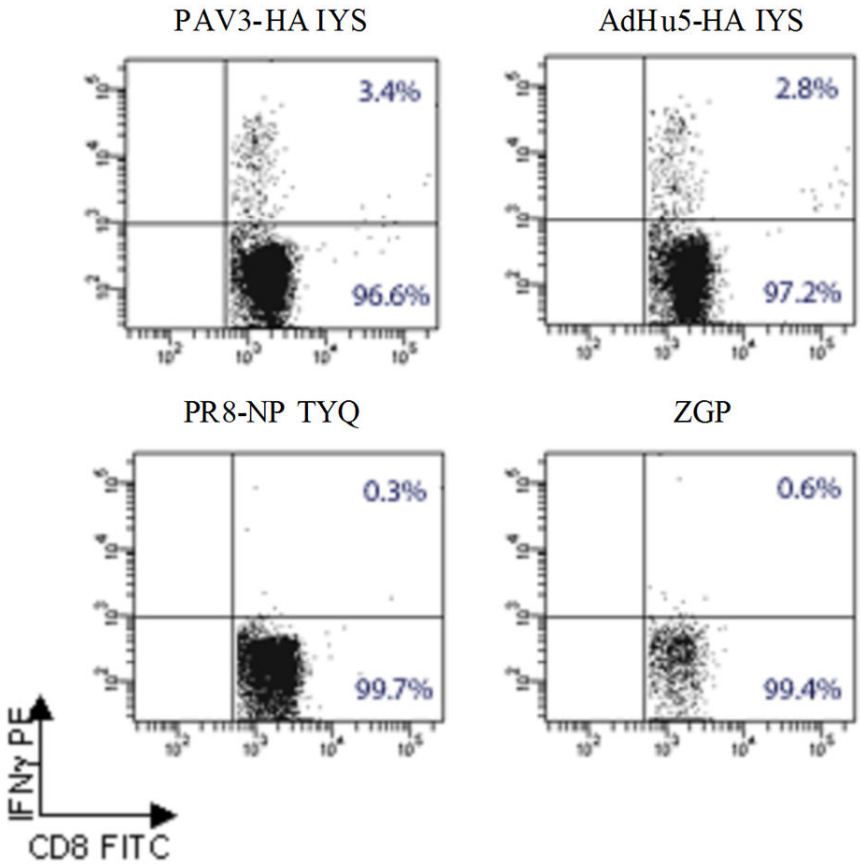



**Figure 4.4 T-cell responses detected by flow cytometery.** Spleens were restimulated with the 9aa peptide corresponding to the HA immunodomoninant epitope (IYSTVASSL, H-2K$^{d}$) or control peptides H1N1-PR8 NP (TYQRTRALV, H-2K$^{d}$) and 8mer ZGP (TELRTFSI, H-2K$^{b}$). The flow cytometry data represents the percentage of CD8$^{+}$IFNγ secreting T-cells ten days post-immunization. The data represent average values and standard deviations from 3 experiments performed with one vector preparation of AdHu5-HA or PAV3-HA.

evaluated in two parts: survival and virus load in the lungs. Groups of BALB/c mice were challenged with $100LD5_0$ of H5N1-H05 on days 5, 8, and 10 post-vaccination (Figure 4.5). Lungs were harvest at day 3 post-infection. Mice from the PAV3-HA and AdHu5-HA groups did not survival lethal challenge 5 days post-vaccination (Figure 4.5a). Interestingly, animals challenged 8 days post-vaccination had significantly greater survival with PAV3-HA than with AdHu5-HA, 66% and 11%, respectively (Figure 4.5b). Fewer signs of disease and less weight loss were also observed with the PAV3-HA vaccine. Both vaccines were fully protective when administered 10 days prior to challenge (Figure 4.5c). Virus titre in the lungs was also significantly lower at all time points compared to control animals (Figure 4.6). Lung titres of $3.57x10^6 \pm 6.26x10^6$, $1.40x10^6 \pm 4.87x10^6$, and $4.82x10^6 \pm 6.57x10^6$ $logTCID_{50}/g$, were detected in PAV3-HA vaccinated mice for days 5, 8, and 10 respectively. In AdHu5-HA vaccinated mice, virus titres of $1.64x10^7 \pm 8.60x10^6$, $1.03x10^7 \pm 6.9x10^6$, and $3.52x10^6 \pm 5.76x10^6$ $logTCID_{50}/g$ were detected. Significantly lower viral load was associated with the PAV3-HA vaccine at both days 5 and 8 compared to AdHu5-HA ($p=0.032$ and $p=0.048$, respectively). Both vaccines had similar virus titres on day 10 ($p=0.72$).

Survival with different doses of each vaccine was also assessed in groups of 10 BALB/c mice vaccinated with $10^8$, $10^9$, or $10^{10}$ vp of recombinant adenovirus PAV3-HA or AdHu5-HA by i.m. Mice were challenged 28 days later with lethal homologous H5N1-H05 virus. Full survival with little signs of disease was observed for the $10^{10}$ vp/mouse dose for both Ad-based vaccines (Figure 4.7a). Both vaccines afforded full survival at a dose of $10^9$ vp/mouse with the PAV3-HA vaccinated mice demonstrating milder signs of disease compared to AdHu5-HA.

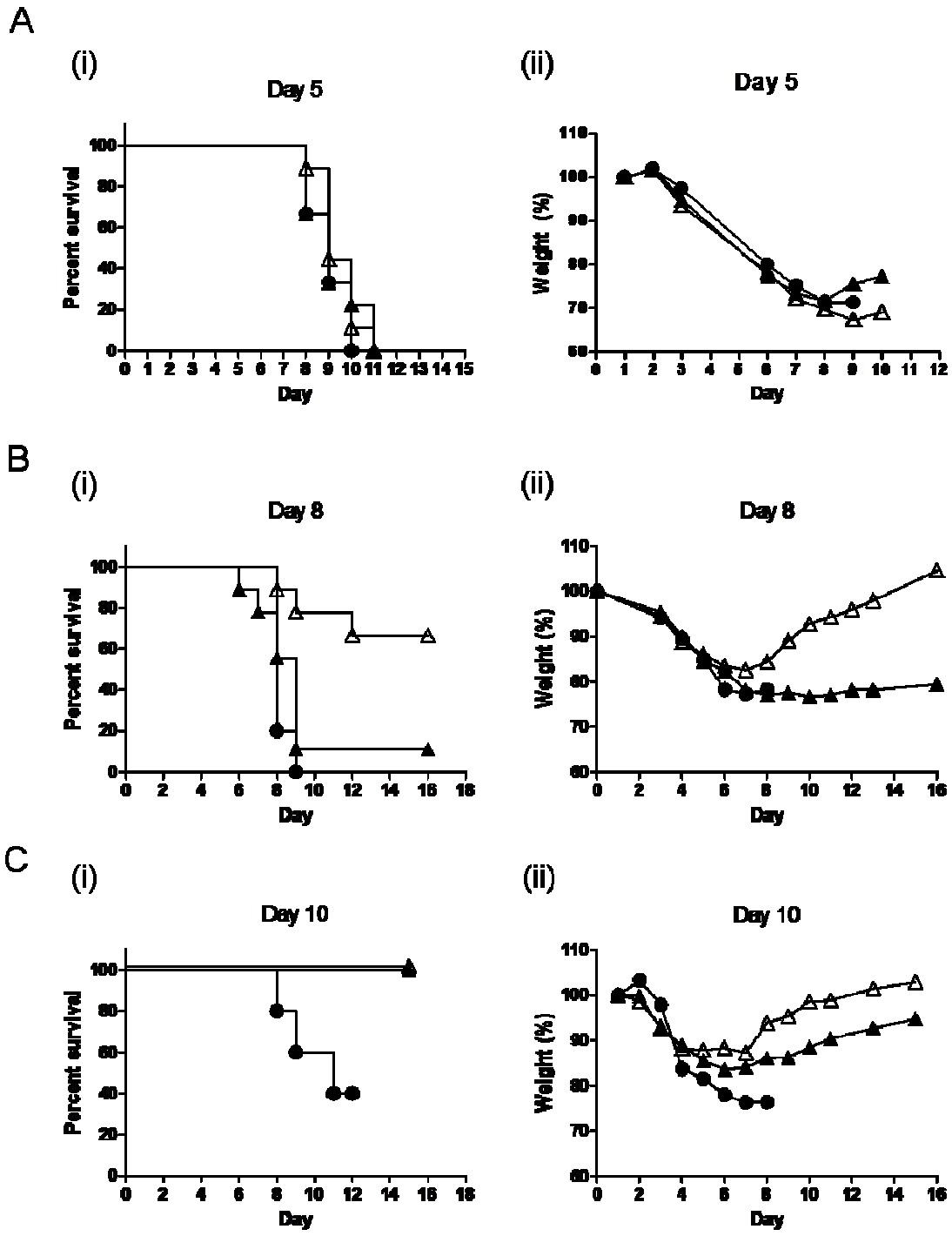



**Figure 4.5 Protection following vaccination with PAV3-HA or AdHu5-HA.** Groups of 9 BALB/c mice were vaccinated with $10^{10}$vp/mouse of PAV3-HA (△), AdHu5-HA (▲), or PBS Control (●) and challenged with homologous H5N1-H05 on (A) day 5, (B) day 8, and (C) day 10 post-vaccination. Mice were observed for (i) survival and (ii) weight loss. The data represent average values from one experiment performed with one vector preparations of each vaccine.

### *4.2.5 Protective efficacy of PAV3-HA and AdHu5-HA following divergent H5N1 challenge*

Protection against the divergent H5N1-HK97 and In05 were also evaluated in groups of 10 BALB/c. Mice were vaccinated with $10^{10}$ vp/mouse of either PAV3-HA or AdHu5-HA vaccines. Mice were challenged 28 days post-immunization with 100LD50 of lethal heterologous H5N1-HK97 or H5N1-In05. Full survival with partial protection from signs of disease was observed against both challenge viruses was observed with the PAV3-HA vaccine (Figure 4.7b). The AdHu5-HA vaccine offered partial protection against both HK97 and In05 (Figure 4.7c).

### *4.2.6 Long-term protection and immunity generated by PAV3-HA and AdHu5-HA following lethal H5N1-H05 challenge*

Several studies have reported the efficacy of Ad5 vaccines to decrease over time. Therefore, long-term protection was also assessed over a period of 12 months. Mice were challenged 12 months following vaccination to assess long-term immunity. Complete protection was offered by the PAV3-HA vaccine in mice challenged with 100LD50 of homologous H5N1-H05 virus (Figure 4.8a). The AdHu5-HA vaccine afforded only 50% survival. HI antibody titres were higher for PAV3-HA compared to AdHu5-HA ($p=0.006$) and this may have contributed to improved survival observed with the PAV3-HA vaccine. NAB titres were similar for both vaccines (Figure 4.8b).

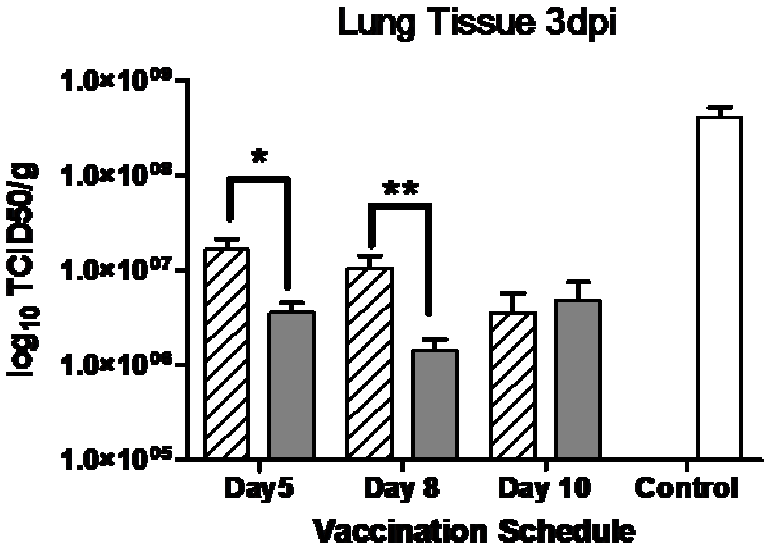
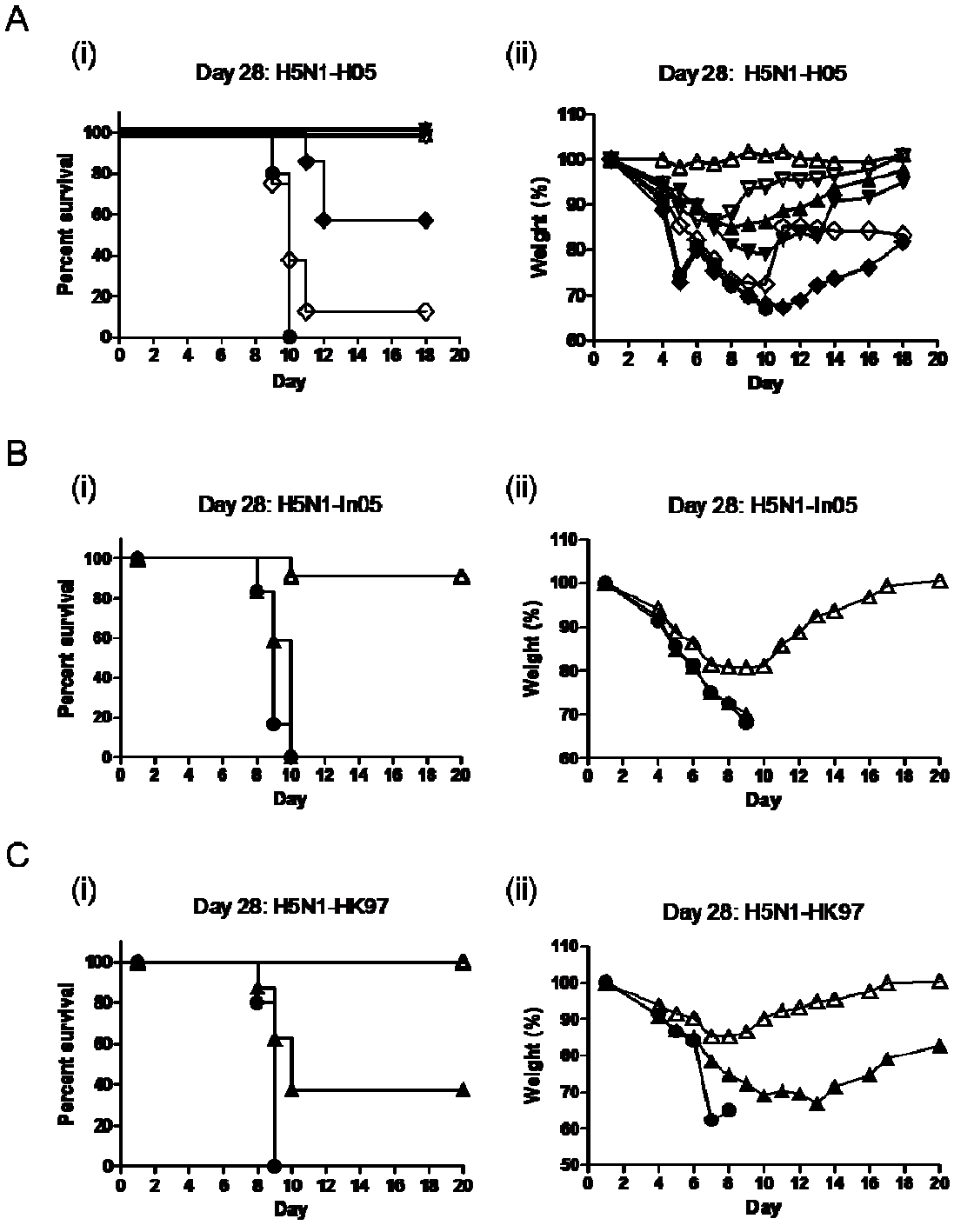



**Figure 4.6**: **Lung virus titres 3 days after lethal challenge.** Groups of 6 BALB/c were vaccinated with $10^{10}$ vp/mouse of PAV3-HA (■) or AdHu5-HA (▨), or no vaccine (Control, (□)) and challenged with $100LD_{50}$ of H5N1-H05 virus on days 5, 8, and 10 post-vaccination. Lungs were harvested from the mice on day 3 post-challenge. Virus titre was determined by $TCID_{50}$ assay using serial dilution of lung homogenates on MDCK cells and monitoring for the presence of CPE over 48 hours. The $TCID_{50}$ titre was calculated by the Reed & Muench method (Reed and Muench, 1938) and normalized/gram of lung tissue. Data is presented as log10 $TCID_{50}$/g of lung tissue. The data represent average values and standard deviations from one experiment performed with one preparation of each vaccine (* and ** represents $p<0.05$).

**Figure 4.7 Protection against homologous and heterologous H5N1 viruses 28 days post-immunization with PAV3-HA or AdHu5-HA.**
(D) Groups of 10 BALB/c mice were vaccinated with PAV3-HA at a dose of $10^8$ (◇), $10^9$ (▽), and $10^{10}$ (△) vp/mouse, AdHu5-HA $10^8$ (◆), $10^9$ (▼), and $10^{10}$ (▲), or PBS Control (●). Twenty-eight days post-vaccination, the mice were challenged with a lethal dose of (A) H5N1-H05, (B) H5N1-In05, or (C) H5N1-HK97 virus and monitored for (i) survival and (ii) weight loss and clinical signs of disease. The data represent average values from two independent experiments performed with 2 different vector preparations of PAV3-HA and AdHu5-HA.


A
(i)
Day 28: H5N1-H05
Percent survival
Day
(ii)
Day 28: H5N1-H05
Weight (%)
Day
B
(i)
Day 28: H5N1-In05
Percent survival
Day
(ii)
Day 28: H5N1-In05
Weight (%)
Day
C
(i)
Day 28: H5N1-HK97
Percent survival
Day
(ii)
Day 28: H5N1-HK97
Weight (%)
Day

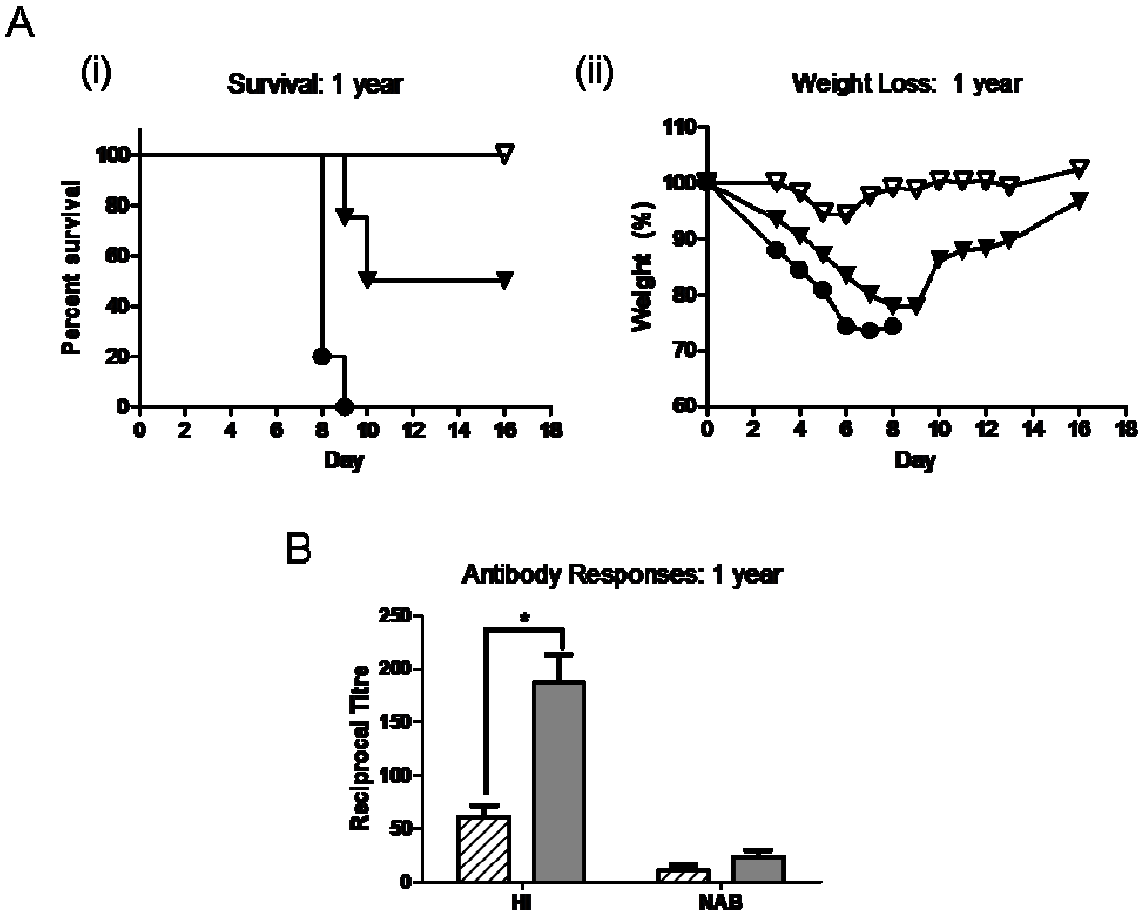



**Figure 4.8 Long-term protection in BALB/c mice.** Long-term protection was evaluated in groups of six BALB/c mice vaccinated with PAV3-HA $10^9$ (▽) vp/mouse, AdHu5-HA $10^9$ (▼) vp/mouse, and PBS Control (●). (A) Mice were challenged 12 months following vaccination with 100LD50 of H5N1-H05 virus and monitored for (i) survival and (ii) weight loss and clinical signs of disease. (B) Long-term antibody responses. Serum was collected 12 months post-vaccination from mice immunized with PAV3-HA and evaluated for HI and NAB responses. The data represent average values and standard deviations from one experiment performed with one preparation of each vaccine (* represents $p<0.05$).

## 4.3 Discussion

Replication-deficient adenovirus-based viral vectors are powerful experimental vaccine platforms capable of generating of protective immune responses against avian influenza (H5N1) viruses. The frequency of pre-existing neutralizing antibodies against AdHu5 vectors in the human population has been associated with undesirable clinical outcomes, therefore, several rare human and mammalian adenoviruses, such as PAV3, with low prevalence of neutralizing antibodies are being evaluated as alternative vaccine vectors. In this study, PAV3 was not neutralized by pooled human sera from an extended population of 10,000-60,000, compared to AdHu5, a finding supported by a previous study evaluating serum from 50 randomly selected individuals (Bangari and Mittal, 2004). However, pooled human serum from thousands of individuals may dilute antibody samples from cross-sections of the population that might otherwise be positive for neutralizing antibody against PAV3. Future studies will need to map the epidemiological profile of the virus in humans in order to determine if localized populations, such as farmers or butchers, may have previous exposure to PAV3 and how the distribution may impact the potential application of PAV3 as a human vaccine vector.

The experiments in Chapter 3 demonstrated that the strongest antibody and T-cell responses are generated against the H5N1 HA protein following vaccination and during the course of natural infection (Patel et al., 2009). The H5N1 model of infection can offer a distinctive strategy to evaluate the kinetics of the immune response following vaccination with PAV3 or AdHu5 vectors, in addition to evaluating protective efficacy following challenge with diverging H5N1 viruses. Antibody and T-cell responses were similar for both vaccines at most time points but interestingly the PAV3-HA vaccine

generated higher NAB responses and T-cell responses faster than AdHu5. The PAV3-HA and AdHu5-HA vaccines offered 100% protection with mild signs of disease against homologous H5N1-H05 when administered 28 days following vaccination with as low as $10^9$vp/mouse. However, the PAV3HA vaccine outperformed AdHu5-HA against divergent heterologous challenges representing different H5N1 lineages. Complete protection and strong HI antibody responses against the H5N1-H05 virus were also observed in a long-term study following vaccination with PAV3-HA.

The relationship between protective immunity and the rapid induction of humoral and cell-mediated immune responses has not been previously addressed by other Ad-based vaccines in the context of H5N1 infection. A DNA-electroporation H5N1 vaccine candidate was able to achieve partial protection when challenge was administered 5 days following infection (Zheng et al., 2009). The ability to induce rapid immunity can be beneficial during a possible outbreak or pandemic. In the current study, PAV3-HA offered better partial protection when mice were challenged 8 days following immunization and significantly lower virus titres were observed in the lungs of mice challenged 5 and 8 days following vaccination with the PAV3-HA vaccine compared with AdHu5-HA. Both PAV3-HA and AdHu5-HA demonstrated full protection against homologous challenge when administered 10 days before challenge. Together, the data suggests two possibilities: first, that undetectable antibody responses may provide sufficient protection against H5N1 viruses. Although partial and full protection were observed following challenge at days 8 and 10 post-vaccination, antibody levels were at or below the limit of detection of the HI and NAB assays, similar to the results observed with the pCAGα-HA DNA vaccine. Secondly, that a strong T-cell response may support

an underdeveloped antibody response and improve the protective efficacy of an H5N1 vaccine. Higher T-cell responses were detected on day 8 following vaccination with the PAV3-HA vaccine compared to AdHu5, perhaps directly translating into better protection by reducing virus replication in the lungs. The PAV3-HA vaccine also provided better protection against divergent H5N1 challenge compared to AdHu5-HA and induced better long-term immunity. Although the influenza HA glycoprotein is antigenically variable, the generation of faster immune responses by PAV3-HA against well-conserved T-cell epitopes may supplement the mismatched antibody response better than AdHu5-HA.

The swift generation of protective immune responses has a potential application in the development of pandemic influenza vaccines and may also be useful in establishing rapid protective immunity following suspected exposure to other emerging infectious pathogens. Conventional vaccines frequently are administered as post-exposure prophylaxis following suspected rabies (Johnson et al., 2010) and hepatitis A (PHAC, 2008) infections. Additionally, several post-exposure immunisation strategies are being developed using viral vectors including VSV and Ad against hemorrhagic fever filoviruses including Ebola and Marburg [(Daddario-DiCaprio et al., 2006; Feldmann et al., 2007), reviewed in (Richardson et al., 2010)]. However, it is possible that the response is antigen specific and the development of immune responses may favour a model that relies predominantly on antibody-mediated protection rather than cell-mediated immunity. Further evaluation of a range of humoral and cell-mediated antigens will be necessary in order to fully assess the ability of PAV3 to offer protection against a wide range of pathogens. Additionally, it will be necessary to evaluate sequential

administration of PAV3 following AdHu5 or itself (Bangari and Mittal, 2004; Moffatt et al., 2000).

Several important considerations will need to be evaluated in order to further develop PAV-based vectors for human vaccine applications, including identification of cellular receptors involved with viral entry, tissue tropism *in vivo*, toxicity, and the long-term persistence in tissues. Recently, Sharma et al reported stronger activation of innate immune responses, pro-inflammatory cytokines, and chemokines following systemic administration of a replication-deficient PAV3 vector expressing eGFP in comparison to similar BAV3 and AdHu5 vectors (Sharma et al., 2009). PAV3 induced more potent activation of innate pattern recognition TLR pathways and adaptor molecules suggesting that it may act as a stronger natural adjuvant compared to AdHu5 (Sharma et al., 2009). The cellular receptors for PAV3 are unclear and may play an important role in the activation of innate immune responses. Preliminary toxicity studies observed lower heaptotoxicity with PAV3 compared to AdHu5 (Hammond and Johnson, 2005; Sharma et al., 2009) however further evaluation at higher doses ($>10^{10}$ vp/mouse) and other routes of immunization, including intramuscular administration, will need to be evaluated. The long-term persistence of PAV3 in tissues is also uncertain and would likely contribute to the establishment and reactivation of memory immune responses. Studies in non-human primates observed differential persistence of simian Ad 21 compared to AdHu5 (Tatsis et al., 2007) over a one year time period.

The data from the current chapter suggests that overall PAV3-HA provides an attractive alternative candidate compared to AdHu5 against diverging H5N1 viruses. To further improve cross-protection against antigenically different H5N1 viruses, several

studies suggest the co-administration of antigenically variable antigens with well-conserved antigens to increase the breadth of the immune response against multiple influenza virus targets. Different combinations can first be systematically evaluated using DNA vaccines to determine the best mix of antigens before further evaluation of combined Ad-based vaccine candidates.

# CHAPTER V

# EVALUATION OF H5N1 ANTIGEN CO-ADMINISTRATION THROUGH COMBINATION OF DNA AND ADENOVIRUS-BASED VACCINES

## 5.1 Introduction

Avian influenza H5N1 viruses are continuously evolving resulting in antigenic variation between different clades, as well as closely related isolates. In Chapters 3 and 4, the experiments described the evaluation of DNA- and Ad-based H5N1 vaccines against homologous and heterologous H5N1 challenges. Full protection against a matched H5N1 infection can be successfully achieved using conventional or experimental vaccines expressing a strain-specific H5N1 antigen. The same vaccines can offer a range of partial to full immunity against infection with a heterologous H5N1 virus depending on antigenic relatedness between the challenge virus and vaccine strain. Complete survival with mild to severe signs of disease was observed against heterologous virus with the Ad-based and DNA-based vaccines, respectively. A broader approach to vaccine design is necessary in order to generate cross-protective immunity responses and full protection against divergent heterologous H5N1 challenge.

The co-administration of HA with other variable (NA) or highly conserved (NP and M2) H5N1 antigens is one strategy to increase the breadth of the immune response. The HA antigen can stimulate both humoral and cell-mediated immune responses however the antigenic diversity among H5N1 HA proteins can limit cross-protection between B and T cell epitopes of different virus isolates (Kuszewski and Brydak, 2000; Welsh and Fujinami, 2007). The results from Chapter 4 suggest that cell-mediated

immune responses may make an important contribution early during H5N1 infection. The generation of additional T-cell responses against highly conserved regions in H5N1 proteins may complement the antibody response and improve virus clearance, prevent signs of disease, and promote recovery. Cross-protection between influenza A subtypes has been observed in mice, ferrets, and pigs (Chang et al., 2010; Van Reeth et al., 2003; Zitzow et al., 2002) naturally infected with a sub-lethal dose of one influenza A virus and challenged with a heterologous virus from a different subtype. Pre-existing immunity to influenza A viruses may have also played a role in prevention of the H1N1-1918 *Spanish Flu* in older adults (Cox and Subbarao, 2000; Hsieh et al., 2006; Reid and Taubenberger, 2003; Taubenberger and Morens, 2010). A protective natural infection can activate a cascade of innate and adaptive immune responses against multiple H5N1 proteins (Guarner and Falcon-Escobedo, 2009), and this combination of antigens may compensate for the considerable degree of variation between influenza A virus subtypes. As well, it is possible that heterosubtypic immunity is achieved through generation of immune responses against well-conserved B and T-cell epitopes. Using the example of natural infection, vaccines can be developed to co-administer external and internal, variable and highly conserved antigens to improve the generation of cross-protective immune responses.

Different combinations of DNA vaccines can be systematically evaluated in order to determine the best candidates to improve protection against homologous and heterologous H5N1 viruses. The pCAGα-H5N1 DNA vaccines, generated as part of the study described in Chapter 3, were mixed together in different combinations in the same vehicle solution in order to produce each multivalent DNA vaccine. Also, the impact of

co-administration of H5N1 DNA vaccines with unrelated antigens (expressed in the same pCAGα DNA platform) was evaluated. Based on the results from these experiments, Ad vectors (PAV3 or AdHu5) expressing the H5N1 antigens can be mixed using optimal combinations as determined by the pCAGα DNA vaccines.

This thesis chapter describes the evaluation of a suboptimal dose of the single pCAGα-HA DNA vaccine combined with one or more additional antigens. The following hypothesis was considered as part of this study:

> Co-administration of H5N1 antigens can improve vaccine efficacy against homologous and heterologous challenges

## 5.2 Results

### *5.2.1 DNA vaccine combinations against lethal homologous and heterologous challenges*

To assess the contribution of additional antigens to overall protection offered by an HA-based vaccine, different H5N1 antigens were combined with the single HA DNA vaccine. A suboptimal dose of 1μg or 5μg of the pCAGα-HA DNA vaccine was mixed with 100μg of pCAGα-NA, -M2, or –NP and co-administered at the same immunization site. Protection was evaluated against lethal homologous H5N1-H05 or heterologous HK97 challenge, respectively. The efficacy of each antigen combination vaccine was compared in parallel to HA alone.

An (HA+NA) combination afforded the best protection against homologous H5N1-H05 challenge, offering 77% survival with moderate signs of disease (Figure 5.1a, p=0.016). (HA+NP) also significantly improved survival to 40% (p=0.036) and immunization with HA alone or (HA+M2) resulted in 10% and 20% survival, respectively. Based on these observations, the combination of (HA+NA) was selected for further evaluation against homologous H5N1-H05 challenge. Different doses of the (HA+NA) vaccine were co-administered at a single immunization site and evaluated against homologous challenge in comparison to the HA DNA vaccine alone. The (HA+NA) combination was fully protective against lethal H05 challenge at all doses of the vaccine (1μg, 5μg, or 10μg per vector), with few observed signs of disease (Table 5.1). A dose-sparing effect was observed with lower doses of the (HA+NA) vaccine in comparison with the HA DNA vaccine alone (p=0.001).

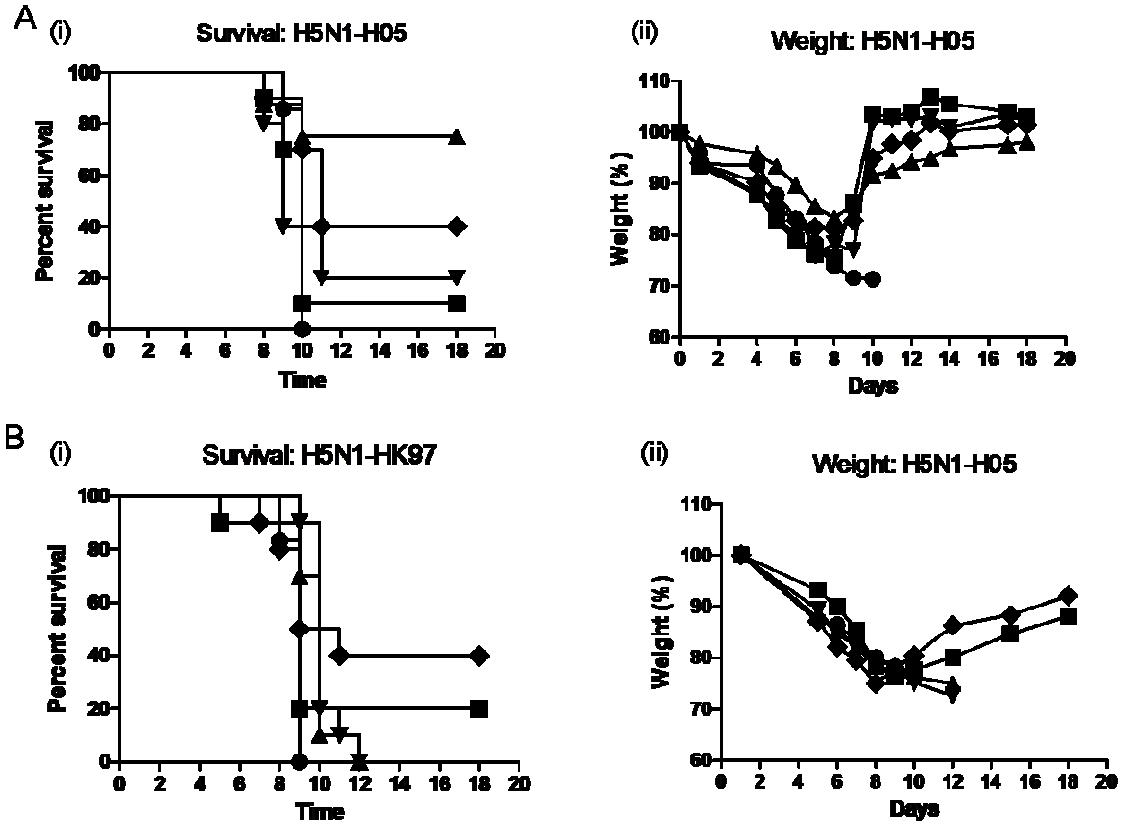



**Figure 5.1 Systematic evaluation of antigen combinations for protection against homologous and heterologous H5N1 viruses.** Groups of 10 BALB/c mice were vaccinated with suboptimal doses of the pCAGα-HA (■) DNA vaccine combined with the pCAGα -NA (▲), -M2 (▼), or –NP (◆) DNA vaccines at a 100μg dose. Control animals were administered an equal volume of PBS (●). (A) Protection following vaccination with 1μg pCAGα-HA and NA, M2, or NP against H5N1-H05 challenge. (B) Protection following vaccination with 5μg of pCAGα-HA and NA, M2, or NP against H5N1-HK97 challenge. (i) survival and (ii) weight loss were monitored for all vaccines. The data represent average values from one experiment performed with at least one different preparation each DNA vaccine.

**Table 5.1: Summary of Homologous (H05) Challenge with different pCAGα-HA and pCAGα-NA combinations.**

| Vaccine | Survival (%) | Weight Loss (%) † | Signs of Disease ‡ |
|---|---|---|---|
| PBS Vehicle | 0 | >25 | Death |
| HA 10ug + NA 10ug | 100 | 4.2 | None |
| HA 5ug + NA 5ug | 100 | 5.2 | None |
| HA 1ug + NA 1ug | 100 | 2.7 | None |
| HA 10ug | 100 | 15 | None |
| HA 5ug | 50 | >25 | Mild |
| HA 1ug | 30 | >25 | Death |

† Weight loss: Percentage difference from pre-challenge weight to the lowest weight observed.
‡ Signs of Disease: Change in observed clinical signs. None: no change from pre-challenge condition. Mild: ruffled fur, slowing activity, loss of body conditions, Severe: laboured breathing, hunched posture, bleeding, death.

The (HA+NP) antigen combination offered the highest survival rate, 40%, against lethal heterologous H5N1-HK97 challenge (Figure 5.1b, p=0.271). (HA+NA) and (HA+M2) offered 20% and 10% protection, respectively, and the suboptimal dose of HA alone was not protective against the H5N1-HK97 virus. Significant weight loss and signs of disease were observed for all vaccine groups. Overall, the (HA+NP) combination offered the best promise for enhancing protection against a heterologous H5N1 virus. Following dose titration of the (HA+NP) DNA vaccine combination, 90% partial protection was afforded against the heterologous H5N1-HK97 virus by the 100µg/vector dose, with mild signs of disease and weight loss following challenge (Table 5.2). Both the 10µg/vector and 50µg/vector doses of the (HA+NP) vaccine offered 80% protection, although signs of disease were more prominent in these animals. Full survival was also achieved by administering 200µg of the single HA DNA vaccine but did not prevent against signs of disease and significant weight loss. Statistical analysis revealed no significant differences between survival and weight loss for the animal groups receiving the (HA+NP) DNA vaccine at the 100µg/vector dose compared to a 200µg single dose of HA alone (p=0.313).

### *5.2.2 Protective efficacy of related and unrelated antigens co-administered at the same immunisation site*

Following the partial success of the bivalent DNA vaccines, it was hypothesized that the addition of more variable and/or conserved influenza antigens could possibly further supplement each combination vaccine. To further evaluate the efficacy of antigen combination, the single pCAGα-DNA vaccines expressing one of four influenza antigens

**Table 5.2: Summary of Heterologous (HK97) Challenge with different pCAGα-HA and pCAGα-NP combinations.**

| Vaccine | Survival (%) | Weight Loss (%) † | Signs of Disease ‡ |
|---|---|---|---|
| PBS Vehicle | 0 | >25 | Death |
| HA 100ug + NP 100ug | 90 | 16 | Mild |
| HA 50ug + NP 50ug | 80 | 22 | Mild |
| HA 10ug + NP 10ug | 80 | 25 | Mild |
| HA 200ug | 100 | 20 | Mild |
| HA 100ug | 70 | 21 | Mild |
| HA 50ug | 70 | >25 | Severe |

† Weight loss: Percentage difference from pre-challenge weight to the lowest weight observed.
‡ Signs of Disease: Change in observed clinical signs. None: no change from pre-challenge condition. Mild: ruffled fur, slowing activity, loss of body conditions, Severe: laboured breathing, hunched posture, bleeding, death.

(HA, NA, M2, and NP) were mixed and co-administered at the same site of immunisation. Combination of the 1µg/vector (HA+NA) vaccine with 1ug/vector of both NP and M2 DNA vaccines offered 90% survival against homologous H5N1-H05 challenge, with minimal signs of disease and weight loss (Figure 5.2a). The addition of NP and M2 antigens offered no significant impact on overall survival compared to (HA+NA) alone (p=0.349).

To assess protection against infection with the heterologous H5N1-HK97 virus, the NA and M2 DNA vaccines were added to the bivalent (HA+NP) combination vaccine. Combination of each of the four H5N1 vaccines at a dose of 50µg/vector afforded 100% survival, although signs of disease and weight loss were observed (Figure 5.2b). While not statistically significant, survival unexpectedly decreased from 80% down to 50% when 10µg of each NA and M2 were added to a dose of 10µg/vector of HA and NP combination vaccine (p=0.133). Overall, the combination of four H5N1 influenza antigens did not significantly enhance the protection offered by the bivalent (HA+NA) or (HA+NP) vaccine candidates against homologous or heterologous challenges, respectively.

To further assess the lack of contribution from additional antigens to each vaccine combination, the Ebola glycoprotein (ZGP) was selected as an unrelated viral antigen and added to (HA+NA) or (HA+NP). Vaccination with (HA+NA) and ZGP (1ug/vector) provided 77% protection against homologous H5N1-H05 challenge, compared to 100% survival observed with (HA+NA) alone. The reduction in protection was not statistically significant (Figure 5.2a, p=0.169) following the addition of the ZGP antigen.

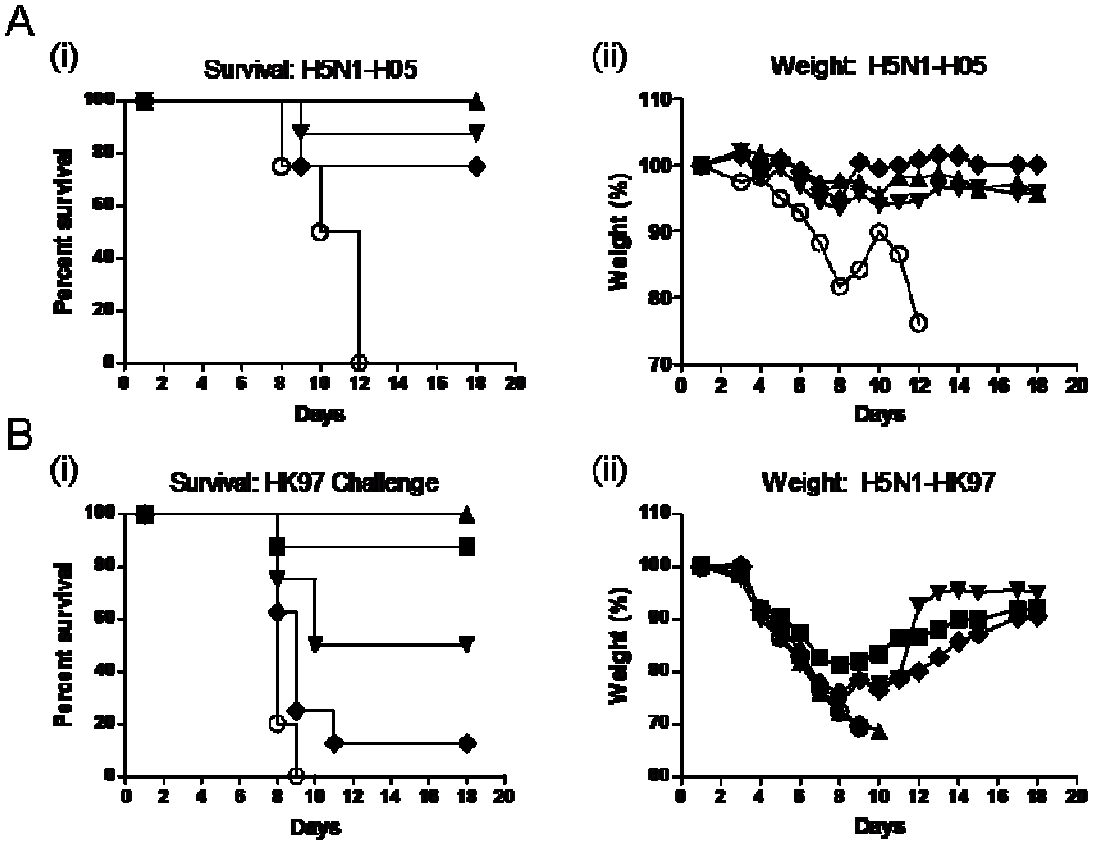



**Figure 5.2 Multivalent antigen combinations administered at a single immunization site.** BALB/c mice were vaccinated with bivalent, trivalent, or tetravalent combinations of the pCAGα DNA vaccines. Mice were challenged 28 days post-vaccination with homologous or heterologous H5N1 viruses. (A) H5N1-H05 Challenge. Control (PBS, ■), HA+NA (1µg , ▲), HA+NA+M2+NP (1µg , ▼), and HA+NA+ZGP (1µg , ◆). (B) H5N1-HK97 Challenge. Control (PBS**,** ○**),** HA+NP (10µg ,■), HA+NA+M2+NP (10µg , ▼ or 50µg , ▲), and HA+NP+ZGP (10µg , ◆) (i) survival and (ii) weight Loss were monitored for each vaccine. The data represent average values from 2-3 independent experiments performed with at least two independent preparations of each DNA vaccine.

A reduction in protection was also observed against lethal heterologous H5N1-HK97 challenge following administration of (HA+NP) combined with the ZGP vaccine (10μg/vector). Overall survival was significantly reduced from 80% with two antigens down to 10% with the addition of the unrelated antigen (Figure 5.2b, p=0.004). Together the data suggest that although certain antigen combinations may improve protection, the co-administration of other combinations of related and unrelated antigens could negatively affect vaccine efficacy. Overall, the bivalent combination of (HA+NA) provided optimal protection against H5N1-H05 challenge and (HA+NP) afforded the best protection against divergent H5N1-HK97 challenge.

### *5.2.3 Protective efficacy of multiple antigens co-administered at different immunisation sites*

Antigen competition at the local site of injection is one possibility for the reduced protection observed following co-administration of three or more antigens at single immunization site. To test this hypothesis, each DNA vaccine in the multivalent combination vaccines was co-administered individually at different sites of injection; a different vaccine vector was administered in each hind limb and/or forelimb at the same time of immunization. A significant decrease in survival was observed when the HA, NA, NP, and M2 DNA vaccines were administered at four different immunization sites (hind left (hl), hind right (hr), fore left (fl), fore right (fr)). HA+NA+M2+NP at a 1ug/vaccine dose offered 25% survival when administered at different sites compared to 90% when the same vaccines were combined at a single injection site (Figure 5.3a p=0.020). The NP and M2 antigens were then replaced with the unrelated ZGP DNA

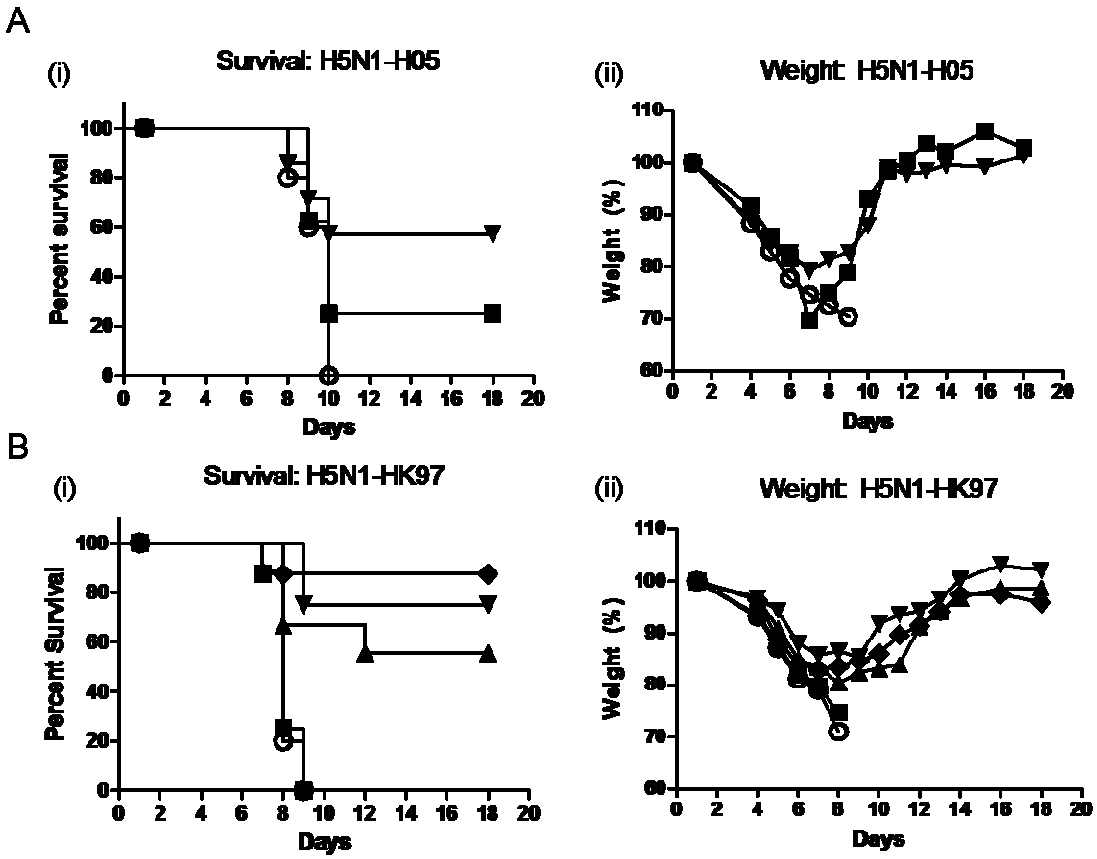



**Figure 5.3 Multivalent antigen combinations administered at different immunization sites.** BALB/c mice were vaccinated with multiple pCAGα DNA vaccines at different sites of immunization. Mice were challenged 28 days post-vaccination with homologous or heterologous H5N1 viruses. (A) H5N1-H05 Challenge. Control (PBS, ■), HA+NA (1µg , ▲), HA+NA+M2+NP (1µg , ▼), and HA+NA+ZGP (1µg , ◆). (B) H5N1-HK97 Challenge. Control (PBS**,** ○**),** HA+NP (10µg ,■), HA+NA+M2+NP (10µg , ▼ or 50µg , ▲), and HA+NP+ZGP (10µg , ◆) (i) survival and (ii) weight Loss were monitored for each vaccine. The data represent average values from 2-3 independent experiments performed with at least two independent preparations of each DNA vaccine.

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vaccine, as well as another unrelated antigen from the Crimean-Congo Hemmorhagic Fever virus M protein (CCHF-M). The co-administration of vaccines expressing two influenza and two unrelated antigens at different sites of immunization offered a survival rate of 57%, with moderate to severe signs of disease. Although significant better survival was observed with this vaccine compared to co-administration of four influenza antigens (p=0.004), overall survival was still reduced compared with of (HA+NA) at a single site of immunization (p=0.059).

Administration of a 10ug/vaccine dose of HA and NP at separate immunization sites (hl, hr) offered a similar survival rate against the heterologous HK97 virus compared to the (HA+NP) vaccine co-administered at a single site (Figure 5.3b p=0.607). However, a significantly lower survival rate of 25% was observed when mice were vaccinated with 10µg/vaccine of HA, NP, NA, and M2 at different locations (hl, hr, fl, fr) compared to the 80% protection offered by (HA+NP) at a single site (p=0.004). Immunization of the 50ug/vaccine dose at different sites of immunization (hl, hr, fl, fr) reduced survival to 50% but this did not meet statistical significance compared to the 80% protection offered by (HA+NP) given at a single site (p=0.051). Eighty-eight percent of animals survived when the NA and M2 antigens were replaced by ZGP and CCHF-M at different sites of immunization and challenged with H5N1-HK97.

These observations suggest that delivery of four H5N1 vaccines simultaneously at different immunization sites does not improve survival against homologous and heterologous viruses. Co-administration of unrelated antigens also did not improve survival. Vaccination with multiple antigens at different sites further reduced overall

protection suggesting that simply separating antigens is not sufficient to overcome possible antigen competition or inhibition.

### *5.2.4 Immune Responses in BALB/c Mice*

In an effort to draw parallels between immune parameters and observed phenotypic changes in mice following infection, humoral and cell-mediated immune responses were monitored following co-administration of the DNA vaccines at a single location or at multiple immunization sites. Serum was collected 25 days post-immunization and antibody levels were evaluated by hemagglutination inhibition and neutralizing antibody assays. T-cell responses were evaluated through detection of the IFNγ recall response following peptide re-stimulation of splenocytes from vaccinated animals using an ELISPOT assay.

#### *5.2.4.1 Hemagglutination inhibition (HI) antibody responses*

The HI antibody response was assessed prior to challenge as a measure of the humoral immune response against homologous and heterologous H5N1 viruses. The HI antibody response was first evaluated following co-administration of vaccines at a single immunization site. The combination of (HA+NA) generated increasing antibody titres against the H5N1-H05 virus ranging from 24±9 to 56±21 for the 1-50μg/vector doses (Figure 5.4a). Combination of (HA+NA) with NP and M2 also resulted in antibody titres that increased in a dose-dependent manner: 36±11, 66±23, and 66±23 for the 1μg/vector, 10μg/vector, and 50μg/vector doses, respectively. A similar reciprocal titres of 46±27 was detected when (HA+NA) was combined with ZGP. By comparison, HI antibody

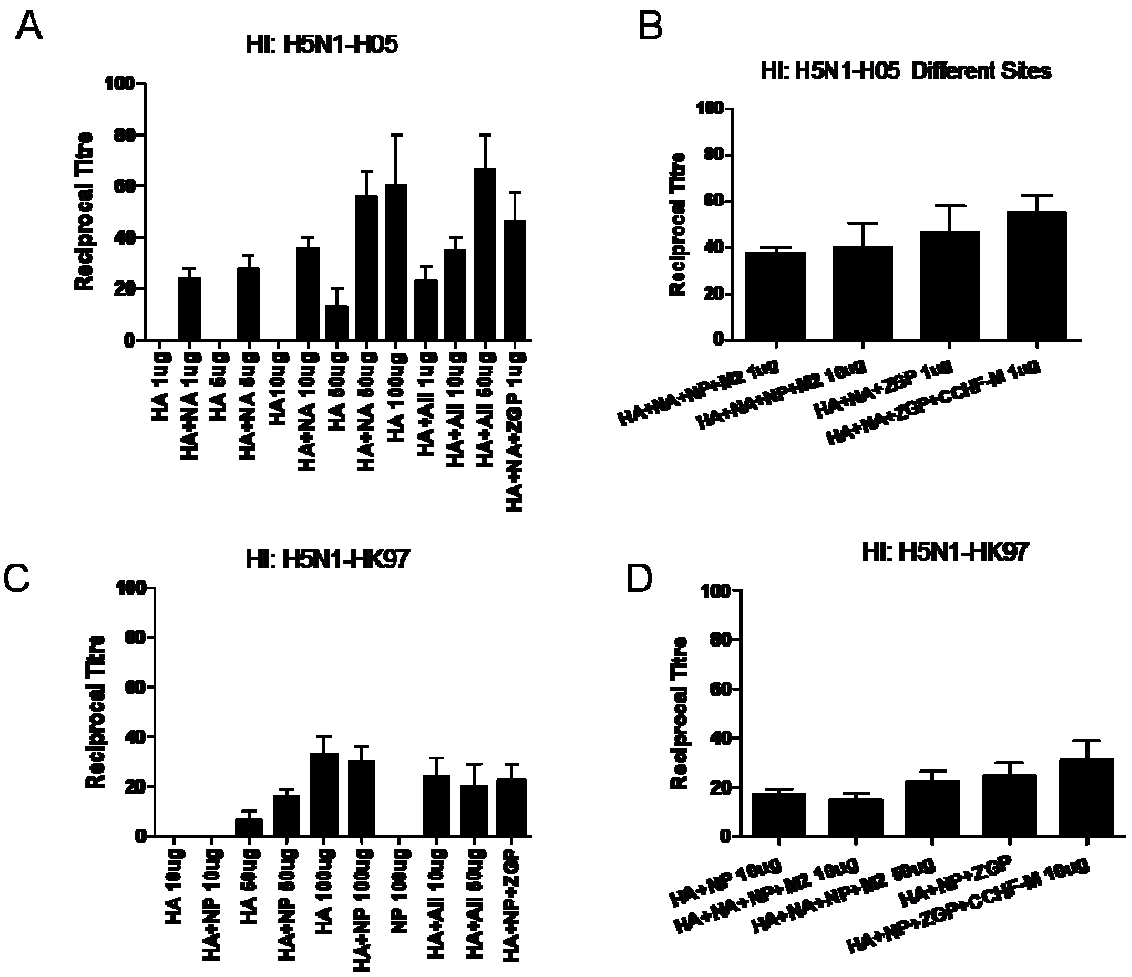


**Figure 5.4 Detection of HI antibody H5N1-H05 and –HK97.** Serum was obtained from groups of 10 BALB/c mice vaccinated with different antigen combinations: HA, HA+NA, HA+NA+M2+NP, HA+NA+GP+CCHF. RDE-treated sera were assayed for HI titres against H5N1-H05 (A) a single site of immunization, (B) different sites of immunization, and H5N1-HK97 (C) a single immunization site and (D) H5N1-HK97 at different immunization sites. A minimum of 10 mice were analyzed for each vaccine. Error bars represent the standard deviation of the data from at least two independent experiments.

responses were not detectable for low doses of the single HA DNA vaccine and titres of 13±11 and 60±34 were detected for the 50ug and 100ug doses, respectively (Chapter 3).

Antigen combination also improved detected of HI antibody titres against the heterologous H5N1-HK97 virus. The (HA+NP) vaccine generated reciprocal HI antibody titres ranging from 0 to 30±14 (Figure 5.4b). HI antibody titres following combination of (HA+NP) with NP and M2 were 24±16 and 20±20 for the 10µg/vector and 50µg/vector doses, respectively. Similarly, a titre of 22±12 against the HK97 virus was detected when (HA+NP) was combined with ZGP a dose of 10µg/vector. However, the detected HI antibody titres were very near or at the limit of detection of the HI assay for most vaccines. HI antibody titres of 6.6±5.7 and 33±11 were detected against the HK97 virus with the HA single DNA vaccine at 50µg and 100µg, respectively.

Additionally, HI antibody titres were also evaluated following co-administration of the DNA vaccines at different sites of immunization. HI titres of 37±7 and 40±30 were detected against the H5N1-H05 virus following administration of the HA, NA, NP, and M2 1ug/vaccine and 10µg/vaccine doses at different locations (Figure 5.4c). A titre of 46±27 was detected following co-administration of HA, NA, and ZGP at different sites of immunization and the HA, NA, ZGP, CCHF-M vaccine had a reciprocal titre of 55±20. Co-administration of 10µg/vaccine of HA and NP generated a titre of 17±4 against the heterologous H5N1-HK97 virus (Figure 5.4d). When HA, NP, NA, and M2 were administered at different locations, 15±7 and 22±11 were detected for the 10µg/vaccine and 50µg/vaccine doses. Co-administration of the HA, NP, and ZGP combination had a titre of 25±12 and HA, NP, GP, and CCHF generated a titre of 31±22, when administered at different injection sites.

Antigen combination improved the detection of HI antibody titres against both homologous and heterologous viruses compared to administration of HA alone. However, no differences in hemagglutination inhibition antibody titres were observed between combination vaccines administered at the same or different sites of immunization.

*5.2.4.2 Neutralizing antibody (NAB) responses*

Levels of virus neutralizing antibodies against H5N1-H05 or –HK97 were also detected from the same serum samples from vaccinated mice as an additional measure of the humoral immune response. NAB titres ranging from 12±4 to 40±24 were detected against the H5N1-H05 virus following vaccination with increasing doses of (HA+NA) combined at a single site of immunization (Figure 5.5a). The (HA+NA)+NP+M2 vaccine generated titres of 23±15 and 33±11for the 10µg/vector and 50µg/vector doses, respectively. As well, mixing 10µg/vector (HA+NA)+ZGP generated an NAB titre of 33±11. Against heterologous H5N1-HK97 virus, NAB titres ranging from 14±5 to 26±13 were also detected for increasing doses of the (HA+NP) combination vaccine that was administered at as single site of injection (Figure 5.5b). In comparison, neutralizing antibody titres following immunization with the HA DNA vaccine were only detectable against H5N1-H05 at a dose of 100µg and could not be detected against the HK97 virus, as previously described (Patel et al., 2009).

Similar NAB titres were observed following co-administration of the different DNA vaccine combinations at different sites of immunization. Reciprocal titres ranging from 20±14 to 33±11 were detected against the H5N1-H05 virus for increasing doses of HA and NA at different locations (Figure 5.5c). Simultaneous administration of HA and

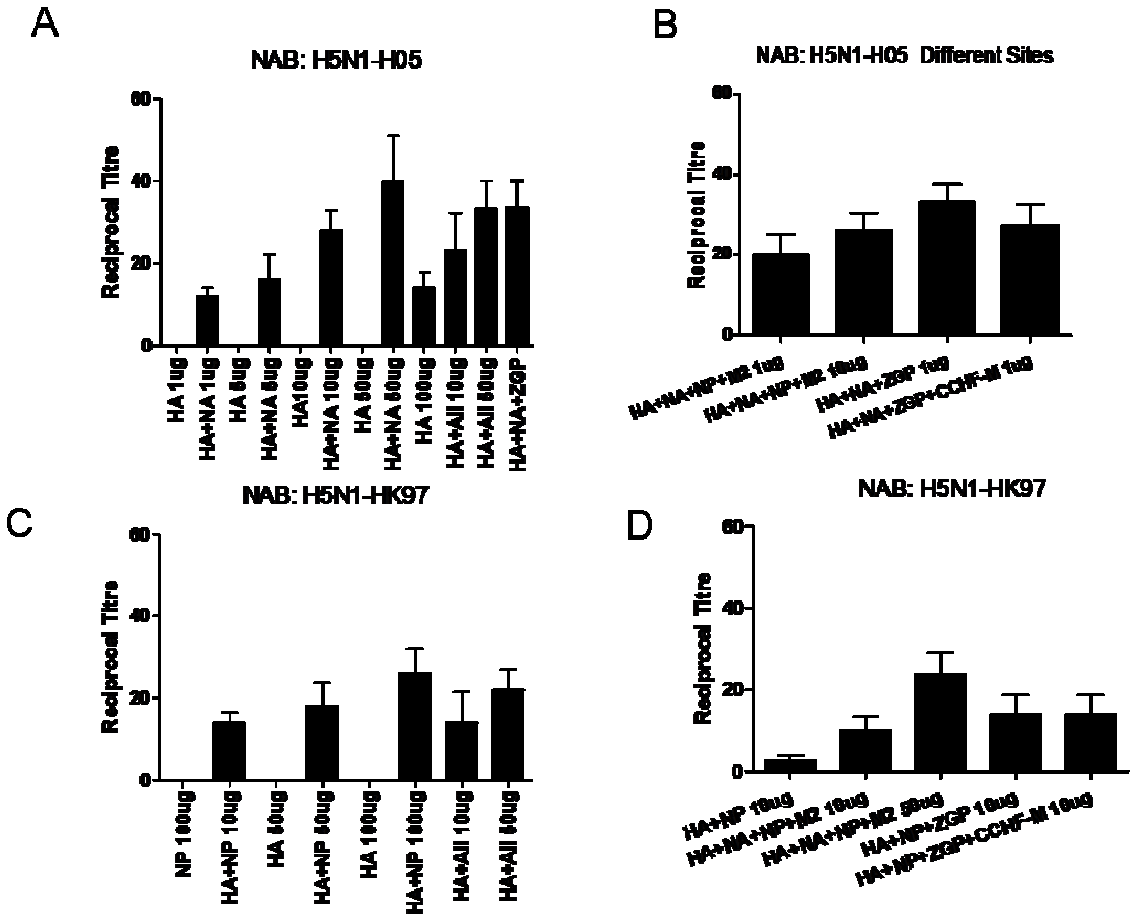


**Figure 5.5 Detection of NAB titres against H5N1-H05 and -HK97.** Serum was obtained from groups of 10 BALB/c mice vaccinated with different antigen combinations: HA, HA+NP, HA+NA+M2+NP, HA+NP+GP+CCHF. RDE-treated sera were assayed for NAB titres against H5N1-H05 at a (A) single site of immunization and (B) different sites of immunization, and against H5N1-HK-97 at (C) single immunization site and (D) different immunization sites. A minimum of 10 mice were analyzed for each assay. Error bars represent the standard deviation of the data from at least two independent experiments.

NA with the ZGP antigen at different sites generated a titre of 33±11 and 27±14 for co-administration of HA, NA, ZGP, and CCHF-M. Lower neutralizing antibody titres were detected against the heterologous H5N1-HK97 virus. A titre of 2±4 was detected when HA and NP were co-administered at different locations (Figure 5.5d). Also, low titres of 10±9 and 23±15 were recorded following vaccination with HA, NP, NA, and M2 at different immunization sites for the 10μg/vaccine and 50μg/vaccine doses, respectively. A titre of 13±14 was detected against H5N1-HK97 when 10μg/vaccine of HA, NP, and ZGP were co-administered individually. The same titre was also observed following co-administration of 10μg/vaccine of HA, NP, ZGP, and CCHF-M.

No significant difference was observed between the neutralizing antibody responses in animals vaccinated at a single site of immunization compared with those vaccinated at multiple locations.

*5.2.4.3 Cell-mediated immune responses*

In addition to humoral immune responses, there is increasing evidence that T-cell responses may play an important supplementary role following influenza virus infection (Hogan et al., 2001; McElhaney et al., 2006; Wiley et al., 2001). T-cell responses were detected using an ELISPOT assay to screen for IFNγ secretion following re-stimulation of mononuclear cells harvested from the spleens of vaccinated mice. The T-cell responses were first screened 10 days post-immunization with the pCAGα-HA alone, (HA+NA), (HA+NP), and (HA+NA+NP+M2) vaccines co-administered at a single injection site. Total pooled T-cell responses were detected following re-stimulation with peptide pools representing the H5N1-H05 HA protein. Responses of 6836±3502, 4829±2046, 5764±3151, and 5616±373 were detected for each respective vaccine (Figure

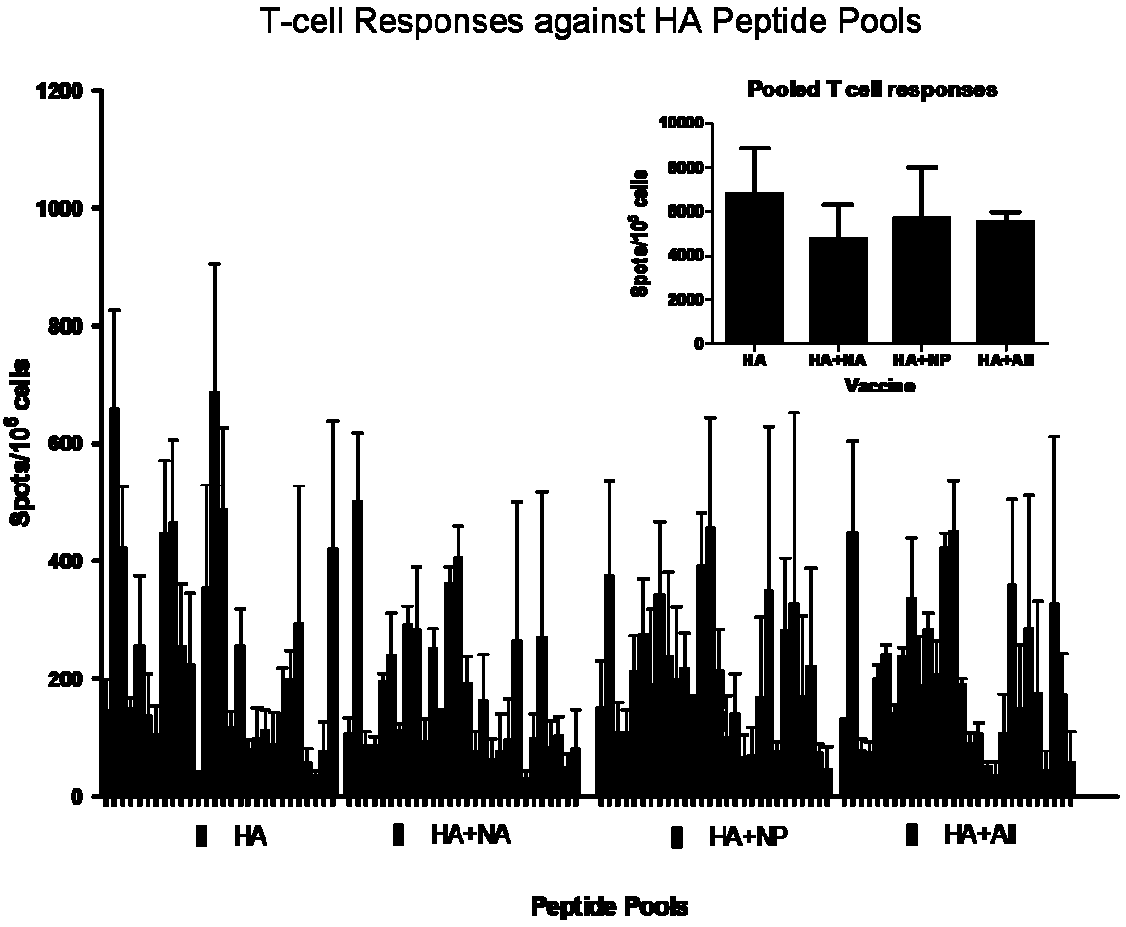


**Figure 5.6 T-cell responses detected by ELISPOT-IFNγ assay against H5N1-H05 HA Peptide Pools.** Groups of 4 BALB/c mice were vaccinated with 50μg of pCAGα-HA, HA+NA, HA+NP, or HA+NA+NP+M2 (All). Mouse spleens were harvested 10 days post-immunization and re-stimulated with 15mer overlapping peptides from an H5N1-H05 HA peptide library. Bars represent the total number of sfc/million cells for 28 pools of HA peptides. The experiment was repeated at least three times and error bars represent the standard deviations of the data. (Inset: pooled T-cell responses. Bars represent the total number of sfc/million cells)

5.6). Splenocytes were also re-stimulated with H5N1-NA or –NP peptide pools. Significantly lower T-cell responses were detected for each combination vaccine when compared in parallel to a single NA or NP vaccine (Figure 5.7, p=0.008 and p=0.007, respectively).

Total pooled T-cell responses were also compared between same and different immunisation sites (Figure 5.8). Administration of the HA+NA or HA+NA+ZGP+CCHF-M vaccines at different sites of administration improved T-cell responses against H5N1-NA peptide pools (p=0.214), although still on average lower than the single NA DNA vaccine. The administration of HA+NP or HA+NP+ZGP+CCHF-M at different sites of administration also increased T-cell responses against H5N1-NP peptide pools to levels similar to the single NP DNA vaccine (p=0.349). However, while overall T-cell responses increased following co-administration of antigens at different sites of administration, protection was not improved.

### *5.2.5 Co-administration of Adenovirus-based Vaccines expressing H5N1 antigens*

Mixing different H5N1 DNA vaccines suggested that a combination of HA and NP can offer similar levels of protection against homologous and heterologous virus challenges. To assess antigen combination in the context of a viral vector-based vaccine platform, replication-deficient porcine adenovirus 3 (PAV3) and human adenovirus 5 (AdHu5)-based expressing the H5N1-H05 HA or NP antigens were developed. (PAV3-HA+PAV3-NP) or (AdHu5-HA+AdHu5-NP) vaccines were mixed together in solution and the co-administered at a single site of immunization. Interestingly, the addition of

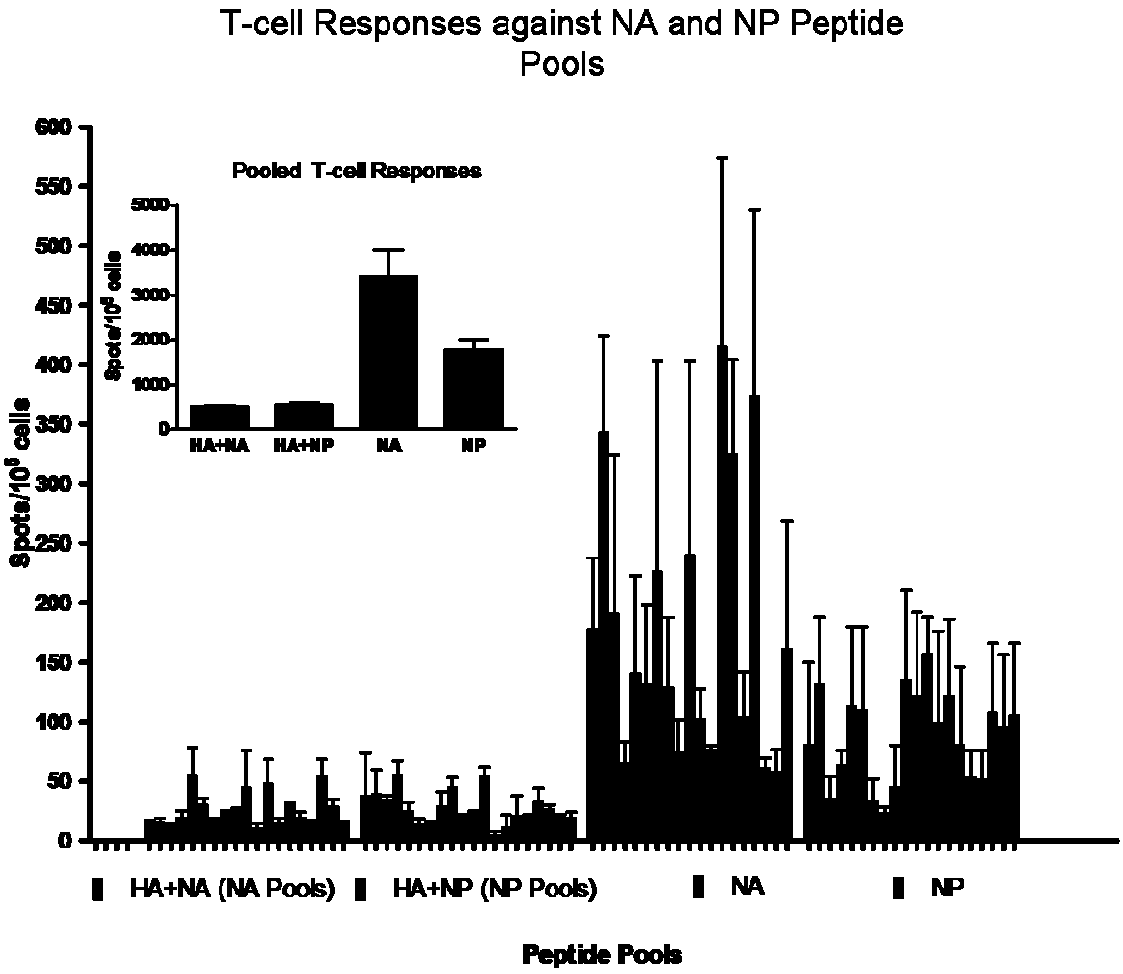


**Figure 5.7 Detection of T-cell responses against H5N1-H05 NA and NP Peptide Pools by ELISPOT-IFNγ assay.** Groups of 4 BALB/c mice were vaccinated with 50µg of HA+NA, HA+NP at a single site of immunization. Mouse spleens were harvested 10 days post-immunization and re-stimulated with 15mer overlapping peptides from an H5N1-H05 NA or NP peptide library. Bars represent the total number of sfc/million cells for 19 pools of NA or 20 NP pools peptides. The experiment was repeated at least three times and error bars represent the standard deviations of the data. (Inset: pooled T-cell responses. Bars represent the total number of sfc/million cells)

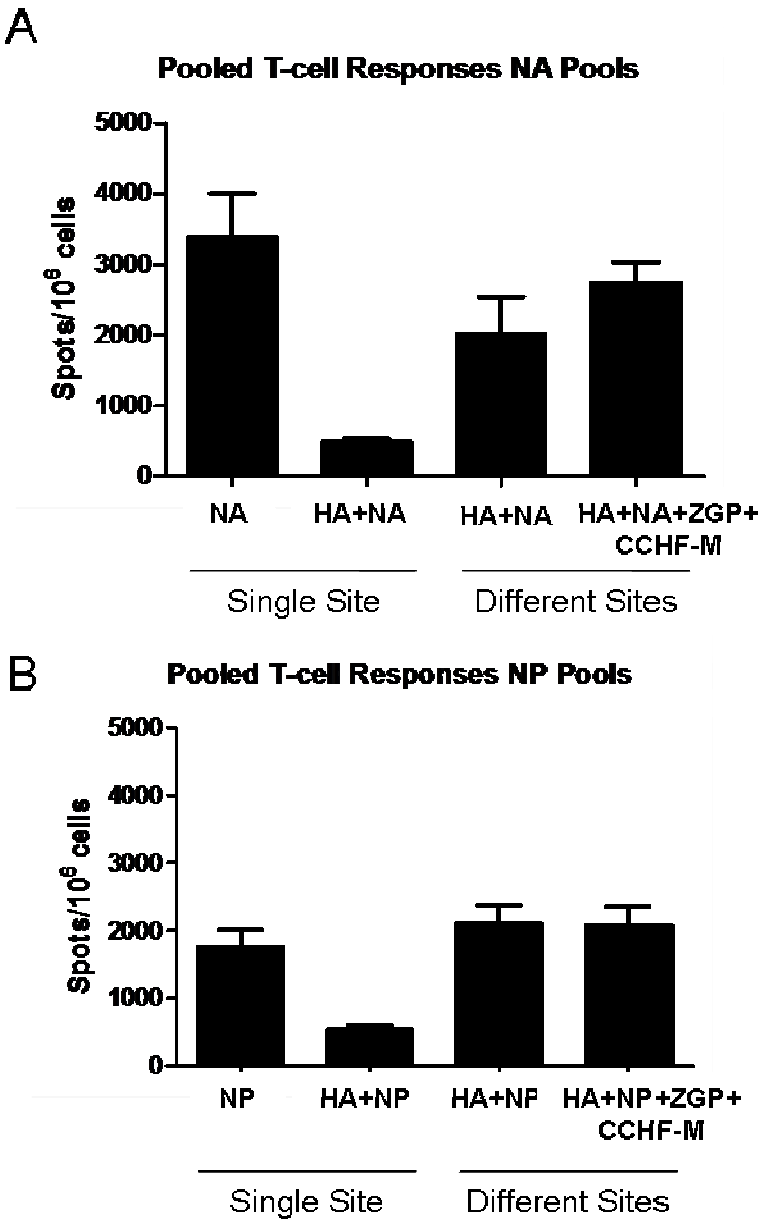


**Figure 5.8 Pooled T cell responses following vaccine administration at different sites of immunization.** Groups of 4 BALB/c mice were vaccinated at different sites of immunization with 50µg of (A) HA+NA and HA+NA+GP+CCHF, or (B) HA+NP and HA+NP+GP+CCHF. Splenocytes were harvested 10 days post-immunization and T-cell responses were detected by ELISPOT-IFNg assay following restimulation with 15mer overlapping peptides from the H5N1-H05 NA or NP peptide libraries. Pooled T-cell responses from single sites of immunization (Figure 5.7) are displayed for comparison. Bars represent the total number of sfc/million cells. Error bars represent the standard deviations of the data and was repeated at least three times.

**Table 5.3:** Efficacy of PAV3-HA+PAV3-NP and AdHu5-HA+AdHu5-NP against homologous (H5N1-H05) and heterologous challenges (H5N1-HK97, H5N1-In05)

| Challenge | Treatment | Protection (%) | Weight Loss (%) † | Signs of Disease ‡ |
|---|---|---|---|---|
| H5N1-H05 | Vehicle | 0 | >25 | Death |
| | PAV3-HA+PAV3-NP | 100 | 15 | Mild |
| | AdHu5-HA+AdHu5-NP | 70 | 23 | Mild to Severe |
| H5N1-HK97 | Vehicle | 0 | >25 | Death |
| | PAV3-HA+PAV3-NP | 100 | 22 | Mild to Severe |
| | AdHu5-HA+AdHu5-NP | 0 | >25 | Severe |
| H5N1-In05 | Vehicle | 0 | >25 | Death |
| | PAV3-HA+PAV3-NP | 80 | 23 | Mild to Severe |
| | AdHu5-HA+AdHu5-NP | 14 | >25 | Severe |

† Weight loss: Percentage difference from pre-challenge weight to the lowest weight observed.
‡ Signs of Disease: Change in observed clinical signs. None: no change from pre-challenge signs.

the NP antigen to either adenovirus-based vaccine did not improve survival. Although the (PAV3-HA+PAV3-NP) combination afforded 100% survival against homologous H5N1-H05 and heterologous HK97 viruses, greater signs of disease and more significant weight loss ($p<0.001$ and $p<0.01$, respectively) were observed following infection compared to PAV3-HA alone (Table 5.3 and Chapter 4). This observation was more evident following vaccination with the (AdHu5-HA+AdHu5-NP) combination vaccine. Reduced survival (70%) and significantly greater weight loss ($p<0.001$) were observed with the bivalent vaccine against homologous H5N1-H05 challenge compared to the single HA-based vaccine (100%). Survival was also significantly reduced against heterologous H5N1-HK97 challenge following vaccination with (AdHu5-HA+AdHu5-NP) ($p=0.032$). The addition of the NP antigen did not significantly affect survival against the divergent clade 2.1.3 H5N1-In05 virus.

Hemagglutination inhibition antibody titres against the H5N1-H05 virus were significantly higher for the (PAV3-HA+PAV3-NP) combination compared with (AdHu5-HA+AdHu5-NP) (Figure 5.9a, $p<0.001$). Titres of 302±53 and 136±75 were detected or each vaccine combination, respectively. Interestingly, although strong HI antibody titres were detected with the PAV3-based vaccine, significantly higher levels were generated by the single PAV3-HA and AdHu5-HA vaccine candidates ($p<0.001$). HI antibody titres were similar for all single and combination vaccines against heterologous HK97 and In05 viruses. Neutralizing antibody titres were similar for all single and combination vaccines against homologous and heterologous H5N1 viruses (Figure 5.9b). The (PAV3-HA+PAV3-NP) vaccine, on average, reported higher neutralizing antibody titres against all three H5N1 viruses, however the differences were not significant when compared with

(AdHu5-HA+AdHu5-NP). Titres of 38±25, 19±15, and 22±18 were detected against H05, HK97, and In05, respectively, following immunization with the (PAV3-HA+PAV3-NP) vaccine. Reciprocal titres against H05, HK97, and In05 following (AdHu5-HA+AdHu5-NP) vaccination were 27±14, 15±10, and 14±15, respectively.

In addition to evaluation of B cell antibody responses prior to challenge, pooled T-cell responses were also assessed following peptide re-stimulation by ELISPOT assay.

Groups of 4 BALB/c mice were immunized with PAV3-NP, (PAV3-HA+PAV3-NP), AdHu5-NP, or (AdHu5-HA+AdHu5-NP) and spleens were harvested 10 days post-vaccination. Splenocytes were re-stimulated with H05-HA or –NP peptide pools to evaluate T-cell responses. Against HA peptide pools, 11 492 and 10 938 sfc/million cells were detected with the (PAV3-HA+PAV3-NP) and (AdHu5-HA+AdHu5-NP) vaccines, respectively. Total T-cell responses against NP peptide pools were decreased in mice vaccinated with (PAV3-HA+PAV3-NP) and (AdHu5-HA+AdHu5-NP). Against NP peptide pools, 6193 and 6291 sfc/million were detected against the control PAV3-NP or AdHu5-NP vaccine, and 4059 or 3019 sfc/million were observed with PAV3(HA+NP) and AdHu5(HA+NP) (Figure 5.10). Overall, a similar trend of decreasing protection and lower T-cell responses against the NP antigen were observed following co-administration of DNA and adenovirus-based vectors expressing the HA and NP antigens.

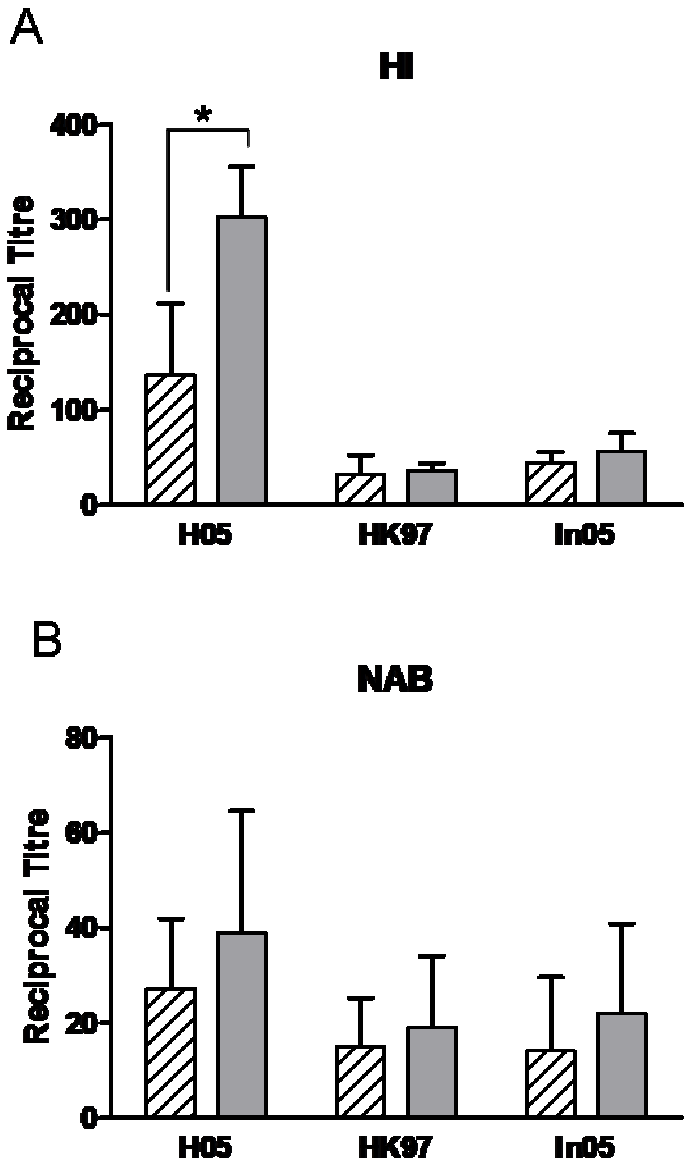


**Figure 5.9 Detection of HI antibody and NAB tires against H5N1-H05, HK97 and –In05 viruses.** Serum was obtained from groups of 10 BALB/c mice vaccinated with PAV3-HA+PAV3-NP (■) and AdHu5-HA+AdHu5-NP (▨) and treated overnight with RDE, with complement inactivation the following day. RDE-treated sera were assayed for (A) HI titres against H5N1–H05, –HK97 and –In05 and (B) NAB titres against H5N1–H05, –HK97 and –In05. Groups of 10 mice were analyzed for each assay and each virus. Error bars represent the standard deviation from one experiment (*represents $p<0.001$).

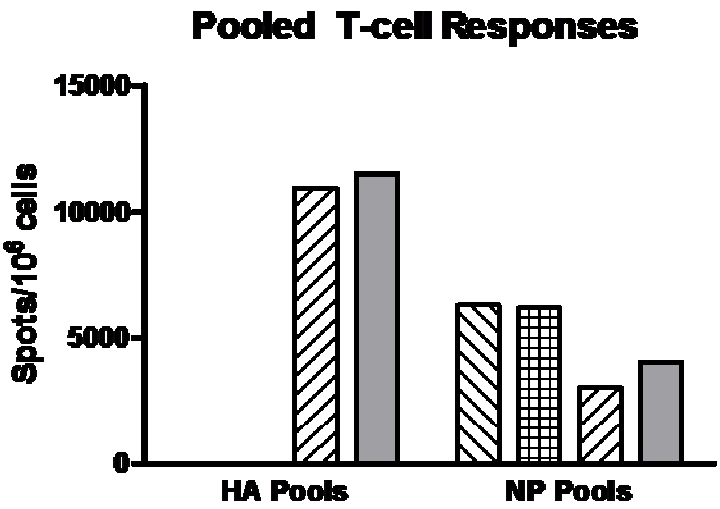


**Figure 5.10 Pooled T-cell responses detected by ELISPOT-IFNγ assay.** Groups of 4 BALB/c mice were vaccinated with $10^{10}$ vp/mouse of either PAV3-NP (▦), PAV3(HA+NP) (▩), AdHu5-NP (▧), or AdHu5(HA+NP) (▨). Mouse spleens were harvested 10 days post-immunization and restimulated with H5N1-H05 HA or NP peptide pools. Bars represent the total number of sfc/million cells for all peptide pools combined. The data represents the average sfc/million cells for one experiment.

## 5.3 Discussion

Conventional and experimental influenza vaccines based on the HA antigen can successfully protect against a matched challenge but may not provide optimal protection against heterologous infection. Protection against diverse H5N1 viruses will likely require the generation of comprehensive adaptive B and T cell responses. The combination of antigenically variable and well-conserved antigens is one strategy to expand cross-protective immunity against diverging H5N1 viruses.

Successful protection against influenza viruses and other pathogens can be achieved through co-administration of DNA vaccines (Carvalho et al., 2010; Chen et al., 1999; Epstein et al., 2002; Kodihalli et al., 1999; Todryk and Hill, 2007). The current chapter describes the co-administration of multiple DNA-based vaccines expressing different influenza antigens to further improve protection against different H5N1 viruses. Improved protection against the H5N1-H05 virus was observed with the HA+NA combination, while better partial protection against H5N1-HK97 challenge was observed with HA+NP. Protection was achievable at lower total doses of vaccine and improved antibody responses against HA were also detected with the bivalent vaccine combinations compared to the single pCAGα-HA DNA vaccine. Interestingly, further protection studies revealed decreased survival when three or more related or unrelated antigens were combined. Immunization of the individual vaccines at different sites improved survival however the bivalent H5N1 vaccines were the optimal candidates.

In the experiments described in this chapter, the generation of antibody responses was improved following multivalent immunization however this did not always correlate with better vaccine efficacy. It is possible that circulating antibodies against additional

antigens may interfere with HA following re-stimulation. A similar study compared the efficacy of HA, HA+NP, and HA+M2+NP DNA vaccines against lethal H5N1 challenge in BALB/c mice (Rao et al., 2009). Although lower survival was observed with the trivalent vaccine, the overall rate was not significant and the authors concluded that all three vaccines were equally effective. Another study observed complete protection in mice when HA+M2+NP were combined in a cationic delivery system (Sharpe et al., 2007). Both vaccine candidates, however, were fully protective when assessed in ferrets. However, the trivalent vaccines generated lower HI and neutralizing antibody responses in both experiments compared to the mono- or bivalent vaccines.

Co-administration of antigens is not a novel concept and has been evaluated for various conventional and subunit vaccine platforms for the past 60 years (Berger et al., 1988; Holman et al., 2008; Jalil et al., 1991; Johansson and Brett, 2007). Older studies suggest that decreased vaccine efficacy may be a consequence of antibody interference between vaccine components. Antibody interference was reported following vaccination with conventional vaccines (MMR, DPT) (Berger et al., 1988; Jalil et al., 1991), subunit vaccines (combined *Pneumococcus*), and the commercial HPV VLP vaccine. However, even with lower antibody titres against several vaccine components the vaccines achieved sufficient seroconversion to receive commercial approval. Interference between MMR components was observed in the commercial live-attenuated vaccine. One study observed an increase in antibody responses and seroconversion when the measles and mumps components were reduced by $1/5^{th}$ and $1/8^{th}$, respectively (Berger and Just, 1988). Braun et al. (1998) also demonstrated that the efficacy of DNA vaccines decreased when the dose of DNA was increased (Braun et al., 1998). External glycoproteins are frequent

targets of antibody immune responses and the development of neutralizing and non-neutralizing antibodies against other antigens in the vaccine may interfere with the secondary anti-HA immune response. Influenza A viruses have been reported to share several cross-reactive epitopes with other RNA viruses such as HIV, Hepatitis C, and Epstein Barr virus (Welsh and Fujinami, 2007). The presence of lower affinity antibodies against shared epitopes may interfere with antibodies of higher affinity to HA. The addition of unrelated GP or CCHF-M could potentially generate immune responses against cross-reactive antibody epitopes and additionally that activate low affinity memory T-cells, leading to changes in virus clearance and possible pathogenesis.

Although antibody responses are frequently reported for influenza vaccines, the correlation between T-cell responses and observed protection has not been previously evaluated in the context of a multivalent H5N1 DNA vaccine. Neither trivalent mouse nor ferret vaccine study presented T-cell responses against antigens other than HA in the experiments (Lalor et al., 2008; Rao et al., 2009). The experiments in this chapter indicated that although T-cell responses against H5N1-H05 HA were similar for single and all multivalent vaccines, significantly lower responses were observed against additional antigens in each vaccine combination. Interference between T-cells may also contribute to differences in overall cell-mediated immune responses. One suggestion is that the HA antigen may be preferentially presented by MHC complexes on antigen presenting cells (APC) over other antigens (Adorini and Nagy, 1990; Kedl et al., 2002) and also affect cross-presentation. Injection of extra antigen-presenting cells at the local site of immunization can improve the priming to T-cells and increased immune response following vaccination with multiple antigens (Kedl et al., 2000; Kedl et al., 2002).

Similar observations were observed with the conventional inactivated H1 influenza vaccine where the HA vaccine component interfered with the ability to generate T-cell responses against NA (Johansson and Kilbourne, 1993). Alternative administration of free NA purified protein circumvented this suggesting that certain immune pathways may interfere with each other (Johansson and Kilbourne, 1996).

At the same time, mixing multiple antigens in a single vaccine candidate may result in antigen dilution and insufficient uptake of antigen by APCs, reducing antibody responses and T-cell priming (Yashima et al., 2010). Vaccinating at different immunisation sites has been recommended to avoid dilution of antigens. A 9-plasmids malaria DNA vaccine observed decreased T-cell responses, interestingly with better responses when administered at different sites (Sedegah et al., 2004). The data in this thesis chapter showed improvement in the T-cell response following immunization at different sites, however, this did not always translate directly to better survival. NA may have acted synergistically to improve the generation of HA antibodies perhaps through mechanisms of attracting higher affinity T-cells or a combination of low affinity and high affinity T-cells (Kedl et al., 2000). This supports the idea that the strength of the immune response may not only be dependent on the type of antigen, dose, affinity and competition between antigens. The idea of antigen dilution suggests that there is a threshold that needs to be overcome in order to achieve protection against heterologous virus. The suboptimal dose of HA in each vaccine may play a crucial role towards overall efficacy. Dilution of a suboptimal dose of HA in a combination vaccine would further reduce the ability of the antigen to stimulate antibody and T-cell responses. However, the administration at different sites of injection may result in the vaccines

being regarded as separate vaccines rather than a combined vaccine and the effect may not be synergistic.

In the current chapter, mice were also vaccinated with the (PAV3-HA+PAV3-NP) and (AdHu5-HA+AdHu5-NP) combined Ad-based vaccines and challenged with a lethal homologous or heterologous virus. Interestingly, lower weight was observed in mice vaccinated with (PAV3-HA+PAV3-NP) and reduced survival was associated with (AdHu5-HA+AdHu5-NP), compared to the PAV3-HA or AdHu5-HA vaccines described in Chapter 4. The HI and NAB antibody titres were also significantly lower for both combination vaccines against matched H5N1-H05 challenge. Other studies have evaluated co-administration of adenovirus vectors with varied rates of success. Recently, Rao et al (2009) described co-administration of an AdHu5 vector expressing H5N1 HA, NA, and M1 antigens. Full protection was achieved in mice and ferrets following two doses of vaccine (Rao et al., 2009). Another study observed 100% protection against lethal homologous challenge with a combination of AdHu5 HA+NP, but did not test the vaccine against heterologous challenges (Hoelscher et al., 2008).

The possibility of antigen competition between adenovirus vectors has also been reported previously (Holman et al., 2008; Tatsis et al., 2006). Competition has also been observed between Ad vectors and other viral-based vaccine platforms like vaccinia (Yashima et al., 2010). Since both vaccines are delivered in an Ad-based vector, it is likely that they are processed in similar ways before translation of the H5N1 genes and processing of the influenza antigens. The CAdVax platform was developed to avoid interference between Ad vectors by incorporating three expression cassettes to deliver additional antigens in a trivalent single Ad vector (Holman et al., 2008). A CAdVax-

based H5N1 vaccine expressing the HA+NA+M1 antigens offered full protection against homologous and heterologous challenges, however higher doses of vaccine were administered: $4x10^{11}$ vp/mouse compared to $1x10^{10}$vp in this thesis and other studies (Holman et al., 2008; Rao et al., 2009; Zhou et al.).

Perhaps differences in the entry and activation of APCs by the PAV3 vaccine vector may lead to better processing of individual expressed antigens than with AdHu5. Alternatively, since reduced survival was experienced with both vaccines, it is also possible that the enhanced levels of antigen expression also contribute to interference between the H5N1 proteins. Similar to the DNA vaccines, lower T-cell responses were observed against the NP antigen. The studies in this thesis suggest that the immune mechanisms involved with combination vaccines will need to be further evaluated in order to fully understand decreases or increases in protection.

Overall, the data supports combining antigens to improve cross-protection, however, antigens cannot be mixed arbitrarily and each vaccine candidate must be carefully assessed in order to ensure optimal protection. The decrease in humoral immunity combined with decreases in T-cell data shown by the studies in this chapter suggest that evaluation of immune responses following combination vaccination should not be overlooked. In order to improve protection offered by additional antigens several other considerations may be necessary including careful selection of antigens, considering the timing of doses, and the overall dosage. The relationship between immune parameters and the degree of protection will need to be considered.

# CHAPTER VI

# FINAL DISCUSSION AND THOUGHTS

From a public health perspective, the prevention of influenza virus infection has been largely successful due to vaccination and the establishment of immunization programmes. Seasonal influenza vaccines can reduce serious illness in young children, older adults, and immunocompromised individuals. Additionally, economic productivity and the general strain on the public health care system have also benefited greatly from influenza vaccination. Moving forward, current basic and clinical research are combining efforts to improve and test various vaccine candidates that can offer better protection against circulating seasonal and potential emerging influenza viruses.

The development of a single *universal* influenza vaccine is one of the goals of influenza vaccine research. To date, no single vaccine has offered full protection against all human influenza viruses. The considerable degree of heterogeneity within the influenza A virus population is a major challenge for developing broad-spectrum vaccines (Johansson and Brett, 2007; Poland et al., 2008). Several vaccines have, however, demonstrated promising levels of cross-protective immunity against heterotypic and heterosubtypic viruses (Arnon and Ben-Yedidia, 2009; Baras et al., 2008; Chen et al., 2000; Keitel and Atmar, 2009). To achieve a universal vaccine, it will be necessary to evaluate different approaches to vaccine design, including the consideration of viral antigens and the generation of innate and adaptive immune responses. A cross-protective vaccine would be a great advantage against antigenically variable strains and possibly against all pandemic viruses.

Classical influenza research has focused primarily on the evaluation of protection and the detection of neutralizing antibody responses. It is becoming increasingly evident from recent studies that other immune parameters may also be involved during the secondary influenza immune response and that the cell-mediated immune response is also likely to be an important supporting factor (Bui et al., 2007; Ellebedy and Webby, 2009). Taking this into consideration, the studies described in this thesis considered the relationship between the generation of humoral and cell-mediated immune responses by various avian influenza H5N1 antigens in the context of experimental DNA and adenovirus-based vaccines, in addition to protection.

Interestingly, the data suggests that both humoral and cell-mediated immune responses contributed to improved survival following lethal challenge with a homologous H5N1 virus. On average, stronger hemagglutination inhibition titres correlated with improved survival following vaccination with DNA or Ad-based vaccines expressing the H5N1 HA antigen. Even in the absence of undetectable or low antibody titres, the rapid generation of early cell-mediated immune responses also was associated with improved recovery and clinical outcome. This was supported by detection of lower virus titres in the lungs of immunized mice following short-term infection. Establishing cross-protection against heterologous infection was more challenging and a combination of strong antibody and cell-mediated immune responses will likely be necessary in order to achieve full protection.

The experimental vaccine candidates developed in this thesis achieved full protection against lethal homologous challenges and in some cases, complete protection against divergent H5N1 viruses. Antibody responses were also detected following

administration of the HA-based DNA and Ad-based vaccine candidates. Interestingly, low doses of the pCAGα-HA DNA vaccine were fully protective in the absence of detectable HI and NAB antibodies. For the first time, the studies in Chapter 3 also identified the dominance of the T-cell immune response in BALB/c mice as strongest against HA, followed by NA, NP, and M2. Early studies evaluating T-cell immune responses focused primarily on the more conserved, internal influenza antigens. Several studies reported strong cell-mediated immune responses against the NP protein following DNA vaccination in C57BL6 mice (Epstein et al., 2005; Luo et al., 2008), an observation that is frequently referenced without additional consideration. This is contrary to the cell-mediated immune profile observed during the experiments of this thesis.

One reason may be the choice of inbred mouse model. In recent years, the BALB/c mouse has been more frequently used to evaluate influenza infection because it can generate strong B cell responses (Bodewes et al., 2010). Additionally, human HPAI H5N1 viruses are highly virulent in BALB/c mice and do not need to be adapted in order to be pathogenic (Bodewes et al., 2010; Vandegrift et al., 2010). C57BL6 mice, in contrast, are generally used for their ability to generate cell-mediated immune responses (Bodewes et al., 2010). Although immunodominant T-cell epitope data is available for BALB/c mice (MHC restriction H2-$K^d$) for HA, very little data is available for HA in C57BL6 mice (MHC restriction H2-$K^b$. Chen et al (1999) compared an HA-based DNA vaccine against H1N1-PR8 using three mouse models: BALB/c, B10, and C3H. Their observations suggested that the HA-based DNA vaccine was poorly immunogenic in B10 and C3H mice (also MHC H2-$K^b$, similar to C57BL6 mice). However, full protection was observed in BALB/c mice (Chen et al., 1999). Charo et al (2004) reported the

efficacy of DNA plasmids expressing H1N1-PR8 HA or NP antigens in C57Bl6 and BALB/c mice. Interestingly, the single HA-based DNA vaccine offered significantly lower survival to the NP-based DNA vaccine in C57BL6 mice (Charo et al., 2004). Further experiments in the study only evaluated the NP antigen in BALB/c mice.

However, as part of the study presented in Chapter 3, a T-cell immunodominance hierarchy of HA>NA>NP>M2 was observed following a natural infection in out-bred non-human primates (Patel et al., 2009). This suggests that perhaps the BALB/c model may be a good representative of natural infection, compared to C57BL6.

Following influenza infection, systemic memory B cell responses are also reactivated. Protection against influenza A viruses is mediated by different antibody subclasses in different mice (Hocart et al., 1989). IgG1 and IgG2a subclasses have been reported for BALB/c and IgG2a and IgG2b for C57BL6 mice.

The co-administration of the bivalent pCAGα-DNA H5N1 vaccines in Chapter 5 offered a promising approach to improve the immunogenicity and reduce the dosage of the single pCAGα-HA vaccine. However, other vaccine candidates like the trivalent and tetravalent DNA vaccines did not confer protective immunity even in the presence of detectable antibody titres. Interestingly, the bivalent Ad-based HA+NP vaccines also did not perform as well as the single HA-based Ad vaccines in Chapter 4. As discussed in Chapters 5 and 6, the differences in vaccine efficacy during co-administration of DNA or Ad-based vaccine may be related to antigen competition at the level of 1) antibodies and/or 2) cell-mediated immune responses. The stability of antigen *in vivo* has been suggested as a possible factor which may affect cross-presentation of antigens for both DNA and Adenovirus vaccines (Gallimore et al., 1998). T-cell dependent antibody

generation by B-2 cells may be decreased by reduced MHC class II priming of CD4+ T-cells.

It has been reported that natural infection with one influenza subtype can provide cross-protection following secondary infection with a different strain or subtype (Mathews et al., 2010; Tamura et al., 2005). Reflecting on this concept, one idea may be to re-consider vaccine formulation in the context of natural influenza infection. The skewed T-cell responses may be a consequence of antigen competition that may not occur during natural influenza infection. Influenza antigens are naturally expressed in variable amounts by influenza viruses, while the vaccine studies in Chapters 5 and 6 co-administered antigens in equal doses. Future vaccine development may need to consider the relative percentage of antigen and formulate the vaccine accordingly, to contain more of certain H5N1 antigens and less of others.

Overall, the studies in this thesis demonstrated that both DNA and adenovirus-based vaccines can provide protection following infection of BALB/c mice with homologous or heterologous H5N1 virus. Interestingly, low doses of the pCAGα-HA DNA vaccine provided comparable levels of protection against homologous challenge to the PAV3-HA or AdHu5-HA vaccines. Strong T-cell responses were also detected with the pCAGα-HA DNA vaccine, although weaker than the Ad-based vaccines. A DNA-DNA prime-boost regimen or a single dose of the PAV3-HA offered the best cross-protection against heterologous H5N1-HK97 challenge. Given the observation of reduced vaccine immunogenicity following antigen co-administration, it may be more effective to administer an H5N1 vaccine as part of a multiple dose regimen. The success of the DNA-DNA (100μg-100μg) prime-boost approach compared to a single, high dose

of pCAGα-HA (200μg) suggests that presentation of antigen, single or combined, may be the rate-limiting step in the establishment of protective immunity.

Taken together, the adenovirus-based vaccines can rapidly generate protective immune responses and may be useful for immediate vaccination during an influenza outbreak or suspected exposure. A DNA prime followed by an Ad-based boost strategy may improve cross-protection against heterologous viruses. It may be interesting to also evaluate administration of the well-conserved NP antigen as part of a multiple dose regimen following priming with HA. One recent study demonstrated that NP-specific cytotoxic T lymphocytes in the lungs and PMBCs from mice that survived lethal challenge had greater proliferative activity, correlating with improved protection (Lin et al., 2010). Additionally, further studies will need to investigate the role of anamnestic immune responses following influenza vaccination. Reactivation of memory B- and T-cell responses will likely play an important role in cross-protective secondary immune responses following infection with heterologous H5N1 and other A influenza viruses.

### 6.1 Limitations with current techniques

One shortcoming of current influenza vaccine research is the lack of standardization and consistency between research methods. Considerable variation in vaccine dose regimens and molecular techniques was frequently noted during the progress of this thesis. The comparison of DNA and Ad-based vaccine candidates from different research groups can be challenging for these reasons. Multi-dose vaccine regimens are often compared with single-dose regimens without appropriate controls.

One concern is the absence of standardization for evaluation of HI antibody titres, a primary diagnostic assay, using horse RBCs. The dilution of turkey and guinea pig

RBCs is fairly standard (generally 0.5%v/v RBCs in saline). However, several studies detected H5N1 HI titres using horse RBCs ranging from 0.5%-2% without additional explanation (Cheng et al., 2009, Stephenson et al, 2004, Suguitan et al., 2006). Additionally, several studies suggesting that horse RBCs are more sensitive for detecting HI antibodies reported the data using two hemagglutinating doses of virus rather than the standard four hemagglutinating doses that is frequently used with turkey red blood cells at diagnostic reference laboratories such as at the National Microbiology Laboratory (Public Health Agency of Canada, Winnipeg, Manitoba, Canada).

The literature review and analysis of the results from this thesis highlighted several instances where vaccines induced significantly stronger antibody responses that did not always correlate with better survival *in vivo*. The immune system is complex and the differences may be due to many additional compounding factors including antigen competition or interference, as mentioned earlier. As well, the use of different animal models may also impact vaccine efficacy. Basic influenza research is frequently conducted using mouse models before evaluation in other animals such as guinea pigs or ferrets, and further studies in larger animal models like non-human primates and human clinical trials. A good animal model for influenza should be a lethal model of infection, display observable signs of infection, mimic natural infection, and be affordable. Mice are attractive models for evaluating H5N1 vaccines since they can be observed in large groups, increasing the statistical power of experiments. The study by Rao et al. (2010) demonstrated that a vaccine that offered high partial protection in mice could achieve full protection in ferrets. Ferrets are often considered a better model for influenza as they share similar sialic acid receptors to humans and can mimic human upper respiratory tract

infection. Although the experiments described in this thesis were performed in mice, it may be interesting to further evaluate the PAV3-HA and AdHu5-HA vaccines in ferrets.

### 6.2 Experimental Adenovirus Vaccines

The generation of more robust, cross-protective immune responses may be generated by intranasal administration of vaccine, similar to the route of natural infection. Similar to influenza viruses, adenovirus vectors can transduce the mucosal epithelium and may be able to generate strong immune responses. Extensive research in recent years has demonstrated that AdHu5 vectors can generate protective immune responses and may be good candidates against H5N1 viruses. The high reported toxicity and death in the OTC gene therapy trial was a reminder that individual considerations need to be assessed for vaccination in humans. The failure of the Merck HIV Step trial also supported the idea that rational design of vaccines is not sufficient and that rigorous *in vivo* testing of various immune parameters should considered. Both studies are reminders that although AdHu5 is a non-pathogenic vector the combined activation of immune responses with a foreign transgene may lead to undesirable side effects. Nevertheless, this has not prevented the further evaluation of several AdHu5-based candidates which are currently in clinical trials for cancer, gene therapy, and infectious diseases. The extensive body of research into Ad vectors indicates well-tolerance and safety in immunocompetent humans despite the drawbacks.

In addition to H5N1 vaccine development, influenza infection can also be a valuable model to evaluate humoral and cell-mediated immune responses against rare and non-human adenoviruses vaccine vectors. A recent report suggests that some human Ad vectors such as Ad26 may not actually be rare in certain populations (Chen et al., 2010).

Overall, a non-pathogenic, non-human adenovirus could be an attractive vaccine vector. Future studies will need to fully evaluate short and long-term safety profile and toxicity in humans, but non-human Ad-based vectors such as PAV3 are promising. The development of Ad-based vaccines in Chapter 4 allowed the generation of stronger B and T-cell responses against the H5N1-HA antigen. The PAV3-HA Ad-based rapidly generated strong T-cell responses and conferred better long-term immunity than AdHu5-HA, although both vaccines offered similar short-term protection. AdHu5-HA also underperformed against divergent challenge compared to PAV3-HA, even with similar titres of antibody.

One advantage is the inability for the PAV3 virus to replication in human cells. Replication-deficient PAV3 viruses have been shown to infect several human cell lines and tissues *in vitro* (Bangari et al., 2005) but are unable to replicate and establish productive infection (Bergen et al., 2004), (Sharma et al., 2009). In tissue culture, cells must express the E1B large genes from porcine adenovirus 3 in order to replicate and amplify a replication-deficient PAV3 vector (Reddy et al., 1999). PAV3 can transduce HEK 293 cells, however cannot replicate in the absence of PAV3 E1B genes (Reddy et al., 1999). This may provide an extra safety-mechanism in favour of using replication-deficient PAV3 vaccine vectors. Other studies suggest that non-human adenovirus vectors like PAV3 and BAV3 can generate more robust innate immune responses, potentially increasing the adjuvant effect of either vector. This could have considerable implications on vaccine design and delivery of antigen. The sequential administration of vaccine could also be improved using this strategy. Studies by Sharma et al suggest that the low toxicity of the PAV3 vector may support the idea of vector re-administration.

This will need to be evaluated in future studies. Other non-human adenoviruses like porcine adenovirus vectors may also hold promise including serotype 5 (PAV5) (Bangari and Mittal, 2006).

The rapid generation of T-cell responses by the PAV3-HA vaccine suggests a possible application for rapid vaccination and potential therapeutic post-exposure vaccines against other pathogens. This would be particularly advantageous during outbreaks, for health care workers and as a potential vaccine for biodefense. Alternatively, novel Ad vectors may be developed to vaccinate livestock and poultry to prevent the transmission of zoonotic pathogens from animals to humans.

### 6.3 Final Thoughts

Influenza vaccination has developed considerably since the virus was first isolated in the 1930s. The rational design of vaccines can greatly improve the speed of evaluation as well as provide valuable information regarding the immune response against individual proteins. The studies in this thesis demonstrated that preliminary evaluation of antigens in a DNA vaccine platform can be used to screen antigens before assessment in other more efficient platforms like adenovirus-based vaccine vectors. In addition to a systematic approach, vaccine design requires some creativity. The number of epitope-based vaccines has increased in the past few years however there have been few successful candidates. To date, some of the most successful vaccines retain the native conformation and functional activity of viral proteins. Deconstruction of immune responses through evaluation of individual antigens or sequences is important however consideration of the relationship between humoral and cell-mediated immune systems may provide interesting ideas for future vaccine development.

Overall, I believe that the studies in my thesis have made several important contributions to the fields of influenza research and vaccine development. Influenza research to-date has focused primarily on the generation of strong antibody responses against the surface HA glycoprotein. In this thesis, I showed that strong cell-mediated immune responses against H5N1 antigens also correlated with protection. T-cell responses were strongest against the HA antigen, followed by NA, NP, and M2. The results from the studies evaluating the PAV3-HA vaccine also suggested a role for cellular responses in protection following the absence of strong antibody titres. I feel that the further evaluation of cell-mediated immune responses in appropriate mouse and other animal models may provide better insight behind the immune mechanisms involved during influenza infection and protection against the virus.

As well, two separate adenovirus platforms, PAV3 and AdHu5, were compared in parallel. The experiments highlighted the efficacy of the PAV3-HA vaccine, demonstrating the rapid generation of better short and long-term protection compared to AdHu5-HA. In addition to assessing protection with respect to H5N1 infection, I believe that the studies also highlight the PAV3 platform as an alternative to AdHu5. PAV3-based vaccines may be promising against other infectious pathogens and could be used to circumvent pre-existing immunity against AdHu5 vectors. However, future studies will need to further characterize PAV3.

Finally, antigen combination was evaluated as an alternative approach to improve vaccine efficacy against H5N1. Combinations of two antigens showed promise but the addition of three or more related and unrelated antigens reduced the efficacy of several vaccine candidates. Careful evaluation of different antigens combinations will be

necessary in order to ensure optimal protection. Further evaluation of the role of antigen competition and interference should be taken into consideration. I also feel that a literature review of older publications may provide valuable insight into possible mechanisms for antigen competition. Older anecdotal evidence and published studies are becoming more readily accessible and current vaccine research can benefit greatly from some of this previous research.

Overall, I think that protection against avian influenza, subtype H5N1, is achievable using experimental DNA and adenovirus-based vaccine platforms. This thesis provides a platform for further development of influenza A vaccines and evaluation of viral pathogenesis and immune responses following infection. The observations from the studies in this thesis can be applied to H5N1, seasonal, and potential pandemic influenza A viruses.

# APPENDIX A

# IMMUNODOMINANT EPITOPES IN BALB/C MICE

## A.1 Introduction

Following expression of antigens by either DNA- or Ad-based vaccines, expressed protein is processed and presented on the surface of APCs on MHC class I or II molecules. Antigenic epitopes are recognized preferentially by different T-cells, giving rise to the immunodominance of certain sequences over others. The identification of T-cell immunodominant epitopes can be useful for evaluating the strength of immune responses following vaccination. A peptide library can be created with overlapping peptides that cover the entire protein. The overlaps allow efficient identification of possible T-cell epitopes by pooling peptides in a matrix organization. The H5N1-05 HA (567aa) and NA (449aa) proteins were divided into 15aa long peptides, with 10aa overlaps.

An ELISPOT-IFNγ assay can provide an efficient way to screen the peptide library and identify T-cell immunodominant epitopes. Once individual 15aa long immunodominant peptides are identified, the 9aa long immunodominant epitope can be determined using the SYFPEITHI Algorithm (Bui et al., 2007; Rammensee et al., 1999; Rammensee et al., 1995) **http://www.syfpeithi.de/**)

## A.2 Results

**Table A.1: HA epitopes**

| Peptide Position | MHC Class I | Peptide Length | Peptide Sequence | SYFPEITHI score |
|---|---|---|---|---|
| 531-539 | H-2K$^d$ | 9 | IYSTVASSL | 30 |
| 206-214 | H-2K$^d$ | 9 | LYQNPTTYI | 27 |
| 212-220 | H-2K$^d$ | 9 | TYISVGTST | 25 |
| 113-121 | H-2K$^d$ | 9 | DYEELKHLL | 24 |
| 159-167 | H-2K$^d$ | 9 | FFRNVVWLI | 20 |
| 554-562 | H-2K$^d$ | 9 | GSLQCRICI | 16 |
| 240-249 | H-2K$^d$ | 9 | RMEFFWTIL | 13 |

**Table A.2: NA epitopes**

| Peptide Position | MHC Class I | Peptide Length | Peptide Sequence | SYFPEITHI score |
|---|---|---|---|---|
| 153-161 | H-2K$^d$ | 9 | RFESVAWSA | 21 |
| 187-195 | H-2K$^d$ | 9 | KYNGIITDT | 19 |
| 387-395 | H-2K$^d$ | 9 | VQHPELTGL | 17 |
| 390-398 | H-2K$^d$ | 9 | PELTGLDCI | 15 |

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