PIV5 AS A NOVEL VACCINE VECTOR FOR PANDEMIC INFLUENZA

by

ALAINA JONES MOONEY

(Under the Direction of S. Mark Tompkins)

ABSTRACT

Highly pathogenic avian influenza virus (HPAI) and the H5N1 subtype in particular, poses a formidable pandemic threat. Current HPAI vaccine candidates suffer from poor immunogenicity, and there are challenges associated with sufficient production and distribution. Parainfluenza virus 5 (PIV5) provides an appealing approach for live virus vectored vaccines. It can be produced quickly and safely in cell culture which is especially important in the event of a pandemic. Here, we have inserted the HA gene of A/Vietnam/1203/04 (H5N1) into the PIV5 genome with the goal of testing the efficacy and mechanism of protection of recombinant PIV5-H5 vaccine vectors. The natural gradient of mRNA translated in PIV5 is dictated by the proximity of the gene to the 3' UTR. Thus, we tested expression and immunogenicity of H5 vaccine vectors where the HA gene is inserted in distinct locations in the PIV5 genome. We show here that vaccination with rPIV5-H5 is safe and effective as a vaccine vector and that it provides protection against highly pathogenic H5N1 challenge. Neuraminidase (NA) is a glycoprotein on the surface of the virus as well as virus-infected cells and is responsible for cleavage of sialic acid residues as the virus buds from the cell at the end of the replication cycle. NA is more conserved than HA, the current influenza vaccine target, which increases the likelihood of achieving

broader protection if used as a vaccine antigen. In this study, we extend the work with rPIV5-H5 to show that vaccination with rPIV5-N1 primes an NA-specific antibody response and T cell response and confers complete protection against homologous influenza virus challenge and significant cross-protection against heterologous influenza virus within the same subtype. There is also evidence of limited cross-protection against virus of a different subtype (H3N2). Because PIV5 is not sialic acid-restricted for replication, it is possible to utilize alternate routes of administration. We continue this work by showing that rPIV5-H5 is effective at providing protection against HPAIV H5N1 when administered intramuscularly as well as intranasally.

INDEX WORDS: Pandemic influenza; Viral vectored vaccine; PIV5

PIV5 AS A NOVEL VACCINE VECTOR FOR PANDEMIC INFLUENZA

by

ALAINA JONES MOONEY

BSA, University of Georgia, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILISOPHY

ATHENS, GEORGIA

2012

PIV5 AS A NOVEL VACCINE VECTOR FOR PANDEMIC INFLUENZA

by

ALAINA JONES MOONEY

Major Professor:	S. Mark Tompkins
Committee:	Biao He
	Ralph A. Tripp
	Elizabeth W. Uhl
	Kimberly Klonowski

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2012

DEDICATION

This work is dedicated to my husband and best friend, Joshua Mooney, my parents, Lorre and Jerry Jones, and my sister, Kyley Jones. Your constant support and love has made all of this work possible.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Mark Tompkins, for all of his guidance and patience throughout the past 5 years. He allowed me to learn how to be a scientist, not simply memorize the science. These are lessons I will carry with me forever. I also thank Dr. Biao He for all of his encouragement and positivity, Dr. Ralph Tripp for all of his useful talks, support, and alternate perspectives. I also extend my thanks to Dr. Betsy Uhl and Dr. Kimberly Klonowski for all of their advice and aid in my development as a scientist.

I would like to extend a special thank you to current and past members of the Animal Health Research Center, especially Jon Gabbard and Cheryl Jones, for their endless help in the lab. I truly don't know if I would have been able to do this without all of you.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Influenza A virus, a negative-strand, enveloped RNA virus in the family *Orthomyxoviridae* [1], is a continuing public health problem, infecting up to 15% of the world's population in its epidemic (seasonal) form [2] and inflicting significant morbidity and mortality in the United States and worldwide[3]. Influenza A pandemics have occurred sporadically over the course of history with varying degrees of severity, but have only been well documented for about the past 100 years. The 1918 "Spanish flu" was responsible for over 50 million deaths worldwide [4]. The recent pandemic H1N1 in 2009 is a milder example, causing under 20,000 (laboratory confirmed) deaths worldwide [5]. Aquatic birds are considered the primary reservoir for influenza A virus[6] and every pandemic virus studied to date has contained gene segments of avian origin [7].

Historically, it was thought that avian viruses would have to reassort or adapt to infect or cause disease in humans until, in 1997, eighteen humans in Hong Kong were infected with highly pathogenic avian influenza virus (HPAIV) subtype H5N1, and 6 of those persons died [8]. Subsequent research has suggested that the 1918 "Spanish flu", the deadliest influenza pandemic in recorded history, was potentially generated by a similar mechanism [9]. HPAI viruses are now considered a potential pandemic threat. Related H5N1 HPAI viruses continue to smolder in Eurasia and as

of November 2011, the WHO has reported 571 human cases with a total of 335 deaths [10]. H5N1 is unable to transmit between humans at this time, however, there is significant concern that the virus could mutate such that efficient spread would be possible [11]. With an approximately 60% case fatality rate, a pandemic HPAI H5N1 virus would cause significant morbidity and mortality, as well as major socioeconomic disruption [12].

Vaccination with an effective vaccine is considered the most effective approach to prevent disease or the transmission of potentially pandemic viruses in humans. The most widely used licensed vaccine for the prevention of seasonal influenza virus infection is an inactivated vaccine, which is grown in embryonated chicken eggs, and requires months of production time between strain identification and vaccine delivery, and millions of eggs [13]. Even though this method represents the only US Food and Drug Administration (FDA)-approved H5N1 vaccine, there are a number of drawbacks in using this method of production for HPAI H5N1, including safety and production issues. Furthermore , these vaccines are poorly immunogenic and require multiple doses to generate a neutralizing antibody response [14]. Similar issues are shared with H5N1 vaccine seed viruses generated by reverse-genetics [15, 16], with some evidence indicating they are actually worse in terms of immunogenicity in the absence of an adjuvant [17]. There is clearly a need for other options for H5N1 vaccines.

There are a number of candidate vaccines in clinical trials at this time (reviewed in [18]), including inactivated viruses formulated with a variety of adjuvants, such

as oil-in-water, and live-attenuated influenza vaccines [19, 20]. These approaches do not, however, address the concerns of using an egg-based vaccine for prepandemic preparedness.

Other vaccine options that are being explored include recombinant DNA and viruses, which have been shown to protect against homologous and heterologous influenza challenge, including H5N1 [21-24]. These finding suggest that virus vectored vaccines may be an effective method of obtaining robust, protective immune responses with the potential of generating a vaccine with cross-protective qualities. This would be ideal in the face of a largely unknown emerging pandemic strain upon a serologically naïve population.

The goal of the studies described here is to develop a novel, safe, and effective vaccine against influenza. The *specific hypothesis* is that parainfluenza virus 5 (PIV5) will be a safe viral vector for the delivery of influenza antigens, and that vaccination with recombinant PIV5 expressing influenza proteins will be immunogenic and provide protection against influenza virus challenge.

PIV5, a non-segmented, negative-stranded RNA virus (NNSV) in the *Rubulavirus* genus in the family *Paramyxoviridae*, was chosen as a vaccine vector based on a number of favorable qualities, including the absence of a DNA phase in the life cycle, which eliminates the likelihood of host genome alteration, as well as a stable genome, making it an ideal candidate for foreign gene insertion and vaccine use [24, 25]. PIV5 is capable of infecting a large range of cell types[26] and, because it causes very little cytopathic effect (CPE) in those cells, is able to grow to high titers in

common cell lines, including VERO cells[27], a vaccine-approved cell line. This would address existing problems associated with egg-based vaccine development. PIV5 also infects a large range of mammals, including humans, without causing clinical disease[28]. PIV5 also has a gradient of gene expression relative to proximity to the leader sequence [29]. It may be possible to exploit this aspect and maximize vaccine immunogenicity by inserting the vaccine antigen close to the leader sequence. Similarly, altering the immune-suppressive mechanisms of PIV5 may enhance vaccine efficacy[30, 31]. A common concern with the use of vaccine vectored vaccines is pre-existing immunity to the vector. Evidence exists that antibodies against PIV5 are not protective, [32] which alleviates many concerns about cross-reacting antibodies of ubiquitous paramyxovirus infections as well as concerns regarding the use of PIV5 in a prime-boost regimen.

The vaccine antigens chosen for expression in the PIV5 vector were chosen based on ubiquitous knowledge that sufficient antibodies to HA are protective against influenza virus infection [33] and that antibodies to NA contributes to resistance to influenza in humans [34] and, because it is more conserved than NA, increases the likelihood of broader immunity [35]. These genes are inserted into PIV5 by reverse-genetics techniques. A similar construct expressing the HA from A/Udorn/72 (H3N2) (rPIV5-H3) has been shown previously to be protective against homologous influenza virus challenge [24]. Here, we propose to examine the safety and protective efficacy of rPIV5 constructs individually expressing the HA

(H5) protein from HPAIV H5N1 and the NA protein from HPAIV H5N1 and the 2009 pandemic H1N1 virus. This proposal examines the following specific aims:

Specific Aim 1: To test the safety and efficacy of a PIV5-vectored vaccine expressing the HA of HPAIV H5N1 (rPIV5-H5). The *working hypotheses* are that the expression of HA is safe and immunogenic, that insertion of the HA closer to the leader sequence will increase vaccine efficacy, and that vaccination with rPIV5-H5 will induce HPAIV H5N1 neutralizing antibodies and protect against challenge.

Specific Aim 2: To test the efficacy of PIV5-vectored vaccines expressing the NA protein of HPAIV H5N1 and the 2009 pandemic H1N1 virus. The *working hypotheses* are that NA will be expressed in its functional form and that immunization with rPIV5-NA will induce robust anti-NA antibodies that protect against homologous and heterologous influenza challenge.

Specific Aim 3: To investigate the efficacy of PIV5 as a vaccine vector when administered intramuscularly as well as intranasally. The *working hypotheses* are that PIV5 will be efficacious as a vaccine vector when administered intramuscularly.

Knowledge obtained from thorough investigation of the specific aims outline above will contribute to the effort for the better design of influenza vaccines, more specifically, to virus vectored influenza vaccines with a focus on the challenges associated with pandemic influenza virus emergence.

References

1. Wright, P.F., G. Neumann, and Y. Kawaoka, *Orthomyxoviruses*, in *Fields Virology*, D.M. Knipe and P.M. Kowley, Editors. 2007, Lippincott Williams and Wilkins: Philadelphia. p. 1691-1740.
2. Girard, M.P., et al., *A review of vaccine research and development: human acute respiratory infections.* Vaccine, 2005. **23**(16154667): p. 5708-5724.
3. Fiore, A.E. *Prevention and control of influenza. Recommendations of the advisory committee on immunization practices (ACIP)*. MMWR Recomm Rep, 2008. **57**, 1-60.
4. Lipatov, A.S., *Influenza: Emergence and Control.* Journal of Virology, 2004: p. 8951-8959.
5. WHO, *Pandemic (H1N1) 2009-update 100*, 2010, World Health Organization. p. 1-3.
6. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiological Reviews, 1992. **56**(1): p. 152-179.
7. Reid, A.H., J.K. Taubenberger, and T.G. Fanning, *Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus.* Nature Rev. Microbiol., 2004. **2**(15494747): p. 909-914.
8. Prevention, C.f.D.C.a., *Update: Isolation of Avian Influenza A(H5N1) Viruses From Humans—Hong Kong, 1997-1998.* JAMA: The Journal of the American Medical Association, 1998. **279**(5): p. 347-348.
9. Taubenberger, J.K., et al., *Characterization of the 1918 influenza virus polymerase genes.* Nature, 2005. **437**(16208372): p. 889-893.
10. WHO. *Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011*. Global Alert and Response (GAR) 2011 29 November 2011 [cited 2011 December 15]; Available from: http://www.who.int/influenza/human_animal_interface/EN_GIP_20111129CumulativeNumberH5N1cases.pdf.
11. WHO. *Current WHO phase of pandemic alert for avian influenza H5N1*. Global Alert and Response (GAR) 2011; Available from: http://apps.who.int/csr/disease/avian_influenza/phase/en/index.html.
12. Whitley, Richard J. and Arnold S. Monto, *Seasonal and Pandemic Influenza Preparedness: A Global Threat.* Journal of Infectious Diseases, 2006. **194**(Supplement 2): p. S65-S69.
13. Gerdil, C., *The annual production cycle for influenza vaccine.* Vaccine, 2003. **21**(16): p. 1776-1779.
14. Centers for Infectious Disease and Research, C. *FDA panel supports H5N1 vaccine approval.* CIDRAP Pandemic Influenza News, 2007. 1-2.
15. Horimoto, T. and Y. Kawaoka, *Strategies for developing vaccines against H5N1 influenza A viruses.* Trends in Molecular Medicine, 2006. **12**(11): p. 506-514.

16. Lipatov, A.S., et al., *Efficacy of H5 Influenza Vaccines Produced by Reverse Genetics in a Lethal Mouse Model.* Journal of Infectious Diseases, 2005. **191**: p. 1216-1220.
17. Treanor, J.J., et al., *Safety and Immunogenicity of an Inactivated Subvirion Influenza A (H5N1) Vaccine.* New England Journal of Medicine, 2006. **354**(13): p. 1343-1351.
18. Poland, G., I. Ovsyannikova, and R. Jacobson, *Immunogenetics of seasonal influenza vaccine response☆.* Vaccine, 2008. **26**: p. D35-D40.
19. Galli, G., et al., *Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine.* Proc Natl Acad Sci U S A, 2009. **106**(19416838): p. 7962-7967.
20. Subbarao, K., *Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics.* Virology, 2003. **305**: p. 192-200.
21. Chen, Z., et al., *Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein.* Journal of General Virology, 1999. **80**: p. 2559-2564.
22. Epstein, S.L., et al., *DNA Vaccine Expressing Conserved Influenza Virus Proteins Protective against H5N1 Challenge Infection in Mice.* Emerging Infectious Diseases, 2002. **8**(8): p. 796-801.
23. Tompkins, S.M., et al., *Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including Subtype H5N1.* Emerging Infectious Diseases, 2007. **13**(3): p. 426-435.
24. Tompkins, S.M., et al., *Recombinant parainfluenza virus 5 (PIV5) expressing the influenza A virus hemagglutinin provides immunity in mice to influenza A virus challenge.* Virology, 2007. **362**(1): p. 139-150.
25. Bukreyev, A., et al., *Nonsegmented Negative-Strand Viruses as Vaccine Vectors.* Journal of Virology, 2006. **80**(21): p. 10293-10306.
26. Arimilli, S., M.A. Alexander-Miller, and G.D. Parks, *A Simian Virus 5 (SV5) P/V Mutant Is Less Cytopathic than Wild-Type SV5 in Human Dendritic Cells and Is a More Effective Activator of Dendritic Cell Maturation and Function.* Journal of Virology, 2006. **80**(7): p. 3416-3427.
27. Choppin, P.W., *Multiplication of a Myxovirus (SV5) with Minimal Cytopathic Effects and without Interference'.* Virology, 1964. **23**: p. 224-233.
28. Cohn, M.L., et al., *T Cell responses to the paramyxovirus simian virus 5: studies in multiple sclerosis and normal populations.* Pathobiology, 1996. **64**(3).
29. Lamb, R.A. and G.D. Parks, *Paramyxoviridae: the viruses and their replication.*, in *Fields Virology*, D.M. Howley and B.N. Knipe, Editors. 2007, Lippincott-Raven: Philadelphia. p. 1449-1496.

30. Sun, M., et al., *Conserved Cysteine-Rich Domain of Paramyxovirus Simian Virus 5 V Protein Plays an Important Role in Blocking Apoptosis.* Journal of Virology, 2004. **78**(10): p. 5068-5078.
31. Lin, Y., et al., *Induction of Apoptosis by Paramyxovirus Simian Virus 5 Lacking a Small Hydrophobic Gene.* Journal of Virology, 2003. **77**(6): p. 3371-3383.
32. Young, D.F., et al., *Clearance of a Persistent Paramyxovirus Infection Is Mediated by Cellular Immune Responses but Not by Serum-Neutralizing Antibody.* Journal of Virology, 1990. **64**(11): p. 5403-5411.
33. Potter, C.W. and J.S. Oxford, *Determinants of immunity to influenza infection in man.* British medical bulletin, 1979. **35**(1): p. 69-75.
34. Murphy, B.R., J.A. Kasel, and R.M. Chanock, *Association of Serum Anti-Neuraminidase Antibody with Resistance to Influenza in Man.* N Engl J Med, 1972. **286**(5027388): p. 1329-1332.
35. Sandbulte, M.R., et al., *Cross-Reactive Neuraminidase Antibodies Afford Partial Protection against H5N1 in Mice and Are Present in Unexposed Humans.* PLoS Med, 2007. **4**(2): p. e59.

CHAPTER 2

LITERATURE REVIEW

Influenza A Virus Overview

Influenza virus is a negative strand, enveloped RNA virus in the family *Orthomyxoviridae* **[1].** *Orthomyxoviridae* includes four genera: influenza A, influenza B, influenza C, and thogotovirus[1]. These categories are based on differences in the nucleoprotein (NP) and matrix protein (M1). Influenza types A and C infect a wide variety of species whereas type B infects only humans. Type A is the only influenza virus associated with pandemics and is the cause of the majority of influenza cases in humans. This review will focus primarily on influenza A.

Influenza A causes significant morbidity and mortality each year. Circulating seasonal influenza strains (H1N1 and H3N2) infect up to 15% of the world's population each year and cause an average of 225,000 hospitalizations and a range of 3349 to 48, 614 deaths in the United States every year over the past 30 years[2, 3, 36]. The annual worldwide influenza burden is estimated by the World Health Organization (WHO) to be approximately 1 billion infections, 3-5 million cases of serious disease, and 300,000 to 500,000 deaths [2]. Influenza pandemics, which have occurred sporadically throughout history, although only well documented in the last century, have been of varying degrees of severity. The most notable was the

"Spanish flu" in 1918, which killed an estimated 50 to 100 million people worldwide[37].

Influenza A has a segmented, negative sense RNA genome encoding eleven proteins. The genome is comprised of eight segments, including surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), matrix (M), nucleoprotein (NP), nonstructural genes (NS), and three polymerase segments (PB1, PB2, and PA)[6]. Multiple gene segments have been demonstrated to be important for infection, replication, and pathogenicity, including the polymerase genes (PA, PB1, and PB2)[9, 38] and hemagglutinin[39].

Influenza A is further subtyped by its two major surface glycoproteins hemagglutinin (HA), neuraminidase (NA). There are 16 HA subtypes[40] and 9 NA subtypes[6]. All 16 HA and 9 NA subtypes were originally isolated from wild aquatic bird populations [41]. Avian influenza viruses have been further classified as highly pathogenic avian influenza viruses (HPAIV), such as H5N1, or low pathogenicity avian influenza viruses (LPAIV) based on the presence of a polybasic cleavage site (some H5 and H7 subtypes, only). This is considered a primary pathogenicity determinant. LPAIV have a monobasic cleavage site.

Infection and Clinical Features: Infection begins when influenza virus infects respiratory epithelial cells. In most cases of human influenza infection, this occurs in the upper respiratory tract. Exceptions include human infection by avian influenza viruses, notably HPAIV H5N1, details and implications of which will be discussed in detail below.

Typical, uncomplicated infection in adults manifests as tracheobronchitis with minor small airway involvement [1]. High fever, cough, myalgias, and malaise are common symptoms and, although the high fever will wane typically within six days of onset of illness, cough and malaise can persist for up to two weeks [1]. Symptoms are similar in children, but can be accompanied by febrile convulsions and there is a higher incidence of gastrointestinal effects [42]. Lower respiratory tract complications are more common in children and the elderly, and include primary viral pneumonia, combined viral-bacterial pneumonia, and secondary bacterial pneumonia [1]. Systemic influenza infection is uncommon in typical epidemic influenza infection, however it appears to occur more commonly with H5N1 HPAIV [43], which will be discussed further below.

Protective immunity: Influenza infection establishes protective and long-term immunity, which is a feature that makes vaccination such a favorable choice for epidemic and pandemic control. Unfortunately, this protection is largely limited to the specific strain of virus and is ineffective against drift and, especially, shift variants, which is problematic for vaccine development. This will be discussed further below. A thorough understanding of the nature of the immune response to influenza (reviewed thoroughly by Mintern et al. [44]) and correlates of protection, are critical for vaccine design.

Immune Response to Influenza A Infection

Recognition: Upon invasion of respiratory mucosa, influenza A viruses are recognized by multiple pattern recognition receptors (PRRs) in the cell [45] which leads to the initialization of signaling leading to activation of innate and cell-mediated immune effectors. The primary signature is the single-stranded RNA (ssRNA) generated by the viral polymerase in the cytoplasm [46] and it is recognized by the host RNA helicase, RIG-1 [47], and toll-like receptor (TLR)-7 in the endosomal compartments of B cells and plasmacytoid dendritic cells (DCs) [48, 49]. TLR-3, expressed on respiratory epithelial cells, is also an important receptor, recognizing dsRNA, leading to activation of the TRIF IRF3 pathway to stimulate interferon [50]. Release of IL-1b, which is important in initiating a number of responses such as cell recruitment and apoptosis, is stimulated upon activation of NOD-like receptor-associated inflammasomes [51-53].

Innate Immune Response: The primary barriers to influenza infection are inhibitory factors in the mucus that reduce the ability of influenza A to infect cells [54]. The initial response to influenza A infection in the lungs is comprised of rapid innate immune mediators including alveolar macrophages (AMs), neutrophils, and natural killer (NK) cells [44]. AMs are the major resident phagocytes in the lungs [55], taking up influenza A antigens [56] and secreting proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and interferon (IFN)-α/β [57, 58] as well as a variety of chemokines [59, 60]. They also play a role in modulating adaptive T cell immunity [61]. AMs also secrete IL-12 which aids in T helper cell development and activates

NK cells [62], which are detectable in the lungs within 48 hours of influenza infection [63]. Upon interaction of NK cell receptors NKp46 [64] and NKp44 [65] with HA on infected cells, protection is likely mediated by expression IFN-γ and TNF-α as well as direct cytotoxicity of infected cells [66].

Although produced by most innate immune mediators, plasmacytoid dendritic cells (pDCs)[67] are the largest producers of type I IFN [68], which is possibly the most important cytokine in the initial response to influenza infection. This is highlighted by the fact that influenza viruses have evolved mechanisms to circumvent the type I IFN response [69]. Pulmonary levels of type I IFNs α and β increase rapidly and directly limit viral replication [70], enhance recruitment of immune mediators[71], and function as parts of a variety of feedback loops leading to enhancement of macrophage function, antigen presentation by APCs, and modulation of adaptive immunity.

Although integral to the early response to influenza, as well as other pathogens, proinflammatory cytokines and chemokines must be tightly regulated as collateral damage can be caused to the already weakened respiratory environment by excessive inflammation [72]. In extreme cases, hyper-induction of proinflammatory cytokines has the potential to lead to a lethal "cytokine shock" [73]. This has been extensively studied in the context of the 1918 "Spanish flu" virus [74, 75] as well as H5N1 and is believed to play a major role in development of acute respiratory distress syndrome (ARDS) and high fatality rate associated with both viruses [76]. This will be discussed in greater detail below.

Other components of the innate immune response against influenza A viruses includes collectins and defensins. Collectins, collagen-like lectins lining the respiratory tract, such as mannan-binding lectin (MBL) [77] and surfactant proteins (SP)-A and –D [78], limit infection and orchestrate viral clearance in a number of ways [79, 80], including compelement activation, opsonization, neutralization, and agglutination. Defensins are cationic host defense peptides produced by leukocytes and epithelial cells that aid in the killing of phagocytized pathogens [81] or act as chemotactic agents to promote immunity.

Adaptive Immunity

Humoral Immune Response: The humoral immune response is mediated by antibodies secreted by B lymphocytes and is the target mechanism for most influenza vaccines. Immunity to respiratory pathogens is induced on two fronts: mucosal and systemic. Because mucosal immunity is the initial site of infection, it is of interest in vaccine research. Protective antibodies against influenza are able to bind directly to the glycoproteins on the surface of the virion and neutralize the virus thus preventing infection.

Upon initial viral entry, constitutive, non-neutralizing low-affinity antibodies of the IgM subtype serve to limit early virus dissemination and to aid in targeting viral antigen to secondary lymphoid organs [82, 83]. Neutralizing antibodies, primarily IgG, are high affinity/avidity antibodies specific for the viral antigen and are rapidly induced. They primarily target the HA protein as neutralization of this glycoprotein impairs viral entry into the cell [84, 85]. NA is also targeted by

neutralizing antibodies [34], although antibodies against NA are not, strictly speaking, capable of preventing infection to the degree that HA-neutralizing antibodies are. This will be discussed further in the discussion of use of HA, NA, and others, as vaccine antigens.

Although discussed primarily the context of cell-mediated protection, CD4+ T-helper cells promote B cell differentiation into immunoglobulin class-switched, antibody-secreting cells, and thus contribute to humoral immunity in an important way. This occurs via the recognition of viral antigen and delivery of an activation signal to the B cell by the CD40 ligand [86].

Mucosal immune system: The inductive sites of the mucosal immune system are the mucosal-associated lymphoid tissues (MALT), where antigen is taken up my APCs and presented to T and B cells for the production of mucosal antibody, IgA. In rodents, the upper respiratory tract where the mucosal immune response is induced is the nasopharyngeal-associated lymphoid tissues (NALT) [87]. Here, antigens are taken up by M cells prime T and B cells, which drain to the cervical lymph nodes and circulate back to the lamina propria of the lining of the respiratory, and other mucosal tracts[88, 89]. Here, secretory IgA (sIgA) antibodies, consisting of dimeric IgA and the secretory component, which is the cleavage product of the major receptor responsible for IgA being transported into mucosal secretions, polymeric IgA (pIgA) [90], are released. sIgA primarily specific to HA and NA in nasal secretions [91] inhibits influenza virus entry at mucosal surfaces

Cell-Mediated Immune Response

Dendritic Cells: A number of dendritic cell (DC) subsets reside in the lungs [92] and the draining lymph node [93]. Upon pulmonary infection, a number of other DC populations are recruited [94] for the antigen uptake, processing, and presentation of antigenic peptides in the context of major histocompatibility complex (MHC) molecules to T cells. They can also be directly infected with virus with similar results [95]. They are required for primary responses [95] as well as memory T cell responses that are required upon subsequent infection [96]. Soon after antigen acquisition, lung DCs migrate to the draining lymph node [94, 97] where antigen presentation ensues.

DC costimulation is an essential function that serves to enhance the antigen-specific signals that are being delivered through the T cell receptor (TCR). The CD28/B7 interaction between DCs and CD8+ T cells is particularly important for influenza A immunity, and known functions include stimulations of T cell expansion [98], cytotoxicity, effector cytokine production, recruitment to the site of infection [99], and survival [100]. Other important costimulatory interactions includes the CD8+ T cell expansion and recall (41BB/41BBL)[101] and accumulation of T cell at the site of infection [98, 102].

CD8+ T Cells: Effector CD8+ T cells, also known as cytolytic or cytotoxic T lymphocytes (CTLs), function in viral clearance [102] by directly targeting and destroying virus-infected cells either by the release of perforin and granzymes or Fas/FasL interaction (the death receptor) [103, 104]. CTLs are primed by antigen

presented in the context of major histocompatibility complex (MHC)-I by APCs, but sometimes cross-presentation by CD4+ T cells is required [105]. After priming, activated CTLs migrate to the lung draining lymph nodes where, in the presence of antigen, they expand approximately within the first 7-10 days of primary infection [106, 107]. Activated CD8+ T cells then traffic to the infected airways to mediate viral clearance [108] by targeting infected cells expressing influenza A peptide in the context of MHC I, the most immunodominant of which are conserved internal proteins PA and NP [109]. Following antigen recognition, CD8+ T cells exert a number of effector functions, including production of cytokines such as IFN-γ, TNF-α, and IL-2 [110], direct cytolysis of virus-infected cells via perforin and granzymes [111], or through Fas-ligand expression [104]. CD8+ T cells also regulate the inflammatory process by producing IL-10 [112].

Following clearance, virus-specific CD8+ T cell levels decrease until they level off approximately 2 months following infection [113], becoming memory pools approximately 10% of the fully expanded population [114]. Upon subsequent infection, memory T cells expand in the lymph nodes and promote viral clearance approximately 2 days earlier than in primary infection [113]. Notably, memory T cells do not provide sterilizing immunity against subsequent infection. This will be discussed further below in the context of targeting T cells in vaccine development.

CD4+ T Cells: CD4+ T cells (T helper cells) are not typically associated with direct virus elimination (although there is evidence that primed CD4 effector cells have a perforin-mediated cytotoxic effect in the early stages of infection [115]), they

are important for regulating humoral (as discussed previously) and CD8+ T cell functions. A strong CD4+ T cell response is induced upon influenza A virus infection, and clonal expansion and differentiation occurs more or less concurrently with CD8+ T cells, with the peak response in the airways occurring 6-7 days following infection [116]. Unlike CD8+ T cells, however, CD4+ T cells require antigen to be presented in the context of MHC class II, which is presented only on a limited number of cells. Following virus clearance, CD4+ T cells contract more quickly than CD8+ T cells [117].

The primary role of CD4+ T cells is their role as T helper cells, in which they mediate B cell differentiation and production of neutralizing antibodies [115, 118], direct CD8 (cytotoxic) T cell responses by secreting Th1 cytokines, such as IFN-γ, IL-2, and TNF-α ([116, 119]reviewed in [120]), and, importantly, aid in the rapid response of CD8+ memory T cells [121]. They also assume a regulatory role expressing IL-10 [112]. Mice lacking functional CD4+ T cells have been shown to be impaired in their response to influenza infection[122].

Emergence of Novel Influenza Strains

Antigenic Drift: Influenza virus infection leads to robust, life-long immunity against the offending influenza strain. Unfortunately, mutations that result from the lack of proof reading ability of the influenza A RNA polymerase, combined with the selective pressure applied by HA- and NA-specific neutralizing antibody [123], lead to poor immune recognition by neutralizing antibodies. HA in particular has a high

amino acid substitution rate and nonsynonymous substitutions significantly exceed synonymous substitutions indicating a selective advantage for novel amino acid sequences in this region[124, 125]. This is commonly referred to as “antigenic drift”. Point mutations in the HAs of drift variants can decrease or eliminate immune recognition by a number of mechanisms, including steric obstruction of receptor binding [126] and by modification of the HA surface such that it is unrecognizable to antibodies [127]. Although T cell epitopes are far more conserved, mostly due to the absence of immune pressure, they too are susceptible to antigenic drift [128]. This can lead to a decrease in functional avidity of the TCR [129] and ultimately limit cross-protective immunity. It is because of antigenic drift in the HA that the seasonal influenza vaccine must be reformulated yearly [130]. This will be discussed further below.

Antigenic Shift: Influenza A can also undergo major antigenic changes referred to as “antigenic shift”, which happens far less frequently than antigenic drift, which is constantly ongoing. This occurs when two distinct influenza viruses infect the same cell and there is reassortment of the viral genomic segments, yielding a novel influenza A virus with an entirely new combination of proteins. When infection occurs in a “mixing vessel”, such as pigs [131], that can be infected by multiple species-adapted viruses, such as avian and human viruses, reassortment can occur between these viruses. This will lead to a novel influenza strain, and in the event that a human influenza virus acquires a major avian virus segment, such as HA or NA, and retained the ability to replicate in humans, there would be no immunity in

the human population and the emergence of a pandemic influenza virus could occur [1, 132].

Pandemic Influenza

A pandemic influenza virus is one that, as previously described, is sufficiently different from seasonal, circulating strains that the populations has little to no immunity to the virus. The virus must also be able to spread easily from person to person [133].

History of Pandemic Influenza: Pandemics have occurred sporadically throughout the 20th century, occurring in 1918, 1957, 1968, and 2009. Three of these four were results of a reassortment event (antigenic shift), with the exception of the 1918 "Spanish flu". The H2N2 virus that caused the 1957 "Asian flu" contained the HA (H2), NA (N2), and PB1 genes from an avian virus, with the remaining gene segments from a previously circulating human virus [38, 134]. The 1968 "Hong Kong influenza" H3N2 virus was composed of avian HA (H3) and PB1 segments with the remaining segments being of human origin [38, 135]. The 2009 swine-origin pandemic influenza was composed of avian NA and M gene segments, swine-origin HA, NP, and NS gene segments, and a human PB1 segment, yielding a triple-reassortant virus[136].

Notably, the H1N1 virus responsible for 1918 "Spanish flu", which is believed to be the most severe pandemic in history, arose by infection and adaptation of an avian influenza virus directly into humans [7, 9].

In addition to humans and the natural reservoir, wild aquatic and shore birds, influenza A is capable of infecting a wide range of mammals and birds, including domestic poultry and swine, horses, seals, dogs, cats, whales, and a number of other species[137](reviewed in [131]). The variety of possible hosts for influenza A increases the number of opportunities for mixed infection and possible reassortment.

Efficient infection and replication play important roles in the emergence of a pandemic influenza virus. As infection begins when the influenza HA binds to the sialic acid (SA) receptor on the host lung epithelial cell surface [6, 39, 138], the specificity and affinity to the receptors are major determinants of host range[139]. Human influenza virus strains, such as circulating H1 and H3 viruses, preferentially recognize SA receptors with α2,6 galactose (GAL) linkages[140], which are found in high quantities on human columnar epithelial cells in the upper respiratory tract[141]. In contrast, avian viruses preferentially bind to α-2,3 GAL sialic acids[140, 142] as these are predominant in the avian intestinal tract [139]. α-2,3-linkages are also found in the upper and lower respiratory tract of humans but are much less abundant [143, 144]. Nonetheless, some avian influenza viruses, such as HPAIV H5N1, have been able to infect humans, which will be discussed further below. The difference in the amino acid sequence of these receptors has been determined to be as few as one to two amino acids [145-147]. The reassortment event leading to the emergence of the 1968 Hong Kong pandemic involved a two amino acid change in an H3 duck strain and a circulating H2N2 human strain [138].

In order for the virus to enter the cell, the HA must cleave into two domains, HA1 and HA2. If this does not occur, the terminal end of the HA1 domain is unable to fuse with the host endosomal membrane and the virus is unable to enter the cell. Therefore, if the appropriate host cell protease, usually a trypsin-like protease, required for cleavage is not present, infection is prevented[148, 149]. Notably, these differences in binding sites and protease cleavage are considered to be integral features in the highly virulent nature of HPAIV H5N1.

Highly pathogenic avian influenza H5N1

Highly pathogenic avian influenza virus (HPAIV), such as H5N1, is defined by the CDC as "a virus that occurs mainly in birds, is highly contagious among birds, and can be deadly to them, especially domestic poultry. Though relatively rare, sporadic human infections with this virus have occurred and cause[d] serious illness and death[150]."

HPAIV H5N1 in Humans: The first HPAIV H5N1 outbreak in humans occurred in Hong Kong in 1997. Until then, the virus was known only to infect birds[151]. Since its emergence, the virus has continued to evolve. Beginning in December 2003, an epizootic of HPAIV H5N1 has spread across a total 38 countries [137]. During this time, 15 countries have reported a total of 571 cases causing 335 deaths (as of December 2011)[10]. Most infections have occurred from close contact with infected birds[152], it is suspected that limited human-to-human transmission has occurred in family clusters[153] in Viet Nam[154], Thailand, and Indonesia[155]. Based on this criteria, the WHO has declared HPAIV a Phase 3 Pandemic Alert Period[11].

As of March 2009, ten genetically and antigenically distinct clades have been identified, although most human cases have been caused by clade one and two with the majority being from clade [156]. A third sublineage, detected through surveillance of live bird markets (LBMs), has been found in southern China, among other places, and has caused infections in both poultry and humans.

Polybasic cleavage site as virulence mechanism: HPAIV H5N1 differs from low pathogenicity avian influenza virus (LPAIV) in that it contains a polybasic cleavage site in the hemagglutinin protein, as discussed above. The multiple basic amino acids at this site are not restricted to cleavage by secreted trypsin-like proteases found only in the respiratory and gastrointestinal tracts, as is LPAIV, but can be cleaved by ubiquitous intracellular subtilisin-like proteases, such as furin, enabling the virus to replicate and spread in a largely unrestricted manner[157, 158]. This has been linked directly to enhanced virulence [159].

Binding site: Like other avian influenza viruses, HPAIV H5N1 preferentially binds to α-2,3-linked sialic acids which are present in low numbers on human nasal epithelial cells and alveolar epithelial cells in the lungs[143, 144]. Replication is most efficient in the alveolar epithelial cells in the lungs with antigen detected in pneumocytes [160] in the deep airways, which could be responsible for the observed inefficient human-to-human transfer[143]. There remains to be debate as to whether or not this is the case as ex vivo data demonstrating nasopharyngeal, adenoid, and tonsillar tissue infection with H5N1 is possible. As these tissues lack α-2,3-linked sialic acids, it suggests other binding sites must be involved [161, 162].

In studying the reassortant 1957 and 1968 pandemic viruses, it was observed that the adaptations of the AIV HA glycoproteins to the α-2,6-linked sialic acids enabled the novel viruses to more readily infect humans and spread more efficiently [138, 163]. Analysis of the 1957 and 1968 viruses further suggests that the AIV PB1 gene is also important [164]. Generally speaking, polymerase genes are

also believed to be important as there are a number of amino acids identified in human viruses but not in avian strains[9].

It is notable that, like the 1918 “Spanish flu” virus, H5N1 is an avian virus that jumped directly into humans, as discussed above. Thus, comparisons between these two viruses are considered especially relevant. Mutations in the PB2 region of the viral polymerase of the “Spanish flu” H1N1 virus appear to play a role in the viruses’ ability to be spread via aerosol [165]. A study on HPAIV H9N2, another potentially pandemic strain, revealed that as few as five amino acid substitutions was sufficient for H9N2, another potential pandemic strain, to be transferred by respiratory droplets in ferrets[166]. Given the ability for influenza viruses to readily mutate, there is a very real possibility that H5N1 could be the next pandemic[137].

Pathogenesis of HPAIV H5N1 in humans: When HPAIV H5N1 infection occurs in humans, usually after close contact with infected birds, rules such as age, underlying clinical factors such as an immune-compromised state, and other factors that are typically associated with an increased risk for seasonal influenza infection do not seem to apply.

HPAIV H5N1 is almost universally associated with a considerably worse clinical outcome (approximated >60% mortality in reported human cases) than seasonal influenza and there seems to exist some variation in severity between H5N1 clades. The 1997 Hong Kong virus (clade 0) and Egypt (clade 2.2) have a milder disease presentation than other H5N1 viruses [167, 168]. Patients with severe H5N1 infection have a rapid onset of primary viral pneumonia associated

with leukopenia, gastrointestinal symptoms, and mild liver and renal dysfunction [168]. Post mortem findings include diffuse alveolar damage with hyaline membrane formation, patchy interstitial infiltrates and pulmonary congestion with varying levels of hemorrhage. These are indicators of acute respiratory distress syndrome (described below). Macrophages, neutrophils, and activated lymphocytes comprise the majority of cellular infiltrates [169, 170].

Pathogenicity Determinants: There are several different mechanisms and combinations of mechanisms that have been postulated to be important pathogenesis determinants in H5N1 infection and may explain the associated severe disease and high mortality. These include the ability of the virus to disseminate beyond the respiratory tract (altered tissue tropism from seasonal influenza), efficient viral replication resulting in higher and prolonged viral replication and burden directly leading to cytolytic damage, and mechanisms for evading and modulating the host immune responses, including the stimulation of a hyper-immune response [137, 171]. The high levels of pro-inflammatory cytokines observed in mice infected with H5N1 as well as the 1918 pandemic virus [76]are believed to play a major role in the development of acute respiratory distress syndrome (ARDS), which is characterized by inflammation in the lung parenchyma causing impaired gas exchange and eventual hypoxemia and multiple organ failure[73]. ARDS is considered to be the ultimate cause of the lethality of these viruses in human [172]. Furthermore, the almost complete lack of cross-reactive

antibodies against H5N1 from previously encountered influenza strains is likely a contributing factor to the inordinately high viral loads.

Vaccine Antigens and Correlates of Protection

Hemagglutinin: Hemagglutinin (HA) is currently the primary target of most influenza vaccines. As discussed previously, high affinity/avidity, neutralizing, receptor-blocking antibodies against the hemagglutinin (HA) glycoprotein on the surface of the virion are effective in preventing influenza infection [33, 173]. Upon recognition of viral invasion, these neutralizing antibodies prevent entry into the cell [84, 85] by binding at the same site as host receptor binding [174]. HA-specific antibodies can also interfere with HA-mediated membrane fusion [175].

Sufficient HA-specific antibody titers are considered to be protective against influenza infection, thus HA-specific antibody titers are the method by which vaccine efficacy is gauged. The standard assay, as determined by the CDC [176], is the hemagglutination inhibition (HI) assay, taking into account both seroconversion as well as the amount of HA contained in the vaccine [130, 177].

In order for neutralization to occur and to maximize vaccine efficacy, the circulating virus strain must mirror the vaccine strain. It is because of this that the seasonal vaccine is reformulated nearly every year as the epidemic strains exhibit antigenic drift, as mentioned previously [178]. The WHO closely monitors and organizes the selection of prevailing strains for any given year. This method is less than desirable for pandemic strains as they emerge very quickly and often without

warning, making it difficult to prepare exactly matched vaccines before the virus has spread. Methods to increase the breadth and strength of immunity, such as adjuvanting, are being investigated for use with potentially pandemic vaccines [179-181]. Adjuvanting could also allow for less antigen to be used, allowing for more efficient vaccine production and administration [182]. This will be discussed further below.

Neuraminidase: Secondary to HA in immunogenicity is the neuraminidase (NA) glycoprotein. NA is also present on the surface of the virus as well as virus-infected cells and is responsible for cleavage of sialic acid residues as the virus buds from the cell at the end of the replication cycle[1]. Approximately four times less NA is expressed on the surface of the virion than HA [183] which is at least partially responsible for the observed skewing of the natural host response towards HA. The disparity in immune pressure could be responsible for the significantly lower mutation rate observed in NA as compared to HA [184, 185]. A higher degree of conservancy, however, does increase the likelihood of the possibility of broader protection if used as a vaccine antigen.

Like HA, NA is targeted by antibodies [34] which, unlike HA-specific-neutralizing antibodies which function primarily to interfere with initial attachment and entry [186-188], function essentially by trapping the progeny virus inside the cell thus reducing viral spread[189]. The antiviral drug oseltamivir works in a similar fashion, which will be discussed in the section on antiviral drugs. By trapping the virus on the surface of infected cells, it can enhance susceptibility of

CTL or NK-cell recognition and killing. NA-inhibiting (NI) properties of NA-specific antibodies are also believed to aid in protection by activation of the classical complement pathway [190], through ADCC [191], and through antibody-mediated opsonophagocytosis [192]. These all contribute to increased resistance to influenza infection in humans [34, 187, 188]. Targeting NA also offers the possibility of some degree of intersubtypic cross-protection against homologous or heterologous NA proteins as has been demonstrated with use of DNA vaccines in the mouse model [35, 193]. There is also evidence that antibodies from previously circulating H1N1 viruses may provide limited protection against HPAIV H5N1 in spite of major antigenic differences [35]. Similar results were obtained when comparing the contribution of seasonal H1N1-specific antibodies to the cross-protection observed in some infected with the 2009 pandemic H1N1 virus [194] Most evidence to date indicates that NA is a poor inducer of heterosubtypic immunity [193] (reviewed in [195]).

Mucosal antibodies, as discussed above, are an important consideration in vaccine design because they are induced at the primary site of infection. sIgA primarily specific to HA and NA in nasal secretions[91] inhibits influenza virus entry at mucosal surfaces and is induced well by live attenuated vaccines[196, 197] but is not well induced by inactivated vaccines[198].

Together, antibody titers against HA and NA correlate with resistance to infection both experimentally [199] and naturally [200]. IgA, IgM, and IgG specific to HA and/or NA can be detected within 10-14 days of primary infection. IgA and

IgM will begin to decline after peaking at 2 weeks post-infection, whereas IgG continues to climb until it peaks at 4-6 weeks[201].

Matrix-2 Protein: The influenza M2 protein is a transmembrane, ion channel forming protein expressed at the surface of the infected cell [202]. Unlike HA and NA, M2 is relatively conserved across influenza subtypes and has been investigated as a candidate for a universal influenza vaccine [203], although evidence suggests that increasing selection pressure on the M2 protein by the presence of M2-specific antibodies may result in further diversification of the M2 sequence, and that the observed conservation of the protein is likely due to the lack of natural selection on the protein [204]. The adamantane anti-viral drugs work to block the M2 ion channel, but are now largely ineffective due to widespread resistance and is now largely ineffective due to widespread resistance [205].

Antibodies against M2 have been demonstrated to be immunogenic and effective at protecting against otherwise lethal challenges in mice, ferrets, and non-human primates [206-209], although these antibodies are not neutralizing and protection conveyed by these antibodies is limited. M2-specific antibodies contribute to NK-cell activity [210] and it is likely that M2-specific antibodies are protective through antibody-dependent cytotoxicity.

Evidence in the mouse model suggests it could be used in conjunction with other antigens and/or in a prime-boost strategy [23, 211]. Some studies have shown evidence that indicates M2 is not protective against lethal challenge in natural

influenza reservoirs such as chickens [212] and pigs [213] and that disease may be exacerbated.

Due to the cross-reactivity observed in M2-specific antibodies, it is possible that M2 could be used in passive monoclonal antibody transfer therapy, which has been shown to protect against lethal challenge in mice [214].

Memory and effector T cells also appear to play a role in M2-based protection, as has been demonstrated by depletion of CD4+ and CD8+ T cells during influenza challenge [23].

Nucleoprotein: Like vaccines targeting M2, vaccines targeting the nucleoprotein (NP) are appealing because the NP is extremely conserved thus they have the potential to induce heterosubtypic immunity. Unlike, M2, which is an external protein, NP is an internal protein, and vaccines targeting conserved internal proteins are primarily interested in the induction of cytotoxic T cells (CTLs) [105], although there is also evidence that non-neutralizing NP-specific IgG plays a limited role in protection [215]. CTLs have been shown to be directed to not only NP [216, 217], but M1, NS1, and the polymerases (PA, PB1, and PB2)[218] as well, making these potentially important targets for broadly protective influenza vaccines [219-222] (reviewed in [105]).

Targeting internal influenza antigens in a vaccine would require endogenous expression of the antigen, such as that observed in viral infection, which limits mechanisms of vaccine delivery to methods that ensure peptide is processed and presented to the CTL in the proper context, which includes plasmid delivery (DNA

vaccine), direct peptide delivery, and viral vectors. This will be discussed further in the discussion of vaccines below.

CTL-based protection is believed to be primarily based on the clearance of infected cells[223] which correlates with T cell levels [224]. T cells do not seem to play a role in prevention of infection[201]. Likewise, NP-based vaccines relying mostly on primed CTLs will not abrogate morbidity entirely as there is an interval of time between infection and virus clearance, although mortality has been shown to be reduced or eliminated [216]. This has been demonstrated on a DNA-based platform [225], when administered with other antigens [226, 227] and appears to be especially promising in a prime-boost system [211, 228]. This could provide a level of immunity against novel pandemic strains in a population that would otherwise likely be completely naïve.

Worthy of mention is the disparity in vaccine success between the mouse model and humans, especially in vaccines with conserved antigens. Although NP and M2-based vaccines have repeatedly provided protection in mice, when used in ferrets, which are a much better influenza model for humans, protection was generated against low doses of H5N1 but not against more rigorous challenge. Further studies are needed [229].

Heterosubtypic Immunity: As discussed above, the most robust natural immunity against influenza (mediated by neutralizing antibodies) is restricted to the specific strain that caused the original infection. Heterosubtypic immunity, which would provide cross-protective responses between serologically distinct

influenza viruses, would obviously be beneficial not only for seasonal influenza vaccination, possibly eliminating the requirement for repeated yearly administration, but also for pandemic preparedness. Pandemics typically occur without warning and spread quickly throughout a naïve population. This is especially troubling in the event that a HPAIV were to mutate such that it could be transmissible between humans. Even a vaccine providing partial protection against such a virus would be advantageous.

Heterosubtypic immunity primarily against conserved viral proteins has been demonstrated in the mouse model for a number of different influenza virus strains [230-233] and has been shown to be long lasting and protective against an otherwise lethal influenza challenge. Mechanisms of heterosubtypic immunity are attributed primarily to CD8+ cytotoxic T lymphocytes (CTL) [234, 235] and CD4+ T helper cells [236], but heterosubtypic immunity is not well understood and evidence shows it is likely a system of redundancy [232].

A cold-adapted, live-attenuated influenza vaccine has been shown to have some potential to yield cross-protective immunity [237]. The generation of a vaccine capable of inducing heterosubtypic immunity, the ultimate, of course, being the "universal" influenza vaccine that could not only lessen disease, but protect completely, is obviously the "holy grail" of influenza vaccine research.

Influenza treatment and prevention

Vaccination is the gold standard influenza intervention strategy because an effective vaccine strategy is able to prevent influenza infection. However, antiviral drugs are also an important countermeasure for both seasonal and potential pandemic influenza viruses as they can reduce the severity and duration of illness and limit spread. Antiviral drugs are also important in bridging gaps in protection caused by immune-compromised vaccine recipients (the elderly, etc).

Antiviral drugs: Currently licensed anti-influenza drugs include two adamantanes, amantadine and rimantadine, and two NA inhibitors, oseltamivir (Tamiflu®) and zanamivir (Relenza ®). Adamantanes work by blocking the M2 ion channel, which prevents virus uncoating and release of genome segments into the cytoplasm [1]. NA inhibitors (oseltamivir and zanamavir) work by interfering with viral release from the cells [238] as well as entry into the cells [239]. NA-inhibitors have been shown to prevent H5N1 viral infection in mice [240]. These drugs can be useful as an adjunct to routine vaccination, however, they can only be used after infection has occurred and resistance is a growing problem[241]. A large fraction of the circulating H5N1 isolates are resistant to adamantanes [205] and, although resistance to NA-inhibitors is less prevalent than resistance to adamantanes among HPAIVs, resistant isolates have been identified [242-244].

Other drugs with anti-influenza effects include Ribavarin (Virazole®), Viramidine, and T-705. These drugs are generally broad spectrum RNA virus

inhibitors and work at various stages of transcription and genome replication (reviewed in [245]).

RNA interference: RNA interference (RNAi) involves the use of antisense (DNA) oligomers and short, interfering RNA (siRNA) molecules to specifically target sequences in influenza virus mRNA, blocking translation, and thus suppressing the expression of viral genes (reviewed in [246]). RNAi has been shown to be efficacious in the treatment of severe influenza in mouse and avian models [247, 248] as well as a number of other virus infections.

Passive immunotherapy: Passive immunotherapy with human or humanized anti-influenza antibodies is also a possibility for treatment of influenza and would be potentially useful in severe cases of pandemic influenza. During the 1918 pandemic, a reduction in mortality was observed in patients treated with serum from convalescent survivors [249] and it has been shown to be a potential option for H5N1 infection [250]. This has also been demonstrated extensively in the mouse model (reviewed in [245]).

Influenza Vaccines

Vaccination remains the most important method of influenza prevention and control in the population. At present, two types if influenza vaccines are licensed for clinical use against seasonal influenza in the United States: inactivated vaccines (whole and subvirion) and live attenuated vaccines (LAIVs). Inactivated vaccines are the most common and are licensed worldwide, although licensure of LAIVs (cold-adapted) for use against seasonal influenza is growing [251].

Both are inactivated vaccines and LAIVs are egg-derived, a process requiring up to seven months, in a worse-case scenario, but on average 5-6 months from strain choice to vaccine administration [252]. The process begins with vaccine strain choice. As mentioned previously, each year, the WHO updates the vaccine strains to match the circulating strains detected by their extensive surveillance network. After vaccine strains are agreed upon, usually in February, "reference strains", which include the HA and NA genes of the circulating viruses as well as other components of the virus that are known to grow well in eggs[253]. In lieu of the former "egg-passaging", in the event of a virus that grows poorly in eggs, reverse-genetics techniques are being used to improve the efficiency of this process[254]. Viruses are then grown in embryonated chicken eggs, purified from the allantoic fluid, and further processed for inactivated or LAI vaccines.

Inactivated vaccines: After grown in eggs and purified, virus can be inactivated using formaldehyde or β-propiolactone (BPL) for whole virus formulations, or purified treated with ether or detergent for split or subunit vaccine formulations to yield the inactivated vaccine, which is the most common of influenza vaccines. There are ongoing efforts to produce cell-based influenza vaccines which will be discussed further below. Inactivated vaccines are delivered intramuscularly or subcutaneously into individuals with the goal of priming neutralizing antibodies against the HA antigen. The vaccine is generally effective, although efficacy is often reduced in the very young, elderly, and otherwise immune compromised individuals[255]. Efficacy can also be reduced in years where the vaccine is poorly

matched to circulating strains. Whole inactivated virus has been shown to prime a broader immune response than more purified formulations (split or subunit), likely due to the presence of the viral RNA which has been shown to activate TLR-7 [256]. Purified formulations, however, are better tolerated.

Live attenuated vaccines: Live attenuated influenza vaccines (LAIVs) are cold-adapted such that their replication is limited. After being grown in and purified from eggs, they are administered intranasally as an aerosol, depositing the live virus in the nasopharynx[257]. Live attenuated strains are composed of the HA and NA of interest (the circulating strain from which we seek protection) and the internal segments of an attenuated master donor strain, of which there are two, and influenza A and an influenza B virus. Attenuation is polygenic, reducing the likelihood of a reassortment event leading to reversion to the fully virulent strain[258]. LAIVs are grown in embryonated hen eggs, as described above. In the case of the 2009 pandemic H1N1 virus, the live attenuated vaccine generated by reverse genetics grew very well[259].

The live attenuated vaccine has been shown to be 78-100% effective in susceptible adults and children [260] but a number of barriers must be overcome before the LAIV can become the primary influenza vaccine, such as approval for use in all ages groups. As of right now, the LAIV vaccine is only approved for healthy individuals ages 2 to 49 years of age and requires a specialized device for administration [36]. Live attenuated vaccines take advantage of mucosal immunity

and thus may provide broader immunity against circulating strains as discussed previously.

Regardless of the advantages of LAIV, there is extreme concern about reassortment with other vaccine or circulating influenza virus strains[261], which could cause an attenuated HPAIV H5N1 virus to revert back to the highly pathogenic phenotype, although it has been tested [20] showing evidence of complete protection against homologous virus challenge in mice and chickens [262]. The success from these studies has not been duplicated in humans (reviewed in [263]). Reverse genetics generated H5N1 strains have also proven to grow quite poorly in eggs, which will be discussed further below [17].

As of late, a new approach to developing a live attenuated vaccine involving the influenza NS1 protein has been taken. The NS1 protein is a nonstructural protein that has been shown to be implicated in suppressing the host immune response, most specifically the innate response [264, 265] by inhibition of the IFN-α/β response [266] by complexing with the cellular sensor of influenza ssRNA, RIG-I [46]. Studies in the mouse model have shown that alteration or deletion of the NS1 protein enhances the humoral and cellular immune response [267] and blocks virus replication [262, 267, 268] and that it is efficacious and appears to be safe when administered intranasally and generates neutralizing antibodies[269].

Current H5N1 vaccines: The current egg-based methods of influenza vaccine generation present a number of problems not only for seasonal influenza, but for pre-pandemic preparedness. In the event of a pandemic, the requirement of having

the strain months in advance of distribution is problematic not only in timing but in resources. Pandemics typically occur with little to no warning. Measures must be taken to reduce the time between strain selection and vaccine distribution, especially for HPAIV vaccines, which come with a host of their own problems.

The same vaccine designs that are used for seasonal influenza vaccination have been attempted for HPAIV but attempts have been met with a number of issues. Compounding the number of drawbacks already associated with seasonal influenza preparation, HPAIV is extremely virulent and kills the embryo in the inoculated egg before the virus reaches the reasonably high titers required for efficient vaccine development [270]. This increases the requirement of eggs to generate sufficient quantities of antigen for vaccination. To complicate the matter further, the primary antigen in influenza vaccine development, hemagglutinin (HA) appears to be poorly immunogenic in the case of H5N1[17, 271]. Early inactivated HPAIV H5N1 vaccines were developed using two Clade 1 viruses, a 2003 H5N1 isolate from Hong Kong and 2004 isolates from Vietnam. These inactivated (2003) and subunit (2004) vaccines required two doses to generate sufficient protective neutralizing antibodies, which further increases the egg requirement, although evidence suggests the MF59 adjuvant increases immunogenicity[272]. Adjuvanting will be discussed further below. The US Food and Drug Administration (FDA) approved an inactivated subunit vaccine for H5N1 in late February 2007 to be used in the event of a pandemic in spite of evidence of limited effectiveness[17]. There appears to be little or no cross-protective neutralizing antibodies between clade 1

and clade 2 vaccines. Overshadowing all of these hurdles is the safety and production issues inherent in working with H5N1, which is classified by the CDC/USDA as a select agent, and the high cost associated.

There are efforts underway to generate cell-based HPAIV vaccines using wild-type virus and this has been met with a reasonable amount of success (reviewed in [273]). Whole inactivated virus vaccines generated from clade 1 and clade 2 H5N1 viruses grown in Vero cells provided cross-protective immunity in the mouse model [274]. A similar vaccine in Vero cells was shown to be immunogenic and well-tolerated in humans [275]. Unfortunately, these methods still require high containment facilities for production with all the accompanying safety issues and high cost.

Reverse genetics has been used to generate live attenuated reassortant viruses to be used as seed viruses in an attempt to circumvent the need to propagate the highly pathogenic original strains [15, 16](reviewed in [15]). Some of these reverse genetics generated seed viruses include cell-adapted strains [273, 276], which would be useful in reducing the pressure on egg-based vaccines, although there are concerns about the introduction of adventitious or oncogenic elements in the absence of the natural filter present in eggs [277]. These viruses contain the immunogenic glycoproteins from HPAIV (HA and NA) on the backbone of a low virulence human influenza strain [278, 279]. These viruses could potentially be used to generate antigen for inactivated vaccine or be used directly as a live-attenuated vaccine. Unfortunately, these viruses have proven to grow as poorly as HPAIV in

eggs and appear to be even less immunogenic than the wild-type protein, requiring as much as six times more antigen than the standard vaccine [17, 271]. There is also evidence that over attenuation *in vivo* can occur [278]. The WHO continues to determine the antigenic characteristics ideally suited for pre-pandemic vaccines and for stockpiling[280].

Immunomodulators: Adjuvants, as mentioned previously, have the ability to enhance immunogenicity of a vaccine by activating the innate immune system directly, by recruiting immune mediators to the vaccine site, or a combination thereof. HA-based adjuvanted vaccines, including phospholipids or oil-in-water emulsions, such as squalene-based MF59 (Novartis [281]) or AS03 (GlaxoSmithKline [282]) [283, 284], have been approved for use in Europe. MF59 works in a TLR-independent fashion by targeting muscle cells to create an immunocompetent environment [285], including the attraction of mononuclear cells and aiding in the differentiation of monocytes into DCs [286]. MF59 has also been shown to be a potent inducer of CD4+ T helper cells [287]. The use of adjuvants is especially important for HPAIV vaccines, such as H5N1, as their wild-type HA proteins are not exceptionally immunogenic [179, 182, 272]. It is also possible that adjuvanting H5N1 vaccines could boost cross-reactivity [180, 181] and since it is so difficult to precisely predict what strain might emerge, in the event of a pandemic, any broadening of protection would be welcome. H5 inactivated vaccines administered with aluminum hydroxide or aluminum phosphate adjuvants [288, 289] have demonstrated enhanced immunogenicity.

Immune stimulating complexes (ISCOMs) represent a subclass of adjuvants. ISCOMS are comprised of phospholipids, cholesterol, and purified saponins from the tree *Quillaja saponaria* Molina [290]. The antigen is either encapsulated in the lipid structure or can simply be administered in tandem. ISCOMs generate a broad and robust immune response (reviewed in [291], and significantly improved an inactivated subunit vaccine against H5N1 [292]. A host of other adjuvants for influenza vaccines are being tested (reviewed in [293]), including toll-like receptors TLR-4[294], purified bacterial proteins [295], cytokines and chemokines[296, 297] and others[298].

Recombinant protein: Recombinant HA protein (rHA)-derived vaccines are also in development and in late stages of clinical trials. This method is not unlike inactivated vaccines in the final stages but circumvents some of the problems associated with egg- and mammalian-cell-based vaccine development. The HA from the selected vaccine strain is cloned into a baculovirus vector, which is used to infect insect cells, generating insect cells that express the HA protein of interest [299]. This HA protein is then purified and used to formulate a trivalent vaccine [300]. Efficacy and safety have been demonstrated, although a large dose was required to generate an appreciable neutralizing antibody response, and this method has been submitted to the FDA for approval [301].

Virus-like particles: Virus-like particles (VLPs) (reviewed in [302]) are non-infectious, non-replicating particles containing immunologically relevant viral structures They are generated in a similar fashion as rHA protein and thus also offer

an alternative to egg- or mammalian-cell-based vaccines. VLPs For influenza, these are generally generated by expressing HA, NA, and the matrix protein (M1) in baculovirus vectors. Yeast, mammalian, or insect cells are then infected with the baculovirus vectors expressing the influenza genes, and due to the presence of the M1 protein, which is a structural protein involved in viral assembly and budding, the influenza proteins spontaneously assemble and bud from infected cells, forming particles that resemble wild-type virions but are not actually infectious. VLPs have been shown to be effective against H9N2 [303], H3N2 [304], and H5N1 [305, 306], in some cases providing protection against heterologous challenge. They have been shown to be protective against homologous and heterologous HPAIV H5N1 challenge in ferrets in a dose-dependent manner [307]. VLPs are generally considered to be safe considering the absence of functional replication machinery and are in Phase I/IIa clinical trials for evaluation of safety and immunogenicity against clade 2 H5N2 (A/Indonesia/05/2005) [277].

VLPs have also been produced in plants, such as *Nicotiana benthiama* expressing the HA from a low pathogenicity H1N1 virus as well as the HA from HPAIV H5N1 and were shown to be immunogenic and protective in low doses against homologous virus challenge as well as heterologous virus challenge when boosted [308]. Other vaccine concepts have been tested with plants (reviewed in [309]), including a transgenic potato expressing a Hepatitis B virus B antigen [310].

DNA Vaccines: DNA-based vaccines (reviewed in [311]) have been studied extensively for the past two decades. DNA vaccines, in short, are bacterial plasmids

containing a strong promoter that is active in eukaryotic cells, the gene of interest[311] (sometimes modified [312]), nucleotide sequences (cytidine phosphate guanosine, or CpG) that stimulate the innate immune system via TLR-9 (reviewed in [313]), and usually a selection marker required for production of the plasmid in bacteria. When administered, traditionally intramuscularly, the plasmid primes the immune system by being transferred into antigen presenting cells (APCs), either by direct transfection or indirectly by transfecting muscle cells [314].

Other methods of administration, including gene gun, epidermal administration [315], and electroporation [316] have been investigated with hopes of increasing cellular uptake.

DNA vaccines have the potential to induce broad, long-term immunity. Trials in animals have shown promising results expressing HA [317] and NA [35, 193], and due to a lack of heterologous protection, much work has been done to broaden protection by expressing more conserved antigens [217], consensus-based HA [318], and a combination thereof [21, 319]. DNA-based vaccines targeting conserved sequences of HPAIV H5N1 have yielded partial protection against HPAIV challenge [225, 320] results of clinical trials have not shown such promise [315, 321, 322], with vaccines achieving some level of protective antibody but only after multiple administrations at high doses. This is likely in part due to insufficient protein expression. DNA vaccines are also being tested for a number of other pathogens and diseases, including SARS, HIV, malaria, and cancer [323].

Drawbacks to use of DNA vaccines include potential integration into host chromosomes, and increasing use of methods such as electroporation to increase cellular uptake of the plasmid has been shown to potentially increase the likelihood of integration [324]. Extensive research has also been done to ensure that anti-DNA antibodies do not induce autoimmune disease [325].

Notably, DNA vaccines have emerged as important priming agents in prime-boost regimens, often using viral vectors as the booster [326]. A prime-boost regimen has a number of advantages, including greater breadth of immunity (humoral and cell-mediated, perhaps), a decrease in the likelihood of escape mutants, and greater antigen recognition in the genetically diverse human population. DNA vaccines have been studied extensively as part of a prime-boost regimen with certain virus vectored vaccines and they appear to complement each other well, as DNA vaccines are often not immunogenic enough on their own and some viral vectors suffer from pre-existing immunity to the vector[327].

Virosomes: Virosomes are spherical unilamellar liposomal vesicles produced by mixing purified influenza virus with phospholipids [328-330]. These vesicles are endocytosed and the antigen is processed as usual, inducing cellular immunity by the MHC I pathway [331]. As reduced immunogenicity is a problem in the elderly, virosomal vaccines have been efficacious in closing the gap [332-334].

Virus-vectored vaccines: Virus-vectored vaccines are the main focus of this work. A variety of vector viruses that are either incapable of replication or replicate without causing disease are being tested as carriers for influenza vaccine antigens.

Virus candidates that have been studied in both replicating and non-replicating form include DNA viruses such as adenoviruses and vaccinia viruses (poxviruses). Primarily replication-defective candidates are positive-strand RNA viruses such as alphaviruses, attenuated or chimeric flaviruses, and replicating vectors include baculovirus and assorted negative-sense, negative-stranded RNA viruses (NNSV). Until the advent of reverse genetics in 1994 [335], NNSVs were not able to be used as viral vectors[336], but now represent a major field of study due to the number of advantages of using such a virus as a vector. Other vectors include adeno-associated viruses and herpesviruses, but, as they are not relevant vectors for influenza, they are outside the scope of this review.

There are a number of inherent advantages of using viruses as vaccine vectors to induce protective immunity against other viruses (and other pathogens as well). Virus vectors are able to embody the benefits of a live, attenuated version of the pathogen itself and are especially useful when, due to any number of possible reasons, a live attenuated version of a pathogen is not feasible. Furthermore, viral vectors can be chosen or engineered to specifically target to a certain cell population to optimize priming of a naturally relevant, protective response.

Virus vectored vaccines are also an important component to the extensively studied prime-boost regimes, especially those involving DNA vaccine priming, as has been discussed previously. Here, virus-vectors studies for use as an influenza vaccine will be reviewed.

Adenovirus: Adenoviruses (genus *Mastadenovirus)* are DNA viruses and are responsible for a wide range of species-specific diseases caused by a wide range of serotypes. The high rate of infection in humans by different serotypes complicates the use of adenovirus vectors, and preexisting immunity is of primary concern.

Replication and Expression features: Recombinant adenoviruses (rAd) have been constructed such that they are replication incompetent in human cells (although they can also be used as replicating vectors). They are able to accommodate gene inserts from 7-10 kb depending on the construction and deletion of viral genes (reviewed in [337] and are able to express the inserted gene (vaccine antigen) at high levels. These viruses can be grown quickly and without the need for eggs in qualified cell lines designed for use with a replication deficient virus[338]. Adenoviruses infect dendritic cells, among a wide range of other cells, leading to efficient antigen presentation to the immune system [339].

As a Vaccine Vector for Influenza: rAd-vectored influenza HA-based vaccines have been shown to be immunogenic in non-human primates against the 2009 pandemic H1N1 virus [340] and in humans against seasonal influenza virus strains via multiple routes of administration (epicutaneous, intranasal, etc) [341]. rAd has also been tested as a vector for HPAIV H5N1 in a number of models. Hoelscher et al. and others have shown that replication-incompetent rAd expressing the HA from H5N1 provides protection against HPAIV H5N1 challenge, homologous and in some cases antigenically distinct [226, 342, 343]. Cross-protection against assorted influenza strains has also been shown using rAd expressing M2 [23], NP [228, 344],

M2+NP [227], as has a system expressing H5N1 HAs from both Clade 1 and Clade 2 in tandem with the conserved NP genes [345]. CAdVax, a next-generation rAd platform involving the removal of certain regions of the adenovirus genome allowing for insertion of multiple target genes, has expanded possibilities for antigen expression. Holman et al. showed that multiple HA antigens, NA, and M1 can be expressed together and in their native form [346]. Non-human rAd strains have also been investigated, and may be important in the use of rAd vectors in the face of preexisting immunity.

The rAd vector has been used not only as an influenza vaccine platform, but with a number of other viruses, including HIV [347-349], hepatitis B virus [350], SARS coronavirus [351], Marburg and Ebola viruses [352, 353], West Nile Virus [354], and Dengue virus [355, 356]. The latter four utilize the CAdVax platform. rAd has also been investigated in the field of cancer vaccine research [357].

Safety and Preexisting Immunity: As a DNA virus, there is concern that viral genes could be integrated into the host genome. Adenovirus seroprevalence in the human population is another area of concern [358]. Evidence in mice, non-human primates, and humans indicates that existing antibodies against the vector might interfere with vaccine efficacy, especially if multiple vaccinations with the same or different antigens are administered [359]. This could reduce initial efficacy, as well as prohibit the use of a prime-boost system using the same vector. Some studies indicate that choosing serotypes sufficiently unlike one another, thereby limiting cross-reactivity, as well as choosing strains that are rare in the human population,

such as a non-human strain [360], may be enough to circumvent the problem of pre-existing immunity reducing vaccine efficacy[361, 362] and has been studied for influenza [363]. Other strategies to avoid the effects of preexisting immunity include a simple increase in vaccine dosage (although dosage of rAd-vectored vaccines required is already relatively high [364]), the use of a heterologous prime-boost system (such as DNA-prime, rAd-boost system [365]), which has been shown to be efficacious in protecting against lethal influenza virus challenge, including H5N1 [225], and alternate routes of administration [359]. This has the potential to work in a similar way for other viral vectors.

Vaccinia virus: Poxviruses are a family of large double-stranded DNA viruses and include smallpox and avipox, such as canarypox and fowlpox, which have been utilized as replication-deficient viral vectors.

Replication and Expression features: They have large genomes capable of maintaining a large amount of transgenic material, especially avipox viruses, which are able to infect, but not replicate in, human cells. They are able to express large amounts of transgene, often using their own promoters. A downside to such a large genome is competition for antigen presentation pathways. Replication of poxviruses occurs in the cytoplasm which, as previously discussed, eliminates the chance of viral gene integration into the host genome. Although replication deficient, it can easily be grown in qualified cell lines [366].

As Vaccine Vectors for Influenza: Particular emphasis has been placed on modified vaccinia virus Ankara (MVA) poxviruses. The original study for use of MVA

as a vector-based vaccine for influenza involved the insertion of the HA and NP gene from A/Puerto Rico/8/34, a circulating H1N1 virus, into a recombinant MVA (recMVA) vector. It was shown to induce neutralizing antibody- and CTL-mediated protection [367]. recMVA has also been explored as a vaccine against HPAIV H5N1, and was shown to protect mice (when given in two high titer doses) [368] and non-human primates against homologous and cross-clade challenge [369]. A multi-valent vaccinia virus-based H5N1 vaccine expressing the HA, NA, and NP from A/VN/1203/04 and the M1 and M2 from A/CK/Indonesia/PA/2003 induced protective neutralizing antibodies in mice when adjuvanted with IL-15 [370].

recMVA has been studied extensively for use as an HIV vaccine as well as other viruses [371], bacteria, including *Mycobacterium tuberculosis [372]*, and malaria caused by *Plasmodium* spp., as well as a variety of tumor-associated antigens for cancer immunotherapy [373] and, interestingly, allergies [374].

Safety and Preexisting Immunity: recMVA has been determined to be safe in humans, including immunocompromised patients, and has already been in use as a smallpox vaccine [375, 376]. Furthermore, unlike adenovirus, repeated vaccination with the same strain is possible because pre-existing antibodies to MVA do not appear to significantly interfere with vaccine efficacy [377, 378]. Nonetheless, studies investigating methods to avoid vector-specific pre-existing immunity have indicated that using a prime-boost system, such as DNA[365], other viral vectors (reviewed in [379]), or other immunomodulators can aid in the circumvention of

interference from vector-based immunity. Similar to adenovirus, a mucosal route of vaccination has also been suggested [380].

Alphavirus: Alphaviruses are single-stranded, positive-sense RNA viruses in the Togavirus family. They are zoonotic, arthropod-vectored viruses only entering human populations sporadically, suggesting low-seroprevalence [326].

Replication and Expression Features: Alphaviruses are naturally targeted to dendritic cells, replicating in the cytoplasm and delivering the vaccine antigen directly to the immune system. They are extremely immunogenic [381], which is attributed to the extremely high level of protein expression [382] and recognition by various pathogen-recognition receptors (PRR) during replication, leading to a robust innate immune response. They also induce apoptosis in infected cells which is important in cross-priming of the immune system [383].

Transgene Expression: Alphavirus-vectored vaccines are typically engineered as non-replicating virus replicon particles (VRP) with the structural genes deleted [384]. This provides room for the inserted vaccine antigen gene and allows for the inserted gene to be the primary immunogen.

As a Vaccine Vector for Influenza. Alphaviruses typically used for vaccine development are Venezuelan equine encephalitis virus (VEE), Sindbis virus (SIN), Semliki forest virus (SFV), and VEE-SIN chimeras [349]. SIN expressing the HA and NP from A/PR/8/34 (H1N1) [385] and VEE expressing the HA from HPAIV H5N1 [386] have been shown to be effective against homologous influenza challenge in mice and chickens, respectively. VEE-VRPs were also tested expressing the NA from

HPAIV in chickens with mixed results [387] and VEE-VRPs expressing the HA from assorted influenza strains was shown to be immunogenic in swine [388]. Alphavirus vectors have also been tested as vaccines against a number of other viruses, including HIV [349], Hendra, and Nipah [389] and have been used in gene therapy as a prophylaxis against tumors [390].

Drawbacks to the use of alphaviruses include cytotoxicity and difficulty and high cost associated with production [390] although recombinant alphaviruses are less so.

Baculovirus: Baculovirus (*Autographa californica* multicapsid nucleopolyhedrovirus) naturally infects insect cells and is capable of transducing mammalian cells in cell culture, which is the primary method by which recombinant HA influenza vaccines are produced (as discussed previously).

Baculoviruses primarily prime the innate branch of the immune system in both TLR-9-dependent and –independent fashions. Baculoviruses also have been shown to have the potential to drive strong systemic and mucosal immune responses by intranasal and oral administration [391]. An oral route of administration is an alternative to intranasal immunization for driving a mucosal antibody response, including the potential for providing protection in the lungs [392], without the concern for patients with respiratory diseases (asthma, etc) [393].

As a Vaccine Vector for Influenza: Pseudotyped baculoviruses are created by integrating the antigen of interest into the fusion protein of the baculovirus, which is

naturally translocated to the plasma membrane and integrated into the envelope [394]. This aspect has been exploited in the investigation of pseudotyped baculoviruses as a vector platform for avian influenza [394], where hemagglutinin-inhibiting antibodies and cellular responses have been observed to be induced in the mouse model [395]and were shown to be protective against HPAIV H5N1 in both the mouse model and non-human primate model [396, 397].

Negative sense, single-stranded RNA viruses (NNSVs): NNSVs (order *Mononegavirales*) includes paramyxoviruses (*Paramyxovirudae*) such as measles virus, mumps virus, Sendai virus, Newcastle disease virus, human respiratory syncytial and metapneumoviruses, human parainfluenza viruses 1-4, and parainfluenza virus 5 (PIV5); filoviruses (*Filoviridae*) Ebola and Marburg viruses; Borna disease virus which is alone in the family *Bornaviridae*, and rhabdoviruses vesicular stomatitis virus (VSV) and rabies virus in the family *Rhabdoviridae*.

As mentioned previously, not until as of late has it been possibly to utilize NNSVs as vaccine vectors due to the inability to manipulate RNA genomes. Using reverse genetics, it is now possible to recover NNSVs from cDNAs by expressing the proteins required for viral transcription and replication simultaneously with a plasmid encoding the RNA genome [398]. Compared to positive strand RNA viruses, the NNSV genome is stable. A recombinant PIV5 expressing GFP maintained expression of the gene for at least 10 generations [399], while positive strand RNA viruses often delete their inserted genes very quickly. NNSVs are also able to accommodate large gene inserts compared to some other potential vector genomes,

while maintaining a relatively small genome such that competition for antigen presenting pathways is minimized. Their genomes are also quite simple and well understood, especially when compared to large, complex genomes such as those found in the *Poxviridae* family. A very provocative feature of NNSVs is the gene transcription gradient, whereby genes closer to the leader sequence are transcribed more abundantly than genes distal to the leader sequence. By inserting a gene closer to the 3' promoter, expression of the gene of interest could be increased

In terms of vaccine production, NNSVs offer another egg-free alternative. Furthermore, most replicate in VERO cells, which is a WHO-approved vaccine cell line. Most can also be administered intranasally, providing the opportunity for mucosal as well as robust systemic immunity, both antibody and cell-mediated. Several NNSVs are currently being used for vaccine development, including vesicular stomatitis virus (VSV) and a number of viruses in the family *Paramyxoviridae* to be discussed in detail below.

Vesicular stomatitis virus: Vesicular stomatitis virus (VSV) is a highly lytic, negative-sense, single-stranded RNA virus (NNSV) in the family *Rhabdoviridae* and is the first NNSV to be discussed in this review. VSV is primarily an infection of livestock [400], although some serotypes do infect humans, causing primarily mild "flu-like" symptoms [401] , although serious conditions such as encephalitis have also been reported [402]. There appears to be low seroprevalence in most areas of the world, however, infection induces a protective neutralizing antibody response to

the envelope glycoprotein which would limit the use for homologous prime-boost[326].

Genome, Replication, and Expression features: VSV has a relatively small genome at 11 kb and can accommodate an insert of up to 4.5 kb [403] and is able to express foreign glycoproteins on the surface of the virion [404]. VSV is a highly lytic virus that targets dendritic cells, and by interacting with TLR-7, stimulates a strong type I interferon response [49]. VSV has also been shown to prime a robust cell-mediated immune response [405].

As a Vaccine Vector for Influenza: rVSV expressing the HA protein from assorted low pathogenicity influenza viruses has been shown to be protective against otherwise lethal challenges [406, 407], including when administered post-exposure [408]. rVSV expressing NP has been shown to induce a robust CD8+ T cell response, although it was not protective on its' own [409]. When tested against HPAIV H5N1, cross-clade neutralizing antibodies were induced and protection was elicited against a similar, but antigenically distinct, H5N1 strain [410]. Taking advantage of the increase in transcription levels proximal to the 3' end of the genome of VSV [411], the authors inserted the H5 gene closer to the leader sequence, and were able to show sterilizing, cross-clade immunity in mice using a prime-boost system [412] as well as cross-clade neutralizing antibodies in non-human primates [413]. rVSV expressing the HA from a HPAIV H7N1 has been shown to be protective against challenge in chickens [414].

VSV has also shown particular promise as a potential HIV vaccine [415] and has also been investigated as a vaccine against hepatitis C virus (HCV), hantavirus, and *Mycobacterium tuberculosis* (reviewed in [400]).

Safety and Preexisting Immunity: Neurotropism and neurovirulence is a major safety concern with VSV as natural and lab-adapted strains have been shown to be neurovirulent in rodents [416]. Attenuated strains have been shown to be less neurovirulent in non-human primates, but not sufficiently [417]. Over-attenuation could lead to reduced immunogenicity, possibly requiring multiple doses of the vaccine, and pre-existing immunity to the vector would limit boosting in most cases, although a heterologous prime-boost system could be used. Replication incompetent strains are also being developed in the event that rVSV cannot be sufficiently attenuated for human use, however, special requirements for growth of these mutants would likely be a limiting factor for mass production [418].

Paramyxoviruses

There are a number of paramyxoviruses that have been explored as vector candidates, including measles virus, Newcastle disease virus, human parainfluenza viruses, and parainfluenza virus 5. Measles virus has been tested as a vector for simian immunodeficiency virus (SIV) [419] and HIV [420]; and human parainfluenza viruses (and chimeras) have been tested as vaccine vectors for Ebola virus [421], RSV [422], and other pathogens. Neither are relevant in the field of influenza research and are therefore outside the scope of this review.

Newcastle Disease Virus: NDV is an avian paramyxovirus in the genus *Avulavirus*. There are three subgroups of NDV strains: velogenic strains, which are extremely virulent and cause systemic infection; mesogenic strains, which also cause systemic infection but it is less severe; and lentogenic strains, which are primarily restricted to the respiratory tract and have been attenuated for use as live-attenuated vaccines in poultry (reviewed in [423]). When mesogenic or lentogenic NDV was administered to non-human primates it was highly attenuated, apparently restricted to the respiratory tract, and expressed high levels of foreign antigen[424]. This is attributed to natural host-range restriction.

As a Vaccine Vector for Influenza: When engineered to express influenza proteins, NDV is another egg-free possibility, as it has been shown to grow to high titers in Vero cells [425]. DiNapoli et al. has shown that a live-attenuated mesogenic strain expressing the HA protein of H5N1 HPAIV (A/VN/1203/04) generated high titers of neutralizing antibodies in serum following a single intranasal (IN) and intratracheal (IT) dose in nonhuman primates[426] and a similar model was effective against HPAIV challenge in mice[427]. rNDV expressing the HA or NA from HPAIV H5N1 was shown to be protective against challenge virus in the lungs in non-human primates [428] As a respiratory virus, NDV requires respiratory tract delivery [429], which is advantageous as a needle-free alternative, but could be problematic in patients with respiratory illnesses (asthma, etc).

NDV has been studied extensively as a vaccine vector in chickens. Nayak et al. demonstrated use rNDVs expressing each HA, NA, and M2 and showed that neither

NA or M2 alone were protective against HPAIV challenge [212]. Importantly, NDV is being used as a dual vaccine in poultry against influenza as well as a number of other avian diseases [430-432].

NDV has also been shown to be immunogenic and induce protective immunity against other viruses, including RSV [433], and was shown to be immunogenic against simian immunodeficiency virus (SIV) [212], SARS-coronavirus [434], and Ebola virus (EBOV) [435]. It has also emerged as a candidate vector for cancer therapy (reviewed in [436]).

Safety and Preexisting Immunity*:* NDV is serologically distinct from human paramyxoviruses thus pre-existing immunity to the vector would not be a problem, although vaccination with NDV-vectored vaccines generates protective immunity to the vector so it is unlikely that repeated use of the vector would be possible [424]. This makes NDV an unlikely candidate for influenza vaccination in humans as repeated vaccination is required in the absence of a universal influenza vaccine antigen.

Parainfluenza virus 5: Parainfluenza virus 5 (PIV5), formerly known as simian virus 5 (SV5) and canine parainfluenza virus 2, is a paramyxovirus in the genus *Rubulavirus*. It is a prototypical paramyxovirus originally isolated from monkey cells in culture in 1956 [27, 437], although it has since been determined to not be a virus of wild monkeys. PIV5 infects a wide range of species, including humans [438, 439] but does not appear to cause disease [438, 440, 441]. The only possible exception is that it is believed to be a cause for kennel cough in canines

[442] although evidence for this is scarce. PIV5 is closely related to human parainfluenza viruses, but antibodies against PIV5 do not appear to be neutralizing [32] although complement has been shown to play a role in the aggregation of virions, but not lysis [443].

Replication and Expression: PIV5 is capable of infection a wide range of cell types, including the vaccine-approved VERO cell line in which it can grow to high titers [29], suggesting it's potential as an egg-free, low cost influenza vaccine alternative. A major factor distinguishing PIV5 from most paramyxoviruses is that PIV5 has only very slight cytopathic effect (CPE) on the cells [27, 444] (although a recent study suggests otherwise[445]) and is able infect nondividing cells. Like other paramyxoviruses, the genome of PIV5 is subject to the gradient of gene expression, which can be utilized to increase transgene expression. Furthermore, the pleomorphic structure of PIV5 provides flexibility to accommodate changes in sizes of PIV5's genome [446], removing many of the restrictions on insert size seen with other vectors.

As a Vaccine Vector for Influenza: Tompkins et al. showed that rPIV5 expressing the H3 from a seasonal H3N2 influenza strain is protective against an otherwise lethal challenge [24]. rPIV5 expressing vaccinia virus (VACV) antigens has also been shown to induce VACV-neutralizing antibodies and partial protection against VACV challenge [447]. As PIV5 is a major component of this research, it will be discussed at length below.

Parainfluenza virus 5

Parainfluenza virus 5 (PIV5), also known as canine PIV2 and formerly known as simian virus 5 (SV5), is a non-segmented negative stranded RNA virus (NNSV) in the *Rubulavirus* genus in the family *Paramyxoviridae*. It is well studied as a prototypical paramyxovirus but not as a viral vector platform and is the primary focus of this review.

PIV5 was first isolated from rhesus and cynomolgus monkey kidney cells in 1956 [27, 437]. Because it was isolated from monkey cells it was assumed to have originated in monkeys, however, serological testing in wild monkeys has shown that there is no exposure to the virus. PIV5 has been shown to infect humans [438, 439] although there is no convincing, reproducible evidence showing that it PIV5 is linked with any human diseases despite speculation that PIV5 might be associated with illnesses such as multiple sclerosis (MS), Creutzfeldt-Jakob disease (CJD), and hepatitis [438, 440, 441]. Despite this, the implication of PIV5 in human diseases has been a subject of controversy and two possible scenarios have been proposed to explain why. The first is that monkey cell lines are commonly used to isolate human viruses and have been shown to be persistently contaminated with PIV5. One would not necessarily detect the presence of the PIV5 as it often shows minimal cytopathic effect (CPE) [27, 441]. Another possibility is the existence of cross-reacting antibodies between other similar human paramyxoviruses, including parainfluenza virus type 2 [448] and mumps virus [449].

Although PIV5 is capable of infecting a wide range of species, including humans, there is no evidence that PIV5 causes clinical disease in humans. PIV5 is believed to be a cause for kennel cough in dogs [442]and killed PIV5 is a component of the commercial vaccine "Vanguard" (Pfizer, Inc).

PIV5 Replication: The PIV5 genome is the smallest of all of the paramyxovirus genomes at 15,246 nt in length, contains seven genes, and encodes eight known viral proteins [446]. The hemagglutinin-neuraminidase (HN), a surface glycoprotein, is involved in virus entry and release from host cells. Upon HN mediated adsorption to the cell, the fusion (F) protein, another surface glycoprotein, mediates cell-to-cell and virus-to-cell fusion in a pH-independent manner, allowing negative sense nucleocapsid release into the cytoplasm.

Once in the cytoplasm, transcription and genome replication begins. The nucleocapsid protein (NP), phosphoprotein (P), and large RNA polymerase (L) proteins comprise the viral ribonucleoprotein (vRNP). Primary transcription begins as the vRNP begins production of leader RNAs and mRNAs from the input virion nucleocapsid. The viral polymerase (P-L) initiates transcription of the positive-sense genome template at the 3' end of each gene, proceeding to the end of each gene where the gene end (GE) region terminates transcription and guides the production of capped and polyadenylated viral mRNAs by a stuttering mechanism. The RNAP continues through the intergenic region (IG) then, upon encountering the gene start (GS) region, restarts mRNA synthesis the start site of the next gene. This process continues down the length of the viral genome, ending at the 5' end.

Reinitiation at the GS region is imperfect, leading to a gradient of transcription that decreases in a step-wise fashion from 3' to 5' [29].

Although there are only seven genes in the PIV5 genome, it encodes eight proteins. V and P mRNAs originate from the same V/P gene which contains two overlapping open reading frames. The V mRNA is faithfully transcribed and the P mRNA is transcribed through pseudo-templated transcription, a process of RNA editing [450]. During transcription of the V protein, the viral RNA polymerase stutters, recognizes a specific sequence in the V/P gene, and inserts two non-templated G residues. As a result, the V/P gene is transcribed into two mRNAs and eventually translated into the V and P protein which share an N-terminus but not a C-terminus [451].

When sufficient viral proteins have been produced in primary transcription, the negative sense genome is replicated to produce a full-length complementary copy called the antigenome, which is then used as a template to direct synthesis of genomic RNA, beginning by synthesis of short trailer RNA. Replication efficiency is dependent on the requirement that the nucleotide chain be in even replicates of six, known as "the rule of six". PIV5 does not require strict adherence to the rule of six, whereas other paramyxoviruses, such as Sendai virus, do [452]. Unassembled N then begins to encapsidate the trailer RNA, leading to the synthesis of encapsidated negative-sense genomes. These can then go on to be additional templates for mRNA synthesis in secondary transcription, templates for the production of additional antigenomes, or for incorporation into budding virions as the helical RNP core [29].

After the genome is replicated, the virions must be assembled and bud from the infected cell. During budding, the nucleocapsid and the M protein are shuttled to the plasma membrane where they join viral glycoproteins HN, F, and SH which arrive via the exocytic pathway (Golgi and ER). The M protein is believed to play multiple important roles in virus assembly at the plasma membrane, including linking the vRNP with the viral glycoproteins as well as recruiting host factors to assist in budding [453, 454]. During virus assembly, the M protein links the NP of the vRNP with the cytoplasmic tails of the glycoproteins (reviewed in [455]), thereby securing the viral envelope (comprised of viral glycoproteins) to the vRNP core. The glycoproteins stud the viral envelope as spike proteins. The neuraminidase (N) portion of the HN protein functions much like the neuraminidase protein of influenza, possessing sialidase activity that cleaves the sialic acid bonds and releasing the virion from the infected cell. Other nonessential proteins include the V protein and the small hydrophobic (SH) protein. These will be discussed further immediately below.

PIV5 immunomodulatory mechanisms: The PIV5 genome includes two nonessential accessory proteins, the V protein and the SH protein, both of which are important in host interactions and immune evasion. The V protein has been shown to be important for RNA synthesis regulation [451] as well as circumvention of the antiviral state. The V protein has been shown to block interferon α and β production and interfere in interferon signaling in a number of ways [456]. During infection of epithelial- and fibroblast-like cells, the V protein has been shown to target STAT1

for degradation. STAT1 is a necessary component for transcription of IFN-stimulated gene factor 3 (ISGF-3) and gamma-activated factor, and without these complexes, IFN I and II-production is inhibited [457, 458]. Polyubiquitination of STAT1 and STAT2 has also been shown [459]. The V protein has also been shown to inhibit IFN-β induction by interference with intracellular dsRNA signaling [460] and by sequestering IRF-3 in the cytoplasm [461]. PIV5 is unable to interfere with interferon induction in the mouse model, rendering growth inefficient [462].

The C-terminal domain of the V protein has also been shown to interact with the DNA binding protein (DDB1), which is involved in the repair of damaged DNA. This interaction results in slowing of the cell cycle [463]. The V protein also appears to have the ability to block the intrinsic apoptotic pathway, as mutation of the conserved cysteine region induced ER stress-related cell death [30].

The small hydrophobic (SH) protein is a 44-AA residue type II integral membrane protein that blocks a tumor necrosis factor (TNF)-mediated extrinsic apoptosis pathway [31, 444, 464].

In addition to the activity of the V protein, PIV5 is able to regulate viral RNA production during replication which reduces host cell responses [465, 466]. PIV5 with a mutation in the V protein establishes a highly productive infection of myeloid dendritic cells but with very little cytokine secretion or upregulation of maturation markers [26], whereas wild type PIV5 generated a robust, TLR-7-dependent IFN-α response, as well as CD80 and CD86 upregulation [467]. Arimilli et al. showed that

rPIV5 expressing flagellin can enhance PIV5-mediated activation of dendritic cells through TLR5 [468].

PIV5 as a vaccine vector: As discussed above, the advent of reverse genetics enabled easy manipulation of the NNSV genome and allowed for insertion and expression of transgenes. There are a number of advantages to using NNSVs as viral vectors. Because of the gradient of gene expression related to distance from the leader sequence, transgenes can be inserted closer to the leader sequence to increase expression [29]. Unlike DNA virus vectors, the cytosolic replication of PIV5 and other NNSVs dictates that there can be no integration of viral genes into the host genome. Furthermore, unlike icosahedral viruses, PIV5 virions have many forms and shapes. This pleomorphic structure provides flexibility to accommodate changes in sizes of PIV5's genome [446]. This is an advantage for insertion of foreign genes as the changes to the viral genome will probably not be tightly restricted by virion structure. The genome of PIV5 is stable, maintaining gene inserts for more than 10 generations [399]. This is an advantage over positive strand RNA viruses as they often quickly delete inserted genes. Recombination in NNSVs is extremely unlikely and has never been observed in nature (reviewed in [469]).

PIV5 is capable of infecting a wide range of cell types, including a variety of human cell lines both established and primary [29]. These cell lines include VERO cells, a cell line approved for vaccine production, in which it can grow to high titers. Unlike most paramyxoviruses, PIV5 has only very slight CPE on the cells [27], although recent evidence suggests otherwise [445].

As with all live replicating vaccine vectors, pre-existing immunity to the vector is a concern. PIV5 is closely related to human parainfluenza viruses, but antibodies against PIV5 do not appear to be neutralizing [32] although complement has been shown to play a role in the aggregation of virions, but not lysis [443].

The advantages of a virus-vectored vaccine, as discussed previously, include the breadth of the possible immune response, both humoral and cell-mediated. Efficient priming of high avidity cytotoxic T lymphocytes is an important part of this goal and numerous studies have shown the importance of a high avidity CTL response [110, 470]. Parks and Alexander-Miller showed that rPIV5 is an efficient inducer of high avidity acute-phase and memory T cells [471], especially when administered intranasally.

Unlike inactivated vaccines which are typically limited to intramuscular administration, PIV5, like other viral vectored vaccines, can be administered intranasally. This is advantageous, as described earlier, because influenza initiates the infection process at a mucosal site. This is beneficial in that it could be administered to patients with respiratory problems (asthma, chronic obstructive pulmonary disorder, etc) that would otherwise be limited by a live replicating vaccine (such as the live attenuated influenza vaccines). Examples of other NNSVs administered intramuscularly include VSV [408, 414], although this can be performed to avoid excessive vector-mediated disease (although it should be noted that even with intramuscular vaccination of VSV, mice still exhibited clinical signs of disease) [408]. NDV has been used as an intramuscular vector for other diseases

[472] but mucosal vaccination has been shown to be necessary for adequate protection against influenza [426, 429] and other viruses [435]. Similar results have been observed using a Sendai virus vector [473, 474].

rPIV5 mutants: It may also be possible to increase potential immunogenicity of PIV5 as a vaccine vector by taking advantage of some of the immune evasion mechanisms PIV5 employs, such as those modulated by the V protein and the SH protein. As discussed above, PIV5 is able to circumvent apoptosis that is typical of paramyxovirus-infected cells. The SH protein is involved in inhibition of TNF-α-mediated apoptosis, which was demonstrated by the creation of a mutant PIV5 virus lacking the SH protein (PIV5ΔSH) [31]. Similarly, a recombinant PIV5 lacking the conserved C-terminus induces apoptosis via the intrinsic apoptotic pathway (PIV5VΔC) [30]. Insertion of a vaccine antigen into either PIV5ΔSH or PIV5VΔC has the potential to increase the possible mechanisms of antigen presentation, as antigens from apoptotic cells are often taken up by professional antigen presenting cells (APCs) and presented in the context of MHC class II.

Previous work: rPIV5-vectored vaccines have been shown to be safe and immunogenic in mice and ferrets when delivered intranasally and to be completely cleared from the lungs within 7-9 days without evidence of systemic infection and inducing minimal pathogenesis [475]. Tompkins et al. showed that a low dose (10^4 PFU) of rPIV5 expressing the H3 (rPIV5-H3) from a seasonal H3N2 influenza strain (A/Udorn/72) is protective against an otherwise lethal challenge [24]. rPIV5

expressing vaccinia virus (VACV) antigens has also been shown to induce VACV-neutralizing antibodies and partial protection against VACV challenge [447].

References

1. Wright, P.F., G. Neumann, and Y. Kawaoka, *Orthomyxoviruses*, in *Fields Virology*, D.M. Knipe and P.M. Kowley, Editors. 2007, Lippincott Williams and Wilkins: Philadelphia. p. 1691-1740.
2. Girard, M.P., et al., *A review of vaccine research and development: human acute respiratory infections.* Vaccine, 2005. **23**(16154667): p. 5708-5724.
3. Fiore, A.E. *Prevention and control of influenza. Recommendations of the advisory committee on immunization practices (ACIP)*. MMWR Recomm Rep, 2008. **57**, 1-60.
4. Lipatov, A.S., *Influenza: Emergence and Control.* Journal of Virology, 2004: p. 8951-8959.
5. WHO, *Pandemic (H1N1) 2009-update 100*, 2010, World Health Organization. p. 1-3.
6. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiological Reviews, 1992. **56**(1): p. 152-179.
7. Reid, A.H., J.K. Taubenberger, and T.G. Fanning, *Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus.* Nature Rev. Microbiol., 2004. **2**(15494747): p. 909-914.
8. Prevention, C.f.D.C.a., *Update: Isolation of Avian Influenza A(H5N1) Viruses From Humans—Hong Kong, 1997-1998.* JAMA: The Journal of the American Medical Association, 1998. **279**(5): p. 347-348.
9. Taubenberger, J.K., et al., *Characterization of the 1918 influenza virus polymerase genes.* Nature, 2005. **437**(16208372): p. 889-893.
10. WHO. *Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011*. Global Alert and Response (GAR) 2011 29 November 2011 [cited 2011 December 15]; Available from: http://www.who.int/influenza/human_animal_interface/EN_GIP_20111129CumulativeNumberH5N1cases.pdf.
11. WHO. *Current WHO phase of pandemic alert for avian influenza H5N1*. Global Alert and Response (GAR) 2011; Available from: http://apps.who.int/csr/disease/avian_influenza/phase/en/index.html.
12. Whitley, Richard J. and Arnold S. Monto, *Seasonal and Pandemic Influenza Preparedness: A Global Threat.* Journal of Infectious Diseases, 2006. **194**(Supplement 2): p. S65-S69.
13. Gerdil, C., *The annual production cycle for influenza vaccine.* Vaccine, 2003. **21**(16): p. 1776-1779.
14. Centers for Infectious Disease and Research, C. *FDA panel supports H5N1 vaccine approval.* CIDRAP Pandemic Influenza News, 2007. 1-2.
15. Horimoto, T. and Y. Kawaoka, *Strategies for developing vaccines against H5N1 influenza A viruses.* Trends in Molecular Medicine, 2006. **12**(11): p. 506-514.

16. Lipatov, A.S., et al., *Efficacy of H5 Influenza Vaccines Produced by Reverse Genetics in a Lethal Mouse Model.* Journal of Infectious Diseases, 2005. **191**: p. 1216-1220.
17. Treanor, J.J., et al., *Safety and Immunogenicity of an Inactivated Subvirion Influenza A (H5N1) Vaccine.* New England Journal of Medicine, 2006. **354**(13): p. 1343-1351.
18. Poland, G., I. Ovsyannikova, and R. Jacobson, *Immunogenetics of seasonal influenza vaccine response☆.* Vaccine, 2008. **26**: p. D35-D40.
19. Galli, G., et al., *Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine.* Proc Natl Acad Sci U S A, 2009. **106**(19416838): p. 7962-7967.
20. Subbarao, K., *Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics.* Virology, 2003. **305**: p. 192-200.
21. Chen, Z., et al., *Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein.* Journal of General Virology, 1999. **80**: p. 2559-2564.
22. Epstein, S.L., et al., *DNA Vaccine Expressing Conserved Influenza Virus Proteins Protective against H5N1 Challenge Infection in Mice.* Emerging Infectious Diseases, 2002. **8**(8): p. 796-801.
23. Tompkins, S.M., et al., *Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including Subtype H5N1.* Emerging Infectious Diseases, 2007. **13**(3): p. 426-435.
24. Tompkins, S.M., et al., *Recombinant parainfluenza virus 5 (PIV5) expressing the influenza A virus hemagglutinin provides immunity in mice to influenza A virus challenge.* Virology, 2007. **362**(1): p. 139-150.
25. Bukreyev, A., et al., *Nonsegmented Negative-Strand Viruses as Vaccine Vectors.* Journal of Virology, 2006. **80**(21): p. 10293-10306.
26. Arimilli, S., M.A. Alexander-Miller, and G.D. Parks, *A Simian Virus 5 (SV5) P/V Mutant Is Less Cytopathic than Wild-Type SV5 in Human Dendritic Cells and Is a More Effective Activator of Dendritic Cell Maturation and Function.* Journal of Virology, 2006. **80**(7): p. 3416-3427.
27. Choppin, P.W., *Multiplication of a Myxovirus (SV5) with Minimal Cytopathic Effects and without Interference'.* Virology, 1964. **23**: p. 224-233.
28. Cohn, M.L., et al., *T Cell responses to the paramyxovirus simian virus 5: studies in multiple sclerosis and normal populations.* Pathobiology, 1996. **64**(3).
29. Lamb, R.A. and G.D. Parks, *Paramyxoviridae: the viruses and their replication.*, in *Fields Virology*, D.M. Howley and B.N. Knipe, Editors. 2007, Lippincott-Raven: Philadelphia. p. 1449-1496.

30. Sun, M., et al., *Conserved Cysteine-Rich Domain of Paramyxovirus Simian Virus 5 V Protein Plays an Important Role in Blocking Apoptosis.* Journal of Virology, 2004. **78**(10): p. 5068-5078.
31. Lin, Y., et al., *Induction of Apoptosis by Paramyxovirus Simian Virus 5 Lacking a Small Hydrophobic Gene.* Journal of Virology, 2003. **77**(6): p. 3371-3383.
32. Young, D.F., et al., *Clearance of a Persistent Paramyxovirus Infection Is Mediated by Cellular Immune Responses but Not by Serum-Neutralizing Antibody.* Journal of Virology, 1990. **64**(11): p. 5403-5411.
33. Potter, C.W. and J.S. Oxford, *Determinants of immunity to influenza infection in man.* British medical bulletin, 1979. **35**(1): p. 69-75.
34. Murphy, B.R., J.A. Kasel, and R.M. Chanock, *Association of Serum Anti-Neuraminidase Antibody with Resistance to Influenza in Man.* N Engl J Med, 1972. **286**(5027388): p. 1329-1332.
35. Sandbulte, M.R., et al., *Cross-Reactive Neuraminidase Antibodies Afford Partial Protection against H5N1 in Mice and Are Present in Unexposed Humans.* PLoS Med, 2007. **4**(2): p. e59.
36. Lambert, L.C. and A.S. Fauci, *Influenza Vaccines for the Future.* New England Journal of Medicine, 2010. **363**(21): p. 2036-2044.
37. Taubenberger, J.K. and D. Morens, *1918 Influenza: the mother of all pandemics.* Emerg Infect Dis, 2006. **12**: p. 15-22.
38. Kawaoka, Y., S. Krauss, and R.G. Webster, *Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics.* J Virol, 1989. **63**(2795713): p. 4603-4608.
39. Yamada, S., et al., *Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors.* Nature, 2006. **444**(17108965): p. 378-382.
40. Fouchier, R.A., *Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls.* Journal of Virology, 2005. **79**: p. 2814-2822.
41. Horimoto, T. and Y. Kawaoka, *Pandemic threat posed by avian influenza A viruses.* Clin Microbiol Rev, 2001. **14**(11148006): p. 129-149.
42. Price, D.A., R.J. Postlethwaite, and M. Longson, *Influenzavirus A2 Infections Presenting with Febrile Convulsions and Gastrointestinal Symptoms in Young Children.* Clinical Pediatrics, 1976. **15**(4): p. 361-367.
43. Beigel, J.H., et al., *Avian influenza A (H5N1) infection in humans.* N Engl J Med, 2005. **353**(16192482): p. 1374-1385.
44. Mintern, J.D., et al., *The Immune Response to Influenza A Viruses Influenza Vaccines for the Future*, R. Rappuoli and G. Del Giudice, Editors. 2011, Springer Basel. p. 173-197.
45. Janeway, C. and R. Jr. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.

46. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates.* Science, 2006. **314**(17038589): p. 997-991001.
47. Hornung, V., et al., *5'-Triphosphate RNA is the ligand for RIG-I.* Science, 2006. **314**(17038590): p. 994-997.
48. Diebold, S.S., *Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA.* Science, 2004. **303**(5663): p. 1529-1531.
49. Lund, J.M., et al., *Recognition of single-stranded RNA viruses by Toll-like receptor 7.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(15): p. 5598-5603.
50. Guillot, L., et al., *Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus.* J Biol Chem, 2005. **280**(15579900): p. 5571-5580.
51. Ichinohe, T., et al., *Inflammasome recognition of influenza virus is essential for adaptive immune responses.* J Exp Med, 2009. **206**(19139171): p. 79-87.
52. Allen, I.C., et al., *The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA.* Immunity, 2009. **30**(19362020): p. 556-565.
53. Thomas, P.G., et al., *The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1.* Immunity, 2009. **30**(19362023): p. 566-575.
54. Yoshikawa, T., et al., *Total viral genome copies and virus–Ig complexes after infection with influenza virus in the nasal secretions of immunized mice.* Journal of General Virology, 2004. **85**(8): p. 2339-2346.
55. Sibille, Y. and H.Y. Reynolds, *Macrophages and polymorphonuclear neutrophils in lung defense and injury.* Am Rev Respir Dis, 1990. **141**(2405761): p. 471-501.
56. Fujimoto, I., et al., *Virus clearance through apoptosis-dependent phagocytosis of influenza A virus-infected cells by macrophages.* J Virol, 2000. **74**(10708457): p. 3399-3403.
57. Hofmann, P., et al., *Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response.* J Leukoc Biol, 1997. **61**(9103226): p. 408-414.
58. Gong, J.H., et al., *Influenza A virus infection of macrophages. Enhanced tumor necrosis factor-alpha (TNF-alpha) gene expression and lipopolysaccharide-triggered TNF-alpha release.* J Immunol, 1991. **147**(1940351): p. 3507-3513.
59. Kaufmann, A., et al., *Defense against influenza A virus infection: essential role of the chemokine system.* Immunobiology, 2001. **204**(11846225): p. 603-613.
60. Tumpey, T.M., et al., *Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice.* J Virol, 2005. **79**(16282492): p. 14933-14944.

61. Wijburg, O.L., et al., *Alveolar macrophages regulate the induction of primary cytotoxic T-lymphocyte responses during influenza virus infection.* J Virol, 1997. **71**(9371606): p. 9450-9457.
62. Monteiro, J.M., C. Harvey, and G. Trinchieri, *Role of interleukin-12 in primary influenza virus infection.* J Virol, 1998. **72**(9573248): p. 4825-4831.
63. Stein-Streilein, J., et al., *Natural killer cells in mouse lung: surface phenotype, target preference, and response to local influenza virus infection.* J Immunol, 1983. **131**(6644021): p. 2699-2704.
64. Mandelboim, O., et al., *Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells.* Nature, 2001. **409**(11234016): p. 1055-1060.
65. Arnon, T.I., et al., *Recognition of viral hemagglutinins by NKp44 but not by NKp30.* Eur J Immunol, 2001. **31**(11536166): p. 2680-2689.
66. Biron, C.A., et al., *Natural killer cells in antiviral defense: function and regulation by innate cytokines.* Annu Rev Immunol, 1999. **17**(10358757): p. 189-220.
67. O'Keeffe, M., et al., *Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus.* J Exp Med, 2002. **196**(12438422): p. 1307-1319.
68. Theofilopoulos, A.N., et al., *Type I interferons (alpha/beta) in immunity and autoimmunity.* Annu Rev Immunol, 2005. **23**(15771573): p. 307-336.
69. Garcia-Sastre, A., *Inhibition of Interferon-Mediated Antiviral Responses by Influenza A Viruses and Other Negative-Strand RNA Viruses.* Virology, 2001. **279**(2): p. 375-384.
70. Husseini, R.H., et al., *Elevation of Nasal Viral Levels by Suppression of Fever in Ferrets Infected with Influenza Viruses of Differing Virulence.* Journal of Infectious Diseases, 1982. **145**(4): p. 520-524.
71. Wyde, P.R., M.R. Wilson, and T.R. Cate, *Interferon production by leukocytes infiltrating the lungs of mice during primary influenza virus infection.* Infect Immun, 1982. **38**(6295944): p. 1249-1255.
72. Herold, S., et al., *Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand.* J Exp Med, 2008. **205**(19064696): p. 3065-3077.
73. La Gruta, N.L., et al., *A question of self-preservation: immunopathology in influenza virus infection.* Immunol Cell Biol, 2007. **85**(17213831): p. 85-92.
74. Kobasa, D., et al., *Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus.* Nature, 2007. **445**(17230189): p. 319-323.
75. Kobasa, D., et al., *Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus.* Nature, 2004. **431**(15470432): p. 703-707.

76. Perrone, L.A., et al., *H5N1 and 1918 Pandemic Influenza Virus Infection Results in Early and Excessive Infiltration of Macrophages and Neutrophils in the Lungs of Mice.* PLoS Pathog, 2008. **4**(8): p. e1000115.
77. Reading, P.C., et al., *A serum mannose-binding lectin mediates complement-dependent lysis of influenza virus-infected cells.* Biochem Biophys Res Commun, 1995. **217**(8554567): p. 1128-1136.
78. Hartshorn, K.L., et al., *Mechanisms of anti-influenza activity of surfactant proteins A and D: comparison with serum collectins.* Am J Physiol, 1997. **273**(9435570): p. 1156-1166.
79. Holmskov, U., S. Thiel, and J.C. Jensenius, *Collections and ficolins: humoral lectins of the innate immune defense.* Annu Rev Immunol, 2003. **21**(12524383): p. 547-578.
80. Crouch, E., K. Hartshorn, and I. Ofek, *Collectins and pulmonary innate immunity.* Immunol Rev, 2000. **173**(10719667): p. 52-65.
81. Doss, M., et al., *Interactions of alpha-, beta-, and theta-defensins with influenza A virus and surfactant protein D.* J Immunol, 2009. **182**(19494312): p. 7878-7887.
82. Ochsenbein, A.F., et al., *Correlation of T cell independence of antibody responses with antigen dose reaching secondary lymphoid organs: implications for splenectomized patients and vaccine design.* J Immunol, 2000. **164**(10843683): p. 6296-6302.
83. Ochsenbein, A.F. and R.M. Zinkernagel, *Natural antibodies and complement link innate and acquired immunity.* Immunol Today, 2000. **21**(11114423): p. 624-630.
84. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. **69**(10966468): p. 531-569.
85. Kwong, P.D. and I.A. Wilson, *HIV-1 and influenza antibodies: seeing antigens in new ways.* Nat Immunol, 2009. **10**(19448659): p. 573-578.
86. Sangster, M.Y., et al., *An early CD4+ T cell-dependent immunoglobulin A response to influenza infection in the absence of key cognate T-B interactions.* J Exp Med, 2003. **198**(14517272): p. 1011-1021.
87. Frieke Kuper, C., et al., *The role of nasopharyngeal lymphoid tissue.* Immunology Today, 1992. **13**(6): p. 219-224.
88. Zuercher, A.W., et al., *Nasal-Associated Lymphoid Tissue Is a Mucosal Inductive Site for Virus-Specific Humoral and Cellular Immune Responses.* The Journal of Immunology, 2002. **168**(4): p. 1796-1803.
89. Tamura, S. and T. Kurata, *Defense mechanisms against influenza virus infection in the respiratory tract mucosa.* Jpn J Infect Dis, 2004. **57 (6)**: p. 236-47.
90. Mostov, K.E., *Transepithelial transport of immunoglobulins.* Annu Rev Immunol, 1994. **12**(8011293): p. 63-84.

91. Rossen, R.D., 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, 1970. **211**(5466966): p. 1157-1161.
92. Sung, S.-S.J., et al., *A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins.* J Immunol, 2006. **176**(16455972): p. 2161-2172.
93. Henri, S., et al., *The dendritic cell populations of mouse lymph nodes.* J Immunol, 2001. **167**(11441078): p. 741-748.
94. Legge, K.L. and T.J. Braciale, *Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection.* Immunity, 2003. **18**(12594953): p. 265-277.
95. Sigal, L.J. and K.L. Rock, *Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation.* J Exp Med, 2000. **192**(11034604): p. 1143-1150.
96. Zammit, D.J., et al., *Dendritic cells maximize the memory CD8 T cell response to infection.* Immunity, 2005. **22**(15894274): p. 561-570.
97. Belz, G.T., et al., *Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus.* Proc Natl Acad Sci U S A, 2004. **101**(15163797): p. 8670-8675.
98. Hendriks, J., Y. Xiao, and J. Borst, *CD27 promotes survival of activated T cells and complements CD28 in generation and establishment of the effector T cell pool.* J Exp Med, 2003. **198**(14581610): p. 1369-1380.
99. Lumsden, J.M., et al., *Differential requirement for CD80 and CD80/CD86-dependent costimulation in the lung immune response to an influenza virus infection.* J Immunol, 2000. **164**(10604996): p. 79-85.
100. Liu, Y., et al., *Distinct costimulatory molecules are required for the induction of effector and memory cytotoxic T lymphocytes.* J Exp Med, 1997. **185**(9016874): p. 251-262.
101. Bertram, E.M., P. Lau, and T.H. Watts, *Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection.* J Immunol, 2002. **168**(11937529): p. 3777-3785.
102. Doherty, P.C., et al., *Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections.* Immunol Rev, 1997. **159**(9416506): p. 105-117.
103. Lowin, B., et al., *Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways.* Nature, 1994. **370**(6491): p. 650-652.
104. Topham, D.J., R.A. Tripp, and P.C. Doherty, *CD8+ T cells clear influenza virus by perforin or Fas-dependent processes.* J Immunol, 1997. **159**(9548456): p. 5197-5200.

105. Schotsaert, M., et al., *Controlling Influenza by Cytotoxic T-Cells: Calling for Help from Destroyers.* Journal of Biomedicine and Biotechnology, 2010. **2010**: p. 1-14.
106. Lawrence, C.W. and T.J. Braciale, *Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection.* J Immunol, 2004. **173**(15240712): p. 1209-1218.
107. Tripp, R., S. Sarawar, and P. Doherty, *Characteristics of the influenza virus-specific CD8+ T cell response in mice homozygous for disruption of the H-2lAb gene.* The Journal of Immunology, 1995. **155**(6): p. 2955-2959.
108. Cerwenka, A., T.M. Morgan, and R.W. Dutton, *Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: homing properties rather than initial frequencies are crucial.* J Immunol, 1999. **163**(10553081): p. 5535-5543.
109. Flynn, K.J., et al., *Virus-specific CD8+ T cells in primary and secondary influenza pneumonia.* Immunity, 1998. **8**(9655482): p. 683-691.
110. La Gruta, N.L., S.J. Turner, and P.C. Doherty, *Hierarchies in Cytokine Expression Profiles for Acute and Resolving Influenza Virus-Specific CD8+ T Cell Responses: Correlation of Cytokine Profile and TCR Avidity.* J Immunol, 2004. **172**(15100298): p. 5553-5560.
111. Moffat, J.M., et al., *Granzyme A expression reveals distinct cytolytic CTL subsets following influenza A virus infection.* Eur J Immunol, 2009. **39**(19404988): p. 1203-1210.
112. Sun, J., et al., *Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10.* Nat Med, 2009. **15**(19234462): p. 277-284.
113. Marshall, D.R., et al., *Measuring the diaspora for virus-specific CD8+ T cells.* Proc Natl Acad Sci U S A, 2001. **98**(11344265): p. 6313-6318.
114. Hogan, R.J., et al., *Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections.* J Immunol, 2001. **166**(11160228): p. 1813-1822.
115. Brown, D.M., et al., *CD4 T Cell-Mediated Protection from Lethal Influenza: Perforin and Antibody-Mediated Mechanisms Give a One-Two Punch.* The Journal of Immunology, 2006. **177**(5): p. 2888-2898.
116. Roman, E., et al., *CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function.* J Exp Med, 2002. **196**(12370257): p. 957-968.
117. Powell, T.J., et al., *CD8+ T cells responding to influenza infection reach and persist at higher numbers than CD4+ T cells independently of precursor frequency.* Clin Immunol, 2004. **113**(15380534): p. 89-8100.
118. Gerhard, W., et al., *Role of the B-cell response in recovery of mice from primary influenza virus infection.* Immunol Rev, 1997. **159**(9416505): p. 95-91103.

119. Graham, M.B., V.L. Braciale, and T.J. Braciale, *Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection.* J Exp Med, 1994. **180**(7931062): p. 1273-1282.
120. Brown, D.M., E. Roman, and S.L. Swain, *CD4 T cell responses to influenza infection.* Semin Immunol, 2004. **16**(15130501): p. 171-177.
121. Krawczyk, C.M., H. Shen, and E.J. Pearce, *Memory CD4 T Cells Enhance Primary CD8 T-Cell Responses.* Infection and Immunity, 2007. **75**(7): p. 3556-3560.
122. Stevenson, P. and P.C. Doherty, *Cell-mediated immune response to influenza virus*, in *Textbook of Influenza*, K.G. Nicholson, R.G. Webster, and A. Hay, Editors. 1998, Blackwell Science Ltd.: Oxford. p. 278-87.
123. Webster, R.G., et al., *Molecular mechanisms of variation in influenza viruses.* Nature, 1982. **296**(6174870): p. 115-121.
124. Suzuki, Y., *Positive selection operates continuously on hemagglutinin during evolution of H3N2 human influenza A virus.* Gene, 2008. **427**(18848975): p. 111-116.
125. Wolf, Y.I., et al., *Long intervals of stasis punctuated by bursts of positive selection in the seasonal evolution of influenza A virus.* Biol Direct, 2006. **1**(17067369): p. 34-34.
126. Fleury, D., et al., *A complex of influenza hemagglutinin with a neutralizing antibody that binds outside the virus receptor binding site.* Nat Struct Biol, 1999. **6**(10360354): p. 530-534.
127. Caton, A.J., et al., *The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype).* Cell, 1982. **31**(6186384): p. 417-427.
128. Price, G.E., et al., *Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia.* J Exp Med, 2000. **191**(10839802): p. 1853-1867.
129. Boon, A.C.M., et al., *The hypervariable immunodominant NP418-426 epitope from the influenza A virus nucleoprotein is recognized by cytotoxic T lymphocytes with high functional avidity.* J Virol, 2006. **80**(16731941): p. 6024-6032.
130. Cox, N.J. and K. Subbarao, *Influenza.* The Lancet, 1999. **354**: p. 1277-1282.
131. Yassine, H.M., et al., *Interspecies and intraspecies transmission of influenza A viruses: viral, host and environmental factors.* Anim Health Res Rev, 2010. **11**(1): p. 53-72.
132. Webby, R.J. and R.G. Webster, *Are we ready for pandemic influenza?* Science, 2003. **302**(14645836): p. 1519-1522.
133. (HHS), U.D.o.H.a.H.S. *Influenza Pandemics: How They Start, How They Spread, and Their Potential Impact*. 2005 October 18, 2005 [cited 2011 December 25]; Available from: http://archive.hhs.gov/nvpo/pandemics/flu2.htm.
134. Schafer, J.R., et al., *Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir.* Virology, 1993. **194**(7684877): p. 781-788.

135. Bean, W.J., et al., *Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts.* J Virol, 1992. **66**(1731092): p. 1129-1138.
136. Garten, R.J., et al., *Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans.* Science, 2009. **325**(5937): p. 197-201.
137. Pappaioanou, M., *Highly pathogenic H5N1 avian influenza virus: Cause of the next pandemic?* Comparative Immunology, Microbiology and Infectious Diseases, 2009. **32**(4): p. 287-300.
138. Harvey, R., et al., *Restrictions to the Adaptation of Influenza A Virus H5 Hemagglutinin to the Human Host.* Journal of Virology, 2003. **78**(1): p. 502-507.
139. Neumann, G. and Y. Kawaoka, *Host range restriction and pathogenicity in the context of influenza pandemic.* Emerg Infect Dis, 2006. **12**(16707041): p. 881-886.
140. Rogers, G.N. and J.C. Paulson, *Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin.* Virology, 1983. **127**: p. 361-373.
141. Couceiro, J.N.S.S., J.C. Paulson, and L.G. Baum, *Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity.* Virus Research, 1993. **29**: p. 155-165.
142. Rogers, G.N., et al., *Differential Sensitivity of Human, Avian, and Equine Influenza A Viruses to a Glycoprotein Inhibitor of Infection: Selection of Receptor Specific Variants.* Virology, 1983. **131**: p. 394-408.
143. Shinya, K., et al., *Avian flu: Influenza virus receptors in the human airway.* Nature, 2006. **440**(7083): p. 435-436.
144. Matrosovich, M.N., et al., *Human and avian influenza viruses target different cell types in cultures of human airway epithelium.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(13): p. 4620-4624.
145. Rogers, G.N., et al., *Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity.* Nature, 1983. **304**(6191220): p. 76-78.
146. Stevens, J., et al., *Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus.* Science, 2006. **312**(16543414): p. 404-410.
147. Glaser, L., et al., *A Single Amino Acid Substitution in 1918 Influenza Virus Hemagglutinin Changes Receptor Binding Specificity.* Journal of Virology, 2005. **79**(17): p. 11533-11536.
148. Garten, W. and H.-D. Klenk, *Understanding influenza virus pathogenicity.* Trends in Microbiology, 1999. **7**(3): p. 99-100.
149. Garten, W. and H. Klenk, *Cleavage Activation of the Influenza Virus Hemagglutinin and Its Role in Pathogenesis*, in *Avian Influenza. Monogr Virol.*, K. H, M. MN, and S. J, Editors. 2008, Basel, Karger. p. 156-167.

150. CDC. *Highly Pathogenic Avian Influenza A (H5N1) Virus*. 2011 [cited 2012 January 4]; Available from: http://www.cdc.gov/flu/avianflu/h5n1-virus.htm.
151. CDC, *Update: isolation of avian influenza A (H5N1) viruses from humans—Hong Kong, 1997–1998.* MMWR, 1998. **46**: p. 1245-1247.
152. Kandun, I.N., et al., *Three Indonesian clusters of H5N1 virus infection in 2005.* N Engl J Med, 2006. **355**(17124016): p. 2186-2194.
153. WHO. *H5N1 avian influenza: Timeline of major events*. 2011 7 November 2011 [cited 2011 December 15]; Available from: http://www.who.int/influenza/human_animal_interface/avian_influenza/H5N1_avian_influenza_update.pdf.
154. Hien, T.T., et al., *Avian Influenza A (H5N1) in 10 Patients in Vietnam.* New England Journal of Medicine, 2004. **350**(12): p. 1179-1188.
155. Ungchusak, K., et al., *Probable person-to-person transmission of avian influenza A (H5N1).* N Engl J Med, 2005. **352**(15668219): p. 333-340.
156. Webster, R.G. and E.A. Govorkova, *H5N1 influenza-a continuing evolution and spread.* New England Journal of Medicine, 2006. **355**: p. 2174-2177.
157. Steinhauer, D.A., *Role of Hemagglutinin Cleavage for the Pathogenicity of Influenza Virus.* Virology, 1999. **258**: p. 1-20.
158. Govorkova, E.A., et al., *Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004.* J Virol, 2005. **79**(15681421): p. 2191-2198.
159. Horimoto, T. and Y. Kawaoka, *Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus.* Journal of Virology, 1994. **68**(5): p. 3120-3128.
160. Uiprasertkul, M., et al., *Influenza A H5N1 replication sites in humans.* Emerging Infectious Diseases, 2005. **11**(7): p. 1036-1041.
161. Nicholls, J.M., et al., *Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses.* Respiratory Research, 2007. **8**(73).
162. Nicholls, J.M., et al., *Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract.* Nat Med, 2007. **13**(2): p. 147-149.
163. Matrosovich, M., et al., *Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals.* J Virol, 2000. **74**(10954551): p. 8502-8512.
164. Belshe, R.B., *The origins of pandemic influenza--lessons from the 1918 virus.* N Engl J Med, 2005. **353**(16306515): p. 2209-2211.
165. Van Hoeven, N., et al., *Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air*. Proceedings of the National Academy of Sciences, 2009. **106**(9): p. 3366-3371.
166. Sorrell, E.M., et al., *Minimal molecular constraints for respiratory droplet transmission of an avian–human H9N2 influenza A virus.* Proceedings of the National Academy of Sciences, 2009. **106**(18): p. 7565-7570.

167. Dudley, J.P. *Age-specific infection and death rates for human A (H5N1) avian influenza in Egypt.* Euro surveill, 2009. **14**.
168. Yuen, K.Y., et al., *Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus.* The Lancet, 1998. **351**(9101): p. 467-471.
169. To, K.-F., et al., *Pathology of Fatal Human Infection Associated With Avian In¯uenza A H5N1 Virus.* Journal of Medical Virology, 2001. **63**: p. 242-246.
170. Korteweg, C. and J. Gu, *Pathology, Molecular Biology, and Pathogenesis of Avian Influenza A (H5N1) Infection in Humans.* American Journal Of Pathology, 2008. **172**(5): p. 1155-1170.
171. Peiris, J.S.M., et al., *Innate immune responses to influenza A H5N1: friend or foe?* Trends in Immunology, 2009. **30**(12): p. 574-584.
172. de Jong, M.D., et al., *Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia.* Nature Medicine, 2006. **12**(10): p. 1203-1207.
173. De Jong, J.C., et al., *Haemagglutinin-inhibiting antibody to influenza virus.* Dev Biol (Basel), 2003. **115**: p. 63-73.
174. Bizebard, T., et al., *Structure of influenza virus haemagglutinin complexed with a neutralizing antibody.* Nature, 1995. **376**(7596443): p. 92-94.
175. Ekiert, D.C., et al., *Antibody recognition of a highly conserved influenza virus epitope.* Science, 2009. **324**(19251591): p. 246-251.
176. Kendal, A.P., et al., *Concepts and procedures for laboratory-based influenza surveillance.* Atlanta, GA: US Department of Health and Human Services, CDC, 1982.
177. Rimmelzwaan, G.F. and J.E. McElhaney, *Correlates of protection: Novel generations of influenza vaccines.* Vaccine, 2008. **26, Supplement 4**(0): p. D41-D44.
178. Smith, D.J., et al., *Mapping the antigenic and genetic evolution of influenza virus.* Science, 2004. **305**(15218094): p. 371-376.
179. Stephenson, I., et al., *Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy.* J Infect Dis, 2005. **191**(15776364): p. 1210-1215.
180. Leroux-Roels, I., et al., *Broad Clade 2 cross-reactive immunity induced by an adjuvanted clade 1 rH5N1 pandemic influenza vaccine.* Plos one, 2008. **3**(18301743).
181. Baras, B., et al., *Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine.* Plos one, 2008. **3**(18167560).
182. Leroux-Roels, I., et al., *Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial.* Lancet, 2007. **370**(17707753): p. 580-589.
183. Webster, R.G., W.G. Laver, and E.D. Kilbourne, *Reactions of antibodies with surface antigens of influenza virus.* J Gen Virol, 1968. **3**(5711432): p. 315-326.

184. Abed, Y., et al., *Divergent evolution of hemagglutinin and neuraminidase genes in recent influenza A:H3N2 viruses isolated in Canada.* J Med Virol, 2002. **67**(12116009): p. 589-595.
185. Kilbourne, E.D., B.E. Johansson, and B. Grajower, *Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins.* Proceedings of the National Academy of Sciences, 1990. **87**(2): p. 786-790.
186. Johansson, B.E., B. Grajower, and E.D. Kilbourne, *Infection-permissive immunization with influenza virus neuraminidase prevents weight loss in infected mice.* Vaccine, 1993. **11**: p. 1037-1039.
187. Kilbourne, E.D., et al., *Antiviral activity of antiserum specific for an influenza virus neuraminidase.* J. Virol., 1968. **2**(4911843): p. 281-288.
188. Powers, D.C., E.D. Kilbourne, and B.E. Johansson, *Neuraminidase specific antibody responses to inactivated influenza virus vaccine in young and elderly adults.* Clin. Diagn. Lab. Immunol., 1996. **3**: p. 511-516.
189. Johansson, B.E., D.J. Bucher, and E.D. Kilbourne, *Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection.* J. Virol., 1989. **63**(3): p. 1239-1246.
190. Mozdzanowska, K., et al., *Enhancement of neutralizing activity of influenza virus-specific antibodies by serum components.* Virology, 2006. **352**(16777168): p. 418-426.
191. Hashimoto, G., P.F. Wright, and D.T. Karzon, *Antibody-dependent cell-mediated cytotoxicity against influenza virus-infected cells.* J Infect Dis, 1983. **148**(6605395): p. 785-794.
192. Hartshorn, K.L., et al., *Neutrophil deactivation by influenza A viruses: mechanisms of protection after viral opsonization with collectins and hemagglutination-inhibiting antibodies.* Blood, 1996. **87**(8605364): p. 3450-3461.
193. Chen, Z., et al., *Cross-protection against a lethal influenza virus infection by DNA vaccine to neuraminidase.* Vaccine, 2000. **18**(28): p. 3214-3222.
194. Marcelin, G., et al., *A Contributing Role for Anti-Neuraminidase Antibodies on Immunity to Pandemic H1N1 2009 Influenza A Virus.* Plos one, 2011. **6**(10): p. e26335.
195. Sylte, M.J. and D.L. Suarez, *Influenza Neuraminidase as a Vaccine Antigen*, in *Vaccines for Pandemic Influenza*, R.W. Compans and W.A. Orenstein, Editors. 2009, Springer Berlin Heidelberg. p. 227-241.
196. Clements, M.L., et al., *Serum and Nasal Wash Antibodies Associated with Resistance to Experimental Challenge with Influenza A Wild-Type Virus.* Journal of Clinical Microbiology, 1986. **24**(1): p. 157-160.
197. Clements, M.L. and B.R. Murphy, *Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine.* Journal of Clinical Microbiology, 1986. **23**(1): p. 66-72.

198. Taylor, H.P. and N.J. Dimmock, *Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG.* J Exp Med, 1985. **161**(2981953): p. 198-209.
199. Murphy, B.R., et al., *Temperature-Sensitive Mutants of Influenza Virus. III. Further Characterization of the ts-1[E] Influenza A Recombinant (H3N2) Virus in Man.* Journal of Infectious Diseases, 1973. **128**(4): p. 479-487.
200. Couch, R.B. and J.A. Kasel, *IMMUNITY TO INFLUENZA IN MAN.* Annual Review of Microbiology, 1983. **37**: p. 529-549.
201. Cox, R.J., K.A. Brokstad, and P. Ogra, *Influenza Virus: Immunity and Vaccination Strategies.Comparison of the Immune Response to Inactivated and Live, Attenuated Influenza Vaccines.* Scandinavian Journal of Immunology, 2004. **59**: p. 1-15.
202. Lamb, R.A., S.L. Zebedee, and C.D. Richardson, *Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface.* Cell, 1985. **40**(3882238): p. 627-633.
203. Frace, A.M., et al., *Modified M2 proteins produce heterotypic immunity against influenza A virus.* Vaccine, 1999. **17**(10403591): p. 2237-2244.
204. Zharikova, D., et al., *Influenza type A virus escape mutants emerge in vivo in the presence of antibodies to the ectodomain of matrix protein 2.* J Virol, 2005. **79**(15890902): p. 6644-6654.
205. Li, K.S., *Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia.* Nature, 2004. **430**: p. 209-213.
206. Neirynck, S., et al., *A universal influenza A vaccine based on the extracellular domain of the M2 protein.* Nat Med, 1999. **5**(10502819): p. 1157-1163.
207. Mozdzanowska, K., et al., *Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2.* Vaccine, 2003. **21**(12744898): p. 2616-2626.
208. Fan, J., et al., *Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys.* Vaccine, 2004. **22**(15297047): p. 2993-3003.
209. Slepushkin, V.A., et al., *Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein.* Vaccine, 1995. **13**(8578816): p. 1399-1402.
210. Jegerlehner, A., et al., *Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity.* J Immunol, 2004. **172**(15100303): p. 5598-5605.
211. Lo, C., et al., *Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: Cold-adapted vaccine versus DNA prime-adenovirus boost strategies☆.* Vaccine, 2008.
212. Nayak, B., et al., *Contributions of the Avian Influenza Virus HA, NA, and M2 Surface Proteins to the Induction of Neutralizing Antibodies and Protective Immunity.* Journal of Virology, 2009. **84**(5): p. 2408-2420.

213. Heinen, P.P., et al., *Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus.* J Gen Virol, 2002. **83**(12124449): p. 1851-1859.
214. Treanor, J.J., et al., *Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice.* Journal of Virology, 1990. **64**(3): p. 1375-1377.
215. LaMere, M.W., et al., *Contributions of Antinucleoprotein IgG to Heterosubtypic Immunity against Influenza Virus.* The Journal of Immunology, 2011. **186**(7): p. 4331-4339.
216. Ulmer, J., et al., *Heterologous protection against influenza by injection of DNA encoding a viral protein.* Science, 1993. **259**(5102): p. 1745-1749.
217. Fu, T.-M., et al., *Dose Dependence of CTL Precursor Frequency Induced by a DNA Vaccine and Correlation with Protective Immunity Against Influenza Virus Challenge.* The Journal of Immunology, 1999. **162**(7): p. 4163-4170.
218. Bennink, J.R., et al., *Anti-influenza virus cytotoxic T lymphocytes recognize the three viral polymerases and a nonstructural protein: responsiveness to individual viral antigens is major histocompatibility complex controlled.* Journal of Virology, 1987. **61**(4): p. 1098-1102.
219. Jameson, J., et al., *Human CD8+ and CD4+ T lymphocyte memory to influenza A viruses of swine and avian species.* J Immunol, 1999. **162**(10358215): p. 7578-7583.
220. Kreijtz, J.H.C.M., et al., *Cross-Recognition of Avian H5N1 Influenza Virus by Human Cytotoxic T-Lymphocyte Populations Directed to Human Influenza A Virus.* J Virol, 2008. **82**(18353950): p. 5161-5166.
221. Zweerink, H.J., et al., *Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses.* Nature, 1977. **267**(194164): p. 354-356.
222. Müllbacher, A., et al., *Cytotoxic T-cell immunity as a target for influenza vaccines.* The Lancet Infectious Diseases, 2006. **6**(5): p. 255-256.
223. Zinkernagel, R.M., *ON NATURAL AND ARTIFICIAL VACCINATIONS.* Annual Review of Immunology, 2003. **21**(1): p. 515-546.
224. McMichael, A.J., et al., *Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes.* J Gen Virol, 1986. **67 (Pt 4)**(3485697): p. 719-726.
225. Epstein, S.L., et al., *DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice.* Emerging Infectious Diseases, 2002. **8**(8): p. 796-801.
226. Hoelscher, M.A., et al., *Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice.* Lancet, 2006. **367**(16473124): p. 475-481.
227. Soboleski, M.R., et al., *Cold-Adapted Influenza and Recombinant Adenovirus Vaccines Induce Cross-Protective Immunity against pH1N1 Challenge in Mice.* Plos one, 2011. **6**(7): p. e21937.

228. Epstein, S.L., et al., *Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein.* Vaccine, 2005. **23**(46–47): p. 5404-5410.
229. Dormitzer, P.R., et al., *Influenza vaccine immunology.* Immunological Reviews, 2011. **239**(1): p. 167-177.
230. Kreijtz, J.H.C.M., et al., *Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice.* Vaccine, 2007. **25**(17005299): p. 612-620.
231. Benton, K.A., et al., *Heterosubtypic immunity to influenza A virus in mice lacking IgA, all Ig, NKT cells, or gamma delta T cells.* J Immunol, 2001. **166**(11390496): p. 7437-7445.
232. Epstein, S.L., et al., *Mechanisms of Heterosubtypic Immunity to lethal Influenza A Virus Infection in Fully Immunocompetent, T Cell-Depleted, [Beta]2-Microglobulin-Deficient, and J Chain-Deficient Mice.* J Immunol, 1997. **158**(9013963): p. 1222-1230.
233. Nguyen, H.H., et al., *Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8(+) cytotoxic T lymphocyte responses induced in mucosa-associated tissues.* Virology, 1999. **254**(9927573): p. 50-60.
234. Lukacher, A.E., V.L. Braciale, and T.J. Braciale, *In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific.* J Exp Med, 1984. **160**(6206190): p. 814-826.
235. Taylor, P. and B.A. Askonas, *Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo.* Immunol, 1986. **58**: p. 417-20.
236. Liang, S., et al., *Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity.* J Immunol, 1994. **152**(8120375): p. 1653-1661.
237. Powell, T.J., et al., *Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against supralethal challenge with heterosubtypic virus.* J Immunol, 2007. **178**(17202366): p. 1030-1038.
238. Gubareva, L.V., L. Kaiser, and F.G. Hayden, *Influenza virus neuraminidase inhibitors.* Lancet, 2000. **355**(10711940): p. 827-835.
239. Matrosovich, M.N., et al., *Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium.* J Virol, 2004. **78**(15507653): p. 12665-12667.
240. Yen, H.-L., et al., *Virulence may determine the necessary duration and dosage of oseltamivir treatment for highly pathogenic A/Vietnam/1203/04 influenza virus in mice.* J Infect Dis, 2005. **192**(16028136): p. 665-672.
241. Hayden, F.G. and M.D. de Jong, *Emerging Influenza Antiviral Resistance Threats.* Journal of Infectious Diseases, 2011. **203**(1): p. 6-10.
242. Kiso, M., et al., *Resistant influenza A viruses in children treated with oseltamivir: descriptive study.* Lancet, 2004. **364**(15337401): p. 759-765.
243. Le, Q.M., et al., *Avian flu: isolation of drug-resistant H5N1 virus.* Nature, 2005. **437**(16228009): p. 1108-1108.

244. Gupta, R.K. and J.S. Nguyen-Van-Tam, *Oseltamivir resistance in influenza A (H5N1) infection.* N Engl J Med, 2006. **354**(16571890): p. 1423-1424.
245. Beigel, J. and M. Bray, *Current and future antiviral therapy of severe seasonal and avian influenza.* Antiviral Research, 2008. **78**(1): p. 91-102.
246. Tan, F.L. and J.Q. Yin, *RNAi, a new therapeutic strategy against viral infection.* Cell Res, 2004. **14**(6): p. 460-466.
247. Tompkins, S.M., et al., *Protection against lethal influenza virus challenge by RNA interference in vivo.* Proceedings of the National Academy of Sciences, 2004. **101**(23): p. 8682-8686.
248. Wu, Y., et al., *Inhibition of highly pathogenic avian H5N1 influenza virus replication by RNA oligonucleotides targeting NS1 gene.* Biochemical and Biophysical Research Communications, 2008. **365**(2): p. 369-374.
249. Luke, T.C., et al., *Meta-Analysis: Convalescent Blood Products for Spanish Influenza Pneumonia: A Future H5N1 Treatment?* Annals of Internal Medicine, 2006. **145**(8): p. 599-609.
250. Zhou, B., N. Zhong, and Y. Guan, *Treatment with Convalescent Plasma for Influenza A (H5N1) Infection.* New England Journal of Medicine, 2007. **357**(14): p. 1450-1451.
251. CDC, *Prevention and Control of Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices (ACIP)*, in *MMWR*2011. p. 1128-1132.
252. Matthews, J., *Egg-based production of influenza vaccine: 30 years of commercial experience.* The Bridge, 2006. **36**(17-24).
253. Kilbourne, E.D., et al., *Related studies of a recombinant influenza-virus vaccine. I. Derivation and characterization of virus and vaccine.* J Infect Dis, 1971. **124**(5115669): p. 449-462.
254. Hoffmann, E., et al., *A DNA transfection system for generation of influenza A virus from eight plasmids.* Proc Natl Acad Sci U S A, 2000. **97**(10801978): p. 6108-6113.
255. Nichol, K., *Efficacy/clinical effectiveness of inactivated influenza vaccines in adults*, in *Textbook of Influenza*, K.G. Nicholson, R.G. Webster, and A. Hay, Editors. 1998, Blackwell Science: Oxford, UK. p. 358-72.
256. Geeraedts, F., et al., *Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling.* PLoS Pathog, 2008. **4**(18769719).
257. Abramson, J.S., *Intranasal, Cold-Adapted, Live, Attenuated Influenza Vaccine.* The Pediatric Infectious Disease Journal, 1999. **18**(12): p. 1103-1104.
258. Murphy, B.R. and K. Coelingh, *Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines.* Viral Immunol, 2002. **15**(12081014): p. 295-323.
259. Chen, Z., et al., *Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs.* J Virol, 2010. **84**(19864389): p. 44-51.

260. Mendelman, P.M., J. Cordova, and I. Cho, *Safety, efficacy and effectiveness of the influenza virus vaccine, trivalent, types A and B, live, cold-adapted (CAIV-T) in healthy children and healthy adults.* Vaccine, 2001. **19**(11257337): p. 2221-2226.
261. Stephenson, I., et al., *Confronting the avian influenza threat: vaccine development for a potential pandemic.* The Lancet Infectious Diseases, 2004. **4**(8): p. 499-509.
262. Steel, J., et al., *Live Attenuated Influenza Viruses Containing NS1 Truncations as Vaccine Candidates against H5N1 Highly Pathogenic Avian Influenza.* Journal of Virology, 2008. **83**(4): p. 1742-1753.
263. Singh, N., A. Pandey, and S.K. Mittal, *Avian influenza pandemic preparedness: developing prepandemic and pandemic vaccines against a moving target.* Expert Rev Mol Med, 2010. **12**: p. e14.
264. Geiss, G.K., et al., *Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza.* Proc Natl Acad Sci U S A, 2002. **99**(12149435): p. 10736-10741.
265. Hale, B.G., et al., *The multifunctional NS1 protein of influenza A viruses.* Journal of General Virology, 2008. **89**(10): p. 2359-2376.
266. Garcia-Sastre, A., et al., *Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems.* Virology, 1998. **252**(9878611): p. 324-330.
267. Ferko, B., et al., *Immunogenicity and protection efficacy of replication-deficient influenza A viruses with altered NS1 genes.* J Virol, 2004. **78**(15542655): p. 13037-13045.
268. Talon, J., et al., *Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach.* Proc Natl Acad Sci U S A, 2000. **97**(10725408): p. 4309-4314.
269. Wacheck, V., et al., *A novel type of influenza vaccine: safety and immunogenicity of replication-deficient influenza virus created by deletion of the interferon antagonist NS1.* J Infect Dis, 2010. **201**(20039806): p. 354-362.
270. Takada, A., et al., *Avirulent Avian influenza virus as a vaccine strain against a potential human pandemic.* J Virol, 1999. **73**(10482580): p. 8303-8307.
271. Treanor, J.J., et al., *Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans.* Vaccine, 2001. **19**: p. 1732-1737.
272. Nicholson, K.G., et al., *Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza.* Lancet, 2001. **357**(11425416): p. 1937-1943.
273. Barrett, P.N., et al., *Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines.* Expert Rev Vaccines, 2009. **8**(19397417): p. 607-618.

274. Kistner, O., et al., *Cell culture (Vero) derived whole virus (H5N1) vaccine based on wild-type virus strain induces cross-protective immune responses.* Vaccine, 2007. **25**(32): p. 6028-6036.
275. Ehrlich, H.J., et al., *A Clinical Trial of a Whole-Virus H5N1 Vaccine Derived from Cell Culture.* New England Journal of Medicine, 2008. **358**(24): p. 2573-2584.
276. Tseng, Y.-F., et al., *Adaptation of High-Growth Influenza H5N1 Vaccine Virus in Vero Cells: Implications for Pandemic Preparedness.* Plos one, 2011. **6**(10): p. e24057.
277. Pandey, A., N. Singh, and S.K. Mittal, *Egg-independent vaccine strategies for highly pathogenic H5N1 influenza viruses.* Human Vaccines & Immunotherapeutics, 2010. **6**(2): p. 178-188.
278. Fan, S., et al., *Immunogenicity and Protective Efficacy of a Live Attenuated H5N1 Vaccine in Nonhuman Primates.* PLoS Pathog, 2009. **5**(5): p. e1000409.
279. Karron, R.A., et al., *A Live Attenuated H9N2 Influenza Vaccine Is Well Tolerated and Immunogenic in Healthy Adults.* Journal of Infectious Diseases, 2009. **199**(5): p. 711-716.
280. Organization, W.H., *Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines*, 2007. p. 164-167.
281. Banzhoff, A., et al., *MF59-adjuvanted vaccines for seasonal and pandemic influenza prophylaxis.* Influenza and other respiratory viruses, 2008. **2**(6): p. 243-249.
282. Leroux-Roels, I., et al., *Priming with AS03A-adjuvanted H5N1 influenza vaccine improves the kinetics, magnitude and durability of the immune response after a heterologous booster vaccination: an open non-randomised extension of a double-blind randomized primary study.* Vaccine, 2010. **28**(3): p. 849-857.
283. Cryz, S.J., J.U. Que, and R. Gluck, *A virosome vaccine antigen delivery system does not stimulate an antiphospholipid antibody response in humans.* Vaccine, 1996. **14**(9004449): p. 1381-1383.
284. Ansaldi, F., et al., *Antibody response against heterogeneous circulating influenza virus strains elicited by MF59- and non-adjuvanted vaccines during seasons with good or partial matching between vaccine strain and clinical isolates.* Vaccine, 2010. **28**(20433807): p. 4123-4129.
285. Mosca, F., et al., *Molecular and cellular signatures of human vaccine adjuvants.* Proc Natl Acad Sci U S A, 2008. **105**(18650390): p. 10501-10506.
286. Tritto, E., F. Mosca, and E. De Gregorio, *Mechanism of action of licensed vaccine adjuvants.* Vaccine, 2009. **27**(19200813): p. 3331-3334.
287. Galli, G., et al., *Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels.* Proc Natl Acad Sci U S A, 2009. **106**(19237568): p. 3877-3882.

288. Bresson, J.L., et al., *Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial.* Lancet, 2006. **367**: p. 1657-1664.
289. Condon, C., *A vaccine in 50 days?* Lancet, 2005. **366**: p. 1686.
290. Rimmelzwaan, G.F. and A.D. Osterhaus, *ISCOMs: Materials, preparation, antigen delivery and immune response.*, in *Antigen delivery systems*, B. Gander, H. Merkle, and G. Corradin, Editors. 1997, Harwood Academic Publishers. p. 123-38.
291. Rimmelzwaan, G.F. and A.D. Osterhaus, *Influenza vaccines: new developments.* Curr Opin Pharmacol 2001. **1**: p. 491-6.
292. Rimmelzwaan, G.F., et al., *ISCOM vaccine induced protection against a lethal challenge with a human H5N1 influenza virus.* Vaccine, 1999. **17**: p. 1355-8.
293. Vogel, F. and S. Hem, *Immunologic adjuvants*, in *Vaccines*, P. SA, O. WA, and O. PA, Editors. 2008, Sanders Elsevier. p. 59-81.
294. Baldridge, J.R., et al., *Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents.* Expert Opin Biol Ther, 2004. **4**(15268679): p. 1129-1138.
295. Stephenson, I., et al., *Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxigenic Escherichia coli enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers.* J Virol, 2006. **80**(16641287): p. 4962-4970.
296. Couch, R.B., et al., *Contrasting effects of type I interferon as a mucosal adjuvant for influenza vaccine in mice and humans.* Vaccine, 2009. **27**(19607949): p. 5344-5348.
297. Kayamuro, H., et al., *The use of a mutant TNF-alpha as a vaccine adjuvant for the induction of mucosal immune responses.* Biomaterials, 2009. **30**(19646748): p. 5869-5876.
298. Wack, A., et al., *Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice.* Vaccine, 2008. **26**(18162266): p. 552-561.
299. Powers, D.C., et al., *Influenza A virus vaccines containing purified recombinant H3 hemagglutinin are well tolerated and induce protective immune responses in healthy adults.* J Infect Dis, 1995. **171**(7769297): p. 1595-1599.
300. Treanor, J.J., et al., *Dose-related safety and immunogenicity of a trivalent baculovirus-expressed influenza-virus hemagglutinin vaccine in elderly adults.* J Infect Dis, 2006. **193**(16586358): p. 1223-1228.
301. Treanor, J.J., et al., *Safety and immunogenicity of a baculovirus-expressed hemagglutinin influenza vaccine: a randomized controlled trial.* JAMA, 2007. **297**(17426277): p. 1577-1582.
302. Kang, S.M., et al., *Influenza virus-like particles as pandemic vaccines.* Curr Top Microbiol Immunol, 2009. **333**(19768411): p. 269-289.

303. Pushko, P., et al., *Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice.* Vaccine, 2005. **23**(16143432): p. 5751-5759.
304. Bright, R.A., et al., *Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin.* Vaccine, 2007. **25**(17337102): p. 3871-3878.
305. Bright, R.A., et al., *Cross-Clade Protective Immune Responses to Influenza Viruses with H5N1 HA and NA Elicited by an Influenza Virus-Like Particle.* Plos one, 2008. **3**(1): p. e1501.
306. Kang, S.-M., et al., *Induction of Long-Term Protective Immune Responses by Influenza H5N1 Virus-Like Particles.* PLoS ONE, 2009. **4**(3): p. 1-8.
307. Mahmood, K., et al., *H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses.* Vaccine, 2008. **26**(42): p. 5393-5399.
308. D'Aoust, M.-A., et al., *Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice.* Plant Biotechnology Journal, 2008. **6**(9): p. 930-940.
309. Rogalska, T., et al., *Current Status of Plants as Vaccine Production Platforms.* J Clin Cell Immunol 2011, S4, 2011. **S4**(003).
310. Thanavala, Y., et al., *Immunogenicity in humans of an edible vaccine for hepatitis B.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(9): p. 3378-3382.
311. Kim, J.H. and J. Jacob, *DNA vaccines against influenza viruses.* Curr Top Microbiol Immunol, 2009. **333**(19768407): p. 197-210.
312. Wang, S., et al., *Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine.* Vaccine, 2006. **24**(16140431): p. 4531-4540.
313. Krieg, A.M., *CpG motifs in bacterial DNA and their immune effects.* Annu. ReV. Immunol, 2002. **20**: p. 709-60.
314. Fu, T.M., et al., *Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes.* Mol Med, 1997. **3**(9234241): p. 362-371.
315. Drape, R.J., et al., *Epidermal DNA vaccine for influenza is immunogenic in humans.* Vaccine, 2006. **24**(16150518): p. 4475-4481.
316. Otten, G.R., et al., *Potent immunogenicity of an HIV-1 gag-pol fusion DNA vaccine delivered by in vivo electroporation.* Vaccine, 2006. **24**(16181711): p. 4503-4509.
317. Kodihalli, S., et al., *DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice.* J Virol, 1999. **73**(9971791): p. 2094-2098.
318. Chen, M.-W., et al., *A consensus–hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses.* Proceedings of the National Academy of Sciences, 2008. **105**(36): p. 13538-13543.

319. Laddy, D.J., et al., *Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens.* Plos one, 2008. **3**(18575608).
320. Kodihalli, S., D.L. Kobasa, and R.G. Webster, *Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines.* Vaccine, 2000. **18**(10775793): p. 2592-2599.
321. Jones, S., et al., *DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial.* Vaccine, 2009. **27**(19368793): p. 2506-2512.
322. Smith, L.R., et al., *Phase 1 clinical trials of the safety and immunogenicity of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin.* Vaccine, 2010. **28**(20117262): p. 2565-2572.
323. Liu, M.A., *DNA vaccines: an historical perspective and view to the future.* Immunological Reviews, 2011. **239**(1): p. 62-84.
324. Wang, Z., et al., *Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation.* Gene Ther, 2004. **11**(14724672): p. 711-721.
325. Mor, G., et al., *Do DNA Vaccines Induce Autoimmune Disease?* Hum Gene Ther, 1997. **8**(3): p. 293-300.
326. Bråve, A., et al., *Vaccine Delivery Methods Using Viral Vectors.* Molecular Pharmaceutics, 2006. **4**(1): p. 18-32.
327. Draper, S.J. and J.L. Heeney, *Viruses as vaccine vectors for infectious diseases and cancer.* Nat Rev Micro, 2010. **8**(1): p. 62-73.
328. Almeida, J.D., et al., *Formation of virosomes from influenza subunits and liposomes.* Lancet, 1975. **2**(53375): p. 899-901.
329. Bron, R., et al., *Preparation, properties, and applications of reconstituted influenza virus envelopes (virosomes).* Methods Enzymol, 1993. **220**(8350760): p. 313-331.
330. Kreijtz, J.H.C.M., A.D.M.E. Osterhaus, and G.F. Rimmelzwaan, *Vaccination strategies and vaccine formulations for epidemic and pandemic influenza control.* Human Vaccines, 2009. **5**(3): p. 126-135.
331. Bungener, L., et al., *Virosome-mediated delivery of protein antigens to dendritic cells.* Vaccine, 2002. **20**(12009284): p. 2287-2295.
332. Pregliasco, F., et al., *Immunogenicity and safety of three commercial influenza vaccines in institutionalized elderly.* Aging (Milano), 2001. **13**(11292151): p. 38-43.
333. Conne, P., et al., *Immunogenicity of trivalent subunit versus virosome-formulated influenza vaccines in geriatric patients.* Vaccine, 1997. **15**(9364699): p. 1675-1679.
334. Gluck, R., et al., *Immunogenicity of new virosome influenza vaccine in elderly people.* Lancet, 1994. **344**(7912766): p. 160-163.
335. Mebatsion and K.K. Conzelmann, *Infectious rabies viruses from cloned cDNA.* EMBO J., 1994. **13**: p. 4195-4203.

336. Suryanarayana, V.V.S., D. Chattopadhyay, and M.S. Shaila, *Non-segmented negative sense RNA viruses as vectors for vaccine development.* Current Science, 2010. **98**(3): p. 379-391.
337. Patterson, L., et al., *Live Adenovirus Recombinants as Vaccine Vectors.*, in *New Generation Vaccines*, M. Levine, et al., Editors. 2004, Marcel Dekker: New York. p. 325-35.
338. Hoelscher, M.A., et al., *New pre-pandemic influenza vaccines: an egg- and adjuvant-independent human adenoviral vector strategy induces long-lasting protective immune responses in mice.* Clin Pharmacol Ther, 2007. **82**(17957181): p. 665-671.
339. J, B. and S. RM, *Dendritic cells and the control of immunity. .* Nature, 1998. **392**: p. 245-252.
340. Wei, C.-J., et al., *Induction of Broadly Neutralizing H1N1 Influenza Antibodies by Vaccination.* Science, 2010. **329**(5995): p. 1060-1064.
341. Van Kampen, K.R., et al., *Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans.* Vaccine, 2005. **23**(15620476): p. 1029-1036.
342. Toro, H., et al., *Protective avian influenza in ovo vaccination with non-replicating human adenovirus vector.* Vaccine, 2007. **25**(15): p. 2886-2891.
343. Gao, W., et al., *Protection of Mice and Poultry from Lethal H5N1 Avian Influenza Virus through Adenovirus-Based Immunization.* Journal of Virology, 2006. **80**(16439551): p. 1959-1964.
344. Roy, S., et al., *Partial protection against H5N1 influenza in mice with a single dose of a chimpanzee adenovirus vector expressing nucleoprotein.* Vaccine, 2007. **25**(39-40): p. 6845-6851.
345. Hoelscher, M.A., et al., *A broadly protective vaccine against globally dispersed clade 1 and clade 2 H5N1 influenza viruses.* J Infect Dis, 2008. **197**(18462165): p. 1185-1188.
346. Holman, D.H., et al., *Multi-antigen vaccines based on complex adenovirus vectors induce protective immune responses against H5N1 avian influenza viruses.* Vaccine, 2008. **26**(18395306): p. 2627-2639.
347. Catanzaro, A.T., et al., *Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector.* J Infect Dis, 2006. **194**(17109335): p. 1638-1649.
348. Prevec, L., et al., *Immune response to HIV-1 gag antigens induced by recombinant adenovirus vectors in mice and rhesus macaque monkeys.* J Acquir Immune Defic Syndr, 1991. **4**(1902514): p. 568-576.
349. Marjorie, R.-G., *Replicating and non-replicating viral vectors for vaccine development.* Current Opinion in Biotechnology, 2007. **18**(6): p. 546-556.
350. Lubeck, M.D., et al., *Immunogenicity and efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus.* Proc Natl Acad Sci U S A, 1989. **86**(2570422): p. 6763-6767.

351. Gao, W., et al., *Effects of a SARS-associated coronavirus vaccine in monkeys.* Lancet, 2003. **362**(14667748): p. 1895-1896.
352. Wang, D., et al., *De novo syntheses of Marburg virus antigens from adenovirus vectors induce potent humoral and cellular immune responses.* Vaccine, 2006. **24**(15): p. 2975-2986.
353. Wang, D., et al., *Development of a cAdVax-based bivalent ebola virus vaccine that induces immune responses against both the Sudan and Zaire species of Ebola virus.* J Virol, 2006. **80**(16501083): p. 2738-2746.
354. Schepp-Berglind, J., et al., *Complex Adenovirus-Mediated Expression of West Nile Virus C, PreM, E, and NS1 Proteins Induces both Humoral and Cellular Immune Responses.* Clinical and Vaccine Immunology, 2007. **14**(9): p. 1117-1126.
355. Holman, D.H., et al., *Two complex, adenovirus-based vaccines that together induce immune responses to all four dengue virus serotypes.* Clin Vaccine Immunol, 2007. **14**(17192403): p. 182-189.
356. RAJA, N.U., et al., *INDUCTION OF BIVALENT IMMUNE RESPONSES BY EXPRESSION OF DENGUE VIRUS TYPE 1 AND TYPE 2 ANTIGENS FROM A SINGLE COMPLEX ADENOVIRAL VECTOR.* The American Journal of Tropical Medicine and Hygiene, 2007. **76**(4): p. 743-751.
357. Bonnet, M.C., et al., *Recombinant viruses as a tool for therapeutic vaccination against human cancers.* Immunol Lett, 2000. **74**(10996623): p. 11-25.
358. Abbink, P., et al., *Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D.* J Virol, 2007. **81**: p. 4654–4663.
359. Doolan, D.L., et al., *Nasal Delivery of an Adenovirus-Based Vaccine Bypasses Pre-Existing Immunity to the Vaccine Carrier and Improves the Immune Response in Mice.* PLoS ONE, 2008. **3**(10): p. e3548.
360. Patel, A., S. Tikoo, and G. Kobinger, *A Porcine Adenovirus with Low Human Seroprevalence Is a Promising Alternative Vaccine Vector to Human Adenovirus 5 in an H5N1 Virus Disease Model.* Plos one, 2010. **5**(12): p. 1-11.
361. Barouch, D.H., et al., *Immunogenicity of Recombinant Adenovirus Serotype 35 Vaccine in the Presence of Pre-Existing Anti-Ad5 Immunity.* Journal of Immunology, 2004. **172**: p. 6290-6297.
362. Thorner, A.R., et al., *Immunogenicity of Heterologous Recombinant Adenovirus Prime-Boost Vaccine Regimens Is Enhanced by Circumventing Vector Cross-Reactivity.* Journal of Virology, 2006. **80**(24): p. 12009-12016.
363. Singh, N., et al., *Bovine Adenoviral Vector-based H5N1 Influenza Vaccine Overcomes Exceptionally High Levels of Pre-existing Immunity Against Human Adenovirus.* Mol Ther, 2008. **16**(5): p. 965-971.
364. Gallo, P., et al., *Adenovirus as vehicle for anticancer genetic immunotherapy.* Gene Ther, 2005. **12**: p. S84-S91.
365. Yang, Z.y., et al., *Overcoming Immunity to a Viral Vaccine by DNA Priming before Vector Boosting.* Journal of Virology, 2003. **77**(1): p. 799-803.

366. Drexler, I., et al., *Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells.* J Gen Virol, 1998. **79 (Pt 2)**(9472619): p. 347-352.
367. Sutter, G., et al., *A recombinant vector derived from the host range-restricted and highly attenuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus.* Vaccine, 1994. **12**(7975844): p. 1032-1040.
368. Kreijtz, J.H.C.M., et al., *Recombinant modified vaccinia virus Ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1.* J Infect Dis, 2007. **195**(17471429): p. 1598-1606.
369. Kreijtz, J.H.C.M., et al., *MVA-based H5N1 vaccine affords cross-clade protection in mice against influenza A/H5N1 viruses at low doses and after single immunization.* Plos one, 2009. **4**(19915662).
370. Poon, L.L.M., et al., *Vaccinia Virus-Based Multivalent H5N1 Avian Influenza Vaccines Adjuvanted with IL-15 Confer Sterile Cross-Clade Protection in Mice.* The Journal of Immunology, 2009. **182**(5): p. 3063-3071.
371. Sutter, G. and C. Staib, *Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery.* Curr Drug Targets Infect Disord, 2003. **3**(14529359): p. 263-271.
372. McShane, H., et al., *Protective immunity against Mycobacterium tuberculosis induced by dendritic cells pulsed with both CD8+- and CD4+-T-cell epitopes from antigen 85A.* Infect Immun, 2002. **70**: p. 1623-1626.
373. Acres, B. and J.-Y. Bonnefoy, *Clinical development of MVA-based therapeutic cancer vaccines.* Expert Review of Vaccines, 2008. **7**(7): p. 889-893.
374. Albrecht, M., et al., *Vaccination with a Modified Vaccinia Virus Ankara-based vaccine protects mice from allergic sensitization.* J Gene Med, 2008. **10**(18816482): p. 1324-1333.
375. Mayr, A. and K. Danner, *Vaccination against pox diseases under immunosuppressive conditions.* Dev Biol Stand, 1978. **41**(223909): p. 225-234.
376. Stittelaar, K.J., et al., *Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques.* Vaccine, 2001. **19**(11395204): p. 3700-3709.
377. Ramirez, J.C., et al., *Attenuated modified vaccinia virus Ankara can be used as an immunizing agent under conditions of preexisting immunity to the vector.* J Virol, 2000. **74**(10906221): p. 7651-7655.
378. Wang, Z., et al., *Recombinant Modified Vaccinia Virus Ankara Expressing a Soluble Form of Glycoprotein B Causes Durable Immunity and Neutralizing Antibodies against Multiple Strains of Human Cytomegalovirus.* Journal of Virology, 2004. **78**(8): p. 3965-3976.
379. Drexler, I., C. Staib, and G. Sutter, *Modified vaccinia virus Ankara as antigen delivery system: how can we best use its potential?* Curr Opin Biotechnol, 2004. **15**(15560976): p. 506-512.

380. Belyakov, I.M., et al., *Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity.* Proc Natl Acad Sci U S A, 1999. **96**(10200293): p. 4512-4517.
381. Davis, N.L., et al., *Alphavirus replicon particles as candidate HIV vaccines.* IUBMB Life, 2002. **53**(12120997): p. 209-211.
382. Liljestro¨m, P. and H. Garoff, *A new generation of animal cell expression vectors based on the Semliki Forest virus replicon.* Bio/Technology, 1991. **9**(12): p. 1356-61.
383. Strauss, J.H. and E.G. Strauss, *The alphaviruses: Gene expression, replication, and evolution.* Microbiol. ReV, 1994. **58**(3): p. 491-562.
384. Schlesinger, S., *Alphavirus expression systems-Promises and problems.* ASM News, 1999. **65**: p. 688-95.
385. Berglund, P., et al., *Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in mice.* Vaccine, 1999. **17**(5): p. 497-507.
386. Schultz-Cherry, S., et al., *Influenza Virus (A/HK/156/97) Hemagglutinin Expressed by an Alphavirus Replicon System Protects Chickens against Lethal Infection with Hong Kong-Origin H5N1 Viruses.* Virology, 2000. **278**(1): p. 55-59.
387. Sylte, M.J., B. Hubby, and D.L. Suarez, *Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection.* Vaccine, 2007. **25**(19): p. 3763-3772.
388. Erdman, M.M., et al., *Alphavirus replicon particle vaccines developed for use in humans induce high levels of antibodies to influenza virus hemagglutinin in swine: Proof of concept.* Vaccine, 2010. **28**(3): p. 594-596.
389. Defang, G.N., et al., *Induction of neutralizing antibodies to Hendra and Nipah glycoproteins using a Venezuelan equine encephalitis virus in vivo expression system.* Vaccine, 2010. **29**(2): p. 212-220.
390. Lundstrom, K., *Alphavirus vectors for vaccine production and gene therapy.* Expert Review of Vaccines, 2003. **2**(3): p. 445-459.
391. Madhan, S., M. Prabakaran, and J. Kwang, *Baculovirus as Vaccine Vectors.* Current Gene Therapy, 2010. **10**(3): p. 201-213.
392. Ruedl, C., et al., *Immune response in the lungs following oral immunization with bacterial lysates of respiratory pathogens.* Clin Diagn Lab Immunol, 1994. **1**(7496936): p. 150-154.
393. Prabakaran, M., et al., *Gastrointestinal delivery of baculovirus displaying influenza virus hemagglutinin protects mice against heterologous H5N1 infection.* J Virol, 2010. **84**(20071572): p. 3201-3209.
394. Yang, D.-G., et al., *Avian Influenza Virus Hemagglutinin Display on Baculovirus Envelope: Cytoplasmic Domain Affects Virus Properties and Vaccine Potential.* Mol Ther, 2007. **15**(5): p. 989-996.
395. Chen, C.-Y., et al., *Baculovirus as an avian influenza vaccine vector: Differential immune responses elicited by different vector forms.* Vaccine, 2010. **28**(48): p. 7644-7651.

396. Jin, R., et al., *Safety and Immunogenicity of H5N1 Influenza Vaccine Based on Baculovirus Surface Display System of <italic>Bombyx mori</italic>.* Plos one, 2008. **3**(12): p. e3933.
397. Tang, X.-C., H.-R. Lu, and T.M. Ross, *Hemagglutinin Displayed Baculovirus Protects Against Highly Pathogenic Influenza.* Vaccine, 2010. **28**(42): p. 6821-6831.
398. Pekosz, A., B. He, and R.A. Lamb, *Reverse genetics of negative-strand RNA viruses: Closing the circle.* Proceedings of the National Academy of Sciences, 1999. **96**: p. 8804-8806.
399. He, B., et al., *Recovery of Infectious SV5 from Cloned DNA and Expression of a Foreign Gene.* Virology, 1997. **237**: p. 249-260.
400. Clarke, D., et al., *Recombinant vesicular stomatitis virus as an HIV-1 vaccine vector.* Springer Seminars in Immunopathology, 2006. **28**(3): p. 239-253.
401. Lyles, D.S. and C.E. Rupprecht, *Rhabdoviridae*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2007, Lippincott Williams and Wilkins, a Wolters Kluwer Business: Philadelphia, PA.
402. Quiroz, E., et al., *A human case of encephalitis associated with vesicular stomatitis virus (Indiana serotype) infection.* Am J Trop Med Hyg, 1988. **39**(2845825): p. 312-314.
403. Haglund, K., et al., *Expression of human immunodeficiency virus type 1 Gag protein precursor and envelope proteins from a vesicular stomatitis virus recombinant: high-level production of virus-like particles containing HIV envelope.* Virology, 2000. **268**(10683333): p. 112-121.
404. Schnell, M.J., et al., *Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles.* Proceedings of the National Academy of Sciences, 1996. **93**(21): p. 11359-11365.
405. Haglund, K., et al., *Robust recall and long-term memory T-cell responses induced by prime-boost regimens with heterologous live viral vectors expressing human immunodeficiency virus type 1 Gag and Env proteins.* J Virol, 2002. **76**(12097563): p. 7506-7517.
406. Roberts, A., et al., *Attenuated vesicular stomatitis viruses as vaccine vectors.* J Virol, 1999. **73**(10196265): p. 3723-3732.
407. Roberts, A., et al., *Vaccination with a Recombinant Vesicular Stomatitis Virus Expressing an Influenza Virus Hemagglutinin Provides Complete Protection from Influenza Virus Challenge.* Journal of Virology, 1998. **72**(6): p. 4704-4711.
408. Barefoot, B.E., et al., *Intramuscular immunization with a vesicular stomatitis virus recombinant expressing the influenza hemagglutinin provides post-exposure protection against lethal influenza challenge.* Vaccine, 2009. **28**(1): p. 79-89.
409. Barefoot, B.E., C.J. Sample, and E.A. Ramsburg, *Recombinant Vesicular Stomatitis Virus Expressing Influenza Nucleoprotein Induces CD8 T-Cell Responses That Enhance Antibody-Mediated Protection after Lethal*

Challenge with Influenza Virus. Clinical and Vaccine Immunology, 2009. **16**(4): p. 488-498.
410. Schwartz, J.A., et al., *Vesicular stomatitis virus vectors expressing avian influenza H5 HA induce cross-neutralizing antibodies and long-term protection.* Virology, 2007. **366**(1): p. 166-173.
411. Iverson, L.E. and J.K. Rose, *Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription.* Cell, 1981. **23**: p. 477-484.
412. Schwartz, J.A., et al., *Potent Vesicular Stomatitis Virus-Based Avian Influenza Vaccines Provide Long-Term Sterilizing Immunity against Heterologous Challenge.* Journal of Virology, 2010. **84**(9): p. 4611-4618.
413. Schwartz, J.A., et al., *Vesicular Stomatitis Virus-Based H5N1 Avian Influenza Vaccines Induce Potent Cross-Clade Neutralizing Antibodies in Rhesus Macaques.* J. Virol., 2011. **85**(9): p. 4602-4605.
414. Kalhoro, N.H., et al., *A recombinant vesicular stomatitis virus replicon vaccine protects chickens from highly pathogenic avian influenza virus (H7N1).* Vaccine, 2009. **27**(19135116): p. 1174-1183.
415. Rose, N.F., et al., *An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants.* Cell, 2001. **106**(11551502): p. 539-549.
416. van den Pol, A.N., K.P. Dalton, and J.K. Rose, *Relative neurotropism of a recombinant rhabdovirus expressing a green fluorescent envelope glycoprotein.* J Virol, 2002. **76**(11773406): p. 1309-1327.
417. Johnson, J.E., et al., *Neurovirulence properties of recombinant vesicular stomatitis virus vectors in non-human primates.* Virology, 2007. **360**(17098273): p. 36-49.
418. Robison, C.S. and M.A. Whitt, *The membrane-proximal stem region of vesicular stomatitis virus G protein confers efficient virus assembly.* J Virol, 2000. **74**(10666254): p. 2239-2246.
419. Zuniga, A., et al., *Attenuated measles virus as a vaccine vector.* Vaccine, 2007. **25**(16): p. 2974-2983.
420. Lorin, C., et al., *A Single Injection of Recombinant Measles Virus Vaccines Expressing Human Immunodeficiency Virus (HIV) Type 1 Clade B Envelope Glycoproteins Induces Neutralizing Antibodies and Cellular Immune Responses to HIV.* Journal of Virology, 2004. **78**(1): p. 146-157.
421. Bukreyev, A.A., et al., *Mucosal parainfluenza virus-vectored vaccine against Ebola virus replicates in the respiratory tract of vector-immune monkeys and is immunogenic.* Virology, 2010. **399**(2): p. 290-298.
422. Schmidt, A.C., et al., *Mucosal Immunization of Rhesus Monkeys against Respiratory Syncytial Virus Subgroups A and B and Human Parainfluenza Virus Type 3 by Using a Live cDNA-Derived Vaccine Based on a Host Range-Attenuated Bovine Parainfluenza Virus Type 3 Vector Backbone.* Journal of Virology, 2002. **76**(3): p. 1089-1099.
423. Hanson, R.P., *Newcastle disease*. 7 ed1978, Ames, Iowa: Iowa State University Press.

424. Bukreyev, A., et al., *Recombinant newcastle disease virus expressing a foreign viral antigen is attenuated and highly immunogenic in primates.* J Virol, 2005. **79**(16227250): p. 13275-13284.
425. Bukreyev, A. and P.L. Collins, *Newcastle disease virus as a vaccine vector for humans.* Curr Opin Mol Ther, 2008. **10**(18228181): p. 46-55.
426. DiNapoli, J.M., et al., *Immunization of primates with a Newcastle disease virus-vectored vaccine via the respiratory tract induces a high titer of serum neutralizing antibodies against highly pathogenic avian influenza virus.* J Virol, 2007. **81**(17715243): p. 11560-11568.
427. Ge, J., et al., *Newcastle disease virus based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses.* J. Virol., 2007. **81**: p. 150-158.
428. DiNapoli, J.M., et al., *Newcastle Disease Virus-Vectored Vaccines Expressing the Hemagglutinin or Neuraminidase Protein of H5N1 Highly Pathogenic Avian Influenza Virus Protect against Virus Challenge in Monkeys.* Journal of Virology, 2010. **84**(3): p. 1489-1503.
429. DiNapoli, J.M., et al., *Delivery to the lower respiratory tract is required for effective immunization with Newcastle disease virus-vectored vaccines intended for humans.* Vaccine, 2009. **27**(10): p. 1530-1539.
430. Huang, Z., et al., *A recombinant Newcastle disease virus (NDV) expressing VP2 protein of infectious bursal disease virus (IBDV) protects against NDV and IBDV.* J Virol, 2004. **78**(15331738): p. 10054-10063.
431. Park, M.-S., et al., *Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease.* Proc Natl Acad Sci U S A, 2006. **103**(16717196): p. 8203-8208.
432. Veits, J., et al., *Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza.* Proc Natl Acad Sci U S A, 2006. **103**(16717197): p. 8197-8202.
433. Martinez-Sobrido, L., et al., *Protection against Respiratory Syncytial Virus by a Recombinant Newcastle Disease Virus Vector.* Journal of Virology, 2006. **80**(3): p. 1130-1139.
434. DiNapoli, J.M., et al., *Newcastle disease virus, a host range-restricted virus, as a vaccine vector for intranasal immunization against emerging pathogens.* Proc Natl Acad Sci U S A, 2007. **104**(17535926): p. 9788-9793.
435. DiNapoli, J.M., et al., *Respiratory tract immunization of non-human primates with a Newcastle disease virus-vectored vaccine candidate against Ebola virus elicits a neutralizing antibody response.* Vaccine, 2010. **29**(1): p. 17-25.
436. Vigil, A., et al., *Recombinant Newcastle Disease Virus as a Vaccine Vector for Cancer Therapy.* Mol Ther, 2008. **16**(11): p. 1883-1890.
437. Hull, R.N., J.R. Minner, and J.W. Smith, *New viral agents recovered from tissue cultures of monkey kidney cells. I. Origin and properties of cytopathogenic agents S.V.1, S.V.2, S.V.4, S.V.5, S.V.6, S.V.11, S.V.12 and S.V.15.* American Journal of Hygeine, 1956: p. 204-215.

438. Hsiung, G.D., *Parainfluenza-5 virus. Infection of man and animal.* Prog. Med. Virol., 1972. **14**: p. 241-274.
439. Goswami, K.K.A., et al., *Does Simian Virus 5 Infect Humans?* Journal of General Virology, 1984. **65**: p. 1295-1303.
440. Vandvick, B. and E. Norrby, *Paramyxovirus SV5 and multiple sclerosis.* Nature, 1989. **338**: p. 769-771.
441. Chatziandreou, N., et al., *Relationships and host range of human, canine, simian and porcine isolates of simian virus 5 (parainfluenza virus 5).* Journal of General Virology, 2004. **85**(10): p. 3007-3016.
442. McCandlish, I.A., et al., *A study of dogs with kennel cough.* Vet. Rec., 1978: p. 293-301.
443. Johnson, J.B., G.A. Capraro, and G.D. Parks, *Differential mechanisms of complement-mediated neutralization of the closely related paramyxoviruses simian virus 5 and mumps virus.* Virology, 2008. **376**(1): p. 112-123.
444. He, B., et al., *The SH Integral Membrane Protein of the Paramyxovirus Simian Virus 5 Is Required To Block Apoptosis in MDBK Cells.* Journal of Virology, 2001. **75**(9): p. 4068-4079.
445. Zhang, L., et al., *Comparison of differing cytopathic effects in human airway epithelium of parainfluenza virus 5 (W3A), parainfluenza virus type 3, and respiratory syncytial virus.* Virology, 2011. **421**(1): p. 67-77.
446. Karron, R.A. and P.L. Collins, *Parainfluenza viruses*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2007, Lippincott Williams and Wilkins, a Wolters Kluwer Business: Philadelphia. p. 1497-1526.
447. Clark, K.M., et al., *Parainfluenza virus 5-based vaccine vectors expressing vaccinia virus (VACV) antigens provide long-term protection in mice from lethal intranasal VACV challenge.* Virology, 2011. **419**: p. 97-106.
448. Randall, R.E. and D.F. Young, *Comparison between Parainfluenza Virus Type 2 and Simian Virus 5: Monoclonal Antibodies Reveal Major Antigenic Differences.* Journal of General Virology, 1988. **69**: p. 2051-2060.
449. Orvell, C., R. Rudbeck, and A. Love, *Immunological relationships between mumps virus and parainfluenza viruses studied with monoclonal antibodies.* Journal of General Virology, 1986. **67**: p. 1929-1939.
450. Thomas, S.M., R.A. Lamb, and R.G. Paterson, *Two mRNAs that differ by two nontemplated nucleotides encode the amino coterminal proteins P and V of the paramyxovirus SV5.* Cell, 1988: p. 891-902.
451. Lin, Y., et al., *The role of simian virus 5 V protein on viral RNA synthesis.* Virology, 2005: p. 270-280.
452. Calain, P. and L. Roux, *The Rule of Six, a Basic Feature for Efficient Replication of Sendai Virus Defective Interfering RNA.* Journal of Virology, 1993. **67**(8): p. 4822-4830.
453. Pei, Z., Y. Bai, and A.P. Schmitt, *PIV5 M protein interaction with host protein angiomotin-like 1.* Virology, 2010. **397**(1): p. 155-166.
454. Schmitt, A.P., et al., *Requirements for Budding of Paramyxovirus Simian Virus 5 Virus-Like Particles.* Journal of Virology, 2002. **76**(8): p. 3952-3964.

455. Schmitt, A.P. and R.A. Lamb, *Escaping from the cell: assembly and budding of negative-strand RNA viruses.* Current topics in microbiology and immunology, 2004. **283**: p. 145-96.
456. Young, D.F., et al., *Paramyxoviridae Use Distinct Virus-Specific Mechanisms to Circumvent the Interferon Response.* Virology, 2000. **269**(2): p. 383-390.
457. Didcock, L., et al., *The V Protein of Simian Virus 5 Inhibits Interferon Signalling by Targeting STAT1 for Proteasome-Mediated Degradation.* Journal of Virology, 1999. **73**(12): p. 9928-9933.
458. Andrejeva, J., et al., *Degradation of STAT1 and STAT2 by the V Proteins of Simian Virus 5 and Human Parainfluenza Virus Type 2, Respectively: Consequences for Virus Replication in the Presence of Alpha/Beta and Gamma Interferons.* Journal of Virology, 2002. **76**(5): p. 2159-2167.
459. Ulane, C., *Paramyxoviruses SV5 and HPIV2 Assemble STAT Protein Ubiquitin Ligase Complexes from Cellular Components.* Virology, 2002. **304**(2): p. 160-166.
460. Poole, E., *The V Proteins of Simian Virus 5 and Other Paramyxoviruses Inhibit Induction of Interferon-β.* Virology, 2002. **303**(1): p. 33-46.
461. He, B., et al., *Recovery of Paramyxovirus Simian Virus 5 with a V Protein Lacking the Conserved Cysteine-rich Domain: The Multifunctional V Protein Blocks both Interferon-β Induction and Interferon Signaling.* Virology, 2002. **303**(1): p. 15-32.
462. Didcock, L., et al., *Sendai Virus and Simian Virus 5 Block Activation of Interferon-Responsive Genes: Importance for Virus Pathogenesis.* Journal of Virology, 1999. **73**(4): p. 3125-3133.
463. Lin, G.Y. and R.A. Lamb, *The Paramyxovirus Simian Virus 5 V Protein Slows Progression of the Cell Cycle.* Journal of Virology, 2000. **74**(19): p. 9152-9166.
464. Wilson, R.L., et al., *Function of Small Hydrophobic Proteins of Paramyxovirus.* Journal of Virology, 2006. **80**(4): p. 1700-1709.
465. Manuse, M.J. and G.D. Parks, *Role for the Paramyxovirus Genomic Promoter in Limiting Host Cell Antiviral Responses and Cell Killing.* Journal of Virology, 2009. **83**(18): p. 9057-9067.
466. Wansley, E.K. and G.D. Parks, *Naturally Occurring Substitutions in the P/V Gene Convert the Noncytopathic Paramyxovirus Simian Virus 5 into a Virus That Induces Alpha/Beta Interferon Synthesis and Cell Death.* Journal of Virology, 2002. **76**(20): p. 10109-10121.
467. Manuse, M.J., C.M. Briggs, and G.D. Parks, *Replication-independent activation of human plasmacytoid dendritic cells by the paramyxovirus SV5 Requires TLR7 and autophagy pathways.* Virology, 2010. **405**(2): p. 383-389.
468. Arimilli, S., et al., *Engineered Expression of the TLR5 Ligand Flagellin Enhances Paramyxovirus Activation of Human Dendritic Cell Function.* Journal of Virology, 2008. **82**(22): p. 10975-10985.
469. Han, G.-Z. and M. Worobey, *Homologous Recombination in Negative Sense RNA Viruses.* Viruses, 2011. **3**(8): p. 1358-1373.

470. Derby, M.A., et al., *High-Avidity CTL Exploit Two Complementary Mechanisms to Provide Better Protection Against Viral Infection Than Low-Avidity CTL.* The Journal of Immunology, 2001. **166**(3): p. 1690-1697.
471. Parks, G.D. and M.A. Alexander-Miller, *High avidity cytotoxic T lymphocytes to a foreign antigen are efficiently activated following immunization with a recombinant paramyxovirus, simian virus 5.* Journal of General Virology, 2002. **83**(5): p. 1167-1172.
472. Kortekaas, J., et al., *Intramuscular inoculation of calves with an experimental Newcastle disease virus-based vector vaccine elicits neutralizing antibodies against Rift Valley fever virus.* Vaccine, 2010. **28**(11): p. 2271-2276.
473. Le, T.-v.L., et al., *Induction of Influenza-Specific Mucosal Immunity by an Attenuated Recombinant Sendai Virus.* Plos one, 2011. **6**(4): p. e18780.
474. Moriya, C., et al., *Intranasal Sendai viral vector vaccination is more immunogenic than intramuscular under pre-existing anti-vector antibodies.* Vaccine, 2011. **29**(47): p. 8557-8563.
475. Capraro, G.A., et al., *Virus growth and antibody responses following respiratory tract infection of ferrets and mice with WT and P/V mutants of the paramyxovirus Simian Virus 5.* Virology, 2008. **376**(2): p. 416-428.
476. WHO. *Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO*. Global Alert and Response (GAR) 2011 June 22, 2011; Available from: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_06_22/en/index.html.
477. Driskell, J.D., et al., *One-step assay for detecting influenza virus using dynamic light scattering*

and gold nanoparticles. Analyst, 2011. **136**(15): p. 3083-3090.
478. Schwartz, J., et al., *Vesicular stomatitis virus vectors expressing avian influenza H5 HA induce cross-neutralizing antibodies and long-term protection.* Virology, 2007. **366**(1): p. 166-173.
479. Dong, J., et al., *Development of a new candidate H5N1 avian influenza virus for pre-pandemic vaccine production.* Influenza Other Respi Viruses, 2009. **3**(6): p. 287-95.
480. DiNapoli, J.M., et al., *Newcastle Disease Virus-Vectored Vaccines Expressing the Hemagglutinin or Neuraminidase Protein of H5N1 Highly Pathogenic Avian Influenza Virus Protect against Virus Challenge in Monkeys.* Journal of Virology, 2009. **84**(3): p. 1489-1503.
481. Forger III, J.M., et al., *Murine infection by vesicular stomatitis virus: initial characterization of the H-2d system.* J Virol, 1991. **65**: p. 4950–4958.
482. Soboleski, M.R., et al., *Cold-adapted influenza and recombinant adenovirus vaccines induce cross-protective immunity against pH1N1 challenge in mice.* Plos one, 2011. **6**(7): p. e21937.
483. Price, G.E., et al., *Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses.* Plos one, 2010. **5**(10): p. e13162.

484. Price, G.E., et al., *Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses.* Vaccine, 2009. **27**(47): p. 6512-21.
485. Lo, C.Y., et al., *Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: cold-adapted vaccine versus DNA prime-adenovirus boost strategies.* Vaccine, 2008. **26**(17): p. 2062-72.
486. Lanthier, P.A., et al., *Live attenuated influenza vaccine (LAIV) impacts innate and adaptive immune responses.* Vaccine, 2011. **29**(44): p. 7849-56.
487. Basha, S., et al., *Comparison of antibody and T-cell responses elicited by licensed inactivated- and live-attenuated influenza vaccines against H3N2 hemagglutinin.* Hum Immunol, 2011. **72**(6): p. 463-9.
488. Subbramanian, R.A., et al., *Pandemic and seasonal H1N1 influenza hemagglutinin-specific T cell responses elicited by seasonal influenza vaccination.* Vaccine, 2010. **28**(52): p. 8258-67.
489. Hancock, K., et al., *Cross-Reactive Antibody Responses to the 2009 Pandemic H1N1 Influenza Virus.* New England Journal of Medicine, 2009. **361**(20): p. 1945-1952.
490. Feng, J., et al., *Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2.* Virology Journal, 2006. **3**(1): p. 102.
491. Tokusumi, T., et al., *Recombinant Sendai viruses expressing different levels of a foreign reporter gene.* Virus Research, 2002. **86**(1-2): p. 33-38.
492. Epstein, S.L. and G.E. Price, *Cross-protective immunity to influenza A viruses.* Expert Review of Vaccines, 2010. **9**(11): p. 1325-1341.
493. Sun, D., et al., *PLK1 down-regulates parainfluenza virus 5 gene expression.* PLoS Pathogens, 2009. **5**(7): p. 1-13.
494. Vandvik, B. and E. Norrby, *Paramyxovirus SV5 and multiple sclerosis.* Nature, 1989. **338**: p. 769-771.
495. Cornwell, H.J., et al., *Isolation of parainfluenza virus SV5 from dogs with respiratory disease.* Vet Rec, 1976. **98**(15): p. 301-2.
496. Spann, K.M., P.L. Collins, and M.N. Teng, *Genetic Recombination during Coinfection of Two Mutants of Human Respiratory Syncytial Virus.* Journal of Virology, 2003. **77**(20): p. 11201-11211.
497. Komada, H., et al., *Immunological relationships between parainfluenza virus type 4 and other paramyxoviruses studied by use of monoclonal antibodies.* Arch Virol, 1991. **116**: p. 277-283.
498. Randall, R.E. and D.F. Young, *Comparison between Parainfluenza Virus Type 2 and Simian Virus 5:*

Monoclonal Antibodies Reveal Major Antigenic Differences. Journal of General Virology, 1988. **69**: p. 2051-2060.

499. Tsurudome, M., et al., *Extensive antigenic diversity among human parainfluenza type 2 virus isolates and immunological relationships among paramyxoviruses revealed by monoclonal antibodies.* Virology, 1989. **171**: p. 38-48.

500. Matrosovich, M., et al., *New low-viscosity overlay medium for viral plaque assays.* Virology Journal, 2006. **3**(1): p. 63.
501. Gabbard, J., et al., *A humanized anti-M2 scFv shows protective in vitro activity against influenza.* Protein Engineering Design and Selection, 2009. **22**(3): p. 189-198.
502. Gillim-Ross, L. and K. Subbarao, *Can Immunity Induced by the Human Influenza Virus N1 Neuraminidase Provide Some Protection from Avian Influenza H5N1 Viruses?* PLoS Med, 2007. **4**(2): p. e91.
503. Chen, Z., et al., *The 2009 pandemic H1N1 virus induces anti-neuraminidase (NA) antibodies that cross-react with the NA of H5N1 viruses in ferrets.* Vaccine, 2012(0).
504. Harley, V.R., C.W. Ward, and P.J. Hudson, *Molecular cloning and analysis of the N5 neuraminidase subtype from an avian influenza virus.* Virology, 1989. **169**(1): p. 239-243.
505. Shi, J., et al., *Protective efficacy of an H1N1 cold-adapted live vaccine against the 2009 pandemic H1N1, seasonal H1N1, and H5N1 influenza viruses in mice.* Antiviral Research, 2012. **93**(3): p. 346-353.

CHAPTER 3

PROTECTION AGAINST H5N1 VIRUS CHALLENGE BY IMMUNIZATION WITH RECOMBINANT PIV5 EXPRESSING HA OF H5N1 (rPIV5-H5)

Alaina Jones Mooney, Zhuo Li, Jon D. Gabbard, Daniel A. Dlugolenski, Scott K. Johnson, Robert J. Hogan, Ralph A. Tripp, Elizabeth MI Uhl, Biao He, S. Mark Tompkins. To be submitted to *Journal of Virology*

Abstract

Highly pathogenic avian influenza virus (HPAI) and the H5N1 subtype in particular, poses a formidable pandemic threat. Current HPAI vaccine candidates suffer from poor immunogenicity, and there are challenges associated with sufficient production and distribution. Parainfluenza virus 5 (PIV5) provides an appealing approach for live virus vectored vaccines. It can be produced quickly and safely in cell culture which is especially important in the event of a pandemic. We have previously shown that using reverse genetics techniques, the hemagglutinin (HA) gene from influenza A virus strain A/Udorn/72 (H3N2) can be inserted as an additional gene between the HN and L genes in the PIV5 genome (rPIV5-H3) and that it provides substantial immunity against influenza A infection in BALB/c mice. Here, we have inserted the HA gene of A/Vietnam/1203/04 (H5N1) into the PIV5 genome with the goal of testing the efficacy and mechanism of protection of recombinant PIV5-H5 vaccine vectors. The natural gradient of mRNA translated in PIV5 is dictated by the proximity of the gene to the 3' UTR. Thus, we tested expression and immunogenicity of H5 vaccine vectors where the HA gene is inserted in distinct locations in the PIV5 genome. The H5 HA was inserted between the SH and HN genes (ZL46), and between the HN and L genes (ZL48). Using a BALB/c mouse model, we show that both PIV5-H5 constructs are avirulent and capable of inducing a broad H5-specific immune response. H5 expression was enhanced in ZL46 as compared to ZL48 which, coupled with greater immunogenicity in mice, suggests that inserting the gene of interest closer to the leader sequence enhances

vaccine efficacy. Vaccination with these constructs reduces weight loss and lung virus titers upon A/VN/1203/04 challenge with protection comparable to vaccination with whole inactivated A/VN/1203/04 or protection conferred following a sub-lethal infection with recombinant A/VN-PR8 infection. The protection conferred by passive transfer of IgG from vaccinated mice indicates that neutralizing antibodies are associated with vaccine efficacy.

Introduction

Influenza is a continuing public health problem, inflicting significant morbidity and mortality each year. Seasonally circulating strains infect up to 15% of the world's population each year and cause an average of 226,000 hospitalizations and 36,000 deaths in the United States every year [3] as well as millions of deaths worldwide. Pandemics have occurred sporadically over the course of history with varying degrees of severity, but have only been well documented for about the past 100 years. The 1918 "Spanish flu" was responsible for over 50 million deaths worldwide (reviewed in [4]). The recent pandemic H1N1 in 2009 is a milder example, causing under 20,000 (laboratory confirmed) deaths worldwide [5]. Aquatic birds are considered the primary reservoir for influenza A virus[6] and every pandemic virus studied to date has contained gene segments of avian origin [7]. Historically, it was thought that avian viruses would have to reassort or adapt to infect or cause disease in humans until, in 1997, eighteen humans in Hong Kong were infected with highly pathogenic avian influenza (HPAI) A virus subtype H5N1 and 6 of those persons died [8]. Subsequent research has suggested that the 1918 'Spanish flu', the deadliest influenza pandemic in recorded history, was potentially generated by a similar mechanism [9]. HPAI viruses are now considered a potential pandemic threat. Related H5N1 HPAI viruses continue to smolder in Eurasia and as of June 22, 2011, the WHO has reported 562 human cases of H5N1 with a total of 329 deaths [476]. With an approximately 60% case fatality

rate, a pandemic HPAI H5N1 virus would cause significant morbidity and mortality, as well as socioeconomic disruption [12].

Vaccination with an effective vaccine is considered the most effective approach to prevent disease or the transmission of potentially pandemic viruses in humans. The most widely used licensed vaccine for the prevention of seasonal influenza virus infection is an inactivated vaccine, which is grown in embryonated chicken eggs, and requires months of production time between strain identification and millions of eggs [13]. There are a number of drawbacks in using this method of production for HPAI H5N1, including safety and production issues. The highly virulent nature of this virus prevents its use as a seed virus for embryonated hen eggs as it kills the embryo before sufficient virus titers can be reached, resulting in the need for other options for H5N1 vaccines. A variety of H5N1 vaccine seed viruses are being generated via reverse-genetics [15, 16], however data thus far suggests that in the absence of an adjuvant, these vaccines are even less immunogenic than inactivated H5N1 and have an even higher antigen requirement[17]. In early 2007, the US Food and Drug Administration (FDA) approved an H5N1 vaccine to be used in the event of a pandemic in spite of evidence of poor immunogenicity and requirement of multiple doses to generate a neutralizing antibody response [14]. There are a number of candidate vaccines in clinical trials at this time (reviewed in [18]), including inactivated viruses formulated with a variety of adjuvants, such as oil-in-water, and live-attenuated influenza vaccines [19, 20]. These approaches do not, however, address the

concerns of using an egg-based vaccine for prepandemic preparedness. Other vaccine options that are being explored include recombinant DNA and viruses, which have been shown to protect against homologous and heterologous influenza challenge, including H5N1 [21-24]. These finding suggest that virus vectored vaccines may be an effective method of obtaining robust, protective immune responses.

Parainfluenza virus 5 (PIV5), formerly known as simian virus 5 (SV5), is a non-segmented negative stranded RNA virus (NNSV) in the *Rubulavirus* genus in the family *Paramyxoviridae*. The benefits of using PIV5 as a vaccine vector have been previously described [24, 25]; and briefly include the absence of a DNA phase in the life cycle, which eliminates the likelihood of host genome alteration, as well as a stable genome, making it an ideal candidate for foreign gene insertion and vaccine use. PIV5 is capable of infecting a large range of cell types[26] and, because it causes very little cytopathic effect (CPE) in those cells, is able to grow to high titers in common cell lines, including VERO cells[27], a vaccine-approved cell line. Use of recombinant PIV5 (rPIV5) as an influenza vaccine vector would address the existing problems associated with the requirement of eggs to grow influenza virus for vaccine development. PIV5 also infects a large range of mammals, including humans, without causing clinical disease[28].

In previous studies, it was shown that the hemagglutinin (HA) gene from influenza A virus strain A/Udorn/72 (H3N2) (rPIV5-H3) could be inserted between the HN and L genes of PIV5, that PIV5 expressing H3 could be recovered, and that

mice immunized with rPIV5-H3 were protected against homologous influenza virus challenge[24]. Here, we show that a rPIV5 expressing the HA from HPAI A/Vietnam/1203/04 (H5N1) (rPIV5-H5) can be rescued and that the HA can be inserted not only between the HN and L genes of PIV5, but alternatively further upstream, between the SH and the HN genes, taking advantage of the transcription gradient based on proximity to the leader sequence observable in all paramyxoviruses. We show that insertion between SH and HN increases HA expression compared to insertion between the HN and L genes and that this increase in expression enhances efficacy of the vaccine. Using a mouse model of influenza virus infection, we show that vaccination with rPIV5-H5 primes both H5-specific T cell and antibody responses, with neutralizing serum antibody being sufficient for protection from HPAI H5N1 challenge.

Results

Rescue of recombinant PIV5-H5 viruses The HA gene of highly pathogenic avian influenza virus A/Vietnam/1203/04 (H5N1) was synthesized using an oligomer-based system. The gene was codon-optimized to ensure maximum expression in mammalian systems and the polybasic cleavage site was removed and replaced with an avirulent sequence (**fig 3.1a**). The codon-optimized HA gene was then inserted into the full length genome of PIV5 between the SH and HN genes (ZL46) and between the HN and L genes (ZL48). After obtaining the rescued virus, full length genome sequencing (**fig 3.1b**) was carried out to confirm the recombinant virus. There is no mutation in ZL46 virus, and there is a single mutation, S21G in the NP protein of ZL48 virus.

Expression of HA in rPIV5-H5 infected cells To confirm HA was being expressed on the surface of infected cells, immunofluorescence staining was performed. VERO cells infected with rPIV5-H5 were stained with an anti-HA (H5) antibody or a monoclonal antibody specific for the V/P proteins of PIV5 (anti-V/P). Expression of H5 as well as V/P was detected in both ZL46 and ZL48-infected cells, confirming that HA is being expressed in cells infected with rPIV5-H5. As expected, PIV5-infected cells were positive only for V/P **(figure 3.1c)**. We hypothesized that there would be a higher level of HA expression in ZL46 compared to ZL48, since the H5 gene was inserted closer to the leader sequence in ZL46. To test this, VERO cells were infected with PIV5, ZL46, or ZL48. Cell lysate was harvested 48 hours post-infection, separated by SDS-PAGE, and analyzed by western blot. A band at 75 kDa,

the size of the HA0 monomer is clearly visible in ZL46 and ZL48 infected lysate, while 46 kDa PIV5 V/P band is present in all infected groups groups. To determine the relative amounts of HA being expressed, we compared the densities of the bands and ZL46 is expressing approximately 3.25 times more HA protein than ZL48 (**figure 3.1e**). To ensure that cells were infected with equivalent amounts of virus, the density of the V/P protein was also measured and determined to be approximately the same for all groups. These results confirm our hypothesis that proximity to the leader sequence affects the expression levels of the inserted HA gene.

Incorporation of HA into the rPIV5-H5 virion We have previously shown that PIV5 expressing recombinant H3 incorporated the influenza HA into the PIV5 virion [24]. To test whether the H5 HA was also incorporated into the virion independent of the location of the gene within the PIV5 genome, we utilized dynamic light scattering (DLS) and gold nanoparticle (AuNP) labels to detect HA on the surface of the virion. Cleared virus culture supernatants of PIV5, ZL46, ZL48, and rg A/VN-PR8 were incubated with AuNP-labeled anti-HA (H5) antibodies and then measuring aggregation of the AuNP probes as previously described [477]. The degree of AuNP aggregation correlates with the presence of virus containing specific HA, with increases in virus increasing aggregation and Z-shift. An increase in the mean hydrodynamic diameter (z-average) of 8 or 14 nm was observed for ZL46 and ZL48, respectively, compared to PIV5, indicating that there was aggregation of the AuNP probes upon introduction of the viruses, suggesting that HA is present on the surface of the virion. The mean diameter observed for PIV5 was approximately the

same size as culture supernatant or allantoic fluid alone. The positive control, rgA/VN-PR8, had a mean diameter of 113.67 nm.

Growth of rPIV5-H5 in vitro and in vivo To determine if insertion of the HA gene caused attenuation or enhanced replication of the recombinant compared to the parent strain, growth curves were performed on PIV5, ZL46, and ZL48. MDBK cells were infected with 0.01 plaque forming units (PFU) per cell and samples of supernatant were taken at 24 hour time points from time zero through day seven and quantified via plaque assay. Insertion of the HA slightly slowed growth *in vitro* compared to the wild-type PIV5, however both of the recombinants achieved peak titers similar to the wild type PIV5, albeit delayed by 1 day **(figure 3.2a).** To determine if there were changes in growth in vivo, BALB/c mice were inoculated with 10^6 pfu of PIV5, ZL46, or ZL48 intranasally (IN), euthanized in groups on days 1, 3, 6, and 8 post-inoculation and their lungs analyzed for virus by plaque assay. No significant difference was observed between PIV5, ZL46, and ZL48 on day 1 post-infection, however by day 3 post-infection, there was significantly more virus in PIV5-infected mice ($p<0.001$) compared to ZL46 oand ZL48, although there was limited virus in all three groups (**figure 3.2b**). By day 6 post-infection there was little difference between PIV5, ZL46, and ZL48 and the virus had cleared by day 8 post-infection (data not shown). Taken together, these data indicates that although insertion of the HA into PIV5 may slightly attenuate the virus *in vivo*, the virus is still able to grow.

Pathology of rPIV5-H5 in BALB/c mice One of the key benefits to the use of PIV5 as a vaccine vector is that it does not cause clinical disease [441]. To confirm that insertion of the H5 gene does not alter the pathogenicity of the virus, BALB/c mice were inoculated with 10^6 PFU PIV5, ZL46 and ZL48 intranasally and monitored daily for weight loss as a symptom of clinical disease and cohorts of mice were euthanized on day 6 or day 13 for histopathologic analysis. There was no weight loss in any of the PIV5-infected mice, while mice inoculated with the attenuated reverse genetics A/VN-PR8 virus lost more than 15% of their starting body weight before recovering (**figure 3.3a**). For histopathologic analysis, sections of lung were H&E stained and levels of pulmonary infiltrate subjectively scored 0-3 with 0=normal (as compared to mock infected) 2=mild, and 3=moderate lymphoid aggregates and total scores determined. In general, the lung histopathology was characterized by mild to medium perivascular and peribronchial infiltrates that were mostly lymphocytes, however PIV5 was associated with the largest inflammatory response, whereas ZL46 and ZL48 were slightly attenuated (**figure 3.3b**). Lungs from ZL46 and ZL48 infected mice showed mild infiltrates (figure 3d and 3e, respectively), while lungs from wild type PIV5 infected mice had extensive perivascular cuffing bronchial infiltrates (Figure 3f), which correlates with the attenuated growth observed in figure 2, and suggesting that the rPIV5 viruses containing the H5 HA maintain an avirulent phenotype.

Immunogenicity of rPIV5-H5 in BALB/c mice We previously demonstrated that vaccination with rPIV5 expressing an H3 HA induced HA-specific, virus

neutralizing antibody responses. To determine the antibody response in rPIV5-H5 immunized mice and to assess whether location of the HA gene within the PIV5 genome altered immunogenicity, BALB/c mice were vaccinated with PIV5, ZL46, ZL48, rg A/VN-PR8 administered IN, or β-propiolactone (BPL)-inactivated A/Vietnam/1203/04 (iA/VN/03/04) administered intramuscularly (IM). The mice were bled on day 21 post-immunization, boosted with the same vaccine on day 28, and bled on day 28 post-boost. The sera were then analyzed for H5-specific IgG by ELISA. As expected, no HA-specific IgG was detected in mice vaccinated with rPIV5, whereas high titers of HA-specific IgG were detected in mice immunized with ZL46 and ZL48 (**figure 3.4a**). These antibody titers were comparable to iA/VN/03/04 and sub-lethal rg A/VN-PR8 infection. Importantly, there was a consistent increase in antibody titer after the second immunization, suggesting that pre-existing antibodies to PIV5 or the HA do not prevent boosting. To assess functionality of antibodies produced, sera were tested for neutralizing activity against rg A/VN-PR8 (H5N1) in a virus micro-neutralization assay. Again, mice vaccinated with PIV5 did not produce antibodies capable of neutralizing rg A/VN-PR8, whereas serum from mice vaccinated with rPIV5-H5 had high H5N1 neutralizing antibody titers at levels comparable to mice infected with rg A/VN-PR8 infection or vaccinated with inactivated A/VN/1203/04 (**figure 3.4b**). One of the benefits of using a live virus-vectored vaccine is the potential to generate a cross-clade protective immune response[478]. To assess this possibility with rPIV5 vaccines, we tested immune sera against a second reverse genetics influenza virus expressing the HA and NA

from A/Anhui/01/2005 (clade 2.3.4) on a A/PR/8 backbone, rg A/Anhui-PR8 [479]. Sera from mice primed and boosted with rPIV5-H5, iA/VN/03/04, rg A/VN-PR8, and rg A/Anhui-PR8 were tested for the presence of neutralizing antibodies against rgA/VN-PR8 as well as rgA/Anhui-PR8. Similar to the control vaccinations, while titers were consistently higher against homologous virus, vaccination with ZL46 and ZL48 primed neutralizing antibody responses to the cross-clade virus (**figure 3.4c**). Thus, vaccination with rPIV5-H5 vaccines induces potent specific and cross-clade virus neutralizing titers.

Another benefit of using a live virus-vectored vaccine is the potential to prime cell-mediated immune responses in addition to antibody responses. To assess the T lymphocyte responses after PIV5 vaccination, BALB/c mice were immunized IN with PIV5, ZL46, or ZL48 or a sub-lethal dose of rg A/VN-PR8. On day 12 post-immunization, mice were euthanized and the mediastinal lymph nodes were isolated, re-stimulated with inactivated A/VN/1203/04 or irrelevant antigen, and assessed for IFN-γ producing lymphocytes by ELISpot assay. There was no significant response to iA/VN/03/04 restimulation by lymphocytes from PIV5-infected mice, however vaccination with either ZL46 or ZL48 primed a significant iA/VN/03/04-specific IFN- γ producing lymphocyte population (**figure 3.4d**). While the T cell response in rg A/VN-PR8 infected mice was about 4-fold greater than the response in ZL46-vaccinated mice, this is certainly attributable to the priming of additional immunodominant T cell responses to other influenza proteins (NP, M1, NA, etc), while the ZL46 and ZL48 responses are restricted to the HA protein alone.

In light of the HA-focused response, these data suggest that vaccination with rPIV5-H5 induces potent a cell-mediated immune responses.

Protection against HPAI H5N1 challenge in mice vaccinated with rPIV5-H5 To determine if vaccination with rPIV5-H5 ZL46 and ZL48 provides effective protection against highly pathogenic H5N1 (A/Vietnam/1203/04) infection, BALB/c mice were immunized IN with PIV5, ZL46, ZL48, or iA/VN/1203/04 IM. Twenty-eight days post-vaccination, mice were challenged with 10 LD_{50} influenza A/VN/1203/04 (IN), and monitored daily for morbidity and mortality. A subset of mice was euthanized on day 3 post-challenge and tissue collected to assess lung virus titers. Mice vaccinated with PIV5 lost nearly 30% of their pre-challenge body weight before succumbing to infection, whereas mice vaccinated with rPIV5-H5 ZL46, ZL48, or inactivated A/VN/1203/04 only lost 3-5% of their pre-challenge body weight (**figure 3.5a**). All mice vaccinated with rPIV5-H5 ZL46 or ZL48 survived, as did control animals vaccinated with inactivated A/VN/1203/04, while 100% of PIV5-immunized control mice succumbed to the infection or had to be euthanized (**figure 3.5b**). Similarly, there was no detectable virus in the lungs of mice vaccinated with ZL46, ZL48, or inactivated A/VN/1203/04 on day three post-challenge, whereas mice vaccinated with wild-type PIV5 had high titers of virus (**figure 3.5c**). This confirms that a single immunization with rPIV5-H5 is effective at protecting against H5N1 HPAI challenge at a level comparable to high-dose (256 HAU) vaccination with inactivated A/VN/1203/04 virus.

Antibody produced in rPIV5-H5 vaccinated mice confers protection against H5N1 HPAI HA-based influenza vaccines, including vaccines against HPAI H5N1 viruses are generally considered to protect against infection by induction of HA-specific neutralizing antibody responses [130, 212]. We found that while PIV5-H5 vaccines induce potent, HA-specific neutralizing antibody responses, vaccination also primed robust T cell responses. Thus it was unclear whether the serum antibody response was sufficient to protect against H5N1 challenge. To test this, immune serum was collected from mice immunized with PIV5, ZL46, ZL48 , or a sub-lethal dose of rg A/VN-PR8 IN, or the equivalent of 256 HAU inactivated A/VN/1203/04 IM. IgG was purified from serum and quantified. Two-hundred μg of purified IgG was transferred to individual BALB/c mice intraperitoneally (IP) and the following day, the mice were challenged with 10 LD_{50} HPAI A/Vietnam/1203/04. Following challenge, mice were monitored daily for morbidity and mortality and a subset of mice was euthanized on day 3 post-challenge and tissues collected to assess lung virus titers. Mice given IgG from control PIV5-vaccinated mice exhibited a steady decrease in body weight and were euthanized or succumbed to the infection by day 9 post-challenge. In contrast, mice receiving antibodies from ZL46-vaccinated mice were largely protected, losing only 10% of their body weight before recovering. Antibodies from ZL48-vaccinated mice delayed and reduced weight loss as compared to the PIV5 group, but protection was incomplete and all of the mice, except for one, succumbed to infection or had to be euthanized (**figure 3.6a, b**). As expected, mice receiving antibodies transferred from

the positive control mice (iA/VN/03/04 or rg A/VN-PR8) experienced limited weight loss and all survived. Influenza virus titers in the lungs reflected the morbidity and mortality data. Although not statistically significant, virus titers in the lungs of mice that received antibodies from ZL46-vaccinated mice were approximately one log lower than mice receiving antibodies from PIV5-vaccinated mice, while there were significant differences between the virus titers of mice receiving antibodies from A/VN-PR8 or iA/VN/03/04-vaccinated mice ($p<0.05$ and $p<0.01$, respectively) (**figure 3.6c**). Since protection was incomplete in some groups, we determined the quantity of neutralizing antibodies present per μg of IgG used for the passive transfer. Twice the amount of serum IgG from ZL48-vaccinated mice was required to neutralize 100 TCID50 of rg A/VN-PR8 as compared to ZL46-vaccinated mice (**figure 3.6d**). This lower amount of neutralizing IgG generated by a single immunization with ZL48 was insufficient to protect against the stringent HPAI challenge., however IgG generated by a single immunization with rPIV5-H5 ZL46was sufficient to protect against lethal HPAI H5N1 infection.

Discussion

There are a variety of live viral vectors being tested as influenza vaccine candidates at this time, including recombinant adenovirus[23, 360] and a number of NNSVs such as vesicular stomatitis virus (VSV)[407, 478] and Newcastle disease virus (NDV)[480]. It is important to find the balance between safety and immunogenicity, as there are specific risks associated with each (reviewed in [336]), for example, VSV is a highly neurotropic virus[481]. The NNSVs have a number of benefits over other vaccine vectors (reviewed in [25]) and among NNSVs, PIV5 has the greatest number, including genomic stability, the ability to replicate to high titer in vaccine approved cell lines (Vero), safety, and limited pre-existing immunity. Here we show that rPIV5 expressing the hemagglutinin from an H5N1 HPAI virus is safe (Fig. 3) and immunogenic in mice, inducing both virus neutralizing serum antibodies and Th1 T cell responses (Fig. 4). Moreover, a single immunization protects mice against a lethal challenge with the highly pathogenic virus A/VN/1203/04 (Fig. 5).

While HA-based vaccines are generally considered to confer antibody mediated protection, live-attenuated and viral vectored vaccines offer the opportunity to induce robust T cell responses, which can contribute to protection [227, 479, 482-488]. Immunization with rPIV5-H5 primed robust influenza virus-specific T cell responses, which produced IFN-γ upon restimulation with whole influenza virus (**figure 3.4d**). While the response was only 25% of the response seen in influenza-infected positive control mice, those animals responding to all of the

antigens in the virus, including the immunodominant nucleoprotein and other conserved antigens, suggesting that the HA-specific response primed by rPIV5-H5 was very robust.

Serum hemagglutination inhibiting (HI) antibodies are considered a correlate of protection for current licensed inactivated vaccines [173]. A four-fold increase in or minimum HI titer of 40 is generally considered to be protective [176]. While not established as a correlate of protection in humans, virus neutralizing antibody (VN) responses are also associated with protection [489]. A single immunization with rPIV5-H5 (ZL46) induced a neutralizing antibody titer of 1:100 (Fig 4b), which has been shown to be protective in mice and ferrets (unpublished results). A second immunization with rPIV5-H5 boosted neutralizing antibody responses, increasing VN titers for both ZL46- and ZL48- immunized mice (Fig. 4b). Moreover, sera from boosted mice contained cross-clade VN antibodies, neutralizing another H5N1 virus rg-Anhui-PR8 (A/Anhui/1/2005; Fig. 4d). Serum from immunized mice was collected, IgG purified, and transferred to naïve mice. Mice given 200μg of IgG from ZL46 immunized mice were completely protected from lethal challenge with A/VN/1203/04. Protection was comparable to protection seen with IgG from mice immunized with high-dose whole, inactivated virus or infected with rg/VN-PR8, where there would be antibodies to the neuraminidase and potentially other viral antigens, that could contribute to protection (i.e. M2 or NP [23] [215, 490]). Taken together, these results demonstrate that rPIV5-H5 primes potent serum VN antibody responses that are sufficient to protect against lethal

H5N1 HPAI challenge and meeting an established correlate of immunity for influenza vaccines.

As with other NNSVs, gene expression in PIV5 increases with relation to proximity to the leader sequence, which is the only de facto promoter of PIV5. Genes closest to the leader sequence are transcribed the most and this decreases in a stepwise manner as distance increases[491]. Therefore, insertion of the HA gene closer to the leader sequence has the potential to increase HA expression in an adjuvant-like fashion. To test this, we inserted the gene between the SH and HN genes (rPIV5-ZL46) as well as between the HN and L genes (rPIV5-ZL48) (**figure 3.1a**) and observed an increase in HA expression greater than three-fold when the gene was inserted between SH and HN (**figure 3.1d**). ZL46 and ZL48 proved to be equally as efficacious in our HPAI H5N1 challenge (**figure 3.5**), however, when equal amounts of IgG from ZL46 or ZL48-vaccinated mice were transferred to naïve recipients, better protection was achieved by ZL46 (**figure 3.6c-d**). ZL46 generally induced higher numbers of IFN-γ T cells and serum IgG titers (Fig. 4) and a micro-neutralization assay performed on the purified IgG used for passive transfer showed that ZL46 immunization induced a higher quality of antibody with a greater amount of VN antibody per microgram of IgG (**figure 3.6a**). This confirms our hypotheisis that movement of the transgene closer to the leader sequence, increasing HA expression provides a boost in vaccine efficacy. It is possible, however, that a point mutation observed in the NP protein of ZL48 could be having an effect on downstream gene expression. It seems unlikely, as we have no indication that the

mutation is hindering expression of other PIV5 genes (**figure 3.1d, e**) or growth (**figure 3.2**). Moerover, we tested correctedZL48 that does not contain this mutation and found that while it is immunogenic and can provide protection from H5N1 challenge, it is inferior to ZL46, like the "mutant" ZL48 described here (data not shown).

The ability to "adjuvant" a vaccine antigen by moving a gene closer to the leader sequence would make PIV5 an especially attractive vector for vaccinating with conserved influenza antigens, such as M2 or NP. Improved expression of these antigens might enable the induction of heterosubtypic immunity with a single immunization, which would be appealing for use "off the shelf" in the face of a looming pandemic while matched vaccines were being generated [492]. As PIV5 does not induce apoptosis, further adjuvanting of the virus could be accomplished by creating a PIV5 mutant that induces apoptosis[30] [31] or increasing the interferon response [493].

Live-attenuated influenza vaccines have shown similar efficacy, albeit not as robust, however there are issues with use of live-attenuated influenza vaccines against potentially pandemic viruses as there is the potential for reassortment with circulating viruses (REF). For other virus-vectored vaccines, there is the potential for reversion to virulence of over-attenuation. rPIV5 cannot reassort with circulating influenza virus. We did not observe any pathology associated with PIV5-vaccination (**figure 3.3**) and there is no conclusive evidence that PIV5 causes disease in humans [438, 441, 494] or any animal with the possible exception of kennel

cough in canines, although it has not been confirmed [442, 495]. This, and the ability to grow for an extended period of time in cell culture without the induction of CPE[27], makes PIV5 an especially promising vaccine candidate.

Especially with the use of a live, replicating vaccine vector, it is crucial that safety be a factor in development. It has been shown previously that insertion of an HA into the genome of PIV5 does not enhance virulence of PIV5[24]. However, the use of an HA from a highly pathogenic strain of avian influenza as well as multiple insertion sites, one that could increase HA expression, prompted us to ensure that these rPIV5-H5 constructs are relatively benign. There was no observed increase in morbidity nor was there an increase in pathology in the lungs (**figure 3.3**). Interestingly, insertion of the HA seems to have reduced lung pathology compared to the parent PIV5 strain. This could be due to mild attenuation *in vivo*, which is further suggested by reduced replication both in the lungs (**figure 3.2b**) as well as *in vitro* (**figure 3.2a**). In light of concerns regarding inflammation in the lungs and the development of asthma, *in vivo* attenuation would be favorable, especially considering adequate protective efficacy is being achieved even with this attenuation.

Another potential safety issue associated with using the H5 from highly pathogenic avian influenza is the extremely remote possibility of recombination. The highly pathogenic phenotype of H5N1 avian influenza is dictated largely by the presence of a polybasic cleavage site in the HA[149]. To address concerns regarding possible, although extremely unlikely, recombination between rPIV5-H5 and a

circulating influenza virus, the polybasic cleavage site was removed from the HA (H5) and replaced with an avirulent sequence. In the event that a new recombinant influenza virus were to arise with this HA as a component, it would not be of the highly pathogenic phenotype. It is important to note that recombination in NNSVs, such as PIV5, is unlikely and has never been observed in nature[496].

There is evidence that some cross-reactivity exists between PIV5 and ubiquitous paramyxoviruses such as mumps and human parainfluenza virus 2[497-499]. It is possible that this cross-reactivity could reduce the efficacy of this vaccine. However, as neutralizing antibodies in mice do not seem to prevent PIV5 infection[32], it seems unlikely that cross-reactive antibodies to PIV5 in humans would prevent PIV5 vaccination. This is reinforced by our observation of a prime-boost effect in rPIV5-H5 vaccinated mice (**figure 3.4a, b**).

While there are a number of live recombinant virus vector vaccines in development, these results provide compelling evidence placing PIV5 above the other candidates. Previous work has shown PIV5 to be safe an effective at priming protective immune responses to seasonal influenza virus infection [24]. Here we confirm safety with a different recombinant gene, that of HPAI H5N1 influenza virus and show potent protective immunity conferred by virus neutralizing serum antibody. Moreover, we can enhance the immunogenicity by moving the recombinant gene closer to the leader sequence, increasing gene expression during infection. These results, combined with the appealing production profile and

extensive use by veterinarians demonstrating safety make rPIV5 a prime candidate as a virus-vectored vaccine for prevention of influenza virus infection.

Materials and Methods

Influenza viruses used include rg A/VN-PR8, rg A/Anhui-PR8 and A/Vietnam/1203/04 (H5N1). A/VN-PR8 and A/Anhui-PR8, provided by Rubin Donis (CDC) were propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48-72 hours. β-propiolactone (BPL)-inactivated A/Vietnam/1203/04 was provided by Richard Webby from St. Jude Children's Research Hospital. The HPAI virus was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 24 hours. All viruses were aliquoted and stored at -80°C. All experiments using live highly pathogenic H5N1 avian influenza viruses were reviewed and approved by the institutional biosafety program at the University of Georgia and were conducted in biosafety level 3, enhanced containment following guidelines for use of Select Agents approved by the CDC.

Mice Female 6 to 8 week old BALB/c mice (Charles River Labs, Frederick, MD) were used for all studies. Mouse immunizations and studies with BSL2 viruses were performed in enhanced BSL2 facilities in HEPA filtered isolators. Mouse HPAI infections were performed in enhanced BSL3 facilities in HEPA filtered isolators *following guidelines approved by the institutional biosafety program at the University of Georgia and for use of Select Agents approved by the CDC.* All animal studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia.

Cells Monolayer cultures of BSR-T7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 400 μg/ml G418. BHK cells were maintained in DMEM containing 10% FBS, 100 IU/ml penicillion, and 100 μg/ml streptomycin. Madin-Darby Bovine Kidney *(*MDBK) and Madin-Darby Canine Kidney (MDCK) cells were cultured in DMEM with 5% FBS, 5% L-glutamine, and an antibiotic/antimycotic solution (10,000 IU/ml penicillin, 10,000 ug/ml streptomycin, and 25ug/ml amphotericin B) (Cellgro Mediatech, Inc). VERO cells were cultured in Minimum Essential Medium (MEM) (Thermo/Hyclone) with 10% FBS and antibiotic/antimycotic. All cells were incubated at 37°C, 5% CO_2.

Construction of recombinant viruses Two recombinant PIV5 plasmids containing HA gene, ZL46 (rPIV5-H5-SH/HN) and ZL48 (rPIV5-H5-HN/L) were generated. To generate ZL46 plasmid, the plasmid BH276 containing full length genome of PIV5 was used as the vector. The unique NgoMIV site in the 5' UTR of HN gene was used for cloning. Because there is a NgoMIV site in HA gene, we introduced the compatible XmaI site into the primers. The gene end (GE), intergenic region and gene start (GS) sequence between SH and HN gene was added into the primer to stop HA gene transcription and start HN gene transcription. The primers pZL137 (5'-CATTGCCCGGGATGGAAAAAATCGTGCTGCTGTT-3') and pZL138 (5'-GCACACCCGGG**GTGTTCGGGCCTATTTTTTCTTTAAAA**TCAAATGCAAATCCTGCACTGC AGGCTTC-3') were applied to amplify HA gene, XmaI site in the two primers were underlined, the GE/GS set between SH and HN gene was in bold. To generate ZL48

plasmid, the plasmid BH311 containing full length genome of PIV5 and an extra EGFP gene insertion between HN and L gene was used as the vector. Two NotI sites flanking the EGFP gene was used for cloning. The primers pZL139 (5'-ATCAGTAGCGGCCGCATGGAAAAAATCGTGCTGCTGTT-3') and pZL141 (5'-ATCAGTAGCGGCCGCATCAAATGCAAATCCTGCACTGCAGGCTTC

-3') were applied to amplify HA gene, NotI sites in the two primers were underlined.

Virus rescue and sequencing The plasmids, pZL46 encoding full length genome of PIV5 with HA gene insertion between SH and HN gene, pZL48 encoding full length genome of PIV5 with HA gene insertion between HN and L gene, and three helper plasmids pPIV5-N, pPIV5-P, and pPIV5-L encoding N, P, and L proteins, were co-transfected into BSR-T7 cells at 95% confluency in 6-cm plates with Plus and Lipofectamine (Invitrogen). The amounts of plasmids used were as follows: 5 μg pZL46/pZL48, 1 μg pPIV5-N, 0.3 μg pPIV5-P, and 1.5 μg pPIV5-L. After 3h incubation, the transfection media was replaced with DMEM containing 10% FBS and 10% TPB. After 72h incubation at 37°C, the media were harvested, and cell debris was pelleted by low speed centrifugation (3,000 rpm, 10 min). Plaque assays on BHK cells were used to purify the single clone of recombinant viruses.

The full length genome of plaque-purified single clone of ZL46 and ZL48 viruses were sequenced. Total RNAs from ZL46 and ZL48 viruses-infected MDBK cells were purified using the RNeasy kit (Qiagen Inc, Valencia, CA). cDNAs were prepared using

random hexamers and aliquots of the cDNA were then amplified in PCR reactions using appropriate oligonucleotide primer pairs.

PIV5 and rPIV5 virus stocks were grown in MDBK cells (<p20) for 5-7 days in DMEM containing 2% FBS until their hema-adsorption titers plateaued. Media was collected and clarified by centrifuging at 3000 rpm for 10 minutes in an Eppendorf tabletop centrifuge (5810 R). Bovine serum albumin (BSA) was added to the clarified supernatant to bring the total solution to 1% BSA. The virus stocks were then aliquoted and frozen quickly in dry ice and stored at -80°C. Virus titers were then determined by plaque assay on VERO cells (described below).

Virus Quantitation: PIV5 titers were determined by plaque assay on VERO cells. VERO cells were incubated with serial dilutions of virus samples made in DMEM with 1% BSA and antibiotic/antimycotic. Virus sample was then removed and overlayed with 1:1 low-melt agarose and DMEM with 2% FBS and antibiotic/antimycotic and incubated at 37°C for 5-6 days. To detect plaques, the monolayers were then fixed with 10% buffered formalin and immunostained. Cells were permeabilized with 1X PBS with 2% FBS, 0.1% sodium azide, and 0.5% saponin (permeabilization buffer). PIV5 was detected using a 1:1000 dilution of antibodies specific to the shared region of the V and P proteins of PIV5 (V/P) for 1hr. Horseradish peroxidase (HRP)-tagged goat-anti-mouse IgG (H&L) secondary antibody (Invitrogen) was then added and incubated for 30min. To visualize plaques, TMB peroxidase substrate (prepared according to manufacturer's instructions) was added (Vector Labs, Inc). The plates were then washed and dried

and the plaques were counted. Influenza titers were determined either by $TCID_{50}$ assay as previous described [227] or by plaque assay on MDCK cells. MDCK cells were incubated for 2 hours at 37°C with serial dilutions of virus samples made in MEM with 1 mg/ml *TPCK*-treated trypsin (Worthington Biochemical). Diluted virus samples were then removed and monolayers were overlayed with 1.2% microcrystalline cellulose Avicel[500] with 1 mg/ml TPCK-treated trypsin. Plates were incubated for 72 hours, the overlay gently washed off with PBS, fixed with cold methanol/acetone (40:60%), air-dried, counter-stained with crystal violet, and plaques visualized.

Fluorescence VERO cells were grown in 24-well plates and infected with PIV5, ZL46, or ZL48 at a multiplicity of infection (MOI) of 5 PFU/cell. At 24 hour post-infection, cells were fixed with 5% buffered formalin for 10min at room temperature. Cells were then permeabilized with permeabilization buffer and then incubated for 1hr with a 1:1000 dilution (1μg/ml) of anti-HA (H5) A/VN/1203/04 monoclonal antibody (BEI Resources). A 1:250 dilution of PE goat anti-mouse Ig (BD Pharmingen) was applied for 45min to detect HA. To detect PIV5, V/P-specific antibodies (diluted 1:1000) were then added and incubated for 1hr. To visualize PIV5, an Alexa Fluor-488-labeled secondary antibody (Invitrogen), diluted 1:500, was added and incubated for 30min and then washed. 0.5mL PBS was added to each well and fluorescence was examined using an AMS EVOS fl fluorescent microscope. Cells were washed extensively between each step with PBS.

Western Blot VERO cells were infected with an MOI of 5 PFU/cell of PIV5, ZL46, and ZL48. Cells were lysed using PBS with 2mM *ethylenediaminetetraacetic acid (EDTA), Roche Complete Mini protease inhibitor (Roche Applied Science), and 1% Triton-X-100 (octyl phenoxy polyethoxyethanol)(Sigma)* 24 hours post-infection. Separation and western blotting was performed as described [501]. Hyper-immune serum from rg A/VN-PR8-infected mice was used as a primary antibody to detect HA and V/P-specific monoclonal antibodies were used to detect V/P. Precision Plus Protein WesternC (BioRad) was used as a standard. Band density was assessed using ImageQuant™ TL software.

Dynamic light scattering (DLS) DLS was performed as described[477]. Anti-HA (H5) A/VN/1203/04 (BEI resources) was used. IgG was purified from serum using a *NAb Protein G Spin Kit (Thermo) according to manufacturer* instructions. Purified IgG was then desalted using zeba spin desalting columns (Thermo) according to manufacturer instructions. Desalted IgG was then concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore) according to manufacturer instructions to a final volume of approximately 2mLs. Protein was quantified using a Pierce BCA (bicinchoninic acid) Protein Assay kit (Thermo) according to manufacturer instructions. PIV5, ZL46, ZL48, rg A/VN-PR8, virus culture supernatant, allantoic fluid, and PBS were assayed.

Virus growth in vitro Virus diluted to a MOI of (0.01 PFU/cell) in DMEM with 1% BSA and antibiotic/antimycotic was added to a confluent monolayer of MDBK cells. The plate was incubated for 1-2 hours at 37°C. Diluted virus was then

removed, the monolayer rinsed with 1X PBS, and replaced DMEM with 1% BSA and antibiotic/antimycotic. Samples of supernatant were collected every 24 hours up to seven days, beginning immediately after fresh medium was placed on the monolayers. Supernatant was quickly frozen in dry ice and stored at -80°C. Virus titer was determined by plaque assay on VERO cells for each time point.

Immunization For vaccination with PIV5 and rPIV5-H5, 10^6 PFU PIV5, rPIV5-ZL46, or rPIV5-ZL48 in 50µl PBS was administered intranasally to mice anesthetized with *2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co).* For sub-lethal rg A/VN-PR8 infection, 2000 PFU virus in 50µl PBS was administered as described for PIV5 vaccination. For BPL inactivated A/VN/1203/04 vaccination, the approximate equivalent of 256 hemagglutination units (HAU)/ml in 50µl PBS was then injected into each of the caudal thigh muscles. Blood was collected on day 21 post-immunization. If boosted, this process was repeated on day 28 post-prime. Mice were monitored daily and, for some experiments, body weights recorded every other day.

Virus growth in vivo 8 week-old BALB/c mice were vaccinated as described above and groups were euthanized on days 1, 3, 6, and 8. Lungs were collected and homogenized in 1.0ml PBS and homogenates cleared by centrifugation. Cleared homogenate was then aliquoted and frozen at -80°C. Cleared homogenate was then assayed for virus titer by plaque assay on VERO cells as described above. 2-way ANOVA with Bonferroni post-test was used to compared ZL46 and ZL48 to PIV5 using Graphpad Prism.

Histopathology Mice were vaccinated with PIV5, ZL46, and ZL48 as described above. Lungs were removed on days 6 and 13 post-immunization, fixed in 10% buffered formalin, sectioned, embedded in paraffin, and sectioned at 4 microns. The sections were then deparaffinized and rehydrated before being stained with hematoxylin and eosin staining. Inflammation was scored by an AVCP board certified veterinary pathologist who was not aware of the treatment groups. Scores of PIV5, ZL46, and ZL48 were compared to PIV5 and compared between construct groups at each time point by two-way RM ANOVA.

ELISA HA (H5)-specific serum antibody titers were measured using an IgG ELISA. Immulon 2 HB 96-well microtiter plates (*ThermoLabSystems)* were coated with 2 μg/ml recombinant H5 protein and incubated at 4°C overnight. Plates were then washed with KPL wash solution (KPL, Inc) and the wells blocked with 200 μl KPL Wash Solution with 5% non-fat dry milk and 0.5% BSA (blocking buffer) for 1hr at room temperature. Serial dilutions of serum samples were made (in blocking buffer) and transferred to the coated plate and incubated for 1hr. To detect bound serum antibodies, 100μl of a 1:1000 dilution alkaline phosphatase-labeled goat anti-mouse IgG (KPL, Inc) in blocking buffer was added per well and incubated for 1hr at room temperature. Plates were developed by adding 100μl pNPP phosphatase substrate (KPL, Inc) per and the reaction allowed to develop at room temperature. Optical density (OD) was measured at 405 nm on a Bio-Tek Powerwave XS plate reader. The IgG titer was determined to be the lowest serum dilution with an OD greater than the mean OD of naïve serum plus 2 standard deviations.

Microneutralization Assay Influenza neutralizing antibody titers were measured in serum by a micro-neutralization assay with an ELISA endpoint. Heat inactivated serum was serially diluted in DMEM with 1% BSA, antibiotic/antimycotic , and 1 μg/ml TPCK trypsin. Diluted serum was then incubated 1000 $TCID_{50}$ rg A/VN-PR8 or rg A/Anhui-PR8 for two hours at 37°C. MDCK cells were then added and incubated at 37°C for 18-24 hours. At the end of the incubation, wells were fixed with ice cold methanol and acetone (80:20 respectively) and an ELISA was performed as described above. The neutralization titer was determined to be the lowest serum dilution capable of neutralizing 1000 $TCID_{50}$ rg A/VN-PR8 or A/Anhui-PR8, as determined by any OD readout higher than the following: ((mean OD of virus only wells-mean OD of cell only wells) divided by (2+mean OD of cell only wells)).

Lymphocyte Harvest 10 days post-vaccination with PIV5, ZL46, ZL48, or rg A/VN-PR8, mediastinal lymph nodes (MLN) from mice were harvested, pooled, and homogenized. Lymphocytes were depleted of erythrocytes using Gey's Balanced Salt solution (Sigma-Aldrich) for 5min at room temperature and debris removed. Cells were then counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter).

Enzyle-linked Immunosorbert Spot (ELISpot) Assay ELISpot to detect T-cell responses in lymphocytes to inactivated A/VN/1203/04 were performed as described [23]. *Cells were re-stimulated with inactivated A/VN/1203/04 (the equivalent of 10 HAU per well), Ebola GP P2 EYLFEVDNL as an irrelevant peptide*

(1µg/ml), and Concanavalin A (2µg/ml) in 50µl Complete Tumour Medium (CTM). Spots were *counted using AID ViruSpot Reader (Cell Technology, Inc).*

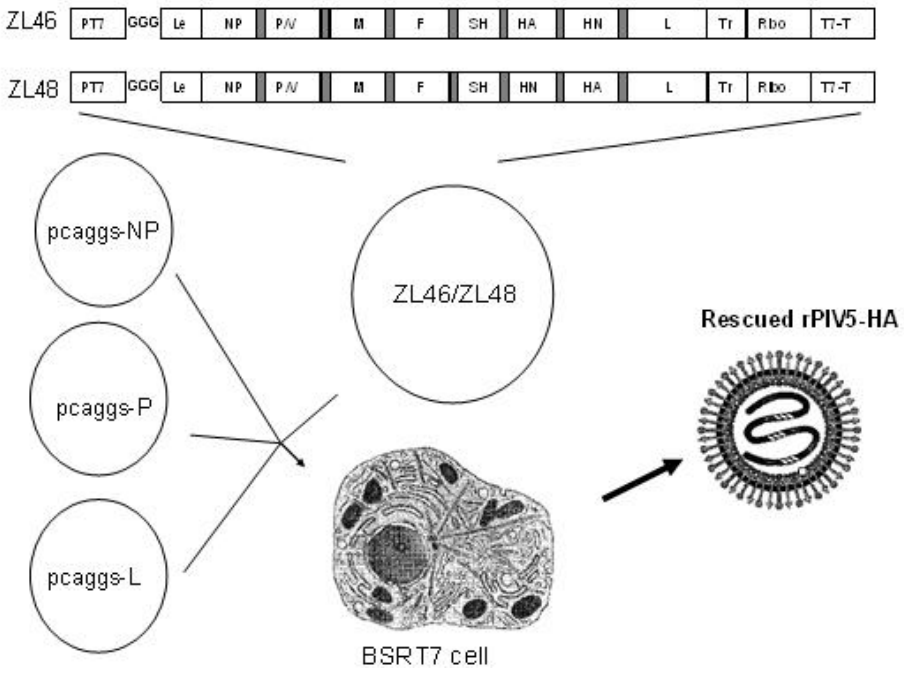
HPAI A/Vietnam/1203/04 (H5N1) challenge experiments BALB/c mice were first vaccinated with wild type PIV5, rPIV5-ZL46, rPIV5-ZL48 IN, or inactivated A/VN/1203/04 IM as described above. 21 days post-vaccination, the mice were bled for serum analysis via the tail vein. On day 24 post-vaccination, mice were anesthetized and inoculated intranasally with 10 LD_{50} A/Vietnam/1203/04 diluted in 50µl PBS. Mice were then monitored daily for morbidity and mortality with body weights measured every other day. On day 3 post-challenge, groups of mice were euthanized and their lungs collected into 1.0ml PBS and homogenized. Homogenate was then cleared by centrifugation. A **$TCID_{50}$ assay** was then used to determine virus titers in cleared homogenate as described [227].

Generation, purification, quantification of hyper-immune serum To generate hyper-immune serum, mice were vaccinated with PIV5, ZL46, ZL48, rg A/VN-PR8, and iA/VN/1203/04 as described above. Mice were boosted on day 28 and serially bled. IgG was purified from serum using a *NAb* Protein G Spin Kit (Thermo) according to manufacturer instructions. Purified IgG was then desalted using zeba spin desalting columns (Thermo) according to manufacturer instructions. Desalted IgG was then concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore) according to manufacturer instructions to a final volume of approximately 2.0mLs. Protein was quantified using a Pierce BCA (bicinchoninic acid) Protein Assay kit (Thermo) according to manufacturer instructions.

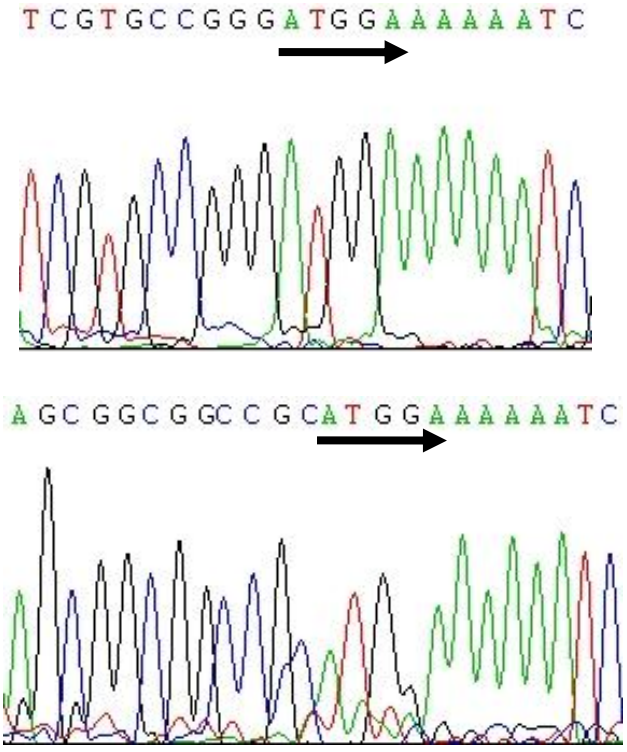
Passive antibody transfer: 200μg purified IgG from PIV5, ZL46, ZL48, iA/VN/03/04, and rgA/VN-PR8 vaccinated mice was administered intraperitoneally to naïve mice. Mice were challenged with HPAI A/Vietnam/1203/04 the day after transfer. Challenge was performed as described. Groups of mice were euthanized on day 3 for assessment of lung virus titers and the remaining mice were monitored as described. Lung virus titers were analyzed using one-way ANOVA followed by pair-wise comparison using Bonferroni's post-test.

Statistical Analyses were performed using GraphPad Prism®.

A


ZL46
PT7
GGG
Le
NP
P/V
M
F
SH
HA
HN
L
Tr
Rbo
T7-T
ZL48
PT7
GGG
Le
NP
P/V
M
F
SH
HN
HA
L
Tr
Rbo
T7-T
pcaggs-NP
pcaggs-P
pcaggs-L
ZL46/ZL48
BSRT7 cell
Rescued rPIV5-HA

B


T C G T G C C G G G A T G G A A A A A A T C
A G C G G C G G C C G C A T G G A A A A A A T C

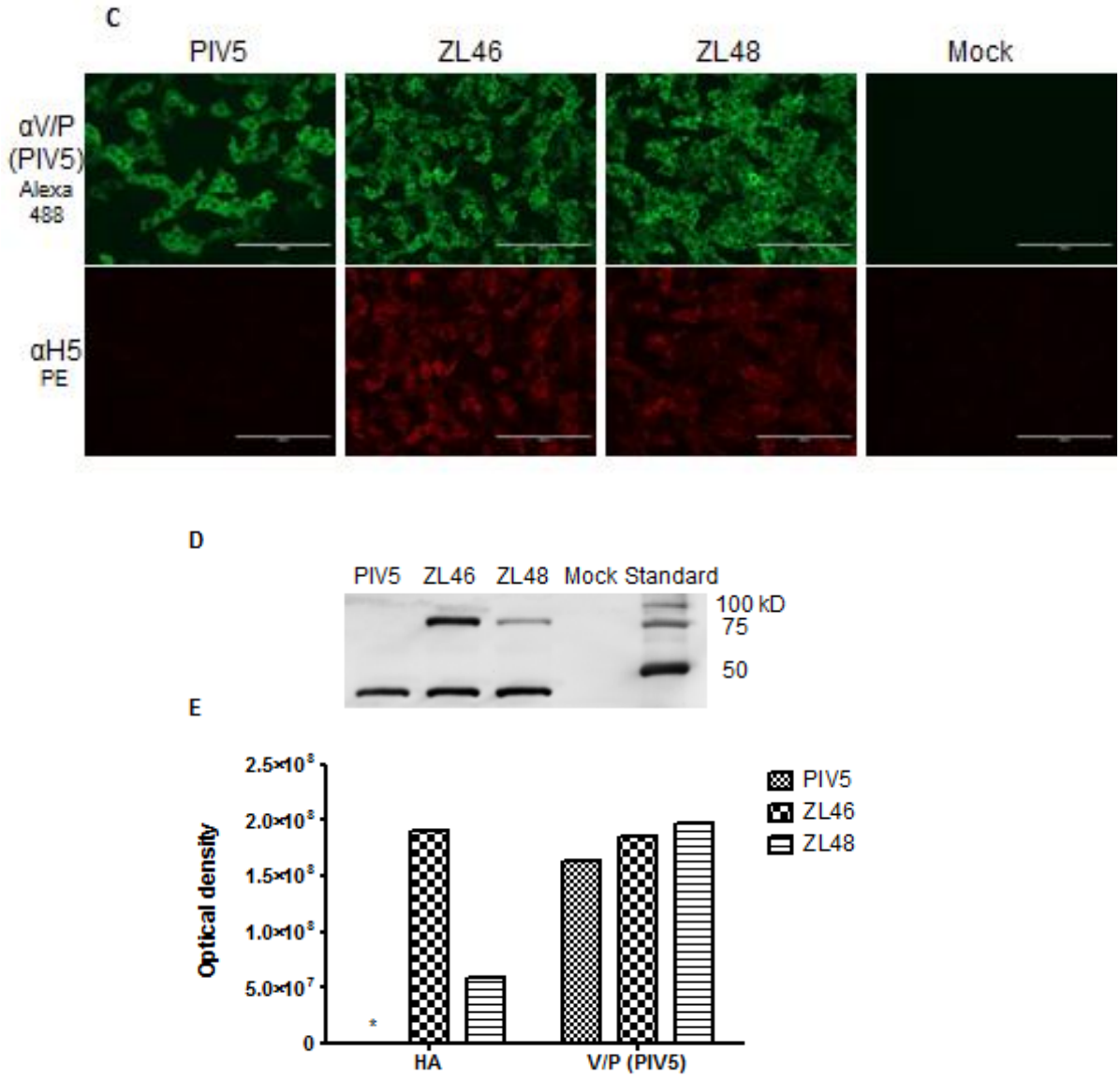


Figure 3.1: Recovery of rPIV5-H5 viruses A. Schematic diagram of the rescue of infectious PIV5 containing HA gene from its cDNA clone. Plasmid pZL46 encoding full length genome of PIV5 with HA gene insertion between SH and HN gene, pZL48 encoding full length genome of PIV5 with HA gene insertion between HN and L gene that was flanked by a bacteriophage T7 RNA polymerase (RNAP) promoter (PT7) and a hepatitis delta virus ribozyme followed by a T7 transcriptional terminator (T7-T). The plasmids contain three extra G residues after the T7 RNAP promoter and prior to the PIV5 leader (Le) sequence to increase T7 RNAP transcription initiation efficiency. A full-length anti-genome sense PIV5 with HA gene insertion RNA transcript can be transcribed from the T7 RNAP promoter with the exact trailer sequence generated by cleavage with hepatitis delta virus ribozyme. The filled boxes

indicate the seven intercistronic regions. The plasmids pCAGGS-NP, pCAGGS-P, and pCAGGS-L each contain the cDNA for the PIV5 NP, P, and L proteins, respectively. BSR-T7 cells were co-transfected with pZL46 or ZL48, and the three helper plasmids pCAGGS-NP, pCAGGS-P, and pCAGGS-L. The transfection media were replaced with DMEM containing 10% FBS and 10% TPB after 3 hr incubation and the cultures were incubated for 72 hr. PT7, T7 RNA polymerase promoter; Le, leader sequence of PIV5; Tr, trailer sequence of PIV5; Ribo, ribozyme from hepatitis delta virus; T7-T, bacteriophage T7 RNA polymerase terminator. (**B)** Sequencing results of HA gene in ZL46 and ZL48 viruses. ZL46 and ZL48 viruses were rescued. The whole genomes of ZL46 and ZL48 viruses were sequenced using appropriate oligonucleotide primers. The sequencing results of HA gene in ZL46 and ZL48 viruses were shown. The arrows indicated the ORF of HA gene**. (C)** Immunofluorescence of VERO cells infected with PIV5, ZL46, and ZL48. At 24 hours post-infection, cells were fixed and stained with anti-H5 (red) and anti-V/P (green) antibodies. Micrographs were taken at 20x magnification and the scale bar is representative of 200μm. **(D)** VERO cells infected with PIV5, ZL46, ZL48 (MOI=5) were lysed, separated on SDS-PAGE gel, transferred to PVDF, and blotted with a monoclonal antibody specific to the V/P proteins of PIV5 and hyper-immune serum from mice infected with rg A/VN-PR8 to detect HA. Sizes (kDa) and positions of HA and V/P are indicated (D). Using ImageQuant software, densitometry analysis was performed on the bands. Data represents relative volume of the bands **(E)**. No HA band was detectable at 75 kD in PIV5-infected VERO cells (*).

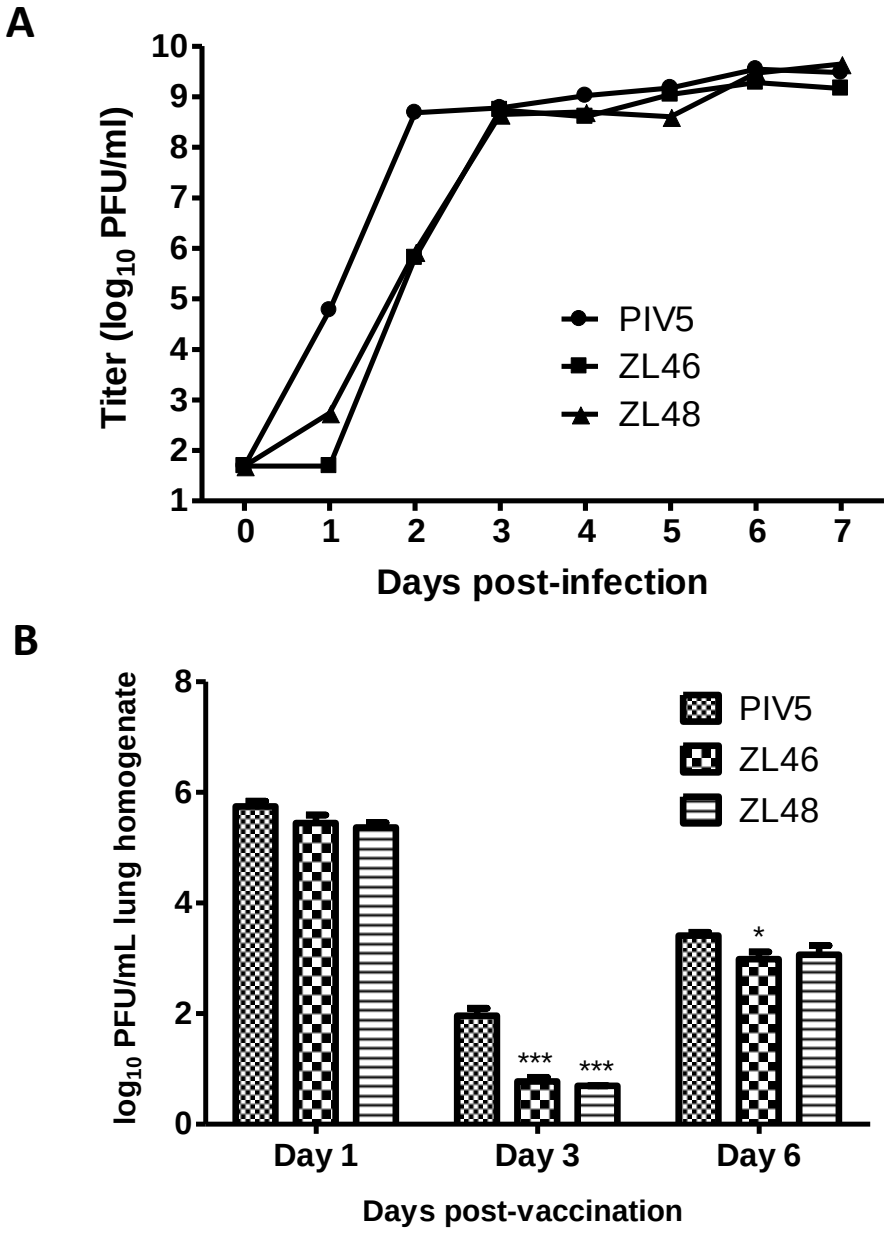


Figure 3.2: Growth of rPIV5-H5 *in vitro* and *in vivo*

(A) MDBK cells were infected with an MOI of 0.01 PFU/cell. Samples of supernatant were collected at 24 hour intervals up to 7 days post-infection. Viral titers in the media were determined via plaque assay on VERO cells. **(B)** BALB/c mice were infected with 10^6 PFU PIV5, ZL46, or ZL48 IN. Groups of mice were euthanized for determination of lung virus titer by plaque assay on days 1, 3, 6, and 8. No virus was detectable in the lungs on day 8 (data not shown). 2-way ANOVA with Bonferroni post-test comparing ZL46 and ZL48 to PIV5. Significance is indicated (*$p<0.05$;*** $p<0.001$).

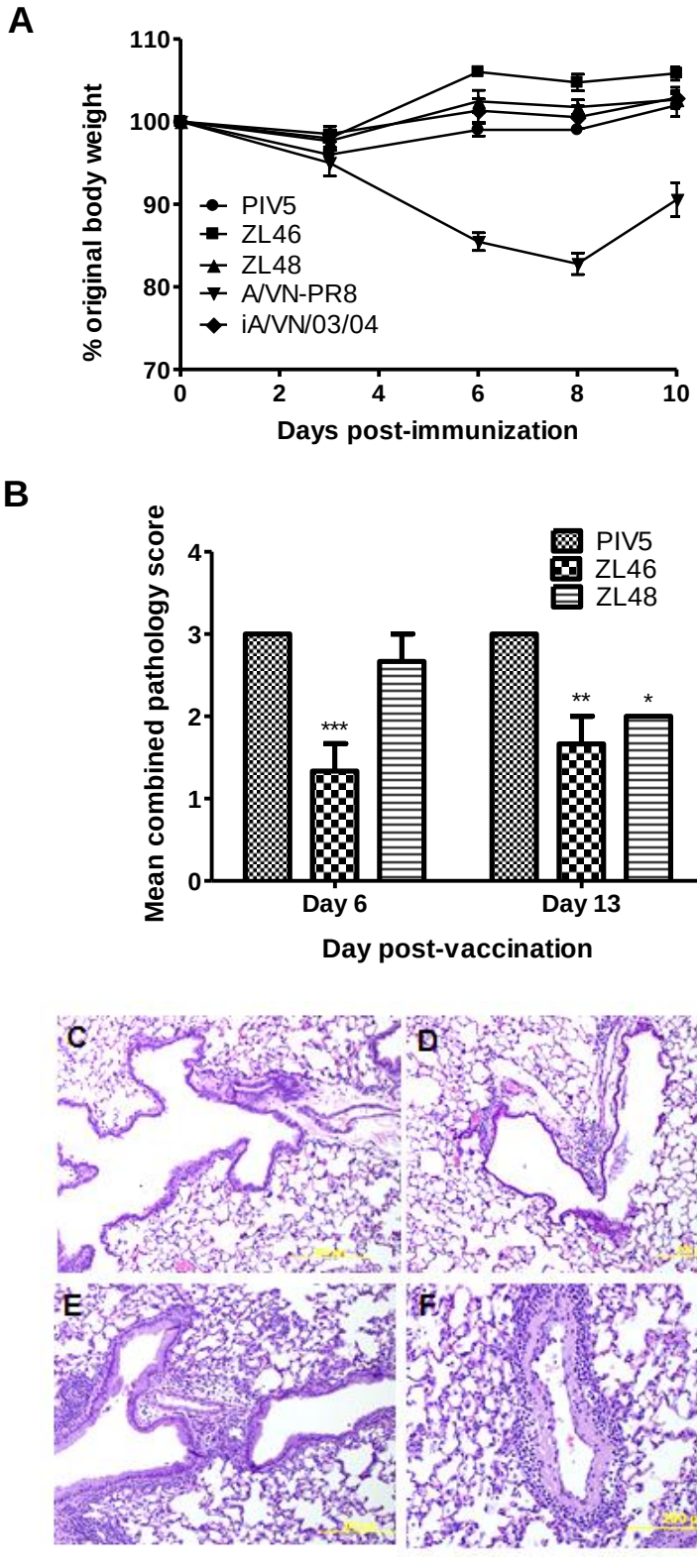
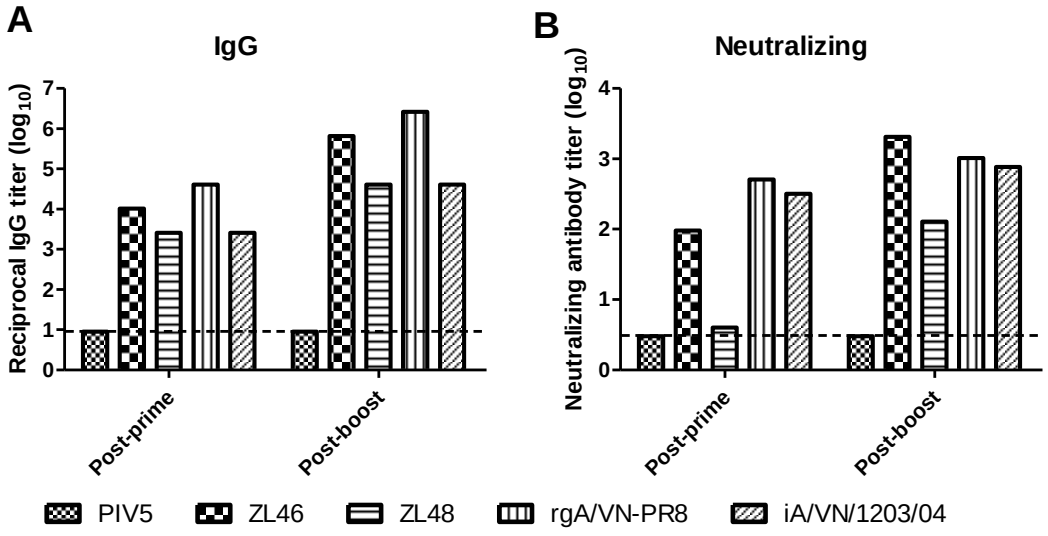
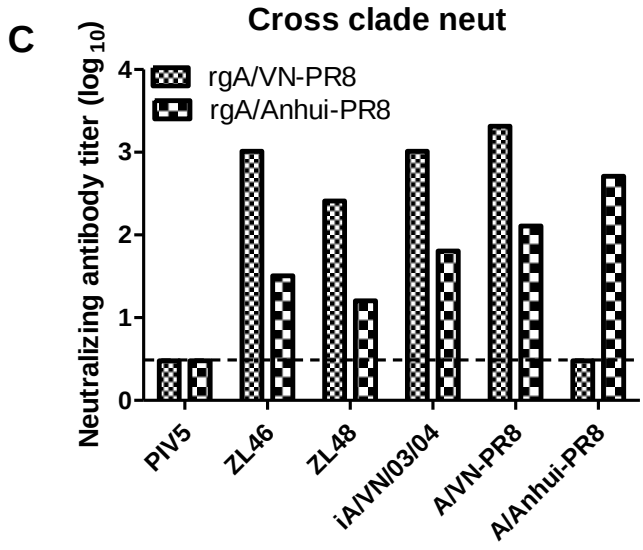

A
% original body weight
110
100
90
80
70
0
2
4
6
8
10
Days post-immunization
PIV5
ZL46
ZL48
A/VN-PR8
iA/VN/03/04
B
Mean combined pathology score
4
3
2
1
0
***
**
*
Day 6
Day 13
Day post-vaccination
PIV5
ZL46
ZL48
C
D
E
F

Figure 3.3: Pathology induced by vaccination with rPIV5-H5 in mice
BALB/c mice (n=5) were immunized with 10^6 pfu PIV5, ZL46, ZL48, 2000 pfu rg A/VN-PR8 i.n., and the equivalent of 512 HAUs inactivated A/VN/1203/04 IM. Weights of the mice were monitored and presented as the mean percentage ± SEM of their pre-challenge body weights (n=5). (B) BALB/c mice (n=3) were infected with PIV5, ZL46, or ZL48 (10^6 PFU in 50 µl PBS). Animals were euthanized on days 6 and 13 p.i. and their lungs removed and fixed. Lungs were sectioned, H&E stained, and analyzed. For each animal, levels of pulmonary infiltrate were subjectively scored 1-3 with 1=normal (as compared to mock infected) 2=mild and 3=medium infiltration and total scores determined. Presented are means of the total scores ± SEM for each group. Scores of PIV5, ZL46, and ZL48 were compared to PIV5 and compared between construct groups at each time point by two-way RM ANOVA and statistical significance is indicated by asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). (C-F) Photographs of stained lung sections (C) Representation of score 1=normal (mock day 6) (D) Rep. of score 2=mild (ZL46 day 13) € Rep. of score 3=medium (PIV5 day 6) (F) Perivascular infiltrates (score 3=medium)(PIV5 day 6)


A
IgG
Reciprocal IgG titer (log10)
0
1
2
3
4
5
6
7
Post-prime
Post-boost
B
Neutralizing
Neutralizing antibody titer (log10)
0
1
2
3
4
Post-prime
Post-boost
PIV5
ZL46
ZL48
rgA/VN-PR8
iA/VN/1203/04


C
Cross clade neut
Neutralizing antibody titer (log10)
rgA/VN-PR8
rgA/Anhui-PR8
0
1
2
3
4
PIV5
ZL46
ZL48
iA/VN/03/04
A/VN-PR8
A/Anhui-PR8

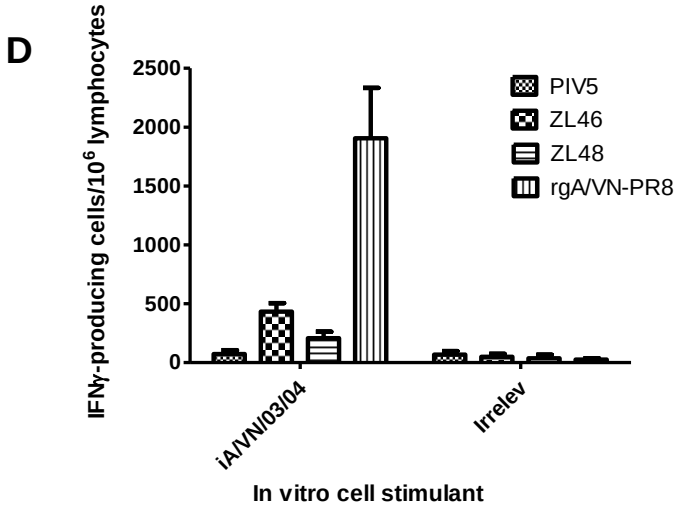

D
IFNγ-producing cells/10^6 lymphocytes
0
500
1000
1500
2000
2500
PIV5
ZL46
ZL48
rgA/VN-PR8
iA/VN/03/04
Irrelev
In vitro cell stimulant

Figure 3.4: Humoral and cell-mediated immune responses to immunization with rPIV5-H5 in mice

BALB/c mice (n=5 per group) were immunized with PIV5, ZL46, ZL48, a sub-lethal dose of rg A/VN-PR8, and with iA/VN/03/04. Mice were bled on day 21 post-immunization, boosted on day 28, and bled on day 21 post-prime. Serum was pooled for analysis. (A) HA (H5) specific antibody titers were measured in serum samples using an IgG (H&L) specific ELISA. 9 represents the limit of detection (B) rg A/VN-PR8-neutralizing antibody titers in post-prime and post-boost serum and (C) rg A/VN-PR8- and rg A/Anhui-PR8-neutralizing antibody titers were measured by virus micro-neutralization assay. 3 represents the limit of detection. (D) IFN-γ producing lymphocytes (pools of n=3 mice per group) in the mediastinal lymph nodes on day 12 post-vaccination as determined by ELISpot analysis. Data is presented as mean $\pm$ SEM.

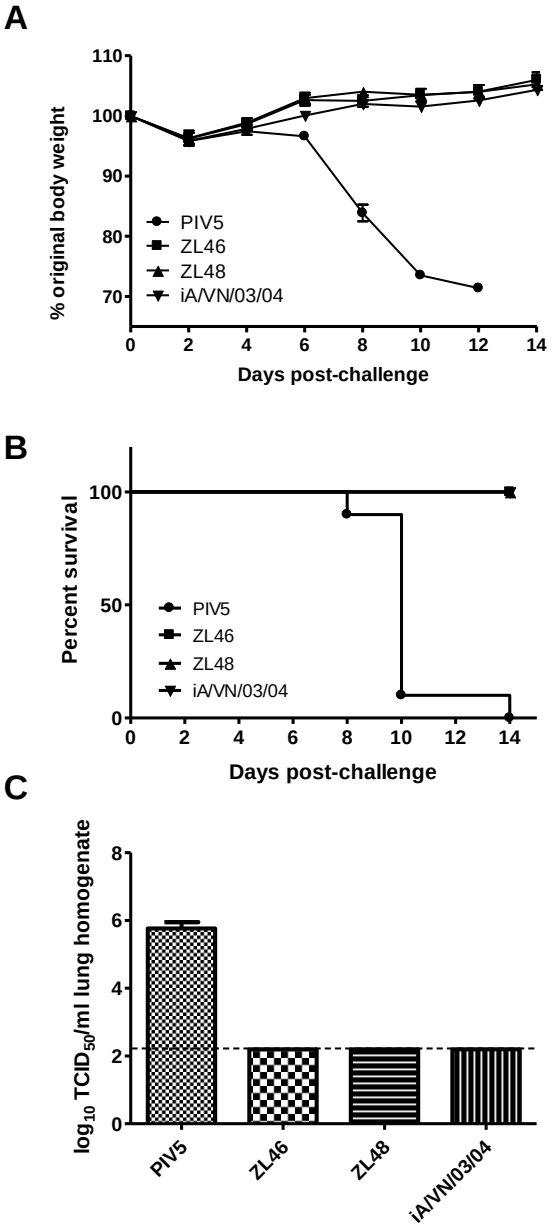


Figure 3.5: Efficacy of rPIV5-H5 as a vaccine vector (protection against HP A/VN/1203/04.

BALB/c mice were immunized IN or IM with rPIV5-H5 or inactivated A/VN/1203/04, respectively. 28 days p.i., the mice were challenged IN with 10 LD_{50} A/VN/1203/04. (A) Weights of the mice were monitored and presented as the mean percentage $\pm$ SEM of their pre-challenge body weights (n=9). (B) Percent of mice surviving post-challenge. (C) Challenge virus titer in the lungs on day 3 post-challenge (n=5 per group) as measured by $TCID_{50}$ on MDCK cells. Data are presented as mean log transformed $TCID_{50}$/ml lung homogenate $\pm$ SEM. The limit of detection was 100 $TCID_{50}$/ml.

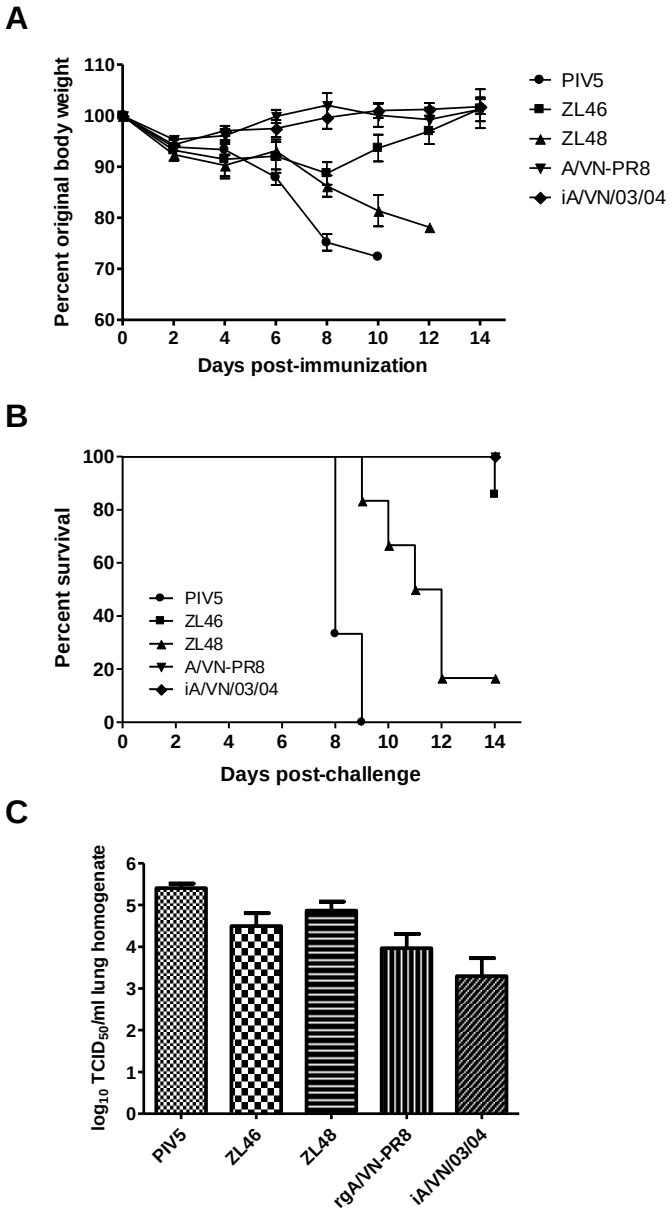


Figure 3.6: Antibody from rPIV5-H5 is sufficient for protection against HPAI H5N1
(A)The amount of neutralizing antibody per µg purified IgG from hyper-immune serum from BALB/c mice vaccinated with PIV5, ZL46, ZL48, rg A/VN-PR8, or iA/VN/03/04 was determined by microneutralization assay. Bars are representative of the reciprocal of ng IgG per ml required to neutralize 1000 TCID50 rgA/VN-PR8. 200ug of purified IgG from each group was transferred to a recipient mouse IP. The following day, the mice were challenged with 10 LD50 HPAI H5N1 A/VN/1203/04 and monitored for (B) weight loss and (C) mortality. On day 3 post-challenge, lungs were harvested and challenge virus determined via $TCID_{50}$ (D). Error bars are representative SEM. Data was analyzed using one-way ANOVA, followed by pair-wise comparison using Bonferroni's post-test.

References

1. Fiore, A.E. *Prevention and control of influenza. Recommendations of the advisory committee on immunization practices (ACIP)*. MMWR Recomm Rep, 2008. **57**, 1-60.
2. Lipatov, A.S., *Influenza: Emergence and Control.* Journal of Virology, 2004: p. 8951-8959.
3. WHO, *Pandemic (H1N1) 2009-update 100*, 2010, World Health Organization. p. 1-3.
4. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiological Reviews, 1992. **56**(1): p. 152-179.
5. Reid, A.H., J.K. Taubenberger, and T.G. Fanning, *Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus.* Nature Rev. Microbiol., 2004. **2**(15494747): p. 909-914.
6. Prevention, C.f.D.C.a., *Update: Isolation of Avian Influenza A(H5N1) Viruses From Humans—Hong Kong, 1997-1998.* JAMA: The Journal of the American Medical Association, 1998. **279**(5): p. 347-348.
7. Taubenberger, J.K., et al., *Characterization of the 1918 influenza virus polymerase genes.* Nature, 2005. **437**(16208372): p. 889-893.
8. WHO. *Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO*. Global Alert and Response (GAR) 2011 June 22, 2011; Available from: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_06_22/en/index.html.
9. Whitley, Richard J. and Arnold S. Monto, *Seasonal and Pandemic Influenza Preparedness: A Global Threat.* Journal of Infectious Diseases, 2006. **194**(Supplement 2): p. S65-S69.
10. Gerdil, C., *The annual production cycle for influenza vaccine.* Vaccine, 2003. **21**(16): p. 1776-1779.
11. Horimoto, T. and Y. Kawaoka, *Strategies for developing vaccines against H5N1 influenza A viruses.* Trends in Molecular Medicine, 2006. **12**(11): p. 506-514.
12. Lipatov, A.S., et al., *Efficacy of H5 Influenza Vaccines Produced by Reverse Genetics in a Lethal Mouse Model.* Journal of Infectious Diseases, 2005. **191**: p. 1216-1220.
13. Treanor, J.J., et al., *Safety and Immunogenicity of an Inactivated Subvirion Influenza A (H5N1) Vaccine.* New England Journal of Medicine, 2006. **354**(13): p. 1343-1351.
14. Centers for Infectious Disease and Research, C. *FDA panel supports H5N1 vaccine approval.* CIDRAP Pandemic Influenza News, 2007. 1-2.
15. Poland, G., I. Ovsyannikova, and R. Jacobson, *Immunogenetics of seasonal influenza vaccine response☆.* Vaccine, 2008. **26**: p. D35-D40.
16. Galli, G., et al., *Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted*

prepandemic vaccine. Proc Natl Acad Sci U S A, 2009. **106**(19416838): p. 7962-7967.

17. Subbarao, K., *Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics.* Virology, 2003. **305**: p. 192-200.
18. Chen, Z., et al., *Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein.* Journal of General Virology, 1999. **80**: p. 2559-2564.
19. Epstein, S.L., et al., *DNA Vaccine Expressing Conserved Influenza Virus Proteins Protective against H5N1 Challenge Infection in Mice.* Emerging Infectious Diseases, 2002. **8**(8): p. 796-801.
20. Tompkins, S.M., et al., *Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including Subtype H5N1.* Emerging Infectious Diseases, 2007. **13**(3): p. 426-435.
21. Tompkins, S.M., et al., *Recombinant parainfluenza virus 5 (PIV5) expressing the influenza A virus hemagglutinin provides immunity in mice to influenza A virus challenge.* Virology, 2007. **362**(1): p. 139-150.
22. Bukreyev, A., et al., *Nonsegmented Negative-Strand Viruses as Vaccine Vectors.* Journal of Virology, 2006. **80**(21): p. 10293-10306.
23. Arimilli, S., M.A. Alexander-Miller, and G.D. Parks, *A Simian Virus 5 (SV5) P/V Mutant Is Less Cytopathic than Wild-Type SV5 in Human Dendritic Cells and Is a More Effective Activator of Dendritic Cell Maturation and Function.* Journal of Virology, 2006. **80**(7): p. 3416-3427.
24. Choppin, P.W., *Multiplication of a Myxovirus (SV5) with Minimal Cytopathic Effects and without Interference'.* Virology, 1964. **23**: p. 224-233.
25. Cohn, M.L., et al., *T Cell responses to the paramyxovirus simian virus 5: studies in multiple sclerosis and normal populations.* Pathobiology, 1996. **64**(3).
26. Driskell, J.D., et al., *One-step assay for detecting influenza virus using dynamic light scattering and gold nanoparticles.* Analyst, 2011. **136**(15): p. 3083-3090.
27. Chatziandreou, N., et al., *Relationships and host range of human, canine, simian and porcine isolates of simian virus 5 (parainfluenza virus 5).* Journal of General Virology, 2004. **85**(10): p. 3007-3016.
28. Schwartz, J., et al., *Vesicular stomatitis virus vectors expressing avian influenza H5 HA induce cross-neutralizing antibodies and long-term protection.* Virology, 2007. **366**(1): p. 166-173.
29. Dong, J., et al., *Development of a new candidate H5N1 avian influenza virus for pre-pandemic vaccine production.* Influenza Other Respi Viruses, 2009. **3**(6): p. 287-95.
30. Nayak, B., et al., *Contributions of the Avian Influenza Virus HA, NA, and M2 Surface Proteins to the Induction of Neutralizing Antibodies and Protective Immunity.* Journal of Virology, 2009. **84**(5): p. 2408-2420.

31. Cox, N.J. and K. Subbarao, *Influenza.* The Lancet, 1999. **354**: p. 1277-1282.
32. Patel, A., S. Tikoo, and G. Kobinger, *A Porcine Adenovirus with Low Human Seroprevalence Is a Promising Alternative Vaccine Vector to Human Adenovirus 5 in an H5N1 Virus Disease Model.* Plos one, 2010. **5**(12): p. 1-11.
33. Roberts, A., et al., *Vaccination with a Recombinant Vesicular Stomatitis Virus Expressing an Influenza Virus Hemagglutinin Provides Complete Protection from Influenza Virus Challenge.* Journal of Virology, 1998. **72**(6): p. 4704-4711.
34. DiNapoli, J.M., et al., *Newcastle Disease Virus-Vectored Vaccines Expressing the Hemagglutinin or Neuraminidase Protein of H5N1 Highly Pathogenic Avian Influenza Virus Protect against Virus Challenge in Monkeys.* Journal of Virology, 2009. **84**(3): p. 1489-1503.
35. Suryanarayana, V.V.S., D. Chattopadhyay, and M.S. Shaila, *Non-segmented negative sense RNA viruses as vectors for vaccine development.* Current Science, 2010. **98**(3): p. 379-391.
36. Forger III, J.M., et al., *Murine infection by vesicular stomatitis virus: initial characterization of the H-2d system.* J Virol, 1991. **65**: p. 4950–4958.
37. Soboleski, M.R., et al., *Cold-Adapted Influenza and Recombinant Adenovirus Vaccines Induce Cross-Protective Immunity against pH1N1 Challenge in Mice.* Plos one, 2011. **6**(7): p. e21937.
38. Soboleski, M.R., et al., *Cold-adapted influenza and recombinant adenovirus vaccines induce cross-protective immunity against pH1N1 challenge in mice.* Plos one, 2011. **6**(7): p. e21937.
39. Price, G.E., et al., *Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses.* Plos one, 2010. **5**(10): p. e13162.
40. Price, G.E., et al., *Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses.* Vaccine, 2009. **27**(47): p. 6512-21.
41. Lo, C.Y., et al., *Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: cold-adapted vaccine versus DNA prime-adenovirus boost strategies.* Vaccine, 2008. **26**(17): p. 2062-72.
42. Lanthier, P.A., et al., *Live attenuated influenza vaccine (LAIV) impacts innate and adaptive immune responses.* Vaccine, 2011. **29**(44): p. 7849-56.
43. Basha, S., et al., *Comparison of antibody and T-cell responses elicited by licensed inactivated- and live-attenuated influenza vaccines against H3N2 hemagglutinin.* Hum Immunol, 2011. **72**(6): p. 463-9.
44. Subbramanian, R.A., et al., *Pandemic and seasonal H1N1 influenza hemagglutinin-specific T cell responses elicited by seasonal influenza vaccination.* Vaccine, 2010. **28**(52): p. 8258-67.
45. De Jong, J.C., et al., *Haemagglutinin-inhibiting antibody to influenza virus.* Dev Biol (Basel), 2003. **115**: p. 63-73.

46. Kendal, A.P., et al., *Concepts and procedures for laboratory-based influenza surveillance.* Atlanta, GA: US Department of Health and Human Services, CDC, 1982.
47. Hancock, K., et al., *Cross-Reactive Antibody Responses to the 2009 Pandemic H1N1 Influenza Virus.* New England Journal of Medicine, 2009. **361**(20): p. 1945-1952.
48. Feng, J., et al., *Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2.* Virology Journal, 2006. **3**(1): p. 102.
49. LaMere, M.W., et al., *Contributions of Antinucleoprotein IgG to Heterosubtypic Immunity against Influenza Virus.* The Journal of Immunology, 2011. **186**(7): p. 4331-4339.
50. Tokusumi, T., et al., *Recombinant Sendai viruses expressing different levels of a foreign reporter gene.* Virus Research, 2002. **86**(1-2): p. 33-38.
51. Epstein, S.L. and G.E. Price, *Cross-protective immunity to influenza A viruses.* Expert Review of Vaccines, 2010. **9**(11): p. 1325-1341.
52. Sun, M., et al., *Conserved Cysteine-Rich Domain of Paramyxovirus Simian Virus 5 V Protein Plays an Important Role in Blocking Apoptosis.* Journal of Virology, 2004. **78**(10): p. 5068-5078.
53. Lin, Y., et al., *Induction of Apoptosis by Paramyxovirus Simian Virus 5 Lacking a Small Hydrophobic Gene.* Journal of Virology, 2003. **77**(6): p. 3371-3383.
54. Sun, D., et al., *PLK1 down-regulates parainfluenza virus 5 gene expression.* PLoS Pathogens, 2009. **5**(7): p. 1-13.
55. Hsiung, G.D., *Parainfluenza-5 virus. Infection of man and animal.* Prog. Med. Virol., 1972. **14**: p. 241-274.
56. Vandvik, B. and E. Norrby, *Paramyxovirus SV5 and multiple sclerosis.* Nature, 1989. **338**: p. 769-771.
57. McCandlish, I.A., et al., *A study of dogs with kennel cough.* Vet. Rec., 1978: p. 293-301.
58. Cornwell, H.J., et al., *Isolation of parainfluenza virus SV5 from dogs with respiratory disease.* Vet Rec, 1976. **98**(15): p. 301-2.
59. Garten, W. and H. Klenk, *Cleavage Activation of the Influenza Virus Hemagglutinin and Its Role in Pathogenesis*, in *Avian Influenza. Monogr Virol.*, K. H, M. MN, and S. J, Editors. 2008, Basel, Karger. p. 156-167.
60. Spann, K.M., P.L. Collins, and M.N. Teng, *Genetic Recombination during Coinfection of Two Mutants of Human Respiratory Syncytial Virus.* Journal of Virology, 2003. **77**(20): p. 11201-11211.
61. Komada, H., et al., *Immunological relationships between parainfluenza virus type 4 and other paramyxoviruses studied by use of monoclonal antibodies.* Arch Virol, 1991. **116**: p. 277-283.
62. Randall, R.E. and D.F. Young, *Comparison between Parainfluenza Virus Type 2 and Simian Virus 5: Monoclonal Antibodies Reveal Major Antigenic Differences.* Journal of General Virology, 1988. **69**: p. 2051-2060.

63. Tsurudome, M., et al., *Extensive antigenic diversity among human parainfluenza type 2 virus isolates and immunological relationships among paramyxoviruses revealed by monoclonal antibodies.* Virology, 1989. **171**: p. 38-48.
64. Young, D.F., et al., *Clearance of a Persistent Paramyxovirus Infection Is Mediated by Cellular Immune Responses but Not by Serum-Neutralizing Antibody.* Journal of Virology, 1990. **64**(11): p. 5403-5411.
65. Matrosovich, M., et al., *New low-viscosity overlay medium for viral plaque assays.* Virology Journal, 2006. **3**(1): p. 63.
66. Gabbard, J., et al., *A humanized anti-M2 scFv shows protective in vitro activity against influenza.* Protein Engineering Design and Selection, 2009. **22**(3): p. 189-198.

CHAPTER 4

VACCINATION WITH RECOMBINANT PIV5 EXPRESSING NEURAMINIDASE PROVIDES HOMOLOGOUS AND HETEROLOGOUS PROTECTION AGAINST INFLUENZA VIRUS CHALLENGE

Alaina Jones Mooney, Zhuo Li, Jon D. Gabbard, Daniel A. Dlugolenski, Scott K. Johnson, Robert J. Hogan, Ralph A. Tripp, Elizabeth MI Uhl, Biao He, S. Mark Tompkins. To be submitted to the Journal of Infectious Diseases

Abstract

Highly pathogenic avian influenza viruses (HPAIV) are considered a potential pandemic threat. Vaccination is considered the most effective measure for controlling influenza. Current vaccination methods suffer from a number of drawbacks, including safety and production issues. Viral-vectored vaccines offer an egg-free alternative. Parainfluenza virus 5 (PIV5), a non-segmented, negative-stranded RNA virus (NNSV) in the family *Paramyxoviridae*, is a favorable vector candidate for a number of reasons, including a simple, stable, and well-understood genome, making it an ideal candidate for foreign gene insertion and vaccine use. Neuraminidase (NA) is a glycoprotein on the surface of the virus as well as virus-infected cells and is responsible for cleavage of sialic acid residues as the virus buds from the cell at the end of the replication cycle. NA is more conserved than HA, the current influenza vaccine target, which increases the likelihood of achieving broader protection if used as a vaccine antigen. In this study, we show that vaccination with rPIV5-N1 primes an NA-specific antibody response and T cell response and confers complete protection against homologous influenza virus challenge and significant cross-protection against heterologous influenza virus within the same subtype. There is also evidence of limited cross-protection against virus of a different subtype (H3N2).

Introduction

Highly pathogenic avian influenza viruses (HPAIV) are considered a potential pandemic threat. As of November 2011, the WHO has reported 571 human cases with a total of 335 deaths [10]. H5N1 is unable to transmit between humans at this time, however, there is significant concern that the virus could mutate such that efficient spread would be possible [11]. With the approximately 60% case fatality rate observed in reported cases, a pandemic HPAI H5N1 virus would cause significant morbidity and mortality, as well as major socioeconomic disruption [12].

Vaccination with an effective vaccine is considered the most effective approach to prevent disease or the transmission of potentially pandemic viruses in humans. The most widely used licensed vaccine for the prevention of seasonal influenza virus infection is an inactivated vaccine, which is grown in embryonated chicken eggs, and requires months of production time between strain identification and vaccine delivery, and and millions of eggs [13]. Even though this method represents the only US Food and Drug Administration (FDA)-approved H5N1 vaccine, there are a number of drawbacks in using this method of production for HPAI H5N1, including safety and production issues. Similar issues are shared with H5N1 vaccine seed viruses generated by reverse-genetics [15, 16], with some evidence indicating they are actually worse in terms of immunogenicity in the absence of an adjuvant [17]. There is clearly a need for other options for H5N1 vaccines.

There are a number of candidate vaccines in clinical trials at this time (reviewed in [18]), including inactivated viruses formulated with a variety of adjuvants, such as oil-in-water, and live-attenuated influenza vaccines [19, 20]. These approaches do not, however, address the concerns of using an egg-based vaccine for prepandemic preparedness.

Other vaccine options that are being explored include recombinant DNA and viruses expressing influenza proteins, which have been shown to protect against homologous and heterologous influenza challenge, including H5N1 [21, 23, 24, 225]. These finding suggest that virus vectored vaccines may be an effective method of obtaining robust, protective immune responses with the potential of generating a vaccine with cross-protective qualities. This would be ideal in the face of a largely unknown emerging pandemic strain upon a serologically naïve population.

Parainfluenza virus 5 (PIV5), a non-segmented, negative-stranded RNA virus (NNSV) in the *Rubulavirus* genus in the family *Paramyxoviridae*, is a favorable vector candidate for a number of reasons, including the absence of a DNA phase in the life cycle, eliminating the chance of host genome alteration, as well as a stable genome, making it an ideal candidate for foreign gene insertion and vaccine use [24, 25]. PIV5 is capable of infecting a large range of cell types[26] and, because it causes very little cytopathic effect (CPE) in those cells, is able to grow to high titers in common cell lines, including VERO cells[27], a vaccine-approved cell line. This would address existing problems associated with egg-based vaccine development.

PIV5 also infects a large range of mammals, including humans, without causing clinical disease[28].

Hemagglutinin (HA) is currently the primary target of most influenza vaccines. High affinity/avidity, neutralizing, receptor-blocking antibodies against the hemagglutinin (HA) glycoprotein on the surface of the virion are effective in preventing influenza infection [33, 173]. The second most abundant protein expressed on the surface of influenza is the neuraminidase (NA) glycoprotein. NA is also present on the surface of the virus as well as virus-infected cells and is responsible for cleavage of sialic acid residues as the virus buds from the cell at the end of the replication cycle[1]. NA is more conserved than HA which increases the likelihood of achieving broader protection if used as a vaccine antigen.

Like HA, NA is targeted by antibodies [34] which, unlike HA-specific-neutralizing antibodies that function primarily to interfere with initial attachment and entry [186-188], function essentially by trapping the progeny virus with the infected cell, thus reducing viral spread[189]. The antiviral drug Oseltamivir, a small molecule NA inhibitor licensed for prophylaxis and treatment of influenza virus infection, works in a similar fashion. In addition, by trapping the virus on the surface of infected cells, it can enhance susceptibility of infected cells to CTL or NK-cell recognition and killing. These all contribute to increased resistance to influenza infection in humans [34, 187, 188].

NA-targeted vaccines have been shown to provide protection against HPAIV H5N1 in mice [35] and in non-human primates [428], among others. Targeting NA

also offers the possibility of some degree of intersubtypic cross-protection against homologous or heterologous NA proteins as has been demonstrated with use of DNA vaccines in the mouse model [35, 193]. There is also evidence that antibodies from previously circulating H1N1 viruses may provide limited protection against HPAIV H5N1 in spite of major antigenic differences [35].

It has previously been demonstrated that vaccination with rPIV5 expressing the hemagglutinin (HA) gene from influenza A virus strain A/Udorn/72 (H3N2) (rPIV5-H3) is safe and protective against homologous influenza virus challenge in mice[24]. Here, we demonstrate that separate rPIV5 constructs expressing the NA from HPAI A/Vietnam/1203/04 (H5N1) (rPIV5-N1 (VN)) and the NA from A/California/04/09 (H1N1) can be rescued and express NA in its active form. Using a mouse model of influenza virus infection, we show that vaccination with rPIV5-N1 primes an NA-specific antibody response and T cell response and confers complete protection against homologous influenza virus challenge and significant cross-protection against heterologous influenza virus within the same subtype. There is also evidence of limited cross-protection against virus of a different subtype (H3N2).

Results

Rescue of recombinant PIV5-N1 viruses The plasmids containing the PIV5 cDNA with NA insertion were flanked by a T7 RNAP promoter and a hepatitis delta virus ribozyme followed by a T7 terminator (T7-T) (**Fig. 4.1A**). pZL108 encoding full length genome of PIV5 with H1N1-N1 gene insertion between HN and L gene, pZL116 encoding full length genome of PIV5 with H5N1-N1 gene insertion between HN and L gene and three helper plasmids, pPIV5-NP, pPIV5-P, and pPIV5-L encoding NP, P, and L proteins respectively, were co-transfected into BSR-T7 cells. After obtaining the rescued virus, full length genome sequencing was carried out to confirm the recombinant virus. There is no mutation in ZL108 and ZL116 viruses. The sequencing results of NA gene in ZL108 and ZL116 viruses were shown in Fig. 1B.

To ensure insertion of the NA gene did not have an effect on viral growth kinetics, an *in vitro* growth curve was performed. There was no apparent effect on viral growth (data not shown). The viruses PIV5-N1(VN) and PIV5-N1(CA), expressing the NA of A/VN/1203/04 and A/CA/04/09, respectively, were then tested for NA expression by infecting Vero cells and assaying NA expression by immunofluorescence using antisera generated against the parent viruses. Both PIV5-N1 viruses express NA during infection (**Fig 4.1b**).

Expression and activity of NA on rPIV5-N1 virions To determine if NA is being expressed on the surface of the recombinant viruses and to determine if it was functional, a neuraminidase assay was performed on equivalent titers of PIV5,

rPIV5-N1 (VN), rPIV5-N1 (CA), and rPIV5-H5 (**figure 4.2**). rgA/VN-PR8 and A/CA/04/09 were included and normalized to relative neuraminidase activity of the chosen rPIV5-N1 groups (data not shown). PIV5 and rPIV5-H5 exhibit baseline neuraminidase activity levels resulting from PIV5 vector HN activity, whereas rPIV5-N1 (VN and CA) neuraminidase levels are significantly elevated, to levels similar to rgA/VN-PR8 and A/CA/04/09 influenza viruses. When treated with a high dose of oseltamivir, an influenza NA-inhibitor, neuraminidase activity in rPIV5-N1 (VN and CA) was decreased to PIV5 background levels (baseline HN activity), whereas rgA/VN-PR8 and A/CA/04/09 were completely inhibited. This indicates that not only is NA expressed on the surface of rPIV5-N1 (VN and CA), but it is expressed in its active and native form.

Immunogenicity of rPIV5-N1 (VN and CA) in BALB/c mice We have shown previously that vaccination with rPIV5 expressing HA from H3N2 or H5N1 (chapter 3) induces an HA-specific, virus neutralizing antibody response. To determine if vaccination with rPIV5-N1 induces an NA-specific antibody response, BALB/c mice were primed and boosted with PIV5, rPIV5-N1 (VN), or rPIV5-N1 (CA) administered intranasally (IN) or rgA/VN-PR8 or A/CA/04/09 influenza viruses administered intramuscularly (IM). Mice were bled on day 21 post-prime, boosted on day 28, and bled again on day 7 post-boost. Serum was then assessed for A/VN/1203/04- and A/CA/04/09- specific IgG antibodies by ELISA. Little to no A/VN/1203/04 or A/CA/04/09-specific IgG was detected in mice vaccinated with PIV5, whereas substantial levels of influenza-specific IgG were detected in mice immunized with

rPIV5-N1 (VN and CA) **(Fig 4.3a).** Although antibody levels specific for homologous viruses were higher, heterologous antibodies were also detected. To determine whether the NA-specific antibodies could block specific neuraminidase activity, serum was analyzed by a 2-(40methylumbeliferyl)-a-D-N-acetylneuraminic acid (MUNANA)-based NA-inhibition assay. Pooled sera from primed and boosted mice were tested against viruses rgA/VN-PR8 and A/CA/04/09homologuous for NA as well as against a second reverse genetics influenza virus expressing the HA and NA from A/Anhui/01/2005 (clade 2.3.4) on a A/PR/8 backbone (rgA/Anhui-PR8 (H5N1))[479]. Sera were also tested against a heterosubtypic virus, A/Philippines/2/82 (H3N2) to further investigate the possibility of cross-inhibition. Serum from PIV5-vaccinated mice did not cause a measurable inhibition of NA activity against any virus strain tested, whereas serum from mice vaccinated with rPIV5-N1 (VN and CA) inhibited neuraminidase activity of homologous and heterologous viruses **(Fig. 4.3b-e)**. In fact, sera from PIV5-N1 vaccinated mice inhibited neuraminidase activity as least as well or better than homologous virus immunization. Thus, vaccination with rPIV5-N1 (VN and CA) vaccines induces potent specific and cross-strain NA-inhibiting antibody titers.

One advantage of live virus-vectored vaccines is the potential to prime a cell-mediated immune response, which can contribute to protection from infection. Thus, we assessed virus-specific T cell responses of PIV5-N1 vaccinated mice and compared them to influenza-infected mice. BALB/c mice were immunized IN with PIV5, rPIV5-N1 (VN or CA), or a sub-lethal dose of rg A/VN-PR8 or A/CA/04/09. On

day 12 post-immunization, mice were euthanized and the mediastinal lymph nodes were isolated, re-stimulated with inactivated A/VN/1203/04, A/CA/04/09, PIV5, or irrelevant antigen, and assessed for IFN-γ producing lymphocytes by ELISpot assay. Vaccination with rPIV5-N1 (VN or CA) induced a cross-specific T cell response, as compared to PIV5-vaccinated mice which showed no significant response **(figure 4.4).** While mice infected with influenza viruses showed more IFN-producing cells, they were responding to the entire virus, as opposed to the NA alone. Also, the influenza primed-lymphocytes had a higher non-specific response, responding to whole PIV5 or irrelevant peptide, suggesting a portion of the response was not antigen specific (fig. 4).

Protection against homologous and heterologous influenza virus challenge To determine if vaccination with rPIV5-N1 (VN or CA) provides effective protection against highly pathogenic H5N1 (A/Vietnam/1203/04), H1N1 (A/California/04/09), and heterosubtypic H3N2 (A/Philippines/2/82) infection, BALB/c mice were immunized IN with PIV5, rPIV5-N1 (VN), rPIV5-N1 (CA). Control animals were immunized IM with 2000 PFU rgA/VN-PR8, A/CA/04/09, or A/Philippines/2/82 X79. Mice were boosted on day 21 post-prime. Seven days post-boost, mice were challenged IN with 10 LD_{50} of influenza A/VN/1203/04 or A/CA/04/09, and monitored daily for morbidity and mortality. A subset of mice from each challenge group was euthanized on day 3 post-challenge and lung tissue collected to assess lung virus titers by TCID50. All mice vaccinated with PIV5 declined in body weight and were euthanized or succumbed to infection. For the

H5N1 challenge, homologous immunization (rPIV5-N1 (VN)) provided complete protection from morbidity and mortality **(Fig. 4.5a and b)**, and reduced H5N1 virus titers in the lungs to below detectable levels **(fig. 5c)**. Mice vaccinated with the heterologous neuraminidase, rPIV5-N1 (CA) were partially protected from morbidity and mortality **(Fig 4.5 a and b)** and had reduced lung virus titers **(Fig. 4.5c)**. For the H1N1 challenge, vaccination with rPIV5-N1 (CA) and rPIV5-N1 (VN) provided complete protection from weight loss and mortality (Fig 5 d and e); however lung virus titers were only slightly reduced **(Fig. 4.5f)**. Curiously, virus was reproducibly detectable in the lungs even in mice vaccinated with homologus virus, A/CA/04/09 **(Fig. 4.5f)**. A subset of vaccinated mice were challenged with 10 LD_{50} of the heterosybtypic virus A/Philippines/2/82 X79 as a subtype control. Strikingly, rPIV5-N1 (CA) immunization provided partial protection against morbidity and mortality following H3N2 challenge **(Fig 4.5g and h)** although there was no reduction in lung virus titers observed **(Fig. 4.5i)**. There was no apparent protection from H3N2 challenge in mice vaccinated with rPIV5-N1 (VN).Taken together, these results indicate that immunization with recombinant PIV5 vaccines expressing influenza neuraminidase can provide robust protection against homologous and cross-strain influenza virus challenges at a level comparable to high-dose IM vaccination with homologous influenza virus strains. Moreover, rPIV5-N1 immunization can potentially induce heterosubtypic immunity.

To determine the mechanism of protection, antibodies were purified from hyper-immune serum and passively transferred to naïve mice. Mice were then

challenged with HPAI H5N1 A/VN/1203/04 or A/CA/04/09 and monitored for morbidity and mortality **(Fig. 4.6)**. Although mice challenged with HPAI H5N1 were not protected by the transfer of antibodies **(Fig. 4.6 a and b)**, antibody transfer was sufficient for partial protection against homologous challenge by A/CA/04/09 **(Fig. c and d)**. It is possible that this difference is a result of the nature of the challenge viruses or insufficient antibody transfer, although it is possible that T cells are playing a significant role in protection. This is yet to be elucidated.

Discussion

The results of this study indicate that the use of NA as a vaccine antigen expressed by the live virus vector PIV5 offers the potential for cross-protective immunity not possible with use of HA. We demonstrate that rPIV5-N1 incorporates functional NA in its virion **(Figure 4.2)** and that rPIV5-N1infected cells express NA **(Figure 4.1)**. Mice immunized with rPIV5-N1 generate robust, cross-reactive IFN-γ producing T cell responses and NAI serum antibody responses **(Figures 4.4 and 4.3, respectively)**. Finally, rPIV5-N1 immunization protects against homologous, heterologous, and in the case of pH1N1 H1 immunization partial heterosubtypic challenge **(Figure 4.5)** and it appears antibody is playing a significant role in protection **(Figure 4.6)**. In all cases, the rPIV5-N1 immunization was at least as effective as immunization with homologous virus, which induces HA-specific neutralizing antibody responses as well at potentially cross-protective responses to

conserved antigens (i.e. M2, NP, etc). Taken together, these results show that rPIV5-N1 is a promising vaccine candidate for seasonal and pandemic preparedness.

Neuraminidase remains largely unexplored as a vaccine antigen for influenza, even though it is only second in immunogenicity to HA. This is likely attributed to the belief that immunity to NA provides incomplete protection against influenza infection. The cross-protective capabilities of NA, however, indicate that it most certainly should be studied, especially in the context of a potentially pandemic influenza virus such as HPAI H5N1, where the human population is largely naïve and any cross-protection would be useful[502]. There is evidence that antibodies resulting from seasonal H1N1 infection may have offered protection in humans against the 2009 pandemic H1N1 [194] and that antibodies from both of the aforementioned viruses may provide limited protection against HPAIV H5N1 [35, 503].

Another possible reason NA has generally not accompanied HA in influenza vaccine preparations is the observed immunodominance of HA. Approximately four times less NA is expressed on the surface of the virion than HA [183] which is at least partially responsible for the observed skewing of the natural host response towards HA. The disparity in immune pressure could be responsible for the significantly lower mutation rate observed in NA as compared to HA [184, 185]. The broader protection observed is attributed to this increased degree of conservancy.

Here, we utilized PIV5 to recombinantly express NA in the absence of HA, avoiding the potential immunodominance of the HA from reducing NA-specific

antibody responses. Indeed, sera from rPIV5-N1consistently inhibited NAI activity better than sera from homologous influenza virus immunized mice (**Figure 4.3 b,c,d**). While we did compare efficacy of heterologous virus in the challenge, we could not eliminated the contribution of conserved antigens to immunity and so could not effectively compare rPIV5-NA to whole influenza immunization in that model.

A number of studies have divided NA subtypes into three groups: N1, N5, and N8; N7 and N9; and N2, which is its own group. Sequence homology between groups ranges from 40-46%, which is consistent with the 46% sequence homology we obtained between A/Philippines/2/82 X79 NA and A/VN/03/04 NA (data not shown). Within groups, the sequence homology is typically between 54 and 68%, and within subtypes it is typically greater than 90% [193, 504]. Most evidence to date indicates that NA is a poor inducer of heterosubtypic immunity [193] (reviewed in [195]). Here, we saw cross reactivity by ELISA and NA inhibition assay between sera from H5N1 NA- and pandemic H1N1 (pH1N1) NA-primed mice and limited heterosubtypic immunity between pH1N1 NA-reactive sera and H3N2 NA. Moreover, this cross-reactive antibody response correlated with protection from lethal challenge. Similar to our findings, Chen *et al* recently demonstrated that sera from pH1N1 vaccinated ferrets cross-reacted with NA from H5N1 in a NA inhibition assay[503]. Chen *et al's*. analysis of homology between H5N1, H1N1, pH1N1 and H3N2 neuraminidases showed 88% homology between A/CA/04/09 and A/VN/1203/04 NA proteins as compared to 81% homology between A/CA/04/09

and A/SD/6/07, a H1N1 seasonal influenza vaccine strain prior to the 2009 pandemic. Many of the shared amino acids were located near the enzymatic site [505], potentially explaining the corss-reactivity and corss-inhibition of NA activity.

The cross-strain-inhibiting antibodies observed in this study, combined with the previous observations by Sandbulte *et al.* [35] and Chen *at al* [503], indicates that it is likely that antibodies play the dominant role in NA-based protection. Here, immunization with rPIV5-N1 did induce robust IFN-γ producing T cell responses. We did not formally test the contribution of these T cell responses to protection, however this possibility could be assessed by a passive antibody transfer study, which would confirm whether antibodies from mice primed with rPIV5-N1 are sufficient for protection. In any event, the addition of the NA-specific T cell responses could provide added benefit, which is one of the advantages of a using a live virus-vectored vaccine.

Neuraminidase as a vaccine antigen would not necessarily stand alone, but could be combined with HA or other conserved viral antigens (e.g. matrix or nucleoprotein). It has been shown that when administered individually, as opposed to together on the virion during a natural infection, the immune response is more balanced between HA and NA [189]. Thus, rPIV5-N1/rPIV5-HA cocktails could be used to prime potent HA and NA-specific antibody responses, providing neutralizing and NAI antibody responses with increased potential for cross-protection. Another potential option worth exploring is the possibility that a rPIV5 construct could be

engineered to express both HA and NA, although the immunodominance profile would have to be carefully studied.

Materials and Methods

Influenza viruses used include rg A/VN-PR8, rg A/Anhui-PR8, A/California/04/09, A/California/07/09, and A/Vietnam/1203/04 (H5N1). A/Philippines/2/82 X79 (H3N2), a 2:6 reassortant with A/PR8/34 was provided by Suzanne Epstein (FDA). rgA/VN-PR8 and rgA/Anhui-PR8, provided by Rubin Donis (CDC) were propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48-72 hours. A/California/04/09 and A/California/07/09, provided by Alexander Klimov (CDC) were grown in cell culture on MDCK cells. β-propiolactone (BPL)-inactivated A/Vietnam/1203/04 was provided by Richard Webby from St. Jude Children's Research Hospital. The HPAI virus was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 24 hours. All viruses were aliquoted and stored at -80°C. All experiments using live highly pathogenic H5N1 avian influenza viruses were reviewed and approved by the institutional biosafety program at the University of Georgia and were conducted in biosafety level 3, enhanced containment following guidelines for use of Select Agents approved by the CDC.

Mice Female 6 to 8 week old BALB/c mice (Charles River Labs, Frederick, MD) were used for all studies. Mouse immunizations and studies with BSL2 viruses were performed in enhanced BSL2 facilities in HEPA filtered isolators. Mouse HPAI infections were performed in enhanced BSL3 facilities in HEPA filtered isolators

following guidelines approved by the institutional biosafety program at the University of Georgia and for use of Select Agents approved by the CDC. All animal studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia.

Cells Monolayer cultures of BSR-T7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB) and 400 μg/ml G418. BHK cells were maintained in DMEM containing 10% FBS, 100 IU/ml penicillion, and 100 μg/ml streptomycin. Madin-Darby Bovine Kidney *(*MDBK) and Madin-Darby Canine Kidney (MDCK) cells were cultured in DMEM with 5% FBS, 5% L-glutamine, and an antibiotic/antimycotic solution (10,000 IU/ml penicillin, 10,000 ug/ml streptomycin, and 25ug/ml amphotericin B) (Cellgro Mediatech, Inc). VERO cells were cultured in Minimum Essential Medium (MEM) (Thermo/Hyclone) with 10% FBS and antibiotic/antimycotic. All cells were incubated at 37°C, 5% CO_2.

Construction of recombinant viruses Two recombinant PIV5 plasmids containing NA gene, ZL108 (rPIV5-H1N1-N1-HN/L) and ZL116 (rPIV5-H5N1-N1-HN/L) were generated. To generate the two recombinant plasmids, the plasmid BH311 containing full length genome of PIV5 with an extra EGFP insertion between HN and L gene was used as the vector. The double NotI site flanking at EGFP ORF was used for cloning. The primers pLW3 (5'-CAGTAGCGGCCGCTAAAATGAATCCAAACCAA-3') and pLW4 (5'-CAGTAGCGGCCGCTTACTTGTCAATGGTAAATGGCAAC- 3') were applied to amplify

H1N1-N1 gene, pZL281 (5'-GGCCAGCGGCCGCCAAAATGAATCCAAATCAGAAGATAA-3') and pZL282 (5'-ATATAGCGGCCGCCTACTTGTCAATGGTGAATG-3') were applied to amplify H5N1-N1 gene, NotI site in the two primers were underlined. Total RNAs from H1N1 and H5N1 viruses-infected MDCK cells were purified using the RNeasy kit (Qiagen Inc, Valencia, CA). cDNAs were prepared using random hexamers and aliquots of the cDNA were then amplified in PCR reactions using the above oligonucleotide primer pairs.

Virus rescue and sequencing The plasmids, pZL108 encoding full length genome of PIV5 with H1N1-N1 gene insertion between HN and L gene, or pZL116 encoding full length genome of PIV5 with H5N1-N1 gene insertion between HN and L gene, and three helper plasmids pPIV5-NP, pPIV5-P, and pPIV5-L encoding NP, P, and L proteins, were co-transfected into BSR-T7 cells at 95% confluency in 6-cm plates with Plus and Lipofectamine (Invitrogen). The amounts of plasmids used were as follows: 5 μg pZL108/pZL116, 1 μg pPIV5-N, 0.3 μg pPIV5-P, and 1.5 μg pPIV5-L. After 3h incubation, the transfection media were replaced with DMEM containing 10% FBS and 10% TPB. After 72h incubation at 37°C, the media were harvested, and cell debris was pelleted by low speed centrifugation (3,000 rpm, 10 min). Plaque assays were used to purify the single clone of recombinant viruses.

The full length genome of plaque-purified single clone of ZL108 and ZL116 viruses were sequenced. Total RNAs from ZL108 and ZL116 viruses-infected MDBK cells were purified using the RNeasy kit (Qiagen Inc, Valencia, CA). cDNAs were

prepared using random hexamers and aliquots of the cDNA were then amplified in PCR reactions using appropriate oligonucleotide primer pairs.

PIV5 and rPIV5 virus stocks were grown in MDBK cells (<p20) for 5-7 days in DMEM containing 2% FBS until their hema-adsorption titers plateaued. Media was collected and clarified by centrifuging at 3000 rpm for 10 minutes in an Eppendorf tabletop centrifuge (5810 R). Bovine serum albumin (BSA) was added to the clarified supernatant to bring the total solution to 1% BSA. The virus stocks were then aliquoted and frozen quickly in dry ice and stored at -80°C. Virus titers were then determined by plaque assay on VERO cells (described below).

Virus Quantitation. PIV5 titers were determined by plaque assay on VERO cells. VERO cells were incubated with serial dilutions of virus samples made in DMEM with 1% BSA and antibiotic/antimycotic. Virus sample was then removed and overlayed with 1:1 low-melt agarose and DMEM with 2% FBS and antibiotic/antimycotic and incubated at 37°C for 5-6 days. To detect plaques, the monolayers were then fixed with 10% buffered formalin and immunostained. Cells were permeabilized with 1X PBS with 2% FBS, 0.1% sodium azide, and 0.5% saponin (permeabilization buffer). PIV5 was detected using a 1:1000 dilution of antibodies specific to the shared region of the V and P proteins of PIV5 (V/P) for 1hr. Horseradish peroxidase (HRP)-tagged goat-anti-mouse IgG (H&L) secondary antibody (Invitrogen) was then added and incubated for 30min. To visualize plaques, TMB peroxidase substrate (prepared according to manufacturer's instructions) was added (Vector Labs, Inc). The plates were then washed and dried

and the plaques were counted. Influenza titers were determined either by $TCID_{50}$ assay as previous described [227] or by plaque assay on MDCK cells. MDCK cells were incubated for 2 hours at 37°C with serial dilutions of virus samples made in MEM with 1 mg/ml TPCK-treated trypsin (Worthington Biochemical). Diluted virus samples were then removed and monolayers were overlayed with 1.2% microcrystalline cellulose Avicel[500] with 1 mg/ml TPCK-treated trypsin. Plates were incubated for 72 hours, the overlay gently washed off with PBS, fixed with cold methanol/acetone (40:60%), air-dried, counter-stained with crystal violet, and plaques visualized.

Virus growth in vitro Virus diluted to a MOI of (0.01 PFU/cell) in DMEM with 1% BSA and antibiotic/antimycotic was added to a confluent monolayer of MDBK cells. The plate was incubated for 1-2 hours at 37°C. Diluted virus was then removed, the monolayer rinsed with 1X PBS, and replaced DMEM with 1% BSA and antibiotic/antimycotic. Samples of supernatant were collected every 24 hours up to seven days, beginning immediately after fresh medium was placed on the monolayers. Supernatant was quickly frozen in dry ice and stored at -80°C. Virus titer was determined by plaque assay on VERO cells for each time point.

Fluorescence VERO cells were grown in 24-well plates and infected with PIV5, rPIV5-N1 (VN), or rPIV5-N1 (CA) at a multiplicity of infection (MOI) of 5 PFU/cell. At 24 hour post-infection, cells were fixed with 5% buffered formalin for 10min at room temperature. Cells were then permeabilized with permeabilization buffer and then incubated for 1hr with a 1:1000 dilution (1µg/ml) of PIV5, V/P-

specific monoclonal antibody. A 1:250 dilution of PE goat anti-mouse Ig (BD Pharmingen) was applied for 45min to detect HA. To detect NA, hyper-immune serum generated to each virus (rgA/VN-PR8 or A/CA/04/09). To visualize NA, an Alexa Fluor-488-labeled secondary antibody (Invitrogen), diluted 1:500, was added and incubated for 30min and then washed. 0.5mL PBS was added to each well and fluorescence was examined using an AMS EVOS fl fluorescent microscope. Cells were washed extensively between each step with PBS.

Immunization For vaccination with PIV5 and rPIV5-N1, 10^6 PFU PIV5, rPIV5-N1 (VN), or rPIV5-N1 (CA) in 50µl PBS was administered intranasally to mice anesthetized with *2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co).* For rgA/VN-PR8, A/CA/04/09, or A/Philippines/2/82 *X79* IM vaccination, 2000 PFU in 50µl PBS of each virus was injected into the caudal thigh muscle. Blood was collected on day 21 post-immunization. For boosting, this process was repeated on day 28 post-prime. Mice were monitored daily and, for some experiments, body weights recorded every other day.

ELISA A/VN/1203/04 or A/CA/04/09-specific serum antibody titers were measured using an IgG ELISA. Immulon 2 HB 96-well microtiter plates (*ThermoLabSystems)* were coated with approximately 10 HAU inactivated A/VN/1203/04 or A/CA/04/09 diluted in PBS and incubated at 4°C overnight. Plates were then washed with KPL wash solution (KPL, Inc) and the wells blocked with 200 µl KPL Wash Solution with 5% non-fat dry milk and 0.5% BSA (blocking buffer) for 1hr at room temperature. Serial dilutions of serum samples were made

(in blocking buffer) and transferred to the coated plate and incubated for 1hr. To detect bound serum antibodies, 100µl of a 1:1000 dilution alkaline phosphatase-labeled goat anti-mouse IgG (KPL, Inc) in blocking buffer was added per well and incubated for 1hr at room temperature. Plates were developed by adding 100µl pNPP phosphatase substrate (KPL, Inc) per and the reaction allowed to develop at room temperature. Optical density (OD) was measured at 405 nm on a Bio-Tek Powerwave XS plate reader. The IgG titer was determined to be the lowest serum dilution with an OD greater than the mean OD of naïve serum plus 2 standard deviations.

Neuraminidase Assay and Neuraminidase Inhibition Assay The neuraminidase (NA) assay and neuraminidase inhibition (NAI) assay were performed using NA-Fluor™ Influenza Neuraminidase Assay kit. Virus stocks (rPIV5 constructs and influenza viruses) were titered for neuraminidase activity according to the NA-Fluor™ Influenza Neuraminidase Assay kit (Applied Biosystems) according to manufacturer's instructions. The virus dilution used for the Neuraminidase Inhibition Assay was normalized for each virus as the highest dilution on the linear range of relative fluorescence units (RFU). To determine NA expression of rPIV5-N1 recombinant viruses, equivalent titers of PIV5 and rPIV5-N1 were assayed with some groups treated with 10,000 nM Oseltamivir and RFU readouts were taken to assess knockdown. NAI assays were also performed to detect neuraminidase-inhibiting serum antibodies. Two-fold dilutions of serum was performed with an

initial dilution of 1:4. The assay was then performed according to manufacturer's instructions. Oseltamivir was included as a control.

Lymphocyte Harvest 12 days post-vaccination with 10^6 PFU PIV5, rPIV5-N1 (VN), rPIV5-N1 (CA), or 1000 PFU rgA/VN-PR8 IN, mediastinal lymph nodes (MLN) from mice were harvested, pooled, and homogenized. Lymphocytes were depleted of erythrocytes using Gey's Balanced Salt solution (Sigma-Aldrich) for 5min at room temperature and debris removed. Cells were then counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter).

Enzyme-linked Immunosorbert Spot (ELISpot) Assay ELISpot to detect T-cell responses in lymphocytes to inactivated A/VN/1203/04 or A/CA/04/09 were performed with lymphocytes harvested above as described [23]. Cells were re-stimulated with inactivated A/VN/1203/04 or A/CA/04/09 (the equivalent of 10 HAU per well), Ebola GP P2 EYLFEVDNL as an irrelevant peptide (1μg/ml), and Concanavalin A (2μg/ml) in 50μl Complete Tumour Medium (CTM). Spots were counted using AID ViruSpot Reader (Cell Technology, Inc).

Influenza virus challenge experiments BALB/c mice were first vaccinated with wild type PIV5, rPIV5-N1 (VN), rPIV5-N1 (CA) IN (as described above), or 2000 PFU rgA/VN-PR8, A/CA/04/09, or A/Philippines/2/82 in 50 μl PBS IM. 21 days post-vaccination, the mice were bled for serum analysis via the tail vein. On day 24 post-vaccination, mice were anesthetized and inoculated intranasally with 10 LD_{50} A/Vietnam/1203/04, A/California/04/09, or A/Philippines/2/82 diluted in 50μl PBS. Mice were then monitored daily for morbidity and mortality with body

weights measured every other day. On day 3 post-challenge, groups of mice were euthanized and their lungs collected into 1.0ml PBS and homogenized. Homogenate was then cleared by centrifugation. A $TCID_{50}$ assay was then used to determine virus titers in cleared homogenate as described [227].

Passive antibody transfer: 200μg purified IgG from PIV5, rPIV5-N1 (VN), rPIV5-N1 (CA), rgA/VN-PR8, and A/CA/04/09 vaccinated mice was administered intraperitoneally to naïve mice. Mice were challenged with HPAI A/Vietnam/1203/04 or A/CA/04/09 the day after transfer. Challenge was performed as described. Mice were monitored for morbidity and mortality and previously described.

Statistical Analyses *were performed using GraphPad Prism®.*

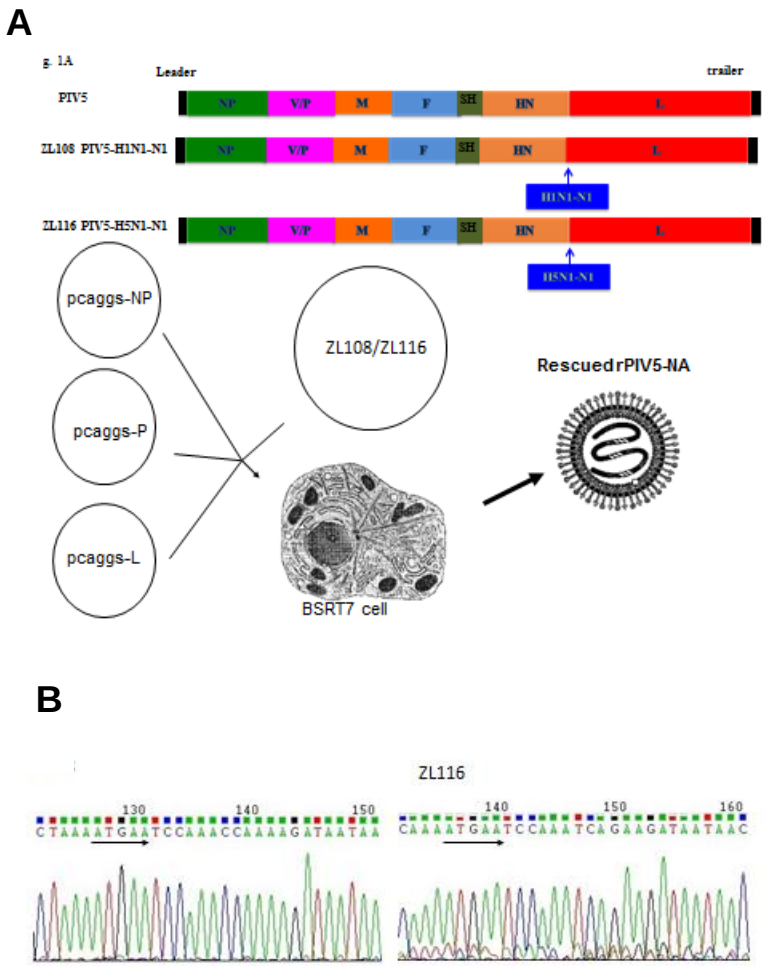


Figure 4.1 Recovery of rPIV5-NA viruses (A) Schematic diagram of the rescue of infectious PIV5 containing NA gene from its cDNA clone. Plasmid pZL108 encodes full length genome of PIV5 with H1N1-NA gene insertion between HN and L gene, pZL116 encodes full length genome of PIV5 with H5N1-NA gene insertion between HN and L gene. The plasmids pCAGGS-NP, pCAGGS-P, and pCAGGS-L each contain the cDNA for the PIV5 NP, P, and L proteins, respectively. BSR-T7 cells were co-transfected with pZL108 or ZL116, and the three helper plasmids pCAGGS-NP, pCAGGS-P, and pCAGGS-L. The transfection media were replaced with DMEM containing 10% FBS and 10% TPB after 3 hr incubation and the cultures were incubated for 72 hr. (B) Sequencing results of NA gene in ZL108 and ZL116 viruses. ZL108 and ZL116 viruses were rescued. The whole genomes of ZL108 and ZL116 viruses were sequenced using appropriate oligonucleotide primers. The sequencing results of NA gene in ZL108 and ZL116 viruses were shown. The arrows indicated the ORF of NA gene.

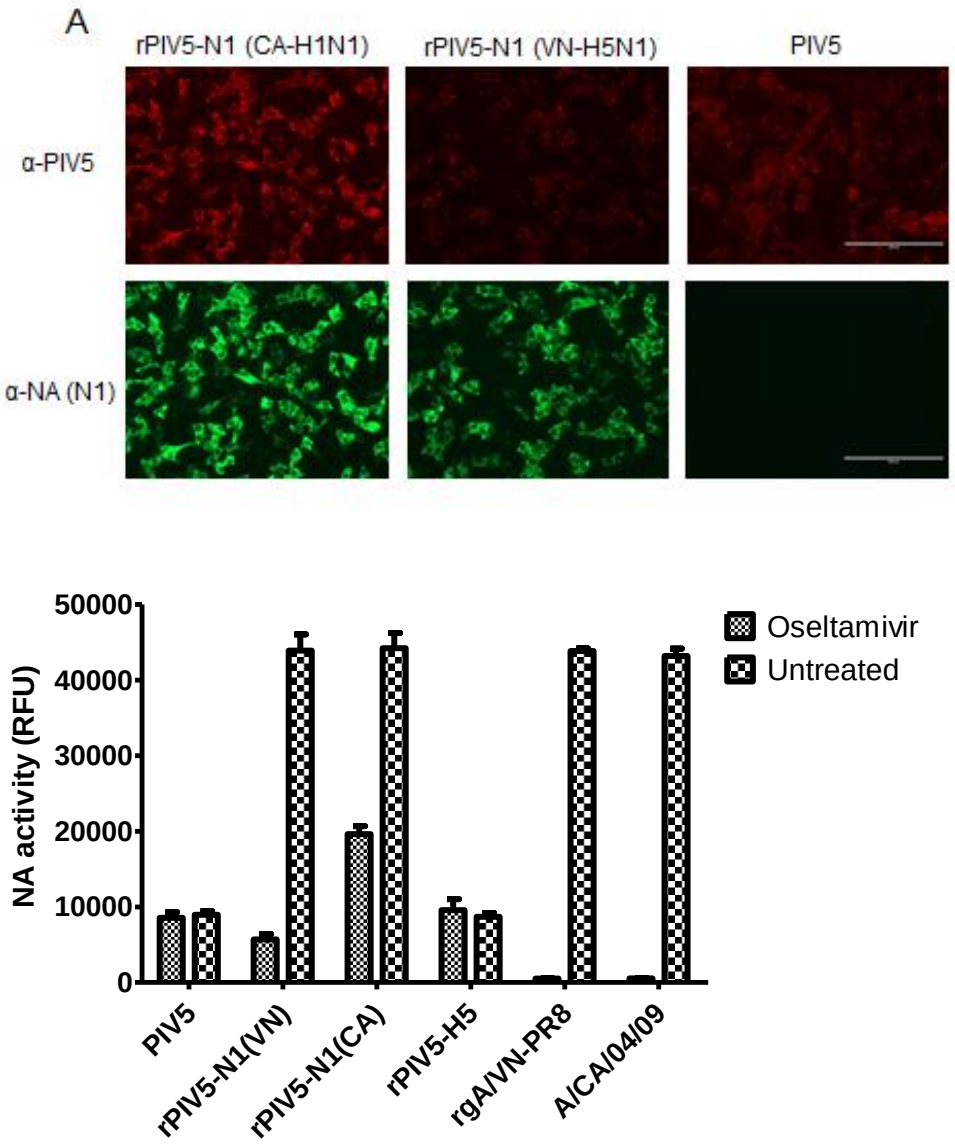


Figure 4.2: NA is being expressed on the surface of the virion in its active form. (A) Immunofluorescence of VERO cells infected with PIV5, rPIV5-N1 (VN), and rPIV5-N1 (CA) at an MOI of 5. At 24 hours post-infection, cells were fixed and stained with anti-PIV5 (V/P)/PE (red) and serum generated against the parent viruses/FITC (green) antibodies. Micrographs were taken at 20x magnification and the scale bar is representative of 200μm. **(B)** Equivalent titers of PIV5, rPIV5-N1 (VN), rPIV5-N1 (CA), and rPIV5-H5; and rgA/VN-PR8 and A/CA/04/09 (normalized to max RFU output of the rPIV5-N1 groups) were incubated with oseltamivir and without. MUNANA substrate was then added and NA activity measured. Treatment with oseltamivir reduced neuraminidase levels in rPIV5-N1 (VN & CA) to PIV5 and rPIV5-H5 baseline levels, whereas rgA/VN-PR8 and A/CA/04/09 NA levels were eliminated completely.

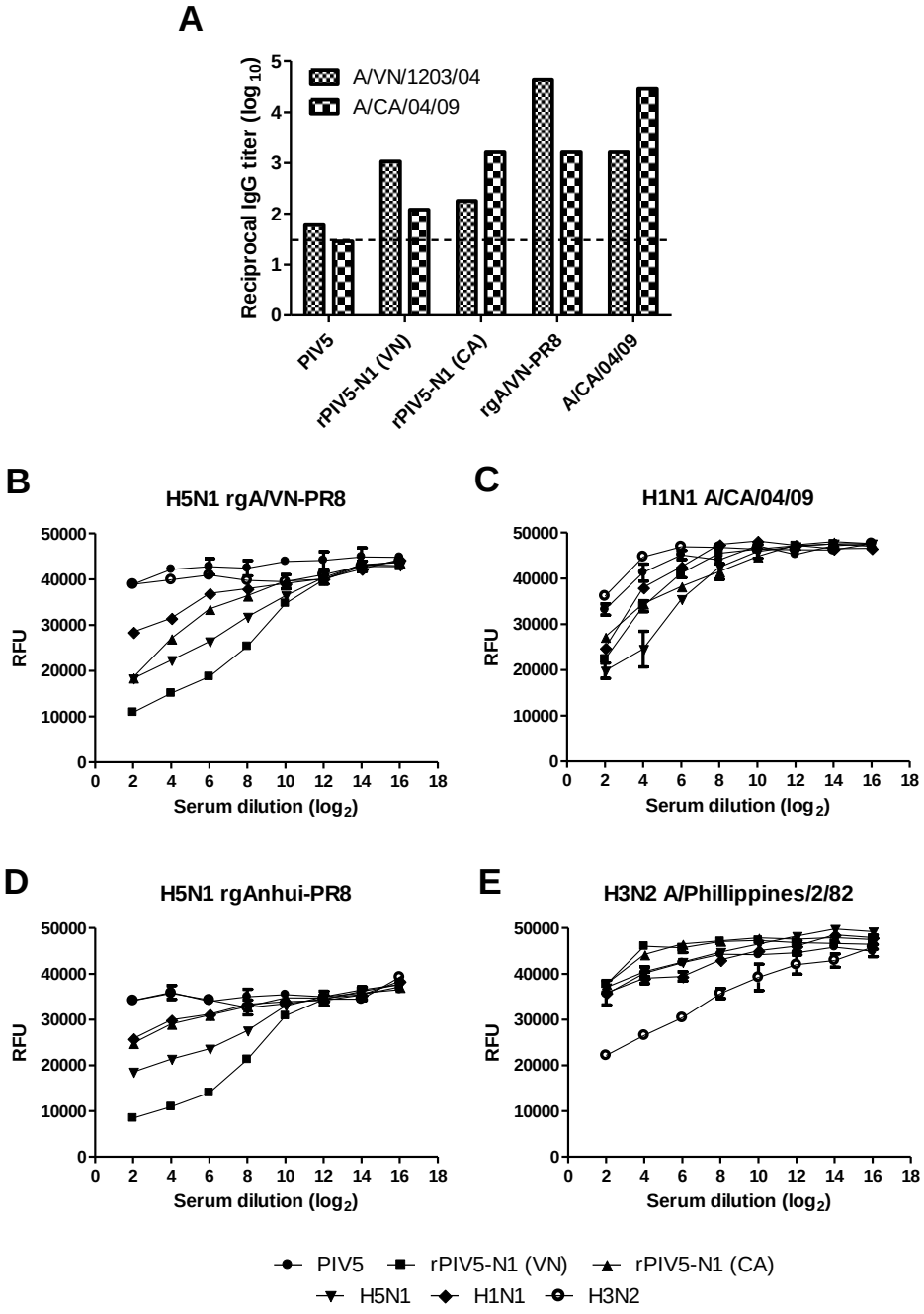


Figure 4.3: Vaccination with rPIV5-N1 induces influenza neuraminidase-inhibiting antibodies. Mice were primed and boosted with 106 PFU PIV5, rPIV5-N1 (VN), rPIV5-N1 (CA) IN, or 2000 PFU rgA/VN-PR8, A/CA/04/00, or A/Philippines/2/82 X79 IM. On day 7 post-boost, mice were bled and their serum antibodies assessed for (A) A/VN/1203/04 or (B) A/CA/04/09-specific IgG antibodies by ELISA. Serum was also tested for antibodies capable of inhibiting neuraminidase of rgA/VN-PR8 (C), rgA/Anghui-PR8 (D), A/CA/04/09 (E), and A/Philippines/2/82 X79 (F).

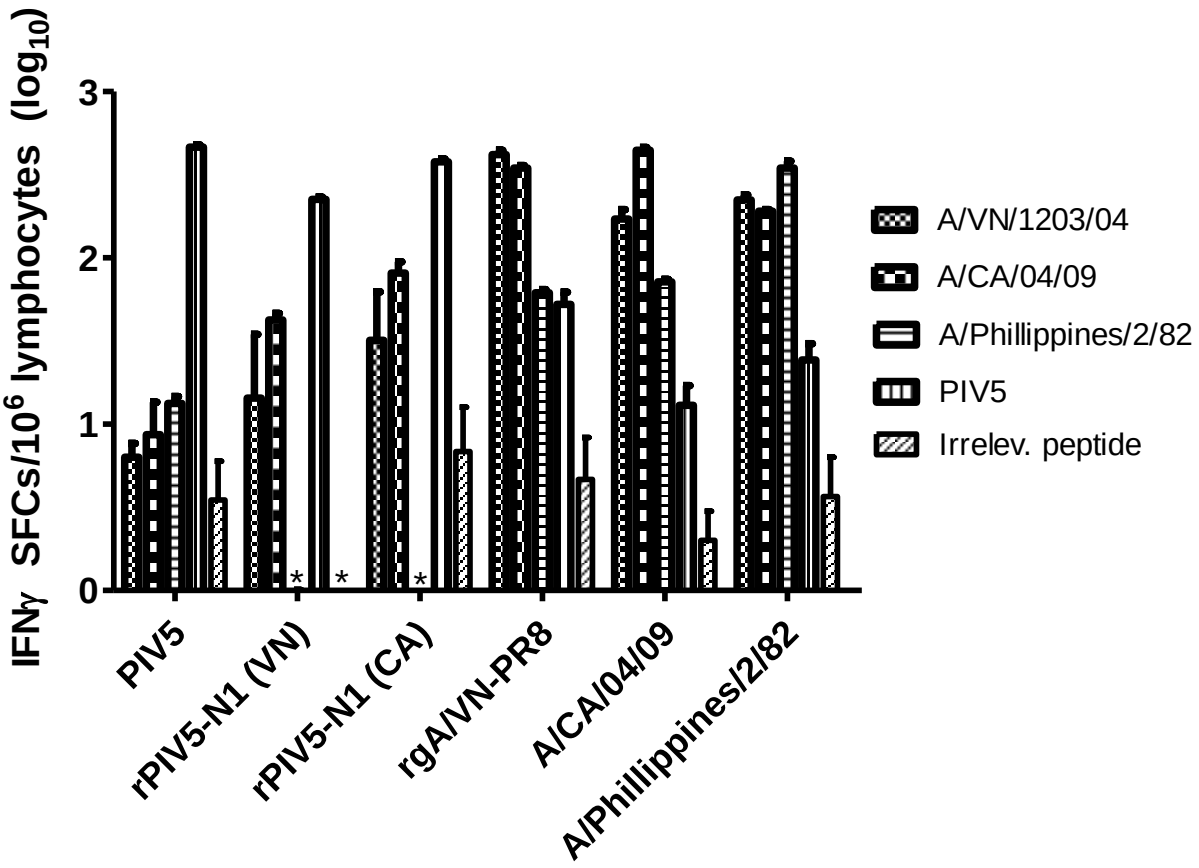


Figure 4.4: rPIV5-N1 primes a cross-reactive T cell response BALB/c mice (n=5 per group) were immunized with PIV5, rPIV5-N1 (VN), rPIV5-N1 (CA), or a sub-lethal dose of rgA/VN-PR8, A/CA/04/09, or A/Philippines/2/82 X79. IFN-γ producing lymphocytes in the mediastinal lymph nodes on day 12 post-vaccination as determined by ELISpot analysis. Data is presented as mean ± SEM.

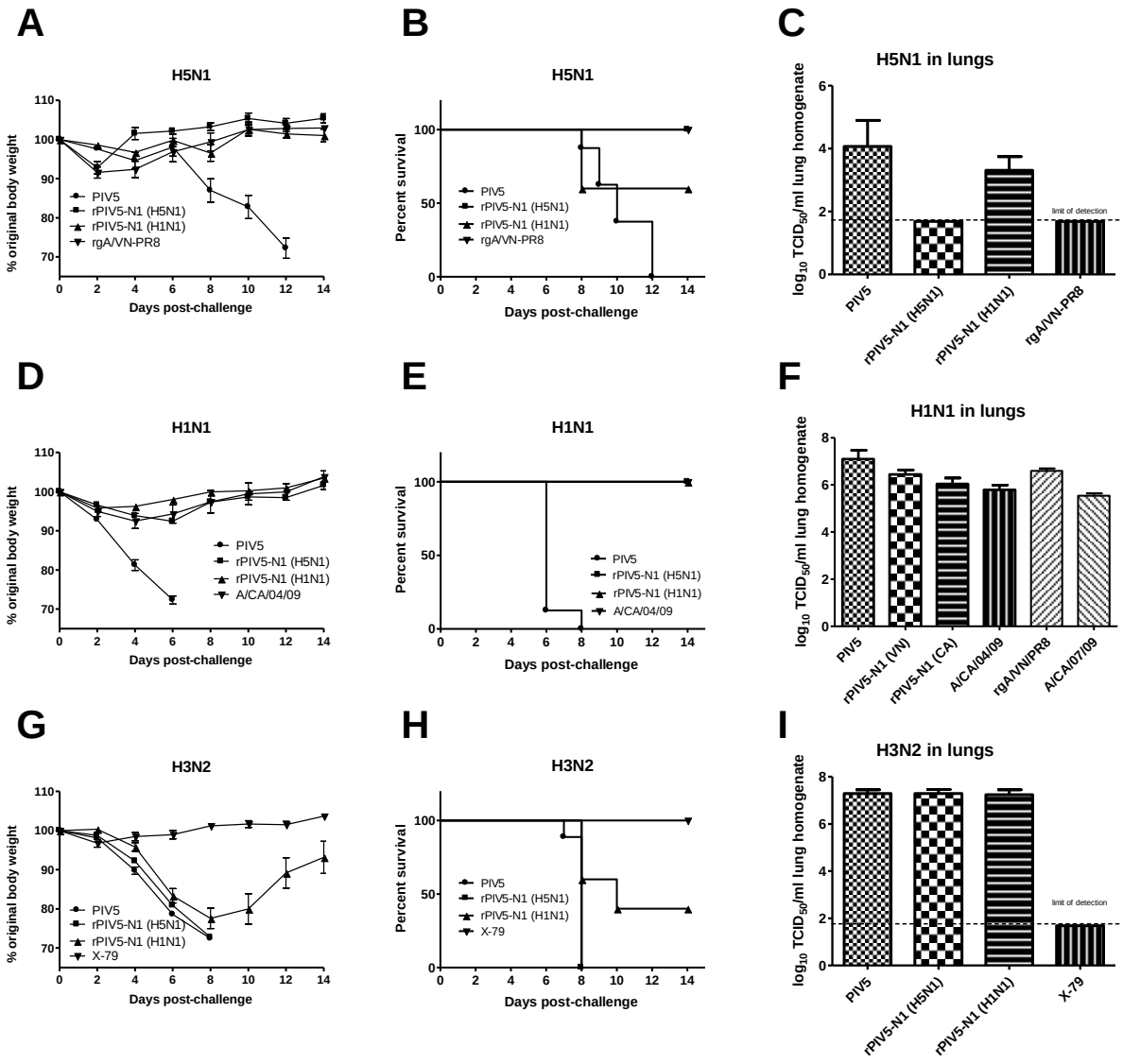


Figure 4.5: Efficacy of rPIV5-N1 as a vaccine vector (protection homologous and heterologous challenge). BALB/c mice were immunized IN or IM with PIV5, rPIV5-N1 (VN), or rPIV5-N1 (CA), or IM with rgA/VN-PR8 or A/CA/04/09. On day 28 post-priming, mice were boosted. Seven days post-boosting, mice were challenged IN with 10 LD_{50} A/VN/1203/04 **(A, B, C)**, A/CA/04/09 **(D, E, F)**, or A/Philippines/2/82 X79 **(G, H, I)**. Weights of the mice were monitored and presented as the mean percentage $\pm$ SEM of their pre-challenge body weights (n=10) **(A, D, G)**. Percent of mice surviving post-challenge **(B, E, F)**. Challenge virus titer in the lungs on day 3 post-challenge (n=5 per group) as measured by $TCID_{50}$ on MDCK cells. Data are presented as mean log transformed $TCID_{50}$/ml lung homogenate $\pm$ SEM. The limit of detection was 100 $TCID_{50}$/ml **(C, F, I)**.

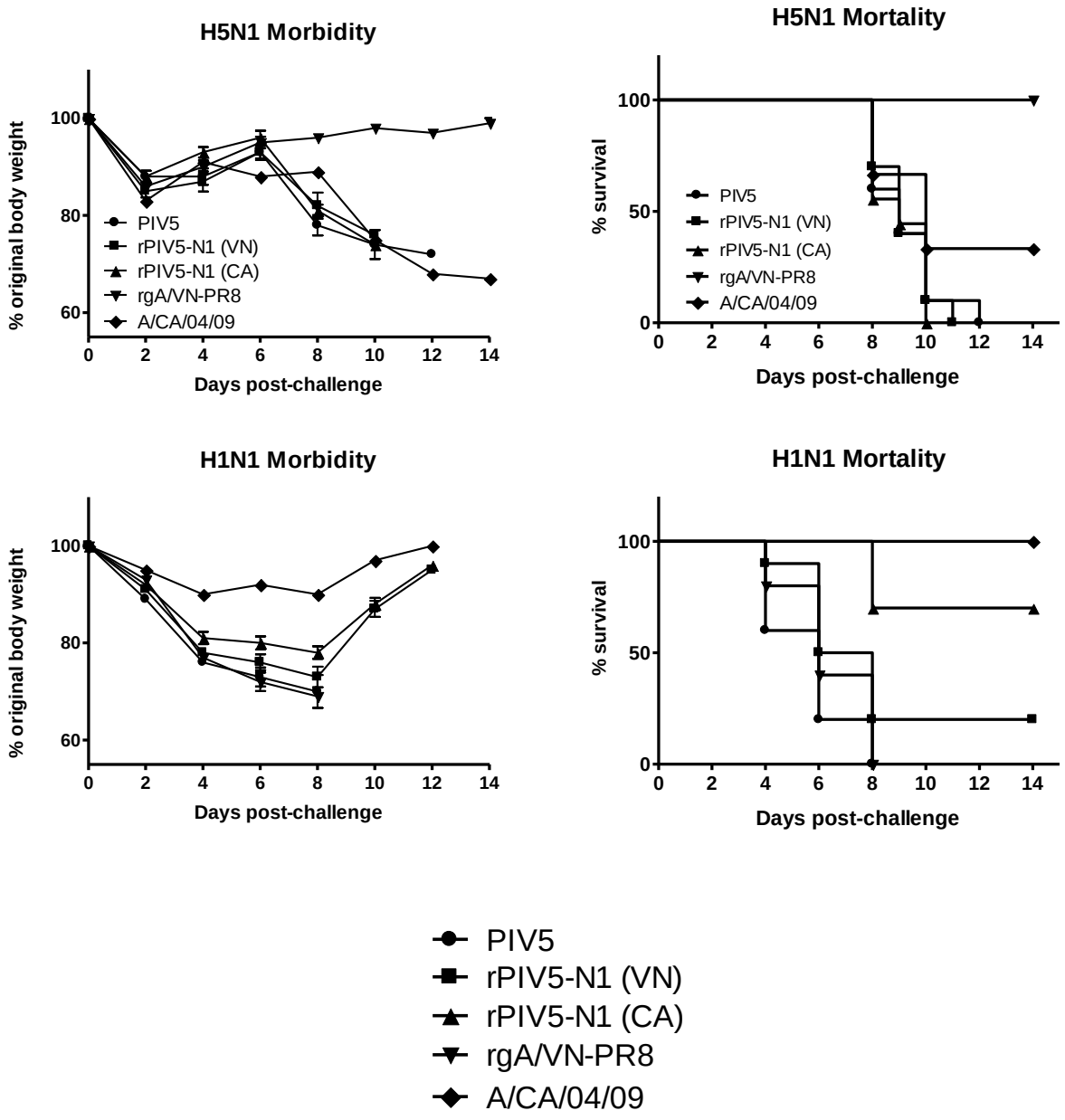


Figure 4.6: Antibodies from rPIV5-N1 play a significant role in protection.
200ug of purified IgG from each group was transferred to a recipient mouse IP. The following day, the mice were challenged with 10 LD50 HPAI H5N1 A/VN/1203/04 (A, B) or A/CA/04/09 (C, D) and monitored for (A, C) weight loss and (B, D) mortality. On day 3 post-challenge, lungs were harvested and challenge virus determined via $TCID_{50}$ (D). Error bars are representative SEM.

References

1. WHO. *Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011*. Global Alert and Response (GAR) 2011 29 November 2011 [cited 2011 December 15]; Available from: http://www.who.int/influenza/human_animal_interface/EN_GIP_20111129CumulativeNumberH5N1cases.pdf.
2. WHO. *Current WHO phase of pandemic alert for avian influenza H5N1*. Global Alert and Response (GAR) 2011; Available from: http://apps.who.int/csr/disease/avian_influenza/phase/en/index.html.
3. Whitley, Richard J. and Arnold S. Monto, *Seasonal and Pandemic Influenza Preparedness: A Global Threat.* Journal of Infectious Diseases, 2006. **194**(Supplement 2): p. S65-S69.
4. Gerdil, C., *The annual production cycle for influenza vaccine.* Vaccine, 2003. **21**(16): p. 1776-1779.
5. Horimoto, T. and Y. Kawaoka, *Strategies for developing vaccines against H5N1 influenza A viruses.* Trends in Molecular Medicine, 2006. **12**(11): p. 506-514.
6. Lipatov, A.S., et al., *Efficacy of H5 Influenza Vaccines Produced by Reverse Genetics in a Lethal Mouse Model.* Journal of Infectious Diseases, 2005. **191**: p. 1216-1220.
7. Treanor, J.J., et al., *Safety and Immunogenicity of an Inactivated Subvirion Influenza A (H5N1) Vaccine.* New England Journal of Medicine, 2006. **354**(13): p. 1343-1351.
8. Poland, G., I. Ovsyannikova, and R. Jacobson, *Immunogenetics of seasonal influenza vaccine response☆.* Vaccine, 2008. **26**: p. D35-D40.
9. Galli, G., et al., *Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine.* Proc Natl Acad Sci U S A, 2009. **106**(19416838): p. 7962-7967.
10. Subbarao, K., *Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics.* Virology, 2003. **305**: p. 192-200.
11. Chen, Z., et al., *Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein.* Journal of General Virology, 1999. **80**: p. 2559-2564.
12. Epstein, S.L., et al., *DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice.* Emerging Infectious Diseases, 2002. **8**(8): p. 796-801.
13. Tompkins, S.M., et al., *Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including Subtype H5N1.* Emerging Infectious Diseases, 2007. **13**(3): p. 426-435.

14. Tompkins, S.M., et al., *Recombinant parainfluenza virus 5 (PIV5) expressing the influenza A virus hemagglutinin provides immunity in mice to influenza A virus challenge.* Virology, 2007. **362**(1): p. 139-150.
15. Bukreyev, A., et al., *Nonsegmented Negative-Strand Viruses as Vaccine Vectors.* Journal of Virology, 2006. **80**(21): p. 10293-10306.
16. Arimilli, S., M.A. Alexander-Miller, and G.D. Parks, *A Simian Virus 5 (SV5) P/V Mutant Is Less Cytopathic than Wild-Type SV5 in Human Dendritic Cells and Is a More Effective Activator of Dendritic Cell Maturation and Function.* Journal of Virology, 2006. **80**(7): p. 3416-3427.
17. Choppin, P.W., *Multiplication of a Myxovirus (SV5) with Minimal Cytopathic Effects and without Interference'.* Virology, 1964. **23**: p. 224-233.
18. Cohn, M.L., et al., *T Cell responses to the paramyxovirus simian virus 5: studies in multiple sclerosis and normal populations.* Pathobiology, 1996. **64**(3).
19. Potter, C.W. and J.S. Oxford, *Determinants of immunity to influenza infection in man.* British medical bulletin, 1979. **35**(1): p. 69-75.
20. De Jong, J.C., et al., *Haemagglutinin-inhibiting antibody to influenza virus.* Dev Biol (Basel), 2003. **115**: p. 63-73.
21. Wright, P.F., G. Neumann, and Y. Kawaoka, *Orthomyxoviruses*, in *Fields Virology*, D.M. Knipe and P.M. Kowley, Editors. 2007, Lippincott Williams and Wilkins: Philadelphia. p. 1691-1740.
22. Murphy, B.R., J.A. Kasel, and R.M. Chanock, *Association of Serum Anti-Neuraminidase Antibody with Resistance to Influenza in Man.* N Engl J Med, 1972. **286**(5027388): p. 1329-1332.
23. Johansson, B.E., B. Grajower, and E.D. Kilbourne, *Infection-permissive immunization with influenza virus neuraminidase prevents weight loss in infected mice.* Vaccine, 1993. **11**: p. 1037-1039.
24. Kilbourne, E.D., et al., *Antiviral activity of antiserum specific for an influenza virus neuraminidase.* J. Virol., 1968. **2**(4911843): p. 281-288.
25. Powers, D.C., E.D. Kilbourne, and B.E. Johansson, *Neuraminidase specific antibody responses to inactivated influenza virus vaccine in young and elderly adults.* Clin. Diagn. Lab. Immunol., 1996. **3**: p. 511-516.
26. Johansson, B.E., D.J. Bucher, and E.D. Kilbourne, *Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection.* J. Virol., 1989. **63**(3): p. 1239-1246.
27. Sandbulte, M.R., et al., *Cross-Reactive Neuraminidase Antibodies Afford Partial Protection against H5N1 in Mice and Are Present in Unexposed Humans.* PLoS Med, 2007. **4**(2): p. e59.
28. DiNapoli, J.M., et al., *Newcastle Disease Virus-Vectored Vaccines Expressing the Hemagglutinin or Neuraminidase Protein of H5N1 Highly Pathogenic Avian Influenza Virus Protect against Virus Challenge in Monkeys.* Journal of Virology, 2010. **84**(3): p. 1489-1503.

29. Chen, Z., et al., *Cross-protection against a lethal influenza virus infection by DNA vaccine to neuraminidase.* Vaccine, 2000. **18**(28): p. 3214-3222.
30. Dong, J., et al., *Development of a new candidate H5N1 avian influenza virus for pre-pandemic vaccine production.* Influenza Other Respi Viruses, 2009. **3**(6): p. 287-95.
31. Gillim-Ross, L. and K. Subbarao, *Can Immunity Induced by the Human Influenza Virus N1 Neuraminidase Provide Some Protection from Avian Influenza H5N1 Viruses?* PLoS Med, 2007. **4**(2): p. e91.
32. Marcelin, G., et al., *A Contributing Role for Anti-Neuraminidase Antibodies on Immunity to Pandemic H1N1 2009 Influenza A Virus.* Plos one, 2011. **6**(10): p. e26335.
33. Chen, Z., et al., *The 2009 pandemic H1N1 virus induces anti-neuraminidase (NA) antibodies that cross-react with the NA of H5N1 viruses in ferrets.* Vaccine, 2012(0).
34. Webster, R.G., W.G. Laver, and E.D. Kilbourne, *Reactions of antibodies with surface antigens of influenza virus.* J Gen Virol, 1968. **3**(5711432): p. 315-326.
35. Abed, Y., et al., *Divergent evolution of hemagglutinin and neuraminidase genes in recent influenza A:H3N2 viruses isolated in Canada.* J Med Virol, 2002. **67**(12116009): p. 589-595.
36. Kilbourne, E.D., B.E. Johansson, and B. Grajower, *Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins.* Proceedings of the National Academy of Sciences, 1990. **87**(2): p. 786-790.
37. Harley, V.R., C.W. Ward, and P.J. Hudson, *Molecular cloning and analysis of the N5 neuraminidase subtype from an avian influenza virus.* Virology, 1989. **169**(1): p. 239-243.
38. Sylte, M.J. and D.L. Suarez, *Influenza Neuraminidase as a Vaccine Antigen*, in *Vaccines for Pandemic Influenza*, R.W. Compans and W.A. Orenstein, Editors. 2009, Springer Berlin Heidelberg. p. 227-241.
39. Shi, J., et al., *Protective efficacy of an H1N1 cold-adapted live vaccine against the 2009 pandemic H1N1, seasonal H1N1, and H5N1 influenza viruses in mice.* Antiviral Research, 2012. **93**(3): p. 346-353.
40. Soboleski, M.R., et al., *Cold-Adapted Influenza and Recombinant Adenovirus Vaccines Induce Cross-Protective Immunity against pH1N1 Challenge in Mice.* Plos one, 2011. **6**(7): p. e21937.
41. Matrosovich, M., et al., *New low-viscosity overlay medium for viral plaque assays.* Virology Journal, 2006. **3**(1): p. 63.

CHAPTER 5

RECOMBINANT PIV5 VACCINE PROTECTS AGAINST HPAI H5N1 INFECTION WHEN DELIVERED INTRANASALLY OR INTRAMUSCULARLY.

Alaina Jones Mooney, Zhuo Li, Jon D. Gabbard, Daniel A. Dlugolenski, Scott K. Johnson, Ralph A. Tripp, Elizabeth MI Uhl, Biao He, S. Mark Tompkins. To be submitted to *Vaccine.*

Abstract

Highly pathogenic avian influenza viruses (HPAIV) are considered a potential pandemic threat. Mass vaccination is considered the most effective method of controlling influenza in humans. There are a number of drawbacks associated with current vaccine strategies, including poor immunogenicity and safety and production issues. Viral-vectored vaccines offer an egg-free alternative. Parainfluenza virus 5 (PIV5), a non-segmented, negative-stranded RNA virus (NNSV) in the family *Paramyxoviridae*, is a favorable vector candidate for a number of reasons, including a simple, stable, and well-understood genome, making it an ideal candidate for foreign gene insertion and vaccine use. We have previously shown that PIV5 expressing HA from H5N1 is protective against HPAIV H5N1 challenge when administered intranasally. Because PIV5 is not sialic acid-restricted for replication, it is possible to utilize alternate routes of administration. Here, we extend our previous work to show that rPIV5-H5 is effective at providing protection against HPAIV H5N1 when administered intramuscularly as well as intranasally.

Introduction

Influenza is a negative-sense, segmented RNA virus in the family Orthomyxoviridae. It is classified into subtypes based on the major antigenic surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Thus far there are 17 different HA subtypes and 9 different NA subtypes [506], all containing segments of avian origin [7]. Influenza has the capacity to reassort, whereby gene segments are exchanged creating a new influenza virus to which the population is immunologically naïve. It was believed reassortment was necessary for human infection until, in 1997, eighteen humans in Hong Kong were infected with highly pathogenic avian influenza (HPAI) A virus subtype H5N1 and 6 of those persons died [8]. It is now believed that the 1918 'Spanish flu', the deadliest influenza pandemic in recorded history, was generated by a similar mechanism [9]. HPAI viruses are now considered a potential pandemic threat and since their emergence in humans there has been a reported total of 571 cases causing 335 deaths (as of November, 2011) [10].

Vaccination is considered the most effective approach of controlling seasonal influenza as well as potentially pandemic viruses in humans. Inactivated vaccines grown in embryonated chicken eggs remain the standard. They are most widely used for prevention of seasonal influenza [13]. There are a number of limitations with this production strategy for HPAI H5N1, including egg-based limitations, which could severely limit the response if this virus were to become a pandemic virus. There are a number of candidate vaccines in clinical trials at this time (reviewed in

[18]), including inactivated viruses formulated with a variety of adjuvants, such as oil-in-water, and live-attenuated influenza vaccines [19, 20]. These approaches do not, however, address the concerns of using an egg-based vaccine for prepandemic preparedness.

We have shown previously that parainfluenza virus 5 (PIV5), a non-segmented negative stranded RNA virus (NNSV), expressing the HA from HPAI H5N1 (rPIV5-H5) is a safe and effective vaccine against HPAI H5N1 (A/VN/1203/04) in mice. Vaccination primes a T cell response as well as a protective neutralizing antibody response. While intranasal (IN) immunization is appealing, having the potential for induction of mucosal antibody responses and avoiding the use of needles for vaccines, there are potential drawbacks. There are potential contraindications regarding the use of live, intranasal virus as a vaccine in immune-compromised populations. An injectable vaccine may avoid this issue and provide opportunity for mass vaccination in agricultural applications. Here, we extend our previous work, comparing the efficacy of rPIV5-H5 vaccines delivered by alternate routes and show that rPIV5-H5 is protective against HPAI H5N1 challenge when administered not only intranasally, but intramuscularly as well.

Results

A recombinant PIV5 construct expressing the HA (H5) from HPAI H5N1 was previously generated and shown to be protective against influenza virus challenge in mice (see chapter 3). Immunization with rPIV5-H5 generated a high serum neutralizing antibody titer that conferred protection with passive transfer. It was shown previously that HA is incorporated into the surface of the rPIV5-H5 virion, so we also sought to determine if inactivated rPIV5-H5 would be efficacious as a vaccine against H5N1 influenza. To determine if rPIV5-H5 is immunogenic when administered intramuscularly (IM), mice were vaccinated with rPIV5-H5 IN with live virus (ZL46), and IM with live or inactivated (iZL46) virus. A last group of mice was given inactivated A/VN/1203/04 (iA/VN/1203/04) as a positive control. Mice were bled on days 7, 14, and 21, and their sera assessed for HA-specific IgG and influenza neutralizing antibodies. Mice vaccinated intransally and intramuscularly with rPIV5-H5 (ZL46) produced high levels of IgG **(figure 5.1a)** and neutralizing antibodies **(figure 5.1b**), with IN administration yielding slightly higher titers. Mice vaccinated with inactivated ZL46 (iZL46) produced barely detectable levels of IgG and neutralizing antibody, indicating that live virus may be required for sufficient immunogenicity. As expected, PIV5-vaccinated mice produced no detectable HA-specific IgG antibodies or rgA/VN-PR8-neutralizing antibodies **(figure 5.1a&b).**

One of the primary benefits of using an intranasal vaccine is the propensity to induce a mucosal immune response. To assess differences in the IgA in mice vaccinated with rPIV5-H5 IM versus IN, mice were vaccinated with PIV5, rPIV5-H5

(ZL46) IM or IN, or inoculated with a sub-lethal dose of rgA/VN-PR8 and nasal washes and bronchial alveolar lavages (BAL) performed on days 14 or 21. No IgA was detectable in the nasal lavage or BAL fluid in mice vaccinated IM with rPIV5-H5 (data not shown). This was not unexpected as this route of administration does not take advantage of the mucosal route. Intranasal administration of rPIV5-H5 induced robust IgA response in both the nasal passages and lungs and was higher at day 14 than at day 21 **(figure 5.1c&d)**. The mucosal IgA response in influenza-inoculated mice continued to rise after day 14, possibly due to the longer time of virus replication before clearance as compared to the rPIV5 (data not shown).

To assess differences in T cell priming with route of administration, mice vaccinated IM or IN with rPIV5-H5 or rgA/VN-PR8 were euthanized on day 12 post-infection and lymph node lymphocytes assayed for influenza-specific, IFN-γ producing T cells. Intranasal vaccination with rPIV5-H5 or influenza virus primed robust influenza-specific T cell responses, but also had increased non-specific activation as compared to IM-immunized mice **(figure 5.1e)**. IM vaccination with rPIV5-H5 (ZL46) primed an A/VN/1203/04-specific T cell response more effectively that IM administration of rgA/VN-PR8, showing and advantage over IM vaccination with influenza virus, as T cells could play a role in protection against influenza virus infection. .

To determine if this mucosal response is necessary for protection against influenza virus infection, i.e. if IM immunization with rPIV5-H5 could protect against challenge, mice were vaccinated with PIV5, rPIV5-H5, or rgA/VN-PR8, delivered IN

or IM. A group of mice were also vaccinated with inactivated PIV5-H5 IM (iZL46) to determine if the weak IgG responses detected (Figure 1a) were protective. On day 28 post-immunization, mice were challenged with 10 LD_{50} HPAI H5N1 A/VN/1203/04. Consistent with observed antibody titers, mice vaccinated with rPIV5-H5 IM were protected from morbidity and mortality associated with HPAI H5N1 challenge similarly to mice vaccinated IN **(figure 5.2a&b)**. Protection was not observed in mice vaccinated with inactivated rPIV5-H5 (iZL46), confirming that live virus, and presumably replication, is required for induction of protective immunity. Interestingly, although virtually no weight loss was observed, there was no reduction in viral load in the lungs on day 3 post-challenge in mice vaccinated IM, whereas mice vaccinated IN had no detectable virus in the lungs **(figure 5.2c)**. So, while both routes of administration are protective (as measured by weight loss and survival), induction of mucosal IgA response and/or the increased IFN-γ T cell numbers associated with IN immunization are limiting infection and virus replication in the lung.

Discussion

Unlike influenza virus, which generally replicates in airway epithelial cells, PIV5 has the potential for broader cellular tropism. This feature makes it an appealing candidate for use as a live intramuscular vaccine. However, this also presents the possibility that it could disseminate to other tissues. Previous studies found no evidence of pathology in other tissues after IN PIV5 infection, although they did not look for virus in these tissues [24], suggesting IM immunization with a rPIV5 vector would be safe, however it would be interesting to assess where virus may be going in the event that it is not restricted to the airway by the route of administration or the immune response.

Inactivated rPIV5-H5 was not efficacious as a vaccine. It is possible that the amount of HA incorporated into the virion is simply insufficient to effectively prime a protective antibody response against influenza infection. Alternatively, the influenza virus may contain other antigens or PAMPs that more effectively prime the response (i.e. act as an adjuvant) and the PIV5 virus lacks these stimulatory molecules. In either case, replication competent rPIV5-H5 overcame this deficiency, priming both T cell and neutralizing antibody responses that protected against homologous HPAI challenge. With the potential for broad cellular tropism, PIV5 is likely replicating in the muscle tissue at the site of immunization; however, it would be interesting to see if the virus is infecting lympjoid cells and replicating in draining lymph nodes, thus priming an enhanced immune response as compared to inactivated virus.

Mucosal antibodies have been associated with protection from both homologous and heterosubtypic immunity ([507-509]. Here, we found that IM administration of a live rPIV5-H5 vaccine failed to induce mucosal IgA responses (figure 1 c and d), but protected against lethal H5N1 challenge **(Figure 5.2 a and b)**. Mucosal immunization (IN) with the same vaccine primed virus neutralizing serum antibody titers equivalent to IM administration **(figure 5.1 a and b)** and also induced virus-specific lung and nasal IgA **(figure 5.1 c and d)**. These mice were also protected from mortality associated with a lethal H5N1 infection, but moreover, had no detectable virus in the lung on day 3 post-infection, whereas mice without detectable IgA (IM immunized groups) has virus titers similar to negative controls **(Figure 5.2c)**. Together, these results show IN and IM administration of the live rPIV5 vaccine to be effective, however the IN delivery is likely the most effective route of administration.

Although we have repeatedly shown intranasal administration of rPIV5-H5 to be safe in mice (chapter 3 and [24]), live replicating virus-vectored vaccines can be of concern for asthmatic or immune-compromised patients. The option of IM administration without modification of the vaccine would be provide an appealing alternative to IN immunization, without a modification of the vaccine platform. This is in contrast to the current alternatives, where either a live-attenuated influenza virus vaccine or a split, inactivated wild type virus vaccine is delivered IN or IM, respectively [251]. It has the potential to marry the advantages of an egg-free influenza vaccine system with the convenience of the choice between IN and IM.

Materials and Methods

Influenza viruses used include rg A/VN-PR8 and A/Vietnam/1203/04 (H5N1). rgA/VN-PR8, provided by Rubin Donis (CDC) were propagated in the allantoic cavity of embryonated hen eggs at 37°C for 48-72 hours. β-propiolactone (BPL)-inactivated A/Vietnam/1203/04 was provided by Richard Webby from St. Jude Children's Research Hospital. The HPAI virus was propagated in the allantoic cavity of embryonated hen eggs at 37°C for 24 hours. All viruses were aliquoted and stored at -80°C. All experiments using live highly pathogenic H5N1 avian influenza viruses were reviewed and approved by the institutional biosafety program at the University of Georgia and were conducted in biosafety level 3, enhanced containment following guidelines for use of Select Agents approved by the CDC.

Mice Female 6 to 8 week old BALB/c mice (Charles River Labs, Frederick, MD) were used for all studies. Mouse immunizations and studies with BSL2 viruses were performed in enhanced BSL2 facilities in HEPA filtered isolators. Mouse HPAI infections were performed in enhanced BSL3 facilities in HEPA filtered isolators following guidelines approved by the institutional biosafety program at the University of Georgia and for use of Select Agents approved by the CDC. All animal studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia.

Cells Madin-Darby Canine Kidney (MDCK) cells were cultured in DMEM with 5% FBS, 5% L-glutamine, and an antibiotic/antimycotic solution (10,000 IU/ml penicillin, 10,000 ug/ml streptomycin, and 25ug/ml amphotericin B) (Cellgro

Mediatech, Inc). VERO cells were cultured in Minimum Essential Medium (MEM) (Thermo/Hyclone) with 10% FBS and antibiotic/antimycotic. All cells were incubated at 37°C, 5% CO_2.

Construction of recombinant viruses rPIV5-H5 (ZL46) was generated as described previously (chapter 3). Briefly, a recombinant PIV5 plasmids containing the HA gene was ZL46 (rPIV5-H5-SH/HN) generated. To generate ZL46 plasmid, the plasmid BH276 containing full length genome of PIV5 was used as the vector. The gene end (GE), intergenic region and gene start (GS) sequence between SH and HN gene was added into the primer to stop HA gene transcription and start HN gene transcription. The HA gene was them amplified. Viruses were then rescued and sequenced as described previously.

PIV5 and rPIV5 virus stocks were grown in MDBK cells (<p20) for 5-7 days in DMEM containing 2% FBS until their hema-adsorption titers plateaued. Media was collected and clarified by centrifuging at 3000 rpm for 10 minutes in an Eppendorf tabletop centrifuge (5810 R). Bovine serum albumin (BSA) was added to the clarified supernatant to bring the total solution to 1% BSA. The virus stocks were then aliquoted and frozen quickly in dry ice and stored at -80°C. Virus titers were then determined by plaque assay on VERO cells (described below).

Virus Quantitation: PIV5 titers were determined by plaque assay on VERO cells. VERO cells were incubated with serial dilutions of virus samples made in DMEM with 1% BSA and antibiotic/antimycotic. Virus sample was then removed and overlayed with 1:1 low-melt agarose and DMEM with 2% FBS and

antibiotic/antimycotic and incubated at 37°C for 5-6 days. To detect plaques, the monolayers were then fixed with 10% buffered formalin and immunostained. Cells were permeabilized with 1X PBS with 2% FBS, 0.1% sodium azide, and 0.5% saponin (permeabilization buffer). PIV5 was detected using a 1:1000 dilution of antibodies specific to the shared region of the V and P proteins of PIV5 (V/P) for 1hr. Horseradish peroxidase (HRP)-tagged goat-anti-mouse IgG (H&L) secondary antibody (Invitrogen) was then added and incubated for 30min. To visualize plaques, TMB peroxidase substrate (prepared according to manufacturer's instructions) was added (Vector Labs, Inc). The plates were then washed and dried and the plaques were counted. Influenza titers were determined either by $TCID_{50}$ assay as previous described [227] or by plaque assay on MDCK cells. MDCK cells were incubated for 2 hours at 37°C with serial dilutions of virus samples made in MEM with 1 mg/ml TPCK-treated trypsin (Worthington Biochemical). Diluted virus samples were then removed and monolayers were overlayed with 1.2% microcrystalline cellulose Avicel[500] with 1 mg/ml TPCK-treated trypsin. Plates were incubated for 72 hours, the overlay gently washed off with PBS, fixed with cold methanol/acetone (40:60%), air-dried, counter-stained with crystal violet, and plaques visualized.

Immunization For vaccination with PIV5 and rPIV5-H5, 10^6 PFU PIV5 or rPIV5-ZL46 in 50µl PBS was administered intranasally to mice anesthetized with 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Aldrich Chemical Co). *For sub-lethal* rg A/VN-PR8 infection, 2000 PFU virus in 50µl PBS was administered as described

for PIV5 vaccination. For rgA/VN-PR8 intramuscular vaccination, 2000 PFU rgA/VN-PR8 was administered in 50 µl PBS in the caudal thigh muscle. Blood was collected on day 21 post-immunization. Nasal washes and bronchial alveolar lavages (BAL) were performed on days 14 or 21 post-vaccination using 0.5 or 1ml PBS respectively.

ELISA HA (H5)-specific serum antibody titers were measured using an IgG ELISA. Immulon 2 HB 96-well microtiter plates (*ThermoLabSystems)* were coated with 2 µg/ml recombinant H5 protein and incubated at 4°C overnight. Plates were then washed with KPL wash solution (KPL, Inc) and the wells blocked with 200 µl KPL Wash Solution with 5% non-fat dry milk and 0.5% BSA (blocking buffer) for 1hr at room temperature. Serial dilutions of serum samples were made (in blocking buffer) and transferred to the coated plate and incubated for 1hr. To detect bound serum antibodies, 100µl of a 1:1000 dilution alkaline phosphatase-labeled goat anti-mouse IgG (KPL, Inc) in blocking buffer was added per well and incubated for 1hr at room temperature. Plates were developed by adding 100µl pNPP phosphatase substrate (KPL, Inc) per and the reaction allowed to develop at room temperature. Optical density (OD) was measured at 405 nm on a Bio-Tek Powerwave XS plate reader. The IgG titer was determined to be the lowest serum dilution with an OD greater than the mean OD of naïve serum plus 2 standard deviations.

Microneutralization Assay Influenza neutralizing antibody titers were measured in serum by a micro-neutralization assay with an ELISA endpoint. Heat inactivated serum was serially diluted in DMEM with 1% BSA, antibiotic/antimycotic , and 1

μg/ml TPCK trypsin. Diluted serum was then incubated 1000 $TCID_{50}$ rg A/VN-PR8 for two hours at 37°C. MDCK cells were then added and incubated at 37°C for 18-24 hours. At the end of the incubation, wells were fixed with ice cold methanol and acetone (80:20 respectively) and an ELISA was performed as described above. The neutralization titer was determined to be the lowest serum dilution capable of neutralizing 1000 $TCID_{50}$ rg A/VN-PR8, as described in chapter 3.

Lymphocyte Harvest 12 days post-vaccination with PIV5, ZL46, or rg A/VN-PR8, mediastinal lymph nodes (MLN) from mice were harvested, pooled, and homogenized. Lymphocytes were depleted of erythrocytes using Gey's Balanced Salt solution (Sigma-Aldrich) for 5min at room temperature and debris removed. Cells were then counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter).

Enzyle-linked Immunosorbert Spot (ELISpot) Assay ELISpot to detect T-cell responses in lymphocytes to inactivated A/VN/1203/04 were performed as described [23]. Cells were re-stimulated with inactivated A/VN/1203/04 (the equivalent of 10 HAU per well), Ebola GP P2 EYLFEVDNL as an irrelevant peptide (1μg/ml), and Concanavalin A (2μg/ml) in 50μl Complete Tumour Medium (CTM). *Spots were* counted using AID ViruSpot Reader (Cell Technology, Inc).

HPAI A/Vietnam/1203/04 (H5N1) challenge experiments BALB/c mice were first vaccinated with wild type PIV5, rPIV5-ZL46, or rgA/VN-PR8 as described above. 21 days post-vaccination, the mice were bled for serum analysis via the tail vein. On day 24 post-vaccination, mice were anesthetized and inoculated

intranasally with 10 LD50 A/Vietnam/1203/04 diluted in 50µl PBS. Mice were then monitored daily for morbidity and mortality with body weights measured every other day. On day 3 post-challenge, groups of mice were euthanized and their lungs collected into 1.0ml PBS and homogenized. Homogenate was then cleared by centrifugation. A TCID50 assay was then used to determine virus titers in cleared homogenate as described [227].

Statistical Analyses were performed using GraphPad Prism®.

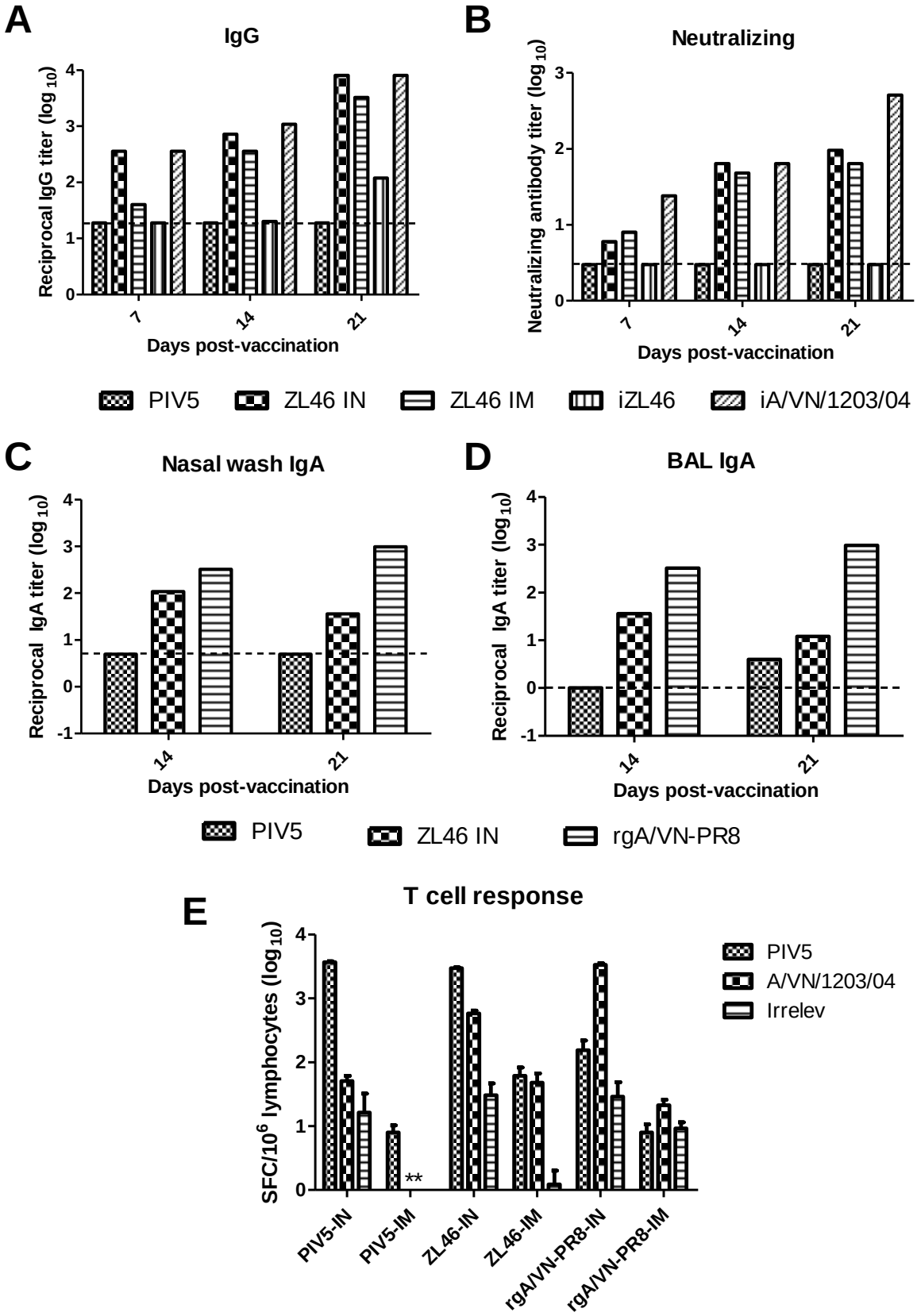

A
IgG
Reciprocal IgG titer (log 10)
Days post-vaccination
B
Neutralizing
Neutralizing antibody titer (log 10)
Days post-vaccination
PIV5
ZL46 IN
ZL46 IM
iZL46
iA/VN/1203/04
C
Nasal wash IgA
Reciprocal IgA titer (log 10)
Days post-vaccination
D
BAL IgA
Reciprocal IgA titer (log 10)
Days post-vaccination
PIV5
ZL46 IN
rgA/VN-PR8
E
T cell response
SFC/10^6 lymphocytes (log 10)
**
PIV5-IN
PIV5-IM
ZL46-IN
ZL46-IM
rgA/VN-PR8-IN
rgA/VN-PR8-IM
PIV5
A/VN/1203/04
Irrelev

Figure 5.1: Humoral and cell-mediated immune responses to immunization with rPIV5-H5 in mice vaccinated intranasally and intramuscularly

BALB/c mice (n=5 per group) were immunized with PIV5 IN, ZL46 IN or IM, inactivated ZL46 IM, or inactivated A/VN/1203/04 IM. Mice were bled on day 21 post-immunization. Serum was pooled for analysis. (A) HA (H5) specific antibody titers were measured in serum samples using an IgG (H&L) specific ELISA. The dotted line represents the limit of detection. (B) rg A/VN-PR8-neutralizing antibody titers in post-immunization serum. Mice were immunized IN or IM with PIV5, ZL46, or a sub-lethal dose of rgA/VN-PR8 and nasal washes (C) and bronchial alveolar lavages (BAL) (D) were performed on days 14 or 21. Samples were pooled for analysis by HA-specific IgG ELISA. (E) IFN-γ producing lymphocytes (pools of n=3 mice per group) in the mediastinal lymph nodes on day 12 post-vaccination as determined by ELISpot analysis. Data is presented as mean $\pm$ SEM.

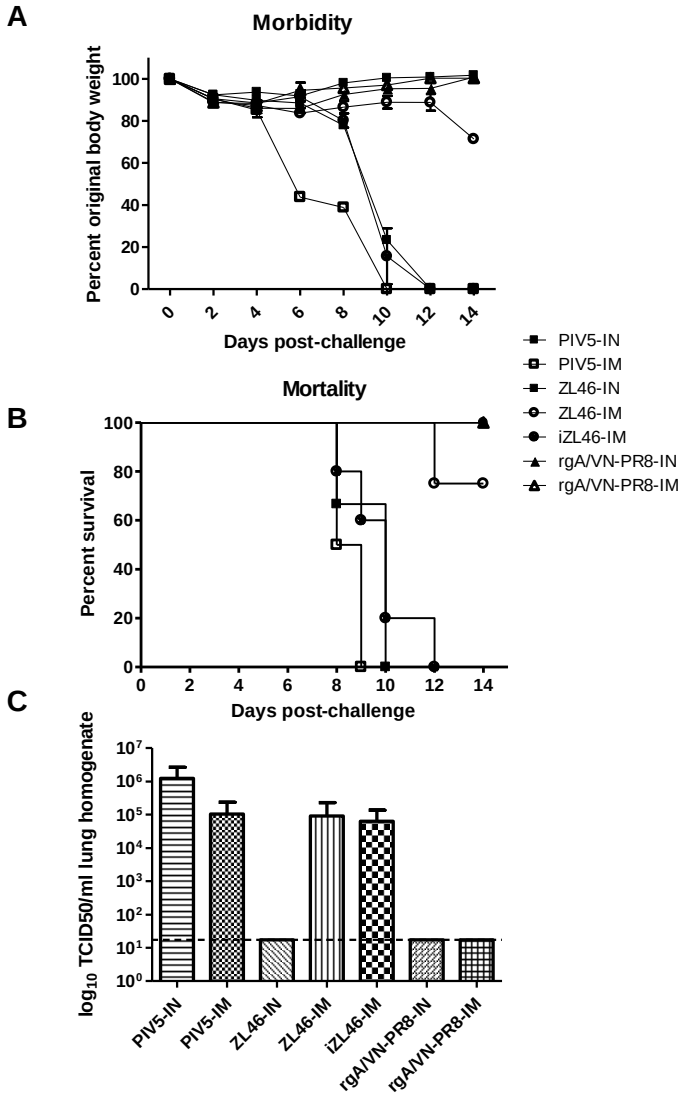


Figure 5.2: Effect of administration route on protection against HP A/VN/1203/04
BALB/c mice were immunized IN or IM with PIV5, rPIV5-H5 (ZL46), inactivated rPIV5-H5 (iZL46) IM, a sublethal dose of rgA/VN-PR8 IN, or rgA/VN-PR8 IM. 28 days p.i., the mice were challenged IN with 10 LD_{50} A/VN/1203/04. (A) Weights of the mice were monitored and presented as the mean percentage $\pm$ SEM of their pre-challenge body weights (n=8). (B) Percent of mice surviving post-challenge. (C) Challenge virus titer in the lungs on day 3 post-challenge (n=5 per group) as measured by TCID50 on MDCK cells. Data are presented as mean log transformed TCID50/ml lung homogenate $\pm$ SEM. The limit of detection was 100 TCID50/ml.

References

1. Tong, S., et al., *A distinct lineage of influenza A virus from bats.* Proceedings of the National Academy of Sciences, 2012.
2. Reid, A.H., J.K. Taubenberger, and T.G. Fanning, *Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus.* Nature Rev. Microbiol., 2004. **2**(15494747): p. 909-914.
3. Prevention, C.f.D.C.a., *Update: Isolation of Avian Influenza A(H5N1) Viruses From Humans—Hong Kong, 1997-1998.* JAMA: The Journal of the American Medical Association, 1998. **279**(5): p. 347-348.
4. Taubenberger, J.K., et al., *Characterization of the 1918 influenza virus polymerase genes.* Nature, 2005. **437**(16208372): p. 889-893.
5. WHO. *Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2011*. Global Alert and Response (GAR) 2011 29 November 2011 [cited 2011 December 15]; Available from: http://www.who.int/influenza/human_animal_interface/EN_GIP_20111129CumulativeNumberH5N1cases.pdf.
6. Gerdil, C., *The annual production cycle for influenza vaccine.* Vaccine, 2003. **21**(16): p. 1776-1779.
7. Poland, G., I. Ovsyannikova, and R. Jacobson, *Immunogenetics of seasonal influenza vaccine response ☆.* Vaccine, 2008. **26**: p. D35-D40.
8. Galli, G., et al., *Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine.* Proc Natl Acad Sci U S A, 2009. **106**(19416838): p. 7962-7967.
9. Subbarao, K., *Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics.* Virology, 2003. **305**: p. 192-200.
10. Tompkins, S.M., et al., *Recombinant parainfluenza virus 5 (PIV5) expressing the influenza A virus hemagglutinin provides immunity in mice to influenza A virus challenge.* Virology, 2007. **362**(1): p. 139-150.
11. Quan, F.-S., et al., *Induction of Heterosubtypic Immunity to Influenza Virus by Intranasal Immunization.* Journal of Virology, 2008. **82**(3): p. 1350-1359.
12. Ye, J., et al., *Intranasal Delivery of an IgA Monoclonal Antibody Effective against Sublethal H5N1 Influe nza Virus Infection in Mice.* Clinical and Vaccine Immunology, 2010. **17**(9): p. 1363-1370.
13. Tumpey, T.M., et al., *Mucosal Delivery of Inactivated Influenza Vaccine Induces B-Cell-Dependent Heterosubtypic Cross-Protection against Lethal Influenza A H5N1 Virus Infection.* Journal of Virology, 2001. **75**(11): p. 5141-5150.
14. CDC, *Prevention and Control of Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices (ACIP)*, in *MMWR*2011. p. 1128-1132.

15. Soboleski, M.R., et al., *Cold-Adapted Influenza and Recombinant Adenovirus Vaccines Induce Cross-Protective Immunity against pH1N1 Challenge in Mice.* Plos one, 2011. **6**(7): p. e21937.
16. Matrosovich, M., et al., *New low-viscosity overlay medium for viral plaque assays.* Virology Journal, 2006. **3**(1): p. 63.
17. Tompkins, S.M., et al., *Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including Subtype H5N1.* Emerging Infectious Diseases, 2007. **13**(3): p. 426-435.

CHAPTER 6

CONCLUSIONS

The studies described here were designed to develop a novel, safe, and effective vaccine against influenza. The hypothesis addressed is that parainfluenza virus 5 (PIV5) will be a safe viral vector for the delivery of influenza antigens, and that vaccination with recombinant PIV5 expressing influenza proteins will be immunogenic and provide protection against influenza virus challenge. The specific aims addressing this hypothesis were:

Specific Aim 1: To test the safety and efficacy of a PIV5-vectored vaccine expressing the HA of HPAIV H5N1 (rPIV5-H5). The *working hypotheses* are that the expression of HA is safe and immunogenic, that insertion of the HA closer to the leader sequence will increase vaccine efficacy, and that vaccination with rPIV5-H5 will induce HPAIV H5N1 neutralizing antibodies and protect against challenge. The data in chapter 3 indicates that the HA from HPAIV H5N1 inserted into PIV5 is expressed at high levels and that relative proximity to the leader sequence increases expression. HA insertion does not increase virulence of the vector. Vaccination with rPIV5-H5 induces a strong HA-specific neutralizing antibody response as well as a T cell response. Immunity generated by vaccination with rPIV5-H5 is protective against HPAIV H5N1 challenge and antibodies are sufficient for protection.

Specific Aim 2: To test the efficacy of PIV5-vectored vaccines expressing the NA protein of HPAIV H5N1 and the 2009 pandemic H1N1 virus. The *working hypotheses* are that NA will be expressed in its functional form and that immunization with rPIV5-NA will induce robust anti-NA antibodies that protect against homologous and heterologous influenza challenge. The data in chapter 4 indicates that rPIV5-N1 expresses neuraminidase in high levels and in its functional form. Vaccination with rPIV5-N1 induces neuraminidase inhibiting antibodies in mice as well as a robust T cell response. Vaccination with rPIV5-N1 is protective against homologous and heterologous challenge with HPAIV H5N1 and the 2009 pandemic H1N1 virus. There is also evidence that vaccination with rPIV5-N1 can induce heterosubtypic protection in mice. Although the studies in chapter 4 indicate that antibody was not sufficient for protection against HPAIV H5N1, antibody was sufficient for protection against the 2009 pandemic H1N1 virus. It is possible that under different circumstances, antibodies generated by rPIV5-N1 vaccination would be sufficient for protection against HPAIV H5N1 (higher antibody dose, etc), although it is possible that T cells play a significant role in protection as well.

Specific Aim 3: To investigate the efficacy of PIV5 as a vaccine vector when administered intramuscularly as well as intranasally. The *working hypotheses* are that PIV5 will be efficacious as a vaccine vector when administered intramuscularly. The data in chapter 5 indicates that rPIV5-H5 induces a robust antibody response when administered intramuscularly, although not as robust as when administered intranasally. Intramuscular vaccination was unable to produce a detectable mucosal

antibody response, whereas intranasal vaccination induced a strong IgA response in the lungs and nasal passages. It is possible this IgA response is responsible for the differences in protection observed when vaccinated mice were challenged with HPAIV H5N1, where mice given the intramuscular vaccine were unable to clear virus by day three post-challenge although there was no virus detectable in mice vaccinated intranasally. It is also possible that this disparity can be explained by a higher quantity or quality in the systemic antibody response as well.

Together, these findings indicate that PIV5 is an excellent viral vaccine vector for pandemic influenza. It induces a strong immune response and protects completely at low doses. It is also able to effectively accommodate more non-traditional antigens such as neuraminidase. PIV5 can also be used via alternate routes of administration, which has the potential to remove the necessity for alternate preparations of vaccine to be administered in different ways.

The knowledge obtained from investigation of the specific aims outlined above demonstrates that PIV5 is a good candidate as a viral vector for influenza virus vaccines. This work has added to our understanding of influenza vaccines and the benefits associated with the use of efficacious viral vectored vaccines in pandemic preparedness. This will aid in better vaccine design for not only pandemic influenza, but influenza in general.