DIGITALCOMMONS
@WAYNESTATE

Wayne State University

Wayne State University Dissertations

1-1-2012

Membrane-Bound Immunomodulators As Adjuvants In A Cell Culture-Based Avian Influenza Vaccine

David Daniel Fischer
Wayne State University,

Follow this and additional works at: http://digitalcommons.wayne.edu/oa_dissertations

Recommended Citation
Fischer, David Daniel, "Membrane-Bound Immunomodulators As Adjuvants In A Cell Culture-Based Avian Influenza Vaccine" (2012). *Wayne State University Dissertations.* Paper 592.

This Open Access Dissertation is brought to you for free and open access by DigitalCommons@WayneState. It has been accepted for inclusion in Wayne State University Dissertations by an authorized administrator of DigitalCommons@WayneState.

MEMBRANE-BOUND IMMUNOMODULATORS AS ADJUVANTS IN A CELL CULTURE-BASED AVIAN INFLUENZA VACCINE

by

DAVID D. FISCHER

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2012

MAJOR: IMMUNOLOGY

MICROBIOLOGY

Approved by:

_____

Advisor Date

_____

_____

_____

© COPYRIGHT BY

DAVID FISCHER

2012

All Rights Reserved

DEDICATION

To my parents Dave and Eileen Fischer.

Thank you for always pushing me to challenge myself even when I protested, encouraging the pursuit of knowledge and always supporting my academic endeavors. I could not have done it without your constant support.

ACKNOWLEDGEMENTS

Throughout the course of my dissertation research I received support from many great people. My advisor Roy Sundick has been a patient and thoughtful mentor and is an excellent teacher. I appreciate his steady support through this long journey. Thank you to my dissertation committee members T.R. Reddy, Harley Tse and P. Michael Long for their insight and guidance from the proposal to the completion of this research. In addition I thank Phil Pellett, Basel Abuaita and Steve Gurczynski for their valuable scientific and technical insight. Finally, special thanks are owed to Radomir Dimivoski. The long and often frustrating hours he spent in the lab assisting and troubleshooting were invaluable and critical to the completion of this project.

TABLE OF CONTENTS

Dedication ii

Acknowledgements iii

List of Figures v

Chapter 1 "General Introduction" 1

Chapter 2 "Vaccine Adjuvant Selection, Design and Production" 39

GMCSF 41

IL2 47

C3d 50

IL4 61

Chapter 3 "Vaccination Studies in Chicks" 66

Materials and Methods 66

Results 70

Chapter 4 "General Conclusions" 86

References 91

Abstract 127

Autobiographical Statement 129

LIST OF FIGURES

Figure 1: Membrane-bound immunomodulator design and development....35

Figure 2: Production of virions bearing membrane-bound immunomodulators 37

Figure 3: GMCSF bioassay....46

Figure 4: IL2 bioassay 51

Figure 5: p29x6 expression on MDCK cells....59

Figure 6: ELISA for p29x6 on H6N2 virions 60

Figure 7: IL4 bioassay 64

Figure 8: Seroconversion levels following vaccination and viral challenge 71

Figure 9: Average geometric mean titers following vaccination....74

Figure 10: Seroconversion following vaccination 75

Figure 11: Virus neutralization following vaccination 76

Figure 12: Average geometric mean titers following booster 77

Figure 13: Seroconversion following booster....78

Figure 14: Average geometric mean titers following challenge 80

Figure 15: Seroconversion following challenge 81

Figure 16: Average HAI titers of chicks testing positive or negative for viral presence....82

Figure 17: RT-PCR of swab samples....83

Figure 18: Weight of chicks following vaccination and challenge....85

CHAPTER 1

General Introduction

Avian Influenza: Avian influenza (AI) viruses are enveloped, segmented, negative-sense RNA viruses belonging to the family *Orthomyxoviridae* genus *influenzavirus* A (1, 2). AI viruses have been isolated from over 100 species covering 12 orders of birds and naturally circulate in their primary natural reservoirs, wetland and aquatic birds, the *Anseriformes* (ducks, geese, and swans) and *Charadriiformes* (gulls, terns, and shorebirds) (1, 3-6). *Anseriformes* and *Charadriiformes* are considered to be the primary natural reservoirs because AI viruses are reliably isolated from particular species in these orders of birds (7). Influenza A viruses are classified by their two major antigenic determinants, the transmembrane glycoproteins hemagglutinin (HA) and neuraminidase (NA) (1). With the exception of the recently isolated H17 from A/bat/Guatemala/09 all HA subtypes (H1-H16), all 9 subtypes of NA (N1-N9) and 105 of the theoretical HA/NA virus combinations have been identified in *Anseriformes* and *Charadriiformes* (5, 8-11).

Wild waterfowl live with AI viruses in evolutionary stasis with carriers typically remaining asymptomatic, indicating an ancient association and an optimal level of adaptation (12-14). The vast majority of AI viruses are considered to be low pathogenic AI (LPAI) or mildly pathogenic AI (MPAI) viruses which produce either subclinical infections or mild respiratory and/or reproductive diseases (3, 15). LPAI viruses replicate in the intestinal and respiratory tracts, spread by the fecal-oral route and are shed at high concentrations through feces (up to $10^{8.7}$ 50% egg-infectious dose (EID_{50}) per gram feces) and at a lower level by the oropharyngeal route (3, 16, 17). Shedding of the virus in feces contaminates water and the environment perpetuating the infection cycle (12). Many AI viruses are stable and remain

infectious in water for periods of up to several months, particularly under favorable conditions of fresh to brackish salinity (0–20,000 parts per million (ppm), temperatures below 17C and a pH range of 7.4-8.2 (18).

Adaptation to Poultry: *Galliformes* (poultry including: turkeys, grouse, chickens, quails, and pheasants) can be infected with AI virus subtypes H5, H6, H7 and H9 by direct contact with wild birds or infected poultry and by indirect contact through fomites, vehicles or contaminated infectious organic material (19). AI viruses transferred to *Galliformes* are often ill-suited to replicate, transmit and cause disease in their new hosts unless multiple adaptation steps take place [6]. Because of the lack of adaptation, transient subclinical infections and the inability to spread between birds and flocks, most of these viruses are unable to persist and infection is only detected via seroconversion (6, 20, 21). Due in part to the diversity of circulating AI viruses, some are better able to establish themselves in the new host allowing for replication and transmission (22). After transfer to the new host, influenza can evolve rapidly leading to an increased possibility of clinical disease and pathogenesis and in rare cases the development of highly pathogenic AI (HPAI) (6, 14, 23).

Changes in AI viruses most indicative of adaptation to *Galliformes* occur in the major surface proteins HA and NA. Changes at the cleavage site of HA, particularly in subtypes H5 and H7, are thought to lead to adaptation and also appear to result in increased virulence (6). Cleavage of the HA0 protein into HA1 and HA2 subunits is necessary for viral infectivity (24). In LPAI viruses HA0 is usually cleaved by trypsin-like proteases which are restricted to the gastrointestinal and respiratory tracts (25). The addition of basic amino acids at the cleavage site by mutation or insertion allows a wider range of proteases to cleave HA0 including putative ubiquitous proteases such as furin (26). Ubiquitous proteases can be located in the endoplasmic

reticulum and are able to cleave HA0 during the translation process resulting in the production of virions that are infectious before they leave the cell, increasing the range of cells that the virus can infect leading to mucosal and systemic replication (6). Systemic replication of the virus leads to infection and damage to major organs and tissues, leading to severe disease and death in birds (27). These viruses are considered to show a highly pathogenic (HP) phenotype (28).

A second major adaptation that takes place upon adaptation to *Galliformes* is the appearance of stalk deletions in the NA gene (6, 29). The stalk of NA located between the trans-membrane and globular head domain and serves to keep the enzymatically active globular head away from the cell membrane or viral surface (6). The domain is usually hydrophilic and around 30 amino acids in length and although stalk length is conserved in single subtypes in wild birds, length can vary between subtypes (6). When AI viruses replicate in poultry, deletion of approximately 12-20 amino acids occurs (6). The enzymatic activity of NA removes sialic acids from viral and host proteins which is needed for efficient release of virus from host cells by preventing virions from sticking to each other (30). While shortening of the stalk does not mutate or disrupt NA's enzymatically active site, it hampers NA activity by decreasing the reach and flexibility of the active site (31). While it seems contradictory that positive selection would choose for a less active protein, it appears changes in HA and NA offset (32). Stalk deletions in NA are typically seen when HA genes have increased numbers of N-glycosolations that reduce receptor affinity for sialic acids in turn making the virus less sticky and reducing need for efficient sialic acid cleavage (6, 32). The less effective NA gene can actually lead to improved viral spread when a lower affinity HA is present (29, 32). It is not known at this time whether the HA or NA mutation occurs first or the exact advantages these changes confer on the virus (33).

The adaptation of a wild bird AI virus to a poultry-adapted AI virus is a complex and frequently unclear event. However, rearing and sales practices in developing nations and Southeast Asia appear to enable this process. (6). Many small-scale backyard or village farmers raise multiple species in close contact, particularly ducks and *Galliformes* and public markets sell live birds under crowded and at times unsanitary conditions (6). Evidence suggests that domestic ducks are more susceptible to wild duck AI viruses than *Galliformes* and duck rearing often allows access to open bodies of water where wild and domestic ducks can interact (6, 34). This leads to a potential chain for wild ducks to pass AI viruses to domestic ducks which in turn spread virus to *Galliformes*. Japanese Quail and turkeys also appear to serve as bridge species in certain geographical areas (6).

Highly Pathogenic Avian Influenza: In contrast to LPAI viruses, which are confined to the intestinal and respiratory organs and shed through the feces, HPAI viruses replicate systemically and are easily shed by nasal and oral routes (35, 36). HPAI is an extremely infectious, highly virulent disease primarily impacting poultry, that can lead to mortality rates of up to 100% in the 48 hours following infection (15). These viruses have emerged following the adaptation and mutation of H5 and H7 subtypes of LPAI viruses after transmission from wild birds to poultry described above (36). While all HPAI strains isolated to date have been from H5 or H7 subtypes, not all H5 and H7 subtypes are highly pathogenic (25). According to the 2009 OIE Terrestrial Manual an AI virus is classified as HPAI when it meets any one of the following criteria in chickens (37):

a) Changes to the HA proteolytic cleavage site of H5 or H7 including: 1) substitutions of the basic amino acids arginine and lysine; 2) insertion of duplications of multiple basic amino acids from codons duplicated from the haemagglutinin cleavage site; 3) short

inserts of basic and non-basic amino acids from unknown sources; 4) lengthening of the proteolytic cleavage site by recombination with other gene segments; and 5) loss of a shielding glycosylation site in combination with basic amino acid substitutions.

b) Is lethal for 75% or more of eight 4- to 8-week-old susceptible chickens within 10 days following inoculation with a standardized dose under laboratory settings.

c) Any virus that has an intravenous pathogenicity index (IVPI) greater than 1.2.

Symptoms of HPAI range from "sudden death with little or no overt clinical signs to disease with clinical presentations including respiratory signs, such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, apathy, reduced vocalization, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination and nervous signs and diarrhea (37)."

Since 1959, 29 epizootics of HPAI have occurred (epizootics are outbreaks of disease affecting many animals of one kind at the same time, analogous to the term epidemic applied to the human population) (38). The largest of these outbreaks is the ongoing panzootic of HPAI H5N1 that first appeared in domestic geese in China in 1996 as A/goose/Guangdong/1/96 (also known as Asian lineage H5N1 and will be referred to as H5N1 hereafter) (38, 39). Prior to this panzootic, HPAI viruses have typically arisen transiently in areas populated with *Galliformes* (36, 40). The majority of these flare-ups were eradicated by mass culling alone and the remainder by a combination of mass culling and vaccination (41). These outbreaks remained limited due to large scale die-offs caused by the high mortality rate of HPAI and the, at the time, inapparent ability of HPAI viruses to persist and spread in wild bird populations (40). Even during the first 5 years of the current H5N1 outbreak, minimal evidence for the presence of the virus in wild birds was found despite endemic infections in *Galliformes* and domestic ducks and

geese (6, 36). Since 2002, the paradigm has shifted. In that year, aquatic and terrestrial birds became infected with and died from H5N1 in China, Indonesia, Vietnam and Egypt (6, 36). In 2005, 9 years after the emergence of H5N1 in domestic birds, a large outbreak of H5N1 of multiple closely related viruses descended from A/goose/Guangdong/1/96 occurred in migratory birds at Lake Qinghaihu in western China (36, 42). Lake Qinghaihu is an important breeding ground for migratory birds that overwinter in Southeast Asia, Tibet and India and other migrant birds that congregate from Southeast Asia, Siberia, Australia and New Zealand (42). During the winter of 2006, the variant from this outbreak spread rapidly westward across Central Asia, Europe, the Middle East and Africa (43). Since then the 10 phylogenetically and antigenically distinct lineages called clades, plus numerous subclades have emerged (6, 36). These subclades of virus have been maintained in the wild bird population, have become endemic in six countries (Bangladesh, China, Egypt, India, Indonesia and Vietnam) and opportunistically spread back to domestic poultry making containment difficult (44-46). From 1997-2011, more than 7,000 H5N1 outbreaks occurred in 63 countries and from January-March 2012 the WHO confirmed 164 outbreaks in poultry in 10 countries and 18 outbreaks in wild birds in 10 countries (46).

Economic Consequences of HPAI: The mass culling and death of over 400 million birds due to H5N1 has led to over $20 billion in economic damage and continued spread threatens the global poultry markets which in 2007 produced 83.7 million metric tons of meat (8.1 million tons were sold through international trade, 2.1 million metric tons were exported from the United States) (46, 47). Countries with H5N1 outbreaks face reductions in consumer sales at home due to fear and lack of confidence along with increased costs due to culling and restocking flocks, increasing biosecurity, increasing surveillance and introduction of vaccination campaigns (47). These countries also face the loss of their export markets due to international

embargoes and loss of market share due to increased production from uninfected countries (47). Importing countries free of H5N1 may also face increased costs due to reduced competition in the market place (47).

The impact has been especially devastating in Southeast Asia where the majority of H5N1 outbreaks have occurred and where ¼ of the world's poultry is produced (47). In 2003, Thailand was the 5th largest poultry exporter in the world (48). After the emergence of H5N1 in 2003-2004, the export market collapsed, with metric tons of poultry exports dropping from 331,044 to 23,953 and income from exports dropping from 22,685 million baht to 1,738 million baht resulting in an estimated reduction of 1.5% of GDP growth (48, 49). Export levels have not recovered.

Thailand, Vietnam and most other Southeast Asian nations have large numbers of small scale poultry producers and as much as one third to one half of the population may depend on poultry production for income (47). While the absolute value of the financial losses in this sector may not be as large as those in the commercial sector, these producers were most impacted relative to assets and income (48). A 2004 study in Vietnam found that in a typical village impacted by an H5N1 outbreak, backyard producers lost on average 2.3 months of production and those loses compounded with loss of poultry for family consumption cost each household $69-108 (US Dollars), a devastating consequence for areas where income per person averages less than $2 per day (50). Furthermore, with the loss of poultry, the prices of alternative meats such as pork rose adding an unknown amount of additional costs (50).

Transmission to Humans: Avian influenza also has the potential to severely impact human health. Influenza has established an extremely wide host range, from birds to various mammalian species including humans, pigs, horses and dogs (51). Evidence indicates that all of

these mammalian influenza viruses diverged from avian ancestors (3). Although AI viruses are normally species specific and infrequently cross species barriers, direct transmission of AI viruses to mammals including pigs, horses, seals, whales, minks and humans has been recorded (13, 52). The specificity and affinity of an influenza virus HA for its sialic acid receptor is a critical factor in host tropism and transmissibility (51). HA binds to host glycans and gangliosides that contain terminal sialic acids (36). Sialic acids bind to cell-membrane sugars through α2,3, α2,6, α2,8 or α2,9 linkages (36, 53). The most common terminal forms of sialic acids are N-acetylneuraminic acid-α2,3-galactose (α2,3-SA) and N-acetylneuraminic acid-α2,6-galactose (α2,6-SA)) and each is expressed in a tissue- and species-specific manner determined by sialyltransferases (31, 36, 53). AI viruses preferentially bind to α2,3-SA which are highly expressed in the avian respiratory and intestinal tract while human influenza viruses preferentially bind α2,6-SA which are highly expressed by human upper-airway epithelia leading to an interspecies barrier (36, 54, 55). It should be noted that humans express α2,3-SA in the lower-respiratory tract on type II pneumocytes, alveolar macrophages, and nonciliated cuboidal epithelial cells in terminal bronchioles. However the relative inaccessibility of this site limits the establishment of infection and limits transmission through sneezing and coughing (56, 57). Swine tracheal epithelia cells express both α2,3-SA and α2,6-SA causing them to be susceptible to both human and avian influenza viruses (36, 58).

An influenza pandemic occurs when a virus with an antigenically unique HA enters the population (59). Pandemics are of zoonotic nature and arise through reassortment of human influenza viruses and those other species or by the direct transmission of a virus from animals to humans (36, 51, 60). Reassortment can occur when two distinct influenza A viruses simultaneous infect a single cell (51). The segmented nature of the influenza genome allows for

the mixing of genes and the packaging and production of a novel strain (51). It is thought that most human pandemic viruses were generated in this manner, including the 1957 H2N2 "Asian flu" (avian/human), the 1968 H3N2 "Hong Kong flu" (avian/human) and the 2009 swine origin H1N1 (avian/swine/human) (35, 36). The reassortment that led to the 2009 pandemic (and possibly the events resulting in the 1957 and 1968 pandemics) occurred in swine which are well suited to serve as a mixing bowl due to their susceptibility to human and avian influenza viruses (36, 61, 62).

The 1918 H1N1 "Spanish flu" pandemic appears to have resulted from the direct adaptation of an unusual avian precursor (35, 63, 64). This pandemic is the most devastating on record, causing clinically apparent illness in an estimated 500 million people (one third of the world's population) with a case fatality rate of >2.5% resulting in 50-100 million deaths (65). In 2005 the US Congressional Budget Office released a report documenting the potential number of infections and fatalities and economic consequences that could be expected if a severe pandemic of the magnitude of the 1918 pandemic or a milder pandemic mimicking the 1957 or 1958 outbreaks occurred today (66). Barring the emergence of an influenza virus with morbidity and mortality rates heretofore unseen, the 1918 projections likely represent a worst case scenario due to significant medical advances in health care including the development of antibiotics to treat secondary pneumonia infections, vaccines, antivirals and respirator technology. Modeling a severe pandemic using an attack rate of 30% and a fatality rate of 2.5% they estimated that in the US alone 90 million people would become clinically ill, 18-45 million would require outpatient care and 2 million would die. 30% of all workers would become ill, 2.5% would die and 30% of all workers would miss an average of 3 weeks due to illness or caring for family members. This could cause the GDP to drop as much as 5% and cost upwards of $675 billion. Using an attack

rate of 25% and a fatality rate of 0.1%, about 75 million would become infected and cause 100,000 deaths (3,000-49,000 typically die from flu related complications due to seasonal influenza). 25% of worked would become ill and miss an average of 4 days. The GDP would decline by 0.5-1% and cost about $70 billion.

Direct transmission of H5N1 (A/Hong/Kong/156/97) to humans was reported in 1997 (67-69). That year, 18 people became infected and 6 died after displaying clinical features including fever and upper-respiratory-tract infections typical of influenza infection along with severe complications, mainly pneumonia, gastrointestinal manifestations, elevated liver enzymes and renal failure (35, 70). The ability of an avian virus to directly infect humans and the high mortality rate sparked fears that a severe pandemic along the lines of 1918 could be on the horizon (67). Since the reemergence of H5N1 in 2003, the WHO has confirmed 608 human cases of H5N1 in 15 countries resulting in 359 fatalities (71). From January-September 2012, 30 WHO confirmed cases occurring in Bangladesh, Cambodia, China, Egypt, Indonesia and Vietnam resulted in 19 deaths (71).

The WHO considers an influenza virus to be a pandemic when the following conditions are met: 1) A new influenza A virus that infects humans; 2) The population at large has little or no immunity to the virus; 3) The virus causes serious illness with high morbidity and mortality and 4) transmits easily from person to person (72). To date H5N1 has not met the fourth criteria of sustained human-to-human transmission. While a handful of cases of H5N1 have occurred from caring for or close contact with an infected individual, these make up less than 1% of confirmed cases with the remaining 99% occurring as a result of direct transmission from infected birds (73-76). The H5 of H5N1 like other AIV HA preferentially binds to α2,3-SA as described above. The molecular changes in HA necessary to confer aerosol and mammalian or

person-to-person transmission are poorly understood. Recently, Imai et al. and Herfst et al. reported the generation of laboratory mutant H5 strains that were able to spread via aerosol between ferrets (Ferrets are widely regarded as the best mammalian laboratory small animal model for the study of influenza, however it should be noted that like all animal models they are imperfect representation of humans and influenza viruses may behave differently in the general population) (73, 77). Imai et al. created a reassortant virus containing the H5 HA from HPAI H5N1 A/Vietnam/1203/2004 and the remaining 7 genes from a 2009 pandemic H1N1 isolate. After introducing random mutations in the H5 they found 2 mutations in the receptor-binding globular head domain known to increase affinity for α2,6-SA, a third that disrupts a N-linked glycosylation site and a fourth in the stalk domain. When this reassortant virus was introduced into ferrets, it replicated efficiently, caused lung lesions and weight loss and could be transmitted in an airborne manner. The virus was neither highly pathogenic nor lethal. Their results show that H5 can convert to a form that supports transmission in mammals, but it is unknown if an entirely avian H5N1 virus would be transmissible and if inclusion of the remaining 7 H5N1 HPAI genes (particularly NS1 and PB1) would confer high pathogenicity or lethality.

Herfst et al. modified the H5 of the HPAI H5N1 A/Indonesia/5/2005 by site-directed mutagenesis and serial passage in ferrets. Unlike Imai et al. the studies in ferrets used a wholly avian virus. After multiple passages the modified H5N1 virus accumulated mutations and was able to be transmitted by aerosol or respiratory droplets and cause disease. 5 consistent amino acid substitutions were found in the transmissible viruses, 4 in HA and one in the polymerase complex protein PB2. In HA 2 mutations were receptor-binding substitutions known to increase α2,6-SA affinity, the third was the same mutation found by Imai et al. at the N-linked

glycosylation site and the fourth was at the HA trimer-interface. The PB2 mutation found is a common mammalian polymerase adaptation.

The results from these two studies indicate that H5N1 may be as few as five amino acid substitutions from being able to spread efficiently in humans. Surveillance data shows that two of these mutations have commonly been found in H5N1 isolates indicating that some circulating viruses may be as few as three substitutions away from becoming transmissible by an aerosol route (78). This strongly indicates that H5N1 surveillance, prevention and control should remain a key focus.

Control of HPAI: Controlling HPAI is critical to the health of animals and humans, safeguarding the food supply and protecting the economic livelihood of many individuals. While mass culling to stamp out HPAI has been used extensively, the multi-continent spread of the disease, maintenance in natural reservoirs, economic costs to producers and regional reliance on small scale producers requires a more comprehensive strategy (79, 80). Furthermore, the OIE and the United Nation's Food and Agricultural Organization (FAO) have taken the position that, "For ethical, ecological and economic reasons, it is no longer acceptable to control and eradicate disease outbreaks mainly by applying a policy of mass slaughter" and that "Vaccines help to improve animal health, public health, animal welfare, and agricultural sustainability, thus protecting the environment, maintaining biodiversity, and protecting consumers of animal products," (79, 81).

Swayne and Suarez of the United States Department of Agriculture (USDA) have described a comprehensive approach for an effective HPAI control or eradication program (15). First, national surveillance and diagnostic programs should be instituted. Second, enhanced biosecurity measures should be practiced at all levels of poultry production to prevent

introduction of HPAI. These measures include eliminating direct contact between poultry and wild birds by physical separation, the use of covered pens to prevent contamination from fecal matter dropped by migrating birds during flyovers, providing clean water sources and not exposing birds to contaminated meat, offal or fomites (6). Third, poultry farmers should be educated to recognize the signs and symptoms of infection and to share information on surveillance and control strategies. Fourth, all AI-infection should be quarantined or moved only in a highly controlled manner. Fifth, stamping-out may be needed in some flocks. Sixth, is the establishment of vaccination programs.

AI vaccines increase host resistance to disease by the generation of neutralizing antibodies and reduce virus shedding curbing spread (79). Preventative vaccination, emergency vaccination or prophylactic vaccination strategies can be appropriate depending on the presence or potential presence of HPAI in a region (79, 82). Preventative vaccines are an option when a region is free from disease but at high risk of introduction (79, 83, 84). This route has been taken in France, the Netherlands and Switzerland where certain difficult to contain birds and zoo birds were vaccinated due to the risk of migratory birds carrying H5N1 passing through (79). The costs of this strategy may be prohibitive in developing nations that do not have a strong veterinary infrastructure (79). Emergency vaccination is conducted during an active outbreak where healthy animals within a certain range of the outbreak are immunized (79). The area of the vaccination zone is determined by transmission rate and spread (79). Prophylactic vaccination is used when HPAI has become endemic in a region (79). Birds are vaccinated in a coordinated and systemic manner against the circulating HA subtype (79). This strategy should involve all levels of poultry producers to be effective and likely needs to be done in conjunction

with the eradication of certain flocks (79). This can be a costly strategy but is necessary when large portions of the poultry production industry are threatened.

Immune Response to Influenza: Development of effective influenza vaccines requires an understanding of the protective immune response generated by infection. Despite the obvious physiologic and structural differences present in the avian and mammalian immune systems, the functional characteristics of these systems are well conserved particularly in peripheral organs, lymphoid cell functions, division, classes, interactions, specificity, and net effect (41, 85-88). The avian immune system generates innate responses and humoral and cell-mediated adaptive responses. Avian T cells homologous to CD3+, CD4+ Th1 and Th2 helper cells and CD8+ CTLs have been identified (41). Chickens have a single gene equivalent to IgG known as IgY (IgY exists as a single subtype unlike mammalian IgG which is present in multiple forms) along with IgM and IgA equivalents (41, 89, 90). Since the immunological response to influenza is far more extensively characterized in mammals, the details of influenza immunity will be described through the human response to infection and appropriate parallels in the avian response will be drawn when available.

Natural infection with influenza can result in decades-long immunity to the original infection virus or an antigenically similar strain (91, 92). Most virus encountered is removed nonspecifically by the mucin layer, cilia and protease inhibitors preventing attachment and uncoating at the respiratory epithelium (60, 93). Influenza virus that escapes these basic defense mechanisms encounters other facets of the innate immune system. During an infection, the innate immune system recognizes pathogens as foreign bodies through microbial-associated molecular patterns (MAMPs) characteristic of infectious agents through pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and generates an appropriate response (94-

96). When influenza infects and replicates in cells, the viral RNA produced activates the RIG-I/IPS-1 pathway (97). When plasmacytoid dendritic cells endocytose virus and incorporate them into their lysosomes, the freed viral RNA is recognized by the TLR7/MyD88 pathway which induces type 1 IFN production (97).

Both the mucosal and systemic arms of the humoral immune system play roles in resistance to influenza infection with secretory IgA (sIgA) involved in protection of the upper respiratory tract and serum IgG in the lower respiratory tract (91). Over the course of an infection in humans, antibodies to all major viral proteins are produced with those specific for HA and NA being the most critical (91, 98, 99). At high enough concentrations in the respiratory tract, strain specific anti-HA antibodies completely neutralize influenza virus infection while anti-NA antibodies slow the spread of infection by hampering viral release from infected cells (100). While antibodies to the well conserved M and NP antigens are generated, they are not protective (100, 101).

Mucosal immunity is important in combating influenza infection as mucosal tissues are the port of entry for the virus. sIgA, and to a lesser extent IgM, are the major neutralizing antibodies towards mucosal pathogens and can prevent entry and act intracellularly to impede viral replication (91, 102). During primary infection, locally produced HA and NA-neutralizing sIgA are present in nasal secretions and IgM, IgG and IgA can be detected in nasal washes with IgM and IgA being most prevalent (91, 103-106). Furthermore local influenza IgA response usually lasts 3-5 months, memory committed IgA secreting B cells can be detected and IgA is the primary Ig subtype detected in secretions after secondary infections (91, 106-108).

Anti-HA antibody levels in serum are the most widely used measure of protection against influenza infection and a hemagglutination inhibition titer of 40 is typically considered protective

(109). This antibody titer is achieved in about 80% of human subjects following infection (91). In humans influenza IgM, IgG and IgA are present in infected individuals and serum levels of anti-HA and anti-NA antibodies correlated with resistance to and recovery from infection (91, 103, 110-113). Within two weeks of primary infection IgM, IgG and IgA can be detected with IgG and IgM being the most abundant. IgA and IgM levels peak and decline at the two week mark while IgG does so at approximately 4-6 weeks. During secondary responses the dominant isotypes are IgG and IgA (91, 106, 114).

While cell-mediated immunity does not play a significant role in sterilizing influenza immunity it has a crucial role in clearance of virus, recovery from infection and the reduction of influenza complications such as lethal secondary infections (91, 115). Influenza-specific CD8+ cytotoxic T lymphocytes (CTLs) are found in the blood and lower respiratory tract of infected individuals and work in concert with influenza specific antibodies and complement to lyse influenza infected cells (91, 116-119). CTL response appears 6-14 days after infection and subsides by day 21 post-infection (91, 120). The majority of CTLs target epitopes on the nucleoprotein (NP) and the acid polymerase (PA) gene, a much lower proportion target basic polymerase subunit 1 (PB1), non-structural protein 2 (NS2) and matrix protein 1 (M1) and a very rare number appear to target HA (115, 121). Additionally, certain CTLs can cross-react between influenza A subtypes (91). Memory CTLs are cross-reactive, appear about 2 days earlier in secondary infection than their primary counterparts, peak at 14 days and persist until returning to baseline at 6 months (91, 120, 122). While memory CTL numbers are not proportional to the rate of infection and illness clearance they do correlate to the pace of viral clearance (115, 119). Despite the inability to prevent illness, vaccines that induce memory CTL responses may play key roles in lessening the severity of highly pathogenic influenza infections (115).

CD4+ T cell responses to influenza infection are predominantly Th1 based leading to the secretion of IFN-γ and IL2 (93, 123). IFNγ and IL-2 drive complement-activating IgG2a antibody production by B cells and enhance proliferation of CTLs (93, 124). It is unclear to what extent CD4+ T cells are necessary or responsible for the development of CTL responses during primary infection but it has been demonstrated that they are needed for proper memory CTL response during secondary infection (115, 125-129). Studies inhibiting Th1 responses show that Th2 based responses, leading to the secretion of IL4 and IL5 which induces B cells to produce non-complement activating IgG1, IgA and IgE, results in a much less robust immune response to influenza (93, 124).

Live-Attenuated vs. Inactivated Vaccines: A significant choice in vaccine development is whether to use a live-attenuated or an inactivated vaccine. Live-attenuated vaccines mimic virulent viruses and bacteria and trigger an immune response by the production of an asymptomatic infection (95, 130). In both human and veterinary practice live-attenuated vaccines generally require only one application, induce major antibody production within 10 to 14 days, stimulate cell-mediated immune responses and use a small amount of antigen (131). While live-attenuated vaccines are generally safe, they do produce active infections and can produce slight signs of infection or severe infection when not properly attenuated (131). Risks associated with their use include failure to fully inactivate the pathogen, residual virulence of the attenuated agent, spread to other subjects and establishment of infection in the immunosuppressed (132). Care must be taken to administer live vaccines to only healthy subjects as vaccinating the ill may result in reduced antibody production or cause an exacerbation of the concomitant disease (131). Additionally, antibiotics or antivirals that would clear the vaccine before immunity can be established must be avoided (131).

Often, an attenuated form of a pathogen can be difficult or unfeasible to attain, unstable, have a risk of reverting to a virulent form or cause burdensome side effects (130). An alternative to a live-attenuated vaccine is an inactivated vaccine consisting of either whole inactivated viruses or bacteria or a purified component of the pathogen such as a protein or carbohydrate. Inactivated vaccines are advantageous because they do not cause disease and often have a longer shelf life than live vaccines (131). Inactivated vaccines are designed to be less reactogenic to reduce adverse reactions; however this results in decreased immunogenicity [52]. These antigens do not replicate, are less immunogenic and are often administrated by an unnatural route of exposure such as intramuscular injection and usually lack MAMPs. As a consequence, these antigens often fail to stimulate a protective reaction in the vaccinated subject (95, 130). This leads to drawbacks including the need for an increased doses or a greater amount of antigen in each dose of the vaccine leading to increased production costs, reduction in compliance and more stress to the subject (131). High levels of antibodies are not usually seen until after a booster and the vast majority of antibody produced is IgG while live vaccines lead to the generation of a wider range of subtypes (131).

Currently Available Influenza Vaccines: Each seasonal influenza vaccine contains 15ug each of HA from H1N1, H3N2 and an influenza B strain. Currently approved vaccines for human use are classified as either cold-adapted live attenuated virus vaccines (LAIV) or inactivated vaccines (93, 133). The LAIV licensed as Flumist® in the United States was approved in 2003 for use in healthy individuals between the ages of 2 and 49 (93). This LAIV is administered intranasally, mimics the natural influenza infection process, induces sIgA and IgG antibody production, induces CTL responses and provides cross-protection amongst some variant types of virus (93, 106, 134, 135). Safety of this vaccine has not been established for

pregnant women, the immunocompromised, those with existing medical conditions that increase risk for influenza complications and other highly susceptible groups such as infants and those aged over 65 (136). Therefore the populations at highest risk for severe influenza infections are not approved to receive this vaccine despite its efficacy in healthy individuals. Additionally, LAIVs are not likely to be used in an avian outbreak or in a prepandemic/early pandemic human outbreak due to the small but extremely dangerous possibility that the novel HA included in the LAIV could recombine with a circulating wild-type virus and create a fully virulent virus containing the HA from the vaccine strain (133).

Parenterally administered inactivated influenza vaccines include the split-product, subunit and whole-virion vaccines. These are the most commonly used influenza vaccines and have been approved for use in individuals ages 6 months or older. When inactivated influenza vaccines are used, innate responses are weakly or not at all stimulated, especially in the case of subunit vaccines (137). When a whole-virus inactivated vaccine is used the TLR7/MyD88 pathway is activated; however, without active production of viral RNA the RIG-I/IPS-1 pathway is not (137). Studies in mice show subunit vaccines lack efficacy in naïve animals that have never been infected with influenza suggesting that subunit vaccines may not be able to provide necessary protection levels in the case of a pandemic when the entire population is naïve to the new influenza strain (137). While whole-virion vaccines are more immunogenic, split and subunit vaccines are more commonly used due to a lower incidence of injection site reactions (91). Inactivated influenza vaccines induce HA-specific serum IgG antibodies and are 60-100% effective in the prevention of morbidity and mortality from homologous viruses in adults, however they offer little to no protection against variant strains (91, 93, 106, 113, 134, 138, 139).

However, inactivated vaccines have reduced efficacy in young immune-naïve populations and are only 30-50% effective in preventing morbidity and mortality in those over 65 (91, 140-142)

As described above, T helper responses to natural infection with influenza are primarily Th1 based. As the LAIVs mimic natural influenza infections they too stimulate Th1 responses (124). On the other hand inactivated split or subunit vaccines generate a predominantly Th2 response with low IgG2a:IgG1 ratios (124, 143). In general inactivated whole-virus vaccine are more immunogenic than split influenza virus vaccines and induce a primarily IgG2a response indicative of a Th1 response in mice (143). Technologies such as the use of adjuvants (described below) must be developed to activate the innate and adaptive immune system and drive Th1 immunity in inactivated influenza vaccines.

As in humans and other mammals, protective immunity to influenza induced by vaccination is primarily antibody mediated in avian species. Neutralizing anti-HA IgY blocks viral attachment and prevents infection and partial protection is generated by anti-NA antibodies (41). Measurable immune responses are generated to nucleoprotein, polymerase and matrix protein, but again do not lead to protective immunity (41, 144, 145). While not extensively examined, limited studies indicate cellular immunity can limit the severity and duration of disease following HPAI virus infection in chickens and turkeys (41).

Almost all (95.5%) AI vaccines manufactured for use in poultry are inactivated whole-virus vaccines administered subcutaneously in the neck or intramuscularly in the thigh (38, 41). To boost immunogenicity, these vaccines are adjuvanted in water-in-oil or water-in-oil-in-water emulsions (41). Vaccines have been used to control LPAI since the 1970s and in recent years have been used to fight LPAI strains of H5 and H7 in the United States, Italy, Mexico, Guatemala and El Salvador (38). From 2002-2012 113 billion doses of AIV vaccines were used

against HPAI (38). Ninety-nine percent of these vaccines were used in China, Egypt, Indonesia and Vietnam against H5N1 and consistently prevented clinical disease and mortality in poultry allowing for the maintenance of livelihood and security standards during disease outbreaks (38). However, a troubling development has been seen in Egypt where agricultural authorities have used vaccination against endemic H5N1 without success (146). Even three doses of inactivated oil-whole-virus emulsion vaccines against the circulating H5N1 clade failed to provide the proper level of protection against the virus (146). Evidence shows that maternal antibodies transferred through the yolk sac neutralize the vaccine before it can stimulate a protective immune response (146). Delaying vaccination until after maternal antibodies disappear runs the risk of exposure to the endemic circulating virus. In these cases, vaccines that effectively stimulate antigen processing and presentation may need to be considered.

The remainder of AI vaccines are recombinant H5 expressing fowlpox or Newcastle disease virus-vectored vaccines (41). Live AIV vaccines are not recommended for use due to the aforementioned potential to mutate into an HPAI and for the need to eradicate LPAI viruses from poultry populations prior to vaccination (41).

Mucosal vaccination by oral or intranasal inoculation using inactivated influenza vaccines would be ideal for both human and poultry populations. In addition to inducing antigen-specific systemic and mucosal immunity and the induction of secretory IgA, mucosal vaccination would increase compliance in needle-phobic humans, allow for easier administration in poultry and decrease the risk of disease spread due to contaminated syringes (147, 148). Unfortunately most soluble antigens are not efficiently taken up when administered by mucosal routes and can induce immune tolerance (147, 149). As of this time oral or intranasal inactivated influenza vaccines are not commercially available.

Vaccine Production: Annually, 250-300 million doses of trivalent influenza vaccines for humans are produced in embryonated hen's eggs through a collaborative process involving WHO Collaborating Centers and vaccine manufacturers (150). Year-round, sentinel physicians collect nasopharyngeal swabs from patients displaying influenza illness and send the samples to National Influenza Centers for isolation and identification of subtype (150). When a new strain is found, it is sent to a WHO Collaborating Center for Influenza Reference and Research. As influenza viruses primarily circulate in winter months, WHO Collaborating Centers review circulating strains for the Northern hemisphere in February and the Southern hemisphere in September (150). After analysis of epidemiological data, the WHO selects variants to be incorporated into the next season's vaccine formulation beginning a 6 month-long manufacturing process. First the new virus strains must be adapted to reduce virulence and maximize growth. This has classically been accomplished by preparing genetic reassortments using the field strain and H1N1 A/PR8/34 (PR8) or PR8-like master strain that grows to high titers in embryonated eggs (150). High-growth hybrids containing the HA and NA from the field strain and internal components from the master strain are isolated and used in the vaccine strain (151). In recent years this step has been aided by the use of reverse genetics (152). After vaccine strains are identified, potency reagent preparation occurs. This step involves the generation of antibodies and reagents by the WHO Collaborating Center for distribution to manufacturers to ensure accurate measurement of vaccine production and dosage (151).

After the vaccine strain is distributed, manufacturers optimize growth conditions in eggs and begin bulk manufacture. Vaccine virus is injected into 9-12 day old specific pathogen free (SPF) fertilized hen's eggs and incubated for 2-3 day. The egg white is harvested and virus is separated, killed chemically (typically with formalin) and concentrated. To produce a split or

subunit vaccine, the purified particles are treated with the detergent Triton and HA and NA are isolated and further purified. The vaccines are tested for sterility and amount of antigen (15ug HA/strain and a detectable amount of NA) then for safety in animals (153). Some countries, including those in Western Europe participating in European Medicines Evaluation Agency (EMEA) require each new influenza vaccine formulation to be evaluated in a clinical study (154).

Veterinary vaccine manufacturers produce a number of inactivated avian influenza vaccines for use in poultry. Vaccines are available for clades of subtypes H5N2, H7N3, H5N9, and H5N1 (155). Additionally reverse-genetics based vaccines are produced for H5N1 with altered H5 cleavage sites to reduce pathogenicity in eggs (155). The manufacturing process is similar to influenza vaccines produced for humans with the exception of an allowance for the use of specific antibody negative eggs and the use of non-purified allantoic fluid containing whole virions to reduce production costs (41, 156). According to OIE guidelines, licensed avian vaccines for high pathogenicity strains should be tested in a minimum of 24 SPF chickens per group with a challenge virus dose that causes 90% or more mortality in non-vaccinated birds (typically 10^6 chicken embryo infectious doses (EID)) (156). For low pathogenicity strains where mortality is not typically a complication from infections, a statistically significant reduction in shedding titer and/or number of birds shedding virus from the oropharynx or cloaca should be seen in the vaccinated groups (156). While HA doses as low as 0.4ug have been shown to protect chickens from challenge, OIE and USDA researchers and the OIE recommend that minimum antigen per dose be 50 50% protective doses (PD_{50}) or 3ug hemagglutinin as the best protection was achieved with 3-8ug of antigen (41, 156-158). Hemagglutination inhibition

titers in vaccinated birds should be at least 1:32 to protect from mortality and 1:128 to result in a reduction in challenge virus replication and shedding (156, 157).

The egg-based influenza vaccine production system has proven to be a reliable way to produce vaccines since the 1950s. However, drawbacks exist particularly pertaining to pandemic preparedness. Viral growth in eggs can be unpredictable and optimization steps lead to delays in production. Even in the best-case scenarios, the egg-based production system takes a minimum of 28 weeks from isolation of a new pandemic strain until release of the vaccine (153). During the 2009 swine H1N1 pandemic, the United States Department of Health and Human Services set a 6-month goal for the production and delivery for the pandemic vaccine. While this goal was met, production was slowed due to a portion of the influenza vaccine production capacity already being in use for the 2009-2010 seasonal influenza vaccine and the pandemic vaccine was not available until after the pandemic peaked (159).

Until recent years, the egg supply was set up only to support seasonal vaccine production leaving periods of 3-4 months where eggs were not available (153). To address this concern, the US government has awarded contracts to manufacturers to maintain a year-round production egg supply (153). Even with a more consistent egg supply, the current 300 million dose vaccine production capacity requires approximately 900 million eggs which must be housed under SPF conditions (153, 160). In a pandemic situation, the best case scenarios where the entire world's vaccine production capacity could be used, the virus grows to high titers in eggs and a single 15ug HA dose achieves efficacy, 900 million-3 billion doses could be produced, enough to protect roughly 13-43% of the world's population (160, 161). Furthermore, avian influenza is infectious to embryonated eggs and egg laying flocks. In the case of a severe avian influenza

pandemic, large scale die-offs of birds due to infection or mass culling could severely strain capacity.

Cell Culture Vaccines: Since the advent of the Salk's killed poliovirus vaccine, most viral vaccines have been produced in cell culture systems (162). To date, there are licensed vaccines for at least 14 different viral diseases (162). The most frequently used cell lines for development and production are: Vero (African green monkey cell line), WI-38 and MRC 5 (secondary human lung fibroblasts), Madin-Darby canine kidney (MDCK), chick embryo fibroblast cells (CEF), PerC6 (immortalized human cell line) and primary monkey kidney (PMK) cells (162). In the USA vaccines produced in the following cell lines have been approved: CEF (measles, mumps, rabies, tick-borne encephalitis), Vero (Japanese encephalitis, poliovirus, vaccinia, rotavirus), WI-38 (rubella), MRC5 (hepatitis A, rabies, herpes zoster) (162). For influenza vaccines the European Union has approved a subunit vaccine produced in MDCK cells and Austria and the Czech Republic have approved an inactivated, split influenza vaccine produced in Vero cells (162).

The cell culture-based system offers several advantages. Established cell lines are fully characterized and come from uniform cell banks ensuring that all are identical unlike the differences between individual embryonated eggs (163). These cell lines use well established protocols and can be grown in a controllable, standardized manner. The production process uses a closed fermenter system which limits the risk of contamination and can eliminate the need for the addition of antibiotics (163). Closed production systems are advantageous in a pandemic context because they are better suited and more easily adaptable to meet the higher biosafety levels needed when using unmodified pandemic strains especially in comparison to the open infection and harvesting process for the egg-based system (163). Additionally, reagents for cell

culture can be stocked and stored months in advance and frozen cell lines are available for immediate use enabling vaccine production at any time in contrast to eggs which requires months of preparation and exact timing of chicken flocks (163). Furthermore, production capacity using suspension cell cultures are limited only by the number and volume of bioreactors and can be scaled accordingly.

Vaccine manufacturers in the US, Japan and Europe have employed the following cell lines for development and manufacture of influenza vaccines: adherent Vero, adherent and suspended MDCK, PerC6, EBx (chicken embryonic stem cell) and EB66 (duck embryonic stem cell) in suspension (163). In response to a 2006 HHS request for proposals, 6 vaccine manufacturers requested and were awarded contacts from the US government totaling more than $1 billion to develop seasonal and pandemic cell-based influenza vaccine platforms (160, 162). 4 of these contracts are still active and 3 systems have reached the clinical trial phase. Inactivated seasonal vaccines produced by Baxter in Vero cells and by Novartis in suspension MDCK cells have completed Phase III clinical trials and applications for licensure are expected to be completed in the near future (162). Additionally, Baxter and Novartis H5N1 pandemic vaccines have completed a Phase I clinical trial and a Phase I trial for Baxter's H9N2 pandemic vaccine has been initiated (162). Currently an inactivated GlaxoSmithKline seasonal vaccine produced in EB66 suspension cells has completed Phase I testing and an H5N1 pandemic vaccine trial is underway and MedImmune is in the preclinical testing stages for a seasonal live-attenuated vaccine produced in adherent MDCK cells (162).

MDCK cells were first isolated and established as a cell line in 1958 and are one of the most investigated, characterized and utilized epithelial cell lines ever. Even after 30 years of in vitro culture, MDCK cells have retained functional characteristics of renal epithelium including

collecting ducts (164). Influenza viruses replicate to high titers in MDCK cells which are permissive for all current human, avian, porcine and equine vaccine strains (163). MDCK have long been the laboratory standard for isolating and replicating influenza virus and are used by the majority of the national Global Influenza Surveillance Network laboratories, all WHO Collaborating Centers and reference laboratories and have been selected by multiple manufacturers for vaccine production trials (162, 163). When influenza viruses are grown in chicken eggs, an adaptation process must take place leading to viral selection of mutants at the antibody sites on HA resulting in variances in antigenicity between the original isolate and the passaged virus (163, 165-167). In MDCK cells, this selective process does not take place, ensuring consistency between viral isolates and vaccine strains (163, 165). This retention of antigenic characteristics in HA may lead to increased vaccine efficiency (168).

In recent years studies have addressed concerns relating to vaccine production in MDCK cells, mainly production limitations due to adherent properties and safety. Standard MDCK cells are highly adherent and the need for large amounts of surface area or carriers limits the ability to scale up production. MDCK cells usually require tissue culture medium supplemented with serum to attach and grow on surfaces increasing the risk of contamination with animal viruses and transmissible spongiform encephalopathies (163). Adherent cells also require trypsin or protease treatment to detach adherent cells during passage further complicating the scaling up process. Moreover, in adherent MDCK cells influenza only buds from polar surfaces containing microvilli limiting virus yield (163).

At the industrial level, suspension cells offer significant advantages to adherent cells lines. When cells grow freely in tissue culture medium, adherence factors from serum supplements are not required, detachment steps utilizing trypsin or proteases are not needed

when passaging cells and suspension cells can be easily expanded by dilution into fresh medium enabling rapid expansion in bioreactors. Currently Novartis's MDCK suspension cell line MDCK 33016-PF is used for the manufacture of a seasonal influenza vaccine in Europe (Optaflu®) and a pandemic H1N1 vaccine (163). These cells are grown in serum-free medium with minimal supplementation, preserve antigen specificity, are resistant to other viruses minimizing the risk of contamination with other respiratory pathogens, are not permissive for most common avian viruses and do not support prion replication (163, 169-171). Extensive studies show that the Optaflu® cell culture-based vaccine is equally well tolerated, immunogenic and safe as egg-based vaccines and has shown 83.8 % efficacy in clinical settings (163, 172-175).

These studies indicate that MDCK cells are an ideal cell line for use in the development of new influenza vaccines.

Adjuvants: Increasing the potency of influenza vaccines is especially critical in the response to an emerging strain or pandemic. First, producing a vaccine that generates a stronger immune response lowers the amount of antigen needed per dose shortening the production time (152). Second, a more potent vaccine may negate the need for a second dose to achieve optimal protection again extending the vaccine supply (152).

To attain long-lasting protective immunity the vaccine needs to stimulate a local response at the injection site and a systemic immune response including activation of antigen presenting cells, production of cytokine and trafficking of lymphocytes [50]. As described above, inactivated vaccines often fail to sufficiently activate the immune system. To overcome this challenge, adjuvants may be added to artificially stimulate immune responses (95). Adjuvants are derived from a variety of sources and typically are not or are weakly immunogenic on their

own, but work to boost immune responses to the vaccine components (94). These molecules can act as carriers or depots, target specific immune responses, or serve immunostimulatory or immunomodulatory functions (176). This lessens the amount of antigen and injections needed making the vaccine more cost effective (94, 95, 176). Additionally, certain adjuvants can shift immune responses to tailor a more appropriate form of immunity (95).

An ideal adjuvant possesses several important qualities. First, it should elicit a proper immune response, such as an appropriately balanced Th1/Th2 response or stimulate the innate immune system via TLRs. Second, it should be compatible with the antigen to ensure a physical interaction. Third, it must be safe to use in an animal or human subject. Fourth, it must be stable and cost effective (94). Adjuvants currently in production or experimental vaccines include multiple aluminum salts (collectively known as alum), oil emulsions, saponins, immune-stimulating complexes (ISCOMs), liposomes, microparticles, polysaccharide derivates, cytokines, and bacterial derivatives (176).

A limited number of adjuvants have been approved for use in human vaccines. As of July 2012, only two adjuvants have been approved for use in the United States, one is alum in the forms of aluminum hydroxide, aluminum phosphate, potassium aluminum sulfate or mixed aluminum salts and the second is AS04, a combination of monophosphoryl lipid A and alum (177). Alum is the most commonly used adjuvant in human vaccines and has been included in tetanus, diphtheria, pertussis, hepatitis A, and inactivated polio vaccines over the course of more than 60 years and has an excellent safety record (94, 177). Alum exerts its adjuvant properties by multiple mechanisms. Alum has long been believed to create a depot effect, allowing for the retention of antigens at specific site resulting in slow release and a prolonged immune response and can keeps antigens in a particulate form which enhances phagocytosis by antigen presenting

cells (94, 178). Additionally alum can induce maturation of monocytes and macrophages and activate the NLRP3 portion of the inflammasome which leads to the production of pro-inflammatory cytokines (94). MS04 is a combination of alum and monophosphoryl lipid A (MPL), a derivative of lipopolysaccharide (LPS) from *Salmonella Minnesota* R595 (94). MPL signals through TLR4 and drives Th1 responses while tempering Th2 responses (179). MS04 is currently used in certain hepatitis B (HBV) and human papilloma virus (HPV) vaccines (177).

Despite its safety and frequency of use, alum is a relatively weak adjuvant with significant drawbacks (95, 130). In human and animal studies it has been shown that alum is not a potent stimulator of antibody production when incorporated into recombinant protein vaccines (180). Alum also biases towards Th2 responses instead of a Th1 response and results in minimal or no generation of CTL responses (132, 181, 182). Furthermore, alum has been shown to induce IgE responses and in some cases cause IgE-mediated allergic reactions (183, 184). These factors make alum particularly unfavorable as an adjuvant for parasitic or viral infections such as influenza necessitating the development of alternative strategies.

Three adjuvants have been approved for use in human influenza vaccines in Europe. Two, AS03 and MF59 are oil-in-water emulsions, microdroplets of oil in water stabilized by surfactants (94, 95). In these two adjuvants the oil utilized is squalene, a type of purified fish oil commonly derived from shark liver oil (185). AS03 is used in the influenza vaccine Prepandrix® and MF59 is used in Fluad®, an influenza vaccine used in adults over age 65 (94). The third, sold as Inflexal®, is a virosome based vaccine consisting of a reconstituted influenza virus envelope that does not contain genetic material or non-surface proteins (94, 186).

Due to poor uptake of antigen, adjuvants are needed for many mucosal vaccinations to be effective. The bacterial enterotoxins cholera toxin (CT) from *Vibrio cholerae* and heat labile

enterotoxin (LT) from *E. coli* are the two most commonly used experimental mucosal adjuvants (147). Despite successful use in mice, these adjuvants are unlikely to be used in human vaccines due to safety concerns (147). When CT was delivered in quantities as low as 2.5ug as an influenza vaccine adjuvant in mice, antigen-specific IgE responses were induced raising concerns of hypersensitivity reactions, along with massive infiltration of mononuclear cells to the lungs (187). CT and its derivative form have also been shown to redirect vaccine delivered antigens to the olfactory nerve and bulb of the brain in mice which has the potential to cause severe neurological reactions (147, 187-189). LT and its nontoxic derivatives have shown negative side effects, primarily the development of Bell's palsy (a weakness or paralysis of facial muscles resulting from damage or trauma to the facial nerves) after intranasal vaccination (147). During the 2000-2001 influenza season an inactivated virosomal-subunit mucosal vaccine containing LT was used in Switzerland. It was withdrawn after it was found to increase the risk of Bell's palsy at least 19 times, a rate which would lead to 13 excess cases per 10,000 vaccines (190). Phase I clinical trials for both an HIV and tuberculosis vaccine utilizing the non-toxic mutant LT LTK63 also had incidents of Bell's palsy in healthy individuals (191). Alternative less toxic adjuvants must be developed for mucosal vaccines to advance.

Naturally derived immunostimulatory molecules such as cytokines are attractive alternatives to the above described adjuvants. Cytokines are master regulators of the immune system that guide the innate and adaptive immune responses and are responsible for establishing and maintaining immunological memory (192). The makeup and extent of the cytokines response to an antigen play key roles in determining if the host will mount an effective immune response (180). Cytokines and chemokines at the site of infection send inflammatory signals that activate resident phagocytes and recruit phagocytic cells, regulate dendritic cell function,

stimulate antigen presentation and drive T and B cell recruitment, effector function and differentiation into memory cells (192). The ability of cytokines to drive favorable immune responses has been exploited in vaccine adjuvant design. Recombinant protein and DNA expressed cytokines have been tested in DNA vaccines, tumor vaccines and killed and live attenuated viral vaccines (96, 193-195). Additionally cytokines have shown great potential as adjuvants for intranasally delivered vaccines and warrant further exploration (147).

Vaccines and Adjuvants in Agricultural Practice: Vaccines are commonly used in the poultry industry and a wide range of livestock animals including cattle, pigs, sheep, goats and farm raised fish. While these vaccines have the same overall goals as human vaccines and work in a similar manner, animal rearing and trade practices present unique challenges (142). According to the United States Department of Agriculture (USDA) biologicals such as vaccines must be "pure, safe, potent and efficacious and not be worthless, contaminated, dangerous or harmful" and "free from properties causing undue local or systemic reactions when used as recommended by the manufacturer" (132). Practical concerns mandate that a vaccine, along with any adjuvant that may be included, have minimal adverse reactions, not significantly impact the comfort and welfare of the animal, not hinder growth and reproduction rate, not result in carcass blemishes or a decline in meat quality, and be easily applicable to large numbers of animals in a short time (142). Additionally vaccines must be cost-effective, especially when the production of tens of billions of doses may be required annually (180). Most current vaccines meet these goals but there is room for improvement (as described below).

The previously discussed vaccination systems, live-attenuated vaccines, inactivated vaccines with alum adjuvants and inactivated vaccines with oil adjuvants, all have shortcomings. With live attenuated vaccines, the response to infection can result in fever with a reduction of

feeding, dullness and reduced milk production (142). Alum adjuvants often fail to stimulate proper, robust immune responses and site reactions can cause irritation and granulomas leading to animal stress and discomfort (180). While oil-based vaccine adjuvants, in particular MF59, have shown promise and in some cases gained approval for use in human vaccines, they are not commonly used in poultry vaccines due to side effects and site reactions (180, 196, 197)

Parenteral administration of vaccines of poultry is typically done by subcutaneous injection in the nape of the neck, intramuscularly in the thigh or by wing web injection. This additional labor costs an average 5 to 7 cents per bird; a significant added expense considering most vaccines alone cost 5 to 10 cents per dose (157, 198). These costs rise quickly in mass production commercial facilities. Alternative methods such as *in ovo* vaccination or posthatch vaccination through the respiratory or alimentary tracts have been proposed (157). In the United States, most broiler chickens are vaccinated *in ovo* for the avian herpesvirus Marek's disease (157). While proof-of-concept studies have shown that inactivated, adenovirus and Newcastle Disease Virus-vectored AIV vaccines can be effective when administered *in ovo*, neutralization of the vaccine by maternal antibodies in areas where AI viruses are endemic is a significant concern (146, 157, 199-201).

Alimentary administered vaccines can be distributed in feed or drinking water. A single study demonstrating protection from infection via an avian influenza vaccine administered through water has been conducted, however to achieve protection, high doses of vaccine had to be delivered up to 9 times (157, 202). Currently, no drinking water or feed AI vaccines are licensed (157).

Vaccines delivered by spray or aerosol are ideal for mass application and are currently used in poultry in the US and many parts of the world for infectious bronchitis virus (IBV),

Newcastle disease virus (NDV), avian metapneumovirus and many *Mycoplasma galliseptum* vaccines (203-205). These vaccines are either mass applied in spray cabinets in hatcheries or by backpack sprayers in production houses (157). Mucosal vaccination also offers the advantages of conjunctival and upper respiratory tract exposure which can induce mucosal and systematic immune responses and can halt infection at the viral entry site (157, 205-209). It should be noted that the three viral vaccines are either attenuated or delivered via live viral vectors. As vaccination with live-attenuated AI viruses is not acceptable for AI, inactivated vaccines must be used. Others have found that that intranasally applied whole inactivated AI vaccines are poorly immunogenic in mice and chickens (205, 208, 210). de Gues et al. found that adjuvanting whole inactivated AIVs with alum, chitosan, cholera toxin B subunit and Stimune® (a commercially available water-in-oil emulsion) did not improve immunogenicity indicating that testing of alternative adjuvants are necessary for intranasal or aerosol vaccination (205).

Membrane-Bound Immunomodulators as Adjuvants in a Cell Culture-Based Avian Influenza Vaccine: To address the shortcomings of current influenza vaccines outlined above, this study examined subcutaneous or intranasally delivered inactivated, whole virus vaccines produced in a mammalian cell culture system, bearing membrane-bound bioactive immunomodulatory adjuvants. To accomplish this, fusion constructs containing the cytoplasmic and transmembrane domains of the influenza surface proteins HA or NA and the immunomodulator of choice were generated using a eukaryotic expression vector. These constructs were stably transfected into MDCK cells and produced immunomodulators that were directed to lipid rafts, anchored to and then expressed on the cell surface (Fig. 1). Afterward, these MDCK cell lines were infected with an influenza virus. As the newly produced virions

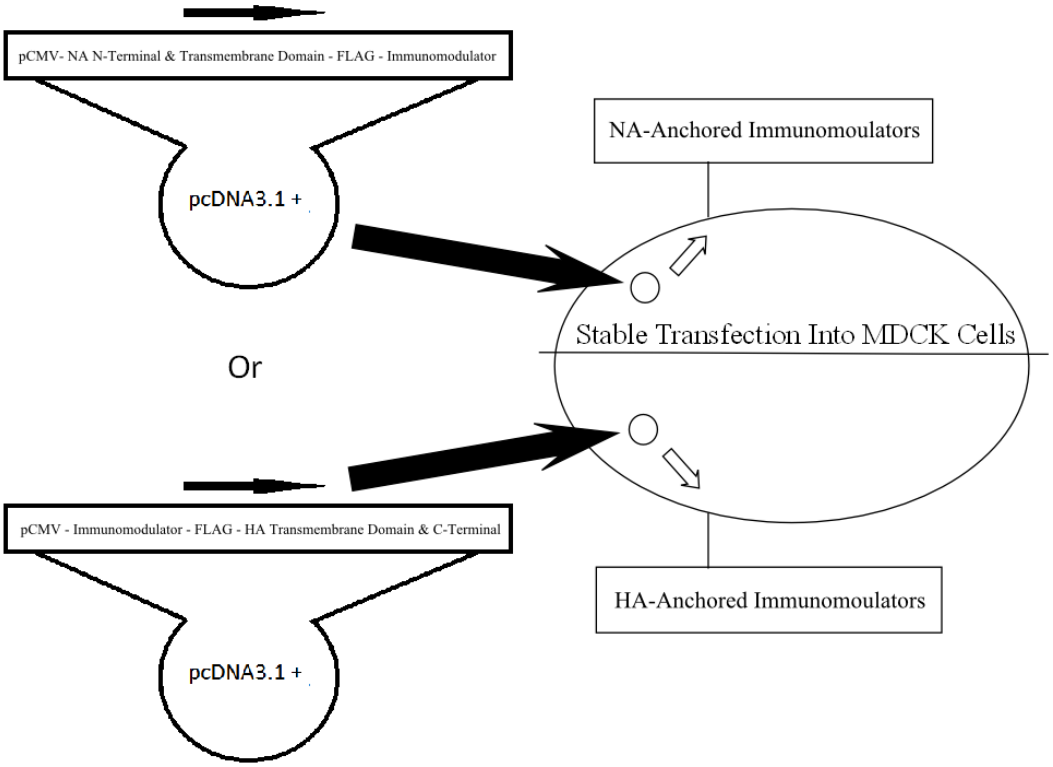


Figure 1. Plasmid design and generation of MDCK cell lines bearing membrane-bound immunomodulators. pcDNA3.1(+) plasmids containing immunomodulators anchored to the NA amino terminal (Type II transmembrane protein) and transmembrane domains or HA carboxyl terminal (Type I transmembrane protein) and transmembrane domains are stably transfected into MDCK cells. Upon expression of the vector, the anchored immunomodulators are directed to lipid rafts at the cell membrane and expressed on the surface of the cell.

budded from the surface of the cell, they incorporated their normal HA and NA proteins along with NA- and HA-anchored immunomodulators. The released virions were collected, purified and inactivated for use as whole virus vaccines (Fig. 2).

This system improves adjuvant delivery in a significant way as tethering the cytokine to the antigen of interest keeps the adjuvant in close contact with the antigen ensuring that immune cells recruited by the adjuvant react with the antigen as well. Tethering the immunomodulator also serves to extend the half-life *in vivo*, a factor that has been a limitation in previous cytokine protein-based immunotherapies (192). Additionally, expressing the immunodulator construct in MDCK cells is cheaper and more efficient than producing and purifying recombinant proteins.

Use of membrane-bound cytokines was first examined by others in tumor vaccine studies. These groups found the membrane-bound versions of mammalian IL2, IL4, IL12 and GMCSF retained bioactivity and augmented immunity to tumor cell lines (211-214). The use of membrane-bound immunomodulators as viral vaccine adjuvants were initially tested in a proof of concept study evaluating the use of a vaccine consisting of the human filamentous H3N2 A/Udorn/72 bearing avian IL2 or GMCSF in a chicken model (215). This study found that IL2 expressed on H3N2 retained IL2 activity *in vitro* and found vaccination with H3N2-IL2 led to elevated antiviral antibody levels *in vivo* when compared to H3N2 alone. Additionally, the GMCSF expressed on H3N2 was found to be bioactive *in vitro*. A second study examined this system in a mouse model using the murine H1N1 A/PR8/34 virions expressing murine IL2, IL4 or GMCSF (216). All three immunomodulators retained bioactivity when conjugated to H1N1 *in vitro* and H1N1-IL2 and H1N1-IL4 protected from lethal challenge in mice.

This dissertation work expands upon previous studies by making and characterizing new immunostimulatory constructs; avian IL4 and C3d (a breakdown product of the third component

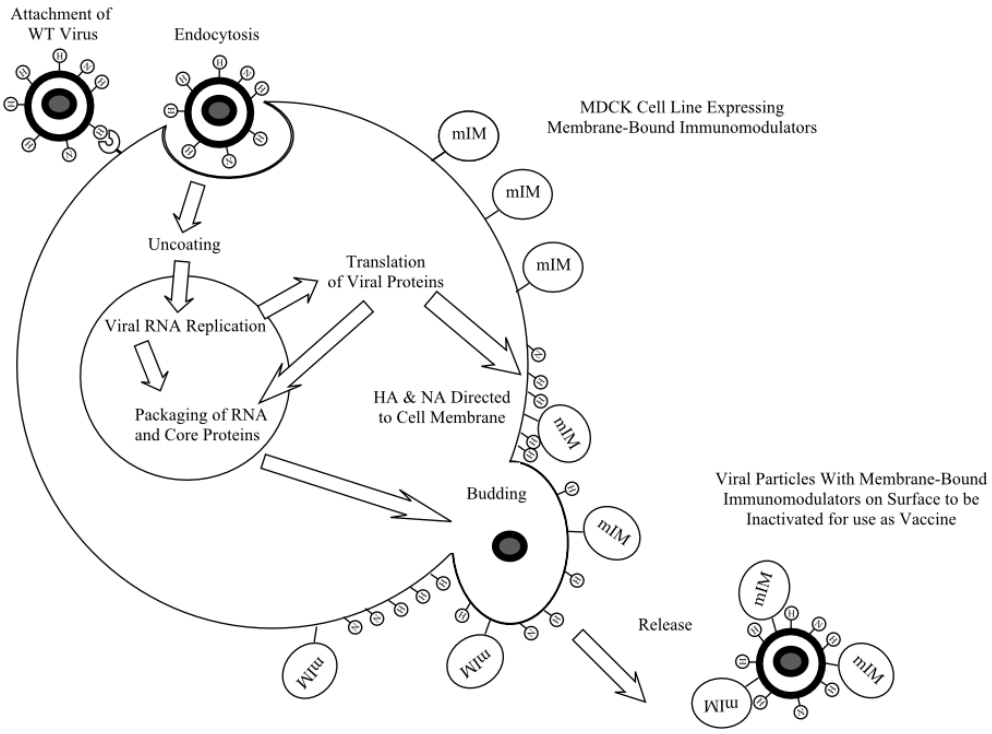


Figure 2: Generation of virions bearing membrane-bound immunomodulators. After generation of stably transfected MDCK cell lines bearing membrane-bound immunomodulators, cells are infected with influenza virus. As new virions assemble and bud from the surface of the cell, they incorporate their endogenous HA and NA surface proteins along with the HA or NA-anchored immunomodulator.

of complement) and testing these constructs and previously produced constructs (IL2 and GM-CSF) expressed on LPAI H6N2.

Chapter 2

Vaccine Adjuvant Selection, Design and Production

H6N2: A/Chick/California/2000-3 (H6N2) was selected for use in this study on the recommendation of the USDA (personal communication from D.E. Swayne, USDA, Southeast Poultry Research Laboratory, Athens, GA). HPAI strains were eliminated from consideration as BSL-3 facilities were not available for use. H6N2 is a LPAI that does not usually produce severe disease in chickens or other laboratory animals and is approved for use in BSL-2 facilities. Symptoms of H6N2 infection include reduced egg production, mild respiratory infection and yolk peritonitis (Yolk peritonitis occurs when eggs are not taken up by the oviduct but are deposited in the abdomen or by rupture of the oviduct. Yolk material can spread over the abdominal organs leading to inflammation and bacterial infection) (217, 218).

H6 viruses circulate in ducks and have been transmitted to chickens (20, 217). In the United States, an outbreak of H6N2 occurred in 2000 in California totaling 12 incidents primarily involving layer-type birds with a lower number affecting backyard chickens and a broiler breeder (217). Cases were detected in California again in 2001-2003 until a killed virus vaccine was deployed to eliminate the virus (20, 217). Jackwood et al. have characterized H6N2 infections in a laboratory setting (217). Chicks were challenged with $1X10^6$ EID_{50} and monitored. Virus shedding was detected at high levels at 2 and 4 days with reduced titers at 7 days. Virus could consistently be recovered by oropharyngeal swabs but was less reliably recovered from cloacal swabs at all time points.

Materials and Methods

Generation of Cytokine-Bearing MDCK Cell Lines: MDCK cells (ATCC) were trypsinized and grown to 80% confluence before transfection. Three ug pcDNA3.1(+) plasmid

DNA (Invitrogen) containing GMCSF-HA, pcDNA3.1-NA-IL2, NA-FLAG-IL4 or NA-FLAG-p29-p29-p29-p29-p29-p29 (p29x6) inserts were transfected using Lipofectamine2000 (Invitrogen) as recommended in the manufacturer's protocol in 6-well plates. Transfectants were selected in growth media (DMEM, 10% fetal calf serum, penicillin, streptomycin and Fungizone) supplemented with geneticin (1.5mg/ml). Stable transfectants were maintained in growth media containing 1mg/ml geneticin.

Virus purification and inactivation: Stable MDCK-immunomodulator cell lines were grown to 90-95% confluence in 100mm tissue culture-treated petri dishes in growth medium without geneticin. Cells were washed with PBS with Ca^{2+} and Mg^{2+} and H6N2 was added to each petri dish at an MOI of 0.05. Virus was adsorbed for 1hr and followed by the addition of DMEM with penicillin, streptomycin and 2ug/ml TPCK trypsin. Viral supernatants were collected after 3 days. Supernatants were pre-cleared by centrifugation at 5,000rpm (4,065.9 X g) for 20min in a GSA rotor (Sorvall). Virus was concentrated by ultracentrifugation at 22,100rpm (88,000 x g) for 1hr in a SW28 rotor (Beckman). Concentrated virus was resuspended in PBS with Ca^{2+} and Mg^{2+}. For use as vaccine constructs, virus was inactivated by heating at 56C for 30min.

Virus was quantified by hemagglutination assay. 100µl virus was added to the first well of each row and 50µl of PBS was added to each remaining well in the row of a V-shaped 96-well microtiter plate. Serial dilutions were made by transferring 50µl from the first well of each row to each successive PBS-containing well. 50µl of a 0.5% chicken red blood cell (RBC) suspension was added to each well. Plates were agitated and then incubated at room temperature until red blood cell controls settled. The reciprocal of the last dilution where hemagglutination occurs was recorded as the viral titer in hemagglutinating units (HAU).

Vaccine Adjuvant Selection:

GMCSF: Granulocyte macrophage colony-stimulating factor (GMCSF), also known as CSF2, is a member of the colony stimulating factor family. GMCSF was initially defined by its ability to generate colonies of granulocytes and macrophages from bone marrow precursor cells in vitro and was later found to influence the behavior of mature myeloid cells (219-222). GMCSF is a secreted, glycosylated, single polypeptide chain produced by a variety of cells including fibroblasts, endothelial cells, macrophages, smooth muscle cells and osteoblasts upon stimulation from inflammatory factors corresponding to infection such as IL-1, TNF and LPS (219, 223). Activation of the GMCSF receptor CSF2R activates the JAK–STAT, MAPK and PI3K pathways (219, 224). GMCSF is polyfunctional and increases the survival, proliferation, differentiation and activation of monocytes, macrophages, neutrophils, eosinophils and basophils and can be used to generate dendritic cell populations (219).

GMCSF has been tested as a therapeutic in many studies. GMCSF (trade name Leukine®, generic name Sargramostim) has been safely used for many years to boost leukocyte levels in immunosuppressed patients and has been shown to decrease infectious complications in those undergoing chemotherapy (94, 225, 226). Numerous groups have incorporated GMCSF into anti-tumor vaccines with varying results (227). In general, when administered in low doses (up to 80ug) for limited periods of time, GMCSF can increase vaccine-induced immune responses and synergize with other adjuvants including MPL, alum and other cytokines while higher doses of GMCSF (100-500ug) tends to suppresses immune responses (227, 228). Immune suppression seen with larger doses of GMCSF is thought to be due to the attraction and proliferation of myeloid suppressor cells, the rapid recruitment of macrophages leading to quick vector clearance or an increase in proapoptotic signaling in CD8+ T cells (228-232).

GMCSF has been tested as an adjuvant for several infectious disease vaccines, primarily as a component of recombinant DNA vaccines. As with tumor vaccines, GMCSF could be either protective or suppressive depending on the immunogen, timing, dosage, vaccine vector and route of administration (233). Several groups have found that GMCSF adjuvantation of experimental DNA vaccines using plasmid or viral vectors led to enhanced immune responses and or protection to pathogens including *Clostridium botulinum* (234), hepatitis B (235-237), Japanese encephalitis virus (238), porcine reproductive and respiratory syndrome virus (PRRV) (239), SIV (240, 241), HIV (242), pseudorabies (243) and haemonchus contortus (244). In a protein-based HIV vaccine, Ahlers et al. found that adjuvanting with 2-5ug recombinant GMCSF enhanced both cellular and humoral immune responses (245).

Previous studies by others relating to GMCSF adjuvantation and intranasal vaccination, mucosal immunity, chicken models and influenza are of particular pertinence to this study. Okada et al. tested three doses of an intranasally delivered HIV DNA vaccine containing 2ug HIV DNA, 2ug plasmid GMCSF and liposomes in mice and found increased anti-HIV serum IgG levels, and fecal IgA levels in comparison to antigen and liposomes alone (246). Bradney et al. vaccinated mice mucosally with 4 doses of 4ug GMCSF and 10ug HIV peptide and observed IgG levels comparable to those induced by a cholera toxin adjuvant (247). Ramsburg et al. used a single dose of VSV vector expressing GMCSF and found increased CD8+ T cells and increased protection from challenge (248). In a similar model Parker et al. used an attenuated HSV-1 viral vector expressing GMCSF and this increased protection from challenge (249). Also, Nambiar et al. noted reduced bacterial loads after mycobacterium challenge following vaccination with BCG expressing GMCSF (250).

Two studies have been conducted in chickens using chicken GMCSF as an adjuvant for infectious bronchitis virus (IBV). Tan et al. vaccinated chickens intramuscularly with 2 doses of 150ug plasmid GMCSF plus 150ug of IBV plasmid DNA and found significantly enhanced cellular and humoral immune responses plus increased protection compared to IBV plasmid DNA alone (251). Zeshan et al. vaccinated chickens in ovo with 10^8 TCID50 of an adenovirus vector carrying GMCSF and IBV antigen and observed increased IBV specific antibodies (252). In addition they found increased spleen cell proliferation and IFN-γ with limited IL-4 production indicating the enhancement of cell-mediated immune responses. Furthermore, following challenge 100% of birds vaccinated with IBV and the GMCSF adjuvant were protected from lesions compared to 70% of those vaccinated with IBV alone. These results indicate that GMCSF can be an effective adjuvant in chickens.

In a series of studies Babai et al. encapsulated H3 and N2 from A/Shangdong/9/93 with GMCSF and or IL2 in liposomes and vaccinated mice and found higher antibody titers, increased survival rates and protection for over 1 year (253-255). Orson et al. delivered a series of 4-5ug of plasmid DNA containing H1 from A/PR8/34, GMCSF and IL12 and observed increased protection levels and the generation of neutralizing cross-protective antibodies (256). Loudon et al. vaccinated macaques 3 times with 1.8ug plasmid encoding H1 from A/New Calendonia/20/99 and 0.2ug of plasmids encoding GMCSF with each dose being delivered intradermally via gene gun (257). They found significantly increased levels of hemagglutination inhibiting (HI) antibodies and cytokine-secreting HA-specific T cells in the periphery of macaques and influenza A-specific mucosal antibodies and T cells in the lung and gut-associated lymphoid tissues. Ramanthan et al. conducted a randomized trial in human cancer patients where 250ug of recombinant GMCSF was administered with seasonal influenza vaccines (258). They did not

find any enhancement of response to influenza vaccines; however the 250ug dose of GMCSF is in the 100-500ug range that is now understood to be suppressive.

These studies showing efficacy of adjuvanting vaccines with GMCSF in different delivery methods and in chickens plus its ability to enhance responses to influenza made it an attractive candidate for study in our model. Previous results from this laboratory demonstrated that chicken GMCSF anchored to murine A/PR8/34 retained biological activity and could stimulate the proliferation of bone marrow cells (215). We hypothesized that chicken GMCSF would act in a similar manner when anchored to an avian strain and enhance the immunogenicity of H6N2.

Materials and Methods:

GMCSF-HA plasmid design and construction: Chicken GMCSF was fused to the transmembrane domain of the influenza HA as described previously (215). Briefly, full length GMCSF was synthesized using 6 oligonucleotides (each approximately 80-100bp including 25bp overhangs) that spanned the entire coding region (GenBank # NM_001007078.1) under conditions described by Dillon and Rosen (259). 5' HindIII and 3' BamHI restriction sites were added to the ends of the full length GMCSF by PCR using the following primers: forward 5′-GCATAAGCTTCCACGATGCTGGCCCAGCTC-3′ and reverse 5′-GCATGGATCCTTAGATGCAGTCTTT CTCCT-3′. The sequence was ligated into pcDNA3.1(+). A 70 amino acid segment of HA derived from the influenza virus A/WSN/33 containing the carboxy-terminal, cytoplasmic tail, transmembrane region and 26 amino acids of the stalk region containing 5' BamHI and 3' EcoRI restriction sites was amplified using the following primers: forward 5′-CCGGATCCAATGGGACTTATGATTATCC-3′ and reverse

5′-CCGAATTCTCAGATGCATATTCTGCACTGC-3′. The HA sequence was ligated into pcDNA3.1-GMCSF creating pcDNA3.1-GMCSF-HA

Bioassay for chicken GMCSF: The bioassays for chicken granulocyte macrophage colony stimulating factor (GMCSF) were performed as previously described (215). Briefly, bone marrow was flushed from the femur of an exsanguinated market chicken (Chase Road Poultry) with Iscove's medium, washed three times and resuspended in Iscove's supplemented with 5% fetal calf serum, 2% autologous chicken serum, 2mM L-glutamine, 1mM pyruvate and penicillin, streptomycin and fungizone. $3X10^5$ cells/150µl were added to 96 well plates containing dilutions of GMCSF-containing COS7 supernatants, heat inactivated H6N2 or H6N2-GMCSF. Plates were incubated at 40C for 72hrs with the addition of 1µCi of [3H] thymidine for the last 18 hours.

Results: GMCSF stimulated the proliferation of bone marrow cells. To confirm that HA-anchored GMCSF was expressed on H6N2 virions and retained bioactivity, heat-inactivated H6N2-GMCSF was incubated with chicken bone marrow cells for 72 hours in a thymidine-uptake proliferation assay (Fig. 3). Incubation of bone marrow cells with a COS7 supernatant containing soluble GMCSF increased thymidine uptake in comparison to untreated cells. Incubation with 400HAU H6N2-GMCSF resulted in an increase in thymidine uptake while incubation with H6N2 was unable to induce a similar increase in proliferation. The ability of H6N2-GMCSF to induce the proliferation of bone marrow cells indicated that GMCSF is expressed on the surface of the virion, retained bioactivity in the anchored-confirmation and was not inactivated by viral inhibition steps.

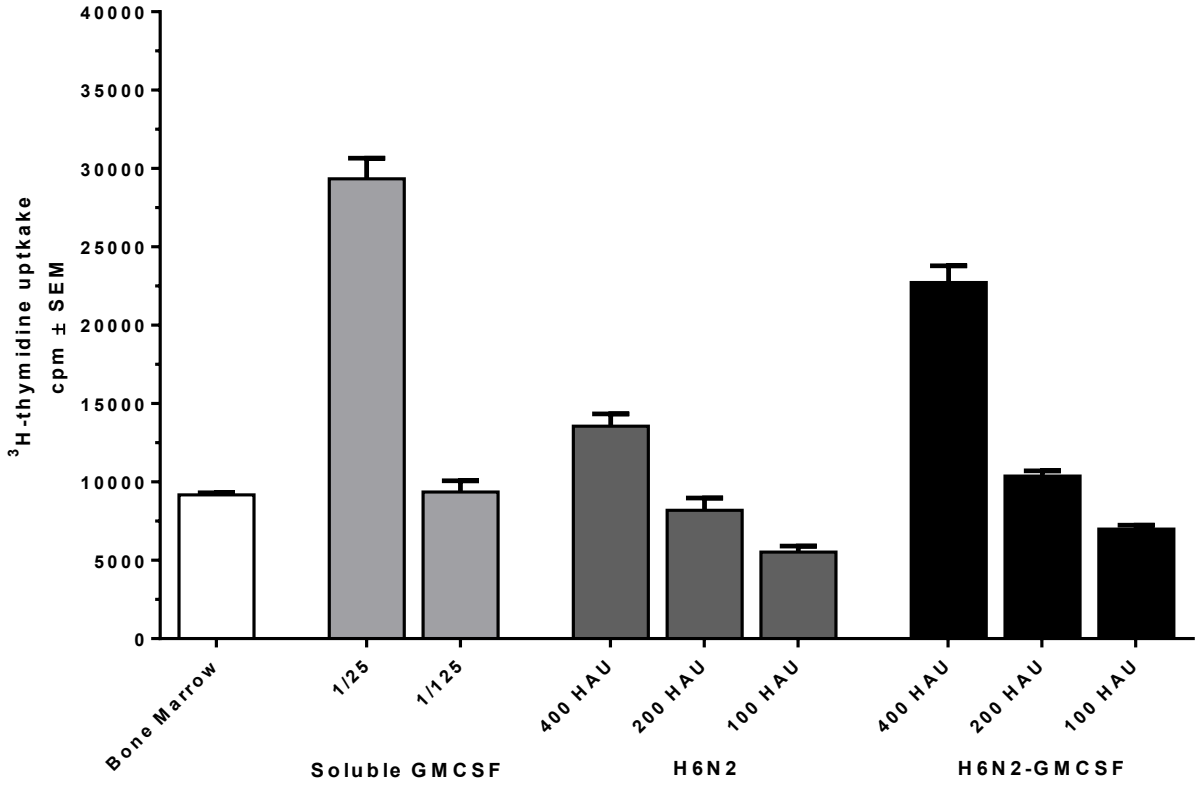


Figure 3. Bioassay for GMCSF activity. Dilutions of soluble GMCSF (light gray bars), H6N2 (dark gray bars) and H6N2-GMCSF (black bars) were incubated with chicken bone marrow cells for 72hrs. White bars (bone marrow cells alone) show normal cell proliferation. ^{3}H-thymidine was added for the last 18hrs. Samples were tested in triplicate and cell proliferation was measured by uptake of radio-labeled thymidine in counts per minute (Representative assay).

IL2: The cytokine interleukin 2 (IL2) influences the homeostasis, differentiation and behavior of many lymphocyte subtypes. Under steady-state conditions, IL2 is primarily produced by CD4+ T helper cells in secondary lymphoid organs (260-262). IL2 is produced at lower levels by CD8+ T cells, natural killer cells (NK) and natural killer T cells (NKT), dendritic cells and mast cells (260, 263, 264). IL2 signals through the high-affinity trimeric IL2 receptor (IL2R) or the lower-affinity dimeric IL2R (260). Cells expressing high levels of dimeric IL2R such as naïve CD8+ T cells, memory CD4+ T cells, memory CD8+ T cells and NK cells are sensitive to exogenous IL2 but typically not responsive to low physiological levels of IL2 found during steady-state conditions (260, 265). After receptor engagement, signal transduction occurs through the JAK-STAT, PI3K-AKT and MAPK pathways and the IL2-IL2R complex is internalized (260, 261, 266, 267).

IL2 signaling impacts CD8+ T cells in all phases of the immune response (primary expansion, contraction, memory cell production and secondary expansion) (260). After acute infection, IL2 levels increase rapidly in the secondary lymphoid organs activating and driving the proliferation of naïve antigen-specific CD8+ T cells (260). Antigen-specific CD8+ T cells that do not receive strong IL2 signals show weakened primary and secondary expansion and insufficient IL2 signals leading to reduced long-lived CD8+ memory T cell production (260).

IL2 significantly influences the differentiation of CD4+ T cells and their polarization into Th1 or Th2 cells. IL2 enhances Th1 cell proliferation by the induction of T-bet expression and an increase in the IL12Rβ2 subunit (260). IL12 signaling leads to the production of Th1 transcription factors and IFNγ, which is enhanced by IL2 produced by responding T cells (260, 268). In Th2 cells, IL2 induces early expression of IL4Rα (260).

IL2 was selected as an adjuvant for this study due to this laboratory's extensive experience in the cloning and characterization of avian IL2 and the success of other groups using avian IL2 as a vaccine adjuvant (269, 270). Hulse and Romero and Li et al. found chicken IL2 administered as a recombinant protein or plasmid DNA was able to enhance antibody responses and increased protection to infectious bursal disease virus (IBDV) (271, 272). Tarpey et al. coadministered a Marek's disease vaccine vector encoding chicken IL2 with IBDV and infectious bronchitis disease vaccines and saw enhanced protection to each vaccine (273). Zhou et al. found intramuscular injection of geese with 300-350ng soluble goose IL-2 plus an inactivated oil-adjuvanted whole-virus H5N2 influenza vaccine enhanced HI antibody titers (274). Xiaowen et al. vaccinated newly hatched chicks intranasally with inactivated whole-virus H5N2 and 50ug recombinant IL2 and observed increased IgA and IgG secreting cells in the respiratory tract (275).

Membrane-bound IL2 has been tested as an influenza vaccine adjuvant by Herbert et al. in a mouse model and by Yang et al. in a chicken model. Herbert et al. vaccinated mice subcutaneously with 0.375ug β-propiolactone inactivated H1N1 A/PR/8/34 bearing murine IL2 (H1N1-IL2) and challenged with a lethal dose (1000 $TCID_{50}$) of H1N1 (216). In comparison to mice vaccinated with H1N1 alone, H1N1-IL2 vaccinated mice had significantly lower viral loads in lungs and significantly enhanced survival. Furthermore, mice exhibited a favorable IgG2a:IgG1 response, indicative of Th1 skewing.

Yang et al. vaccinated chicks subcutaneously with 10ug H3N2 A/Udorn/72 bearing NA anchored IL2 (H3N2-IL2) at 1 week and boosted with the same dose at 4 weeks (215). After booster vaccination, serum ELISA assays demonstrated chicks vaccinated with H3N2-IL2 had significantly ($p < 0.05$) higher antiviral titers than those vaccinated with H3N2 alone. HI assays

indicated that 100% of chicks vaccinated with H3N2-IL2 had titers of 20 or more while only 42.8% of those vaccinated with H3N2 reached the same threshold ($\chi 2$ value of $p = 0.018$).

We hypothesize IL2 anchored to H6N2 will behave similarly to the membrane-bound IL2 in Herbert et al. and Yang et al.

Materials and Methods:

NA-IL2 Plasmid Design and Construction: A 51 amino acid region containing the full amino-terminal cytosolic and transmembrane domains and 17 amino acids of the stalk domain were derived from the NA segment human H1N1 influenza A/WSN/33 segment (Genbank L25817) and fused to the full length coding sequence of mature chicken IL2 using PCR based cloning techniques described previously (215). The following primer pairs and restriction endonuclease sites were used (restriction sites underlined): NA forward 5' GACTGGATCCCTGCCATGAATCCAAAC 3' (BamHI), NA reverse 5' ACTGCCTTGGTTGCATAT 3' (StyI), IL2 Forward 5' GCATCCAAGGCGCATCTCTATCA 3'(StyI), and IL2 reverse primer 5' GCTAGAATTCTTATTTTTGCA 3' (EcoRI). The NA-IL2 coding sequence was ligated into the plasmid pcDNA3.1(+) (Invitrogen) creating pcDNA3.1-NA-IL2.

Bioassay for chicken IL2: The bioassays for chicken IL2 activity were performed as previously described (215, 276). Briefly, spleens were removed from market chickens, passed through stainless steel mesh, washed three times in Iscove's medium (Gibco) and cultured at 10^7 cells/ml in Iscove's medium with bovine serum albumin (BSA), 2mg/mL, Concanavalin A (ConA),10µg/mL, and penicillin, streptomycin and fungizone at 40°C. At 24hrs ConA was neutralized with 0.05 M α-methyl mannopyrannoside, the media diluted 2-fold and supplemented with autologous serum (final concentration of 2%) and incubated for 2 additional days. Viable

chicken T-cell blasts were isolated following separation on Histopaque, washed, counted, and used as indicator cells. To assay the bioactivity of IL-2 expressed on H6N2 virions, 400, 200 and 100 HAU of H6N2-IL2 and H6N2 viral particles were added to 96 well round bottom plates and chicken T-cell blasts were added at 5 X 10^4 cells/well in Iscove's medium supplemented with 2% autologous chicken serum, 2 mg/mL bovine serum albumin, penicillin, streptomycin and fungizone. Recombinant chicken IL2 was added at a concentration of 10^{-10} M as a positive control. Cultures were incubated overnight at 40C in 5% CO_2, the last 6h with 1 μCi of [3H] thymidine. Cells were harvested on glass fiber filters using an automated harvester and counted in a LKB Betaplate B-counter. Controls included blast cells alone and blast cells stimulated with soluble recombinant IL2.

Results: IL2 is a potent stimulator of T cell growth. To confirm NA-IL2 anchored to H6N2 retained bioactivity as it did in Yang et al. and Herbert et al., it was tested in a T cell proliferation assay (Fig. 4). Recombinant soluble IL2 served as a positive control and it stimulated thymidine uptake about 8 fold. 400HAU H6N2-IL2 also stimulated T cell blasts, while 400HAU of wild-type H6N2 moderately inhibited T cell proliferation. These results indicate NA-anchored IL2 was expressed on inactivated H6N2 virions and retained its bioactivity.

C3d: The complement system is part of the innate immune system and consists of more than 30 proteins, which exist as zymogens, present in plasma and on cell surfaces (277, 278). Complement plays three major physiologic roles: 1) Acts as a host defense against infection by the opsonization of pathogens, chemotaxis and activation of leukocytes and lysis of bacteria and infected cells, 2) Serves as a bridge between the innate and adaptive immune system by

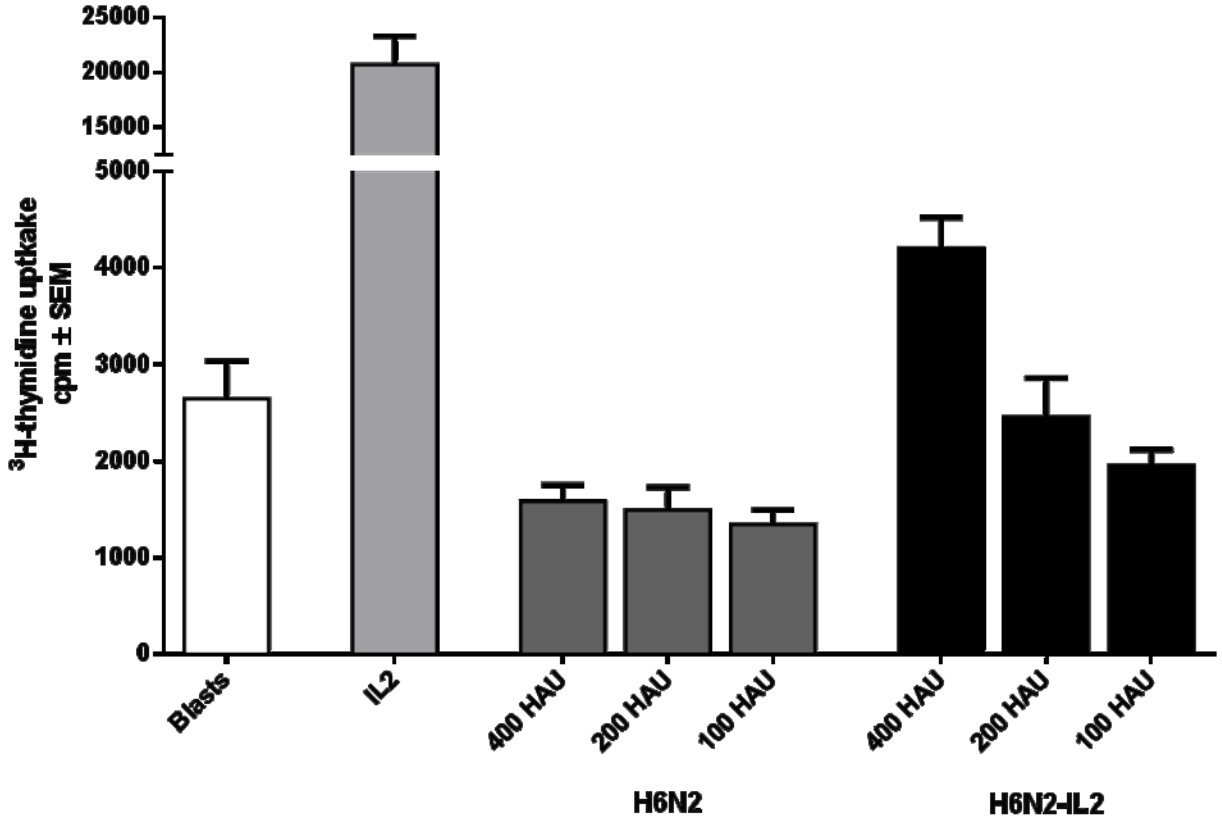


Figure 4. Bioassay for IL2 activity. Dilutions of recombinant IL2 (light grey bars), H6N2 (dark gray bars) and H6N2-IL2 (black bars) were incubated with chicken T cell blasts overnight. White bars (T-cell blasts) show normal cell proliferation. ^{3}H-thymidine was added for the last 6hrs. Samples were tested in triplicate and cell proliferation was measured by uptake of radio-labeled thymidine in counts per minute (Representative assay).

augmentation of antibody responses and enhancement of immunologic memory and 3) Disposes of waste by clearing immune complexes and apoptotic cells (279). The complement cascade can be activated through three separate mechanisms, the classical, lectin, and alternative pathways. The classical pathway responds to IgM- and IgG-tagged immune complexes, Apoptotic cells, certain viruses and gram-negative bacteria and ligands bound to C-reactive protein (277). The lectin pathway is triggered when the pattern-recognition molecule mannose-binding lectins binds to terminal mannose groups found on many types of bacteria. The alternative pathway is initiated by the spontaneous hydrolysis of C3 and after contact with multiple types of bacteria, fungi, viruses and tumor cells (277, 278). The end products of these pathways, either C4bC2a or C3bBb, react with the C3 convertases and trigger the remainder of the complement pathway (278). C3 is hydrolyzed into C3b, then converted by Factors I and H to ic3b and then to C3c and C3d by Factor I and CR1 (278).

The C3 degradation factor C3d is an opsonizing agent that binds foreign antigen and complement receptor 2 (CR2, CD21) (280, 281). CR2 is found on B cells, follicular dendritic cells (FDCs) and a subset T cells and serves as a link between the innate and adaptive immune systems (282). FDCs are present in the germinal center of lymph nodes and have a critical role in antigen presentation (283). CR2 on FDCs binds antigens coated with C3d and retain these antigens for extended periods of time enhancing antigen presentation (278, 284, 285). In addition, this trapping of opsonized antigen is important for the production of high-affinity antibodies and generating and maintaining memory B cells (282, 286, 287). CR2 is also part of the B-cell antigen receptor complex that also includes CD19 and CD81 (278). B cells are normally activated when antigen binds to the B-cell recptor, surface IgM, Igα or Igβ (BCR) (278, 288). When antigen is linked to C3d, the CR2/CD19/CD81 complex is engaged along with the

BCR resulting in cross-talk between pathways and enhanced cell activation (278, 288, 289). The dual signaling of these receptor complexes has a strong molecular adjuvant effect and can lower the threshold of antigen needed for B-cell activation 10,000-fold, counter inhibitory signals and reduce apoptosis (288-290). Other effects of the crosslinking of the BCR and CR2 include enhanced antigen uptake and improved presentation by MHCII molecules (291, 292)

The ability of C3d to link the innate and adaptive immune responses, lower antigen threshold, improve antigen presentation and enhance phagocytosis suggests that it would serve as an excellent adjuvant. The first study utilizing C3d as an adjuvant was done in 1996 by Dempsey et al. (290). Mice were immunized with recombinant hen egg lysozyme (HEL) fused to two or three copies of full-length murine C3d. The HEL-C3d fusion constructs were found to be 1,000-10,000-fold more immunogenic than HEL alone. In the time since then, human, chicken, bovine and porcine C3ds have been cloned and tested as adjuvants (293-296). Additionally many methods have been developed to link C3d repeats (typically 3 copies (C3d3)) to antigenic proteins by plasmid DNA gene fusions, covalently, by biotinylation, the baculovirus expression vector system and immune-stimulating complexes (ISCOMs) (100, 294, 297-299). Numerous C3d3-antigen vaccine constructs have been tested in murine models and many have displayed increased immunogenicity when compared to vaccination with antigen alone including HIV ENV and gp120, influenza HA and M2, measles HA, bovine viral diarrhea virus E2, Hepatitis E HEV-p179, pseudorabies virus gC, Rift Valley Fever virus Gn, *Bacillus anthracis* PA and *Streptococcus pneumonia* capsular polysaccharide (298, 300-314).

Bower and Ross (315) found that the 28 amino acid segment of C3d that binds the CR2 receptor (P28) is sufficient for adjuvant activity. In that study they found that 4 repeats of murine P28 conjugated to the N-terminus of HIV gp120 elicited enhanced cellular and humoral

immune responses that were statistically similar to those generated by linking 3 copies of full length C3d to gp120. This further indicated that C3d adjuvant activity acts through the CR2 receptor and also confirmed that a smaller adjuvant fragment may be used. Other groups have successfully tested murine P28 as an adjuvant for PRRV, West Nile virus, foot-and-mouth disease virus and *Plasmodium berghei* (296, 316-318). These groups found 4 to 6 repeats of P28 conjugated to antigens resulted in the strongest immune responses. Additionally, Liu and Niu (295) found the 29 amino acid chicken CR2 binding domain of C3d (P29) enhanced immune responses to Newcastle disease virus when conjugated to NDV-F protein, with the highest responses coming when 6 copies of p29 (p29x6) were used.

Of particular relevance to this study are the previous studies done with C3d and influenza antigens. Ross et al. (297) generated a DNA vaccine encoding 3 copies of murine C3d to the secreted form of HA from the murine influenza strain A/PR/8/34 (H1N1) (sHA-mC3d3) that was administered by gene gun to BALB/c mice. They found sHA-mC3d3 DNA accelerated avidity maturation of antibodies to HA and the appearance of hemagglutinin- inhibition activity. These quicker responses also correlated with earlier appearances of protective immunity. Additionally, complete protection from live virus challenge could be achieved by a single vaccination with sHA-3C3d DNA than with a non-adjuvanted form of HA DNA. In a later study Mitchell et al. (305) found that sHA-mC3d3 could induce heterosubtypic immunity. BALB/c mice were vaccinated with plasmids containing 3 copies of C3d and the HA from A/Puerto Rico/8/34 (H1N1) or A/Aichi/2/68-x31 (H3N2) by gene gun. While sera from the vaccinated mice did not neutralize the heterologous virus, the mice were protected from heterosubtypic challenge with a lethal dose of virus. Li et al. (307) tested a DNA vaccine in mice containing a plasmid expressing C3d as above and the equivalent sHA region of the swine influenza

A/Swine/Guangdong/164/06 (H3N2). In contrast to the previous studies, BALB/c mice were vaccinated intramuscularly. The vaccinated mice were challenged with either A/Swine/Heilongjiang/74/2000 (H3N2) or A/Swine/Guangdong/96/06 (H1N1). Antibody titers and affinity resulting from immunization with sHA-mC3d3 were higher and stronger than those vaccinated with HA alone. One week after challenge with H3N2, no virus was isolated from the mice immunized with any of the HA expressing plasmids. In mice challenged with heterologous H1N1, only mice immunized with sHA-mC3d3 did not show lesions in the kidneys or brain, again due to increased antibodies and stronger affinity maturation.

C3d has also been tested as an adjuvant in recombinant-protein influenza vaccines. Watanabe et al. (306) tested C3d as an alternative to cholera toxin B (CTB) or lymphotoxin (LT) for the induction of mucosal immunity. Intranasal vaccination of BALB/c mice with the recombinant protein sHA-mC3d3 resulted in locally secreted IgA and serum IgG antibodies and led to complete protection against homologous virus challenge while sHA alone was unable to do so. Zhang et al. (318) fused 2 copies of chicken C3d to the matrix protein M2 gene of A/chicken/Guangdong/2000 (H9N2). While the sequence of M2 is highly conserved amongst a wide variety of avian influenza viruses and expressed on infected cells, it is weakly immunogenic and is present in limited numbers on virus particles (318). This group hoped C3d would boost the immunogenicity of M2 as it did for HA in the aforementioned studies. They found anti-sM2 antibody was elevated in chickens intramuscularly vaccinated with the C3d adjuvant, however, protection ratios only reached 13.3% and further studies were not pursued.

The previous studies suggest that C3d has great promise as a membrane-bound adjuvant due to its ability to 1) enhance humoral and mucosal immune responses to HA 2) increase survival and induce heterosubtypic immunity in mice and 3) act effictively when delivered

subcutaneously or intranasally. While the chicken p29x6 construct has not been tested with influenza, its efficacy in NDV vaccines in chickens suggests potential use as an adjuvant for other chicken vaccines. Although C3d adjuvanted M2 did not result in acceptable protection levels in chickens, its use as an adjuvant offers advantages over the system utilized by Zhang et al. (318). First, only 2 copies of C3d were conjugated to M2 which may not be sufficient for increasing immunogenicity. The other previously mentioned studies found 3 copies of C3d to be effective while Liu et al. (295) found 6 repeats of the CR2 binding domain elicited the strongest responses. Additionally, attaching p29 to the whole virion allows its adjuvant effects to act on all viral proteins, including the highly immunogenic HA and NA along with M2 and the remaining internal proteins allowing for the possibility of immune responses to a wider range of antigens and the increased potential for heterosubtypic protection.

Materials and Methods:

NA-p29x6 Plasmid Design and Construction: Hexamers of the 29 amino acid CR2-recpetor binding sequence of chicken C3d (p29) were generated using a protocol modified from Liu and Niu (295). The sequence of p29 containing 5' HindIII and BamHI restriction sites and a 3' BglII restriction site, FLAG tag and a KpnI restriction site was generated in 2 PCR reactions using overlapping oligonucleotides. The initial PCR reaction used the following two primers with 25 base pair overlaps: forward 5'-AAGCTTGGATCCAAAGTCCTGATGAGTTCTCCAAAGATGGCACCCACTGGGCGGAACGCAACGCCCACACCTACAACATCG-3' and reverse 5'-GGTACCTTACTTGTCATCGTCGTCCTTGTAGTCAGATCTAGCGTAGGACGTCCCCTCGATGTTGTAGGTGTGGGCGTTGCG-3'. The PCR product was gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and reamplified with the following primers:

forward 5'-GCATAAGCTTGGATCCAAAGTCCTG-3' and reverse 5'-GTCAGGTACCTTACTTGTCATCGTC-3'. The sequence was digested with HindIII and KpnI and inserted into puc19 (Invitrogen). 2 aliquots of puc19-p29-FLAG were made. The first was cut with BglII and EcoRI and the large fragment containing the puc19 backbone and p29 minus the FLAG tag and stop codon was recovered. The second was cut with BamHI and EcoRI and the small fragment containing p29 with FLAG and the stop codon were recovered. The two fragments were ligated together (BamHI and BglII cut sites can be ligated together and then cannot be cut again by either enzyme) yielding puc19-p29-p29-FLAG. 2 aliquots of the puc19-p29x2-FLAG plasmid were digested with BglII and EcoRI or BamHI and EcoRI. The fragments recovered from these digests, the large segment from BglII/EcoRI containing the p19 backbone and 2 copies of p29 and the small segment from BamHI and EcoRI containing 2 copies of p29, the FLAG tag and stop codon were ligated together to make puc19-p29x4-FLAG. The 4x repeat of p29 was cut from the plasmid with BglII and EcoRI and ligated into the puc19-p29x2-FLAG plasmid to create puc19-p29X6-FLAG. The FLAG-tagged p29X6 repeat was cut from the plasmid using BamHI and EcoRI and ligated into pcDNA3.1-NA (Derived by excising IL2 with BamHI and EcoRI from pcDNA3.1-NA-IL2) to create pcDNA3.1-NA-p29x6-FLAG.

Immunofluorescence Detection of p29x6 on MDCK Cells: $1X10^5$ MDCK and MDCK-p29X6 cells were adhered to coverslips in 24 well plates. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed with PBS. Coverslips were blocked with 10% BSA in PBS for 30min at 37C then incubated with mouse anti-FLAG primary antibody (Sigma) at a 1:100 dilution in 3% BSA. After washing, coverslips were incubated with a 1:500 dilution of FITC conjugated goat anti-mouse secondary antibody (Anaspec) for 45 min. in the dark at 37C. Coverslips were covered with a small drop of Slow

Fade Gold® Reagent with DAPI (Invitrogen) and mounted to microscope slides. Images were acquired using a Nikon E600 microscope with epifluorescence at 40X magnification.

ELISA for Detection of p29x6 on H6N2 Virions: H6N2 and H6N2-p29x6 were diluted to 100HAU/100µl or 10HAU/100µl and adsorbed to 96-well ELISA plates (Immulon II, Dynatech Laboratories Inc.) overnight at 4C. Coated wells were then blocked (2% BSA in PBST) overnight at 4C. After washing, wells were incubated with mouse anti-FLAG IgG (1:1000; Sigma) in blocking buffer overnight at 4C. Following washing wells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:2000; Sigma) for 3hrs at 25C. Wells were washed and incubated with 200µl p-Nitrophenyl phosphate (pNPP; Sigma) in the dark for 30min at room temperature. The reaction was halted by the addition of 50µl 3 N NaOH and absorbance for the stopped was read at 405 nm using a BioTek Epoch microplate spectrophotometer.

Results: To ensure p29x6 was expressed on MDCK cells and H6N2 virions immunofluorescence and ELISA assays were conducted. To detect the presence of FLAG-labeled p29x6 on the surface of MDCK cells, cells were grown on coverslips and stained with an anti-FLAG primary antibody and a FITC-labeled secondary antibody (Fig. 5). No wild type MDCK cells were found to be FITC positive while a majority of MDCK-p29x6 cells were stained indicating that p29x6 is expressed on H6N2-p29x6. Clusters of p29x6 were seen near the outer edges of many cells possibly indicating concentration at lipid rafts.

To detect the presence of FLAG labeled p29x6 on H6N2 virions, H6N2 and H6N2-p29x6 were coated on ELISA plates and stained with anti-FLAG primary antibody and alkaline phosphatase-conjugated secondary antibody (Fig. 6). Alkaline phosphatase activity was elevated

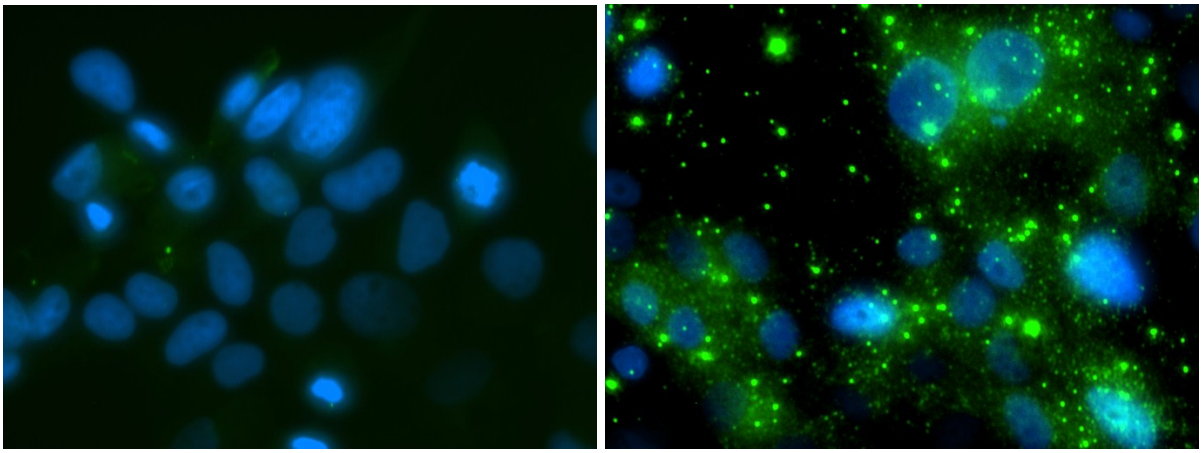


Figure 5. Immunofluorescence labeling of MDCK cell lines expressing NA-p29x6. Left panel: Wild type MDCK. Right panel: MDCK-p29X6. Green = FITC positive for p29x6 expression. Blue = DAPI counterstained nuclei.

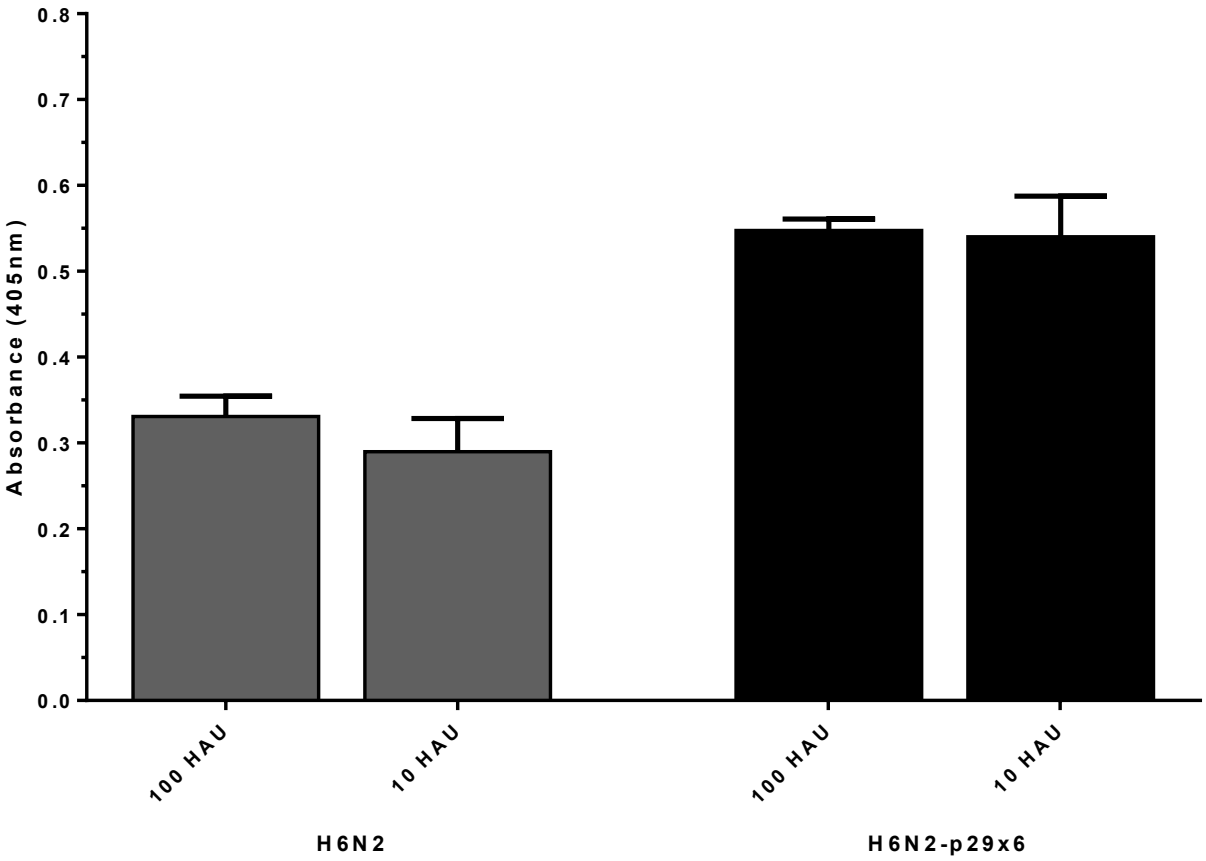


Figure 6. ELISA for detection of p29x6 on H6N2 virions. Wild type H6N2 (gray bars) and H6N2-p29x6 (black bars) were labeled with anti-FLAG primary antibodies and alkaline phosphatase-conjugate secondary antibodies. Alkaline phosphatase activity was measured by the addition of pNPP and absorbance at 405nm. Samples were tested in triplicate and SEM was calculated.

at both 100HAU and 10HAU dilutions of H6N2-p29x6 in comparison to wild type H6N2indicating that p29x6 is expressed on inactivated H6N2 virions. The similar absorbance measurements for both 100HAU and 10HAU are likely due to saturation of the available sites on the microtiter plate at the lower concentration.

IL4: Interleukin 4 is a pleiotropic type II cytokine produced primarily by CD4+ Th2 cells and to a lesser extent by basophils, mast cells, NK1.1 T cells and eosinophils (319-323). IL4 drives the differentiation of antigen-stimulated naïve T cells from Th0 cells into Th2 cells leading to the production of IL5, IL10 and IL13 while suppressing Th1 cell responses (319, 324, 325). IL4 also controls facets of immunoglobulin class switching, determining if human B cells switch to IgE and IgG4 production or if murine B cells shift to IgE and IgG1 (319). IL4 plays prominent roles in the development of protective responses to hemlminths and extracellular parasites (319, 326). IL-4 also plays a role in macrophage activation (327). Additionally, IL4 serves as a B cell growth factor, increases expression of MHC class II molecules in B cells, increases expression of CD23, upregulates production of the IL-4 receptor and stimulates changes in the vascular endothelium to favor recruitment of T cells and eosinophils (319).

While endogenous IL4 drives a Th2 response that is unfavorable for mounting an immune response to influenza vaccines, as an adjuvant it has displayed surprising behaviors. Biedermann et al. found co-delivering IL4 with *Leishmania major* enhanced IL12 production and Th1 responses (328). Eguchi et al. demonstrated similar results in a transfected tumor vaccine (329). Most importantly, when Herbert et al. vaccinated mice with H1N1-IL4, mouse serum antibody IgG1:IgG2a ratios were indicative of a Th1 immune response (216). Furthermore, of all of the constructs tested in their study, mice vaccinated with membrane-bound

IL4 adjuvanted vaccines has the lowest average lung viral titers and highest rate of survival. These results made IL4 an obvious candidate for this study.

It should be noted that concerns have been raised over use of IL4 as an adjuvant. In 2001, Jackson et al. found that a mousepox virus (ectromelia) expressing IL4 was able to overcome genetic resistance and was also able to infect previously vaccinated mice resulting in acute disease with high mortality. This led to concerns that mutant human poxviruses (such as smallpox) resistant to vaccination could be produced by the inclusion of IL4. The generation of a higher virulence influenza virus is highly unlikely to result from our study for a number of reasons. As described above, IL4 adjuvantation increased efficacy of a killed H1N1 murine vaccine. Additionally, our vaccine particles do not actively synthesize IL4 reducing the risk of complications that could potentially arise due to excessive expression of the cytokine. Furthermore, poxviruses are highly reliant on Th1 mechanisms for clearance (Th1 responses were hampered by IL4 in Jackson et al.) while Th1 or Th2 responses can clear influenza as seen with current seasonal influenza vaccines.

Materials and Methods:

NA-IL4 Plasmid Design and Construction: Chicken IL4 was fused to the transmembrane NA segment previously described in the NA-IL2 plasmid design section. Mature IL4, excluding the IL4 signal sequence was amplified by PCR from pCI-neo-IL4 (a gift from P. Kaiser). 5' StyI and 3' EcoRI restriction sites were added using the following primers: forward 5'-GATCCCTTGGCCTGTGCTTACAGCTCTCAGT-3" and reverse 5'-GATCGAATTCTCACTTATTTTTAGCTAGTT-3'. A FLAG sequence was added to the 3' end of IL4 using the previously described forward primer and the reverse primer

5'-GATCGAATTCTCATTTGTCGTCGTCGTCTTTATAGTC-3'. The IL4-FLAG sequence was ligated into pcDNA3.1-NA creating pcDNA3.1-NA-IL4-FLAG.

Bioassay for IL4 activity: IL4 activity was measured by its ability to stimulate nitric oxide (NO) production in macrophages as described by He et al. (330). HD11 chicken macrophages (A gift from H. He, USDA, College Station, TX) were maintained in DMEM supplemented with 10% chicken serum, penicillin, streptomycin, fungizone, and 1.5 mM L-glutamine at 39 °C. 100µl aliquots of cell suspension (2×10^6 cells/ml) were seeded into each well of a round-bottom 96-well plate and allowed to grow to about 85% confluence. Before stimulation, cells were replaced with fresh medium containing no phenol red. Cells were stimulated with or without 64, 125, 250, 1000 and 1500 HAU H6N2, H6N2-NA-IL4 and H6N2-IL4-HA overnight. NO levels were measured using its stable metabolite, nitrite using the Griess assay (Promega). Briefly, 100 µl culture supernatant from each well was transferred to the wells of a flat-bottom 96-well ELISA plate and combined with 50µl of 1% sulfanilamide and 50µl of 0.1% naphthylenediamine. After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD595) using a BioTek Epoch microplate spectrophotometer. Readings were compared to a standard curve generated with sodium nitrite (Sigma).

Results: To demonstrate the presence and bioactivity of IL4 on H6N2 virions, HD11 macrophages were incubated with H6N2 or H6N2-IL4. Both NA and HA-anchored IL4 molecules were tested for their ability to activate NO production in chicken macrophages. NO levels in wells incubated with H6N2 were similar to background. 125-1000HAU/well of H6N2-NA-IL4 were able to induce NO production to levels similar to those produced by 500ng of IL4. H6N2-IL4-HA was only able to induce high levels of NO production at 1000HAU/well. These

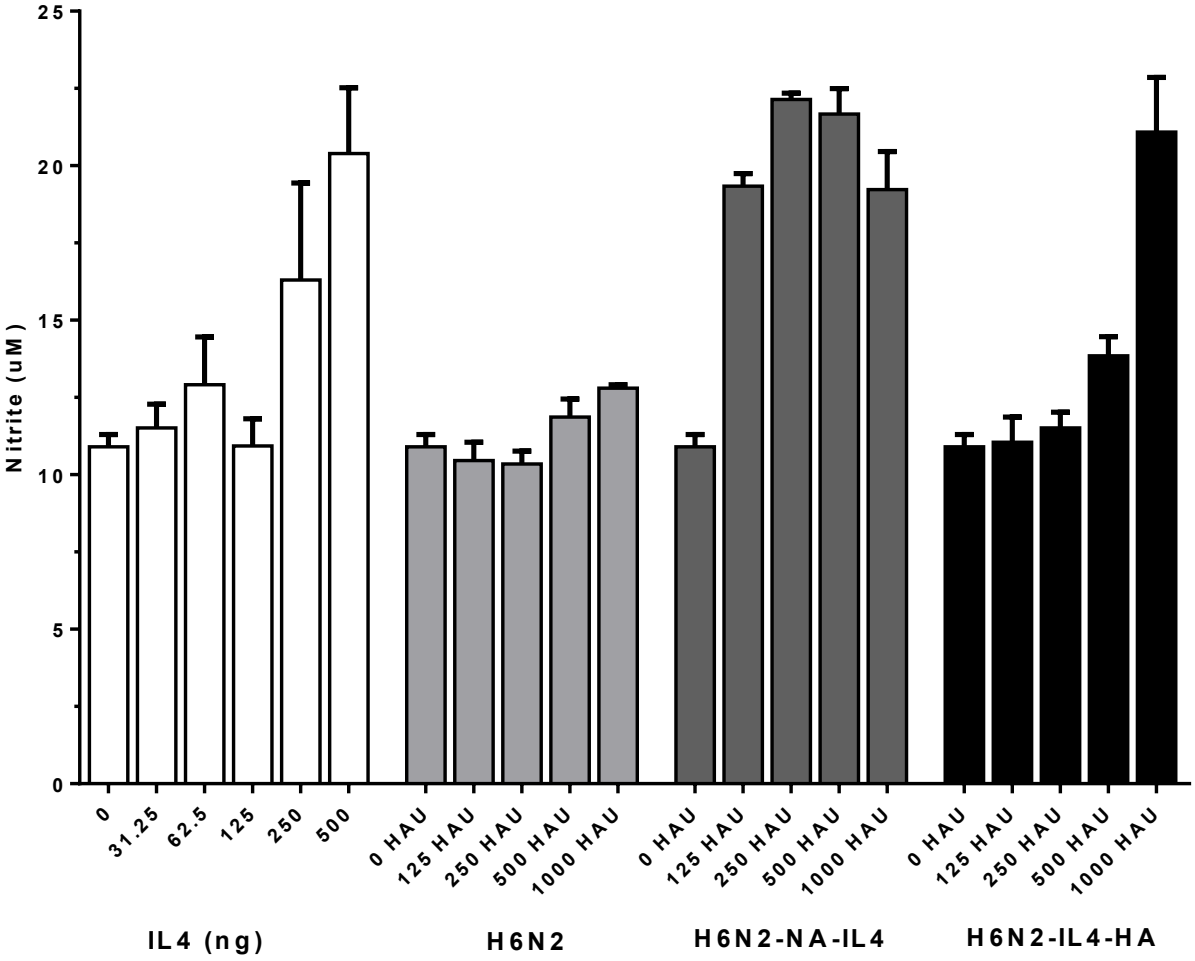


Figure 7. Bioassay for IL4 activity. Dilutions of recombinant IL4 (white bars), wild type H6N2 (light gray bars), H6N2-NA-IL4 (dark gray bars) and H6N2-IL4-HA were incubated with HD11 chicken macrophages. IL4 activity was measured by the induction of NO production in the HD11 cells, a sign of macrophage activation. NO levels were measured using its stable metabolite, nitrite, with the Griess assay. Samples were tested in triplicate and SEM was calculated (Representative assay).

results confirm the presence and activity of IL4 on H6N2 virions. H6N2-NA-IL4 was selected for use in *in vivo* studies as it was bioactive across a wide range of doses.

Chapter 3

Vaccination Studies in Chicks

Materials and Methods: All chicks used in vaccination studies were ISA Brown pullet egg layers purchased as day old chicks locally (Townline Farms, Zeeland, MI) and maintained in Division of Laboratory Animal Resources (DLAR) facilities at Wayne State University in accordance with institutional protocols. In the preliminary vaccination trial 72 ISA Brown female chicks were divided into 8 experimental groups: saline vaccination control, no virus challenge (n=6), saline vaccination control with virus challenge (n=6), subcutaneous vaccination with H6N2 (n=12), H6N2-IL2 (n=12), H6N2-GMCSF (n=6) followed by live virus challenge, intranasal vaccination with H6N2 (n=12), H6N2-IL2 (n=12), H6N2-GMCSF (n=6) followed by live virus challenge. 21-day-old chickens were vaccinated subcutaneously at the base of the neck or intranasally with the inactivated A/Chick/California/2000-3 (H6N2) vaccines described above. Vaccines contained 970 hemmaglutinating units (HAU) (approximately 8ug total viral protein) of virus resuspended in 100µl PBS. 10 days following the primary vaccination, chickens were challenged intranasally with $5X10^6$ $TCID_{50}$ resuspended in 100µl PBS. 3 days following challenge oropharyngeal and cloacal swabs were collected and resuspended in viral transport medium (tissue culture medium 199 containing 0.5% BSA, penicillin ($2X10^6$ U/liter), streptomycin 200 mg/liter, polymyxin B ($2X10^6$ U/liter), gentamicin (250 mg/liter), nystatin ($0.5X10^6$ U/liter), ofloxacin HCI (60 mg/liter), and sulfamethoxazole (0.2 g/liter)). 5 days post-challenge serum was collected and the chicks were sacrificed.

In the second vaccination trial a total of 91 chickens were used for immunization studies and divided into 10 experimental groups: saline control vaccination, no virus challenge (n=8), subcutaneous vaccination with saline control (n=9), H6N2 (n=10), H6N2-IL2 (n=9), H6N2-IL4

(n=9), H6N2-p29x6 (n=11), H6N2-GMCSF (n=10) followed by virus challenge, intranasal vaccination with H6N2 (n=10), H6N2-p29x6 (n=9) and H6N2-IL4 (n=6) followed by virus challenge. Briefly, 3-week-old chickens were vaccinated subcutaneously at the base of the neck or intranasally with the inactivated A/Chick/California/2000-3 (H6N2) vaccines described above. Vaccines contained 2000 hemmaglutinating units (HAU) of virus resuspended in 100µl PBS. Immunized chickens were boosted 21 days later with the same dose of vaccine delivered by the same route. 2 weeks following the booster vaccination, chickens were challenged intranasally with $5X10^6$ $TCID_{50}$ resuspended in 100µl PBS. Serum was collected from each chicken 2 weeks following each vaccination and 1 week post-challenge. Oropharyngeal swabs were collected in 1ml PBS 3 days post-challenge, stored at -80 C, and thawed only once for RNA extraction.

Protective antibody assays: Hemagglutination inhibition (HAI) responses were measured. Sera were treated with receptor-destroying enzyme (RDE) (Cholera Filtrate; Sigma-Aldrich) as described previously to remove non-specific inhibitors of hemagglutination (331, 332). Lyophillized RDE was reconstituted with 5ml sterile distilled water and diluted to 100ml with calcium saline, pH 7.2m aliquoted and stored at -20C. RDE was added to each serum sample at a 4:1 ratio (0.2 ml RDE + 0.05ml serum) and incubated overnight at 37C. Following the overnight incubation 5 volumes of 1.5% sodium citrate (0.25 ml) were added to each sample and incubated at 56C for 30 min to inactivate remaining RDE giving a starting serum dilution of 1:10.

Alternatively, if RDE treatment failed to sufficiently remove non-specific inhibitors, sera were treated with trypsin, heat and periodate as described elsewhere (333). 20µl of serum were combined with 10µl of 8 mg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) in 0.1M phosphate buffer (pH 8.2) and incubated at 56°C for 30 min. After

samples cooled to room temperature, 30µl 0.011M periodic acid (Sigma) were added, and the mixture was incubated for 15 min. 30µl of 1% glycerol saline was added, and the mixture was incubated at room temperature for 15 min. Lastly, 2 volumes of phosphate-buffered saline were added to give a 1:10 dilution of the initial sample.

HAI assays were performed by standard protocols (331). Briefly, 25µl of wild-type H6N2 in PBS with an HA titer of 8 HA units/50µl was mixed with 25µl of twofold dilutions of each RDE-treated serum in V-bottomed 96-well plates. After 30 min of incubation at room temperature, 50µl of 0.5% chicken red blood cells were added to the mixtures. The plates were incubated at room temperature until red blood cells in non-virus-containing control wells settled. The HAI titer was calculated as the reciprocal of the last dilution of antiserum that completely inhibited hemagglutination.

Influenza virus neutralization assays were also used. MDCK cells were grown to confluence in 96 well flat bottom tissue culture plates. Twofold serial dilutions of sera in viral growth medium were combined with 100 $TCID_{50}$ H6N2, added to MDCK cells and incubated for 2hrs. The virus-antibody mixture was removed, plates were washed and fresh viral growth medium was added. After 3 days, cells were observed for cytopathic effect.

Titration of virus in swab samples: MDCK cells were plated at $2X10^4$ cells per well in a 96 well flat bottom tissue culture plate. Tracheal and cloacal swab supernatants were diluted 1/25, 125, 625, 3125, 15625 and 78,125 in PBS with Ca^{2+} and Mg^{2+} and penicillin, streptomycin, fungizone and ciprofloxacin and added to washed MDCK cells. Plates were incubated for 1hr at 37C then rinsed and incubated with DMEM, penicillin, streptomycin and TPCK trypsin (2ug/ml) for 3 days at 37C.

RNA extraction and quantitative, real-time reverse-transcriptase polymerase chain reaction (RT-PCR): Viral particles from oropharyngeal swabs were pelleted by ultracentrifugation in an SW55 Ti rotor (Sorvall) at 30,000 rpm (~85,300 x g) for 1hr at 4C. Viral RNA was prepared from lysates of the pelleted virus using the RealTime ready Cell Lysis Kit (Roche Inc.). Lysates were treated with RNAse inhibitor according to the manufacturer's instructions. The Transcriptor Universal cDNA Master (Roche Inc.) was used to generate cDNA in a 20µl reaction mixture containing the following reagents: 12.5µl PCR grade water, 4µl reaction buffer, 1µl reverse transcriptase, 0.5µl thermolabile DNAse and 2µl viral RNA lysate. Reactions were performed in a Mastercycle pro thermal cycler (Eppendorf) with the following cycling conditions: primer annealing and DNAse degradation 10min at 29C, reverse transcription 10min at 55C and denaturation 5min at 85C.

Quantitative real-time PCR was carried out using the Roche FastStart Universal Probe Master (ROX) kit. Reaction mixtures contained 5µl cDNA template, 25µl Roche FastStart Universal Probe Master Mix, 0.5µl FAM-labeled hydrolysis probe, 0.5µl forward primer, 0.5µl reverse primer and 18.5µl PCR-grade water. Primer and probe sequences for influenza A/Chick/California/2000-3 (H6N2) M1 matrix protein were: forward primer 5'-CGCCACATGTGAGCAGATT-3', reverse primer 5'-ATTGTCACCATTTGCCTGTG-3' and FAM-490 labeled Roche Universal Probe #159, 5'-CCAGCATC-3'. Reactions were performed using a BioRad *i*Cycler with the following cycling conditions: 1 cycle 50C for 2min, 1 cycle 95C for 10min, 45 cycles of 95C 15sec, 60C 1min. A melting curve was performed at the end of the experiment. DNA was quantitated by the levels of hydrolyzed FAM-490.

Statistical Analysis: To determine whether seroconversion rates and viral clearance rates among the groups were significantly different from each other, Fisher's two sided exact test

was performed and a value of $p<0.05$ was considered significant. To determine whether HAI titers among the groups of chickens were significantly different from each other, analysis of variance (ANOVA) and Bonferoni's Multiple Comparison Test (Prism, Graphpad) were performed and a value of $p<0.05$ was considered significant.

Results:

Preliminary Vaccination Trial: This preliminary vaccination study was undertaken to assess the ability of heat-inactivated H6N2 avian influenza vaccines bearing GMCSF or IL2 delivered subcutaneously or intranasally to strengthen immune responses to influenza in chickens. To achieve this, 21 day old ISA Brown Pullet egg layers were vaccinated with H6N2, H6N2-IL2 or H6N2-GMCSF. 10 days after vaccination, chicks were challenged with live H6N2. 3 days after viral challenge, tracheal and cloacal swabs were taken to test for viral replication. 5 days after challenge, sera was collected to analyze antibody titers. Chicks with HAI titer of 80 or higher after challenge were considered to be seroconverted and highly reactive to the challenge virus (Fig. 8). None of the chicks mock-vaccinated with saline achieved seroconversion. 1/12 chicks vaccinated with H6N2 subcutaneously seroconverted. 6/12 chicks vaccinated with H6N2-IL2 subcutaneously, an increase over H6N2 alone ($p=0.06$) and a significant increase over mock vaccination ($p<0.05$). 4/6 chicks vaccinated with H6N2-GMCSF seroconverted, a significant increase over both mock vaccination and H6N2 ($p<0.05$). After intranasal vaccination 1/12 chicks vaccinated with H6N2 and 0/12 chicks vaccinated with H6N2-IL2 seroconverted. 6/12 chicks vaccinated intranasally with H6N2-GMCSF seroconverted, a moderate increase ($p=0.08$) compared to H6N2 delivered intranasally. Titration of virus recovered from tracheal and cloacal swabs on MDCK cells was attempted however, results were unreliable due to a non-specific cytopathic effect unrelated to influenza infection.

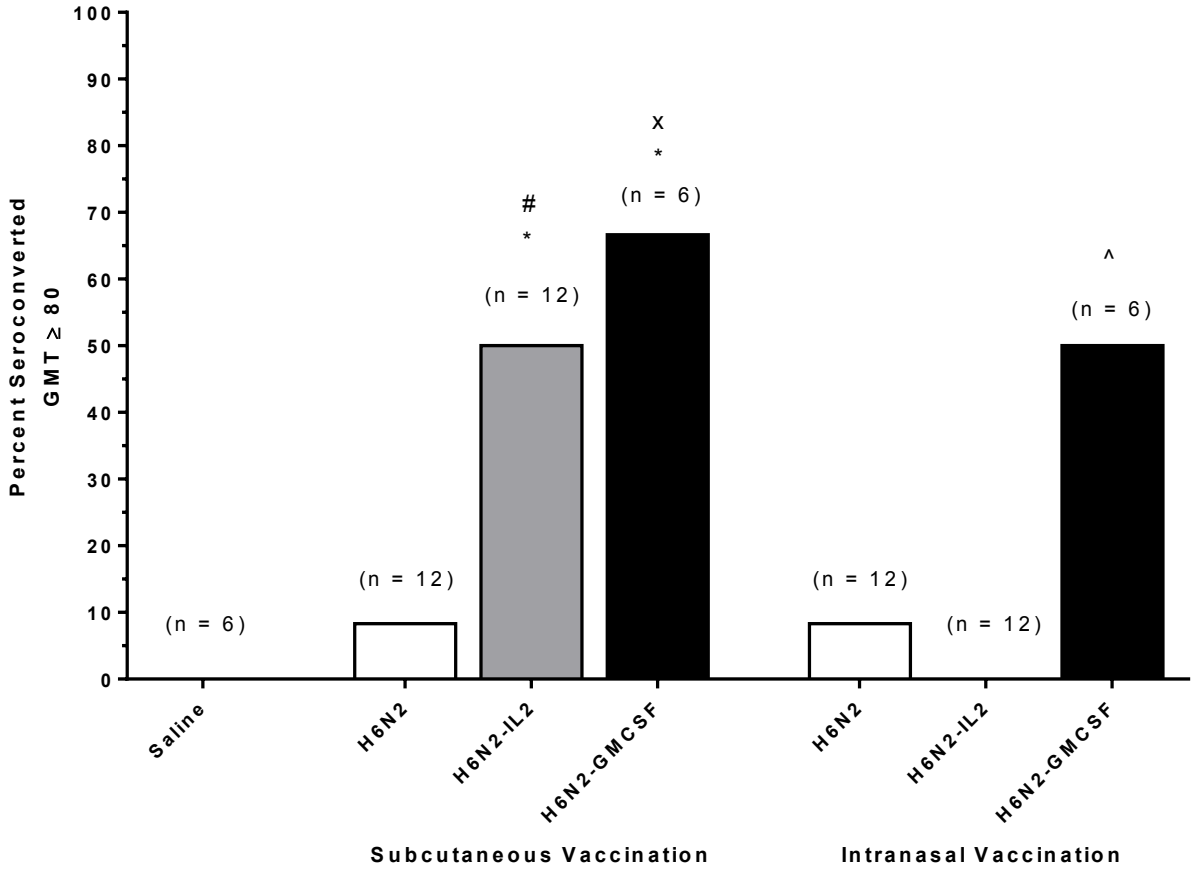


Figure 8. Seroconversion levels following vaccination and viral challenge. Serum was collected 5 days after challenge and analyzed by HAI assay. Chicks with GMTs of 80 or above were considered to be highly reactive and seroconverted. (* = $p<0.05$ compared to saline, x = $p<0.05$ compared to H6N2 subcutaneous, # = $p=0.06$, ^ = $p=0.08$)

These results indicated that adjuvanting subcutaneously delivered influenza vaccines with membrane-bound IL2 or GMCSF could increase antibody production in response to live viral challenge compared to vaccination with inactivated vaccine alone. The data also indicated that adjuvanting intranasal influenza vaccines with membrane-bound GMCSF may moderately strengthen antibody responses while IL2 fails to do so.

***In vivo* testing of H6N2 vaccine constructs:** A second vaccination trial was conducted with modifications from the preliminary study to further analyze H6N2-IL2 and H6N2-GMCSF and to test H6N2-IL4 and H6N2-p29x6. All four constructs plus wild type H6N2 were tested as subcutaneous vaccinations. Due to capacity and animal number limitations and failure of intranasal vaccination with H6N2-IL2 and H6N2-GMCSF to significantly improve seroconversion rates in the preliminary study, only wild type H6N2, H6N2-IL4 and H6N2-p29x6 were tested intranasally. Additional design changes include the use of a higher vaccine dose (2000HAU vs. 970HAU), the addition of a booster vaccination, serum collection after each vaccination and a longer time period between vaccination and challenge. Others determined that RT-PCR could be used to reliably test oropharyngeal swabs for viral presence at its peak replication titers 3 days after challenge (217, 334). This technique was utilized instead of viral titration of swab material in MDCK cells to eliminate the contamination issues seen in the preliminary study. Furthermore, an HAI titer of 40 was considered to be positive for seroconversion, a standard more in line with USDA and WHO recommendations.

Chicks were vaccinated 21 days after hatching. At day 35 (14 days after vaccination) sera were collected to measure antibody response to the primary vaccination and the booster vaccine was administered. On day 49 sera were collected to measure antibody response to the booster vaccine and chicks were challenged with live H6N2. On day 52, oropharyngeal swabs

were collected to test for viral replication (or clearance). On day 56 sera were collected to analyze post-challenge antibody titers.

Serum Antibody Response to Vaccination: After primary vaccination, average antibody titers determined by HAI were similar for unvaccinated controls and all subgroups of vaccinated chicks (Fig. 9). A minimal number of chicks had HAI titers equal to or above the seroconversion level of 40 GMT (Fig. 10). An influenza virus neutralization assay was also used after primary vaccination. Chicks were considered to have increased protection if a 1/40 dilution of serum could neutralize 100 $TCID_{50}$ H6N2. In comparison to saline vaccinated chicks, chicks vaccinated with H6N2-IL4 ($p<0.005$), H6N2-p29x6 ($p<0.00005$) and H6N2-GMCSF ($p<0.0005$) subcutaneously and H6N2-p29x6 intranasally ($p<0.05$) had increased protection (Fig. 11). In comparison to chicks vaccinated with H6N2 subcutaneously, those vaccinated with H6N2-GMCSF ($p<0.05$) and H6N2-p29x6 ($p<0.0005$) subcutaneously had increased protection.

Following booster vaccination groups vaccinated with H6N2-p29x6 and H6N2-GMCSF subcutaneously and H6N2-IL4 and H6N2-p29x6 intranasally averaged HAI titers greater than 40 (Fig. 12). In comparison to saline vaccinated chicks, chicks vaccinated with H6N2-p29x6 ($p<0.05$) and H6N2-GMCSF ($p<0.0005$) subcutaneously and H6N2-p29x6 intranasally ($p<0.0005$) had significantly elevated antibody titers as measured by HAI. Furthermore, in comparison to chicks vaccinated with H6N2 subcutaneously, those vaccinated with H6N2-GMCSF ($p<0.005$) subcutaneously and H6N2-p29x6 intranasally ($p<0.005$) had significantly higher antibody titers. Chicks vaccinated with H6N2-IL4 ($p<0.05$), H6N2-p29x6 ($p<0.0005$) and H6N2-GMCSF ($p<0.0005$) subcutaneously and H6N2 ($p<0.05$), H6N2-IL4 ($p<0.05$) and H6N2-p29x6 ($p<0.0005$) intranasally had significantly increased rates of seroconversion than those receiving saline (Fig. 13). In comparison to chicks vaccinated with H6N2 subcutaneously,

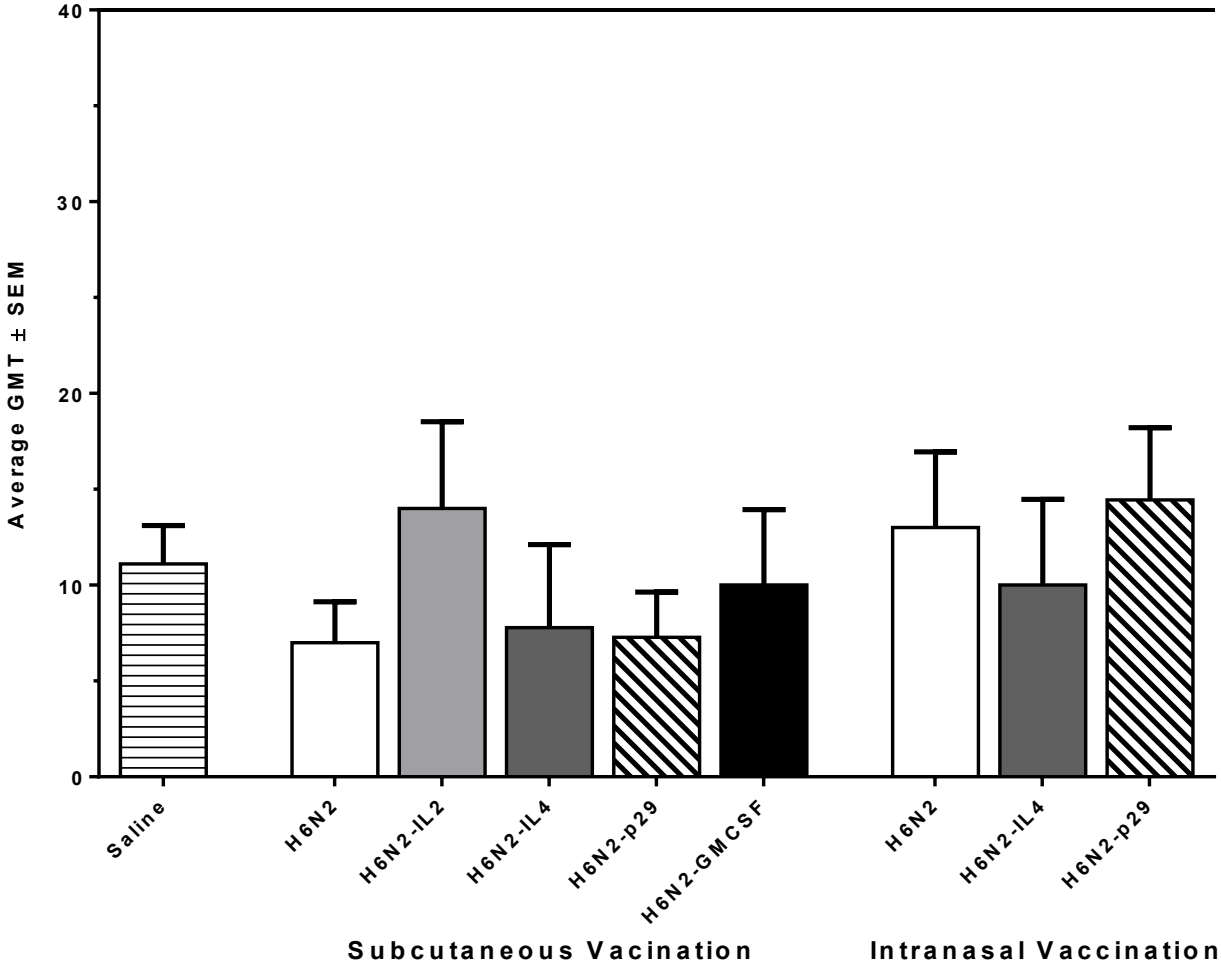


Figure 9. Average GMT following vaccination with 2000HAU at 21 days of age. Serum was collected 14 days after vaccination and titers were determined by HAI assay. HAI titers of 40 or greater were considered to be seroprotective.

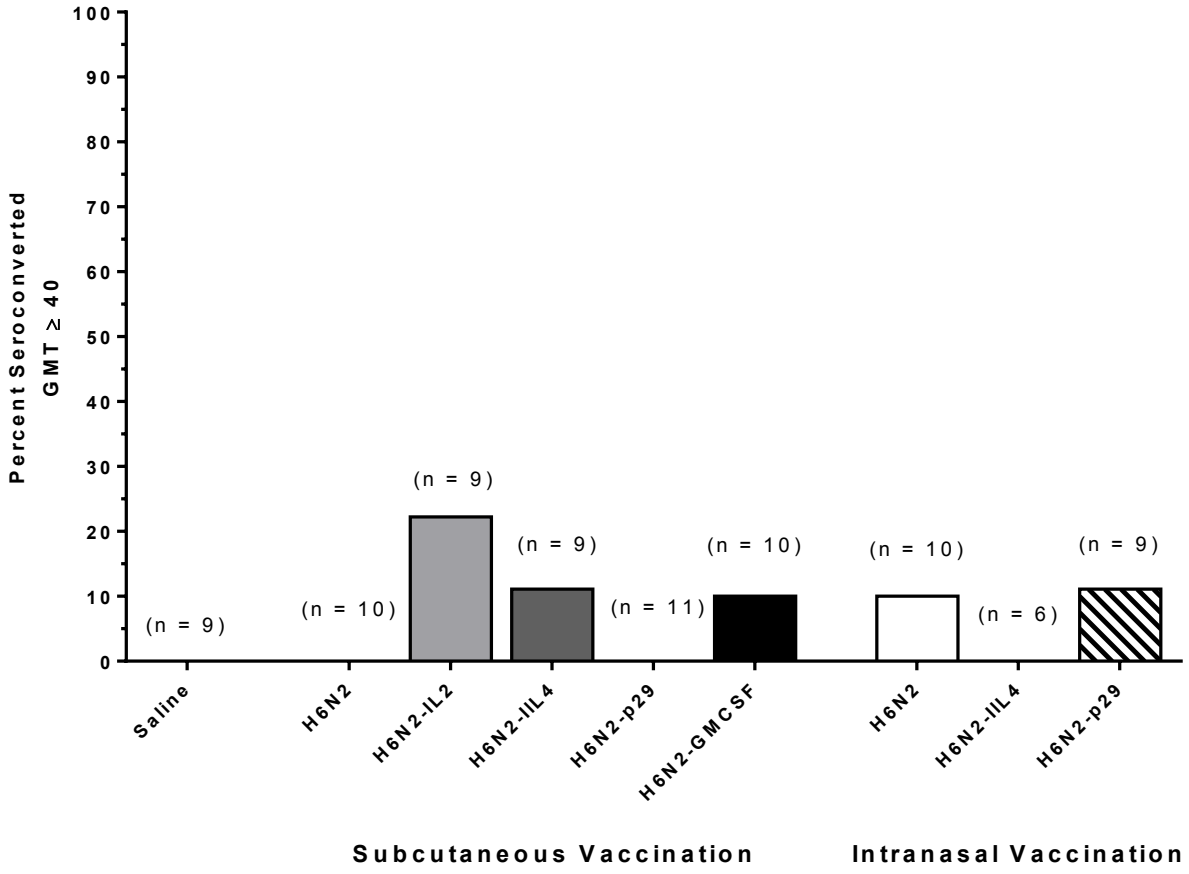


Figure 10. Percentage of chicks per group considered to have seroprotective titers as determined by HAI assay following primary vaccination.

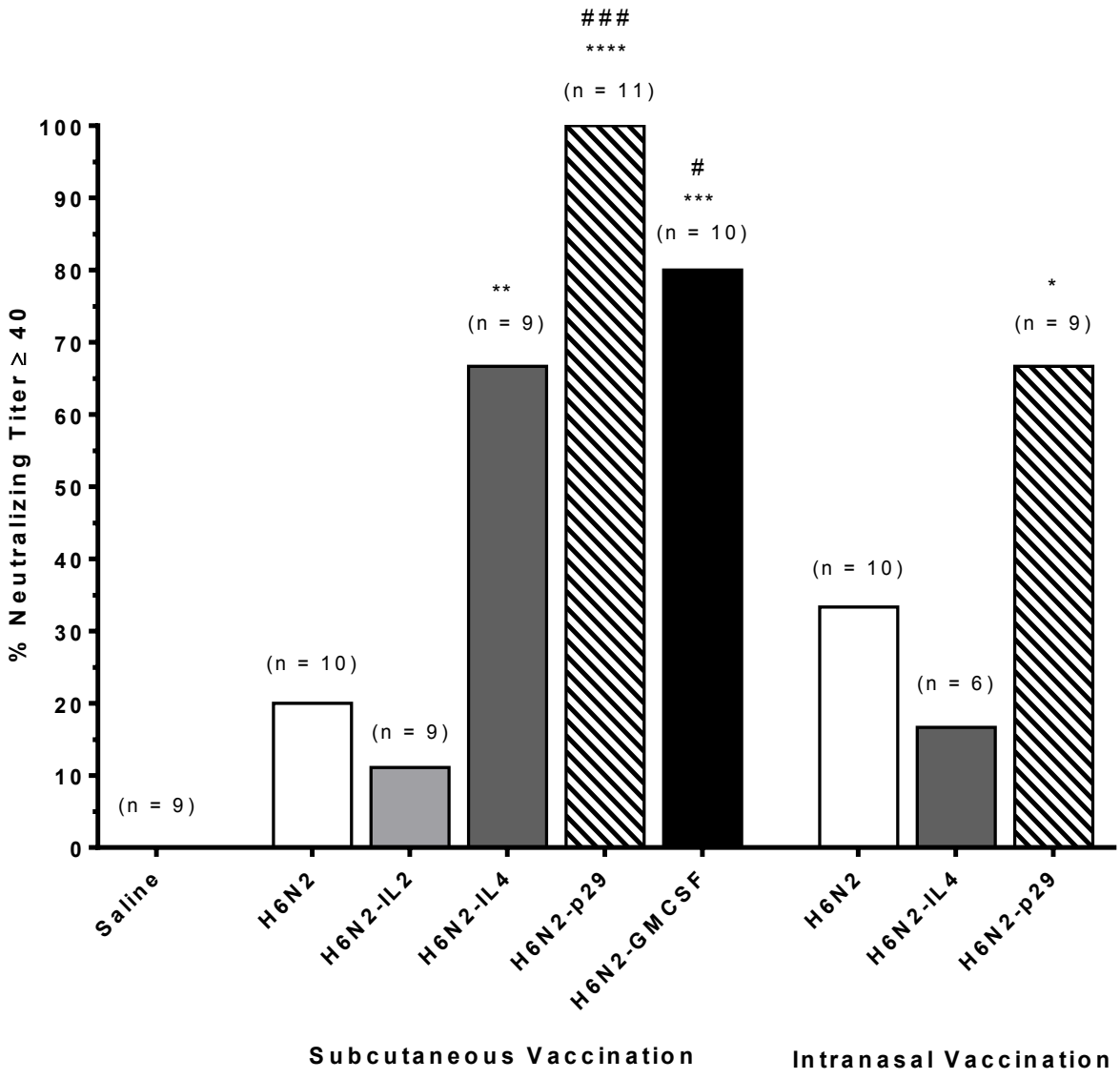


Figure 11. Percentage of chicks per group considered to have increased protective titers as determined by influenza virus neutralization assay following primary vaccination. (* = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$, **** = $p<0.00005$ compared to saline vaccinated chicks, # = $p<0.05$, ### = $p<0.0005$ compared to H6N2 subcutaneous vaccinated chicks)

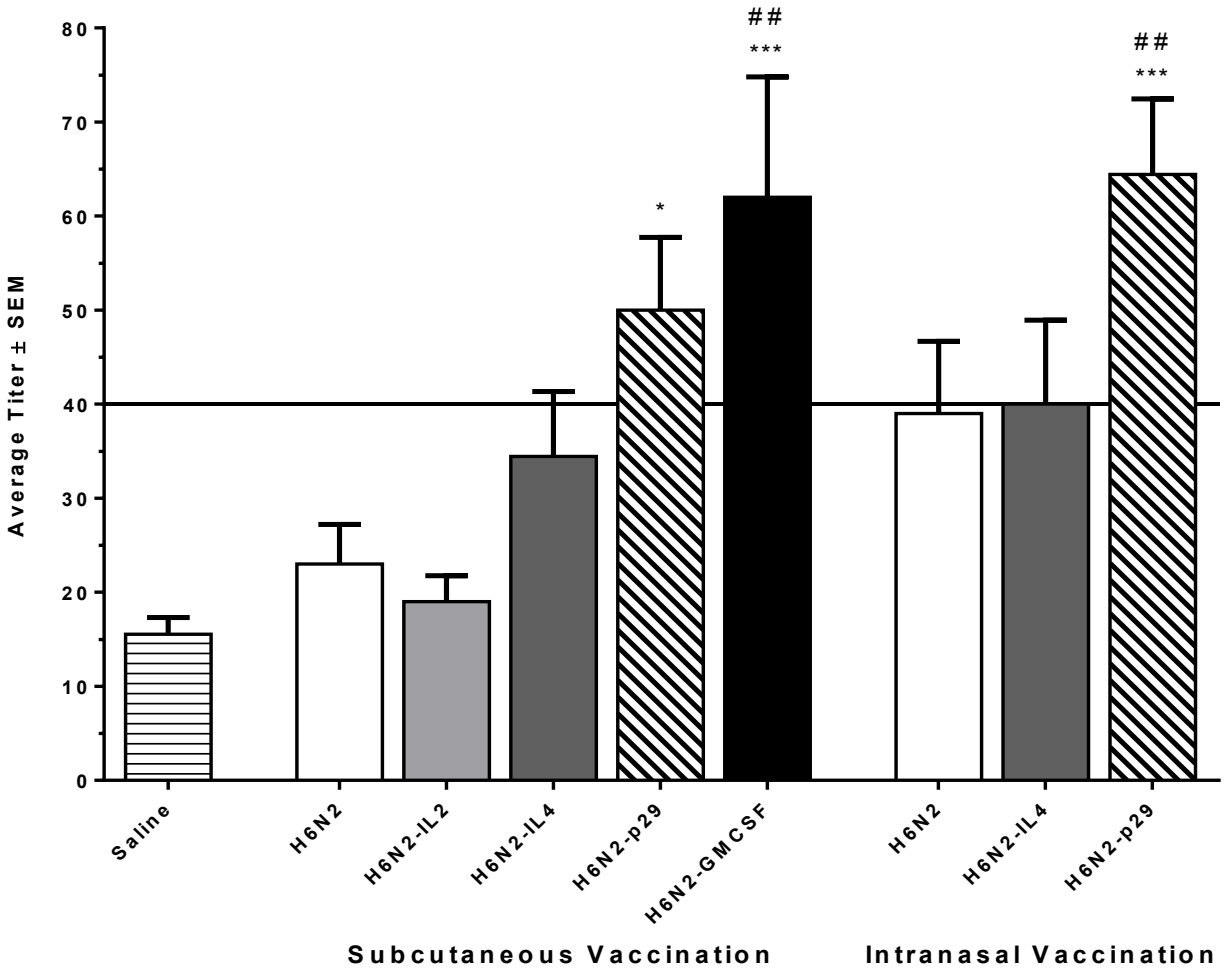


Figure 12. Average GMT following booster vaccination with 2000HAU at 35 days of age. Serum was collected 14 days after booster vaccination and titers were determined by HAI assay. HAI titers of 40 or greater were considered to be seroprotective. (* = $p<0.05$, *** = $p<0.0005$ compared to saline, ## = $p < 0.005$ compared to WT-H6N2 subcutaneous vaccinated chicks)

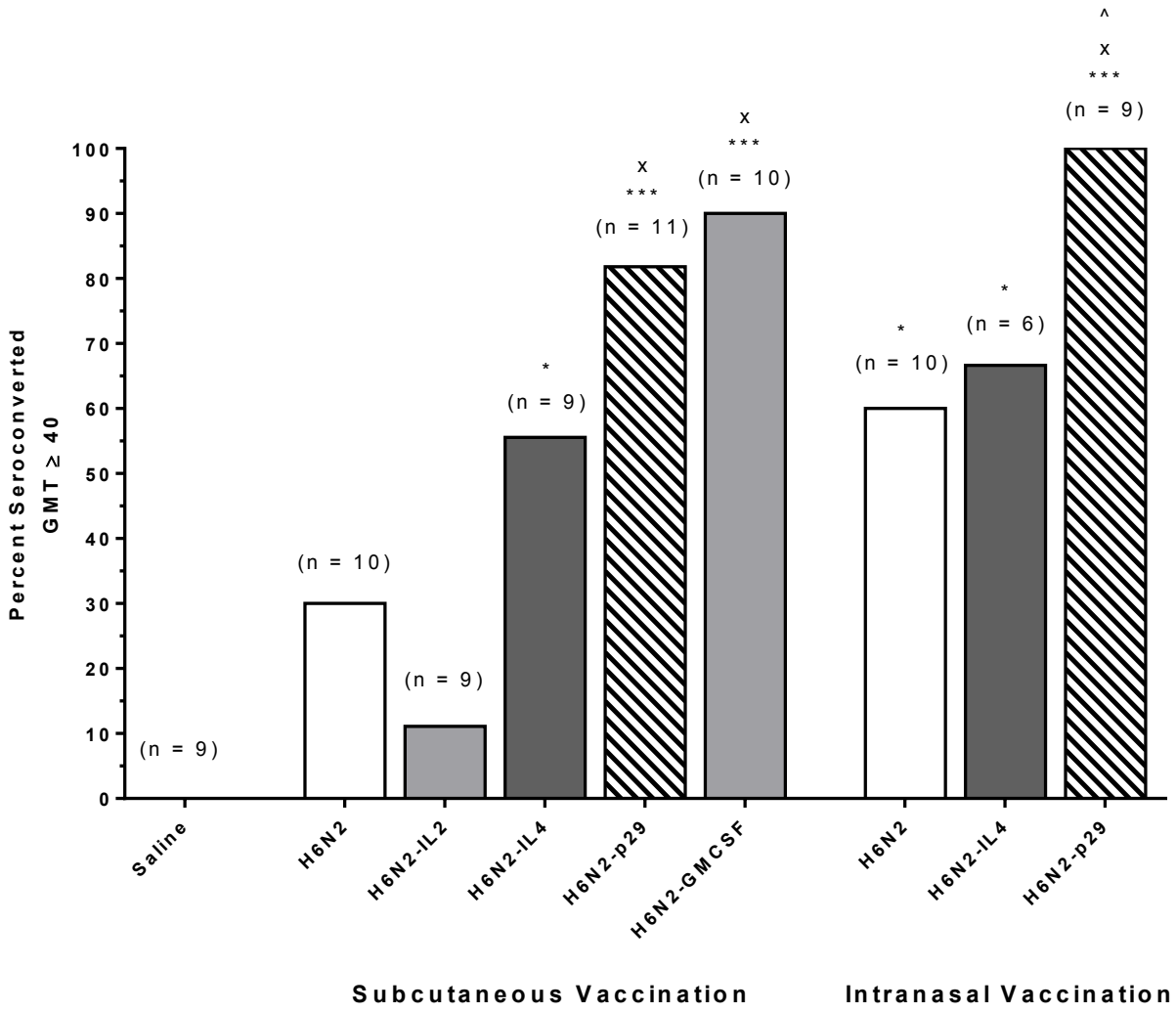


Figure 13. Percentage of chicks per group considered to have seroprotective titers as determined by HAI assay following booster vaccination. (* = $p<0.05$, *** = $p<0.0005$ compared to saline, x = $p<0.05$ compared to H6N2 subcutaneous, ^ = $p<0.05$ H6N2 intranasal vaccinated chicks)

those vaccinated subcutaneously with H6N2-p29x6 ($p<0.05$) and H6N2-GMCSF ($p<0.05$) and H6N2-p29x6 ($p<0.05$) had significantly higher seroconversion rates. Additionally, intranasal vaccination with H6N2-p29x6 resulted in a significantly higher seroconversion rate ($p<0.05$) than intranasal vaccination with H6N2.

Following challenge with live H6N2 all groups with the exception of saline controls had average HAI titers over 40 and at least 90% seroconversion (Figs. 14 & 15).

These results demonstrate that inactivated H6N2 vaccines with membrane-bound p29x6 intranasally and subcutaneously enhances antibody titers and seroconversion compared to standard inactivated H6N2. Additionally, adjuvanting with membrane-bound GMCSF results in a subcutaneous vaccine that increases antibody titers and seroconversion of subcutaneous produced by subcutaneous vaccination.

Viral Shedding Following Vaccination and Challenge: To assess the ability of the vaccine constructs to eliminate viral replication and shedding, oropharyngeal swabs were collected three days after challenge and tested for presence of H6N2 by RT-PCR. On average, chicks that were positive for virus had HAI titers of 21 while those that were negative averaged an HAI titer of 45 (Fig. 16). All saline vaccinated chicks challenged with live H6N2 were positive for H6N2 (Fig. 17). Each vaccinated group exhibited a significantly reduced shedding in comparison to saline treated chicks (Fig. 17). All chicks vaccinated with H6N2-p29X6 subcutaneously and H6N2 and H6N2-p29x6 intranasally completely eliminated viral shedding, a significant improvement over vaccination with subcutaneous H6N2 alone ($p<0.05$). Interestingly, H6N2 was more effective in eliminating viral shedding when delivered intranasally. These results indicate that p29x6 is an effective adjuvant for the reduction of viral shedding when delivered by either a subcutaneous or mucosal route.

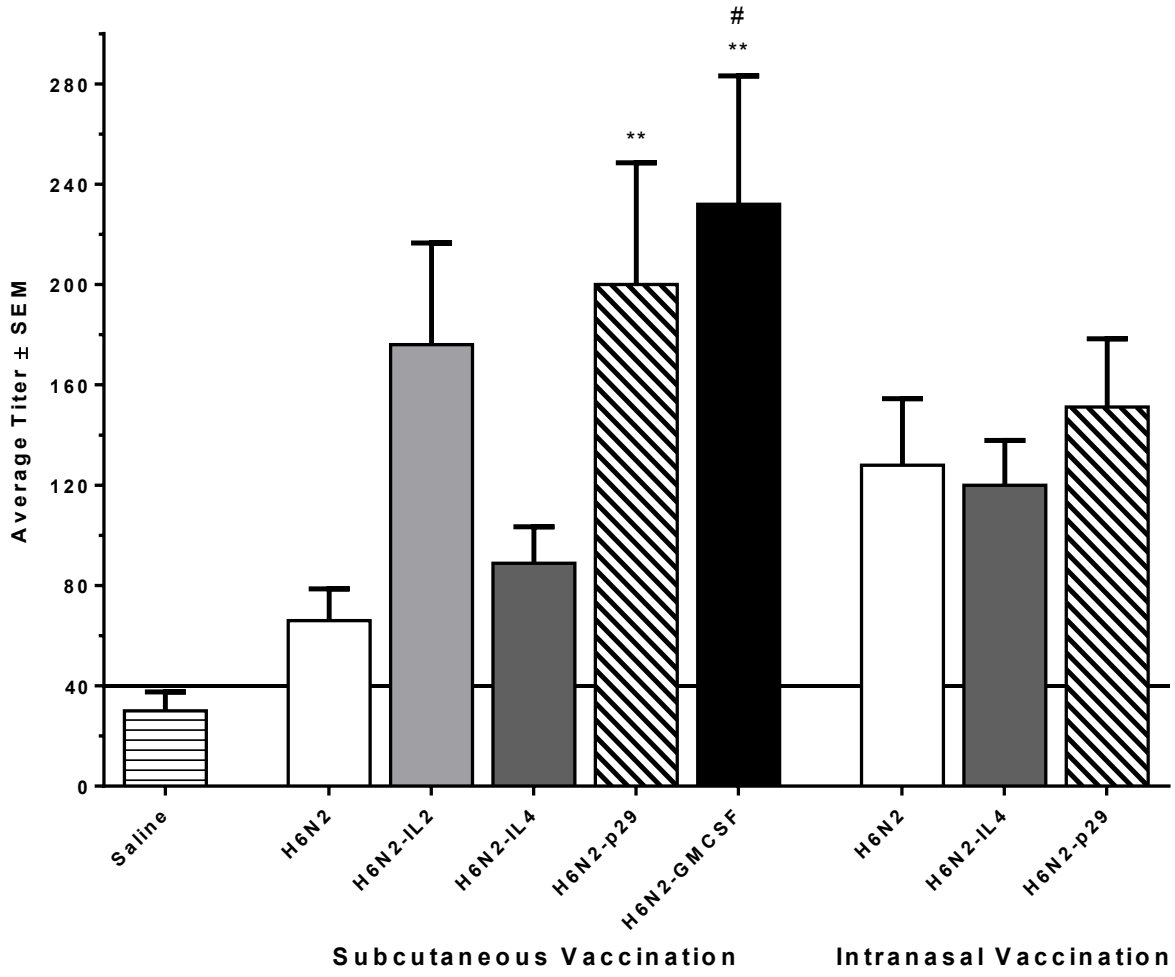


Figure 14. Average GMT following challenge with $5X10^6$ $TCID_{50}$ at 49 days of age. Serum was collected 7 days after challenge and titers were determined by HAI assay. HAI titers of 40 or greater were considered to be seroprotective. (** = $p<0.005$ compared to saline, # = $p < 0.05$ compared to WT-H6N2 subcutaneous vaccinated chicks). Note: all chicks, including the saline control group, were challenged with live virus.

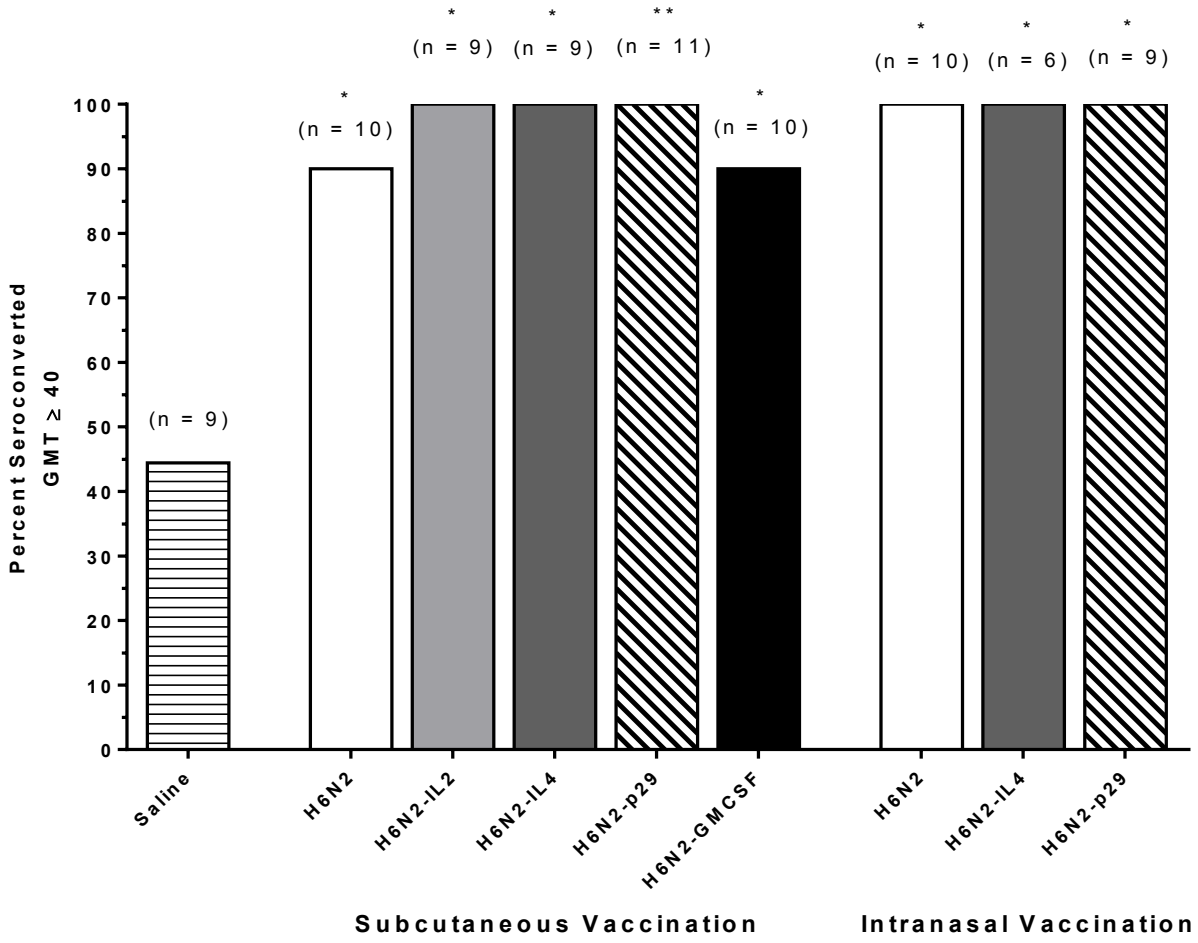


Figure 15. Percentage of chicks per group considered to have seroprotective titers as determined by HAI assay following live virus challenge. (* = $p<0.05$, ** = $p<0.005$ compared to saline vaccinated chicks). Note: all chicks, including the saline control group, were challenged with live virus.

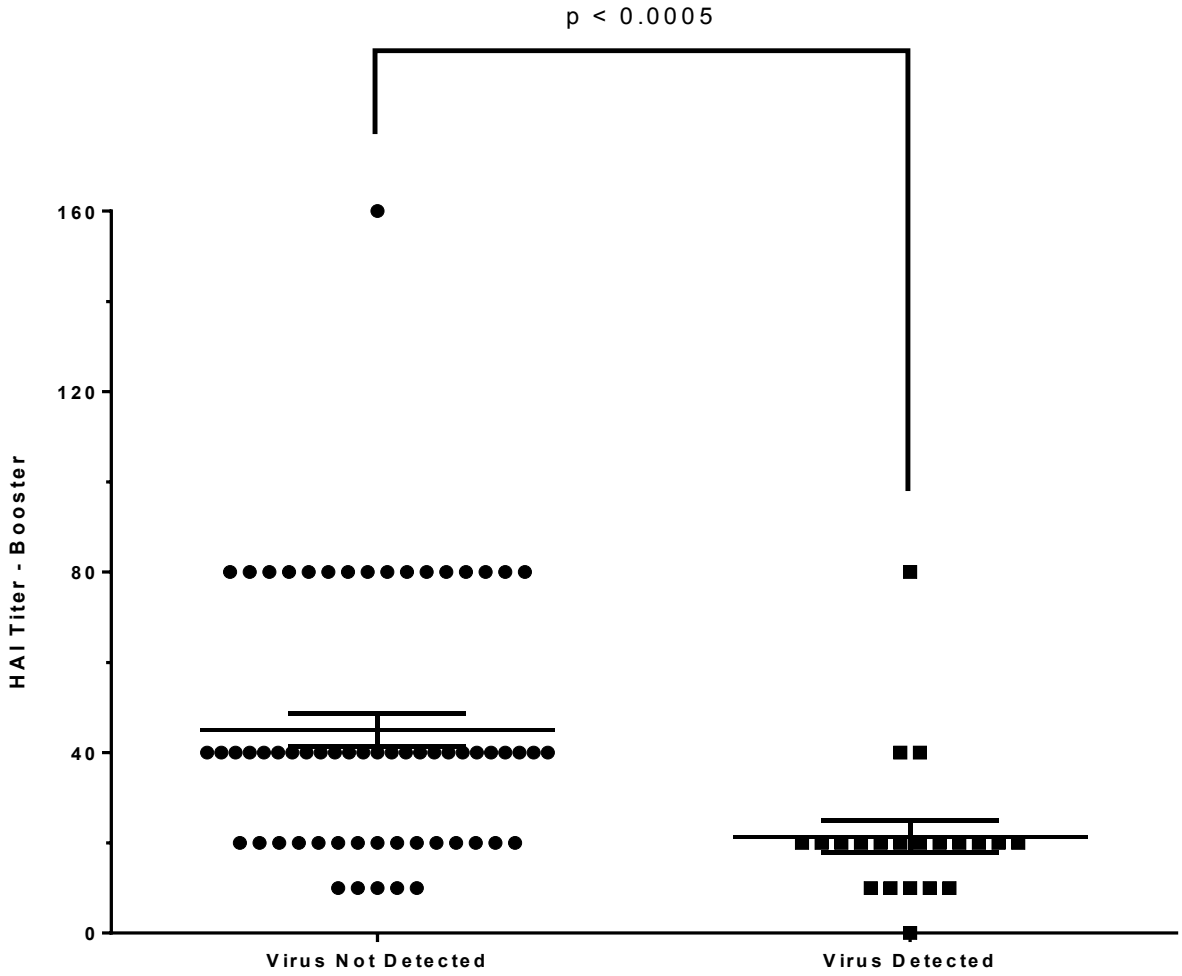


Figure 16. HAI titers following booster vaccination of chicks negative or positive for H6N2 three days following challenge. Oropharyngeal swabs were collected 3 days after challenge and tested for viral presence by RT-PCR.

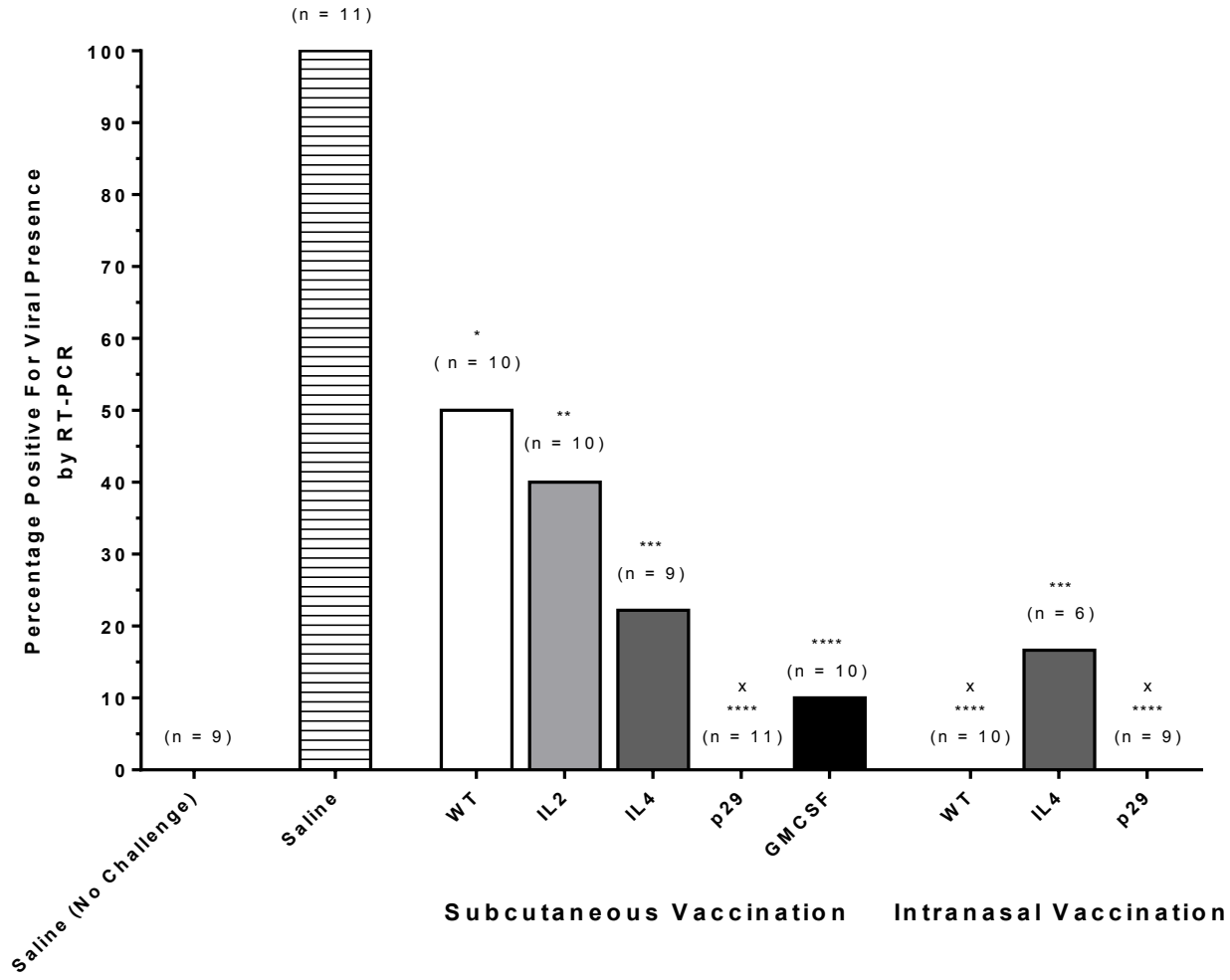


Figure 17. Percentage of birds testing positive for H6N2 by RT-PCR three days after challenge. (* = p<0.05, ** = p<0.005, *** = p <0.0005, **** = p <0000.5 compared to challenged birds vaccinated with saline, x = p<0.05 compared to WT-H6N2 subcutaneous vaccination)

Morbidity and Mortality: H6N2 typically results in mild or inapparent illness. All chicks were monitored for signs of illness, discomfort and weight loss. As expected, no chicks displayed signs of illness and all continued to gain weight after viral challenge (Fig. 18).

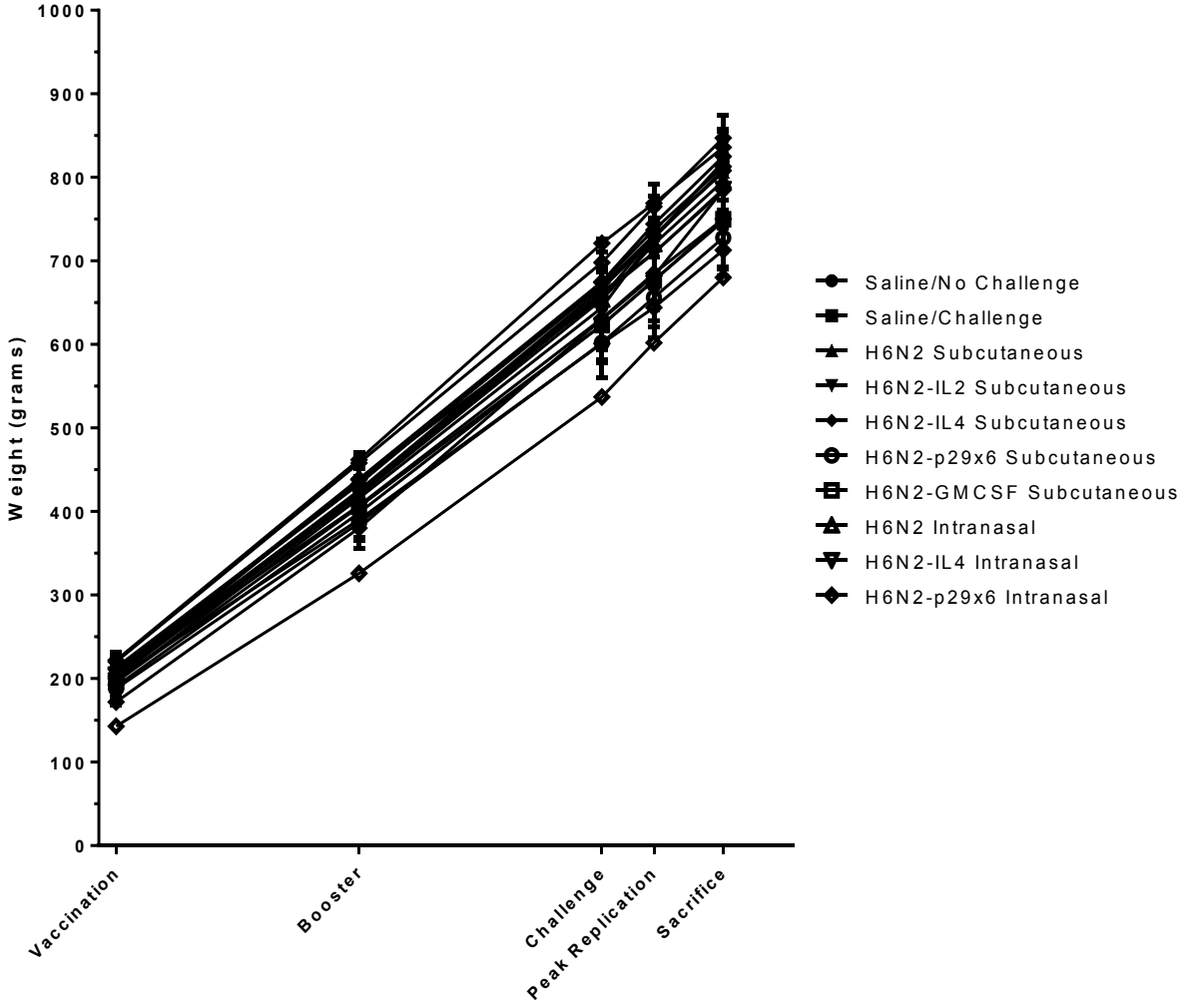


Figure 18. Weights of chicks in grams at vaccination (day 21), booster vaccination (day 35), live virus challenge (day 49), peak replication (day 52) and sacrifice (day 56).

Chapter 4

General Conclusions

Avian influenza viruses are a threat to global human and animal health. This danger has been exemplified by the multi-continent spread of highly pathogenic H5N1 and infections in both bird and human populations. The development of improved vaccines is crucial for control of influenza in poultry and for pandemic prevention and response in humans.

This study examined the efficacy of inactivated whole-virus avian influenza vaccines bearing four membrane-bound immunomodulators, produced in a mammalian cell culture-based system and tested in chickens. It was demonstrated previously that avian GMCSF and IL2 could be incorporated into filamentous and spherical influenza virions. This prior study found two doses of an inactivated vaccine consisting of filamentous human influenza virus bearing membrane-bound avian IL2 elicited stronger antibody responses than an unadjuvanted vaccine in chickens (215). This system was also successfully tested in a mouse model with murine IL2 and IL4 (216). This study builds upon these previous results by examining the effects of membrane-bound GMCSF, C3d, IL2 and IL4 on antibody response and viral clearance using avian influenza vaccine in an avian model. This study found that incorporation of membrane-bound avian GMCSF and C3d adjuvants into inactivated whole-virus avian influenza vaccines resulted in significantly enhanced antibody responses and significantly increased viral clearance when delivered subcutaneously (GMCSF and C3d) and intranasally (C3d) in comparison to an inactivated whole-virus vaccine without adjuvant.

A drawback of classic inactivated vaccines is the failure to effectively stimulate innate immune responses and lack of efficient uptake by antigen-presenting cells. The two most successful immunomodulators used in the study, C3d and GMCSF, both act on antigen

presenting cells. GMCSF stimulates the growth and activation of macrophages and dendritic cells and C3d links the innate and adaptive immune systems and increases uptake by follicular dendritic cells. The enhanced immune responses seen when C3d and GMCSF were incorporated into vaccine particles suggest that stimulating the growth and activation of antigen presenting cells and enhancing antigen uptake is key for improving inactivated vaccines. An additional reason to suspect that these immunomodulators act on APC's is that they are attached to killed influenza particles, which are not expected to persist for extended periods in the chickens. Consequently, it is suspected that virus particle interaction with APC's could be influenced by immunomodulators affecting APC's. However, it is also possible that C3d on the virus particles interacts with naïve or cross-reactive B cells of low affinity for influenza viral antigens.

In this study, IL4 and IL2 adjuvants were not able to significantly enhance immune responses as GMCSF and C3d did. IL4 moderately boosted antibody titers and seroconversion rates and increased viral clearance in subcutaneous vaccinations, however these results did not achieve statistical significance. These results did not match those of Herbert et al., which indicated that IL4 was a stronger adjuvant than GMCSF (216). This suggests differences in activity and potency of murine and avian membrane-bound IL4. Other possible explanations are that IL4 dosing must be refined in this system or that efficacy of IL4 adjuvantation may vary between influenza strains.

The failure of IL2 as an adjuvant in the subcutaneously delivered vaccine was surprising following the results from Yang et al. (215). Yang et al. found a significant increase in antibody titers in chicks following two doses of inactivated filamentous influenza bearing avian IL2. With the exception of the induction of post-challenge antibody titers, IL2 was a weak or completely ineffective adjuvant in our study. It should be noted that IL2 also boosted antibody responses

following challenge in our preliminary vaccination study however antibody levels were not tested following vaccination and prior to challenge. It is possible that the filamentous H3N2 A/Udorn/72 used in Yang et al. presents IL2 more efficiently than the spherical H6N2 used in this study. Testing of membrane-bound IL2 in other spherical influenza viruses should be undertaken. Additionally, dosage should be examined. Yang et al. used a lower dose of virus (and in turn IL2). As discussed above, high doses of certain cytokines can be inhibitory due to the induction of regulatory cells. A broad range of vaccine doses and IL2 levels should be examined to determine if an optimal, stimulatory dose can be found. Furthermore, IL2 and IL4 do not actively facilitate the uptake of antigen, a factor that may hinder their usefulness as solitary adjuvants. Future studies using this system should investigate combinations of adjuvants, particularly ones that stimulate antigen uptake in conjunction with those that act on T cells and B cells. For example, one might consider the use of APC immunomodulators in a primary vaccination followed by T and/or B cell immunomodulators in a booster vaccination.

An effective mucosal influenza vaccine would offer advantages in both agricultural and human practice. For poultry, this would allow for mass administration by aerosol reducing handling and labor costs. For humans it would have the potential to increase compliance rates in needlephobic patients. For both groups, a mucosal vaccination offers the potential for improved mucosal immune responses and neutralization of virus at the infection site. An important finding of this study was that C3d was highly effective as an adjuvant in intranasal vaccination with inactivated influenza virus. Each chick receiving H6N2-C3d via the intranasal route seroconverted and cleared the challenge virus and as a group, average antibody titers were significantly higher than inactivated virus without adjuvant delivered by subcutaneous and intranasal routes. These results indicate that C3d should be pursued further as a mucosal

adjuvant. Another interesting finding was that intranasal vaccination with inactivated virus resulted in a moderate (but not statistically significant) increase in antibody titers and a significant increase in viral clearance when delivered intranasally compared to subcutaneously. This suggests that even if manufacturers choose not to pursue adjuvantation, further study of inactivated intranasal influenza vaccines is warranted.

The standard egg-based influenza vaccine propagation system has many drawbacks, particularly long production times and capacity limitations. A key feature of this study is the utilization of a mammalian cell culture line for production of both virions and adjuvants. MDCK cells were successfully transfected with membrane-bound immunomodulators and virions derived from these cells were successfully used in a vaccine model. Membrane-bound immunomodulators should next be incorporated into suspension MDCK cell lines and MDCK cells modified to grow in serum-free medium to assess scalability and mass production.

While this study further validated the membrane-bound immunomodulator adjuvant model in influenza, in addition to the aforementioned suggestions, steps need to be taken before it can be approved for use on a wide scale in poultry. First, combinations of immunomodulators should be tested. Finding the right mix of these adjuvants could lead to even more effective vaccines. Second, this model must be tested using high pathogenicity AI viruses such as H5N1, H7N7 and H9N2 in BSL3 facilities before it can be deployed for widespread use. Third, studies should be undertaken to determine how much of each immunomodulator is expressed on the surface of each virion. Protocols will have to be standardized to produce vaccine particles bearing a consistent amount of adjuvant to appease regulatory agencies. Furthermore, it needs to be determined how the expression of HA or NA anchored immunomodulators impacts the quantity of HA or NA antigens on a virion. This is particularly important for the HA anchored

constructs as a significant drop in HA could severely reduce the immunogenicity of the vaccine. Finally, significant clinical and safety testing would need to be completed before consideration for use as a human vaccine.

In conclusion C3d and GMCSF are highly effective immunomodulatory adjuvants when tethered to the surface of influenza virions. These adjuvants and the cell culture-based production system offer to significantly enhance current influenza vaccines and have the potential for widespread use in the control of avian influenza.

REFERENCES

1. Lamb R, Krug R. Orthomyxoviridae: the viruses and their replication. In: Knipe D, Howley P, Griffin D, editors. Fields Virology 4th edn: Lippincott Williams & Wilkins; 2001. p. 1487 - 531.

2. Swayne DE, Halvorson DA. Influenza. In: Saif YM, Fadly AM, Glisson JR, McDougald LR, Nolan LK, Swayne DE, editors. Diseases of Poultry 12 ed. Ames, IA: Blackwell Publishing; 2008. p. 153-84.

3. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiological reviews. 1992;56(1):152-79. Epub 1992/03/01.

4. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science. 2006;312(5772):384-8. Epub 2006/04/22.

5. Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, et al. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS pathogens. 2007;3(5):e61. Epub 2007/05/16.

6. Suarez DL. Avian influenza: our current understanding. Animal health research reviews / Conference of Research Workers in Animal Diseases. 2010;11(1):19-33. Epub 2010/07/02.

7. Stallknecht DE, Brown JD. Ecology of avian influenza in wild birds. In: Swayne DE, editor. Avian Influenza. Ames, IA2008. p. 43–58.

8. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal of virology. 2005;79(5):2814-22. Epub 2005/02/15.

9. Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, et al. Influenza A viruses of migrating wild aquatic birds in North America. Vector Borne Zoonotic Dis. 2004;4(3):177-89. Epub 2005/01/06.

10. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, et al. A distinct lineage of influenza A virus from bats. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(11):4269-74. Epub 2012/03/01.

11. Cinatl J, Jr., Michaelis M, Doerr HW. The threat of avian influenza A (H5N1). Part I: Epidemiologic concerns and virulence determinants. Medical microbiology and immunology. 2007;196(4):181-90. Epub 2007/05/12.

12. van den Berg T, Lambrecht B, Marche S, Steensels M, Van Borm S, Bublot M. Influenza vaccines and vaccination strategies in birds. Comparative immunology, microbiology and infectious diseases. 2008;31(2-3):121-65. Epub 2007/09/25.

13. Horimoto T, Kawaoka Y. Pandemic threat posed by avian influenza A viruses. Clinical microbiology reviews. 2001;14(1):129-49. Epub 2001/01/09.

14. Webster RG, Peiris M, Chen H, Guan Y. H5N1 outbreaks and enzootic influenza. Emerging infectious diseases. 2006;12(1):3-8. Epub 2006/02/24.

15. Swayne DE, Suarez DL. Highly pathogenic avian influenza. Rev Sci Tech. 2000;19(2):463-82. Epub 2000/08/10.

16. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology. 1978;84(2):268-78. Epub 1978/02/01.

17. Keawcharoen J, van Riel D, van Amerongen G, Bestebroer T, Beyer WE, van Lavieren R, et al. Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). Emerging infectious diseases. 2008;14(4):600-7. Epub 2008/04/09.

18. Brown JD, Goekjian G, Poulson R, Valeika S, Stallknecht DE. Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. Veterinary microbiology. 2009;136:20-6.

19. Capua I, Marangon S. Control of avian influenza infections in poultry with emphasis on vaccination. Expert review of anti-infective therapy. 2006;4(5):751-7. Epub 2006/12/05.

20. Senne D. Avian influenza in North and South America, 2002–2005. Avian Diseases. 2007;51(Supplement 1):167-73.

21. Alexander DJ. Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian Diseases. 2007;51(Supplement 1):161-6.

22. Swayne DE, Slemons RD. Using mean infectious dose of high- and low-pathogenicity avian influenza viruses originating from wild duck and poultry as one measure of infectivity and adaptation to poultry. Avian Dis. 2008;52(3):455-60. Epub 2008/10/23.

23. Suarez DL. Evolution of avian influenza viruses. Veterinary microbiology. 2000;74(1-2):15-27. Epub 2000/05/09.

24. Perdue ML. Molecular determinants of pathogencity for avian influenza viruses. In: Swayne DE, editor. Avian Influenza. Ames, IA: Blackwell Publishing; 2008. p. 23-41.

25. Capua I, Alexander DJ. Human health implications of avian influenza viruses and paramyxoviruses. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 2004;23(1):1-6. Epub 2003/12/10.

26. Stieneke-Grober A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, et al. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. The EMBO journal. 1992;11(7):2407-14. Epub 1992/07/01.

27. Rott R. The pathogenic determinant of influenza virus. Veterinary microbiology. 1992;33(1-4):303-10. Epub 1992/11/01.

28. Suarez DL. Influenza A virus. In: Swayne DE, editor. Avian Influenza. Ames, IA: Blackwell Publishing; 2008. p. 3-22.

29. Matrosovich M, Zhou N, Kawaoka Y, Webster R. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. Journal of virology. 1999;73(2):1146-55. Epub 1999/01/09.

30. Liu C, Eichelberger MC, Compans RW, Air GM. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. Journal of virology. 1995;69(2):1099-106. Epub 1995/02/01.

31. Suzuki Y. Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biological & pharmaceutical bulletin. 2005;28(3):399-408. Epub 2005/03/04.

32. Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, Kobasa D, et al. Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. Journal of virology. 2000;74(13):6015-20. Epub 2000/06/14.

33. Munier S, Larcher T, Cormier-Aline F, Soubieux D, Su B, Guigand L, et al. A genetically engineered waterfowl influenza virus with a deletion in the stalk of the neuraminidase has increased virulence for chickens. Journal of virology. 2010;84(2):940-52. Epub 2009/11/06.

34. Spackman E, Swayne DE, Suarez DL, Senne DA, Pedersen JC, Killian ML, et al. Characterization of low-pathogenicity H5N1 avian influenza viruses from North America. Journal of virology. 2007;81(21):11612-9. Epub 2007/08/31.

35. Horimoto T, Kawaoka Y. Influenza: lessons from past pandemics, warnings from current incidents. Nature reviews Microbiology. 2005;3(8):591-600. Epub 2005/08/03.

36. Watanabe Y, Ibrahim MS, Suzuki Y, Ikuta K. The changing nature of avian influenza A virus (H5N1). Trends in microbiology. 2012;20(1):11-20. Epub 2011/12/14.

37. Avian Influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011: World Organisation for Animal Health.

38. Swayne DE, Pavade G, Hamilton K, Vallat B, Miyagishima K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. Rev Sci Tech. 2011;30(3):839-70. Epub 2012/03/23.

39. Xu X, Subbarao, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology. 1999;261(1):15-9. Epub 1999/09/15.

40. Webster RG. Influenza: an emerging disease. Emerging infectious diseases. 1998;4(3):436-41. Epub 1998/08/26.

41. Swayne DE, Kapczynski D. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunological reviews. 2008;225:314-31. Epub 2008/10/08.

42. Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang XW, et al. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science. 2005;309(5738):1206. Epub 2005/07/08.

43. Wang G, Zhan D, Li L, Lei F, Liu B, Liu D, et al. H5N1 avian influenza re-emergence of Lake Qinghai: phylogenetic and antigenic analyses of the newly isolated viruses and roles of migratory birds in virus circulation. The Journal of general virology. 2008;89(Pt 3):697-702. Epub 2008/02/15.

44. Lvov DK, Shchelkanov MY, Prilipov AG, Vlasov NA, Fedyakina IT, Deryabin PG, et al. Evolution of highly pathogenic avian influenza H5N1 virus in natural ecosystems of northern Eurasia (2005-08). Avian Dis. 2010;54(1 Suppl):483-95. Epub 2010/06/05.

45. Sharshov K, Silko N, Sousloparov I, Zaykovskaya A, Shestopalov A, Drozdov I. Avian influenza (H5N1) outbreak among wild birds, Russia, 2009. Emerging infectious diseases. 2010;16(2):349-51. Epub 2010/02/02.

46. H5N1 Highly Pathogenic Avian Influenza - Monthly Global Overview. 2012; Available from: http://www.fao.org/docrep/015/an388e/an388e.pdf.

47. Chmielewski R, Swayne DE. Avian Influenza: Public Health and Food Safety Concerns. Annual Review of Food Science. 2011;2:20.

48. McLeod A, Morgan N, Prakash A, Hinrichs J. Economic and Social Impacts of Avian Influenza. 2005 [cited 2012 August 25]; Available from: http://www.fao.org/docs/eims/upload//211939/Economic-and-social-impacts-of-avian-influenza-Geneva.pdf.

49. Chantong W, Kaneene JB. POULTRY RAISING SYSTEMS AND HIGHLY PATHOGENIC AVIAN INFLUENZA OUTBREAKS IN THAILAND: THE SITUATION, ASSOCIATIONS, AND IMPACTS. SOUTHEAST ASIAN J TROP MED PUBLIC HEALTH. 2011;42(3):13.

50. Delquigny T. Evolution and Impact of Avian Influenza Epidemic and Description of the Avian Production in Vietnam. Rome, Italy: 2004.

51. Medina RA, Garcia-Sastre A. Influenza A viruses: new research developments. Nature reviews Microbiology. 2011;9(8):590-603. Epub 2011/07/13.

52. Cox N, Uyeki TM. Avian Influenza. In: Swayne DE, editor. Avian Influenza. Ames, IA: Blackwell; 2008. p. 453-84.

53. Angata T, Varki A. Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. Chemical reviews. 2002;102(2):439-69. Epub 2002/02/14.

54. Ito T, Kawaoka Y. Host-range barrier of influenza A viruses. Veterinary microbiology. 2000;74(1-2):71-5. Epub 2000/05/09.

55. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual review of biochemistry. 2000;69:531-69. Epub 2000/08/31.

56. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Avian flu: influenza virus receptors in the human airway. Nature. 2006;440(7083):435-6. Epub 2006/03/24.

57. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, et al. H5N1 Virus Attachment to Lower Respiratory Tract. Science. 2006;312(5772):399. Epub 2006/03/25.

58. Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, et al. Dating the emergence of pandemic influenza viruses. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(28):11709-12. Epub 2009/07/15.

59. Poland GA. Vaccines against avian influenza--a race against time. The New England journal of medicine. 2006;354(13):1411-3. Epub 2006/03/31.

60. Wright P, Webster R. Orthomyxoviruses. In: Knipe D, Howley P, Griffin D, editors. Fields Virology 4th edn: Lippincott Williams & Wilkins; 2001. p. 1533 - 79.

61. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. Journal of virology. 1998;72(9):7367-73. Epub 1998/08/08.

62. Scholtissek C, Burger H, Kistner O, Shortridge KF. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology. 1985;147(2):287-94. Epub 1985/12/01.

63. Reid AH, Taubenberger JK, Fanning TG. The 1918 Spanish influenza: integrating history and biology. Microbes and infection / Institut Pasteur. 2001;3(1):81-7. Epub 2001/02/28.

64. Taubenberger JK, Reid AH, Janczewski TA, Fanning TG. Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2001;356(1416):1829-39. Epub 2002/01/10.

65. Taubenberger JK, Morens DM. 1918 Influenza: the mother of all pandemics. Emerging infectious diseases. 2006;12(1):15-22. Epub 2006/02/24.

66. A Potential Influenza Pandemic: Possible Macroeconomic Effects and Policy Issues. 2005.

67. de Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL. A pandemic warning? Nature. 1997;389(6651):554. Epub 1997/10/23 22:33.

68. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science. 1998;279(5349):393-6. Epub 1998/02/07.

69. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. 1998;351(9101):472-7. Epub 1998/03/03.

70. Yuen KY, Chan PK, Peiris M, Tsang DN, Que TL, Shortridge KF, et al. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet. 1998;351(9101):467-71. Epub 1998/03/03.

71. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2012. World Health Organization; 2012 [cited 2012 August 10]; Available from: http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html.

72. Doshi P. The elusive definition of pandemic influenza. Bulletin of the World Health Organization. 2011;89:532-8.

73. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. Science. 2012;336(6088):1534-41. Epub 2012/06/23.

74. Kandun IN, Wibisono H, Sedyaningsih ER, Yusharmen, Hadisoedarsuno W, Purba W, et al. Three Indonesian clusters of H5N1 virus infection in 2005. The New England journal of medicine. 2006;355(21):2186-94. Epub 2006/11/25.

75. Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). The New England journal of medicine. 2005;352(4):333-40. Epub 2005/01/26.

76. Wang H, Feng Z, Shu Y, Yu H, Zhou L, Zu R, et al. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. Lancet. 2008;371(9622):1427-34. Epub 2008/04/11.

77. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature. 2012;486(7403):420-8. Epub 2012/06/23.

78. Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, et al. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. Science. 2012;336(6088):1541-7. Epub 2012/06/23.

79. Peyre M, Fusheng G, Desvaux S, Roger F. Avian influenza vaccines: a practical review in relation to their application in the field with a focus on the Asian experience. Epidemiology and infection. 2009;137(1):1-21. Epub 2008/08/15.

80. Bouma A, Chen H, Erasmus B, Jones P, Marangon S, Domenech J. Avian Influenza Vaccination. OIE/FAO/IZSVe Scientific Conference; March 20-22,2007; Verona, Italy2007.

81. Schudel AA, Lombard M. Recommendations of the OIE International Conference on the Control of Infectious Animal Diseases by Vaccination, Buenos Aires, Argentina, 13 to 16 April 2004. Rev Sci Tech. 2007;26(2):519-21. Epub 2007/09/26.

82. Capua I, Marangon S. Vaccination policy applied for the control of avian influenza in Italy. Developments in biologicals. 2003;114:213-9. Epub 2003/12/18.

83. Capua I, Marangon S. Control and prevention of avian influenza in an evolving scenario. Vaccine. 2007;25(30):5645-52. Epub 2006/12/16.

84. Bruschke C, Bruckner G, Vallat B. International standards and guidelines for vaccination of poultry against highly pathogenic avian influenza. Developments in biologicals. 2007;130:23-30. Epub 2008/04/17.

85. Bourlet Y, Behar G, Guillemot F, Frechin N, Billault A, Chausse AM, et al. Isolation of chicken major histocompatibility complex class II (B-L) beta chain sequences: comparison with mammalian beta chains and expression in lymphoid organs. The EMBO journal. 1988;7(4):1031-9. Epub 1988/04/01.

86. Kaiser P, Poh TY, Rothwell L, Avery S, Balu S, Pathania US, et al. A genomic analysis of chicken cytokines and chemokines. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2005;25(8):467-84. Epub 2005/08/20.

87. Ratcliffe MJ, Lassila O, Reynolds J, Pink JR, Vainio O. A re-evaluation of the function of the bursa of Fabricius. Progress in clinical and biological research. 1987;238:3-14. Epub 1987/01/01.

88. Sharma JM. Introduction to poultry vaccines and immunity. Advances in veterinary medicine. 1999;41:481-94. Epub 1999/01/16.

89. Avery S, Rothwell L, Degen WD, Schijns VE, Young J, Kaufman J, et al. Characterization of the first nonmammalian T2 cytokine gene cluster: the cluster contains functional single-copy genes for IL-3, IL-4, IL-13, and GM-CSF, a gene for IL-5 that appears to be a pseudogene, and a gene encoding another cytokinelike transcript, KK34. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2004;24(10):600-10. Epub 2005/01/01.

90. Lundqvist ML, Middleton DL, Radford C, Warr GW, Magor KE. Immunoglobulins of the non-galliform birds: antibody expression and repertoire in the duck. Developmental and comparative immunology. 2006;30(1-2):93-100. Epub 2005/09/10.

91. Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scandinavian journal of immunology. 2004;59(1):1-15. Epub 2004/01/16.

92. Potter C. Chronicle of influenza pandemics. In: Nicholson K, Webster R, Hay A, editors. Textbook of Influenza. Oxford, UK: Blackwell Science; 1998. p. 3-18.

93. Ainai A, Tashiro M, Hasegawa H. Cross-protective immunity against influenza virus infections induced by intranasal vaccination together with a TLR3-mucosal adjuvant. Human vaccines. 2011;7 Suppl:174-82. Epub 2011/02/16.

94. Nicholls EF, Madera L, Hancock RE. Immunomodulators as adjuvants for vaccines and antimicrobial therapy. Annals of the New York Academy of Sciences. 2010;1213:46-61. Epub 2010/10/16.

95. Spickler AR, Roth JA. Adjuvants in veterinary vaccines: modes of action and adverse effects. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine. 2003;17(3):273-81. Epub 2003/05/31.

96. Tovey MG, Lallemand C. Adjuvant activity of cytokines. Methods Mol Biol. 2010;626:287-309. Epub 2010/01/26.

97. Akira S. Innate immunity and adjuvants. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2011;366(1579):2748-55. Epub 2011/09/07.

98. Cretescu L, Beare AS, Schild GC. Formation of antibody to matrix protein in experimental human influenza A virus infections. Infection and immunity. 1978;22(2):322-7. Epub 1978/11/01.

99. Potter CW, Oxford JS. Determinants of immunity to influenza infection in man. British medical bulletin. 1979;35(1):69-75. Epub 1979/01/01.

100. Barrault DV, Steward M, Cox VF, Smith RA, Knight AM. Efficient production of complement (C3d)3 fusion proteins using the baculovirus expression vector system. Journal of immunological methods. 2005;304(1-2):158-73. Epub 2005/08/20.

101. Askonas B, McMichael A, Webster R. The immune response to influenza viruses and the problem of protection against infection. In: Beare AS, editor. Basic and Applied Influenza Research. Boca Raton, FL: CRC Press; 1982. p. 159-88.

102. Artenstein MS, Bellanti JA, Buescher EL. Identification of the Antiviral Substances in Nasal Secretions. Proc Soc Exp Biol Med. 1964;117:558-64. Epub 1964/11/01.

103. Murphy BR, Chalhub EG, Nusinoff SR, Kasel J, Chanock RM. Temperature-sensitive mutants of influenza virus. 3. Further characterization of the ts-1(E) influenza A recombinant (H3N2) virus in man. The Journal of infectious diseases. 1973;128(4):479-87. Epub 1973/10/01.

104. Mazanec MB, Coudret CL, Fletcher DR. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. Journal of virology. 1995;69(2):1339-43. Epub 1995/02/01.

105. Rossen RD, Butler WT, Waldman RH, Alford RH, Hornick RB, Togo Y, et al. The proteins in nasal secretion. II. A longitudinal study of IgA and neutralizing antibody levels in nasal washings from men infected with influenza virus. JAMA : the journal of the American Medical Association. 1970;211(7):1157-61. Epub 1970/02/16.

106. Murphy BR, Clements ML. The systemic and mucosal immune response of humans to influenza A virus. Current topics in microbiology and immunology. 1989;146:107-16. Epub 1989/01/01.

107. Wright PF, Murphy BR, Kervina M, Lawrence EM, Phelan MA, Karzon DT. Secretory immunological response after intranasal inactivated influenza A virus vaccinations: evidence for immunoglobulin A memory. Infection and immunity. 1983;40(3):1092-5. Epub 1983/06/01.

108. Brokstad KA, Cox RJ, Eriksson JC, Olofsson J, Jonsson R, Davidsson A. High prevalence of influenza specific antibody secreting cells in nasal mucosa. Scandinavian journal of immunology. 2001;54(1-2):243-7. Epub 2001/07/06.

109. Cox NJ, Subbarao K. Influenza. Lancet. 1999;354(9186):1277-82. Epub 1999/10/16.

110. Cox RJ, Brokstad KA, Zuckerman MA, Wood JM, Haaheim LR, Oxford JS. An early humoral immune response in peripheral blood following parenteral inactivated influenza vaccination. Vaccine. 1994;12(11):993-9. Epub 1994/08/01.

111. Callard RE. Specific in vitro antibody response to influenza virus by human blood lymphocytes. Nature. 1979;282(5740):734-6. Epub 1979/12/13.

112. Yarchoan R, Murphy BR, Strober W, Schneider HS, Nelson DL. Specific anti-influenza virus antibody production in vitro by human peripheral blood mononuclear cells. J Immunol. 1981;127(6):2588-94. Epub 1981/12/01.

113. Couch RB, Kasel JA. Immunity to influenza in man. Annual review of microbiology. 1983;37:529-49. Epub 1983/01/01.

114. Brown TA, Murphy BR, Radl J, Haaijman JJ, Mestecky J. Subclass distribution and molecular form of immunoglobulin A hemagglutinin antibodies in sera and nasal secretions after experimental secondary infection with influenza A virus in humans. Journal of clinical microbiology. 1985;22(2):259-64. Epub 1985/08/01.

115. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. Emerging infectious diseases. 2006;12(1):48-54. Epub 2006/02/24.

116. Quinnan GV, Ennis FA, Tuazon CU, Wells MA, Butchko GM, Armstrong R, et al. Cytotoxic lymphocytes and antibody-dependent complement-mediated cytotoxicity induced by administration of influenza vaccine. Infection and immunity. 1980;30(2):362-9. Epub 1980/11/01.

117. Ruben FL, Bachmayer H, Fireman P. Parameters of humoral and cellular immunity of influenza A/USSR/90/77 (H1N1) virus in various age groups. The Journal of infectious diseases. 1980;142(5):699-703. Epub 1980/11/01.

118. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. The New England journal of medicine. 1983;309(1):13-7. Epub 1983/07/07.

119. McMichael AJ, Michie CA, Gotch FM, Smith GL, Moss B. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. The Journal of general virology. 1986;67 (Pt 4):719-26. Epub 1986/04/01.

120. Ennis FA, Rook AH, Qi YH, Schild GC, Riley D, Pratt R, et al. HLA restricted virus-specific cytotoxic T-lymphocyte responses to live and inactivated influenza vaccines. Lancet. 1981;2(8252):887-91. Epub 1981/10/24.

121. Sun Y, Liu J, Yang M, Gao F, Zhou J, Kitamura Y, et al. Identification and structural definition of H5-specific CTL epitopes restricted by HLA-A*0201 derived from the H5N1 subtype of influenza A viruses. The Journal of general virology. 2010;91(Pt 4):919-30. Epub 2009/12/04.

122. Ennis FA, Yi-Hua Q, Schild GC. Antibody and cytotoxic T lymphocyte responses of humans to live and inactivated influenza vaccines. The Journal of general virology. 1982;58(Pt 2):273-81. Epub 1982/02/01.

123. Brown DM, Roman E, Swain SL. CD4 T cell responses to influenza infection. Seminars in immunology. 2004;16(3):171-7. Epub 2004/05/08.

124. Moran TM, Park H, Fernandez-Sesma A, Schulman JL. Th2 responses to inactivated influenza virus can Be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection. The Journal of infectious diseases. 1999;180(3):579-85. Epub 1999/08/07.

125. Belz GT, Wodarz D, Diaz G, Nowak MA, Doherty PC. Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. Journal of virology. 2002;76(23):12388-93. Epub 2002/11/05.

126. Belz GT, Liu H, Andreansky S, Doherty PC, Stevenson PG. Absence of a functional defect in CD8+ T cells during primary murine gammaherpesvirus-68 infection of I-A(b-/-) mice. The Journal of general virology. 2003;84(Pt 2):337-41. Epub 2003/02/01.

127. Riberdy JM, Christensen JP, Branum K, Doherty PC. Diminished primary and secondary influenza virus-specific CD8(+) T-cell responses in CD4-depleted Ig(-/-) mice. Journal of virology. 2000;74(20):9762-5. Epub 2000/09/23.

128. Cardin RD, Brooks JW, Sarawar SR, Doherty PC. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. The Journal of experimental medicine. 1996;184(3):863-71. Epub 1996/09/01.

129. Brooks JW, Hamilton-Easton AM, Christensen JP, Cardin RD, Hardy CL, Doherty PC. Requirement for CD40 ligand, CD4(+) T cells, and B cells in an infectious mononucleosis-like syndrome. Journal of virology. 1999;73(11):9650-4. Epub 1999/10/09.

130. Bowersock TL, Martin S. Vaccine delivery to animals. Advanced drug delivery reviews. 1999;38(2):167-94. Epub 2000/06/06.

131. Responsible use of vaccines and vaccinations in farm animal production. 2006 [cited 2012 July 24]; Available from: http://www.ruma.org.uk/guidelines/vaccines/long/farm%20vaccine%20long.pdf.

132. Roth JA. Mechanistic bases for adverse vaccine reactions and vaccine failures. Advances in veterinary medicine. 1999;41:681-700. Epub 1999/01/16.

133. Hai R, Garcia-Sastre A, Swayne DE, Palese P. A reassortment-incompetent live attenuated influenza virus vaccine for protection against pandemic virus strains. Journal of virology. 2011;85(14):6832-43. Epub 2011/05/06.

134. Murphy BR. Mucosal Immunity to Viruses. In: Ogra P, Mestecky J, Lamm ME, Strober W, McGee J, Bienenstock J, editors. Handbook of Mucosal Immunology. San Diego, CA: Academic Press; 1994. p. 333-43.

135. Keitel W, Piedra P. Live cold-adapted, reassortant inflenza vaccines (USA). In: Nicholson K, Webster R, Hay R, editors. Textbook of Influenza. London: Blackwell Scientific Publications; 1998. p. 373-90.

136. Fiore AE, Shay DK, Broder K, Iskander JK, Uyeki TM, Mootrey G, et al. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control. 2009;58(RR-8):1-52. Epub 2009/08/01.

137. Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, Uematsu S, et al. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol. 2007;179(7):4711-20. Epub 2007/09/20.

138. Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Japanese journal of infectious diseases. 2005;58(4):195-207. Epub 2005/08/24.

139. Beyer WE, Palache AM, de Jong JC, Osterhaus AD. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis. Vaccine. 2002;20(9-10):1340-53. Epub 2002/01/31.

140. Jefferson T, Rivetti D, Rivetti A, Rudin M, Di Pietrantonj C, Demicheli V. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. Lancet. 2005;366(9492):1165-74. Epub 2005/10/04.

141. Nichol KL, Wuorenma J, von Sternberg T. Benefits of influenza vaccination for low-, intermediate-, and high-risk senior citizens. Archives of internal medicine. 1998;158(16):1769-76. Epub 1998/09/17.

142. Heegaard PM, Dedieu L, Johnson N, Le Potier MF, Mockey M, Mutinelli F, et al. Adjuvants and delivery systems in veterinary vaccinology: current state and future developments. Archives of virology. 2011;156(2):183-202. Epub 2010/12/21.

143. Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scandinavian journal of immunology. 2005;62(1):36-44. Epub 2005/08/12.

144. Brown DW, Kawaoka Y, Webster RG, Robinson HL. Assessment of retrovirus-expressed nucleoprotein as a vaccine against lethal influenza virus infections of chickens. Avian Dis. 1992;36(3):515-20. Epub 1992/07/01.

145. Webster RG, Kawaoka Y, Taylor J, Weinberg R, Paoletti E. Efficacy of nucleoprotein and haemagglutinin antigens expressed in fowlpox virus as vaccine for influenza in chickens. Vaccine. 1991;9(5):303-8. Epub 1991/05/01.

146. Kim JK, Kayali G, Walker D, Forrest HL, Ellebedy AH, Griffin YS, et al. Puzzling inefficiency of H5N1 influenza vaccines in Egyptian poultry. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(24):11044-9. Epub 2010/06/11.

147. Thompson AL, Staats HF. Cytokines: the future of intranasal vaccine adjuvants. Clinical & developmental immunology. 2011;2011:289597. Epub 2011/08/10.

148. Pizza M, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan G, et al. Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. Vaccine. 2001;19(17-19):2534-41. Epub 2001/03/21.

149. Pavot V, Rochereau N, Genin C, Verrier B, Paul S. New insights in mucosal vaccine development. Vaccine. 2012;30(2):142-54. Epub 2011/11/17.

150. Gerdil C. The annual production cycle for influenza vaccine. Vaccine. 2003;21(16):1776-9. Epub 2003/04/11.

151. Pandemic influenza vaccine manufacturing process and timeline. 2009 [updated 6 August 2009; cited 2012 August 21]; Available from: http://www.who.int/csr/disease/swineflu/notes/h1n1_vaccine_20090806/en/index.html.

152. Ellebedy AH, Webby RJ. Influenza vaccines. Vaccine. 2009;27 Suppl 4:D65-8. Epub 2009/10/20.

153. Matthews J. Egg-Based Production of Influenza Vaccine: 30 Years of Commercial Experience. The Bridge. 2006;36(3):17-24.

154. Wood JM, Levandowski RA. The influenza vaccine licensing process. Vaccine. 2003;21(16):1786-8. Epub 2003/04/11.

155. Avian Influenza Vaccine Producers and Suppliers for Poultry. 2009 [22 August 2012]; Available from: ftp://ftp.fao.org/docrep/fao/011/ai326e/ai326e00.pdf.

156. Avian Influenza. OIE Terrestrial Manual 2012: World Organisation for Animal Health; 2012.

157. Swayne DE, Kapczynski D. Vaccines, vaccination, and immunology for avian influenza viruses in poultry. In: Swayne DE, editor. Avian Influenza. Ames, IA: Wiley-Blackwell; 2008. p. 407-51.

158. Garcia A, Johnson H, Srivastava DK, Jayawardene DA, Wehr DR, Webster RG. Efficacy of inactivated H5N2 influenza vaccines against lethal A/Chicken/Queretaro/19/95 infection. Avian Dis. 1998;42(2):248-56. Epub 1998/06/30.

159. An HHS Retrospective on the 2009 H1N1 Influenza Pandemic to Advance All Hazards Preparedness. 2012.

160. Vaccine Production in Cells. United States Department of Health and Human Services; [cited 2012 August 22]; Available from: http://www.flu.gov/planning-preparedness/federal/vproductioncells.html.

161. Pandemic influenza vaccines: current status. Geneva, Switzerland2009 [cited 2012 August 10]; Available from: http://www.who.int/csr/disease/swineflu/notes/pandemic_influenza_vaccines_20090924/en/index.html.

162. Perdue ML, Arnold F, Li S, Donabedian A, Cioce V, Warf T, et al. The future of cell culture-based influenza vaccine production. Expert review of vaccines. 2011;10(8):1183-94. Epub 2011/08/23.

163. Gregersen JP, Schmitt HJ, Trusheim H, Broker M. Safety of MDCK cell culture-based influenza vaccines. Future microbiology. 2011;6(2):143-52. Epub 2011/03/04.

164. Devuyst O, Beauwens R, Denef JF, Crabbe J, Abramow M. Subtypes of Madin-Darby canine kidney (MDCK) cells defined by immunocytochemistry: further evidence for properties of renal collecting duct cells. Cell and tissue research. 1994;277(2):231-7. Epub 1994/08/01.

165. Oxford JS, Corcoran T, Knott R, Bates J, Bartolomei O, Major D, et al. Serological studies with influenza A(H1N1) viruses cultivated in eggs or in a canine kidney cell line (MDCK). Bulletin of the World Health Organization. 1987;65(2):181-7. Epub 1987/01/01.

166. Schild GC, Oxford JS, de Jong JC, Webster RG. Evidence for host-cell selection of influenza virus antigenic variants. Nature. 1983;303(5919):706-9. Epub 1983/06/23.

167. Robertson J. Clinical influenza virus and the embryonated hen's egg. Medical Virology. 1993;3:97-106.

168. Katz JM, Webster RG. Efficacy of inactivated influenza A virus (H3N2) vaccines grown in mammalian cells or embryonated eggs. The Journal of infectious diseases. 1989;160(2):191-8. Epub 1989/08/01.

169. Trusheim H, Wilms BRR, Jung S, Muth R, Eggers M, Enders M, et al., editors. The MDCK 33016PF cell line is not only suitable for production of a cell-based influenza vaccine but is also an ideal substrate for influenza virus isolation. Options for Control of Influenza VI Conference; 2007; Toronto, Canada.

170. Gregersen JP. A quantitative risk assessment of exposure to adventitious agents in a cell culture-derived subunit influenza vaccine. Vaccine. 2008;26(26):3332-40. Epub 2008/05/20.

171. Onions D, Egan W, Jarrett R, Novicki D, Gregersen JP. Validation of the safety of MDCK cells as a substrate for the production of a cell-derived influenza vaccine. Biologicals : journal of the International Association of Biological Standardization. 2010;38(5):544-51. Epub 2010/06/12.

172. Ambrozaitis A, Groth N, Bugarini R, Sparacio V, Podda A, Lattanzi M. A novel mammalian cell-culture technique for consistent production of a well-tolerated and immunogenic trivalent subunit influenza vaccine. Vaccine. 2009;27(43):6022-9. Epub 2009/08/12.

173. Szymczakiewicz-Multanowska A, Groth N, Bugarini R, Lattanzi M, Casula D, Hilbert A, et al. Safety and immunogenicity of a novel influenza subunit vaccine produced in mammalian cell culture. The Journal of infectious diseases. 2009;200(6):841-8. Epub 2009/08/14.

174. Reisinger KS, Block SL, Izu A, Groth N, Holmes SJ. Subunit influenza vaccines produced from cell culture or in embryonated chicken eggs: comparison of safety, reactogenicity, and immunogenicity. The Journal of infectious diseases. 2009;200(6):849-57. Epub 2009/08/14.

175. Frey S, Vesikari T, Szymczakiewicz-Multanowska A, Lattanzi M, Izu A, Groth N, et al. Clinical efficacy of cell culture-derived and egg-derived inactivated subunit influenza vaccines in healthy adults.

Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2010;51(9):997-1004. Epub 2010/09/28.

176. Guy B. The perfect mix: recent progress in adjuvant research. Nature reviews Microbiology. 2007;5(7):505-17. Epub 2007/06/15.

177. Vaccine Safety and Availability. Silver Spring, MD2011 [updated July 7, 2011; cited 2012 July 16]; Available from: http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/VaccineSafety/ucm187810.htm.

178. Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. Vaccine. 2005;23(13):1588-95. Epub 2005/02/08.

179. Wheeler AW, Woroniecki SR. Allergy vaccines--new approaches to an old concept. Expert opinion on biological therapy. 2004;4(9):1473-81. Epub 2004/09/01.

180. Asif M, Jenkins KA, Hilton LS, Kimpton WG, Bean AG, Lowenthal JW. Cytokines as adjuvants for avian vaccines. Immunology and cell biology. 2004;82(6):638-43. Epub 2004/11/20.

181. O'Hagan DT. Recent advances in vaccine adjuvants for systemic and mucosal administration. The Journal of pharmacy and pharmacology. 1998;50(1):1-10. Epub 1998/03/21.

182. Cox JC, Coulter AR. Adjuvants--a classification and review of their modes of action. Vaccine. 1997;15(3):248-56. Epub 1997/02/01.

183. Gupta RK. Aluminum compounds as vaccine adjuvants. Advanced drug delivery reviews. 1998;32(3):155-72. Epub 2000/06/06.

184. Gupta RK, Siber GR. Adjuvants for human vaccines--current status, problems and future prospects. Vaccine. 1995;13(14):1263-76. Epub 1995/10/01.

185. Squalene-based adjuvants in vaccines. 2006 [updated December 2008; cited 2012 July 18]; Available from: http://www.who.int/vaccine_safety/topics/adjuvants/squalene/questions_and_answers/en/.

186. Herzog C, Hartmann K, Kunzi V, Kursteiner O, Mischler R, Lazar H, et al. Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine. Vaccine. 2009;27(33):4381-7. Epub 2009/05/20.

187. Hodge LM, Marinaro M, Jones HP, McGhee JR, Kiyono H, Simecka JW. Immunoglobulin A (IgA) responses and IgE-associated inflammation along the respiratory tract after mucosal but not systemic immunization. Infection and immunity. 2001;69(4):2328-38. Epub 2001/03/20.

188. Armstrong ME, Lavelle EC, Loscher CE, Lynch MA, Mills KH. Proinflammatory responses in the murine brain after intranasal delivery of cholera toxin: implications for the use of AB toxins as adjuvants in intranasal vaccines. The Journal of infectious diseases. 2005;192(9):1628-33. Epub 2005/10/06.

189. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J Immunol. 2000;165(9):4778-82. Epub 2000/10/25.

190. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. The New England journal of medicine. 2004;350(9):896-903. Epub 2004/02/27.

191. Lewis DJ, Huo Z, Barnett S, Kromann I, Giemza R, Galiza E, et al. Transient facial nerve paralysis (Bell's palsy) following intranasal delivery of a genetically detoxified mutant of Escherichia coli heat labile toxin. PloS one. 2009;4(9):e6999. Epub 2009/09/17.

192. Chabalgoity JA, Baz A, Rial A, Grille S. The relevance of cytokines for development of protective immunity and rational design of vaccines. Cytokine & growth factor reviews. 2007;18(1-2):195-207. Epub 2007/03/10.

193. Kumar S, Koul M, Rai A. Role of Immunostimulatory Molecules in Poultry Vaccines. Recent patents on biotechnology. 2010. Epub 2010/07/03.

194. Boyaka PN, Lillard JW, Jr., McGhee J. Interleukin 12 and innate molecules for enhanced mucosal immunity. Immunologic research. 1999;20(3):207-17. Epub 2000/03/31.

195. Boyaka PN, McGhee JR. Cytokines as adjuvants for the induction of mucosal immunity. Advanced drug delivery reviews. 2001;51(1-3):71-9. Epub 2001/08/23.

196. Droual R, Bickford AA, Cutler GJ. Local reaction and serological response in commercial layer chickens injected intramuscularly in the leg with oil-adjuvanted Mycoplasma gallisepticum bacterin. Avian Dis. 1993;37(4):1001-8. Epub 1993/10/01.

197. Leenaars M, Koedam MA, Hendriksen CF, Claassen E. Immune responses and side effects of five different oil-based adjuvants in mice. Veterinary immunology and immunopathology. 1998;61(2-4):291-304. Epub 1998/06/05.

198. Swayne DE. Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1 high pathogenicity avian influenza. Annals of the New York Academy of Sciences. 2006;1081:174-81. Epub 2006/12/01.

199. Steel J, Burmakina SV, Thomas C, Spackman E, Garcia-Sastre A, Swayne DE, et al. A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine. 2008;26(4):522-31. Epub 2007/12/21.

200. Stone H, Mitchell B, Brugh M. In ovo vaccination of chicken embryos with experimental Newcastle disease and avian influenza oil-emulsion vaccines. Avian Dis. 1997;41(4):856-63. Epub 1998/02/10.

201. Toro H, Tang DC, Suarez DL, Sylte MJ, Pfeiffer J, Van Kampen KR. Protective avian influenza in ovo vaccination with non-replicating human adenovirus vector. Vaccine. 2007;25(15):2886-91. Epub 2006/10/24.

202. Crawford JM, Garcia M, Stone H, Swayne D, Slemons R, Perdue ML. Molecular characterization of the hemagglutinin gene and oral immunization with a waterfowl-origin avian influenza virus. Avian Dis. 1998;42(3):486-96. Epub 1998/10/20.

203. De Wit JJ, Swart WA, Fabri TH. Efficacy of infectious bronchitis virus vaccinations in the field: association between the alpha-IBV IgM response, protection and vaccine application parameters. Avian pathology : journal of the WVPA. 2010;39(2):123-31. Epub 2010/04/15.

204. Ley DH. Mycoplasmosis. In: Saif YM, Barnes H, Glission JR, Fadly AM, McDougald LR, Swayne D, editors. Diseases of poultry. 12th ed. Ames, Iowa: Blackwell Pub. Professional; 2003. p. 722–44.

205. de Geus ED, van Haarlem DA, Poetri ON, de Wit JJ, Vervelde L. A lack of antibody formation against inactivated influenza virus after aerosol vaccination in presence or absence of adjuvantia. Veterinary immunology and immunopathology. 2011;143(1-2):143-7. Epub 2011/06/21.

206. Atmar RL, Keitel WA, Cate TR, Munoz FM, Ruben F, Couch RB. A dose-response evaluation of inactivated influenza vaccine given intranasally and intramuscularly to healthy young adults. Vaccine. 2007;25(29):5367-73. Epub 2007/06/15.

207. Tseng LP, Chiou CJ, Chen CC, Deng MC, Chung TW, Huang YY, et al. Effect of lipopolysaccharide on intranasal administration of liposomal Newcastle disease virus vaccine to SPF chickens. Veterinary immunology and immunopathology. 2009;131(3-4):285-9. Epub 2009/05/15.

208. Worrall EE, Sudarisman, Priadi A. Sialivac: an intranasal homologous inactivated split virus vaccine containing bacterial sialidase for the control of avian influenza in poultry. Vaccine. 2009;27(31):4161-8. Epub 2009/05/02.

209. Yoshikawa T, Matsuo K, Suzuki Y, Nomoto A, Tamura S, Kurata T, et al. Total viral genome copies and virus-Ig complexes after infection with influenza virus in the nasal secretions of immunized mice. The Journal of general virology. 2004;85(Pt 8):2339-46. Epub 2004/07/23.

210. Hagenaars N, Mastrobattista E, Glansbeek H, Heldens J, van den Bosch H, Schijns V, et al. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. Vaccine. 2008;26(51):6555-63. Epub 2008/10/14.

211. Nizard P, Gross DA, Babon A, Chenal A, Beaumelle B, Kosmatopoulos K, et al. Anchoring cytokines to tumor cells for the preparation of anticancer vaccines without gene transfection in mice. J Immunother. 2003;26(1):63-71. Epub 2003/01/07.

212. Kim YS, Sonn CH, Paik SG, Bothwell AL. Tumor cells expressing membrane-bound form of IL-4 induce antitumor immunity. Gene therapy. 2000;7(10):837-43. Epub 2000/06/14.

213. Nagarajan S, Selvaraj P. Glycolipid-anchored IL-12 expressed on tumor cell surface induces antitumor immune response. Cancer research. 2002;62(10):2869-74. Epub 2002/05/23.

214. Poloso NJ, Nagarajan S, Mejia-Oneta JM, Selvaraj P. GPI-anchoring of GM-CSF results in active membrane-bound and partially shed cytokine. Molecular immunology. 2002;38(11):803-16. Epub 2002/03/30.

215. Yang Y, Leggat D, Herbert A, Roberts PC, Sundick RS. A novel method to incorporate bioactive cytokines as adjuvants on the surface of virus particles. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2009;29(1):9-22. Epub 2008/11/19.

216. Herbert AS, Heffron L, Sundick R, Roberts PC. Incorporation of membrane-bound, mammalian-derived immunomodulatory proteins into influenza whole virus vaccines boosts immunogenicity and protection against lethal challenge. Virology journal. 2009;6:42. Epub 2009/04/28.

217. Jackwood MW, Suarez DL, Hilt D, Pantin-Jackwood MJ, Spackman E, Woolcock P, et al. Biologic characterization of chicken-derived H6N2 low pathogenic avian influenza viruses in chickens and ducks. Avian Dis. 2010;54(1):120-5. Epub 2010/04/23.

218. Caruso KJ, Cowell RL, Meinkoth JH, Klaassen JK. Abdominal effusion in a bird. Veterinary clinical pathology / American Society for Veterinary Clinical Pathology. 2002;31(3):127-8. Epub 2002/08/22.

219. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. Nature reviews Immunology. 2008;8(7):533-44. Epub 2008/06/14.

220. Handman E, Burgess AW. Stimulation by granulocyte-macrophage colony-stimulating factor of Leishmania tropica killing by macrophages. J Immunol. 1979;122(3):1134-7. Epub 1979/03/01.

221. Hamilton JA, Stanley ER, Burgess AW, Shadduck RK. Stimulation of macrophage plasminogen activator activity by colony-stimulating factors. Journal of cellular physiology. 1980;103(3):435-45. Epub 1980/06/01.

222. Whetton AD, Dexter TM. Myeloid haemopoietic growth factors. Biochimica et biophysica acta. 1989;989(2):111-32. Epub 1989/12/17.

223. Petersdorf SH, Dale DC. The biology and clinical applications of erythropoietin and the colony-stimulating factors. Advances in internal medicine. 1995;40:395-428. Epub 1995/01/01.

224. Fleetwood AJ, Cook AD, Hamilton JA. Functions of granulocyte-macrophage colony-stimulating factor. Critical reviews in immunology. 2005;25(5):405-28. Epub 2005/09/20.

225. Beveridge RA, Miller JA, Kales AN, Binder RA, Robert NJ, Harvey JH, et al. A comparison of efficacy of sargramostim (yeast-derived RhuGM-CSF) and filgrastim (bacteria-derived RhuG-CSF) in the therapeutic setting of chemotherapy-induced myelosuppression. Cancer investigation. 1998;16(6):366-73. Epub 1998/07/29.

226. Biesma B, de Vries EG, Willemse PH, Sluiter WJ, Postmus PE, Limburg PC, et al. Efficacy and tolerability of recombinant human granulocyte-macrophage colony-stimulating factor in patients with chemotherapy-related leukopenia and fever. Eur J Cancer. 1990;26(9):932-6. Epub 1990/01/01.

227. Decker WK, Safdar A. Cytokine adjuvants for vaccine therapy of neoplastic and infectious disease. Cytokine & growth factor reviews. 2011;22(4):177-87. Epub 2011/08/25.

228. Parmiani G, Castelli C, Pilla L, Santinami M, Colombo MP, Rivoltini L. Opposite immune functions of GM-CSF administered as vaccine adjuvant in cancer patients. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2007;18(2):226-32. Epub 2006/11/23.

229. Schell JB, Bahl K, Rose NF, Buonocore L, Hunter M, Marx PA, et al. Viral vectored granulocyte-macrophage colony stimulating factor inhibits vaccine protection in an SIV challenge model: protection correlates with neutralizing antibody. Vaccine. 2012;30(28):4233-9. Epub 2012/04/28.

230. Faderl S, Harris D, Van Q, Kantarjian HM, Talpaz M, Estrov Z. Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces antiapoptotic and proapoptotic signals in acute myeloid leukemia. Blood. 2003;102(2):630-7. Epub 2003/03/29.

231. Lechner MG, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. J Immunol. 2010;185(4):2273-84. Epub 2010/07/21.

232. Wunderlich G, Moura IC, del Portillo HA. Genetic immunization of BALB/c mice with a plasmid bearing the gene coding for a hybrid merozoite surface protein 1-hepatitis B virus surface protein fusion protects mice against lethal Plasmodium chabaudi chabaudi PC1 infection. Infection and immunity. 2000;68(10):5839-45. Epub 2000/09/19.

233. Chen H, Gao N, Fan D, Wu J, Zhu J, Li J, et al. Suppressive effects on the immune response and protective immunity to a JEV DNA vaccine by co-administration of a GM-CSF-expressing plasmid in mice. PloS one. 2012;7(4):e34602. Epub 2012/04/12.

234. Li N, Yu YZ, Yu WY, Sun ZW. Enhancement of the immunogenicity of DNA replicon vaccine of Clostridium botulinum neurotoxin serotype A by GM-CSF gene adjuvant. Immunopharmacology and immunotoxicology. 2011;33(1):211-9. Epub 2011/02/03.

235. Qing Y, Chen M, Zhao J, Hu H, Xu H, Ling N, et al. Construction of an HBV DNA vaccine by fusion of the GM-CSF gene to the HBV-S gene and examination of its immune effects in normal and HBV-transgenic mice. Vaccine. 2010;28(26):4301-7. Epub 2010/05/01.

236. Pichichero ME. Improving vaccine delivery using novel adjuvant systems. Human vaccines. 2008;4(4):262-70. Epub 2008/04/10.

237. Chou HY, Lin XZ, Pan WY, Wu PY, Chang CM, Lin TY, et al. Hydrogel-delivered GM-CSF overcomes nonresponsiveness to hepatitis B vaccine through the recruitment and activation of dendritic cells. J Immunol. 2010;185(9):5468-75. Epub 2010/10/05.

238. Zhai YZ, Li XM, Zhou Y, Ma L, Feng GH. Intramuscular immunization with a plasmid DNA vaccine encoding prM-E protein from Japanese encephalitis virus: enhanced immunogenicity by co-

administration of GM-CSF gene and genetic fusions of prM-E protein and GM-CSF. Intervirology. 2009;52(3):152-63. Epub 2009/06/13.

239. Wang X, Li J, Jiang P, Li Y, Zeshan B, Cao J. GM-CSF fused with GP3 and GP5 of porcine reproductive and respiratory syndrome virus increased the immune responses and protective efficacy against virulent PRRSV challenge. Virus research. 2009;143(1):24-32. Epub 2009/05/26.

240. Lai L, Kwa S, Kozlowski PA, Montefiori DC, Ferrari G, Johnson WE, et al. Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine. The Journal of infectious diseases. 2011;204(1):164-73. Epub 2011/06/02.

241. Robinson HL, Montefiori DC, Villinger F, Robinson JE, Sharma S, Wyatt LS, et al. Studies on GM-CSF DNA as an adjuvant for neutralizing Ab elicited by a DNA/MVA immunodeficiency virus vaccine. Virology. 2006;352(2):285-94. Epub 2006/06/03.

242. Xu HZ, Li A, Yu Y, Li JF, Liu X, Wang XH, et al. [Expression of G-CSF and GM-CSF receptors on CD34 positive cells in aplastic anemia and myelodysplastic syndrome patients and its significance]. Zhongguo shi yan xue ye xue za zhi / Zhongguo bing li sheng li xue hui = Journal of experimental hematology / Chinese Association of Pathophysiology. 2008;16(6):1308-11. Epub 2008/12/23.

243. Yoon HA, Aleyas AG, George JA, Park SO, Han YW, Lee JH, et al. Cytokine GM-CSF genetic adjuvant facilitates prophylactic DNA vaccine against pseudorabies virus through enhanced immune responses. Microbiology and immunology. 2006;50(2):83-92. Epub 2006/02/24.

244. Yen HH, Scheerlinck JP. Co-delivery of plasmid-encoded cytokines modulates the immune response to a DNA vaccine delivered by in vivo electroporation. Vaccine. 2007;25(14):2575-82. Epub 2007/01/17.

245. Ahlers JD, Dunlop N, Alling DW, Nara PL, Berzofsky JA. Cytokine-in-adjuvant steering of the immune response phenotype to HIV-1 vaccine constructs: granulocyte-macrophage colony-stimulating factor and TNF-alpha synergize with IL-12 to enhance induction of cytotoxic T lymphocytes. J Immunol. 1997;158(8):3947-58. Epub 1997/04/15.

246. Okada E, Sasaki S, Ishii N, Aoki I, Yasuda T, Nishioka K, et al. Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. J Immunol. 1997;159(7):3638-47. Epub 1997/10/08.

247. Bradney CP, Sempowski GD, Liao HX, Haynes BF, Staats HF. Cytokines as adjuvants for the induction of anti-human immunodeficiency virus peptide immunoglobulin G (IgG) and IgA antibodies in serum and mucosal secretions after nasal immunization. Journal of virology. 2002;76(2):517-24. Epub 2001/12/26.

248. Ramsburg E, Publicover J, Buonocore L, Poholek A, Robek M, Palin A, et al. A vesicular stomatitis virus recombinant expressing granulocyte-macrophage colony-stimulating factor induces enhanced T-cell responses and is highly attenuated for replication in animals. Journal of virology. 2005;79(24):15043-53. Epub 2005/11/25.

249. Parker JN, Pfister LA, Quenelle D, Gillespie GY, Markert JM, Kern ER, et al. Genetically engineered herpes simplex viruses that express IL-12 or GM-CSF as vaccine candidates. Vaccine. 2006;24(10):1644-52. Epub 2005/10/26.

250. Nambiar JK, Ryan AA, Kong CU, Britton WJ, Triccas JA. Modulation of pulmonary DC function by vaccine-encoded GM-CSF enhances protective immunity against Mycobacterium tuberculosis infection. European journal of immunology. 2010;40(1):153-61. Epub 2009/10/16.

251. Tan B, Wang H, Shang L, Yang T. Coadministration of chicken GM-CSF with a DNA vaccine expressing infectious bronchitis virus (IBV) S1 glycoprotein enhances the specific immune response and protects against IBV infection. Archives of virology. 2009;154(7):1117-24. Epub 2009/06/23.

252. Zeshan B, Mushtaq MH, Wang X, Li W, Jiang P. Protective immune responses induced by in ovo immunization with recombinant adenoviruses expressing spike (S1) glycoprotein of infectious bronchitis virus fused/co-administered with granulocyte-macrophage colony stimulating factor. Veterinary microbiology. 2011;148(1):8-17. Epub 2010/09/21.

253. Babai I, Barenholz Y, Zakay-Rones Z, Greenbaum E, Samira S, Hayon I, et al. A novel liposomal influenza vaccine (INFLUSOME-VAC) containing hemagglutinin-neuraminidase and IL-2 or GM-CSF induces protective anti-neuraminidase antibodies cross-reacting with a wide spectrum of influenza A viral strains. Vaccine. 2001;20(3-4):505-15. Epub 2001/10/24.

254. Ben-Yehuda A, Joseph A, Barenholz Y, Zeira E, Even-Chen S, Louria-Hayon I, et al. Immunogenicity and safety of a novel IL-2-supplemented liposomal influenza vaccine (INFLUSOME-VAC) in nursing-home residents. Vaccine. 2003;21(23):3169-78. Epub 2003/06/14.

255. Ben-Yehuda A, Joseph A, Zeira E, Even-Chen S, Louria-Hayon I, Babai I, et al. Immunogenicity and safety of a novel liposomal influenza subunit vaccine (INFLUSOME-VAC) in young adults. Journal of medical virology. 2003;69(4):560-7. Epub 2003/02/26.

256. Orson FM, Kinsey BM, Densmore CL, Nguyen T, Wu Y, Mbawuike IN, et al. Protection against influenza infection by cytokine-enhanced aerosol genetic immunization. The journal of gene medicine. 2006;8(4):488-97. Epub 2006/01/04.

257. Loudon PT, Yager EJ, Lynch DT, Narendran A, Stagnar C, Franchini AM, et al. GM-CSF increases mucosal and systemic immunogenicity of an H1N1 influenza DNA vaccine administered into the epidermis of non-human primates. PloS one. 2010;5(6):e11021. Epub 2010/06/15.

258. Ramanathan RK, Potter DM, Belani CP, Jacobs SA, Gravenstein S, Lim F, et al. Randomized trial of influenza vaccine with granulocyte-macrophage colony-stimulating factor or placebo in cancer patients. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2002;20(21):4313-8. Epub 2002/11/01.

259. Dillon PJ, Rosen CA. A rapid method for the construction of synthetic genes using the polymerase chain reaction. BioTechniques. 1990;9(3):298, 300. Epub 1990/09/01.

260. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nature reviews Immunology. 2012;12(3):180-90. Epub 2012/02/22.

261. Malek TR. The biology of interleukin-2. Annual review of immunology. 2008;26:453-79. Epub 2007/12/08.

262. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. The Journal of experimental medicine. 2005;201(5):723-35. Epub 2005/03/09.

263. Granucci F, Vizzardelli C, Pavelka N, Feau S, Persico M, Virzi E, et al. Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. Nature immunology. 2001;2(9):882-8. Epub 2001/08/30.

264. Hershko AY, Suzuki R, Charles N, Alvarez-Errico D, Sargent JL, Laurence A, et al. Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. Immunity. 2011;35(4):562-71. Epub 2011/10/11.

265. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science. 2006;311(5769):1924-7. Epub 2006/02/18.

266. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. Cell. 1993;73(1):5-8. Epub 1993/04/09.

267. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nature reviews Immunology. 2006;6(8):595-601. Epub 2006/07/27.

268. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annual review of immunology. 2010;28:445-89. Epub 2010/03/03.

269. Stepaniak JA, Shuster JE, Hu W, Sundick RS. Production and in vitro characterization of recombinant chicken interleukin-2. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 1999;19(5):515-26. Epub 1999/07/01.

270. Sundick RS, Gill-Dixon C. A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. J Immunol. 1997;159(2):720-5. Epub 1997/07/15.

271. Hulse DJ, Romero CH. Partial protection against infectious bursal disease virus through DNA-mediated vaccination with the VP2 capsid protein and chicken IL-2 genes. Vaccine. 2004;22(9-10):1249-59. Epub 2004/03/09.

272. Li J, Liang X, Huang Y, Meng S, Xie R, Deng R, et al. Enhancement of the immunogenicity of DNA vaccine against infectious bursal disease virus by co-delivery with plasmid encoding chicken interleukin 2. Virology. 2004;329(1):89-100. Epub 2004/10/13.

273. Tarpey I, van Loon AA, de Haas N, Davis PJ, Orbell S, Cavanagh D, et al. A recombinant turkey herpesvirus expressing chicken interleukin-2 increases the protection provided by in ovo vaccination with infectious bursal disease and infectious bronchitis virus. Vaccine. 2007;25(51):8529-35. Epub 2007/11/13.

274. Zhou JY, Chen JG, Wang JY, Wu JX, Gong H. cDNA cloning and functional analysis of goose interleukin-2. Cytokine. 2005;30(6):328-38. Epub 2005/06/07.

275. Xiaowen Z, Qinghua Y, Xiaofei Z, Qian Y. Co-administration of inactivated avian influenza virus with CpG or rIL-2 strongly enhances the local immune response after intranasal immunization in chicken. Vaccine. 2009;27(41):5628-32. Epub 2009/08/04.

276. Kolodsick JE, Stepaniak JA, Hu W, Sundick RS. Mutational analysis of chicken interleukin 2. Cytokine. 2001;13(6):317-24. Epub 2001/04/09.

277. Walport MJ. Complement. First of two parts. The New England journal of medicine. 2001;344(14):1058-66. Epub 2001/04/05.

278. Toapanta FR, Ross TM. Complement-mediated activation of the adaptive immune responses: role of C3d in linking the innate and adaptive immunity. Immunologic research. 2006;36(1-3):197-210. Epub 2007/03/06.

279. Walport MJ. Complement. Second of two parts. The New England journal of medicine. 2001;344(15):1140-4. Epub 2001/04/12.

280. Weis JJ, Tedder TF, Fearon DT. Identification of a 145,000 Mr membrane protein as the C3d receptor (CR2) of human B lymphocytes. Proceedings of the National Academy of Sciences of the United States of America. 1984;81(3):881-5. Epub 1984/02/01.

281. Iida K, Nadler L, Nussenzweig V. Identification of the membrane receptor for the complement fragment C3d by means of a monoclonal antibody. The Journal of experimental medicine. 1983;158(4):1021-33. Epub 1983/10/01.

282. Roozendaal R, Carroll MC. Complement receptors CD21 and CD35 in humoral immunity. Immunological reviews. 2007;219:157-66. Epub 2007/09/14.

283. Park CS, Choi YS. How do follicular dendritic cells interact intimately with B cells in the germinal centre? Immunology. 2005;114(1):2-10. Epub 2004/12/21.

284. Aubry JP, Pochon S, Graber P, Jansen KU, Bonnefoy JY. CD21 is a ligand for CD23 and regulates IgE production. Nature. 1992;358(6386):505-7. Epub 1992/08/06.

285. Yoshida K, van den Berg TK, Dijkstra CD. Two functionally different follicular dendritic cells in secondary lymphoid follicles of mouse spleen, as revealed by CR1/2 and FcR gamma II-mediated immune-complex trapping. Immunology. 1993;80(1):34-9. Epub 1993/09/01.

286. Fang Y, Xu C, Fu YX, Holers VM, Molina H. Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. J Immunol. 1998;160(11):5273-9. Epub 1998/05/30.

287. Guo RF, Ward PA. Role of C5a in inflammatory responses. Annual review of immunology. 2005;23:821-52. Epub 2005/03/18.

288. Toapanta FR, DeAlmeida DR, Dunn MD, Ross TM. C3d adjuvant activity is reduced by altering residues involved in the electronegative binding of C3d to CR2. Immunology letters. 2010;129(1):32-8. Epub 2010/01/13.

289. van den Elsen JM, Isenman DE. A crystal structure of the complex between human complement receptor 2 and its ligand C3d. Science. 2011;332(6029):608-11. Epub 2011/04/30.

290. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science. 1996;271(5247):348-50. Epub 1996/01/19.

291. Cherukuri A, Cheng PC, Pierce SK. The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. J Immunol. 2001;167(1):163-72. Epub 2001/06/22.

292. Prechl J, Baiu DC, Horvath A, Erdei A. Modeling the presentation of C3d-coated antigen by B lymphocytes: enhancement by CR1/2-BCR co-ligation is selective for the co-ligating antigen. International immunology. 2002;14(3):241-7. Epub 2002/02/28.

293. Firth MA, Prando Moore D, Pei Y, Shewen PE, Lo RY, Yoo D, et al. Cloning of a gene fragment encoding bovine complement component C3d with expression and characterization of derived fusion proteins. Veterinary immunology and immunopathology. 2006;114(1-2):61-71. Epub 2006/08/15.

294. Henson SE, Smith D, Boackle SA, Holers VM, Karp DR. Generation of recombinant human C3dg tetramers for the analysis of CD21 binding and function. Journal of immunological methods. 2001;258(1-2):97-109. Epub 2001/10/31.

295. Liu D, Niu ZX. Cloning of a gene fragment encoding chicken complement component C3d with expression and immunogenicity of Newcastle disease virus F gene-C3d fusion protein. Avian pathology : journal of the WVPA. 2008;37(5):477-85. Epub 2008/09/18.

296. Fan H, Tong T, Chen H, Guo A. Immunization of DNA vaccine encoding C3d-VP1 fusion enhanced protective immune response against foot-and-mouth disease virus. Virus genes. 2007;35(2):347-57. Epub 2007/05/15.

297. Ross TM, Xu Y, Bright RA, Robinson HL. C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge. Nature immunology. 2000;1(2):127-31. Epub 2001/03/15.

298. Test ST, Mitsuyoshi J, Connolly CC, Lucas AH. Increased immunogenicity and induction of class switching by conjugation of complement C3d to pneumococcal serotype 14 capsular polysaccharide. Infection and immunity. 2001;69(5):3031-40. Epub 2001/04/09.

299. Moore DP, Hodgins DC, Firth MA, McBey BA, Shewen PE. Incorporation of antigens from Mannheimia haemolytica culture supernatant, and recombinant bovine C3d into ISCOM matrix using

neutravidin-biotin interaction. Biotechnology and applied biochemistry. 2011;58(3):198-202. Epub 2011/06/18.

300. Bower JF, Sanders KL, Ross TM. C3d enhances immune responses using low doses of DNA expressing the HIV-1 envelope from codon-optimized gene sequences. Current HIV research. 2005;3(2):191-8. Epub 2005/04/28.

301. Bower JF, Yang X, Sodroski J, Ross TM. Elicitation of neutralizing antibodies with DNA vaccines expressing soluble stabilized human immunodeficiency virus type 1 envelope glycoprotein trimers conjugated to C3d. Journal of virology. 2004;78(9):4710-9. Epub 2004/04/14.

302. Green TD, Montefiori DC, Ross TM. Enhancement of antibodies to the human immunodeficiency virus type 1 envelope by using the molecular adjuvant C3d. Journal of virology. 2003;77(3):2046-55. Epub 2003/01/15.

303. Liu F, Mboudjeka I, Shen S, Chou TH, Wang S, Ross TM, et al. Independent but not synergistic enhancement to the immunogenicity of DNA vaccine expressing HIV-1 gp120 glycoprotein by codon optimization and C3d fusion in a mouse model. Vaccine. 2004;22(13-14):1764-72. Epub 2004/04/08.

304. Ross TM, Xu Y, Green TD, Montefiori DC, Robinson HL. Enhanced avidity maturation of antibody to human immunodeficiency virus envelope: DNA vaccination with gp120-C3d fusion proteins. AIDS research and human retroviruses. 2001;17(9):829-35. Epub 2001/06/29.

305. Mitchell JA, Green TD, Bright RA, Ross TM. Induction of heterosubtypic immunity to influenza A virus using a DNA vaccine expressing hemagglutinin-C3d fusion proteins. Vaccine. 2003;21(9-10):902-14. Epub 2003/01/28.

306. Watanabe I, Ross TM, Tamura S-i, Ichinohe T, Ito S, Takahashi H, et al. Protection against influenza virus infection by intranasal administration of C3d-fused hemagglutinin. Vaccine. 2003;21(31):4532-8.

307. Li GX, Tian ZJ, Yu H, Jin YY, Hou SH, Zhou YJ, et al. Fusion of C3d with hemagglutinin enhances protective immunity against swine influenza virus. Research in veterinary science. 2009;86(3):406-13. Epub 2008/11/18.

308. Green TD, Newton BR, Rota PA, Xu Y, Robinson HL, Ross TM. C3d enhancement of neutralizing antibodies to measles hemagglutinin. Vaccine. 2001;20(1-2):242-8. Epub 2001/09/25.

309. Wang L, Sunyer JO, Bello LJ. Fusion to C3d enhances the immunogenicity of the E2 glycoprotein of type 2 bovine viral diarrhea virus. Journal of virology. 2004;78(4):1616-22. Epub 2004/01/30.

310. Wang L, Oriol Sunyer J, Bello LJ. Immunogenicity of a bovine viral diarrhea virus E2-C3d fusion protein containing a bovine homolog of C3d. Developmental and comparative immunology. 2005;29(10):907-15. Epub 2005/06/28.

311. Tong T, Fan H, Tan Y, Xiao S, Ling J, Chen H, et al. C3d enhanced DNA vaccination induced humoral immune response to glycoprotein C of pseudorabies virus. Biochemical and biophysical research communications. 2006;347(4):845-51. Epub 2006/07/22.

312. Yang S, Wang C, Fang X, Zhai L, Dong C, Ding L, et al. Fusion of C3d molecule with neutralization epitope(s) of hepatitis E virus enhances antibody avidity maturation and neutralizing activity following DNA immunization. Virus research. 2010;151(2):162-9. Epub 2010/05/11.

313. Bhardwaj N, Heise MT, Ross TM. Vaccination with DNA plasmids expressing Gn coupled to C3d or alphavirus replicons expressing gn protects mice against Rift Valley fever virus. PLoS neglected tropical diseases. 2010;4(6):e725. Epub 2010/06/29.

314. Kolla RV, Chintalapati S, Sabet M, Santelli E, Liddington RC, David M, et al. Complement C3d conjugation to anthrax protective antigen promotes a rapid, sustained, and protective antibody response. PloS one. 2007;2(10):e1044. Epub 2007/10/18.

315. Bower JF, Ross TM. A minimum CR2 binding domain of C3d enhances immunity following vaccination. Advances in experimental medicine and biology. 2006;586:249-64. Epub 2006/08/09.

316. Bergmann-Leitner ES, Duncan EH, Leitner WW, Neutzner A, Savranskaya T, Angov E, et al. C3d-defined complement receptor-binding peptide p28 conjugated to circumsporozoite protein provides protection against Plasmodium berghei. Vaccine. 2007;25(45):7732-6. Epub 2007/10/13.

317. Dunn MD, Rossi SL, Carter DM, Vogt MR, Mehlhop E, Diamond MS, et al. Enhancement of anti-DIII antibodies by the C3d derivative P28 results in lower viral titers and augments protection in mice. Virology journal. 2010;7:95. Epub 2010/05/14.

318. Zhang D, Xia Q, Wu J, Liu D, Wang X, Niu Z. Construction and immunogenicity of DNA vaccines encoding fusion protein of murine complement C3d-p28 and GP5 gene of porcine reproductive and respiratory syndrome virus. Vaccine. 2011;29(4):629-35. Epub 2010/12/08.

319. Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. Annual review of immunology. 1999;17:701-38. Epub 1999/06/08.

320. Seder RA, Paul WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annual review of immunology. 1994;12:635-73. Epub 1994/01/01.

321. Yoshimoto T, Paul WE. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. The Journal of experimental medicine. 1994;179(4):1285-95. Epub 1994/04/01.

322. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. Nature. 1995;373(6511):255-7. Epub 1995/01/19.

323. Dubucquoi S, Desreumaux P, Janin A, Klein O, Goldman M, Tavernier J, et al. Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. The Journal of experimental medicine. 1994;179(2):703-8. Epub 1994/02/01.

324. Seder RA, Paul WE, Davis MM, Fazekas de St Groth B. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. The Journal of experimental medicine. 1992;176(4):1091-8. Epub 1992/10/01.

325. Hsieh CS, Heimberger AB, Gold JS, O'Garra A, Murphy KM. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(13):6065-9. Epub 1992/07/01.

326. O'Garra A, Steinman L, Gijbels K. CD4+ T-cell subsets in autoimmunity. Current opinion in immunology. 1997;9(6):872-83. Epub 1998/03/11.

327. Gordon S. Alternative activation of macrophages. Nature reviews Immunology. 2003;3(1):23-35. Epub 2003/01/04.

328. Biedermann T, Zimmermann S, Himmelrich H, Gumy A, Egeter O, Sakrauski AK, et al. IL-4 instructs TH1 responses and resistance to Leishmania major in susceptible BALB/c mice. Nature immunology. 2001;2(11):1054-60. Epub 2001/10/16.

329. Eguchi J, Hiroishi K, Ishii S, Baba T, Matsumura T, Hiraide A, et al. Interleukin-4 gene transduced tumor cells promote a potent tumor-specific Th1-type response in cooperation with interferon-alpha transduction. Gene therapy. 2005;12(9):733-41. Epub 2005/03/18.

330. He H, Genovese KJ, Kogut MH. Modulation of chicken macrophage effector function by T(H)1/T(H)2 cytokines. Cytokine. 2011;53(3):363-9. Epub 2011/01/07.

331. Webster R, Cox N, Stöhr K. WHO Manual on Animal Influenza Diagnosis and Surveillance. Geneva, Switzerland: 2002.5.

332. Wang S, Taaffe J, Parker C, Solorzano A, Cao H, Garcia-Sastre A, et al. Hemagglutinin (HA) proteins from H1 and H3 serotypes of influenza A viruses require different antigen designs for the induction of optimal protective antibody responses as studied by codon-optimized HA DNA vaccines. Journal of virology. 2006;80(23):11628-37. Epub 2006/09/22.

333. Lowen AC, Steel J, Mubareka S, Carnero E, Garcia-Sastre A, Palese P. Blocking interhost transmission of influenza virus by vaccination in the guinea pig model. Journal of virology. 2009;83(7):2803-18. Epub 2009/01/21.

334. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of clinical microbiology. 2002;40(9):3256-60. Epub 2002/08/31.

ABSTRACT

MEMBRANE-BOUND IMMUNOMODULATORS AS ADJUVANTS IN A CELL CULTURE-BASED AVIAN INFLUENZA VACCINE

by

DAVID D. FISCHER

December 2012

Advisor: Dr. Roy Sundick

Major: Immunology & Microbiology

Degree: Doctor of Philosophy

Inactivated viral vaccines often generate suboptimal immune responses. Adjuvants are incorporated into vaccines to increase their immunogenicity, however currently available adjuvants have shortcomings which have limited their use in human and veterinary medicine. This necessitates the development of new adjuvants and delivery systems. Cytokines have been extensively tested as adjuvants in vaccines but challenges such as diffusion from antigen, short half-lives and production costs have been encountered. To address this, we developed a technology that efficiently produces inactivated, whole-virus influenza vaccine bearing membrane-bound cytokines. Tethering the cytokine to the antigen of interest keeps the immunomodulator in close contact with the antigen ensuring that immune cells recruited or activated by the adjuvant react with the antigen as well. Influenza can be used to test this model since its surface proteins HA and NA contain conserved signal sequences that direct these proteins to the infected cell surface where they are picked up by budding virions. Plasmids containing cytokines fused to these signal sequences are transfected into MDCK cell lines and, upon expression, the fusion proteins are directed to the lipid islands on the cell membrane. When the cells are infected with influenza, new virus assembles at the lipid islands and incorporates the fusion proteins into the viral envelope. Furthermore, this *in vitro* cell culture-

based production system bypasses many of the current limitations found in the egg-based influenza vaccine production system.

This model was tested in a chicken model using membrane-bound C3d, GMCSF, IL2 and IL4 as adjuvants. GMCSF adjuvanted vaccines delivered subcutaneously and C3d adjuvanted vaccines delivered subcutaneously and intranasally resulted in significantly higher antibody titers than vaccines with inactivated virus alone. Vaccination with C3d adjuvanted virions by either route completely eliminated viral shedding after challenge with live virus and the GMCSF vaccine reduced the number of birds shedding virus. In conclusion, these data support our hypothesis that this technology improves the efficacy of killed influenza vaccines. More studies seem warranted to test additional immjunomodulators, influenza strains and other enveloped viruses.

AUTOBIOGRAPHICAL STATEMENT

I was born in Kalamazoo, Michigan and graduated from Comstock High School in 2002. In 2002 I began my research career as an undergraduate in Dr. Kurt Hankenson's lab in the Department of Orthopaedic Surgery at the University of Michigan, Ann Arbor. I graduated from the University of Michigan in 2006 with a B.S. degree in Cellular & Molecular Biology. In the fall of 2006 I began graduate studies in the Department of Immunology & Microbiology at the Wayne State University School of Medicine in Detroit. In 2007 I joined Dr. Roy Sundick's lab and completed dissertation work utilizing a novel influenza vaccine adjuvant system in chickens. In October, 2012 I will begin postdoctoral research involving rotavirus vaccines in Dr. Linda Saif's laboratory at the Ohio Agricultural Research and Development Center in Wooster (an affiliate of The Ohio State University). My career goal is to become a principal investigator in an academic research laboratory focusing on vaccine and adjuvant development.