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Adenovirus Vector-based Vaccine Approach for Emerging Influenza Viruses

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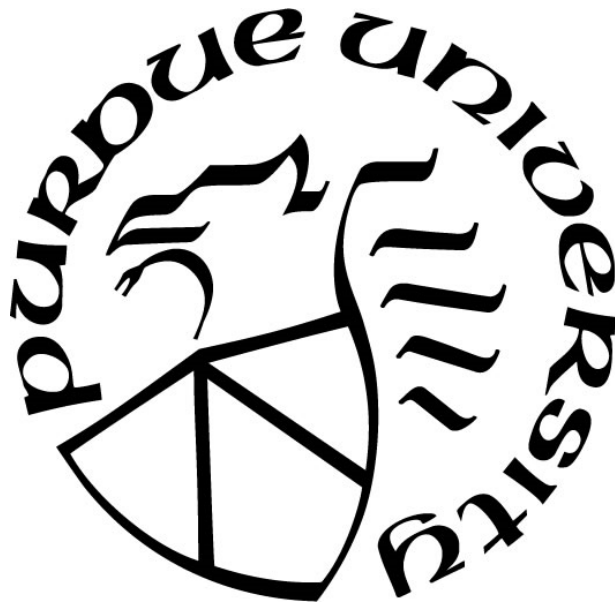# ADENOVIRUS VECTOR-BASED VACCINE APPROACH FOR EMERGING INFLUENZA VIRUSES

by

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To my family Nelly, Nelly and Bassil.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

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Since 1996, there have been numerous reports of human infections with avian influenza A viruses of subtypes H5N1, H7N7 and H9N2. In 2013, a new avian influenza virus strain of H7N9 subtype emerged in China causing more than 1559 infections in humans resulting in 616 deaths so far. Although human-to-human transmission has been infrequent and limited, genetic reassortment between avian and human/porcine influenza viruses or mutations in some of the genes leading to virus replication in the upper respiratory tract in humans could result in the generation of a novel pandemic influenza virus strain that not only can infect but also effectively transmit among the human population which would have little or no immunity to the new virus.

Various strategies to develop effective vaccines against H5, H7, and H9 viruses have been evaluated. In general, these strategies yielded low to no cross-reactivity immune responses against antigenically distinct heterologous viruses from other clades. Hence, newer vaccine approaches with the potential to induce both humoral and cellular immune responses are needed to confer protection against a broad range of influenza viruses and their clades and subclades emerging from avian reservoirs. The main aim of this project is to determine whether bovine adenovirus (Ad) [BAd] vector-based influenza vaccine approach will be better than that of the human Ad [HAd] vector-based strategy. The proposal is based on the hypothesis that intranasal immunization with BAd vector-based influenza vaccine may serve a better option due to the presence of sialic acid receptors (the primary receptors for BAd internalization) in the respiratory tract.

The goals of this proposal are: i) Determination of bovine adenoviral vector-based H5N1 influenza vaccine immunogenicity and protection efficacy in a mouse model (Aim 1); ii) Determination of the decline of Ad vector immunity with time and its effect on repeat immunization with the same

Ad vector (Aim 2); and iii) Potential approach to obtain consistency in titration of various adenoviral vectors (Aim 3).

Since the nature of the next pandemic influenza virus is unknown, Dr. Mittal's laboratory overall efforts will be directed towards the generation of universal influenza vaccines that are broadly protective against H5, H7 and H9 influenza subtypes (as well as H1 and H3 subtypes) and thus could significantly lower morbidity, hinder transmission and prevent mortality in a pandemic situation before a strain-matched vaccine can be produced.

CHAPTER 1.   INTRODUCTION

1.1   Abstract

Introduction: Annual vaccination is one of the most efficient and cost-effective strategies to prevent and control influenza epidemics. Most of currently available influenza vaccines are strong inducer of antibody responses against viral surface proteins, hemagglutinin (HA) and neuraminidase (NA), but are poor inducers of cell-mediated immune responses against conserved internal proteins. Moreover, due to the high variability of viral surface proteins because of antigenic drift or antigenic shift, many of the currently licensed vaccines confer little or no protection against drift or shift variants.

Areas covered: Next generation *influenza* vaccines that can induce humoral immune responses to receptor-binding epitopes as well as broadly neutralizing conserved epitopes, and cell-mediated immune responses against highly conserved internal proteins would be effective against variant viruses as well as a novel pandemic influenza until circulating strain-specific vaccines become available. Here we discuss vaccine approaches that have potential to provide broad spectrum protection against influenza viruses.

Expert opinion: Based on current progress in defining cross-protective influenza immunity, it seems that the development of a universal influenza vaccine is feasible. It would revolutionize the strategy for influenza pandemic preparedness, and significantly impact the shelf-life and protection efficacy of seasonal influenza vaccines.

1.2 Introduction

Despite being a vaccine-preventable disease, influenza continues to remain *a major public health problem* worldwide. As per the World Health Organization (WHO) estimates, influenza viruses infect 5-15% of the global population annually resulting in 250,000 to 500,000 deaths[1]. In the United States alone, influenza viruses are estimated to infect more than 50 million people every year resulting in over 200,000 hospitalizations and 30,000 -50,000 deaths[2-4]. Influenza affects people of all age groups, but the highest risk of complications occurs among children under the age of two years, adults over 65 years old, pregnant women, and people with certain medical conditions such as cancer, chronic lung disease, heart disease, diabetes, and the blood, lung, or kidney disorders.

Moreover, since 1997 there have been several reports of human infections with novel avian influenza viruses from subtypes H5N1, H7N7, H7N1, H7N3, H7N9, and H9N2 **(Fig. 1)**. As of June 2017, these viruses have resulted in over 2450 cases of human infections in more than fifteen countries. Of these, H5N1 viruses have so far accounted for over 859 cases and 453 deaths (case fatality rate of over 52%), while H7N9 virus has accounted for 1582 cases and 610 deaths (case fatality rate over 38%)[5]. Transmission of avian influenza viruses between humans has been rare, however, reassortment between circulating human influenza virus strains (e.g. H1N1, H3N2 etc.) and an avian influenza virus could generate a novel influenza virus with pandemic potential.

There have been three major influenza pandemics in the 20th century - the "Spanish flu" in 1918, the "Asian flu" in 1957 and the "Hong Kong flu" in 1968 **(Fig. 1)**. Among these, the "Spanish flu" was the most devastating resulting in about 675,000 deaths in the United States and over 50 million around the world [6,7]. Almost four decades after the last major influenza pandemic, a new swine/human/avian-origin H1N1 influenza A virus emerged in Mexico in April 2009 **(Fig. 1)[8]**. Within weeks, it spread around the world resulting in the first influenza pandemic of the 21st century. While the H1N1 pandemic was not as lethal as initially feared, its ability to spread worldwide in a short period highlighted the public health threat posed by novel influenza viruses originating from non-human reservoirs.

1.3   Current influenza vaccines and their limitations

Vaccination remains the most effective and economical way to prevent influenza infections and their complications. There are three types of seasonal influenza vaccines that are currently licensed for use in humans in the U.S.: 1) inactivated; 2) live attenuated; and 3) recombinant HA influenza vaccines [9-13]. Inactivated influenza vaccines and recombinant HA vaccines are administered intramuscularly (i.m.) and are either split-virion or subunit vaccines prepared from purified inactivated influenza viruses. Live attenuated influenza vaccines contain influenza virus strains that are adapted to grow at a lower temperature and are administered intranasally (i.n.). Recombinant and inactivated vaccines used for seasonal influenza are either trivalent or quadrivalent containing two influenza A virus strains (H1N1 and H3N2) and one or two influenza B virus strains of Yamagata or Victoria lineages. Similarly, live attenuated vaccines are trivalent and contain internal proteins from the donor strains, cold-adapted (ca) A/Ann Arbor/6/60 and ca B/Ann Arbor/1/66 and the surface proteins, HA and NA, from the circulating strains from influenza A (H1N1 and H3N2) and B viruses. Whereas, stockpiled pandemic influenza vaccines are monovalent and contain only the virus strain that has the potential to cause the pandemic. Such inactivated or live attenuated vaccines are prepared from reverse genetics-derived influenza viruses containing the HA (with a modified cleavage site) and NA from the target influenza virus and the remaining six gene segments from a donor strain, A/Puerto Rico/8/1934 (H1N1) [A/PR/8/34 (H1N1)] which was adapted to grow well in eggs. Live attenuated pandemic influenza vaccines use ca A/Ann Arbor/6/60 (H2N2) or A/Leningrad/134/17/57 (H2N2) as the background strain [14].

The effectiveness of influenza vaccines is largely dependent on the antigenic closeness of the vaccine virus strain with that of the circulating virus as well as the attack rate. A meta-analysis showed the pooled vaccine effectiveness of 70% (95% CI 55- 80) for matched viruses and 55% (95% CI: 42-65) for unmatched strains [15]. However, the effectiveness of influenza vaccine in young children and older adults is comparatively lower due to weaker immune systems in these populations [16-18]. In the context of an influenza pandemic, the seasonal influenza vaccine approach is of limited importance mainly because it is not possible to predict the nature of pandemic virus before its emergence. The time it takes to first generate a vaccine strain and to produce a pandemic vaccine during a pandemic, uncertainty of the availability of billions of

embryonated chicken eggs, and the lack of enhanced biosafety facilities to produce pandemic influenza vaccines pose obvious problems. Moreover, manufacturing and stockpiling large quantities of strain-specific pre-pandemic vaccines against various avian influenza strains with pandemic potential would be impractical. In addition, challenges exist to ensure that the stockpiled vaccines retain the potency during the storage period until they are needed. Hence, there is an urgent need to develop novel influenza vaccines which can induce broad cross-reactive immune responses that may provide some level of protection against the pandemic virus before a strain-matched vaccine can be produced. Several approaches to develop such broadly protective vaccines have been evaluated in preclinical and clinical studies. This review discusses various vaccine strategies that have demonstrated broad cross-protective immunity against influenza viruses (Table 1 & Fig 2).

1.4 Vaccine approaches targeting the HA stem region

HA is a homotrimeric integral membrane glycoprotein present on the viral envelope (Fig. 3). It consists of two domains: the globular head domain comprising the middle portion of HA1, and a stem domain that includes N-and C-terminal regions from HA1 (~50 amino acids) and the ectodomain of HA2. The head domain is responsible for the binding of the virus to the host cell surface receptor, while HA2, a membrane-proximal stem domain contains the membrane fusion domain which is necessary for the fusion of the viral envelope and the host cell membrane during the influenza virus life cycle. Currently licensed influenza seasonal vaccines as well as approved and stockpiled pandemic vaccines mainly induce antibodies directed against the globular head domain of the HA molecule. This domain is continuously under selection pressure due to the induction of virus-neutralizing antibodies following natural infection or vaccination, thus it is highly prone to mutations [19]. Hence, immunity induced by these vaccines is most effective only against homologous influenza viruses.

Unlike the HA head region, the HA stem region has been shown to be fairly conserved across influenza A subtypes [20]. The high level of conservation in the HA stem region is due to less exposure to the host immune system. Based on amino acid similarity and stem structure, influenza A viruses are divided into two major phylogenetic groups: Group 1 (subtypes H1, H2, H5, H6, H8,

H9, H11, H12, H13, H16, H17, and H18) and Group 2 (subtypes H3, H4, H7, H10, H14, and H15)[21].

Vaccine approaches targeting the conserved stem region could have potential to provide protection against novel emerging strains of influenza viruses [20,22-31]. Interestingly, several recently identified monoclonal antibodies (mAbs) demonstrating broad neutralizing activity against influenza viruses have been shown to bind to the stalk region of HA [26]. These antibodies bind to the fusion domain, a highly conserved portion in the HA stem, to prevent fusion between the viral envelope and endosomal membrane during the influenza virus life cycle. Furthermore, several studies have underlined the importance of HA stem-specific antibodies in providing heterosubtypic protection [27-34].

A peptide-based vaccine targeting the long alpha helix region (LAH) of the HA stem region showed broad protection against multiple influenza viruses [30]. The immunogen contained a linear epitope recognized by the broadly neutralizing mAb 12D1 conjugated to a carrier protein keyhole limpet hemocyanin (KLH). Immunization of mice with the peptide vaccine induced high levels of cross-reactive antibodies against HA of several Group 1 and Group 2 influenza virus subtypes. Furthermore, vaccinated mice were protected against lethal challenge with A/Hong Kong/1/68-PR8 (H3N2) [X-31], A/PR/8/34 (H1N1), or A/Vietnam/1203/2004 (H5N1) [A/VN/1203/04 (H5N1)] influenza viruses, although the immunization did not prevent morbidity.

The conserved stem portion of HA was evaluated for its ability to confer broad protection against influenza virus challenge in mice. Immunized mice exhibited high levels of cross-reactive antibodies against multiple HA subtypes and were protected against lethal challenge with A/PR/8/34 (H1N1) [32]. In a subsequent study, a similar HA2-based subunit vaccine was found to be highly immunogenic in mice conferring protection against lethal challenge with A/Hong Kong/68 (H3N2) [33]. Mallajosyula et al. developed a headless HA stem vaccine based on the group 1 A/PR8 virus [34]. Immunized mice developed broadly cross-reactive antibodies that neutralized diverse virus strains from H1, H3, and H5 influenza A subtypes and conferred protection against a homologous A/PR8, although was not able to protect against morbidity. Furthermore, this vaccine construct was not able to confer protection against heterosubtypic group

2 virus challenge with A/Hong Kong/68 (H3N2). Gong et al. evaluated a norovirus (NoV) particle-based HA2 vaccine for its ability to induce cross-protection against viruses from H1N1 and H3N2 influenza A, and Influenza B viruses [35]. The HA2 immunogen tested here contained HA2 region from 90-105 amino acid residues of subtypes H1, H3 and B in the loops 1, 2 and 3 of the protrusion (P) domain, respectively. In vaccinated mice, the trivalent HA2-P immunogen induced high levels of HA2-specific antibody responses that were further enhanced by a virus booster vaccination. Furthermore, there was good neutralization of H3N2 and B viruses, and immune mice were protected against challenge with a H3N2 virus.

Immunization with plasmid DNA or an adenovirus (Ad) vectored vaccine expressing HA protein has been shown to elicit HA stem-specific antibodies in various animal models [36]. In a recent study, priming with plasmid DNA vaccine encoding H1N1 HA and boosting with either a H1N1 influenza vaccine or an Ad-based vaccine expressing H1N1 HA elicited high levels of cross-neutralizing antibodies directed to the HA stem region. Interestingly, the prime/boost approach was found to be more effective in inducing protective immune responses against diverse H1N1 isolates than immunization with either approach alone. A modified vaccinia Ankara (MVA)-based mosaic H5 HA-based vaccine expressing the recombinant immunogen representing 2,145 H5N1 field isolates was developed [37,38]. Vaccinated mice were completely protected against challenge with H5N1 viruses from clades 0, 1, and 2 and against A/PR/8/34 (H1N1).

Sequential immunization of mice with plasmid DNAs encoding HA proteins of four different H3N2 viruses (A/Hong Kong/1/68, A/Alabama/1/81, A/Beijing/47/92, and A/Wyoming/3/03) elicited broadly-neutralizing antibodies which reacted with H3 influenza viruses originated from 1968 to 2003[24]. Interestingly, the epitopes recognized by these antibodies were shown to be present in the stem region. In another study, immunization of mice with Ad vector-based vaccines encoding HA proteins from H1, H5, H7, and H9 influenza subtypes induced very high levels of antibodies directed against linear epitopes recognized by two broadly neutralizing antibodies, 12D1 and CR6261, both of which were shown to be present in the conserved stem region of HA2[36]. Although the neutralizing abilities of these HA stem-directed antibodies were not tested, but the potential of this vaccine approach in inducing stem-specific antibodies was demonstrated.

Fusion of the viral envelope and the endosomal membrane during the influenza virus life cycle is mediated by a hydrophobic stretch of amino acids known as the fusion domain, which is present towards the amino (N)-terminal end of HA2. Since the fusion domain is not exposed on the HA surface, it is less prone to mutations, and thus it is a suitable target for developing universal vaccine approaches against influenza. A H3N2 fusion domain tagged to KLH was evaluated for its efficacy in inducing broad protective immunity against influenza viruses [39]. Immunized mice had significantly higher levels of fusion domain-specific antibodies and were protected against challenge with a low dose of either a homologous virus A/Mississippi/1/85 (H3N2) or a heterosubtypic virus A/PR/8/34 (H1N1).

A HA stem only immunogen (mini-HA) based on H1 subtype sequence of A/Brisbane/59/2007 (H1N1) demonstrated the structural and broadly neutralizing antibody (bnAb) properties similar to the whole-length HA protein [40]. Vaccination of mice with the mini-HA vaccine induced antibodies against multiple influenza virus strains from group 1 (H2, H5, H9) and group 2 (H3, H7). Furthermore, good protection was observed against heterologous A/PR/8/34 (H1N1) and heterosubtypic A/Hong Kong/156/97 (H5N1) viruses. Vaccination of cynomolgus monkeys with the mini-HA vaccine induced antibodies that cross-reacted with multiple group 1 HAs. Interestingly, both in mice and cynomolgus monkeys this vaccine induced antibodies which competed with a broadly neutralizing stem-binding mAb CR9114 and elicited antibody-dependent cell-mediated cytotoxicity (ADCC) responses to multiple HAs. In another study, a HA stem nanoparticle-based vaccine (HA-SS-np) containing the ectodomain of A/New Caledonia/20/1999 (H1N1) virus was designed [41]. Vaccination of mice and ferrets with HA-SS-np in combination with a monophosphoryl lipid A-synthetic dicorynomycolate adjuvant induced antibodies that reacted with influenza viruses from H1, H2, H5, and H9 subtypes. Following challenged with a highly pathogenic A/Vietnam/1203/2004 (H5N1) virus, complete protection was observed in the vaccinated mouse group. Ferrets vaccinated with HA-SS-np demonstrated partial protection following H5N1 virus challenge with 4/6 animals surviving. Efforts have been made to design chimeric HA-based vaccines containing the head and stem regions from different influenza A and/or B viruses. A chimeric HA immunogen (cHA/B) that contained globular head region from multiple influenza A virus subtypes: H5 (A/Vietnam/1203/2004), H7 (A/mallard/Alberta/24/2001), H8 (A/mallard/Sweden/24/2002), and stem region from influenza B virus (B/Yamagata/16/88)

was developed [42]. Vaccination of mice with cHA/B resulted in broadly cross-reactivity antibodies and protection against diverse influenza A and B viruses, and the protection was mainly due to antibody Fc-mediated effector functions.

Immune responses generated against the HA stem region appear sufficient to confer protection against low doses of challenge with heterologous influenza viruses. Further studies are needed to determine whether complete protection against higher doses of a challenge virus could be achieved and whether the level of cross-protective antibodies could be increased using an adjuvant or a novel vaccine delivery system. To enhance the protective efficacy of HA stem or fusion domain-based vaccines, inclusion of other conserved epitopes in the vaccine formulation may be necessary.

1.5   Vaccine strategies targeting matrix 2 (M2) protein

M2 is a 97 amino acid residues type III integral membrane protein sparsely present on the surface of mature virions (Fig. 3). It forms a pH activated tetrameric proton-selective ion channel and plays an important role during influenza virus replication. It consists of three structural domains: 1) a 24-residue N-terminal ectodomain (M2e) necessary for the incorporation of the M2 into mature virions, 2) a 19-residue transmembrane domain necessary for the ion channel activity, and 3) a 54 amino acids C-terminal intravirion/intracellular domain necessary for the assembly of influenza virions. Unlike HA and NA, M2e is relatively conserved across human influenza A viruses, whereas in H5N1 and H7N9, the first 10 amino acid residues of M2e are same as of H1N1 and H3N2, but the remaining 14 residues have approximately 78 and 42% amino acid identity with H5N1 and H7N9 M2e, respectively [43], therefore, it is a potential candidate for a broadly protective vaccine.

Antiviral activity of anti-M2 antibodies was first reported on the basis that a mAb (14C2) directed against M2 reduced the plaque size of several influenza virus strains. Treatment of mice with 14C2 monoclonal antibody resulted in a significant reduction in influenza A virus replication in the lungs [44,45]. Based on these early findings, several groups have since evaluated the potential of M2 as a target for universal influenza vaccines. In one of the earliest studies, immunization of mice with a baculovirus-expressed recombinant M2 protein resulted in induction of high levels of anti-M2 antibody titers leading to protection against lethal challenge either with a homologous virus

[A/Ann Arbor/6/60 (H2N2)] or a heterologous virus [A/Hong Kong/1/68 (H3N2)][46]. In another study, immunization of mice with a M2-GST fusion protein vaccine with M2-derived from a A/Aichi/2/68 (H3N2) virus induced high levels of M2-specific antibodies and conferred protection against challenge either with a homologous A/Hong Kong/1/68 (H3N2) or heterosubtypic A/Ann Arbor/6/60 (H2N2) or A/Taiwan/1/86 (H1N1) viruses[47].

A recombinant M2 protein vaccine containing three tandem copies of M2e, NP epitope and hepatitis B virus core antigen expressed in E. coli was shown to confer cross-protection in mice against challenge either with A/Beijing/501/2009 (H1N1) or A/Ostrich/SuZhou/097/2003 (H5N1)[48]. Along these lines, M2e has been conjugated with carrier proteins including KLH, bovine serum albumin, Neisseria meningitiditis outer membrane protein complex, human papillomavirus L protein, and the leucine zipper domain of yeast transcription factor GCN4 to enhance immunogenicity and protective efficacy of M2e-based vaccines by several investigators [43,49]. DNA and viral vectored vaccines encoding M2 either alone or in combination with other influenza virus proteins have also been evaluated. Mice primed with a DNA vaccine encoding M2 and boosted with an Ad-based M2 vaccine exhibited broadly cross-reactive antibodies and M2-specific T cell responses which conferred protection against challenge with A/PR/8/34 (H1N1) or A/Thailand/SP-83/04 (H5N1)[50]. In another study, immunization of mice with chimpanzee Ad vector-based vaccines encoding M2e domains from H1, H5, H7 influenza A virus subtypes fused to NP of an H1 subtype virus resulted in robust M2e-specific antibody responses and provided protection against challenge with H1N1 influenza virus strains A/PR/8/34 or A/Fort Monmouth/1/47[51].A DNA vaccine expressing a fusion product of both the H1N1 HA and M2e exhibited high levels of HA-specific and M2e-specific antibodies and CD8 T cell responses inducing cross-protection in mice against challenge with a H5N2 virus [A/aquatic bird/Korea/W81/05][52].

 It appears that M2e-specific antibodies do not prevent influenza virus infection, but are mainly responsible for the virus clearance following infection through ADCC [53]. The studies described above indicate that M2e is an interesting target for developing universal vaccines targeting influenza, although additional studies are needed to determine the full impact of M2e-based vaccine approaches.

1.6   Vaccine strategies targeting NP

NP is a major internal virion protein which encapsulates the viral genome (Fig. 3). Apart from being the most abundant protein in infected cells and virions, NP is known to play diverse roles in the influenza virus life cycle [54]. Unlike the HA and NA, NP is conserved (>90%) across influenza A viruses [55]. The cytotoxic T lymphocyte (CTL) responses induced against NP have been shown to aid in virus clearance and are critical for recovery from influenza virus infections [56-58]. Due to the high level of conservation in NP across influenza viruses, immune responses induced by NP have been shown to be cross-reactive. It is widely believed that such a cross-reactive NP-specific CD8+ T cell–mediated immunity has tremendous potential in reducing the impact of an influenza pandemic, thus making it a good candidate for developing broadly protective vaccine approaches.

Cross-protective efficacy of NP either alone or in combination with HA, M2e or M1 has been evaluated. Immunization of mice with purified NP of H3N2 influenza A virus (X31) resulted in significant cross-protection against lethal challenge with a heterosubtypic H1N1 influenza virus, A/PR/8/34[59-63]. Co-administration of ferrets with a DNA-based vaccine encoding HA from A/Hawaii/01/91 (H3N2) and NP and M1 from A/Beijing/353/89 (H3N2) was shown to provide better protection against challenge with antigenic drift variants A/Georgia/03/93 (H3N2) or A/Johannesburg/33/94 (H3N2) compared to an inactivated vaccine produced for the 1992-93 influenza season[64]. Immunization of mice with a DNA vaccine encoding the NP and matrix protein of A/PR/8/34 (H1N1) virus was shown to reduce replication of A/Hong Kong/483/97 (H5N1) and conferred protection against a lethal challenge with A/Hong Kong/156/97 (H5N1)[63].

Although DNA vaccines encoding NP have been shown to confer some level of cross-protection against influenza virus challenge in animal models, their potency needs to be enhanced before they can be used for human application. One of the approaches to enhance the efficacy of DNA vaccines is to prime with DNA vaccine and to boost with a recombinant viral vector encoding the same antigen. Mice primed with a DNA vaccine and boosted with an Ad vectored vaccine [both expressing NP of A/PR/8/34 (H1N1) virus] exhibited stronger T cell and humoral responses compared to mice immunized with either vaccine alone [62]. This prime-boost regimen provided complete protection against challenge with A/Philippines/2/82 (H3N2) virus and partial cross-

protection against highly pathogenic H5N1 viruses (A/Hong Kong/156/97 or A/Hong Kong/483/97). This prime-boost approach was also effective in significantly reducing virus titers in the lungs. To broaden the efficacy of an Ad-based HA vaccine, inclusion of NP completely protected mice from challenge with a homologous A/Indonesia/05/2005 (H5N1) virus as well as antigenically distinct A/VN/1203/04 (H5N1)[65]. Immunized mice had significantly higher levels of both HA and NP-specific CD8 T cells which apparently could be crucial for imparting protection against antigenically distinct H5N1 viruses. In a similar study, mice vaccinated i.n. with an Ad vector expressing the NP gene of A/PR/8/34 (H1N1) exhibited high levels of NP-specific humoral and cellular immune responses that conferred protection against lethal challenge either with a homologous virus [A/PR/8/34 (H1N1)] or heterosubtypic viruses [A/Philippines/2/82 (H3N2) or A/VN/1203/04 (H5N1)][66]. A chimpanzee Ad-vectored vaccine encoding the NP gene of H1N1 influenza A virus A/PR/8/34 (H1N1) was used to immunize mice[67]. Intramuscular vaccination resulted in a robust anti-NP T-cell response, although no virus-neutralizing antibodies were detected. Vaccinated mice showed an improved survival rate following challenge with a homologous virus strain [A/PR/8/34(H1N1)] as well as heterosubtypic virus strains [A/VN/1203/04 (H5N1) or A/Hong Kong/483/1997 (H5N1)]. In another study, a simian Ad-vectored vaccine expressing the fusion protein of consensus NP and M1 induced high levels of NP-specific cellular and humoral immune responses that protected mice against challenge with A/Fort Monmouth/1/47-MA (H1N1)[68].

Viral vectors based on parainfluenza virus 5 (PIV5) and modified vaccinia virus Ankara (MVA) have also been evaluated by several investigators to deliver conserved influenza A viral proteins to induce broad protection against influenza A viruses. A promising vaccine candidate, MVA/HA1/C13L/NP [MVA expressing HA of A/California/04/09 (H1N1) and NP of A/VN/1203/04 (H5N1)], fused to a secretory signal of the vaccinia virus was shown to induce cross-protective immunity against several influenza virus subtypes in mice [64, 69]. Vaccinated mice were completely protected against challenge with A/VN/1203/04 (H5N1), A/Norway/3487-2/09 (H1N1) or A/PR/8/34(H1N1), and were partially (57%) protected against challenge with A/Aichi/68 (H3N2) virus. MVA-based vectors expressing conserved influenza viral proteins have successfully been evaluated in a series of phase 1 and 2a human clinical trials and found to be safe and immunogenic. Berthoud et al. evaluated a MVA-based vaccine expressing M1 and NP (MVA-

NP+M1) in healthy adults [70]. Intramuscular vaccination with MVA-NP+M1 was well tolerated and high levels of NP and M1-specific T cells were observed in vaccinated individuals. In a subsequent study conducted in adults aged between 50-85 years, MVA-NP+M1 vaccine was also found to be well tolerated and immunogenic after a single i.m. vaccination [71-74]. Similarly, the PIV5-NP-HN/L vaccine [a PIV5-based vector expressing NP from A/VN/1203/04 (H5N1)] conferred partial protection in mice against challenge either with a homologous, A/VN/1203/04 (H5N1) or a heterosubtypic, A/PR/8/34(H1N1) virus [75, 76].

Another approach that has been evaluated to enhance the potency of NP-based vaccine involves genetic fusion or co-administration with molecular adjuvants known to chemo-attract and stimulate antigen presenting cells (APC). Fusion of NP of A/PR/8/34 (H1N1) with the herpes simplex virus type 1 protein 22 (VP22) in a DNA vaccine formulation elicited high levels of NP-specific humoral and cellular immune responses in mice [77]. Furthermore, the immunized animals were protected against lethal challenge with either a homologous virus A/PR/8/34 (H1N1) or a heterosubtypic virus A/Udorn/72 (H3N2).

1.7   Multivalent vaccination to broaden vaccine coverage

Multivalent or polyvalent vaccination involving the combined administration of two or more vaccine antigens is another approach that has been evaluated to induce broad protective immunity against influenza viruses. We developed an Ad-based multivalent influenza vaccine encoding HA genes from H5N1, H7N2, and H9N2 avian influenza virus subtypes. Vaccination of mice with this multivalent vaccine induced robust HA-specific cellular and humoral immune responses, and provided protection against heterologous H5N1, H7N2, and H9N2 influenza virus strains. Interestingly, inclusion of H5N1 NP in the vaccine formulation induced NP-specific CD8 T cell responses, and conferred heterosubtypic protection against H1N1 and H3N2 influenza viruses [36].

Similarly, an Ad vector-based multivalent vaccine incorporating HA, NA and M1 from an avian H5N1 virus [A/Chicken/Thailand/CH-2/04)] and 1918 pandemic virus [A/South Carolina/1/18 (H1N1)] inducted strong humoral and cellular immune responses against both pandemic influenza viruses in mice[78]. Immunized animals were fully protected from challenge with H5N1 strains A/VN/1203/04 or A/Indo/05/2005. A MVA-based vaccine encoding HA, NA, and NP of clade 1

A/VN/1203/04 (H5N1) virus and M1 and M2 of A/chicken/Indonesia/PA/03 (H5N1) virus along with interleukin (IL)-15 was shown to confer cross-clade protection in mice against challenge with a clade 2.2 H5N1 virus (A/chicken/Indonesia/BL/03)[79].

Several groups have evaluated co-administering the currently licensed seasonal or pandemic influenza vaccines in combination with viral vectored or DNA vaccines encoding conserved virus proteins in an effort to broaden vaccine-induced protection against diverse influenza virus strains [72]. Co-administration of a seasonal influenza vaccine with MVA-NP+M1 (a MVA vector based vaccine encoding NP and M1 proteins from H1N1 virus strain) in adults older than 50 years was shown to induce potent humoral and cellular immune responses with cross reactivity against several influenza A virus subtypes[72]. In another study, supplementation of split 2009 pandemic H1N1 vaccine with a VLP-based M2e vaccine (M2e5x VLP: VLP-based vaccine containing 5 tandem copies of M2e) induced significant cross-protection in ferrets against challenge with a seasonal H1N1 strain BR/59 compared to vaccination with the split vaccine alone[80]. Song et al. evaluated M2e5x VLP for its ability to induce cross-protective immune responses against H5N1 viruses in chickens [2]. Chickens vaccinated with H5N1-inactivated vaccine supplemented with M2e5x VLPs had improved M2e-specific antibody responses compared to vaccination with an inactivated H5N1 vaccine alone. A significantly better cross-protection was observed against antigenically distinct H5N1 viruses in the M2e5x VLP vaccine group [81].

1.8   Influenza virus-like particles vaccines

Virus-like particles (VLPs) are non-infectious macromolecular structures derived from the self-assembly of viral structural proteins. VLPs display the viral antigens mimicking the structure of live virus particles, but are devoid of the viral genetic material. The VLP-based vaccine platform has the potential for delivering multiple vaccine antigens to broaden the vaccine coverage. A VLP-based vaccine containing HA derived from H5N1, H7N2, and H2N3 strains conferred protection in ferrets against challenge with homologous viruses from H5, H7 and H2 subtypes [21]. Similarly, VLPs containing HA, NA and M1 derived from seasonal influenza viruses (H1N1, H3N2 and influenza B viruses) elicited high levels of HI antibody titers against homologous and heterologous viruses and were protected against influenza virus challenge in mice and ferrets [82]. Several groups have evaluated supplementing inactivated influenza vaccines with VLP-based M2e

vaccines in an effort to broaden protection against challenge with heterosubtypic viruses. Supplementation of an inactivated A/ PR/8/34 (H1N1) virus vaccine with VLPs containing M2 from A/WSN/33 (H1N1) induced high levels of M2e-specific antibodies in vaccinated mice and provided complete protection against challenge with a heterologous virus [A/California/04/09 (H1N1)] and heterosubtypic viruses [A/VN/1203/04 (H5N1) and A/Philippines/82 (H3N2)][83]. Addition of an alum formulated A/Wuhan/359/95 (H3N2) inactivated vaccine with a synthetic M2e peptide vaccine conferred cross-protection in mice against lethal challenge with a heterosubtypic virus A/PR/8/1934 (H1N1). Priming with a DNA vaccine encoding HA from an A/Thailand/1Kan/2004 (H5N1) virus and boosting with a VLP-based vaccine induced broad cross-neutralizing ability against all reported clades and subclades of H5N1 viruses and protected mice from a lethal dose of H5N1 challenge following both active and passive immunizations [84]. VLP-based platform appears to be ideal for simultaneous delivery of antigens from multiple strains/subtypes in one vaccine formulation thereby broadening the vaccine coverage. Immunogenicity issues associated with VLP-based vaccines, especially when using a multivalent/multiantigen vaccine formulation could be addressed using adjuvants currently licensed for other vaccines. Moreover, the performance of VLP-based vaccines has successfully been demonstrated in human clinical studies against 2009 H1N1 pandemic virus and highly pathogenic H7N9 avian influenza virus [79, 85, 86].

1.9 Enhancing vaccine efficacy using adjuvants

Identification of novel adjuvants with potential to induce broad cross-protection against antigenically diverse influenza viruses has been one of the major goals of vaccine researchers. A variety of plant, bacterial, insect and pharmaceutical compounds including immune stimulating complexes (ISCOMs), heat-labile enterotoxin (LT), cholera toxin (CT), chitosan, liposomes have been evaluated in animal models for their potential to enhance the cross-protective efficacy of influenza vaccines.

ISCOMs are spherical open cage-like structures formed by mixing quillaja saponins, cholesterol, phospholipids and vaccine antigens. Immunization of mice with an ISCOM-formulated formalin-inactivated H1N1 vaccine induced high levels of H1N1-specific cellular and humoral immune responses and provided protection against challenge with A/Japan/305/57(H2N2) or

A/Philippines/1/82(H3N2) influenza viruses [87,88]. Interestingly, vaccination with the ISCOM-formulated H1N1 vaccine induced heterosubtypic protection against challenge with A/Hong Kong/1073/99(H9N2) or A/Hong Kong/156/97(H5N1)] avian influenza viruses [89,90]. Significantly higher levels of HI antibodies were observed in vaccinated animals receiving ISCOM-formulated vaccines following i.n. and subcutaneous vaccine delivery. Efficacy of an ISCOM-formulated influenza vaccine in a Phase 1 clinical trial in healthy adults induced higher CTL activity than the unformulated vaccine against A/Taiwan/1/86 (H1N1) or A/Philippines/1/82 (H3N2)[91].

An inactivated X-31 influenza vaccine formulated with mutant derivative of LT from E. coli (R192G) was used to immunize mice i.n. and challenged with a lethal virus [A/Hong Kong/483/97 (H5N1)] and a nonlethal virus [A/Taiwan/1/86 (H1N1)][92]. Immunized mice were fully protected, and the observed heterosubtypic protection appeared to be mediated by cross-reactive HA antibodies. Similarly, i.n. immunization of an inactivated A/PR8 vaccine formulated with CT was shown to confer cross-protection in mice against lethal challenge with a heterosubtypic X-79 virus [93].

Chitosan, a carbohydrate biopolymer derivative of chitin found in mushrooms and in the exoskeletons of insects and crustaceans (shrimp, crab, and shell fish) has been shown to possess adjuvant activity in many preclinical studies. A subunit M1 vaccine containing A/chicken/Jiangsu/7/02 (H9N2) formulated with chitosan and delivered i.n. to mice induced strong M1-specific humoral and cellular immune responses and completely protected mice against challenge with a homologous virus A/chicken/Jiangsu/7/02 (H9N2) and partially protected against challenge with heterosubtypic viruses [A/PR/8/34 (H1N1) or A/chicken/Henan/12/04 (H5N1)]. In a Phase I clinical trial, a chitosan formulated inactivated trivalent seasonal influenza vaccine was well tolerated and induced a four-fold or greater increase in HI antibody titers against seasonal influenza viruses in more than 40% of the volunteers[94].

Liposomes are artificially prepared vesicles consisting of natural or synthetic phospholipids. Formulation of seasonal influenza vaccine with liposome-based adjuvants has been shown to induce cross-protection in animal studies [83]. A novel cationic liposome-based adjuvant JVRS-

100 [comprising double-stranded plasmid DNA and octadecenoyloxy[ethyl-2-heptadecenyl-3-hydroxyethyl] chloride (DOTIM) and cholesterol as liposome–DNA complexes (CLDC)] has been shown to possess adjuvant activity in several studies in mice and non-human primates[77,95,96]. An inactivated A/PR/8/34 (H1N1) influenza vaccine formulated with JVRS-100 adjuvant was shown to induce cross-protective immunity in mice against challenge with a sublethal dose of X-31 influenza virus. Similarly, an inactivated split-virion H5N1 clade 1 vaccine formulated with JVRS-100 and delivered i.m. to mice induced strong antibody responses and enhanced cross-protective efficacy [89].

MF59 is an oil-in-water emulsion adjuvant licensed for seasonal, pandemic and pre-pandemic vaccines, which has been shown to induce cross-reactive immunity against divergent influenza strains in animal models and humans. Significantly higher levels of antigen-specific cellular and humoral responses were observed when the vaccine was co-administered with MF59 compared with those receiving the vaccine without the adjuvant. A H5N1 pandemic influenza vaccine against A/turkey/Turkey/01/2005 (H5N1) when administered with MF59 induced cross-reactive antibody responses against the heterologous H5N1 virus strains [A/Indonesia/5/2005 and A/Vietnam/1194/2004] in pediatric subjects, adults (18 to 60 years) and elderly subjects ($\geq$61 years)[90]. AS03, an oil-in-water tocopherol-based emulsion adjuvant has also been evaluated to induce cross-reactive immune responses in human clinical studies [92, 97-107]. Langley et al. evaluated immunogenicity and safety of AS03-adjuvanted split-virion prepandemic H5N1 influenza vaccine [A/Indonesia/ 5/05 (IBCDC-RG2) (clade 2.1)] in a Phase 1/2 study involving 680 adults [104]. Intramuscular administration of 2 doses (3.75 µg of HA) of AS03-adjuvanted recombinant H5N1 vaccine induced significantly higher levels of antibodies that neutralized H5N1 viruses from clades 1, 2.2, and 2. Overall, the adjuvanted vaccines were well tolerated, with no major safety concerns. In another study, Leroux-Roels et al. assessed variable dosage (3.8, 7.5, 15, and 30 µg HA) of a split virion A/Vietnam/1194/2004 NIBRG-14 vaccine with or without AS03 in healthy volunteers aged 18-60 years [106-108]. The adjuvanted vaccine formulation was well tolerated with no serious adverse events. Interestingly, high levels of neutralizing antibodies were observed against a heterologous clade 2 A/Indonesia/5/2005 (H5N1) isolate in 37 of 48 (77%) participants. Overall these results indicate the cross-protective and dose-sparing efficacy of AS03 adjuvanted vaccines. Efficacy of an oil-in-water nano-emulsion (NE) to enhance the

immunogenicity of an H5N1 subvirion vaccine was evaluated [109]. Immunized mice elicited high levels of HA-specific antibodies and were protected against challenge with both clade 1 and 2 H5N1 viruses.

1.10 Consensus antigenic domain-based influenza vaccine strategies

The use of antigenic domains of relatively conserved proteins across different influenza virus strains has been evaluated to develop broadly protective influenza vaccines. A consensus HA-based DNA vaccine encoding conserved HA sequences of circulating H5N1 viruses produced cross-neutralizing antibodies against clades 1, 2.1, 2.2, 2.3.2, and 2.3.4 H5N1 reassortant viruses and immunized animals were protected against lethal challenge with H5N1 viruses [A/Vietnam/1194/04 (clades 1), A/turkey/Turkey/03 (clade 2.2) or A/Indonesia/5/05 (clade 2.1)][110]. Similarly, an Ad vector-based vaccine (HA1-con) encoding a synthetic centralized HA1 region based on 21 H1N1 HA sequences representing the main branches of H1N1 HA phylogenetic tree. This vaccine conferred protection against challenge with several diverse influenza viruses of H1N1 subtype, including the 2009 H1N1 pandemic influenza virus[111]. Surprisingly, the HA1-con vaccine induced protection against A/PR/8/34 (H1N1) as early as Day 3 post-vaccination. In another study, mice and ferrets were immunized with DNA vaccines encoding consensus H5HA (based on consensus H5N1 HA sequences), N1NA (based on consensus H1N1 and H5N1 NA sequences), or NP (containing a consensus M2e peptide fused to a consensus NP) antigens. These vaccines were shown to induce substantial cross-protection against lethal challenge with A/Hanoi/30408/05 (H5N1), A/VN/1203/04 (H5N1) or A/PR/8/34 (H1N1) [112]. In addition, a VLP-based vaccine displaying a consensus HA protein that was designed using a computationally optimized broadly reactive antigen (COBRA) strategy was effective in inducing protective immune responses against diverse H5N1 viruses in cynomolgus macaques [113,114]. Most of the consensus antigen-based vaccine approaches have demonstrated protective potential against antigenic drift variants of seasonal and avian H5N1 influenza A viruses. However, their utility against novel emerging influenza virus strains from a different subtype needs to be further assessed. Inclusion of conserved viral proteins or co-administration with adjuvants might enhance the cross-protective potential of these vaccines.

1.11 Passive immunization with monoclonal antibodies inducing broad cross-protective immunity

Passive immunization using mAbs with broad heterosubtypic neutralizing ability has gained more interest in recent years due to its potential to rapidly deliver a universal therapeutic and prophylactic alternative against pandemic influenza viruses. Several novel approaches to rapidly isolate such broadly-neutralizing influenza virus-specific mAbs have been explored, and these mAbs have demonstrated great potential in pre-clinical studies in animal models [115-118].

Using a human antibody phage display approach, a panel of broadly neutralizing HA-specific mAbs utilizing IgM+ memory B cells were identified from the donors vaccinated with a seasonal influenza vaccine[116]. These mAbs displayed cross-neutralizing activity against influenza viruses from subtypes H1, H2, H5, H6, H8 and H9. Prophylactic treatment of mice with the most potent mAb CR6261 conferred protection against lethal challenge with homologous influenza virus A/Vietnam/1194/04 (H5N1) or heterosubtypic influenza virus A/WSN/33 (H1N1). Inoculation of mice with CR6261 one day following challenge with A/Hong Kong/156/97 (H5N1) or A/WSN/33 (H1N1) also conferred complete protection elucidating its therapeutic potential. Similarly, a family of high affinity human neutralizing antibodies which displayed broad neutralization ability against several influenza viruses from Group 1 was isolated [23]. Prophylactic or therapeutic inoculation of mice with three mAbs (F10, A66, and D8) conferred protection against lethal challenge with H5N1 viruses (A/VN/1203/04 or A/Hong Kong/483/97) or H1N1 viruses (A/WSN/33 or A/PR/8/34). Interestingly, the broad-spectrum neutralization activity of these mAbs was primarily due to their binding to a highly conserved pocket in the stem region of HA, which has been shown to be conserved across human and nonhuman influenza viruses.

Another mAb CR8020 demonstrated neutralization activity against a wide spectrum of H3 influenza strains as well as viruses from subtypes H7 and H10. Passive transfer of CR8020 conferred protection in mice against challenge with H3 or H7 viruses demonstrating its prophylactic and therapeutic efficacy [117]. Using a novel single-plasma cell screening technique, the mAb FI6v, which showed broad neutralization activity in mice against a wide range of Groups 1 and 2 influenza viruses, was isolated [119]. Therapeutic inoculation of mice and ferrets with

FI6v3 conferred complete protection from lethal challenge with diverse influenza viruses including A/PR/8/34 (H1N1), X31 (H3N2), and A/VN/1203/04 (H5N1).

Sequential administration of mice with HA proteins from antigenically different influenza A viruses has been shown to induce selective expansion of B cells producing broadly cross-reactive antibodies [29]. Two broadly cross-neutralizing mAbs, 12D1 and 6F12, which were generated following sequential administration of plasmids encoding HA proteins of H3N2 or H1N1 viruses, respectively have demonstrated potent broad-spectrum efficacy in animal studies[29]. The mAb 13D4 that was isolated following sequential immunization of mice with formalin inactivated H5N1 virus, thus demonstrating potent therapeutic efficacy in mice against lethal challenge with several antigenically distinct H5N1 viruses[19].

In a recent study, HA cross-reactive memory B cells were isolated from subjects immunized with an H5N1 DNA/inactivated virus prime-boost influenza vaccine [120]. Three antibody classes were recognized, and each of them were able to neutralize distinct subtypes of group 1 and group 2 influenza A viruses. Co-crystallization of these antibodies with HA elucidated that they bind to overlapping epitopes in the stem region.

Several influenza A mAb are being evaluated in Phase 1 and Phase 2 clinical studies and have demonstrated good safety and efficacy [121]. Some of these mAb include CR6162 and CR8020 (Janssen Pharmaceutical K. K.), MHAA4549A (Genentech Inc.), MED18852 (Medimmune LLC), VIS410 (Visterra Inc.), CT-P27 (Celltrion), and TCN-032 (Theraclone Sciences Inc.).

1.12 Expert commentary

Currently licensed influenza vaccines are serotype-specific and offer little or no protection against seasonal influenza virus variants and novel viruses emerging from non-human reservoirs. Development of a universal influenza vaccine that can provide protection against all influenza A viruses could be stockpiled in preparation for a future influenza pandemic. The importance of having such a vaccine was evident during the 2009 H1N1 pandemic, as strain-matched pandemic vaccines were not available until six months after the declaration of the pandemic. Although the 2009 pandemic was not as deadly compared to previous influenza pandemics, its worldwide spread

in a short period highlighted the need to develop vaccine approaches which offer cross-protective immune responses. It is feared that a pandemic caused by an influenza virus from avian subtypes including H5, H7, or H9 against which most of the human population is immunologically naïve could be more deadly than the 2009 H1N1 pandemic.

Vaccine approaches targeting conserved proteins, NP and M or relatively conserved regions of HA, have demonstrated broad cross-protective immunity/heterosubtypic immunity in pre-clinical animal models. VLP-based vaccine platforms are well suited for delivery of multiple viral antigens in a single vaccine formulation. Moreover, formulation of current influenza vaccines with novel adjuvants derived from plant, insect and synthetic pharmaceutical compounds has been shown to induce some level of cross-protection in animal models, although toxicity issues, especially when used at higher doses, needed to be addressed. Passive immunization using neutralizing monoclonal antibodies targeting the conserved regions of HA has been shown to provide broad cross-protective potential, however, their efficacy at lower doses needed to be demonstrated in humans. Identification of new conserved influenza-specific B and T cell epitopes would certainly help in developing a universal influenza vaccine that could provide protection against many diverse influenza subtypes. Although we are moving in the right direction in our pursuit for a universal influenza vaccine, our target still appears to be far away but seems achievable. It is important to keep evaluating newer approaches that offer cross-protection against emerging seasonal variants and other emerging avian influenza viruses.

1.13 Five-year view

The efficacy of seasonal influenza vaccines varies year-to-year against circulating viruses due to antigenic mismatch, and the emergence of novel influenza viruses on a regular basis is a potential concern for an influenza pandemic. Therefore, the concept of universal influenza vaccine was proposed some years ago, but multiple strategies towards this objective have gained momentum in recent years and it is expected to continue until a suitable universal influenza vaccine is developed and licensed for general use. For a regulatory point of view, the commercialization of a new influenza vaccine is largely dependent on the HAI endpoint, but now we have started thinking out of the box and slowly preparing ourselves for other immune correlates to monitor vaccine efficacy. The requirement for a large field study to evaluate the efficacy of a new influenza vaccine

formulation is needed to be changed to allow introduction of new vaccines in a shorter timeframe. The complexity of new vaccine delivery systems or formulations will be amended to pursue a simple vaccine design or formulation for the development and release of a universal influenza vaccine to the marketplace. The role of cell-mediated immunity and non-neutralizing antibodies in conferring broad protection against heterologous and hererosubtypic influenza viruses will be further elucidated and considered for the novel vaccine design or formulation. Novel delivery systems, new adjuvants, and broadly protective neutralizing monoclonal antibodies will be further refined for the development of new vaccine formulations or therapeutic agents. Individual or a combination of HA, NP, NA, or M2 protein/s or immunogenic domain/s will serve as antigen/s for the universal influenza vaccine.

1.14 Key issues

- Influenza virus surface proteins, HA and NA, undergo constant change due to antigenic drift and shift.
- Currently available seasonal influenza vaccines do not provide protection against emerging H5, H7 or H9 avian influenza viruses.
- The HA stem region contains conserved epitopes that could serve as target for cross-protective immunity against heterologous as well as heterosubtypic influenza viruses.
- M2 and NP could also function as target for cross-protective immunity against heterologous as well as heterosubtypic influenza viruses.
- Influenza VLPs and new adjuvants for influenza vaccines could also assist in generating cross-protective influenza immunity.
- Targeting conserved regions of influenza viruses could provide broad protection.
- Broadly cross-neutralizing antibodies have demonstrated cross protection in preclinical studies.
- Passive immunization with monoclonal antibodies with broad cross-protective efficacy would be useful as a therapy following influenza infections.

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Table 1.1: Select broadly protective vaccine candidates targeting the conserved regions of Influenza viruses.

	Vaccine target	Vaccine approach [antigen]	Test species	Immune response mediating protection	Protection conferred against influenza viruses	Challenge influenza virus dose	References
1	HA stem	Peptide vaccine [LAH portion of A/HK/1/68 (H3N2)]	Mice	Antibodies to HA stem	PR8/H1N1 A/VN/1203/04 (H5N1) A/HK/1/68-PR8 (H3N2)	10-15 MLD_{50}	[30]
2	HA fusion peptide	Peptide vaccine [Fusion peptide of A/Mississippi/1/85 (H3N2)]	Mice	Antibodies to HA fusion peptide	PR8/H1N1 A/Mississippi/1/85 (H3N2)	1 MLD_{50}	[39]
3	M2	Recombinant protein [M2 of A/Ann Arbor/6/60 (H2N2)]	Mice	Antibodies to M2	A/Ann Arbor/6/60 (H2N2) A/HK/1/68 (H3N2)	10 MLD_{50}	[46]
4	M2	Recombinant protein [M2 of A/ Aichi/ 2/68 (H3N2)]	Mice	Antibodies to M2	A/Ann Arbor/6/60 (H2N2) A/Taiwan/1/86 (H1N1) A/HK/1/68 (H3N2)	$1 \times 10^5 - 5 \times 10^6$ EID_{50}	[47]
5	M2	DNA/Ad [M2 protein of PR8/H1N1]	Mice	Antibodies to M2	PR8/H1N1 A/Thailand/Sp-83/04 (H5N1)	1.5×10^4 MLD_{50} of PR8/H1N1 virus, and 10 MLD_{50} of A/Thailand/Sp-83/04 (H5N1)	[50]
6	M2, HA	DNA [M2 and HA of H1N1 virus]	Mice	Antibodies to HA and M2	A/Korea/W81/05 (H3N2)	5 MLD_{50}	[52]
7	M2, NP	Ad [M2 of H1N1, H5N1, and H7N2 and NP of H1N1]	Mice	Antibodies to M2 and CMI to NP	PR8/H1N1 A/Fort Monmouth/1/47 (H1N1)	10-150 MLD_{50} of PR8/H1N1, and 3-10 LD_{50} of A/Fort Monmouth/1/47 (H1N1)	[51]
8	M2e, NP	Recombinant protein [M2e and NP epitopes fused to HBc]	Mice	Antibodies to M2 and CMI to NP	A/Beijing/501/2009 (H1N1) A/ostrich/Suzhou/097/2003 (H5N1)	50 LD_{50} of A/Beijing/501/2009 (H1N1), and 10 LD_{50} of A/ostrich/ Suzhou/097/2003 (H5N1)	[48]
9	NP	Recombinant protein [NP of X31 (H3N2) virus]	Mice	CMI to NP	PR8/H1N1	8-16 LD_{50}	[59]

10	HA, NP, and M1	DNA [HA of A/Hawaii/01/91 (H3N2) and NP & M1of A/Beijing/ 353189 (H3N2)]	Ferrets	Antibodies to HA, and CMI to NP and M1	A/Georgia/03193 (H3N2) A/Johannesburg/3 3194 (H3N2)	$200 \times 50\%$ ferret infectious dose	[61]
11	NP, M1	DNA [NP and M1 of PR8/H1N1]	Mice	CMI to NP and M1	A/Hong Kong/483/97 (H5N1) A/Hong Kong/156/97 (H5N1)	100-10,000 MID_{50}	[63]
12	NP	DNA-Ad [NP of PR8/H1N1]	Mice	CMI to NP	PR8/H1N1 A/Hong Kong/483/97 (H5N1) A/Hong Kong/156/97 (H5N1)	10-10,000 MID_{50}	[32]
13	NP	Ad [NP of PR8/H1N1]	Mice	CMI to NP	PR8/H1N1 A/Philippines/2/8 2 (H3N2) A/VN/1203/04 (H5N1)	10 MLD_{50}	[62]
14	NP	Ad [NP of PR8/H1N1]	Mice	CMI to NP	PR8/H1N1 A/VN/1203/04 (H5N1) A/Hong Kong/ 483/1997 (H5N1)	100 MLD_{50}	[67]
15	NP, M2	Ad [Consensus NP and M2 genes]	Mice	CMI to NP and antibodies to M2	A/Fort Monmouth/1/47-ma (H1N1)	10^4 $TCID_{50}$	[122]
16	HA, NP	MVA [HA of A/ California/04/0 9 (H1N1) virus, and NP of A/VN/1203/04 (H5N1)]	Mice	Antibodies to HA. and CMI to NP	A/VN/1203/04 (H5N1) A/Norway/3487-2/09 (H1N1) PR8/H1N1 A/Aichi/68 (H3N2)	100 MLD_{50}	[64,69]
17	NP	PIV5 [NP from A/VN/1203/04 (H5N1)]	Mice	CMI to NP	A/VN/1203/04 (H5N1) PR8/H1N1	10 MLD_{50}	[75,76]

Abbreviations: HA, hemagglutinin; M2, M2 ion channel protein; M2e, ectodomain of M2 protein; M1, M1 matrix protein; NP, nucleoprotein; LAH, long alpha helix; CMI, cell-mediated immunity; MLD_{50}, 50% mouse lethal dose; MID_{50}, 50% mouse infectious dose; EID_{50}, 50% egg infectious dose; Ad, adenoviral vector; MVA, Modified vaccinia Ankara *virus;* HBc, hepatitis B core antigen; *PR8/H1N1, A/Puerto Rico/8/1934 (H1N1); A/VN/1203/04 (H5N1), A/Vietnam/1203/2004 (H5N1); A/HK/1/1968 (H3N2), A/Hong Kong/1/1968 (H3N2); X-31,* A/Hong Kong/1/68-PR8 (H3N2); A/X-47 (H3N2), A/Victoria/3/75(H3N2)×PR8.
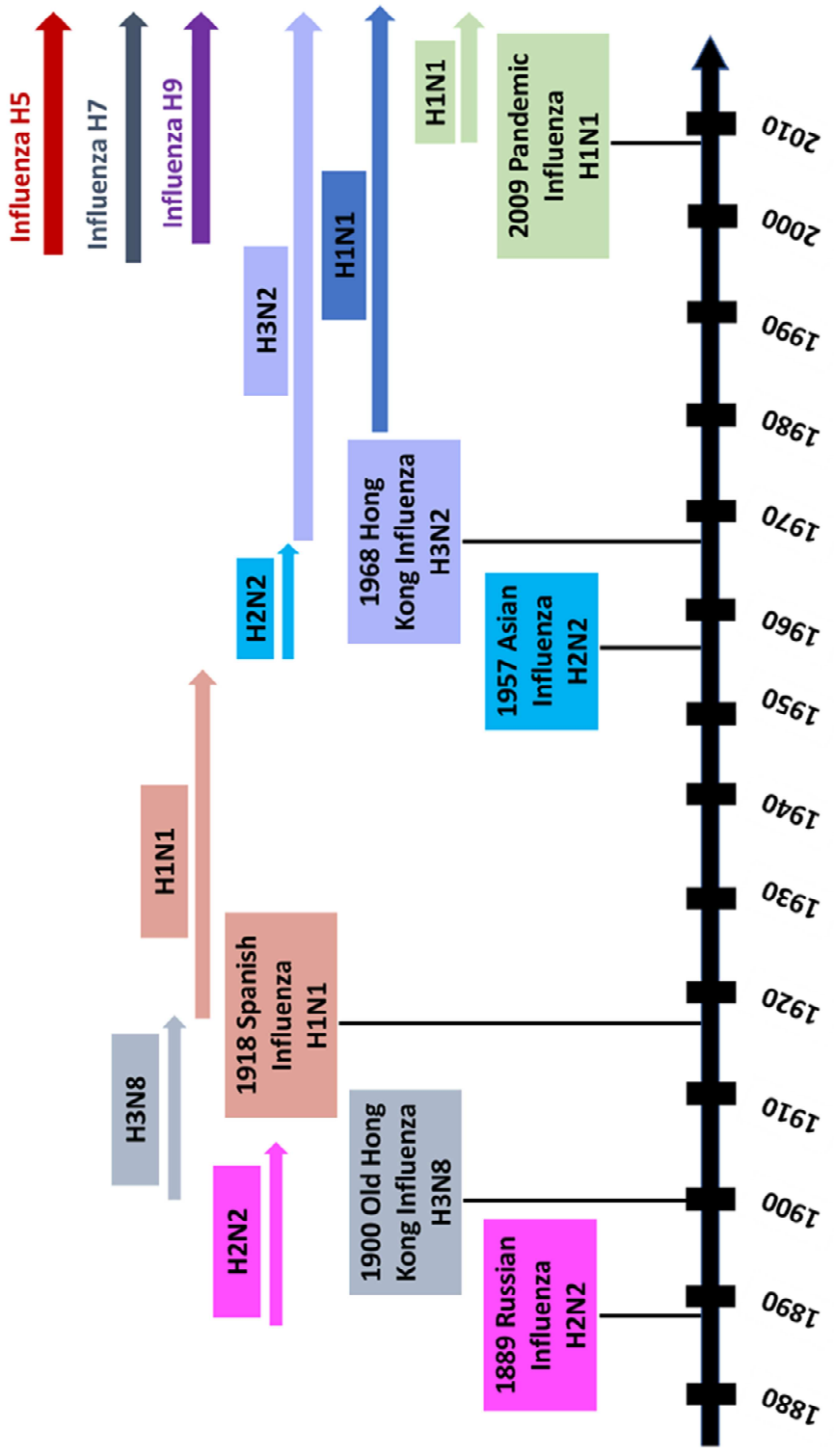
Figure 1.1: Timeline of influenza A pandemics and human infections by emerging influenza A viruses.
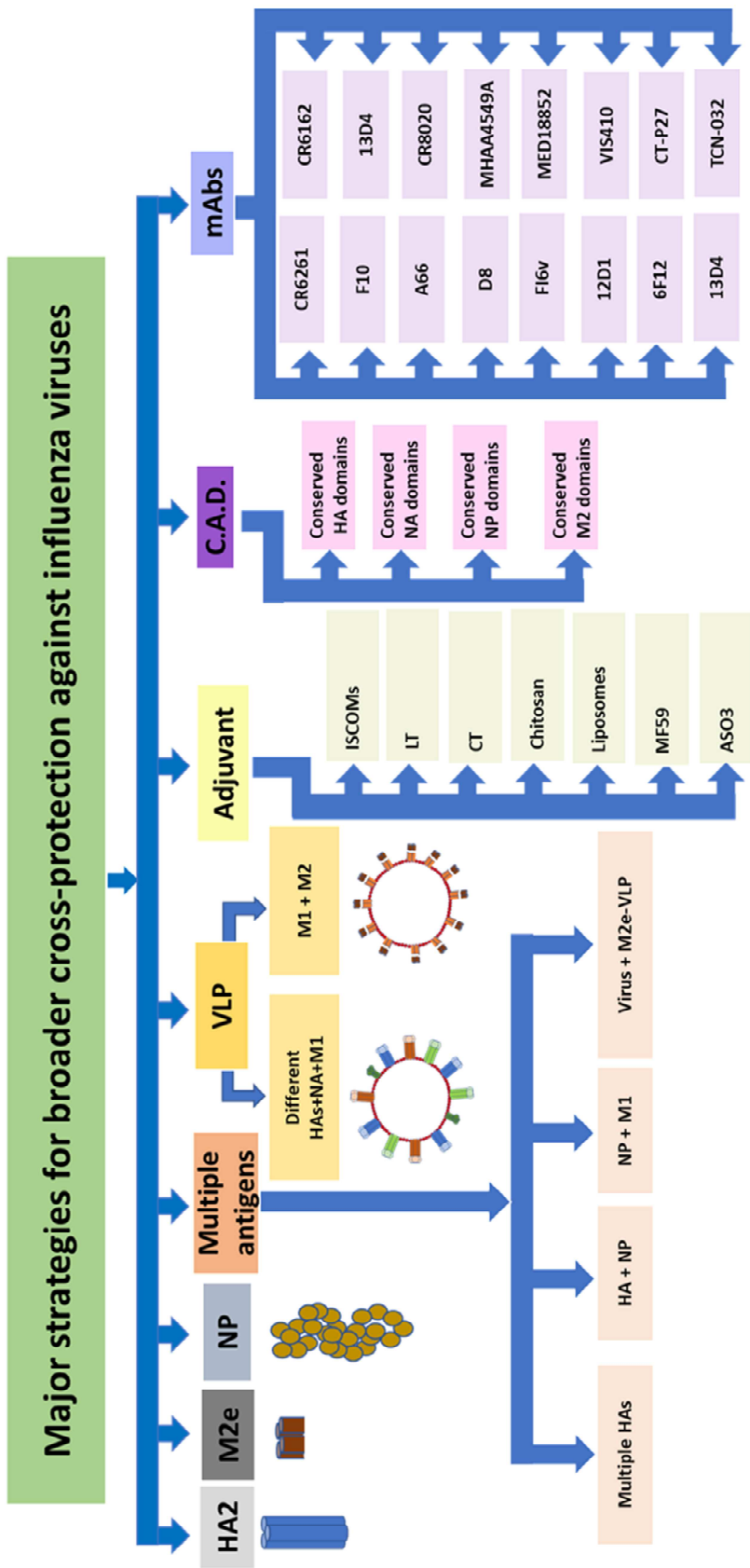
Figure 1.2: Major strategies for broader cross-protection against influenza viruses.
HA, hemagglutinin; HA2, HA stem domain; M2e, matrix protein 2 ectodomain; NP, nucleoprotein; NA, neuraminidase; M1, matrix protein 1; VLP, virus-like particle; C.A.D. consensus antigenic domain; ISCOMs, immune stimulating complexes; LT, heat-labile enterotoxin; CT, cholera toxin and mAbs, monoclonal antibodies.
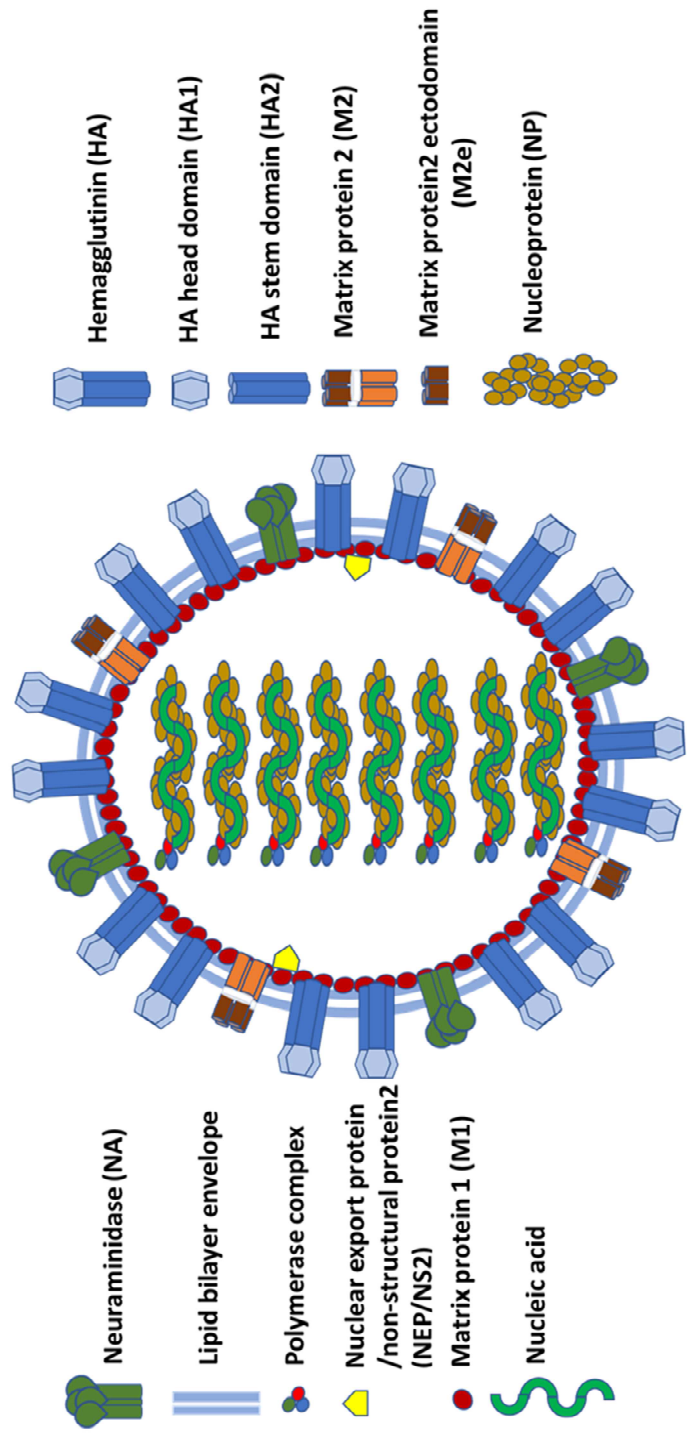
Figure 1.3: Schematic diagram of influenza A virus illustrating the virus components.

CHAPTER 2.   CURRENT USE OF ADENOVIRUS VECTORS AND THEIR PRODUCTION METHODS

2.1   Abstract

Various adenovirus (AdV) vector systems have been a lucrative option for gene delivery. They can serve as potential vaccine candidates for the prevention of several prevailing infectious diseases and also hold the promise for gene therapy especially for targeting a number of cancer types. Several AdV vector-based therapies are current at various stages of clinical trials worldwide leading to immense interest of both the clinicians and researchers. Since these vectors are easy to manipulate, have broad tropism and have the capability to grow to high titers, this delivery system has a wide range of application in different clinical settings. This chapter emphasizes on some of the current usage of AdV vectors and their production methods.

2.2   Introduction

Adenoviruses (AdVs) are non-enveloped double stranded DNA viruses having a genome of approximately 34-44 kilobase pairs (kbp). Initially, a human AdV was first isolated in 1953 from the adenoid tissues [1] and hence was named AdV [2]. They are known to cause inapparent and symptomatic infections of the upper or lower respiratory tract, gastrointestinal tract or eyes, which are usually self-limiting in healthy individuals. Although AdVs were known for a long time, their therapeutic potential as a gene delivery vehicle was realized only with the advent of recombinant technology. With continual advancement in the biology of AdV, it became the first viral gene transfer vector to be used in human. More than 60 serotypes of human AdVs have been described, of which the vector backbone of human AdV type C5 has been used extensively for gene delivery [3].

2.3   AdV vectors: pros and cons

AdV vectors have several advantages which make them ideal for gene delivery. The biology of the virus has been deciphered, which makes the molecular manipulation of the genome easier. Moreover, several AdVs have low or no virulence in humans and have high transduction efficiency for both replicating as well as non-replicating cell types. The vector can also be grown and purified

in very high titers and in large quantities at a reasonable cost. Furthermore, AdV vectors possess minimal risk of insertional mutagenesis because of their inability to integrate into the host genome. The transient transgene expression by AdV vectors have been harnessed for oncolytic therapy and also for expression of vaccine antigens [4-9].

However, the transient nature of transgene expression by AdV vectors sometimes limits their use where continuous transgene expression is necessary for a desired therapeutic effect. Apart from this limitation, AdV vectors are known to activate innate immunity, which can lead to severe toxicity at a very high vector dose. One such evidence is the death of a patient enrolled in the ornithine transcarbamylase (OTC) deficiency clinical trial due to high vector dosage leading to multiple organ failure [10].

Due to high prevalence of human AdVs, nearly 80% of human population is exposed to one or more AdV types multiple times in their lives [11-13], thereby developing AdV neutralizing antibodies popularly known as 'pre-existing vector immunity' [14]. The issue of pre-existing vector immunity can be addressed to some extent by increasing the vector dosage without increasing toxicity [13, 15, 16]. Alternatively, pre-existing vector immunity can also be circumvented using nonhuman AdV vectors and heterologous prime-boost approaches. Innovation in vector engineering strategies and the use of different immunosuppressive agents can also be used to overcome some of these limitations in the existing vector systems [17-19].

2.4   Non-human AdV vectors

Nonhuman AdV vector systems based on bovine AdV, simian AdV, porcine AdV, ovine AdV, Canine AdV, avian AdV and murine AdV [20, 21] were developed in search of safe and efficient gene delivery vehicles to overcome the shortcomings of human AdV vector systems, especially the concern of pre-existing vector immunity. For example, bovine AdV vectors are not neutralized by human AdV-specific neutralizing antibodies and the prevalence of bovine AdV cross-neutralizing antibodies were not detected in human serum samples [22, 23]. Moreover, various nonhuman AdV vectors use different receptors for internalization thereby broadening the range of cell types that can be targeted [24, 25].

2.5 AdV vectors: usage and current status

Initially, when the therapeutic potential of AdV vectors was realized, they were evaluated for a broad range of medical conditions including genetic diseases and metabolic disorders. However, soon it was realized that transgene expression is usually maintained for a short duration [26, 27]. This limits the use of AdV vectors to conditions where transient transgene expression is required for the desired effects, such as recombinant vaccines and cancer therapeutics.

2.5.1 AdV vector-based vaccines for infectious diseases

With the advancement in the field of viral vectored vaccines, various AdV vectors have been tested both in pre-clinical as well as clinical studies [6, 28, 29] for different infectious diseases. This is due to the fact that AdV vector-based vaccines induce a balanced humoral and cell-mediated immune (CMI) responses [30, 31] by stimulating innate immunity through pathways that are both Toll-like receptor (TLR)-dependent and TLR-independent [32, 33]. AdV vectors encoding for different antigens from influenza virus have been tested in different animal models and have shown high protection efficiency against homologous and heterologous influenza viruses [34-39]. A vaccine construct expressing hemagglutinin (HA) of a H5N1 influenza virus provided cross protection in mice following challenge with different strains of highly pathogenic H5N1 influenza viruses [40]. Similarly in a clinical trial, immunization with an AdV vector encoding the HA gene of influenza virus increased hemagglutination inhibition (HI) titers in more than 75% of the participants [41].

With time, there have been several modifications incorporated in the AdV vector system to overcome the existing limitations of pre-existing vector immunity and inadequate antigen-specific immunogenicity. For this purpose, other AdV types from both human and nonhuman AdVs have been evaluated. An AdV35 vector-based HIV vaccine was evaluated in a clinical trial and was found very effective and safe [42]. AdV26, another less common type of human AdV, has been recently evaluated for Ebola vaccine in a clinical trial and it elicited a favorable antibody response [43]. In addition to less prevalent human AdVs, several nonhuman AdVs, in particular chimpanzee AdV (ChAdV) vectors have shown very encouraging results in clinical trials for malaria [44], leishmania [45] and Ebola [46]. Recently, ChAdV vector, ChAd3-EBOZ, encoding for the Ebola G glycoprotein gene of the Zaire strain showed strong antibody and T cell responses in Phase I

and II clinical trials [47]. Another approach which has been adopted to improve transgene immunogenicity is the use of single cycle AdV vectors having the deletion of pIIIa protein coding gene. A single cycle AdV vector encoding influenza HA was assessed for immunogenicity in both cotton rats and hamsters leading to enhanced immune responses at a low dosage [48].

2.5.2 Oncolytic AdV vector-based therapies for cancer

The oncolytic nature of AdV has been utilized to combat various forms of cancer. To achieve effective oncolysis, the virus should infect and replicate within the cancer cells. Most of human AdVs require Coxsackievirus and Adenovirus Receptor (CAR) for virus internalization, but in many forms of cancer, there is marked downregulation or complete absence of CAR [49] leading to marked reduction in cell transduction with AdV vectors. To increase the interaction between the virus and cancer cell surface molecules, introduction of a motif like RGD in the knob region of AdV fiber improves the interaction with integrins which are expressed on the cancer cell surface [50]. In some cases, complete swapping of fiber is done for its preferred interaction with a cell surface molecule such as desmoglien 2 which is expressed in large number on cancer cells [51-54]. It seems very assuring that only tumor cells can be lysed by these oncolytic AdV vectors since their replication competency is dependent on the presence of a specific tumor antigen. A successful oncolytic AdV therapy also requires some other vector modifications to overcome the immunological as well as structural barriers of tumor microenvironment. Oncolytic AdV vector expressing relaxin, facilitates better vector spread in the dense extracellular matrix (ECM) [55]. VCN-01, is another armed oncolytic AdV vector which expresses hyaluronidase and is currently being tested in Phase 1 clinical trials [56]. Recently, oncolytic AdV vector ONCOS 102 expressing GM-CSF demonstrated a potent therapeutic effect with minimal side effects [57]. Many other molecules like interferon alpha, tumor necrosis factor alpha and other interleukins are also being investigated as delivery molecules with oncolytic AdV vectors. It seems that targeting the tumor microenvironment, in addition to the tumor cell lysis, is a better approach for cancer therapeutics using oncolytic AdV vectors.

2.6 AdV vector types

Several changes have been made in the AdV vector design methodology to improve vector recovery, transgene expression and safety. AdV vectors can be broadly classified into three types based on the deletions of the viral genes.

2.6.1 First and second generation AdV vectors

First generation AdV vectors contain the deletion of the E1 region or E1 & E3 regions of the viral genome. Deletion of the E1 region results in a replication incompetent vector, and also serves the purpose of increasing the capacity of the foreign gene cassette [58]. E1-deleted AdV vectors can only be grown in a cell line (e.g., HEK 293) that constitutively expresses E1 proteins [59]. However, anchorage-dependent cell lines can be used only for small-scale production of vector preparations. To achieve scalability and batch-to-batch consistency, a suspension cell culture bioreactor system is used with a variant of the HEK 293 cell line capable of growing cultures in suspension without serum. A bioreactor with 10,000 L capacity is projected to yield 10^9 -10^{10} viral particle/milliliter (vp/mL) [60]. However, the usage of HEK 293 cells can result in the production of contaminating replication competent virus due to homologous recombination. The PER.C6 [61] and SL0036 cell lines [62] have been developed with a minimal E1 region to eliminate the possibility of homologous recombination. The major drawback of first generation AdV vector system is high immunogenicity in the host, which raises safety concern in situations where a very high vector dose is required for desired effects.

Second generation AdV vectors were created to minimize the shortcomings of first generation vectors. Second generation AdV vectors were designed with deletion of two more gene regions, E2 and/or E4, along with E1 and E3 deletions. The idea was to reduce the vector immunogenicity by reducing the leaky expression of viral genes [63]. Apart from this, the deletion/s also increase/s the transgene carrying capacity of vector. However, these multiple regions deleted vectors require an appropriate complimentary cell line for their propagation.

2.6.2   Third generation AdV vectors

Third generation AdV vectors include the helper-dependent vectors, also known as gutless vectors, which are designed by the removal of all of the AdV genes. These vectors retain only the packaging signal along with the inverted terminal repeat (ITR) sequences from the viral genome [64-67]. Due to the complete absence of protein coding regions in the helper-dependent vectors, they result in significant reduction in vector immunogenicity leading to improved safety in patients. Moreover, the transgene carrying capacity of helper-dependent vector system can be up to 36 kbp. Production of these vectors requires a helper virus which is a transgene-empty first generation AdV vector. Initially, the low yield of helper-dependent vector and the contamination with the helper virus were two major concerns with this vector system [64]. Both of these concerns were addressed by replacing the helper virus with the AdV vector in which the packaging sequences are flanked with a site-specific recombination sequences, e.g. loxP, and the helper-dependent vector is grown with this novel helper vector in a cell line that expresses an appropriate recombinase, e.g., Cre recombinase for loxP sites [67, 68]. The loxP-Cre recombinase or equivalent system will results in the generation of novel helper virus genomes without the packaging sequences thereby allowing efficient packaging of the helper-dependent vector genomes. Third generation AdV vectors have shown promising results in different animal models with minimal adverse effects [69].

2.7   Construction of AdV vectors

Several techniques were developed to construct AdV vectors and are broadly divided into two approaches: 1) direct insertion of the foreign gene into the viral genomic DNA, in a plasmid form, using unique restriction enzymes [30, 70]; and 2) recombination between two plasmids through homologous recombination either in bacteria or in a permissive cell line [71-75]. The two plasmids system includes a genomic plasmid that contains nearly the complete AdV genome with appropriate deletion/s, and a shuttle plasmid carrying the foreign gene cassette and AdV sequences that are essential for homologous recombination and generation of an infectious AdV vector. Below are detailed protocols that can be used to create and purify infectious AdV vectors using these two different production techniques.

2.7.1   Materials

2.7.1.1 Generation of AdV vector by homologous recombination in bacteria

1. Genomic plasmid (pAdV-ΔE1E3)

2. Shuttle plasmid containing the desired transgene (pAdV-shuttle-T)

3. E. coli BJ5183 strain: kept in aliquots at -80ºC

4. E. coli DH5α strain: kept in aliquots at -80ºC

5. Lysogeny broth or Luria-Bertani (LB) broth and LB agar

6. Minimum essential medium (MEM)

7. Opti-MEM (Gibco)

8. Fetal bovine serum (FBS)

9. Ampicillin 50mg/mL

10. Lipofectamine 2000 (Invitrogen)

11. Enzymes- PacI, HindIII, and Antarctic phosphatase

12. 50× 1% TAE: Tris 242 g, glacial acetic acid 57.1 mL, 0.5 M EDTA, pH 8.0 100 mL, and MilliQ water to adjust the volume to 1 L

13. GENECLEAN® III Kit (MP Biomedicals)

14. Maxi Fast Ion Plasmid Kit (IBI Scientific)

15. Appropriate cell line that supports the replication of the desired AdV vector

2.7.1.2 Generation of AdV vector using Cre/loxP recombination system

1. Genomic plasmid pAdVΔψ,E1,E3/loxP

2. Shuttle plasmid pAdV-shuttle/loxP containing a transgene (pAdV-shuttle/loxP/T)

3. E. coli DH5α strain: kept in aliquots at -80ºC

4. Lysogeny broth or Luria-Bertani (LB) broth and LB agar.

5. Minimum essential medium (MEM)

6. Fetal bovine serum (FBS)

7. Ampicillin 50mg/mL

8. CaCl2 2.5 M

9. Hepes buffer saline (HBS): HEPES 5g/L, NaCl 8g/L, KCl 0.37 g/L, Na2HPO4.2H2O 0.125 g/L, glucose 1 g/L; final pH 7.1.

10. Carrier DNA: salmon sperm DNA (SSDNA) 1mg/mL

11. GENECLEAN® III Kit (MP Biomedicals)

12. Maxi Fast Ion Plasmid Kit (IBI Scientific)

13. 293Cre4 cell line or any other appropriate cell line.

2.7.1.3   Purification of AdV vectors

1. Minimum Essential Medium (MEM) Eagle (Corning).

2. 10× Phosphate buffer saline (PBS) pH 7.4: NaCl 80 g, KCl 2 g, Anhydrous Na2HPO4 14.4 g, KH2PO4 2.4 g, MilliQ H2O 900 mL

3. PBS++: PBS containing 0.1% MgCl2 & 0.1% CaCl2

4. Fetal bovine serum (FBS)

5. 0.1M Tris, pH 8.0.

6. 5 % Sodium deoxycholate solution

7. Saturated cesium chloride (CsC1) solution in 0.01M Tris and 0.001M EDTA

8. AdV vector permissive cell line, e.g. HEK 293 for human AdV vectors.

2.8   Methods

2.8.1   Generation of AdV vector by homologous recombination in bacteria

For construction of first generation of AdV vectors, the genomic plasmid, pAdV-ΔE1E3, comprises the entire AdV genome flanked with a unique restriction enzyme (e.g., PacI) and contains appropriate E1 and E3 deletions. The shuttle plasmid, pAdV-shuttle-T, contains both ends of the AdV genome, E1 deletion replaced with a eukaryotic promoter (e.g., human cytomegalovirus (HCMV) promoter), multiple cloning sites (MCS) for foreign gene insertion, a polyadenylation (poly A) signal (e.g., bovine growth hormone (BGH) polyA), AdV sequences for homologous recombination, and a unique restriction enzyme site (e.g. PacI) at both ends of AdV sequences. The genomic DNA is released from pAdV-ΔE1E3 by PacI digestion followed by gel purification. Alternatively, genomic DNA extracted from a purified preparation of AdV-ΔE1E3 (empty vector) can be used. The linearized shuttle plasmid containing a transgene (pAdV-shuttle-T) and the AdV genomic DNA are used for co-transformation of Escherichia coli (E. coli) BJ5183. This bacterial strain is recBC sbc positive and thus is capable of homologous recombination. The desired result will produce pAdV-ΔE1E3 plasmid carrying the AdV genome with a foreign insert

in the E1 region (Fig. 1). The transfection of an appropriate cell line with PacI-digested pAdV-ΔE1E3 will result in the generation of infectious AdV vector expressing the desired transgene.

2.8.1.1 Preparation of plasmid DNA for bacterial transformation

1. Digest 5 µg of genomic plasmid pAdV-ΔE1E3 using the PacI restriction enzyme to release AdV-ΔE1E3 sequences from the plasmid backbone.

2. Digest 5 µg of the pAdV-shuttle-T shuttle plasmid containing the transgene with HindIII to linearize the plasmid and dephosphorylate it using Antarctic phosphatase.

3. Purify AdV-ΔE1E3 and the linearized pAdV-shuttle-T by running onto 1% TAE agarose gel, collecting the appropriate bands and then recovering the DNA using the GENECLEAN III Kit following the manufacturer's instructions.

2.8.1.2 Transformation of E. coli BJ5183 for homologous recombination and selection of positive clones

1. Mix the gel purified AdV-ΔE1E3 DNA (insert) to the linearized dephosphorylated pAdV-shuttle-T DNA (vector) at a 2:1 molar ratio (insert:vector). For example, 0.1 µg linearized and dephosphorylated pAdV-shuttle-T DNA would be combined with 1 µg of Ad-ΔE1E3 DNA.

2. Co-transform the E. coli BJ5183 with the insert and vector mixture using either electroporation or heat shock.

3. The transformed bacteria is incubated in 1 mL of LB medium for 15 min at 37ºC in a shaking incubator.

4. The volume of culture medium is reduced to 250 µL by centrifugation at 1,000×g for 5 min.

5. Spread the transformed bacteria onto LB agar plates containing ampicillin or carbenicillin as a selection antibiotic, and incubate the plates at 37ºC for 18 h.

6. Using standard culturing techniques, pick small colonies and grow them in LB broth containing the appropriate antibiotic at 37ºC overnight.

7. Extract DNA from miniprep cultures using the plasmid isolation kit according to the manufacturer's instructions. Following, digest with appropriate restriction enzyme/s to confirm the positive recombinant clones.

8. Transform E. coli Dh5α with one or more positive recombinant clones to obtain high yield of recombinant plasmid DNA for transfection of an appropriate cell line.

2.8.1.3 Generation of infectious AdV vector containing the desired transgene by transfection of an appropriate cell line with the recombinant DNA clones

1. Digest 50-100 μg of recombinant DNA clones with PacI to release the AdV genome containing the transgene cassette.

2. Clean the digested DNA by phenol/chloroform/isoamyl alcohol (25:24:1) solution, precipitate the DNA with absolute alcohol, and resuspend it in sterile water.

3. Grow the appropriate cells in 4.5mL of complete cellular medium (specific to the respective cells utilized) overnight in 60 mm tissue culture plates making sure that the cells will not attain more than 70% confluencey at the time of transfection.

4. Change the media to Opti-MEM, 4.5 mL/plate, 2 h before transfection and keep the plates in a CO2 incubator at 37ºC.

5. Dilute 5 μg of PacI-digested recombinant plasmid DNA into 150 μl Opti-MEM in a 1.5 ml sterile tube.

6. Dilute 15 μL of lipofectamine 2000 in 150 μL Opti-MEM in another 1.5 mL sterile tube.

7. Incubate these tubes at room temperature for 20-30 min.

8. Add the Opti-MEM containing the DNA drop by drop into the tube containing Opti-MEM + lipofectamine.

9. Incubate the DNA/lipofectamine mixture at room temperature for 20 min.

10. Increase the volume of DNA/lipofectamine mixture to 500 μL with Opti-MEM and add drop by drop onto the cells in a 60 mm plate.

11. Incubate the plates at 37ºC for 5-6 h, change the medium to MEM containing 5% fetal bovine serum and continue the incubation at 37ºC.

12. Change the media of the transfected plates every 3-4 days.

13. The visible cytopathic effects (CPE) due to generation of infectious AdV vector will be observed any time after 10 days post-transfection.

14. For downstream processing please refer to section 3.3.

2.8.2   Generation of AdV vector using Cre/loxP recombination system

In this method the genomic plasmid, pAdVΔψ, E1, E3/loxP, contains the entire AdV genome except the packaging signal (ψ) sequences, E1 and E3 regions, and a loxP site is added just after the E1 deletion for recombination. The shuttle plasmid, pAdV-shuttle/loxP, carries both ends of the AdV genome, the ψ sequences, the E1 region deletion is replaced with the HCMV promoter, multiple cloning sites, and the BGH poly A. Just after the BGH ploy A, a loxP site is added. To obtain human AdV vectors by Cre/loxP recombination, HEK 293 cell line carrying the Cre recombinase gene (293Cre4) can be transfected with the genomic plasmid and the shuttle plasmid containing a transgene cassette [75, 76].

 2.8.2.1 Co-Transfection of 293Cre4 to recover infectious AdV vector

1. Purify the genomic (pAdVΔψ, E1, E3/loxP) and shuttle plasmid containing the transgene (pAdV-shuttle/loxP/T) by Maxiprep using a kit.

2. Grow 293Cre4 (or appropriate cells) overnight in 60 mm tissue culture plates making sure that the cells will attain a confluency of approximately 70-90% at the time of transfection.

3. Change the medium with 4.5 mL of MEM containing 10% FBS before transfection.

4. 2 ml of HBS containing 20 μg SSDNA (10μg/mL HBS) is sheared by vortex for 1 minute and 0.5 mL of this solution is aliquoted in four 1.5 mL sterile tubes.

5. Add 10μg of pAdVΔψ, E1, E3/loxP and 5μg of pAdV-shuttle/loxP/T to each 1.5 mL tube from step 4. Incubate HBS and plasmid DNA mixture for 30-45 min at room temperature.

6. Add 25 μL of 2.5 M CaCl2 to each 1.5 mL tube and incubated them for 20-30 min at room temperature.

7. Add the content of each 1.5 mL tube drop by drop onto 293Cre4 cells in a 60 mm plate, incubate the plates at 37ºC in a CO2 incubator for 4-5 h, and then change the medium with 5 mL of MEM containing 5% FBS.

8. The media should be changed every 3-4 days and the CPE depicting the generation of infectious AdV vector should appear any time after 7 days post-transfection.

9. For downstream processing please refer to section 3.3.

2.8.3 Purification of AdV vectors

Purified stocks of AdV vectors are required for all preclinical and clinical studies to evaluate efficacy of the desired AdV vectors. Cesium chloride (CsCl) density-gradient centrifugation technique is the most common and reliable method for AdV vector purification for preclinical studies in animal models.

2.8.3.1 Preparation of a large stock of AdV vector for purification

1. Split HEK 293 cells into 30x150 mm tissue culture plates in MEM containing 10% FCS and incubate them overnight at 37ºC in a CO2 incubator.

2. When the cells are approximately 90% confluent (usually after 24 h incubation), infect the cells at a multiplicity of infect (MOI) of 5 plaque forming units (PFU) of the desired AdV vector after diluting the working vector stock in PBS++.

3. For infection, remove the media from each plate and add 3 mL of PBS++ containing the appropriate amount of vector.

4. Incubate the infected plates at 37ºC in a CO2 incubator for 30 minutes and then add the 25 mL of maintenance medium (MEM + 2% FCS) per plate.

5. Incubate the infected plates at 37ºC in a CO2 incubator till complete cytopathic effect (CPE) is observed. The CPE is characterized by cell rounding and detaching and is achieved usually within 48 h post-infection.

6. Harvest the infected cells and the culture medium using a cell lifter and collect in a sterile 500 mL centrifuge bottle.

7. Centrifuge the contents at 2000 ×g for 10 min at 4ºC to pellet the infected cells.

8. Discard the supernatant in another container and autoclave the discarded supernatant.

9. Resuspend the pellet in 15 mL 0.1 M Tris pH 8.0 and transfer to a 50 mL tube.

10. Add 1.5 mL of 5% sodium deoxycholate solution, vortex for 30 seconds (the content will be more viscous due to cell lysis and the release of cellular DNA), and incubate the tube on ice for 30 min and vortex every 10 min.

11. Use tissue homogenizer 3 times at the medium speed for 10 seconds and 2 times at the high speed for 5 seconds to shear the DNA. This step should be done in a hood and the sample should be kept on ice during processing.

2.8.3.2 AdV vector purification by CsCl density gradient centrifugation

1. Add 0.58 mL saturated CsC1 solution for each 1 mL of the homogenized vector suspension.
2. Mix well and distribute the mixture into two 13 mL Beckman polyallomer tubes and spin in SW40 rotor for 16 h at 4°C at 35,000 rpm.
3. Collect the vector bands and pool into a 5 mL Beckman polyallomer tube (use saturated CsC1 0.58 ml for each 1 ml of 0.1 M tris PH 8 for a balance tube).
4. Centrifuge the pooled vector in SW50.1 rotor at 35,000 rpm at 4°C for 16 h.
5. Collect the vector band and keep at 4°C till dialysis (do not keep the vector with CsCl for longer than 1 day at 4°C).
6. Prepare the dialysis tube by boiling in 500 mL of 1mM EDTA, pH 8.0, for 10 min.
7. After cooling of the dialysis tube, clip the tube from one end and transfer the purified vector in CsCl solution to the tube and clip the other end.
8. Dialyze the vector in 1 L PBS for 2 h twice and in 1 L PBS++ for 2 h once at 4°C.
9. After dialysis, collect the purified AdV vector from the dialysis tube into a sterile tube in a biosafety cabinet.
10. Add 1/10 volume of sterile glycerol in the purified AdV vector preparation, make aliquots (0.5- 1 mL each), label the vials and store at -80ºC.
11. Vector titration can be done by plaque assay in a suitable cell line. The vector particle count can be done by measuring OD at 260 nm using a spectrophotometer [77-79].

2.9   Notes

1. AdV vector can be modified in any part of its genome by homologous recombination in bacteria.

2. Electrophoresis of the DNA in an agarose gel containing ethidium bromide should be done in dark to avoid the shearing of DNA, if the DNA will be used for cloning or transfection.

3. Dephosphorylation of the vector plasmid for recombination in bacteria is essential to decrease the empty vector colonies during selection.

4. Colonies with the right recombinant plasmid are usually of small size following bacterial recombination in E. coli BJ5183.

5. All DNA samples for transfection should be dissolved in sterile water under sterile conditions.

6. The Cre/loxP transfection method differs from lab to lab in the ratio of the big plasmid (genomic) and the small plasmid (shuttle). The best results we are getting are with 10 μg of genomic plasmid + 5 μg of shuttle plasmid.

7. After adding 2.5 M CaCl2 into the HBS/DNA mixture, fine precipitates are expected making the solution somewhat whitish in color. If coarse white precipitates are formed, it may adversely impact the transfection efficiency.

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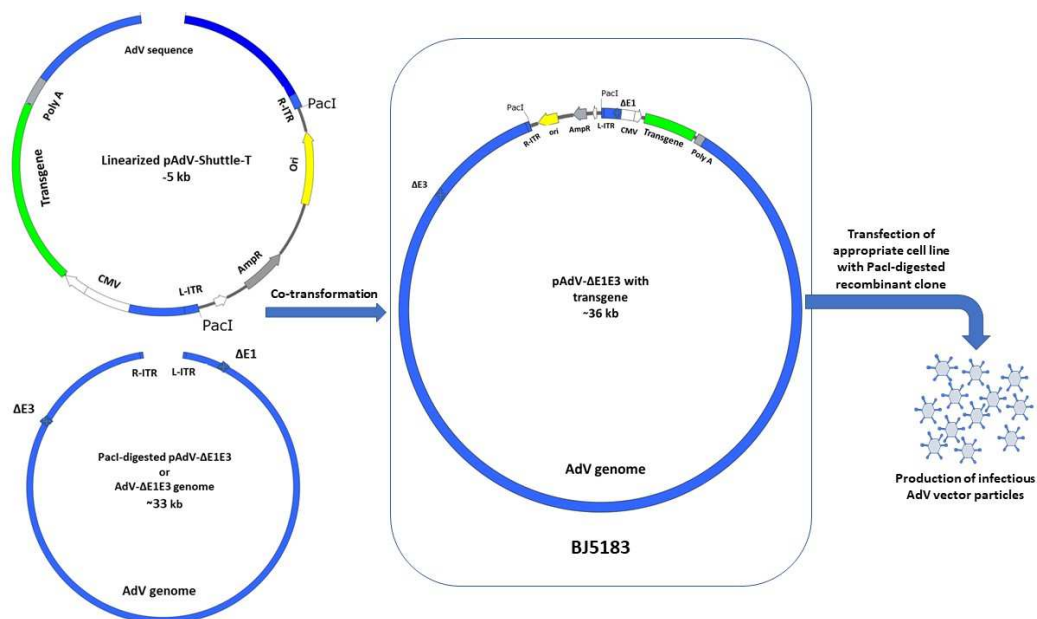
Figure 2.1: Generation of AdV vector using homologous recombination in bacteria.

After insertion of the desired transgene into AdV vector shuttle plasmid (pAdV-shuttle-T), the pAdV-shuttle-T is linearized by restriction digestion between the right and left ends of AdV of genome. AdV vector genomic plasmid with deletion in E1 and E3 sequences (pAdV-ΔE1E3) is digested to release AdV-ΔE1E3 sequence from the plasmid backbone. The linearized pAdV-shuttle-T and AdV-ΔE1E3 co-transformation into BJ5183 will result in combination between the homologous sequences and create recombinant plasmid clones contain the whole sequence of AdV-ΔE1E3 with the transgene (pAdV-ΔE1E3-transgene) inside an expression cassette. Transfection of a cell line expressing E1 proteins of AdV with pAdV-ΔE1E3-transgene digested with appropriate restriction enzyme (i.e. PacI) to remove the plasmid backbone, will develop AdV vector expressing the inserted transgene protein or peptide.

AdV, adenovirus; R-ITR, right inverted terminal repeats of adenovirus genome; L-ITR, left inverted terminal repeats of adenovirus genome; CMV, cytomegalovirus promoter; PolyA, polyadenylation signal; AmpR, ampicillin resistance gene; Ori, plasmid bacterial origin of replication; ΔE1, Adenovirus genome without the E1 region; ΔE3, Adenovirus genome without the E1 region; BJ5183, E.coli bacterial strain for homologous recombination.
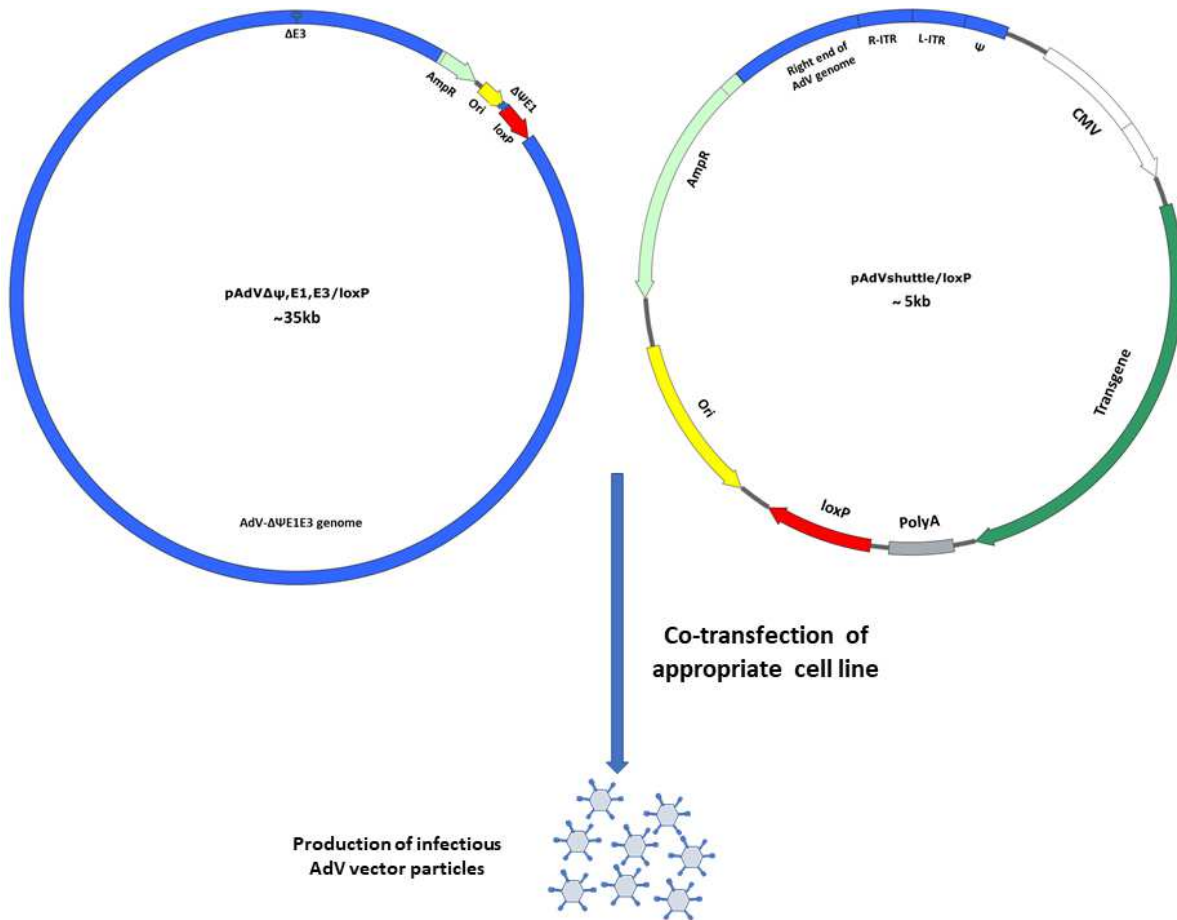
Figure 2.2: Generation of AdV vector using Cre/loxP recombination system.

Co-transfection of pAdVΔψ,E1,E3/loxP and pAdVshuttle/loxP (AdV vector shuttle plasmid for cre/loxP recombinase system) containing the desired transgene, into a cell line expressing Cre recombinase protein and E1 proteins of AdV, AdV vector expressing the inserted transgene protein or peptide will be generated.

AdV, adenovirus; R-ITR, right inverted terminal repeats of adenovirus genome; L-ITR, left inverted terminal repeats of adenovirus genome; CMV, cytomegalovirus promoter; PolyA, polyadenylation signal; AmpR, ampicillin resistance gene; Ori, plasmid bacterial origin of replication; ΔE1, Adenovirus genome without the E1 region; ΔE3, Adenovirus genome without the E1 region; ψ, AdV packaging signal.

CHAPTER 3. ENHANCED IMMUNOGENICITY AND PROTECTION WITH A BOVINE ADENOVIRAL VECTOR-BASED H5N1 VACCINE

3.1 Abstract

Several human and nonhuman adenovirus (AdV) vectors including bovine AdV type 3 (BAdV-3) were developed as gene delivery vectors to supplement and/or to elude human AdV (HAdV)-specific neutralizing antibodies (vector immunity). Here we evaluated the vaccine immunogenicity and efficacy of BAdV-3 vector (BAd-H5HA) expressing hemagglutinin (HA) of a H5N1 influenza virus in a dose escalation study in mice with intranasal (i.n.) or intramuscular (i.m.) route of inoculation in comparison with the HAdV type C5 (HAdV-C5) vector (HAd-H5HA) expressing HA of a H5N1 influenza virus. Dose-related increases in responses were clearly noticeable. A single i.m. inoculation with BAd-H5HA resulted in enhanced cellular immune responses compared to that of HAd-H5HA and conferred complete protection following challenge with a heterologous H5N1 virus at the dose of 3×10^7 plague-forming units (PFU), whereas significant amount of influenza virus was detected in the lungs of mice immunized with 1×10^8 PFU of HAd-H5HA. Similarly, compared to that of HAd-H5HA, a single i.n. inoculation with BAd-H5HA produced significantly enhanced humoral (IgG and its subclasses as well as IgA) and cellular immune responses and conferred complete protection following challenge with a heterologous H5N1 virus. Complete protection with BAd-H5HA was observed with the lowest vaccine dose (1×10^6 PFU), but similar protection with HAd-H5HA was observed at the highest vaccine dose (1×10^8 PFU). These results suggest that at least 100-fold dose-sparing can be achieved with BAd-H5HA vector compared to HAd-H5HA vaccine vector.

3.2 Introduction

Adenovirus (AdV) vector-based vaccines have been shown to elicit a balanced humoral and cell-mediated immune (CMI) responses[1, 2] by activating innate immunity through both Toll-like receptor (TLR)-dependent as well as TLR-independent pathways[3, 4]. Due to the high prevalence of AdV in humans, the development of AdV-specific neutralizing antibodies, known as 'pre-existing vector immunity' [5-7], is one of the potential concerns for several human AdV (HAdV) vector-based vaccine delivery systems. To address this concern a number of less prevalent HAdVs

or nonhuman AdVs have been developed as vaccine delivery vectors [8, 9]. These nonhuman AdV vectors are based on bovine AdV (BAdV), simian AdV (SAdV), porcine AdV (PAdV), ovine AdV (OAdV), Canine AdV (CAdV), avian AdV (AAdV) and murine AdV (MAdV)[8, 9].

It has been demonstrated that there were no reductions in humoral and CMI responses against the vaccine immunogen and the resultant protection efficacy of a BAdV type 3 (BAdV-3) vector-based H5N1 influenza vaccine even in the presence of exceptionally high levels of pre-existing HAdV vector immunity [10]. In addition, pre-existing HAdV-neutralizing antibodies in humans did not cross-neutralize BAdV-3[11], and HAdV-specific CMI responses did not cross-react with BAdV-3[4]. Bio-distribution, pathogenesis, transduction, and persistence studies in animal models and cell lines have suggested that the safety aspects of BAdV vectors are similar to that of HAdV vectors [12, 13]. It has been illustrated that the cell internalization of BAdV-3 is independent of the HAdV type C5 (HAdV-C5) receptors [Coxackievirus-AdV receptor (CAR) and αvβ3 or αvβ5 integrin][14], but it is indicated that the α(2,3)-linked and α(2,6)-linked sialic acid receptors serve as major receptors for BAdV-3 internalization[15]. It appears that BAdV-based vectors may serve as excellent vaccine vectors for humans without any concerns of pre-existing HAdV vector immunity.

Vaccine formulation features that are important for developing effective pre-pandemic influenza vaccine strategies include the development of a balanced humoral and CMI responses that could offer cross-protection, safety and efficacy with a single dose, dose sparing for vaccine delivery to a large number of individuals, and the capacity to produce a large number of vaccines at short notice. In our previous studies we have demonstrated that AdV vector-based vaccines could elicit potent humoral and CMI responses in mice conferring cross-protection depending on the immunogen/s of choice [16, 17] . Since AdV vectors have been evaluated for their efficacy as gene delivery vehicles in many clinical trials in humans[18-21], it is well understood how to produce a clinical grade of purified AdV vector lots in exceptionally large quantity under GLP conditions in certified cell lines in a short time span[1, 22-24].

In this study we have compared the immunogenicity and efficacy of BAdV-3 vector (BAd-H5HA) expressing hemagglutinin (HA) of a H5N1 influenza virus with that of HAdV-C5 vector (HAd-

H5HA) expressing HA of a H5N1 influenza virus in a mouse model. The vaccine doses [1×10^6, 3×10^6, 1×10^7, 3×10^7, or 1×10^8 plague-forming units (PFU)] and the routes of immunization [intranasal (i.n.) or intramuscular (i.m.)] were evaluated to determine whether the BAdV vector system will serve as a better vaccine vector compared to the HAdV vector. Overall, significantly higher levels of humoral and CMI responses, and higher vaccine efficacy, in mice were observed with the BAd-H5HA vector compared to that of HAd-H5HA. The best protection efficacy, with a significantly lower vaccine dose, was observed in the mouse group inoculated i.n. with BAd-H5HA. These results suggest that the BAdV-3-based vector system is a better vaccine delivery vehicle for developing pre-pandemic influenza vaccines.

3.3   Material and methods:

Cell lines, AdV vectors, and influenza viruses BHH3 (bovine-human hybrid clone 3)[25], BHH2C (bovine-human hybrid clone 2C)[25], 293 (human embryonic kidney cells expressing HAdV-C5 E1 proteins)[26], and MDCK (Madin-Darby canine kidney) cell lines were propagated as monolayer cultures in minimum essential medium (MEM) (Life Technologies, Gaithersburg, MD) containing either 10% reconstituted fetal bovine serum or fetal calf serum (Hyclone, Logan, UT) and gentamycin (50 µg/ml).

The construction and characterization of BAd-H5HA [BAdV-3 E1 and E3 deleted vector expressing HA of A/Hong Kong/156/97(H5N1) (HK/156)][10], BAd-ΔE1E3 (BAdV-3 E1 and E3 deleted empty vector)[11] , HAd-H5HA [HAdV-C5 E1 and E3 deleted vector expressing HA of HK/156][27], HAd-ΔE1E3 (HAdV-C5 E1 and E3 deleted empty vector)[28], have been described previously. BAd-H5HA and BAd-ΔE1E3 were grown and titrated in BHH3 cells as described previously[10], and HAd-H5HA and HAd-ΔE1E3 were grown in 293 cells and titrated in BHH2C cells as described previously[17]. These vectors were purified by Cesium chloride density gradient ultracentrifugation as described [29].

A/Vietnam/1203/2004(H5N1)-PR8/CDC-RG [VN/1203/RG] that was created by reverse genetics (RG) in the A/PR/8/1934(H1N1) [PR8] background was grown in embryonated hen eggs and titrated in the eggs and/or MDCK. The HA gene in the vaccine vectors was from HK/156, which is antigenically distinct from the challenge virus VN/1203/RG.

Immunogenicity and protection studies in mice:

All immunization and protection studies in mice were performed in a USDA-approved BSL-2+ facility with the approvals of the Institutional Animal Care and Use Committee (IACUC), and the Institutional Biosafety Committee (IBC). Six-to-eight-week-old BALB/c mice (Harlan Sprague Dawley Inc., Indianapolis, IN) were served as the subjects for immunization and protection studies following approved guidelines.

The mouse groups (10 animal/group) were mock-inoculated (with phosphate-buffered saline (PBS), pH 7.2) or inoculated i.n. or i.m. with 1×10^6, 3×10^6, 1×10^7, 3×10^7, or 1×10^8 PFU of BAd-H5HA or HAd-H5HA. The mouse groups inoculated i.n. or i.m. with 1×108 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 were served as vector controls. Four weeks post-inoculation, 5 animals/group were anesthetized with ketamine-xylazine (90 mg/kg ketamine and 10 mg/kg xylazine) by intraperitoneal injections, the blood samples were collected via retro-orbital puncture, nasal washes were collected by washing the nasal passage with 0.5 ml of PBS, and the lung washes were prepared after homogenizing one lung from each animal in 1 ml of PBS as described previously [30]. The serum samples, nasal washes and lung washes were used to evaluate the humoral immune responses. The second lung was processed to collect CD3+ T cells from the lung cells using MagniSort® Mouse CD3 Positive Selection Kit following the manufacturer's instructions (Affymetrix eBioscience San Diego, CA), and used to monitor CMI responses. The spleens, respiratory area lymph nodes (RLN) and inguinal lymph nodes (ILN) were also collected to evaluate CMI responses.

The remaining five animals per group were challenged i.n. with 100 mouse infectious dose 50 (MID50) of VN/1203/RG. Three days post-challenge, the animals were euthanized under ketamine-xylazine anesthesia as described above, and the lungs were collected for determination of the lung virus titers as described previously [17].

Enzyme-linked immunosorbent assay (ELISA):

ELISA was performed as described earlier [31, 32]. 96-well ELISA plates (eBioscience, San Diego, CA) were coated with purified HA protein (0.5 μg/ml) of HK/156 (MyBioSource, Inc., San Diego, CA, USA) and incubated overnight at 4ºC. Following blocking with 1% bovine serum albumin

(BSA) in PBS, diluted serum samples (1:500 dilution for IgG & IgG1 and 1:50 for IgG2a and IgG2b), 1:5 diluted nasal washes, or 1:10 diluted lung washes were added and incubated at room temperature for 2 h. During the standardization process, various dilutions of each type of sample were tested to establish the best dilution. The horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgA antibodies, (Invitrogen| Fisher Scientific Corp.) at a recommended dilution for each antibody was added and incubated at room temperature for 2 h. The color development was obtained with a BD OptEIA™ ELISA sets TMB substrate (Fisher Scientific Corp.). The reaction was stopped with 2N sulfuric acid solution and the optical density readings were obtained at 450 nm using a SpectraMax® i3x microplate reader (Molecular Devices, Sunnyvale, CA).

ELISpot Assays:

The ELISpot assays were performed as described previously [27]. The splenocytes, lung lymphocytes, RLN, and ILN were used for interferon-gamma (INFγ) ELISpot assays after stimulating the cells with HA518 (IYSTVASSL) peptide (H-2Kd-restricted CTL epitope for HA). The number of spot-forming units (SFU) were counted using a dissection microscope.

Statistical analyses:

Two-way ANOVA with Bonferroni post-test were performed to determine statistical significance. The statistical significance was set at $p < 0.05$.

3.4   Results

Induction of enhanced humoral immune responses with BAd-H5HA compared to HAd-H5HA:

The mouse groups inoculated i.n. or i.m. once with 1×10^6 (Fig. 1A), 3×10^6 (Fig. 1B), 1×10^7 (Fig. 1C), 3×10^7 (Fig. 1D) or 1×10^8 (Fig. 1E) PFU of HAd-H5HA or BAd-H5HA elicited dose-dependent increases in anti-HA IgG antibody levels. These levels in the i.m.-inoculated HAd-H5HA groups were similar to or slightly better than those observed in the similarly inoculated BAd-H5HA groups. In the i.n.-inoculated BAd-H5HA groups, however, anti-HA IgG antibody levels were significantly higher than those in the similarly or i.m.-inoculated HAd-H5HA groups

or the i.m.-inoculated BAd-H5HA groups. The control groups inoculated i.n. or i.m. with PBS, HAd-ΔE1E3, or BAd-ΔE1E3 did not yield anti-HA IgG antibody levels above background (Fig. 1).

We have shown previously that animals immunized with Ad vectors expressing HA produce high levels of hemagglutination inhibition (HI) and virus-neutralizing (VN) antibody titers against homologous influenza virus strains[16, 17, 27, 33, 34]; therefore, here we only tried to determine HI and VN titers against an antigenically distinct influenza virus strain (VN/1203/RG), and noticed that none of the vaccinated groups developed any detectable HI and VN titers above the empty vector controls (data not shown). These findings were not unexpected since similar results were observed in our previous studies [17, 35]

To determine whether the route of immunization or the vector type will influence the levels of IgG subclasses, the serum samples collected from the mouse groups inoculated i.n. or i.m. once with 1×10^6 (Figs. 2A, 3A & 4A), 3×10^6 (Figs. 2B, 3B & 4B), 1×10^7 (Figs. 2C, 3C & 4C), 3×10^7 (Figs. 2D, 3D & 4D) or 1×10^8 (Figs. 2E, 3E & 4E) PFU of HAd-H5HA or BAd-H5HA were analyzed for anti-HA IgG1 (Fig. 2), IgG2a (Fig. 3) and IgG2b (Fig. 4) levels by ELISA, respectively. As expected, there were dose-dependent increases in anti-HA IgG1, IgG2a and IgG2b antibody levels. These levels in the i.m.-inoculated HAd-H5HA groups were similar to or slightly better or lower than those observed in the i.m.-inoculated BAd-H5HA groups. However, in the i.n.-inoculated BAd-H5HA groups, anti-HA IgG1, IgG2a and IgG2b antibody levels were significantly higher than those in the i.n.- or i.m.-inoculated HAd-H5HA groups or the i.m.-inoculated BAd-H5HA groups (Figs. 2, 3 & 4). Control groups inoculated i.n. or i.m. with PBS, HAd-ΔE1E3, or BAd-ΔE1E3 did not yield anti-HA IgG1 (Fig. 2), IgG2a (Fig. 3) or IgG2b (Fig. 4) antibody levels above background.

Furthermore, to ascertain if the route of immunization or the vector type will influence the development of HA-specific IgA responses at the mucosal level, lung washes (Fig. 5) as well as nasal washes (Fig. 6) from the mouse groups inoculated i.n. or i.m. once with 1×10^6 (Figs. 5A & 6A), 3×10^6 (Figs. 5B & 6B), 1×10^7 (Figs. 5C & 6C), 3×10^7 (Figs. 5D & 6D) or 1×10^8 (Figs. 5E & 6E) PFU of HAd-H5HA or BAd-H5HA were analyzed for anti-HA IgA levels by ELISA.

As expected, there were dose-dependent increases in anti-HA IgA antibody levels in the lung washes as well as nasal washes. IgA antibody levels in the lung washes and nasal washes in the i.m.-inoculated BAd-H5HA or HAd-H5HA groups were detected only with doses of 1×10^7 PFU and onwards (Figs. 5 & 6). IgA antibody levels in the lung washes and nasal washes of the i.m.-inoculated BAd-H5HA groups were similar to or slightly higher than those in the i.m.-inoculated HAd-H5HA, whereas IgA antibody levels in the lung washes and nasal washes in the i.n.-inoculated BAd-H5HA or HAd-H5HA groups were detected with the lowest dose of 1×106 PFU and onwards. In the i.n.-inoculated BAd-H5HA groups, anti-HA IgA antibody levels in the lung washes (Fig. 5) and nasal washes (Fig. 6) were significantly higher than those in the i.n.- or i.m.-inoculated HAd-H5HA groups or the i.m.-inoculated BAd-H5HA groups. The control groups inoculated i.n. or i.m. with PBS, HAd-ΔE1E3, or BAd-ΔE1E3 did not yield anti-HA IgA antibody levels above background (Figs. 5 & 6).

Induction of enhanced CMI responses with BAd-H5HA compared to HAd-H5HA:

CMI responses against influenza viruses are important for virus clearance following infection and play an important role in heterologous as well as heterosubtypic protection against influenza viruses [17, 36]. To verify whether the route of immunization or the vector type will impact the development of the CMI responses against influenza in vaccinated mice, we analyzed splenocytes (Fig. 7), pooled RLN cells (Fig. 8), pooled ILN cells (Fig. 9), and pooled lung lymphocytes (Fig. 10) from AdV vector-inoculated groups for HA-specific CMI responses following in vitro stimulation with HA518 using an IFNγ-specific ELISpot assay. The numbers of INFγ-secreting HA518-specific CD8 T cells from the mouse groups inoculated i.n. or i.m. once with 1×10^6 (Figs. 7A, 8A, 9A & 10A), 3×10^6 (Figs. 7B, 8B, 9B & 10B), 1×10^7 (Figs. 7C, 8C, 9C & 10C), 3×10^7 (Figs. 7D, 8D, 9D & 10D) or 1×10^8 (Figs. 7E, 8E, 9E & 10E) PFU of HAd-H5HA or BAd-H5HA are shown. There were dose-dependent increases in the numbers of INFγ secreting HA518-specific CD8 T cells in splenocytes (Fig. 7), pooled RLN cells (Fig. 8), pooled SLN cells (Fig. 9), and pooled lung lymphocytes (Fig. 10) of the vaccinated groups compared with the empty vector (Ad-ΔE1E3) or PBS control groups. There were significantly higher numbers of INFγ secreting HA518-specific CD8 T cells in splenocytes of the i.m.- or i.n.-inoculated BAd-H5HA groups compared to the i.m.- or i.n.-inoculated HAd-H5HA groups (Fig. 7), and the numbers were

consistently higher in the i.m.-inoculated vaccine groups compared to that of the i.n. inoculated vaccine groups.

There were substantially higher numbers of INFγ secreting HA518-specific CD8 T cells in the RLN of the i.m.- or i.n.-inoculated BAd-H5HA groups compared to those of the i.m.- or i.n.-inoculated HAd-H5HA groups (Fig. 8), and the numbers were consistently higher in the i.n.-inoculated vaccine groups compared to those of the i.m.-inoculated vaccine groups. Whereas in ILN of the i.m.-inoculated vaccine groups, higher numbers of INFγ secreting HA518-specific CD8 T cells were detected compared to those of the empty vector (Ad-ΔE1E3) or PBS control groups or the i.n.-inoculated vaccine groups (Fig. 9), and the numbers were consistently higher in the i.m.-inoculated vaccine groups compared to those of the i.n.-inoculated vaccine groups. Furthermore, considerably elevated numbers of INFγ secreting HA518-specific CD8 T cells in the lung lymphocytes of the i.m.- or i.n. inoculated BAd-H5HA groups compared to those of the i.m.- or i.n.-inoculated HAd-H5HA groups, respectively, were visualized (Fig. 10), and the numbers were consistently higher in the i.n.-inoculated vaccine groups compared to those of the i.m.-inoculated vaccine groups.

Development of enhanced protection in mice immunized with BAd-H5HA compared to HAd-H5HA:

Since the influenza virus VN/1203/RG, which was used as a challenge virus to evaluate the efficacy of protection, does not cause significant morbidity or mortality in mice, significant reductions in lung viral titers in vaccinated animals following challenge is a useful measure of the vaccine protective efficacy. The mouse groups were immunized i.n. or i.m. once with 1×10^6 (Fig. 11A), 3×10^6 (Fig. 11B), 1×10^7 (Fig. 11C), 3×10^7 (Fig. 11D) or 1×10^8 (Fig. 11E) PFU of HAd-H5HA or BAd-H5HA, and subsequently challenged with 100 MID50 of VN/1203/RG. For the i.n. route of inoculation, the lowest vaccine dose of 1×10^6 PFU of BAd-H5HA conferred complete protection following challenge (Fig. 11A). The lowest vaccine dose for i.n.-inoculated HAd-H5HA that yielded complete protection following challenge was 3×10^7 PFU (Fig. 11E). For the i.m. route of inoculation, the lowest vaccine dose for BAd-H5HA that conferred complete protection following challenge was 3×10^7 PFU (Fig. 11D). For the i.m.-inoculated HAd-H5HA animal group, the log lung virus titer with a dose of 1×10^8 PFU was 1.84±1.14, compared to 4.90±0.42

and 4.6±0.46 log lung virus titers in the empty vector (HAd-ΔE1E3) or PBS control groups, respectively (Fig. 11E). Even at a challenge dose of 3×10^8 PFU per animal for i.m.-inoculated HAd-H5HA animal group, log lung virus titers of 0.84±0.5 was observed.

3.5   Discussion

Several studies using AdV vector-based influenza vaccines have been conducted both in animal models [27, 33, 37] and as clinical trials in humans [19, 38] to explore the potential of this vector system. Previously we have shown that Ad vector-based vaccines can provide complete protection against challenge with homologous and heterologous (antigenically distinct) influenza virus strains [27]. Moreover, Ad vector vaccines containing multiple HA from different HA subtypes, or expressing nucleoprotein (NP) of H5N1 influenza, conferred either complete protection or significant reduction in lung virus titers, respectively, following challenge with pandemic H5, H7 or H9 influenza viruses[16, 17].

The purpose of this study was to determine some of the parameters that are important for a pre-pandemic influenza vaccine including route of immunization, dose sparing, protection from a single dose, and protection against a heterologous influenza virus strain. This study was based on the hypothesis that a combination of HA-specific CMI responses and cross-reactive (though not necessarily cross-neutralizing) humoral immune responses will provide heterologous protection against an antigenically distinct H5N1 influenza virus. In addition, this study also focused on determining the vector type and route of immunization for enhanced immunogenicity conferring efficient protection.

Overall, there were no significant differences in the serum anti-HA IgG, IgG1, IgG2a, and IgG2b or mucosal IgA responses in mouse groups inoculated i.m. either with BAd-H5HA or HAd-H5HA. However, mice inoculated with BAd-H5HA had higher increases in the numbers of INFγ secreting HA518-specific CD8 T cells in the spleen, lung and RLN than did those inoculated with HAd-H5HA. In contrast, there were no significant differences in the numbers of INFγ secreting HA518-specific CD8 T cells in the ILN of the mouse groups inoculated i.m. either with BAd-H5HA or HAd-H5HA. An intravenous bio-distribution study in mice has demonstrated that the BAd vector genome persists longer and with higher copy numbers in the spleen and lungs than that of the HAd

vector [12]. Similar bio-distribution following the i.m. route of immunization may be responsible for the higher level of CMI responses with the BAd-H5HA vector. Additional studies will be required to determine the mechanism/s of these observations.

The HK/156 HA expressed in the AdV vectors and the HA in the challenged virus VN/1203/RG are antigenically different, so it was not surprising that the serum samples from either BAd-H5HA- or HAd-H5HA-inoculated animal groups did not have detectable levels of HI or VN titers against the challenge virus. We purposely opted for antigenically distinct HAs as an immunogen and a challenge virus to test our hypothesis that a combination of CMI responses and non-neutralizing antibodies will provide heterologous protection. The mouse group inoculated i.m. with 3×10^7 PFU of BAd-H5HA were completely protected from the challenge with VN/1203/RG, whereas detectable levels (0.84±0.5 log) of lung virus titers were observed in the mouse group i.m.- inoculated with 3×10^8 PFU of HAd-H5HA. These observations suggest that significantly higher levels of CMI responses and non-neutralizing antibodies elicited with BAd-H5HA may be responsible for enhanced protection at a significantly lower dose.

In mouse groups inoculated i.n. with BAd-H5HA, levels of serum anti-HA IgG, IgG1, IgG2a, IgG2b, mucosal anti-HA IgA, and the numbers of INFγ secreting HA518-specific CD8 T cells in the spleen, lung and RLN, were significantly higher than in the groups inoculated i.m. with BAd-H5HA or inoculated i.m. or i.n. with HAd-H5HA. Additional experiments are required to determine whether the high levels of humoral (systemic and mucosal) and CMI responses are due to better transduction of and/or the levels and duration of persistence of the BAd vector genomes following immunization with BAd-H5HA. Since BAd-3 utilizes the α(2,3)-linked and α(2,6)- linked sialic acid receptors as the major receptors for virus internalization[15], while HAd-C5 uses CAR[39-41] for virus entry in susceptible cells, we expect that BAd-H5HA will better transduce the respiratory tract following i.n. inoculation compared to HAd-H5HA. Moreover, the levels and duration of persistence of the BAd vector genome copy numbers in the lungs were found to be significantly higher than that of the HAd vector in an intravenous bio-distribution study [12]. There is a possibility that a similar situation may occur following the i.n. inoculation.

Complete protection from challenge with an antigenically distinct H5N1 influenza virus VN/1203/RG was observed even in the mouse group inoculated i.n. with 1×10^6 PFU of BAd-H5HA, whereas similar level of protection with HAd-H5HA was only obtained with a much higher vector dose of 1×10^8 PFU. These observations suggest that BAd vector-based i.n. vaccine delivery system has considerable promise for dose sparing, because an approximately 100-fold lower vaccine dose of BAd-H5HA compared to HAd-H5HA conferred complete protection. Dose sparing not only lowers vaccine costs, but also increases the capacity to produce a large number of doses especially in an event similar to the influenza pandemic. Of course, additional studies will be needed to determine the long-term efficacy of cross-protective humoral and CMI responses induced by BAdV-3-based vectors.

The results described in this manuscript suggest that the BAdV-3-based vector system has many advantages over HAd systems as a vaccine delivery vehicle for developing pre-pandemic influenza vaccines. Further studies in another animal model of influenza, such as ferrets, will be essential to fully explore the potential of this vaccine delivery system. Additional studies will be required to determine the best combinational antigens or immunogenic domains that could elicit broadly cross-protective immune responses when delivered through the BAd vector system for developing a universal influenza vaccine for pandemic preparedness and to offer a better vaccine option against seasonal influenza viruses.

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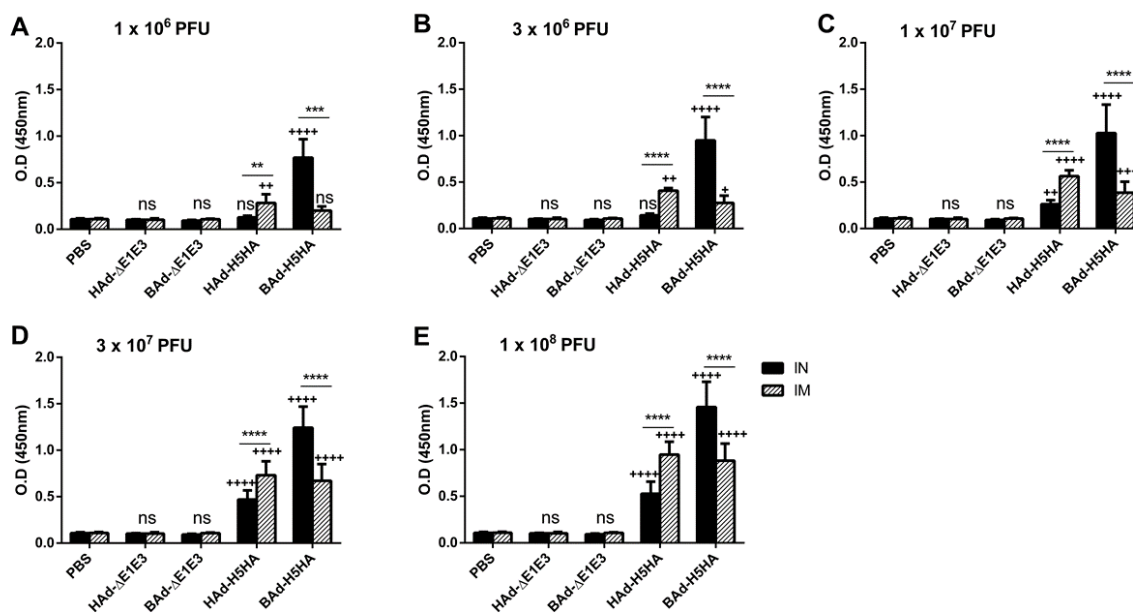
Figure 3.1: HA-specific serum IgG antibody responses in mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×108 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:500, and the development of HA-specific IgG antibody responses were monitored by ELISA. Data are represented as the mean ± standard deviation (SD) of the optical density (OD) readings. Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at $p<0.05$; ** or ++, significant at $p<0.01$; *** or +++, significant at $p<0.001$; and **** or ++++; significant at $p<0.0001$. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; and ns, no significance at $p>0.05$.

Figure 3.2: HA-specific serum IgG1 antibody responses in mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:500, and the development of HA-specific IgG antibody responses were monitored by ELISA. Data are represented as the mean ± standard deviation (SD) of the optical density (OD) readings. Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at p<0.05; ** or ++, significant at p<0.01; *** or +++, significant at p<0.001; and **** or ++++; significant at p<0.0001. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; and ns, no significance at p>0.05.
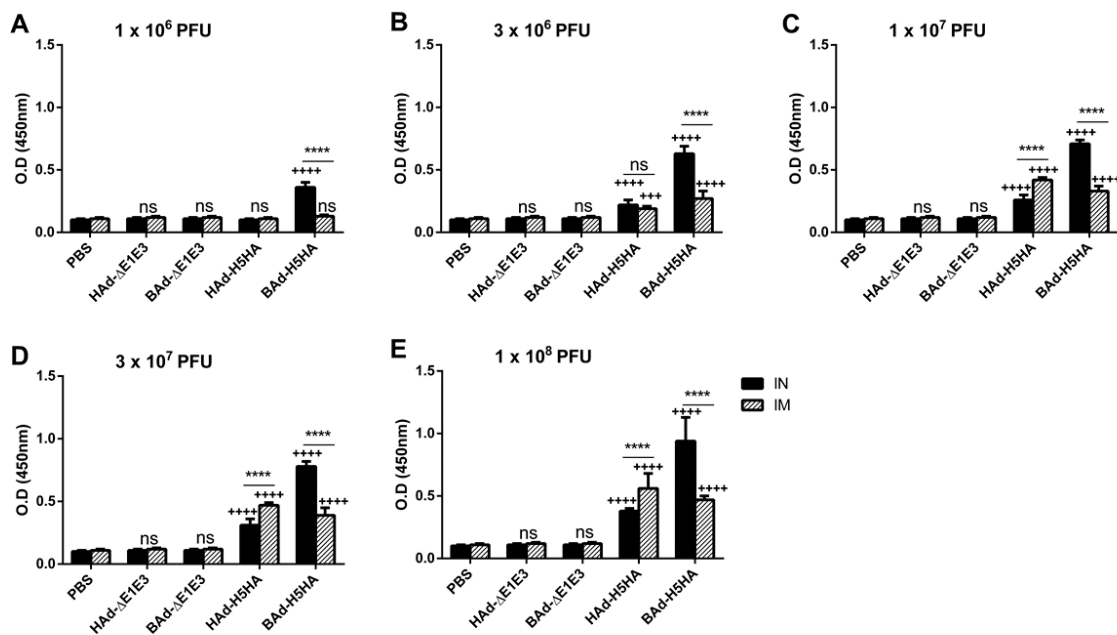
Figure 3.3: HA-specific serum IgG2a antibody responses in mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:500, and the development of HA-specific IgG antibody responses were monitored by ELISA. Data are represented as the mean $\pm$ standard deviation (SD) of the optical density (OD) readings. Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at $p<0.05$; ** or ++, significant at $p<0.01$; *** or +++, significant at $p<0.001$; and **** or ++++; significant at $p<0.0001$. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; and ns, no significance at $p>0.05$.
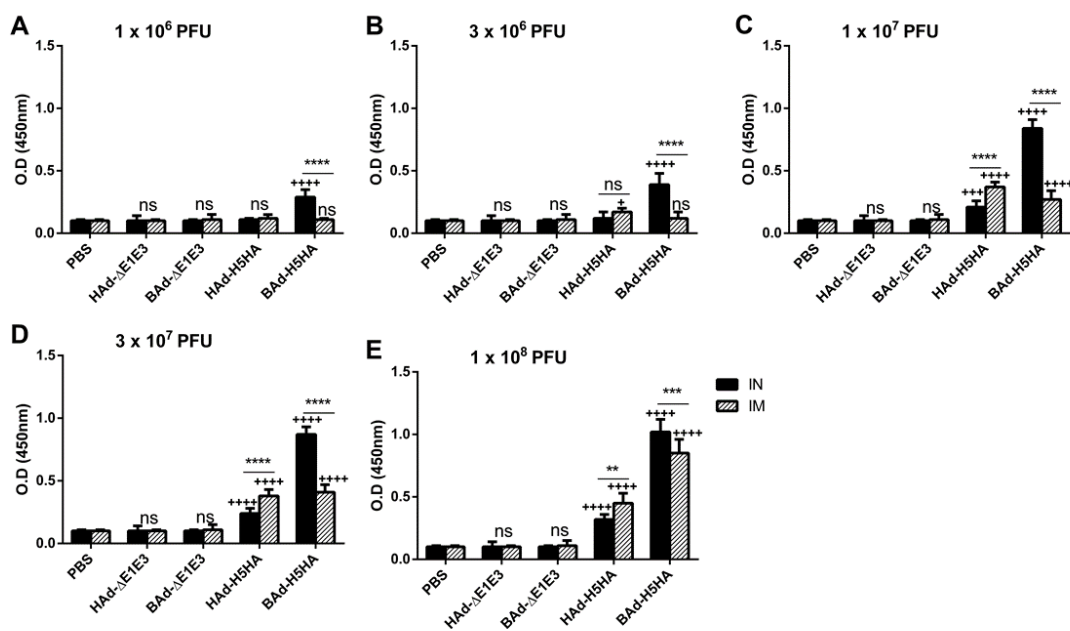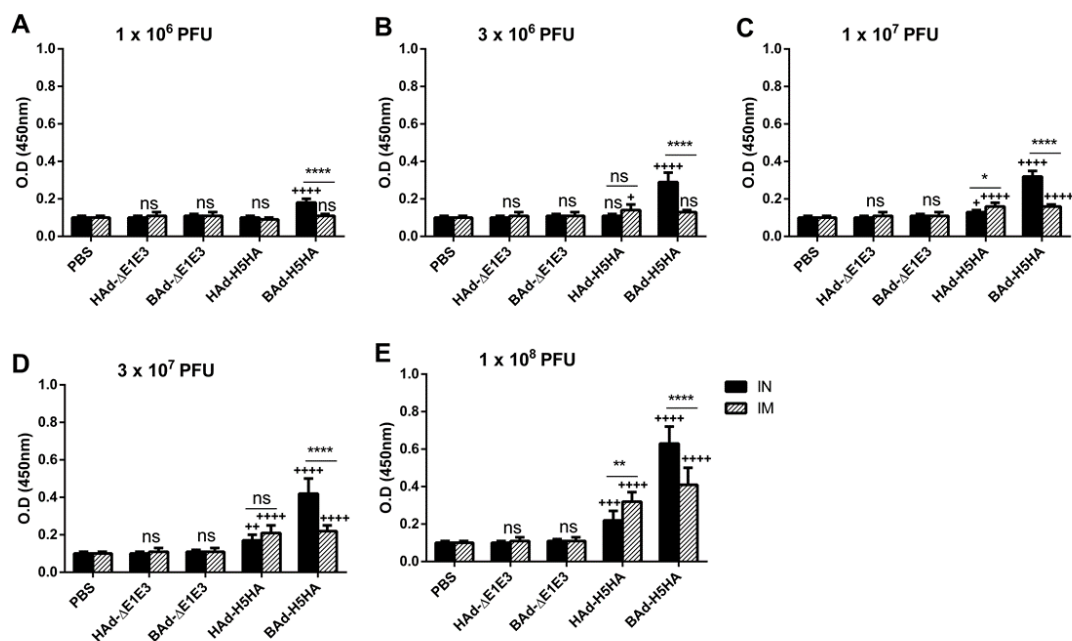
Figure 3.4: HA-specific serum IgG2b antibody responses in mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, serum samples were collected, diluted to 1:500, and the development of HA-specific IgG antibody responses were monitored by ELISA. Data are represented as the mean ± standard deviation (SD) of the optical density (OD) readings. Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at p<0.05; ** or ++, significant at p<0.01; *** or +++, significant at p<0.001; and **** or ++++; significant at p<0.0001. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; and ns, no significance at p>0.05.

Figure 3.5: HA-specific IgA antibody responses in nasal washes of mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, nasal wash samples were collected, diluted to 1:5, and the development of HA-specific IgG antibody responses were monitored by ELISA. Data are represented as the mean ± standard deviation (SD) of the optical density (OD) readings. Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at p<0.05; ** or ++, significant at p<0.01; *** or +++, significant at p<0.001; and **** or ++++; significant at p<0.0001. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; and ns, no significance at p>0.05.
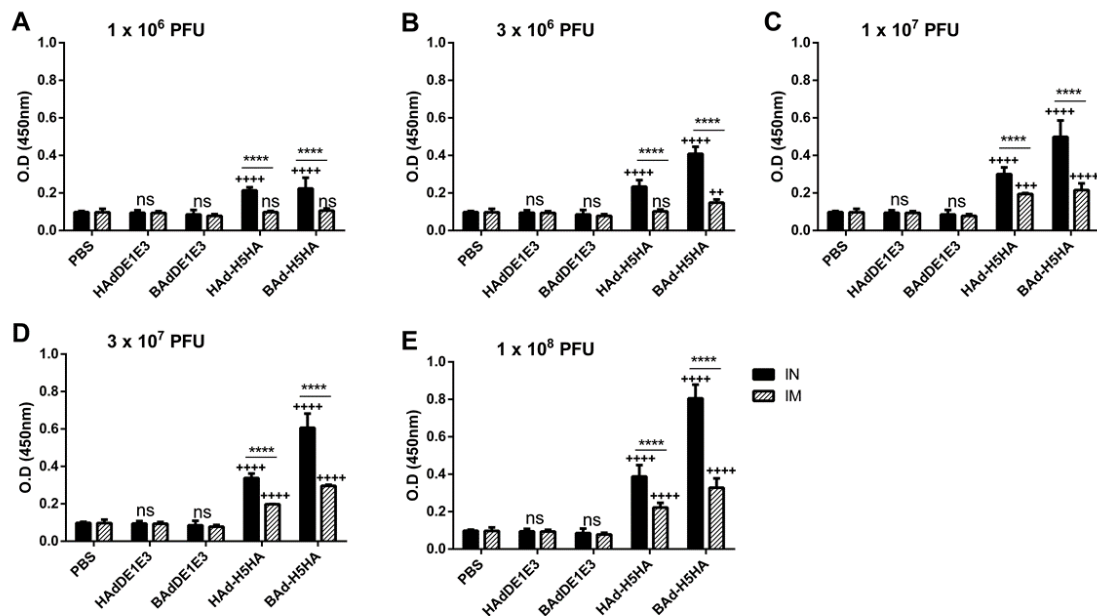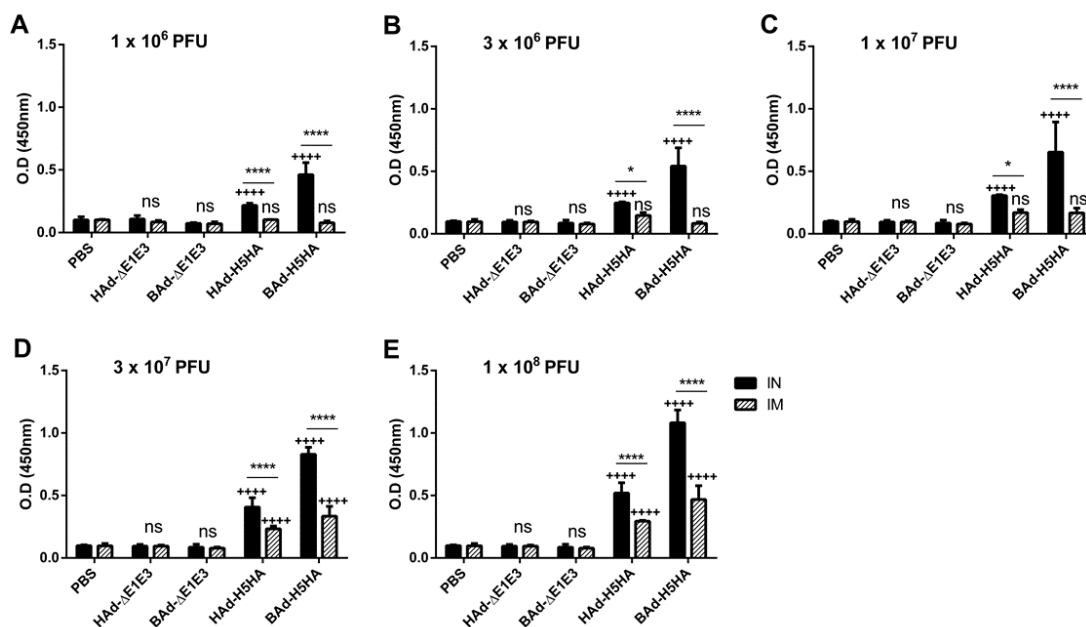
Figure 3.6: HA-specific IgA antibody responses in lung washes of mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, lung wash samples were collected, diluted to 1:10, and the development of HA-specific IgG antibody responses were monitored by ELISA. Data are represented as the mean ± standard deviation (SD) of the optical density (OD) readings. Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at p<0.05; ** or ++, significant at p<0.01; *** or +++, significant at p<0.001; and **** or ++++; significant at p<0.0001. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; and ns, no significance at p>0.05.
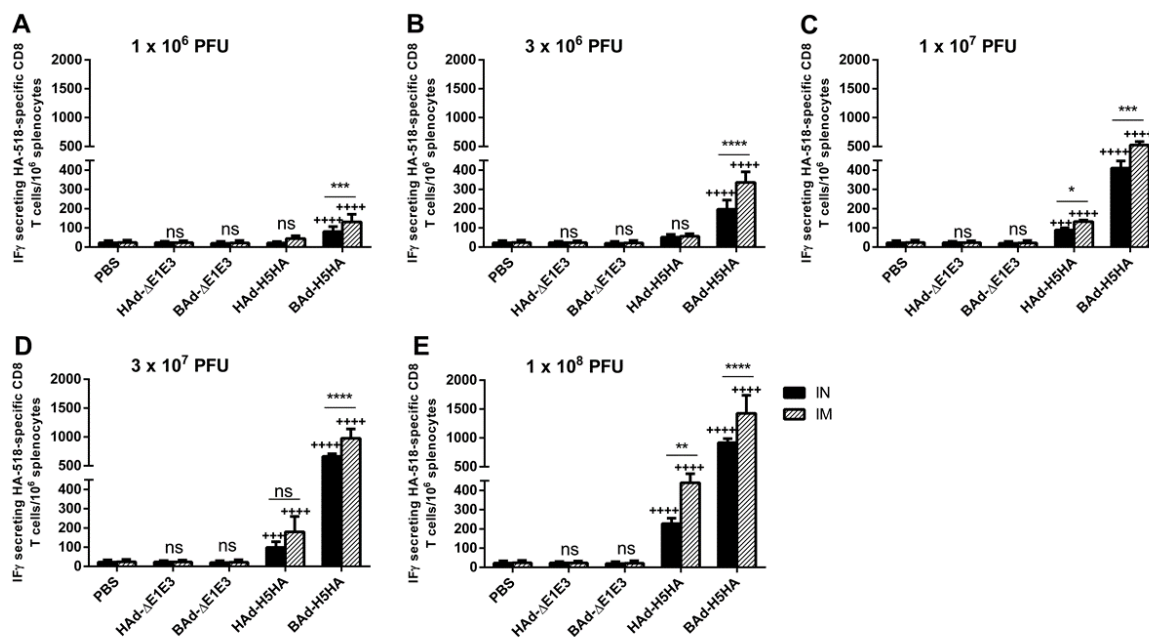
Figure 3.7: HA518 epitope-specific IFNγ secreting CD8+ T cells in the spleens of mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the spleens were collected, and the splenocytes were evaluated for HA-specific cell-mediated immune responses using INFγ-ELISpot assay. The data represent mean ± standard deviation (SD) of the number of spot-forming units (SFU). Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at $p<0.05$; ** or ++, significant at $p<0.01$; *** or +++, significant at $p<0.001$; and **** or ++++; significant at $p<0.0001$. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; and ns, no significance at $p>0.05$.

Figure 3.8: HA518 epitope-specific IFNγ secreting CD8+ T cells in the respiratory lymph nodes (RLN) of mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the RLN were collected, and the pooled RLN cells were evaluated for HA-specific cell-mediated immune responses using INFγ-ELISpot assay. The data represent mean number of spot-forming units (SFU) from pooled samples. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of a A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline.
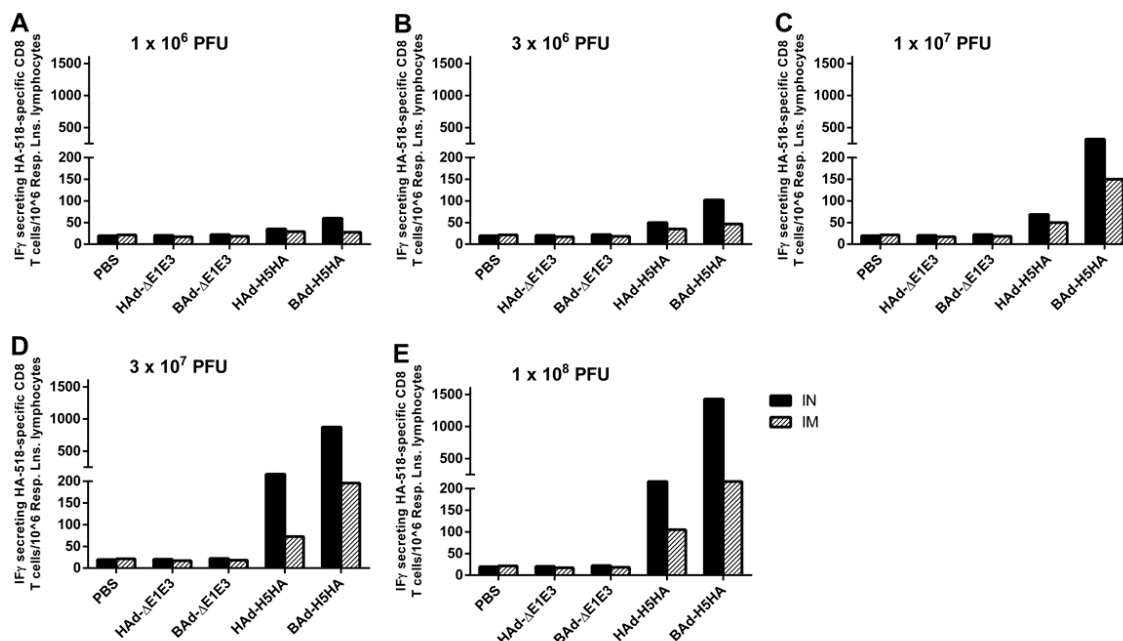
Figure 3.9: HA518 epitope-specific IFNγ secreting CD8+ T cells in the inguinal lymph nodes (ILN) of mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the ILN were collected, and the pooled ILN cells were evaluated for HA-specific cell-mediated immune responses using INFγ-ELISpot assay. The data represent mean number of spot-forming units (SFU) from pooled samples. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of a A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline.
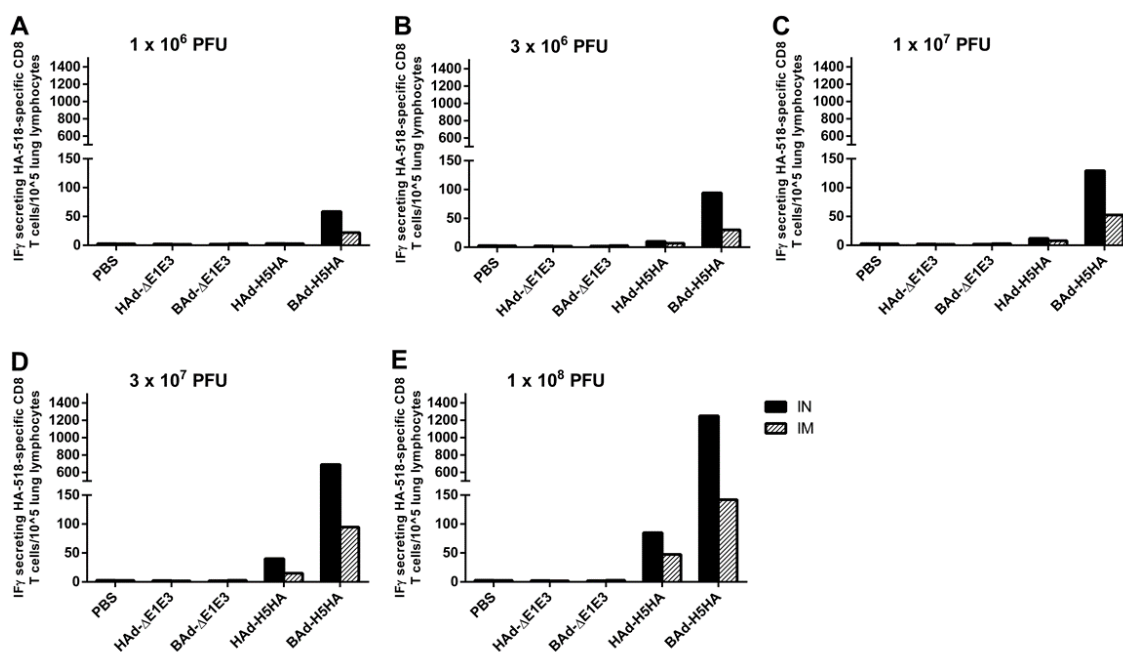
Figure 3.10: HA518 epitope-specific IFNγ secreting CD8+ T cells in the lungs of mice
immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after inoculation, the lungs were collected, and the pooled lung lymphocytes were evaluated for HA-specific cell-mediated immune responses using INFγ-ELISpot assay. The data represent mean the number of spot-forming units (SFU) from pooled samples. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of a A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline.
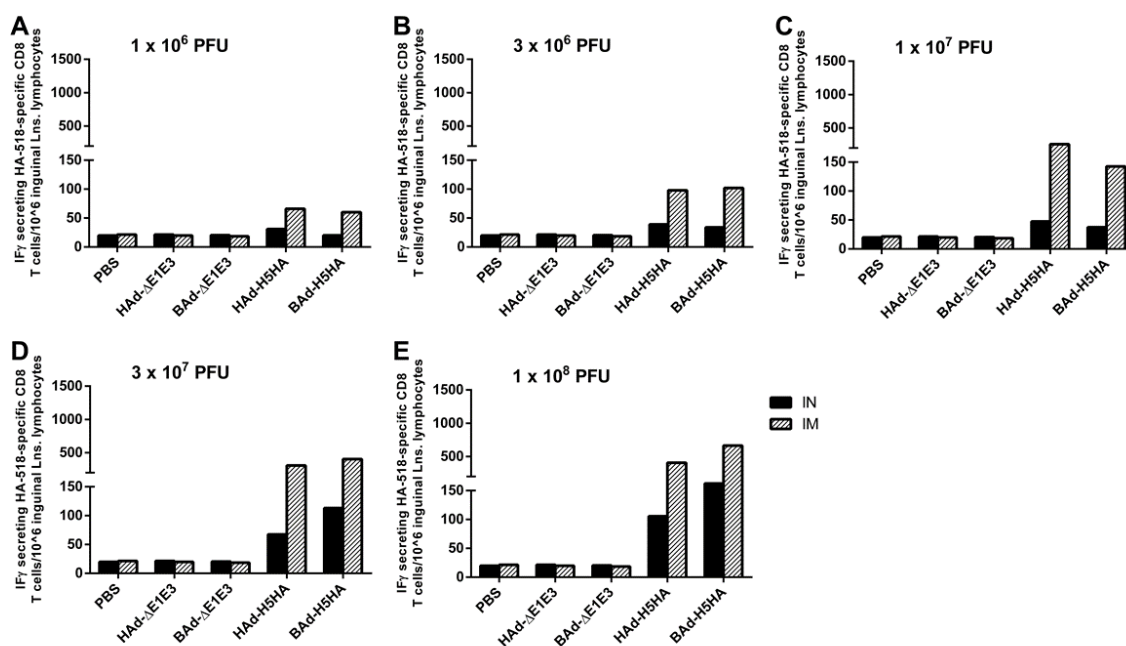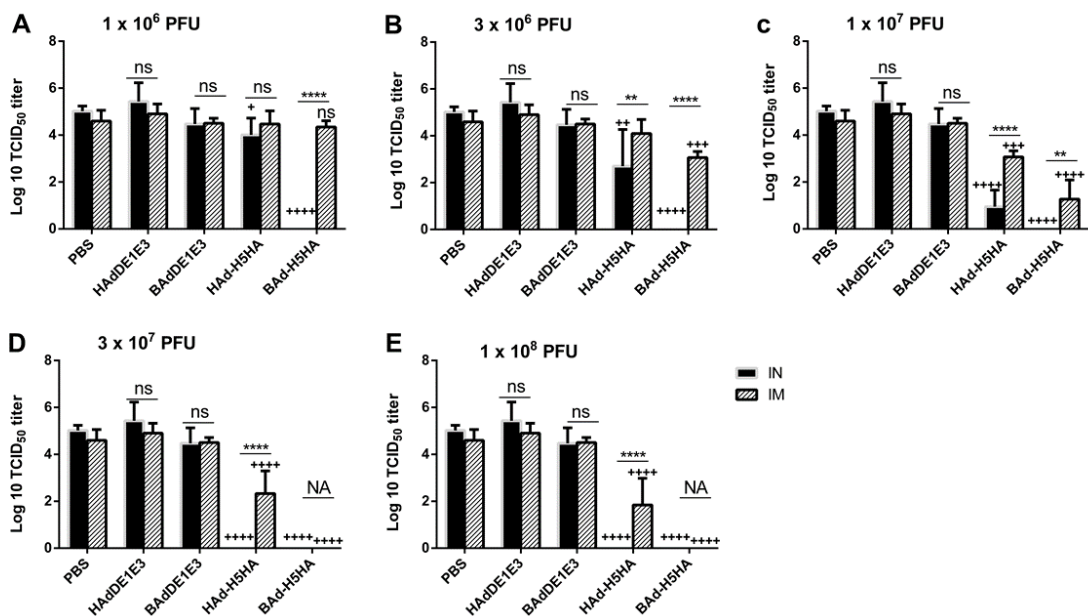
Figure 3.11: Lung influenza virus titers in mice immunized once with BAd-H5HA or HAd-H5HA.

Mice were immunized intramuscularly (i.m.) or intranasally (i.n.) once with 1×10^6 (A), 3×10^6 (B), 1×10^7 (C), 3×10^7 (D) or 1×10^8 (E) PFU of BAd-H5HA or HAd-H5HA. For all dose groups, mice inoculated i.m. or i.n. with PBS or 1×10^8 PFU of BAd-ΔE1E3 or HAd-ΔE1E3 served as negative or internal controls, respectively. Four weeks after immunization, mice were challenged with 100 MID50 of A/Vietnam/1203/2004(H5N1)-PR8/CDC-RG influenza virus, and three days after the challenged, mice were euthanized, and the lungs were collected to determine lung virus titers. The data are shown as mean Log10 TCID$_{50}$±SD and the detection limit was 0.5 Log10 TCID$_{50}$/ml. Statistically significant responses are shown as compared to PBS group (+) or i.n. versus i.m. route of inoculation in the same group (*). * or +, significant at $p<0.05$; ** or ++, significant at $p<0.01$, *** or +++, significant at $p<0.001$; and **** or ++++, significant at $p<0.0001$. The statistical analyses were done by Bonferroni post-test and two-way ANOVA using Graph Pad Prim 6. BAd-H5HA, bovine adenoviral vector expressing hemagglutinin (HA) of a A/Hong Kong/156/97(H5N1) influenza virus; HAd-H5HA, human adenoviral vector expressing HA of a A/Hong Kong/156/97(H5N1) influenza virus; BAd-ΔE1E3 (BAd empty vector); HAd-ΔE1E3, (HAd empty vector); PBS, phosphate-buffered saline; ns, no significance at $p>0.05$; NA, not applicable.

CHAPTER 4.   LONGEVITY OF ADENOVIRUS VECTOR IMMUNITY AND ITS IMPLICATION ON VACCINE EFFICACY

4.1   Abstract

There is high occurrence of adenovirus (AdV) infections in humans due to the presence of more than 60 human AdV types. The majority of individuals get exposed to one or more HAdV types early in their lives leading to the development of AdV type-specific neutralizing antibodies popularly known as 'vector immunity', which has been considered a potential concern for AdV vector-based applications for vaccines or gene therapy. The objective of this investigation was to determine the prevalence of HAdV type C5 (HAdV-C5), chimpanzee AdV type 7 (chAdV-7) and bovine AdV type 3 (BAdV-3) in 60 human serum samples, and to establish whether annual vaccination with an AdV vector-based vaccine will be possible due to sufficient decline in AdV neutralizing antibody titers within a year. There was high prevalence of virus-neutralizing antibodies against HAdV-C5, whereas, low levels of cross-neutralizing vector immunity against ChAdV-7 and no occurrence of cross-neutralizing vector immunity against BAdV-3 were observed. Naïve or HAdV-C5-primed mice were mock-inoculated (with PBS) or inoculated i.m. with 108 p.f.u. of either HAd-GFP [HAdV-C5 vector expressing green fluorescent protein (GFP)] to mimic the conditions for the first inoculation with an AdV vector-based vaccine. At 1, 3, 6, and 10 months post-HAd-GFP inoculation, naïve- or HAdV-primed animals were vaccinated i.m. with 108 p.f.u. of HAd-H5HA [HAdV-C5 vector expressing hemagglutinin (HA) of H5N1 influenza virus]. There were significant continual decreases in vector immunity titers with time, thereby leading to significant continual increases in the levels of HA-specific humoral and cell-mediated immune responses. Following challenge with an antigenically heterologous H5N1 virus, the level of protection was also improved with time and well-aligned with the reductions in vector immunity titers. These results indicate that the annual immunization with the same AdV vector will be effective due to significant decline in vector immunity.

4.2   Introduction

Adenovirus (AdV) vector-based vaccines induce excellent humoral and cell-mediated immune (CMI) responses[1, 2] due to the adjuvant-like effect of Ad vectors in stimulating the innate immune system through both Toll-like receptor (TLR)-dependent and TLR-independent pathways[3, 4]. Ad vector-based influenza vaccines have elucidated excellent potential in both animal models [5-7] and clinical trials in humans [8, 9]. Our immunogenicity and protective efficacy studies in mice demonstrate that Ad vector-based vaccines provide complete protection against challenge with homologous and antigenically distinct strains of influenza viruses [6].

There is a high incidence of AdV infections in the general population due to the circulation of more than 60 human AdV (HAdV) serotypes. The development of Ad-specific neutralizing antibodies, popularly known as 'pre-existing vector immunity', in the majority of individuals [10-12] has been considered a potential concern for Ad vector-based vaccine efficacy. The high level of virus neutralizing antibody titers in humans in the U.S. was found to be in the range of 256-512 in 16% of the samples [10]. In Sub-Saharan children, a median HAdV-C5 neutralizing antibody titer of 512 was observed [13]. However, it is unclear what levels of vector immunity may have significant negative impact on development of effective immune responses and the decline of vector immunity with time.

Earlier we have evaluated the role of HAd-C5-neutralizing antibodies or vector immunity in impacting the immunogenicity and protection efficacy of a HAd vector (HAd-HA-NP) expressing the HA and NP genes of A/Vietnam/1203/04 (H5N1) influenza virus [14]. The mouse groups were primed either intranasally (i.n.) or intramuscularly (i.m.) with varying doses of HAdV-C5, and following the development of vector immunity the animal groups were immunized with HAd-HA-NP via the i.n. or i.m. route. The immunogenicity and protection results suggested that high levels of vector immunity [520 virus-neutralization (VN) titer] did not adversely impact the protective efficacy of the vaccine. Further increases in vector immunity (up to 2240 VN titers) were overcome by either increasing the vaccine dose by 5× or using an alternate route of vaccination. In the presence of exceptionally high levels of vector immunity (~3040 VN titers), immunization with a 5× vaccine dose resulted in approximately 3.3-3.7 logs reduction in lung virus titers.

Canarypox virus-based vaccines are routinely used in pet animals on an annual basis suggesting that the development of immunity against a canarypox vector due to yearly exposure does not negatively impact the vaccine efficacy [15, 16]. In this manuscript we tried to address whether annual vaccination with an AdV vector-based vaccine will be possible due to the decline in AdV neutralizing antibody titers below 500 within a year. This study is important for determining the practical utility of AdV vector-based vaccines for human use. Our results of HAd-specific neutralizing antibody levels in humans suggests that approximately 32% individuals have vector immunity titer above 200. There was continual decline in vector immunity with time leading significant increases in humoral and cell-mediated immune responses with decreases in vector immunity in the mouse model. In addition, effective immunogenicity and protection was observed in HAdV-C5-primed animal groups immunized with a HAd vector (HAd-H5HA) expressing the HA gene of A/Hong Kong/156/97(H5N1) (HK/156)] at 6-month and onwards.

4.3   Material and methods

Cell lines and viruses:

All cell lines were grown in minimum essential medium (MEM) (Life Technologies, Gaithersburg, MD) with 10% fetal calf serum (Hyclone, Logan, UT) and 50 µg/ml gentamycin. 293 (human embryonic kidney cells expressing HAdV-C5 E1 proteins) [17] and BHH2C (bovine-human hybrid clone 2C)[18] were used to grow and titrate human adenovirus vectors (HAdVs) and chimpanzee adenovirus type 7 empty vector (ChAd-ΔE1E3). BHH3 (bovine-human hybrid clone 3) [18] were used to grow and titrate bovine adenovirus type 3 empty vector (BAd-ΔE1E3). MDCK (Madin-Darby canine kidney) cell line was used to titrate influenza virus titers in the lungs for protection studies. ChAdC7-ΔE1E3 was kindly provided by Dr. Stefan Worgall, Department of Pediatrics, Weill Cornell Medical College, New York, NY.

The construction of HAdV-C5 empty vector (HAd-ΔE1E3) [19], HAdV-C5 vector expressing green fluorescent protein (HAd-GFP) [10], HAdV-C5 vector expressing HA of A/Hong Kong/156/H5N1 (HAd-H5HA)][6], BAd-ΔE1E3 [20], and ChAd-ΔE1E3 [21] have been described previously. All AdV vectors were purified using cesium chloride density gradient ultracentrifugation as described [14].

The influenza virus A/Vietnam/1203/2004(H5N1)-PR8/CDC-RG [VN/1203/RG] which was created by reverse genetics, was grown in embryonated chicken eggs and quantified as tissue culture infectious dose 50 ($TCID_{50}$) in MDCK cells. VN/1203/RG was used for challenge studies in mice as described in the experimental design. The HA gene in HAd-H5HA vector is from HK/156 influenza virus, which is antigenically distinct from the HA of VN/1203/RG (the challenge virus).

Virus neutralization assays:

To study the sero-prevalence of virus-neutralizing or cross-neutralizing antibodies against three different AdV (HAdV-C5, ChAdV-7 and BAdV-3) in human, randomly chosen sixty human serum samples were purchased from the Indiana University Simon Cancer Center Tissue Procurement Facility and used according to the institutional biosafety committee guidelines. The human serum samples were inactivated at 56ºC for 30 minutes. The inactivated serum samples were serially two-fold diluted in MEM in 96-well tissue culture plates starting with the dilution 1:4. Then 100 PFU of HAd-ΔE1E3, ChAd-ΔE1E3 or BAd-ΔE1E3 were added to each well. After 1 h of incubation, 104 cells in 100 µl of MEM with 10% fetal bovine serum were added to each well as described previously [10]. For these neutralization assays, BHH-2C cells were used to determine virus-neutralization titer for HAd-ΔE1E3 and ChAd-ΔE1E3, while BHH3 cells were used for BAd-ΔE1E3.

For determining virus-neutralizing or cross-neutralizing antibodies among HAdV-C5, ChAdV-7 and BAdV-3), serum samples collected from mice (5 animals/group) inoculated either with HAd-ΔE1E3, ChAd-ΔE1E3 or BAd-ΔE1E3 were used. Each serum sample was serially two-fold diluted in MEM in 96-well tissue culture plates starting with the dilution 1:10 and the remaining steps were same as described above.

The neutralization titer was calculated as the reciprocal of the highest dilution of the serum that prevented the virus cytopathic effect (CPE) in the cells using Reed and Muench method [22].

Animal inoculation and experimental design:

The animal experiments were operated in BSL-2+ lab at Purdue University approved by USDA. The Institutional Animal Care and Use Committee (IACUC), and the Institutional Biosafety Committee (IBC) have approved the protocols for animal inoculations and protection studies.

Five to 6-week-old female BALB/c mice (purchased from Harlan Sprague Dawley Inc., Indianapolis, IN) were used to examine the longevity of HAdV-C5 neutralizing antibodies and its impact on the immune responses and protection of the HAd-H5HA vaccine. The diagrammatic representation of experimental plan is depicted (Fig. 1).

Control groups. At Day 0, all control groups were inoculated intranasally (i.n.) with 20 μl/animal of phosphate buffer saline (PBS) under anesthesia. One month later all Control groups were inoculated intramuscularly (i.m.) with 50 μl of PBS. Subsequently at 1, 3, 6 and 10 months post-second inoculation with PBS, 10 animals per group were inoculated i.m. with 1×10^8 PFU of HAd-ΔE1E3.

Naïve vaccinated groups. At Day 0, all naïve-vaccinated groups were inoculated i.n. with 20 μl of PBS under anesthesia and one month later, all animals received 50 μl of PBS i.m. Subsequently at 1, 3, 6, and 10 months post-second PBS inoculation, 10 animals were inoculated i.m. with 1×10^8 PFU of HAd-H5HA.

Primed vaccinated groups. At Day 0, all primed-vaccinated groups were inoculated i.n. with 1×10^7 PFU of HAdV-C5 and one month later, all animals were inoculated i.m. with 1×10^8 PFU of HAd-GFP. Subsequently at 1, 3, 6, and 10 months post-inoculation with HAd-GFP, 10 animals were inoculated with 1×10^8 PFU of HAd-H5HA.

Blood samples were collected from the cheek vein from all mice 3 days before the second and third inoculations for the evaluation of HAdV-C5 neutralizing antibodies and GFP antibody levels using the plaque assay and ELISA, respectively. Blood samples and spleens were collected under anesthesia from 5 mice in each group (control, naïve-vaccinated or primed-vaccinated) at 4 weeks post-third inoculation either with HAd-ΔE1E3, HAd-H5HA or HAd-H5HA, respectively. Serum samples were used to determine the humoral immune responses by ELISA, and splenocytes were

used to determine the cell mediated immune responses by ELISpot. The remaining 5 mice in each group (control, naïve-vaccinated or primed-vaccinated) were challenged at 4 weeks post-third inoculation i.n. with 100 mouse infectious dose 50 (MID50) of A/VN/1203-RG under anesthesia. Three days after the challenge mice were euthanized, and the lungs were collected for evaluating the protection efficacy by determining the lung virus titers.

Vector-neutralizing antibody titration:

Serum samples collected from the mouse study (Fig. 1) before the second or third inoculation were used to monitor HAdV-C5-neutralization antibody titers by virus-neutralization assay as described previously[18]. Briefly, serum samples were diluted 1:10 and inactivated at 56ºC for 30 minutes. The inactivated sera were incubated with 100 PFU HAdV-C5 at 37ºC for 1 h. Each serum/virus mixture was added to BHH-2C cells at 90% confluency in 60-mm tissue culture plates in triplicate. After 30 minutes at 37ºC, the agarose overlay (MEM containing 0.5% agarose, 5% fetal calf serum and 1% yeast extract) was added into each plate and incubated at 37ºC in 5 % CO2 incubator for 7 days. The number of AdV plaques were counted to determine virus-neutralization titers. The highest serum dilution that reduced the number of virus plaques by 50% compared to the control virus-infected cells was considered as the HAdV-C5-neutralization antibody titer.

Enzyme-linked immunosorbent assay (ELISA):

For measuring the humoral immune responses to GFP or HA, ELISA was used as described previously [23, 24]. Briefly, 96-well ELISA plates (Thermo Scientific Clear Flat-Bottom Immuno Nonsterile 96-Well Plates) were coated with 0.5 µg/ml of purified GFP protein (Upstate, Temecula, CA) or HA protein of HK/156 (MyBioSource, Inc., San Diego, CA), incubated overnight at 4ºC, and blocked with 1% bovine serum albumin (BSA) in PBS. Various serial dilutions of a few mouse serum samples were used to determine the best dilution for all serum samples. Finally, all serum samples were diluted to 1:200, added into the wells containing GFP or HA and incubated for 2 h at room temperature. The plates were washed 4 times with PBST (PBS + Tween 0.5%) and horseradish peroxidase-conjugated goat anti-mouse IgG (anti-mouse IgG-HRP) [Invitrogen|Fisher Scientific Corp.) at a dilution of 1:5000 in PBS containing 0.5% BSA was added into each well and incubated at room temperature for 2 h. The plates were washed 4 times with PBST and the color development was achieved with BD OptEIA™ ELISA set TMB substrate (Fisher Scientific

Corp.) following the manufacture recommendations. Equal volume of 2N sulfuric acid solution was used to stop the reaction. The optical density readings were measured at 450 nm using a SpectraMax® i3x microplate reader (Molecular Devices, Sunnyvale, CA).

ELISpot Assays:

The assays were performed as described previously [6]. The splenocytes were extracted from the spleen tissues and used for mouse anti-interferon-gamma (anti-INFγ) ELISpot assays. Different dilution of the splenocytes in triplicates were stimulated with HA518 (IYSTVASSL) peptide (H-2Kd-restricted CTL epitope for HA) at a concentration of 0.25 µg/ml. The number of the spot-forming units (SFU) were counted using AID ELiSpot reader 8.0 (Autoimmun Diagnostika GmbH, Germany).

Statistical analyses:

One and two-way ANOVA with Bonferroni post-test were conducted using GraphPrism 6.0 to determine the statistical significance between groups. The statistical significance was set at $p < 0.05$.

4.4   Results

Prevalence of HAdV-C5, ChAdV-7 or BAdV-3 neutralizing antibodies in humans:

It is well-known that HAdV-C5-neutralizing antibodies are prevalent in high percentage in humans worldwide [10, 25-28]. In addition to prevalence of HAdV-C5-neutralizing antibodies in humans, here we were also interested in determining whether there is prevalence of cross-neutralizing antibodies against ChAdV-7 or BAdV-3 – two most distinct groups of non-human AdVs which have excellent potential for developing gene delivery vectors for a range of applications. In 60 randomly collected serum samples from normal healthy individuals, HAdV-C5 neutralizing antibody titers over 500, 200, 100, 50, 25 or 4 were observed in 21.7, 23.3, 30, 40, 45, and 60% of the serum samples, respectively (Table 1). It was also recognized that ChAdV-7 cross-neutralizing antibody titers above 50, 25 or 4 were detected in 1.7, 5 and 32% of the serum samples, respectively (Table 1). There was no correlation between the samples having high levels of HAdV-C5 neutralizing antibody titers and the samples showing reasonable levels of ChAdV-7 cross-neutralizing antibody titers suggesting that there is absence of cross-neutralization between HAdV-

C5 and ChAdV-7 antibodies. In addition, none of the serum samples showed detectable levels of BAdV-3 cross-neutralizing antibodies (Table 1).

Cross-neutralizing ability of anti-HAdV-C5, anti-ChAdV-7 and anti-BAdV-3 antibodies:

Serum samples collected from the mouse groups (5 animals/group) inoculated with either HAd-ΔE1E3, ChAd-ΔE1E3 or BAd-ΔE1E3 were developed HAd-ΔE1E3-, ChAd-ΔE1E3- or BAd-ΔE1E3-neutralizing antibody titers >1028, 209±27.14 and >1028, respectively (Table 2). The mouse serum samples having more than 1028 neutralizing antibody titers against HAdV-C5 did not cross-neutralize either with ChAdV-7 or BAdV-3. Similarly, the mouse serum samples having more than 1028 neutralizing antibody titers against BAdV3 failed to demonstrate detectable levels of cross-neutralization of HAdV-C5 or ChAdV-7. Furthermore, a neutralizing titer of 209±27.14 against ChAdV-7 did not yield detectable levels of cross-neutralization of HAdV-C5 or BAdV-3.

Decline in HAdV-C5-neutralizing antibodies with time in a mouse model:

To mimic the pre-existing AdV vector immunity status of humans in the mouse model, we first inoculated mice i.n. with 1×10^7 PFU of HAdV-C5 to develop reasonable levels of HAdV-C5-neutralizing antibodies. A single inoculation of HAdV-C5 resulted in AdV-neutralization titers in the range of 333±67 at 4 weeks post-inoculation (data not shown). Since the AdV vector immunity titer above 200 is considered as high level [29], HAdV-C5-neutralization titers in our mouse model closely mimic the situation in humans. Mouse groups having AdV-neutralizing antibodies were inoculated with 1×10^8 PFU of HAd-GFP to mimic the normal human conditions where individuals having pre-existing AdV vector immunity were immunized first time with an AdV vectored vaccine. HAdV-C5-neutralizing antibody titers in mice were reached to 1127±128 at 4 weeks post-inoculation with HAd-GFP (Fig. 2). To monitor the decline in vector immunity with time, HAdV-C5-neutralizing antibody titers in mice were determined at 3, 6 and 10 months post-HAd-GFP inoculation. The HAdV-C5-neutralizing antibody titer started to decline by time and reached to 907±137, 648±102, and 288±71.5 after 3, 6 and 10 months, respectively post-inoculation with HAd-GFP (Fig. 2). The decline curve shows that the half-life of HAdV-C5-neutralizing antibodies was approximately 6 months.

Inhibition in GFP-specific antibody response in HAdV-C5-primed mice compared to naïve mice following inoculation with HAd-GFP:

To determine the impact of vector immunity on the resultant humoral immune response, HAdV-C5-primed group having vector neutralizing antibody titers of 333±67 or naïve group were inoculation with HAd-GFP. There was approximately 26.6% inhibition in GFP-specific ELISA antibody levels in the HAdV-C5-primed group compared to the naïve group (Fig. 3A) suggesting that significant levels of reporter-specific humoral immune response occurred even in the presence of high levels of vector immunity. Furthermore, to monitor the decline in reporter-specific antibody levels with time, GFP-specific ELISA antibody levels in HAdV-C5-primed group were determined at 3, 6 and 10 months post-HAd-GFP inoculation. The GFP-specific antibody levels started to decline by time and reached to 81.8, 72, and 67.8% after 3, 6 and 10 months, respectively compared to the level one month post-HAd-GFP inoculation (Fig. 3B) indicating that AdV vector immunization provides long lasting humoral immune responses.

Impact of vector immunity and first vector inoculation on the development of HA-specific humoral and cell-mediated immune responses following immunization with HAd-H5HA: Improvement in HA-specific humoral and cell-mediated immune responses following decline of HAdV-C5 neutralizing antibody titers with time:

To mimic the situation in human after the first vector inoculation, HAdV-C5-primed mouse groups following the development of vector immunity were subjected to the first vector inoculation with HAd-GFP. This led to the development of very high titers of vector neutralizing antibodies in the range of 1127±128 at 4 weeks post-inoculation with HAd-GFP. These levels of HAdV-C5-neutralizing antibodies allowed us to monitor the improvement in HA-specific humoral and cell-mediated immune responses following the decline of HAdV-C5 neutralizing antibody titers with time. Age-matched naïve or HAdV-C5-primed mouse groups were immunized i.m. once with 1 × 108 PFU of HAd-H5HA at 1, 3, 6, and 10 months post-inoculation with HAd-GFP. There were significant and continual increases in HA-specific ELISA antibody levels in primed vaccinated groups with time (Fig. 4). As expected, the maximum inhibition in HA-specific antibody levels were observed in the primed vaccinated group that was immunized 1-month post-inoculation with HAd-GFP. At 10 months post-inoculation with HAd-GFP, vector neutralizing antibodies titers were declined to 288±71.5, thereby the HA-specific ELISA antibody levels in the primed

vaccinated group at 10 months were significantly better than the primed vaccinated groups at 1, 3 or 6 months. Although the HA-specific ELISA antibody levels in the primed vaccinated group at 10 months were slightly lower than that of the naïve vaccinated group at 10 months, but these differences were not statistically significant (Fig. 4). HA-specific ELISA antibody levels in naïve vaccinated groups at 1, 3, 6 and 10 months were somewhat similar (Fig. 4).

AdV vectors are known to induce significant levels of CMI responses to the antigenic protein, and the CMI responses to influenza antigens are essential for conferring heterologous as well as heterosubtypic protection against influenza viruses [30-33]. Because of the importance of induction of CMI responses, the HA518 epitope-specific CD8+ immune response was also monitored to determine the improvement in HA-specific CMI responses following decline of HAdV-C5 neutralizing antibody titers with time. There were significant and continual increases in the number of INFγ-secreting CD8+ T cells in the spleens of the primed vaccinated groups following decline in vector immunity with time (Fig. 5). The maximum inhibition in the number of INFγ-secreting CD8+ T cells in the spleens was observed in the primed vaccinated groups that was immunized 1 or 3 months post-inoculation with HAd-GFP (Fig. 5). At 10 months post-inoculation with HAd-GFP when vector neutralizing antibodies titers were declined to 288±71.5, the numbers of INFγ-secreting CD8+ T cells in the spleens of the primed vaccinated group at 10 months were significantly better than the primed vaccinated groups at 1, 3 or 6 months, but these numbers were lower than that of the naïve vaccinated group at 10 months (Fig. 5). The number of INFγ-secreting CD8+ T cells in the spleens of naïve vaccinated groups at 1, 3, 6 and 10 months were showed a rising trend until the 6-month group and then somewhat declined in the 10-month group (Fig. 5).

Enhancement in heterologous H5N1 influenza protection efficacy due to improvement in HA-specific humoral and cell-mediated immune responses following decline of HAdV-C5 neutralizing antibody titers with time:

Impact of vector immunity on the vaccine efficacy can be best judged by determining the protection efficacy following challenge. To better determine the differences in protection efficacy between the primed vaccinated groups and the naïve vaccinated groups following decline of HAdV-C5 neutralizing antibody titers with time, we used an antigenically distinct H5N1 virus

(VN/1203/RG) for challenge studies since challenge with VN/1203/RG will not confer complete protection even in the naïve vaccinated groups. This allowed us better comparison between naïve vaccinated and primed vaccinated groups. For evaluating influenza virus protection efficacy, monitoring of the lung viral titers in the primed vaccinated groups and comparing them with the lung viral titers of the naïve vaccinated or control groups. Significant reductions in lung viral titers is an important parameter of the vaccine protective efficacy and it is also a strong indicator of the inhibition of virus transmission.

To determine enhancement in heterologous H5N1 influenza protection efficacy due to improvement in HA-specific humoral and cell-mediated immune responses following decline of HAdV-C5 neutralizing antibody titers with time, the control, naïve vaccinated and primed vaccinated mouse groups were inoculated with either HAd-ΔE1E3, HAd-H5HA or HAd-H5HA, respectively at 1, 3, 6, and 10 months post-inoculation with HAd-GFP. The control, naïve vaccinated and primed vaccinated groups were challenged with 100 MID50 of VN/1203/RG one-month post-vaccination. In the control groups, the lung virus titers at 1, 3, 6 and 10 months were 5.22, 4.48, 4.74 and 5.09 logs, respectively, and in the naïve vaccinated groups, the lung virus titers at 1, 3, 6 and 10 months were 2.57, 1.18, 2.07 and 2.11 logs, respectively (Fig. 6). Whereas, in the primed vaccinated groups, the lung virus titers at 1, 3, 6 and 10 months were 4.29, 2.97, 2.81 and 2.86 logs, respectively (Fig. 6). Significant and continual reductions in the lung virus titers in the primed vaccinated groups were observed following decline in vector immunity with time (Fig. 6). There was a significant reduction in the lung virus titers in the naïve vaccinated group compared to the control or the primed vaccinated groups at 1 and 3 months. At 6 and 10 months groups the differences in the lung virus titers between the primed and naïve vaccinated groups were statistically similar (Fig. 6). The protection results correlate with the improvement of both humoral and CMI responses after 6 months post-inoculation with HAd-GFP.

4.5   Discussion

Pre-existing AdV vector immunity is considered one of the important factors that could significantly inhibit the efficacy of an AdV vector, where its uptake by susceptible cells in a host is compromised due to the presence of vector-neutralizing or vector cross-neutralizing antibodies. Since AdV is a common human pathogen and there are more than 60 different types of HAdVs

that are known to infect humans. Due to high prevalence of AdV in humans, the majority of individuals get infected with one or more AdV type early in their lives leading to high occurrence of pre-existing AdV vector immunity [11, 12, 34]. Therefore, several less prevalent HAdV types as well as nonhuman AdVs including ChAdV, BAdV and others have developed to circumvent pre-existing vector immunity [1, 35-37].

Here we examined the prevalence of HAdV-C5, ChAdV-7 or BAdV-3 neutralizing antibodies in 60 serum samples collected from normal healthy individuals since they represent three unique AdV vector systems. As expected, there was high prevalence of HAdV-C5-neutralizing antibodies with over 500 neutralization titers in approximately 21% of the samples. High prevalence of HAdV-C5-neutralizing antibodies in humans has been observed previously [10]. Low levels of ChAdV-7 cross-neutralizing antibodies in the range of 4-64 were detected an approximately 32% of the samples, confirming earlier findings where the prevalence of about 13% in healthy adults and 31% in hepatic carcinoma patients were reported [38]. Whereas, no detectable levels of BAdV-3 cross-neutralizing antibodies were observed in any of the serum samples supporting earlier results [20]. These results suggest that except HAdV-C5-based vectors, pre-existing vector immunity will not impact vaccine efficiency of either ChAdV-7- or BAdV-3-based vectors. In addition, using anti-HAdV-C5, anti-ChAdV-7 or anti-BAdV-3 mouse serum samples, virus-specific neutralization was observed with no cross-neutralization any other virus suggesting that HdV-C5-, chAdV-7- and BAdV-3-based vectors could be used for sequential administration in the presence of even exceptionally high levels of vector-specific neutralizing antibodies. It has been demonstrated that sequential administration of HAdV-C5 and BAdV-3 vectors overcomes vector immunity in an immunocompetent mouse model of breast cancer [39].

Earlier we have demonstrated that high levels of vector immunity [520 virus-neutralization (VN) titer] did not adversely impact the protective efficacy of a two dose HAdV-C5-based influenza vaccine [14]. Further increases in vector immunity (up to 2240 VN titers) were taken care of either by increasing the vaccine dose by 5-fold or using an alternate route of vaccination. Furthermore, in the presence of exceptionally high levels of vector immunity (~3040 VN titers), immunization with a 5-fold vaccine dose resulted in approximately 3.3-3.7 log reductions in lung virus titers.

Similarly, in a separate study, the impact of vector immunity on a HAdV-C5-based HIV vaccine in a phase I clinical trial was shown to be overcome by increasing the vaccine dose [40].

In addition to pre-existing AdV vector immunity, the development of AdV vector-specific neutralizing antibodies could play an important role in inhibiting the vaccine efficacy of the same vector carrying the same or a different inset the second time around. This issue will be applicable to any AdV vector or any other virus vector. For many vaccines, for example influenza, it is important to determine whether annual immunization with the same vector is feasible. To address this critical need, we mimicked the pre-existing AdV vector immunity in the mouse model, immunized first time with the same vector containing the GFP gene, and subsequently at 1, 3, 6, and 10 months post-HAd-GFP inoculation animals were immunized with the same vector expressing HA of a H5N1 influenza virus. The use of a single dose of the vaccine vector and challenge with an antigenically heterologous H5N1 influenza virus are important aspects of our experimental design.

There was a persistent decline in HAdV-C5-neutralizing antibody titers with time reaching below 300 at 10 months post-HAd-GFP inoculation suggesting that immunization with the same vector will be effective. The pre-existing vector titers of 333±67 only inhibited the development of GFP-specific antibody levels to approximately 26.6% compared to the naïve animals inoculated with HAd-GFP. As expected, there was a continual decline in GFP-specific antibody levels with time, but significant levels persisted at least for 10 months signifying that immunogen-specific immune responses with AdV vectors are usually long lasting. Earlier in naïve mice immunized twice with HAdV vector expressing HA of a H5N1 virus, significant levels of humoral and CMI responses conferring complete protection from morbidity and mortality following challenge with a lethal H5N1 influenza virus were demonstrated even after one year [7].

With the decline vector immunity with time in this study, there were significant enhancement in the levels of humoral as well as CMI immune responses following immunization with HAd-H5HA especially at 6 and 10 months post-HAd-GFP inoculation. These immune responses particularly at 6 and 10 months resulted in significant protection as measured by reduction in the lung virus titers following challenge with an antigenically heterologous H5N1 influenza virus. Decreases in

the lung virus titers in the primed vaccinated group at10-month post-HAd-GFP inoculation were close to the titers observed in the naïve vaccinated group implying that the annual vaccination with an AdV vector-based vaccine will be possible due to the decline in AdV-specific neutralizing antibody titers within a year mainly due to waning of vector immunity below the level (~300 VN titer) that may not have significant impact on vector efficacy. The decay of vector immunity with time in humans may vary significantly from the mouse model, therefore, it will be necessary to conduct a study in humans with a less prevalent HAdV or nonhuman AdV vector to determine the decay kinetics of vector immunity. In addition, the decay kinetics in humans may differ depending on the choice of AdV vector type. It may not be easy to conduct a vector immunity decline study for an AdV that is a common human pathogen due to likelihood of natural exposure during the study period.

In summary, it is clear from this study that both pre-existing AdV vector immunity and the development of vector-neutralizing antibodies following the first inoculation with any AdV vector are important in determining a successful strategy for an AdV vector-based gene delivery. Development of a number of less prevalent HAdV types as well as nonhuman AdVs as gene delivery vehicles are essential to have optimum options for various applications ranging from the vaccine to gene therapy. Our results suggest that it may be better to use a different AdV vector for the second inoculation if the time period between two inoculations is less than 6 months. However, the same vector may be used again successfully if the time period between two inoculations is more than 10 months at least for the vaccine applications.

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Table 4.1: Prevalence of virus neutralizing antibodies in humans against HAdV-C5, chAdV-7) or BAdV-3.

	Titer above 4 (% samples)	Titer above 25 (% samples)	Titer above 50 (% samples)	Titer above 100 (% samples)	Titer above 200 (% samples)	Titer above 500 (% samples)
HAdV-C5	60	45	40	30	23.3	21.7
ChAdC7	32	5	1.7	0	0	0
BAd3	0	0	0	0	0	0

Randomly collected 60 serum samples from normal healthy individuals were used to evaluate neutralizing antibody levels against HAdV-C5, chAdV-7 or BAdV-3 using virus neutralization assays. HAdV-C5, human adenovirus type 5; chAdV-7, chimpanzee adenovirus type 7; and BAdV-3, bovine adenovirus type 3.

Table 4.2: HAdV-C5, chAdV-7 or BAdV-3 cross-neutralizing antibodies in mouse sera.

	Anti-HAdV-C5	Anti-chAdV-7	Anti-BAdV-3
HAdV-C5	>1028	<10	<10
chAdV-7	<10	209±27.14	<10
BAdV-3	<10	<10	>1028

Anti-HAdV-C5, anti-chAdV-7 and anti-BAdV-3 mouse serum samples (5 animals/group) were used to determine cross-neutralizing antibody titers against HAdV-C5, chAdV-7 or BAdV-3 by virus neutralization assays. HAdV-C5, human adenovirus type 5; chAdV-7, chimpanzee adenovirus type 7; and BAdV-3, bovine adenovirus type 3.
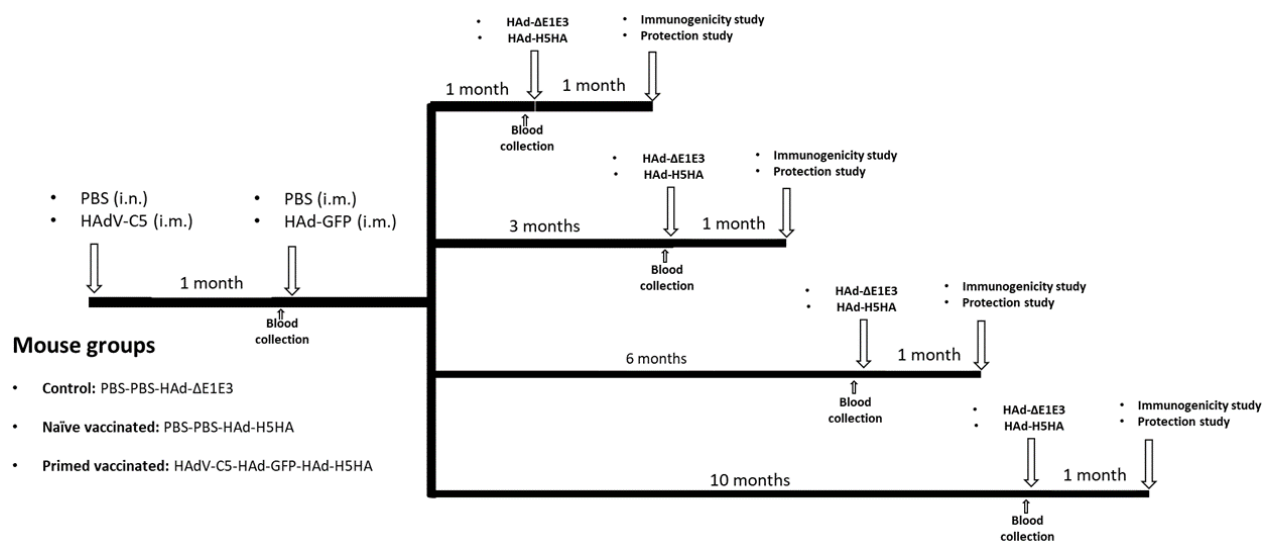
Figure 4.1: Diagrammatic representation of animal immunization and challenge studies.

To mimic pre-existing vector immunity and the development of vector-specific immune responses following first time vaccination with an Ad vector-based vaccine, 5 to 6-week-old BALB/c mice were mock-inoculated (with PBS) or inoculated with 107 p.f.u. of HAdV-C5 via the i.n. route (the natural route of HAdV-C5 infection in humans) to develop high levels (>200 virus-neutralizing antibody titers) of pre-existing vector immunity. At 1-month post-inoculation, the primed animals were inoculated i.m. with 108 p.f.u. of HAd-GFP to mimic the conditions for a first inoculation with the AdV vector-based vaccine. Subsequently, at 1, 3, 6, and 10 months post-HAd-GFP inoculation, naïve- or HAdV-primed animals (10 mice/group) were vaccinated i.m. with 108 p.f.u. of HAd-ΔE1E3 or HAd-H5HA. Before each immunization, blood samples were collected from the cheek vein. For immunogenicity studies, five animals from each group were euthanized under anesthesia at 4 weeks after immunization and the blood and spleen were collected to monitor humoral and cell-mediated immune responses. For protection studies, the remaining five immunized animals were challenged i.n. with 100 mouse infectious dose 50 (MID50) of VN/1203/RG, euthanized at 3 days post-challenge, and the lungs were collected to determine the lung virus titers. PBS, phosphate buffer saline; i.n., intranasal; i.m., intramuscular; HAdV-C5, Human adenovirus type 5; HAd-ΔE1E3, HAdV-C5 empty vector with deletions in E1 and E3 regions; HAd-GFP, HAd-ΔE1E3 vector with the GFP gene inserted in the E1 region; HAd-H5HA, HAd-ΔE1E3 vector with the HA gene from A/HK/156/H5N1 influenza virus inserted in the E1 region.
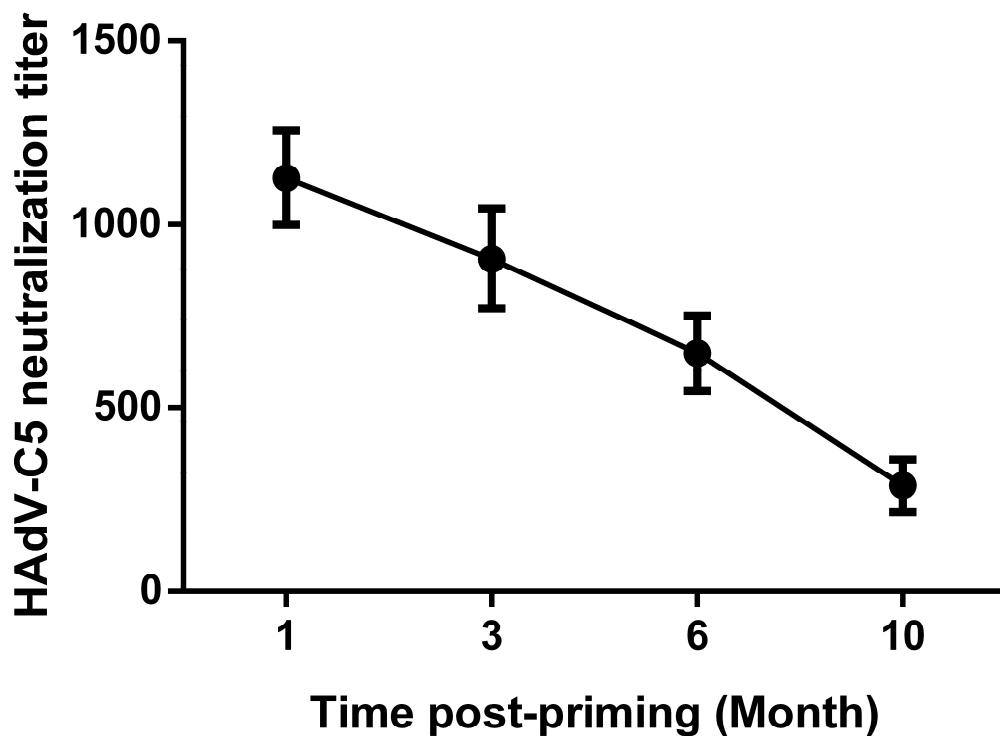
Figure 4.2: Decline in vector immunity with time in the mouse model.

Six to 8-week-old BALB/c mice were inoculated with 107 p.f.u. of HAdV-C5 via the i.n. route and at 1-month post-inoculation, the primed animals were inoculated i.m. with 108 p.f.u. of HAd-GFP. Following 1, 3, 6, and 10 months post-HAd-GFP inoculation, blood samples were collected from the cheek vein to monitor the development of HAdV-C5-neutralizing titers by virus neutralization assay. The data are depicted as mean ± SD from 10 mice. HAdV-C5, Human adenovirus type 5.
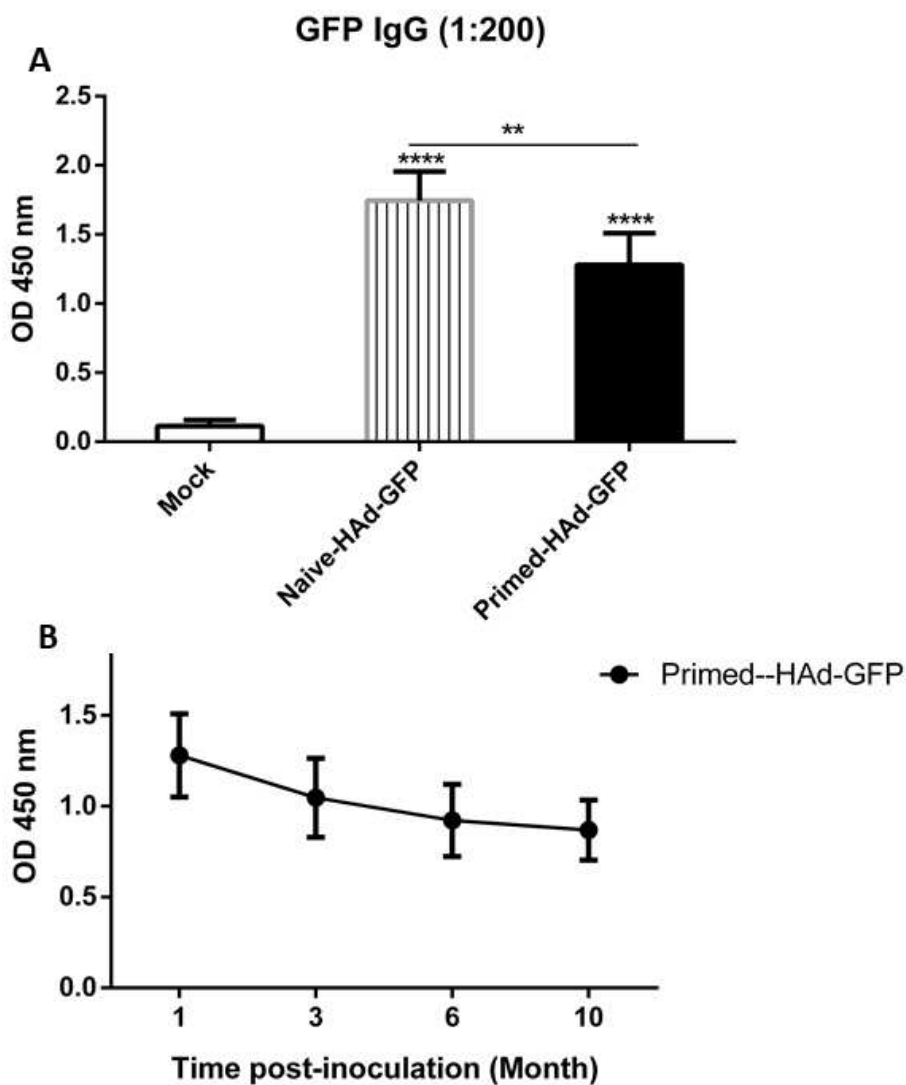
Figure 4.3: Inhibition in GFP-specific antibody response in HAdV-C5-primed mice, compared to naïve mice.

Inhibition in GFP-specific antibody response in HAdV-C5-primed mice compared to naïve mice following inoculation with HAd-GFP. A) Naïve or HAdV-C5-primed mice were immunized with HAd-GFP, and blood samples were collected at 1 month post-immunization. B) HAdV-C5-primed mice were immunized with HAd-GFP, and blood samples were collected at 1, 3, 6 and 10 months post-immunization. GFP-specific ELISA antibody levels were determined in all serum samples. **, significant at $p<0.01$ and ****, significant at $p<0.0001$. The statistical analysis was done by Bonferroni post-test and one-way ANOVA using GraphPad Prim 6.

Figure 4.4: Increases in HA-specific antibody levels following immunization with HAd-H5HA with decline in vector immunity with time.

At 1, 3, 6, and 10 months post-HAd-GFP inoculation, naïve or HAdV-primed BALB/c mice were immunized i.m. with 108 p.f.u. of HAd-H5HA, and at 4 weeks after the immunization with HAd-H5HA, the blood samples were collected to monitoring the development of HA-specific antibody levels by ELISA. Ns, non-significant at $p<0.05$; *, significant at $p<0.05$; and ****, significant at $p<0.0001$. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prim 6.
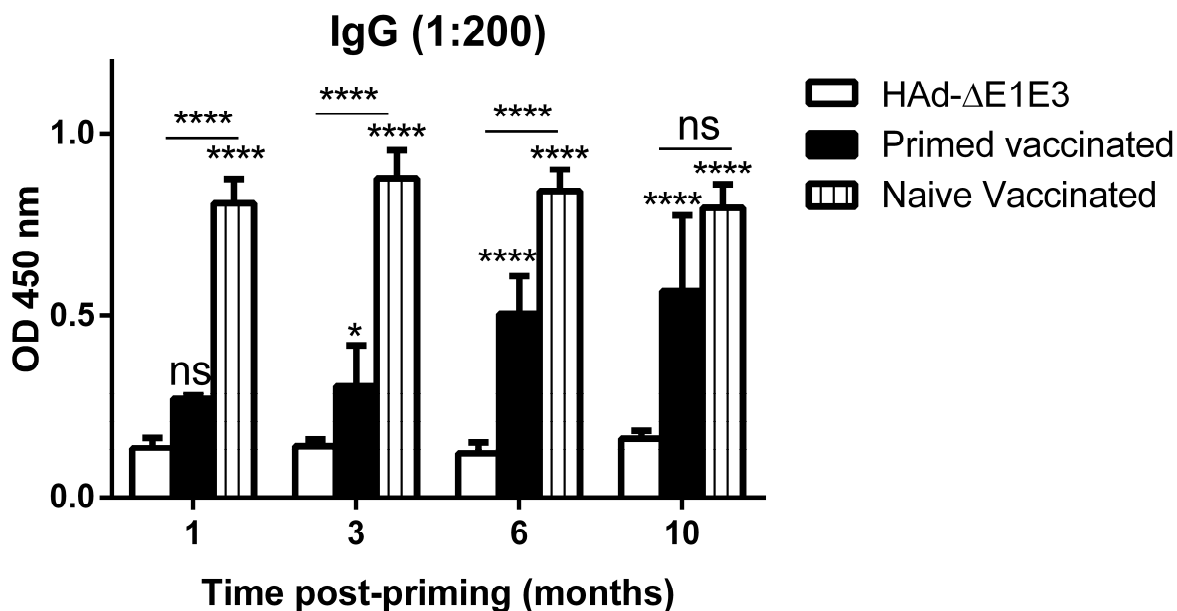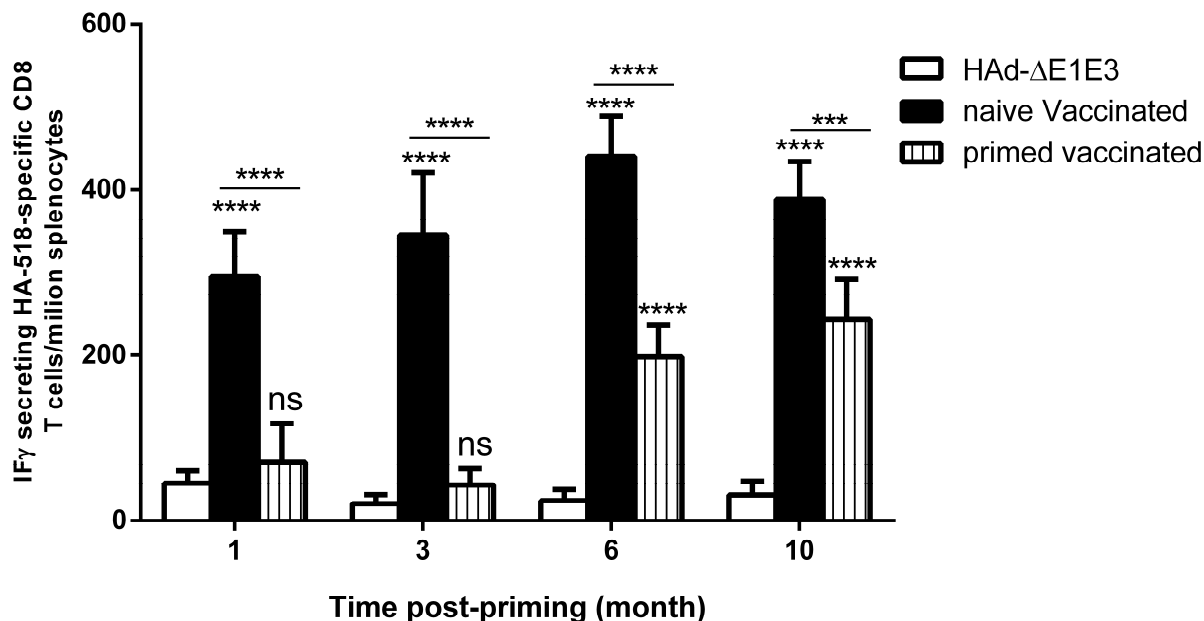
Figure 4.5: Increases in the number of HA-specific IFN-□ secreting CD8 T cells following immunization with HAd-H5HA with decline in vector immunity.

At 1, 3, 6, and 10 months post-HAd-GFP inoculation, naïve or HAdV-primed BALB/c mice were immunized i.m. with 108 p.f.u. of HAd-H5HA, and at 4 weeks after the immunization with HAd-H5HA and the spleens were collected to monitoring the number of HA-specific IFN-□ secreting CD8 T cells by ELISpot. The data are represented as the mean ± standard deviation (SD) of spot-forming units (SFU). Ns, non-significant at p<0.05; *, significant at p<0.05; **, significant at p<0.01; ***, significant at p<0.001; and ****, significant at p<0.000; the statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prim 6.
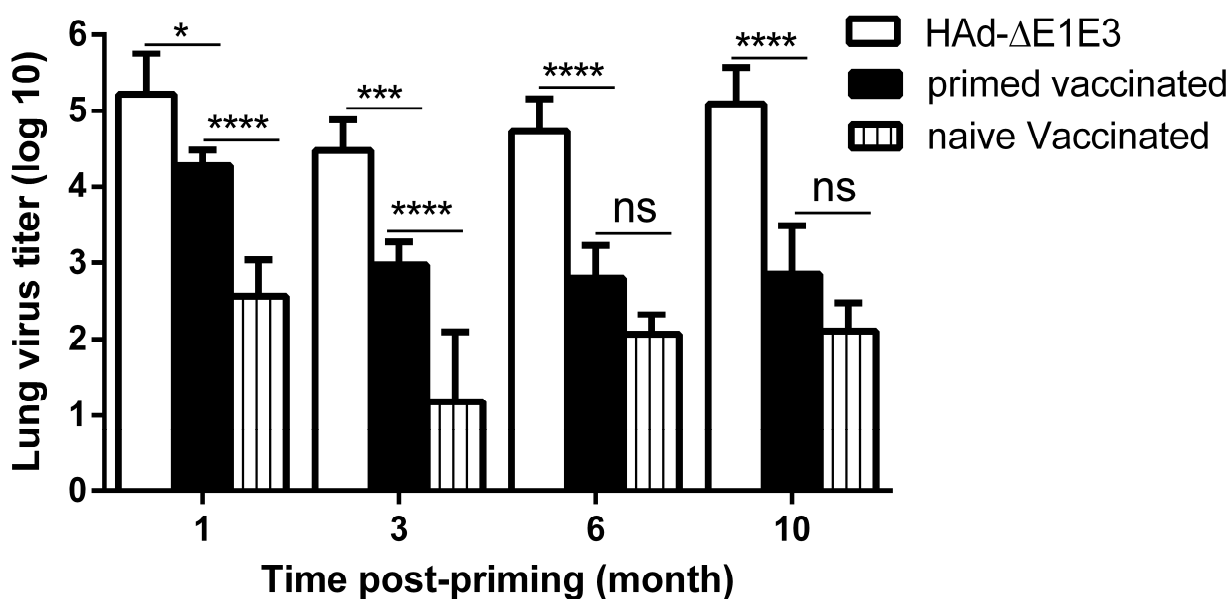
Figure 4.6: Enhancement in heterologous H5N1 influenza protection efficacy following decline of HAdV-C5 neutralizing antibody titers with time.

At 1, 3, 6, and 10 months post-HAd-GFP inoculation, naïve or HAdV-primed BALB/c mice were immunized i.m. with 108 p.f.u. of HAd-H5HA or HAd-ΔE1E3, and at 4 weeks after the immunization with HAd-H5HA and the animals were challenged i.n. with 100 mouse infectious dose 50 (MID_{50}) of VN/1203/RG, euthanized at 3 days post-challenge, and the lungs were collected and processed for lung virus titers to monitor protection efficacy. The data are shown as mean Log10 $TCID_{50}\pm$SD and the detection limit was 0.5 Log10 $TCID_{50}$/ml. ns, non-significant at p<0.05; *, significant at p<0.05; **, significant at p<0.01; ***, significant at p<0.001; and ****, significant at p<0.000. The statistical analysis was done by Bonferroni post-test and two-way ANOVA using GraphPad Prism 6.

CHAPTER 5. POTENTIAL APPROACH TO OBTAIN CONSISTENCY IN TITRATION OF VARIOUS ADENOVIRAL VECTORS

5.1 Abstract

Various types of human and nonhuman adenoviral (AdV) vectors are being used for preclinical and clinical investigations as a gene delivery vectors. To determine an approach that can accurately titrate various AdV vectors to obtain consistent results in preclinical and clinical studies with different AdV vectors. In this manuscript, we compared plaque-forming unit (PFU), tissue culture infectious dose 50 ($TCID_{50}$), focus-forming unit (FFU), virus particle (VP) count, and genome copy number (GCN) of purified preparations of human AdV type C5 (HAdV-C5), bovine AdV type 3 (BAdV-3) and porcine AdV type 3 (PAdV-3) to determine a correlation between infectious and noninfectious virus particles. Our results suggest that a ratio of VP: GCN followed by VP:FFU could provide a better analysis to accurately reflect AdV titration for achieving similar responses using different vector stocks or vector systems.

5.2 Introduction

Adenoviruses (AdVs) are non-enveloped viruses with icosahedral capsids of 90-100nm in size containing the dsDNA genome ranging from 26 to 46 Kb [1]. A number of AdVs belonging to human, simian, bovine, porcine, canine, ovine, avian and other species have been developed as gene delivery systems for recombinant vaccines and gene therapy applications ranging from metabolic disorders to cancer therapy [2-8]. Several AdV vectors are currently being evaluated for their potential as gene delivery vehicles in clinical trials [9-11]. These human or non-human AdV vectors can be of various types such as replication competent (e.g., E3-deleted), replication defective (e.g., E1-, E4-, E1 & E3-, E1 & E4-, or E1, E3 & E4-deleted), conditional replication competent (e.g., expression of E1A under an inducible promoter) and helper-dependent/gutless (e.g., containing at least the AdV inverted terminal repeats and the packaging sequences) vectors[12].

AdV titration by plaque assay to determine plaque-forming units (PFU) is still considered as a widely accepted method to titrate infectious AdV particles. For AdVs that do not easily develop

plaques in infected cell monolayers, tissue culture infectious dose 50 (TCID$_{50}$) by TCID$_{50}$ assay is believed to be one of the preferred assay for AdV titration. Following the development of conditional replication competent and helper-dependent vectors, AdV vector titration by either by plaque assay or TCID$_{50}$ assay lost their applicability. To fill this void, virus particle (VP) count assay was developed to quantitate number of AdV particles in purified preparations [13-15]. Now VP assay is based on spectrophotometrically estimation of the amount of protein in a purified AdV vector preparation to calculate the number of VP using a formula; virus particle counts (VP) = A260nm × dilution factor ×3.5 × 109 [15, 16]. A ratio of VP: PFU is preferred for AdV vector titration, wherever it is applicable. Since VP count is based on the total protein content in a virus preparation, this assay will include the empty VP (without AdV genome sequences), mature VP (containing AdV genome sequences) and ruptured particles. The ratio of empty and ruptured VP: mature VP can be in the range of 11-2300 [17-19]. Suggesting that, the majority of VP in an AdV preparation represents the empty virus particles. For the majority of AdV vector-based gene delivery systems, the number of mature VP in a vector preparation are critical for the desired foreign gene expression.

5.3 Results and discussion

In an effort to determine an appropriate AdV vector titration system which is independent of the cell line used and the type of AdV, we selected human AdV type C5 (HAdV-C5), bovine AdV type 3 (BAdV-3), and porcine AdV type 3 (PAdV-3) and propagated them in HEK 293[20], MDBK, and PK-15 cell lines, respectively by infecting with a multiplicity of infection (MOI) of 5 PFU per cell. The cell receptors for HAdV-C5, BAdV-3 and PAdV-3 internalization are distinct [21-24]. Each AdV preparation was purified by cesium chloride (CsCl) density gradient centrifugation [25]. The purified AdV preparations were titrated for PFU, TCID$_{50}$, FFU (focus-forming units), VP, and GCN (genome copy number). Titration of HAdV-C5, BAdV-3, and PAdV-3 for PFU, TCID$_{50}$, and FFU were conducted using BHH2C [26], MDBK, and PK-15 cell lines, respectively. For PFU, serially log-diluted HAdV-C5, BAdV-3 or PAdV-3 were used to infect BHH2C, MDBK or PK-15 cell lines and the plaques were counted on Day 7, 10 or 12 post-infection, respectively. For TCID50, serially log-diluted HAdV-C5-, BAdV-3-, or PAdV-3-infected cells were examined for cytopathic effect (CPE) on Day 5, 7, or 10 post-infection and the TCID$_{50}$ titers were determined by Reed Muench formula;

$Proportionat\;\;i\;tan\;\;=\dfrac{Dilution\ of\ 50\%\ CPE-Dilution\ just\;\;elow\ 50\%\ CPE}{Dilution\ a\;\;ove\ 50\%\ CPE-Dilution\ just\;\;elow\ 50\%\ CPE}$ [27]. For FFU,

serially log-diluted HAdV-C5, BAdV-3 or PAdV-3 were used to infect BHH2C, MDBK or PK-15 cell lines, respectively, and at 3 days post-infection, infected cell monolayers were fixed with cold methanol and virus-infected individual cells or cell clusters (FFU) were identified using HAdV-C5-, BAdV-3- or PAdV-3-specific rabbit antibodies[28] followed by incubation with anti-rabbit HRP antibody, the plate were further incubated with a solution containing 0.015% hydrogen peroxide solution and 0.05% diaminobenzidine(DAB) for brown color development[29]. Numbers of VP in purified preparations of HAdV-C5, BAdV-3, and PAdV-3 were determined spectrophotometrically by measuring optical density at 260 nm. To determine GCN, 1 ml of purified preparations of HAdV-C5, BAdV-3 or PAdV-3 were used to extract viral DNA [30], and the amount of DNA was determined spectrophotometrically by measuring OD at 260 nm. The GCN was finally determined using the genome size in nucleotides in the following formula;

$Numb\;r\ of\ viru\;\;g\;nom\;\;\;opi\;\;=\dfrac{DNA\ amount(ng)\times 6.022\times 10^{23}}{DNA\ length(\;p)\times 10^{9}\times 650}$ [31]. Each assay was done in

triplicate and three independent preparations of each virus were used with similar results.

PFU, TCID$_{50}$, FFU, VP, and GCN titers (log mean ± SD) with the purified preparation of HAdV-C5 were 11.19±0.04, 11.26±0.08, 11.5±0.08, 13.18±0.01, and 12.43±0.02, respectively (Fig. 1A). Similarly, PFU, TCID$_{50}$, FFU, VP, and GCN titers (log mean ± SD) with the purified preparation of BAdV-3 were 10.27±0.03, 10.26±0.10, 10.4±0.10, 12.56±0.03, and 12.29±0.02, respectively (Fig. 1B). Whereas, PFU, TCID$_{50}$, FFU, VP, and GCN titers (log mean ± SD) with the purified preparation of PAdV-3 were 9.53±0.32, 10.39±0.21, 10.42±0.20, 12.57±0.03, and 12.52±0.03, respectively (Fig. 1C).

Since the plaque formation by PAdV-3 in PK-15 cells was not as efficient as that of HAdV-C5 in BHH2C cells or BAdV-3 in MDBK cells, PAdV-3 titer in PFU was significant lower than that of TCID50 or FFU titers of PAdV-3 preparation. In this study, PAdV-3 served as an AdV with less efficient plaque-forming ability, which may be due to less efficient cell-to-cell virus transfer and/or overgrowth of PK-15 cells with time suggesting that plaque assay is not a best choice for titration of every AdV or AdV vector. Both TCID$_{50}$ and FFU seems to be a good alternative to plaque assay for titration of any AdV or AdV vector that replicates in a cell line, however, our results with

HAdV-C5, BAdV-3 and PAdV-3 suggest that FFU could provide more consistent results for replicative AdVs or AdV vectors compared to PFU or $TCID_{50}$. Unlike PFU or $TCID_{50}$, FFU seems independent of the cell-to-cell virus transfer. FFU titration can easily be adapted for foreign gene expression in place of viral gene expression for conditional replication competent or helper-dependent vectors. Apparently, FFU titration is more tedious compared to titration by PFU or TCID50, but the results are available earlier than PFU or $TCID_{50}$ since FFU can be performed after a few days post-infection.

The other two assays, VP and GCN, which were included in this study are not dependent on virus replication. The protocol for VP is very simple and quick due to directly estimating VP from the OD readings of a purified preparation of AdV or AdV vector, whereas, GCN is a bit time consuming because there is a need to extract the viral genomic DNA from purified preparation before estimating GCN from the OD readings of a purified preparation of AdV or AdV vector genome. Since VP assay is based on the total protein estimation, it counts both empty and ruptured (non-infectious) and mature AdV particles (infectious), whereas, GCN assay is based on the total DNA estimation, it counts only mature AdV particles (infectious). Therefore, the VP titer will always be higher than the GCN titer of the same AdV preparation. For titration of various AdV vectors, the VP titers are widely used for preclinical and clinical studies [32, 33].

Due to a variety of AdV vectors deferring in the vector type, the type and species origin of AdV, and the cell line for vector propagation, it is difficult to anticipate that a single vector titration method will be applicable for all AdV vectors. To better address this issue, a ratio between two titration assays such as VP: PFU is often used for AdV vectors [19, 34]. The VP:PFU ratios for purified preparations of HAdV-C5, BAdV-3, and PAdV-3 were 93.33, 206.91, and 1047.50 respectively, the VP:$TCID_{50}$ ratios for HAdV-C5, BAdV-3, and PAdV-3 were 68.29, 215.15, and 134.94, respectively, the VP:FFU ratios for HAdV-C5, BAdV-3, and PAdV-3 were 35.47, 139.67, and 134.08, respectively, and the VP:GCN ratios for HAdV-C5, BAdV-3, and PAdV-3 were 4.14, 1.96, and 1.08, respectively (Figs. 1 D-F). Various other combinations of ratios were examined, but the ratios either with VP or GCN provided similar conclusions. Since PAdV-3 was not efficient in producing plaques in PK-15 cells, a higher VP: PFU ratio of 2187.5 compared to 93.33 and 206.91 for HAdV-C5 and BAdV-3, respectively clearly indicated poor plaque-forming ability of

PAdV-3. The VP: $TCID_{50}$ or VP: FFU ratio provided somewhat similar results, but FFU is applicable to all type of vectors including conditional replication competent and helper-dependent vectors, and therefore, the VP: FFU ratio will provide the best outcome compared to the VP:PFU or VP:$TCID_{50}$ ratio. However, the best scenario was observed with the VP: GCN ratio and it will be applicable to all types of AdV vector systems.

This study examines five methods of AdV titration, PFU, $TCID_{50}$, FFU, VP and GCN, in an attempt to determine the best ratio that can be applied to various AdV preparations to accurately reflect some level of consistency in virus titration. We found that the VP: GCN ratio could serve as a mean to adjust batch-to-batch variability in vector titers or to adjust the vector dose for other type of AdV vectors for preclinical or clinical evaluations. The research outcomes of this study could help in developing a universal approach for AdV vector titration to better interpret and analyze the results from various studies that are conducted in various laboratories using different AdV vectors. Additional studies with various type of AdV vectors in cell culture as well as in animal models are required to fully explore the advantages and challenges of the best titration method described in this manuscript.

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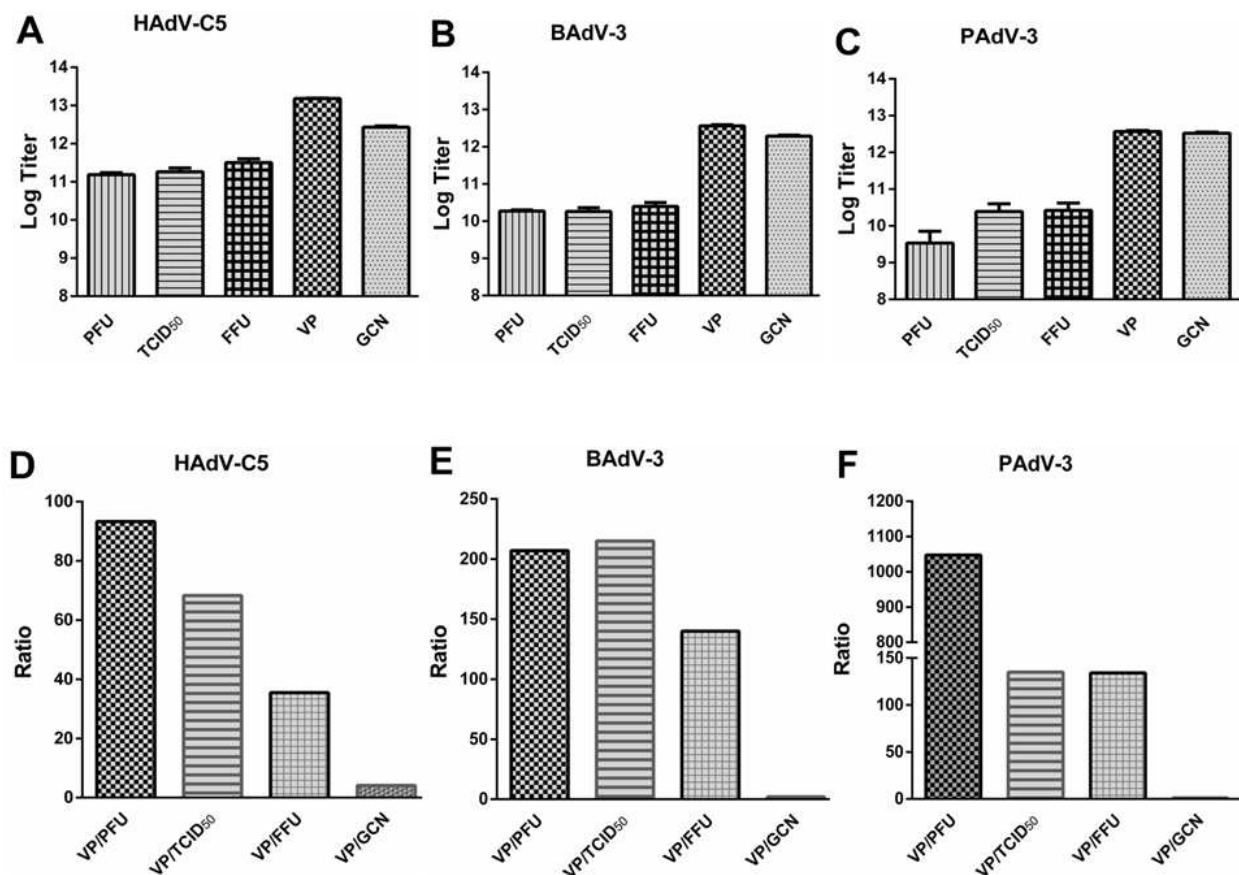
Figure 5.1: Titration of HAdV-C5, BAdV-3, and PAdV-3 by PFU, TCID50, FFU, VP and GCN.

293, MDBK, or PK-15 cells were infected with log-diluted purified preparation of HAdV-C5, BAdV-3, or PAdV-3, respectively, to determine virus titers in PFU by plaque assay, in TCID50 by TCID50 assay, and in FFU by FFU assay. The VP titers of purified preparation of HAdV-C5, BAdV-3, and PAdV-3 were calculated by VP assay using the OD values of diluted preparations as determined spectrophotometrically. The VP titers of purified preparation of HAdV-C5, BAdV-3, and PAdV-3 was calculated by GCN assay using the OD values of diluted viral DNA preparations as determined spectrophotometrically. Titers of A) HAdV-C5, B) BAdV-3, and C) PAdV-3 by various assays, and the VP ratios with other titration assays for D) HAdV-C5, E) BAdV-3, and F) PAdV-3 are shown. HAdV-C5, human adenovirus type C5; BAdV-3, bovine adenovirus type 3; PAdV-3, porcine adenovirus type 3; PFU, plaque-forming units; TCID50, tissue culture infectious dose 50; FFU, focus-forming units; VP, virus particle count; GCN, genome copy number; OD, optical density.

VITA

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EDUCATION

2013 -Present PhD student in the Laboratory of Molecular Virology and Gene Therapy, Department of Comparative Pathobiology, College of Veterinary, Purdue University. Expected date of completion: April 2018.

2006 - 2010 Master's in Veterinary Medical Sciences, Poultry Diseases Department, Veterinary Medicine School, Benha University, Egypt.

1999-2004 DVM, Veterinary Medical Sciences, Veterinary Medicine School, Benha University, Egypt.

AWARDS

• Fully funded PhD scholarship from the Egyptian minister for education, 2013-18.

• Research assistant fellowship from Purdue Graduate School, 2013-18.

• Phi Zeta PVM Graduate Student Research Award   2018

• Purdue Society of Sigma Xi Poster Competition, Life Sciences Division   2018

RESEARCH EXPERIENCE

• Tissue culture techniques.

• Growing and purification of viral stocks.

• Quantification of virus particles.

• Evaluation of vaccines efficacy and safety.

• Basic virology, immunology and molecular techniques.

• Construction of adenovirus vectors for vaccine and gene therapy.

• Protein expression and purification.

• Working in BSLA2+ labs.

• Growing, quantification and purification of influenza viruses.

• Planning and working in animal experements.

• Lab manger.

TEACHING EXPERIENCE

2005-2013   Teaching assistant of Avian and Rabbit Diseases, College of Veterinary Medicine, Benha University, Egypt.

 2005-2013   Teaching instructor of Farm Animal's Disease Diagnosis and Treatment. College of Veterinary Medicine, Benha University, Egypt.

 2015-present   Presented a few lectures in Veterinary Virology course. Taught molecular virology laboratory techniques to the summer undergraduate students. College of Veterinary medicine, Purdue University, USA.

PUBLICATIONS

1. Vemula SV*, **Sayedahmed EE***, Sambhara S, Mittal SK. Vaccine approaches conferring cross-protection against influenza viruses. Expert Rev Vaccines. 2017;16(11):1141-54. Epub 2017/09/20. doi: 10.1080/14760584.2017.1379396. PubMed PMID: 28925296. **(*Co-first authors)**

2. Hassan AO, Amen O, **Sayedahmed EE**, Vemula SV, Amoah S, York I, Gangappa S, Sambhara S, Mittal SK. Adenovirus vector-based multi-epitope vaccine provides partial protection against H5, H7, and H9 avian influenza viruses. PLoS One. 2017; 12(10):e0186244. Epub 2017/10/13. doi: 10.1371/journal.pone.0186244. PubMed PMID: 29023601.

3. **Sayedahmed EE**, Hassan AO, Kumari R, Cao W, Gangappa S, York I, Sambhara S, Mittal SK. Bovine adenoviral vector-based H5N1 influenza vaccine provides enhanced immunogenicity and protection at a significantly lower dose compared to human adenoviral vector-based vaccine in a mouse model. **Ready for Submission**

4. **Sayedahmed EE**, Shukla S, Kumari R, Hassan AO, Mohammed SI, York I, Gangappa S, Sambhara S, Mittal SK. Longevity of adenovirus vector immunity and its implication on vaccine efficacy. **Ready for Submission**

5. **Sayedahmed EE**, Mittal SK. Potential approach to obtain consistency in titration of various adenoviral vectors. **Ready for Submission**

6. **Sayedahmed EE**, Kumari R, Mittal SK. Adenoviral Vectors for Gene Therapy. In: Methods in Molecular Biology. Editors: Manfredsson F and Benskey M. Humana Press. 2018. (Under revision) (**Book Chapter**)

PRESENTATIONS

Department Presentations

1. Implication of adenoviral vector immunity in immunization. Nov. 19, 2017. Purdue University Comparative Pathobiology Department, West Lafayette Indiana.

2. Bovine adenoviral vector system for developing effective vaccines against emerging avian influenza viruses. April 27, 2017. Purdue University Comparative Pathobiology Department, West Lafayette Indiana.

3. Consistency in adenovirus vector quantification for immunization and gene therapy. April 21, 2016. Purdue University Comparative Pathobiology Department, West Lafayette Indiana.

Conference Presentations (O, Oral; P, Poster)

1. Hassan AO, Amen O, Vemula SV, Sayedahmed EE, York I, Sambhara S, Mittal SK. (2016). Development of a broadly protective adenovirus vector-based vaccine against A/H5, A/H7 and A/H9 avian influenza viruses. (Presented at the 35th Annual Meeting of American Society for Virology, Virginia Polytechnic Institute & State University, Blacksburg, VA).[O]

2. Mittal SK, Sayedahmed EE, Hassan AO. (2016). Prospects of bovine adenoviral vector system for gene delivery. (Presented at the 12th International Adenovirus Meeting, Barsinghausen, Germany). [O]

3. Sayedahmed EE, Hassan AO, Kumari R and Mittal SK. (2016). Bovine adenoviral vector system for developing effective vaccines against emerging avian influenza viruses (Presented at the 97th Annual Conference of Research Workers in Animal Diseases, Chicago, IL). [O]

4. Hassan AO, Amen O, Vemula SV, Sayedahmed EE, York I, Sambhara S, Mittal SK. (2016). Efforts towards developing a universal vaccine against emerging influenza viruses. (Presented at the 97th Annual Conference of Research Workers in Animal Diseases, Chicago, IL). [O]

5. Mittal SK, Hassan AO, Amen O, Vemula SV, Sayedahmed EE, York I, Gangappa S, Sambhara S. (2016). Emerging avian influenza viruses: Their implications and control strategies. (Presented as a keynote address at the 97th Annual Conference of Research Workers in Animal Diseases, Chicago, IL).^{O}

6. Hassan AO, Amen O, Vemula SV, Sayedahmed EE, York I, Sambhara S, Mittal SK. (2017). Adenovirus vector-based multi-epitope vaccines provides partial protections against multiple subtypes of avian influenza viruses. (Presented at the Viral Immunity: Mechanisms and Consequences, Santa Fe, NM; February 19-23, 2017).^{O}

7. E.E. Sayedahmed, A.O. Hassan, R. Kumari, S.K. Mittal. Longevity of adenovirus vector immunity and its implication for annual immunization. (Presented at the Conference of Research Workers in Animal Diseases, Chicago, IL; December 4, 2017).^{O}

8. Mboko WP, Cao W, Liepkalns J, Sayedahmed EE, Kamal RP, Mishina M, McCoy J, Amoah S, Bohannon C, Ranjan P, Gangappa S, York I, Mittal SK, Sambhara S. (2018). Non-replicating adenoviral empty vector induces a protective immune response against H7N9 infection (presented at the Keystone Symposium on Immunological Memory: Innate, Adaptive and Beyond, Austin, TX, February 2018) ^{P}

9. Ekramy E Sayedahmed, Ahmed O Hassan, Rashmi Kumari, Weiping Cao, Shivaprakash Gangappa, Ian York, Suryaprakash Sambhara, and Suresh K. Mittal. .A bovine adenoviral vector-based H5N1 influenza vaccine produced a complete protection against a heterologous influenza virus challenge.(Presented at Sigma Xi Graduate Student Research Poster Award Competition, West Lafayette, IN, February 21, 2018) ^{P}

10. Ekramy E Sayedahmed, Ahmed O Hassan, Rashmi Kumari, Weiping Cao, Shivaprakash Gangappa, Ian York, Suryaprakash Sambhara, and Suresh K. Mittal. Bovine adenoviral vector-based H5N1 influenza vaccine provides enhanced immunogenicity and protection at a significantly lower dose compared to human adenoviral vector-based vaccine in a mouse model. (Presented at 2018 Health and Disease: Science, Culture and Policy Research Poster Session, West Lafayette, IN, March 1, 2018)^P