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Broad cross-protection by recombinant influenza viruses expressing conserved M2-
hemagglutinin chimera and virus-like particles in young and aged mice

by

Bo Ryoung Park

Under the Direction of Sang-Moo Kang, Ph.D.

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the Institute for Biomedical Sciences

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2021

ABSTRACT

Current influenza virus hemagglutinin (HA)-based vaccines leave a significant gap to improve the vaccine effectiveness against mismatched strains and for the elderly. The goal of my dissertation research projects was to develop influenza vaccination strategies for inducing more effective cross-protection in mouse animal models.

In chapter one, I investigated whether enhanced cross-protection would be induced after vaccination with recombinant influenza H3N2 virus expressing extra conserved M2e epitopes in a chimeric HA molecule (rgH3N2 4xM2e). Intranasal vaccination of BALB/c mice with live rgH3N2 4xM2e virus induced humoral and cellular immune responses to M2e and conferred broad cross-protection against the different subtypes of influenza viruses. These findings provide evidence that new recombinant virus vaccination can enhance the cross-protective efficacy by inducing immunity to HA and M2e.

The concerns about negative impacts on the efficacy of repeated influenza vaccination have been raised. In the second chapter, I studied the impact of heterologous prime-boost vaccinations on cross-protection utilizing recombinant H1N1 and H3N2 subtype influenza viruses expressing chimeric HA (4xM2e-HA) in C57BL/6 mice known to be a lower responder than BALB/c mice. I demonstrated that heterologous prime-boost immunization with recombinant 4xM2e-HA viruses was more effective in inducing cross-protective M2e and HA stalk specific IgG antibodies and cross-protection than homologous prime-boost immunization. The research outcomes from project two support the positive impacts of a heterologous prime-boost strategy on conferring more effective cross-protection.

In the last chapter, I determined whether influenza HA virus-like particle (HA VLP) vaccination would induce homologous and heterologous protection in aged (18-month-old) BALB/c mice, in comparison with young adult (6-8 weeks old) mice. I found that higher doses of HA VLP were required in aged mice to induce homologous and heterologous protection. Aged mice displayed a lower capacity to induce IgG antibodies cross-reactive to different viral antigens and HA stalk domains, suggesting limited diversity of de novo B cell repertoire. Notably, incorporation of cytokine molecular adjuvants onto HA VLP enhanced protective efficacies as evidenced by more effective lung viral controls in aged mice. These findings provide insights into developing an effective vaccination strategy in the elderly.

INDEX WORDS: Influenza virus, vaccine, live-attenuated virus (LAIV), virus-like particle (VLP), M2e, cross-protection

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2021

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Office of Academic Assistance – Graduate Programs

Institute for Biomedical Sciences

Georgia State University

November 2021

DEDICATION

I dedicate this to my husband, Park/Kwon families, and three aunts from the Chi family
who inspire me to be the best version of myself.

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First and foremost, I would like to express my sincere gratitude to my Ph.D. mentor Dr. Sang-Moo Kang, for his generous supports and valuable scientific expertise. I truly appreciate Dr. Kang for putting in the extra time and effort to help me grow and succeed as an independent scientist.

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LIST OF ABBREVIATIONS

aa	amino acid
ADCC	Antibody dependent cellular cytotoxicity
ADP	antibody-dependent phagocytosis
ADP	antibody-dependent phagocytosis
BALF	bronchoalveolar lavage fluid
ca	cold adapted
CTL	cytotoxic T lymphocytes
DMEM	Dulbecco's Modified Eagle Medium media
EID ₅₀	50% egg infectious doses
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
GM-CSF	granulocyte macrophage colony-stimulating factor
GPI	glycosylated phosphatidylinositol
HA	hemagglutinin
HAI	hemagglutination inhibition
HAU	hemagglutination activity unit
HRP	horseradish peroxidase
i.m.	intramuscular
i.n.	intranasal
IFN	Interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IIV	inactivated influenza virus vaccine
IL	Interleukin
ISM	immunostimulatory molecule
LAIV	live-attenuated influenza virus vaccine
LD ₅₀	median lethal dose
LRT	lower respiratory tract
M2e	extracellular domain of Matrix 2
MALT	mucosal-associated lymphoid tissue
MDCK	Madin-Darby Canine Kidney
MDV	master donor virus
NA	neuraminidase
NAe	100% conserved epitope in the NA (aa222–230)
OAS	original antigenic sin
PBS	phosphate-buffered saline
rBV	recombinant baculovirus
RDE	receptor destroying enzyme

rg	reverse genetic
SA	sialic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHM	somatic hypermutation
slgA	secretory immunoglobulin A
TCID50	Median Tissue Culture Infectious Dose
TM	transmembrane
TMB	tetramethylbenzidine substrates
TNF	tumor necrosis factor
URT	upper respiratory tract
VE	vaccine effectiveness
VLPs	virus-like particles

1 INTRODUCTION

1.1 Influenza virus

Influenza virus causes a contagious respiratory disease accompanied by moderate to severe symptoms that can lead to death. The CDC estimates 12,000 to 61,000 annual deaths since 2010 in the U.S. Out of 4 genera of influenza (types A, B, C, and D), influenza A and influenza B are clinically related in human disease and cause seasonal influenza epidemics annually. Influenza A has been evolving faster and more diverse than influenza B accompanied by changes in their amino acid and antigenic properties (Nobusawa and Sato, 2006). Influenza A belonging to the Orthomyxoviridae family possesses eight segmented genomes of negative-sense single-stranded RNA in the form of viral ribonucleoprotein particles (polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural (NS)). Influenza A viruses are subdivided into antigenically different subtypes based on the two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). 16 HAs and 9 NAs have been described to involve influenza A reassortment from a total of 18 HAs (H1-H18) and 11 NAs (N1-N11) that include bat influenza A-like viruses (Krammer et al., 2018b). Influenza A is solely responsible for a pandemic that occurs due to the emergence of novel strains/subtypes of viruses with its broad variety of animal species reservoirs (Long et al., 2019). Prior history of pandemic records includes 1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1 influenza A viruses (Taubenberger et al., 2019). The 1918 H1N1 pandemic caused an estimate of 50 million deaths worldwide, while the 1957 H2N2 pandemic and the 1968 H3N2 pandemic each reported approximately one million lives (Taubenberger et al., 2019). The most recent 2009 H1N1

pandemic claimed about 284,000 deaths (Nickol and Kindrachuk, 2019). The influenza A viruses can be divided into two groups based on the phylogenetic differences in HA proteins. H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18 belong to Group 1 and group 2 comprises H3, H4, H7, H10, H14, and H15 (Sutton et al., 2017).

1.2 Influenza virus life cycle

Influenza virus infects respiratory tracts and mainly replicates in epithelial cells. Virus infection starts with binding to the host epithelial cells in the upper respiratory tracts. HA plays a role for this attachment by recognizing sialic acid on the cellular surface with host specificity. HA proteins from human adapted influenza viruses bind preferentially to α -2,6 sialic acid (SA) that abundant in the human upper respiratory tract (URT) while the avian influenza viruses tend to bind to α -2,3 SA that found on epithelial cells of the human lower respiratory tract (LRT) and birds' intestinal tract (Shao et al., 2017, Krammer et al., 2018b). Viruses are then internalized into the cells by endocytosis. The conformational change of HA induced by acidification involves the fusion between the viral envelop and cellular membrane. Released eight viral genetic materials: PB2, PB1, PA, HA, NP, NA, M, and NS in the cytoplasm are imported into the nucleus of the host cells for transcription and replication. The RNA polymerase complex composed of three subunits such as PB2, PB1, and PA is responsible for both transcription and replication. The 8th RNA gene segment, NS encodes two proteins including NS1 and NS2, and RNA segment 7 (M) carrying to open reading frames encodes M1 and M2 proteins. Budding of new virions from the host plasma membrane is facilitated by NA that separates and release the viruses through SAs removal following the hydrolysis of the glycosidic linkage (Dou et al., 2018).

1.3 Targets for influenza vaccines

Vaccine components are annually updated by WHO with the prediction of virus strains that would be circulated for the upcoming influenza season. However, the influenza vaccine effectiveness was estimated between 10% and 60% during 2004-2020 season (CDC, 2021e). Current hemagglutinin (HA)-based seasonal vaccines are suboptimal in providing cross-protection against antigenically distinct influenza viruses due to the hypervariability of HA. If the vaccine strains mismatch with circulating viruses, the vaccine effectiveness can be dramatically reduced. The shortcoming of current influenza virus vaccines necessitates the development of more effective and universal vaccines such as by targeting highly conserved viral epitopes to confer cross-protection against newly emerging influenza viruses.

1.3.1 Hemagglutinin (HA)

Hemagglutinin (HA) is most abundant surface glycoprotein of influenza virus (Wu and Wilson, 2020) with 300-400 HA spikes. HA0 is a HA precursor and must be cleaved into HA1-HA1 dimer form that is linked by single disulfide bonds for viral infection. The presence of a polybasic HA cleavage site is associated with high virulence. Immunodominant head domain located central part of HA1 is the primary target of the antibody response (inducing neutralizing antibodies) but antigenically highly variable. All antigenic sites are presented in the globular head domain in H1 and H3 HAs. By contrast, immunosubdominant HA stalk found in HA2 is the highly conserved (Nachbagauer and Palese, 2020). Antibodies specific to highly conserved HA stalk domain have been described to confer broad protection with neutralizing activity and antibody-dependent cell-mediated cytotoxicity (ADCC). To enhance the immunogenicity of the HA stalk domain, headless HA constructs have been generated (Impagliazzo et al., 2015, Yassine et al., 2015). Also,

a sequential chimeric HA (HAs carrying identical stalk domain but antigenically different HA subtype head domains) immunization strategy has been developed (Hai et al., 2012, Chen et al., 2016). A phase I clinical study reporting the safety and ability of chimeric HA-based vaccines to induce cross-reactive anti-HA stalk domain antibodies suggested the potential as a universal vaccine development (Nachbagauer et al., 2021). Recently, a conserved protective determinant in the HA head domain from the interface of the HA trimer has been newly found. The antibodies to the conserved head domain showed inhibition of cell-to-cell virus spread (Bangaru et al., 2019).

1.3.2 Neuraminidase (NA)

Neuraminidase (NA) is the second major glycoprotein (10- 20%) on the virion surface, with about 40-50 NA spikes (McAuley et al., 2019). NA is less variable and shows lower antigenic mutation rates compared to HA (Sandbulte et al., 2011). Antibodies to NA are mostly non-neutralizing and have been known to inhibit virus budding from the host cells. Moreover, anti-NA antibodies induce cross-protective immunity within a subtype and confer protection against lethal infection in animal models. In research performed with guinea pigs showed that NA-based vaccination prevented the transmission of the virus (McMahon et al., 2019). An independent role in the protection of antibodies to viral NA has been suggested (Monto et al., 2015, Walz et al., 2018). Almost 100% conserved epitope in the NA (NAe, NA amino acids 222–230, located close to the enzymatic active site) was demonstrated in all influenza A subtypes and influenza B viruses (Stadlbauer et al., 2019, Gravel et al., 2010). This universally conserved NA epitope has been suggested as a potential cross-protective target for vaccine design. The current vaccines including inactivated virus and live attenuated virus vaccines, are poorly immunogenic to induce NA2e

specific antibodies. To enhance the immunogenicity of NAe, recombinant influenza virus carrying chimeric HA conjugates with NAe epitope was generated, and the contribution of NAe for cross-protection was recently reported (Kim et al., 2020).

1.3.3 Matrix Protein 2 (M2)

Matrix protein 2 (M2, 97 amino acids) found in influenza A virus is an integral transmembrane protein that consists of the N-terminal ectodomain (M2e, aa2-24), transmembrane (TM) domain (aa25-46), and amphiphilic C-terminal cytoplasmic tail (aa47-97) (Du et al., 2021). The M2 protein is a proton-selective ion channel responsible for internal acidification (by fluxing of H⁺ ions) of the influenza virus, which is crucial for viral entry and egress (Liang et al., 2016). The low pH of the endosome induces the HA-mediated membrane fusion. The extracellular domain M2e is evolutionary highly conserved, contains its eight N-terminal residues (aa2-9, SLLTEVET) which co-translated with matrix protein and thus exhibit more than 99% similarity in all influenza A viruses (Liu et al., 2005). The antibodies specific for M2e were detected during virus infection (Nachbagauer and Palese, 2020) and M2e antibodies have been shown to be protective in animals including mice and ferrets (El Bakkouri et al., 2011, Mezhenkaya et al., 2021b, Wise et al., 2012). M2e-based immunity is infection permissive that does not neutralize the virus, while reducing the clinical symptoms. (Schotsaert et al., 2013). The mechanism of protection is known to rely on Fc-mediated antibody functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent phagocytosis (ADP) and antibody-dependent complement activation (Vandervan and Kent, 2020). Also, T cell responses of M2e-specific CD4⁺ and CD8⁺ have been found to contribute to protection (Wu et al., 2007, Eliasson et al., 2018). The M2 protein is abundantly expressed on the infected cell surface, however, natural

influenza virus infection induces a weak anti-M2e antibody response (Feng et al., 2006). A possible reason for this low immunogenicity can be the small size of M2e with its low number of copies incorporated into the virion compared to other large surface glycoproteins such as HA and NA (Hutchinson et al., 2014). Therefore, M2e based vaccines have been focusing on improving the reactivity of M2e, for example M2e presenting virus-like particles (VLPs), flagellin-linked M2e, or DNA or RNA vectors are used (Schepens et al., 2018). M2e-based vaccines are not available yet, but they have been undergoing extensive trials in preclinical animal models and also clinical trials (Mezhenskaya et al., 2019). Although the induced antibodies specific for the conserved domains have been suggested to contribute to cross-protection, their protective efficacy tends to be weak. Antibodies against these conserved domains are relatively rare in humans because those epitopes are immunosubdominant. M2e- or NA-based vaccines induce non-neutralizing immunity that is less effective for protection compared to neutralizing immunity elicited by conventional HA globular head-based vaccines. Although HA stalk-based vaccine induces neutralizing antibodies, it is insufficient to confer strong protection possibly due to the poor accessibility of the antibodies to the HA stalk domain on the virus and inability to block the entry of virus infection. In contrast to the HA head specific antibodies, the antibodies against M2e, HA stalk, and NA do not inhibit the viral entry into the host cells and this might be able to provide a higher chance of allowing virus replication (Jang and Seong, 2014).

1.4 Reverse genetics

The nature of the influenza virus allowing the reassortment due to its segmented genomes has been utilized in the laboratory to generate reassortant viruses for their use as vaccines (either inactivated or live-attenuated influenza vaccines). The reverse genetics system using eight

plasmids (encoding influenza viral RNA segments) enabled to creation of recombinant influenza viruses (Neumann et al., 1999). The cold-adapted (ca) A/Ann Arbor/6/60 (USA/Canada, and Europe) and ca A/Leningrad/134/17/57 (in Russia, India, and China) have been approved as a master donor virus (MDV) for human usage.

The PR8-based (A/PR/8/34) reverse genetics technique is widely used in the laboratory to generate reassorted virus strains. The mutations in polymerase genes (PB1 and PB2) have been known to be critical for attenuation of A/PR8 (He et al., 2013).

1.5 Immune responses of intranasal vaccination versus intramuscular vaccination

Route of vaccination can affect immune responses. While intramuscular (i.m.) vaccination mainly induces a systemic immune response, intranasal (i.n.) vaccination can elicit both mucosal (at the local/distal mucosal sites) and systemic immune responses. The muscle blood rapidly takes the intramuscularly administered vaccine antigens and travels to the draining lymphoid tissues of the body (Oberdan Leo, 2011). However, non-invasive intranasal vaccines spread into the body at a relatively slower rate. Conventionally, i.m. vaccination mainly induces the secretion of IgG and IgA antibodies into the systemic blood systems. Meanwhile, i.n. vaccination induces systemic IgG (at relatively low levels) and IgA antibodies at local mucosal sites where are considered as frontline immunity by protecting respiratory or mucosal pathogens at the initial infection sites. Mucosal-associated lymphoid tissue (MALT) is a specialized tissue for the mucosal immune system (Mohn et al., 2018). Mucosal IgA shows cross-reactivity against virus variants. In mucosal immunity, dimeric secretory IgA (sIgA) fulfills a crucial role as a significant immunoglobulin on mucosal surfaces, which confer local protection against bacterial and viral pathogens in the nasal mucosa. Polymeric sIgA is more effective than monomeric serum such as

IgA or IgG since it can transfer to the mucous actively by transcytosis, and it is protected against degradation by the secretory components. sIgA recognizes commensal microbes and inhibits their overgrowth. Also, sIgA binds to luminal microbes and toxins to protect them from attaching to mucosal epithelial cells (Corthesy, 2013). Mucosal IgG also plays essential role in neutralizing the pathogens or mediating opsonization. The most common route of vaccination for the influenza vaccine is i.m. with an inactivated influenza virus vaccine (IIV). After vaccine administration, antigens are picked up by antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages. APCs move towards lymph nodes draining the site of injection. Upon the antigen encountering, the adaptive immune system is initiated and effector cells, including CD4 T cells, cytotoxic T cells and antibodies are generated. These effector cells circulate the bloodstream and back to the site of injection. Systemic IgG antibodies and local mucosal IgG antibodies diffused from systemic IgG play a critical role in protection after IIV application via an intramuscular route (Sridhar et al., 2015). Whereas licensed live-attenuated influenza virus vaccine (LAIV) administration via i.n. can induce cross-reactive mucosal IgA and CD8+ cytotoxic T lymphocytes (CTL) in addition to IgG. Once LAIV is delivered intranasally mimicking natural infection, antigens taken up by DCs are transferred to the tonsils/adenoids. At this location, activation and proliferation of T and B cells are initiated by CD4 T cells. Also, affinity maturation and isotype switching of antibodies are carried out. Activated T and B cells back to the site of vaccination or infection and then enter the circulation system. Plasma cells secrete specific mucosal antibodies and local antigen specific antibodies spread into circulation. Antibody secreting cell response remains longer with LAIV than IIV, and this can be explained by the extended duration of stimulation with LAIV due to its local administration and virus replication

(higher antigen content) in the nasal mucosa (Mohn et al., 2018). Also, LAIV induces lung tissue-resident memory T cells, which provides long-term protection. LAIV can induce innate immune responses more intensively by providing multiple pathogen-associated molecules including viral RNAs which can be detected by pathogen recognition receptors (PRRs) such as TLR7 and TLR8 which are recognizing single-strand RNA. In children, LAIV induces higher immunogenic responses and better efficacy than IIV. LAIV elicits a T-cell response in children. Since pre-existing immunity in adults might inhibit virus replication of LAIV, immunologically naïve children allow better replication of LAIV and induce more protective immune responses. The status of pre-existing immunity might influence the efficacy of influenza vaccination.

1.6 Immunosenescence in the elderly

Annually, influenza results in approximately 650,000 deaths globally and people 65 years and older are at overwhelmingly higher risk with occupying 70 – 85 percent of seasonal flu-related deaths (CDC estimation in the United States) (CDC, 2021b). Annual vaccination is known to be the most effective strategy to reduce the risk of influenza in the elderly by decreasing the risk of influenza from 6% to 2.4% (Tanner et al., 2021). However, the vaccine effectiveness (VE) in over-65 years (17-53%) is disproportionately dropping compared to young adults (70-90%) (Goodwin et al., 2006). Conventional influenza vaccine is less immunogenic (only 30-40%) in the elderly, and this results in at least 20% of failure in elderly recipients to induce hemagglutination inhibition (HAI) antibodies (Keren et al., 1988) known to be correlated with protection (Dunning et al., 2016). The low overall effectiveness is believed mainly due to an altered immune response that is different from children and young adults who are the primary targets for designing influenza vaccine (Ciabattini et al., 2018). The age-associated deleterious changes in the immune

system including immunosenescence and inflammaging are responsible for low vaccine effectiveness (Tanner et al., 2021). Immunosenescence refers an age-related immunological dysfunction accompanied by gradual deterioration in innate and adaptive immune responses. For example, downregulation of primary lymphoid organs causes decreased production of B and T cells progenitors; reduction of phagocytosis and chemotaxis and increased production of pro-inflammatory cytokines and dysfunctional memory cells (Ciabattini et al., 2018). Inflammaging is the state of chronic hyperinflammation. The constantly increased basal levels of inflammation interfere with detecting and generating truly protective inflammatory responses to pathogens. Therefore, inflammaging substantially contributes to increased susceptibility in the elderly to infection (Lambert et al., 2012).

Recipients' viral history of influenza infection and vaccination would be considered for the vaccine effectiveness in the elderly. This can be described by the concept of original antigenic sin (OAS) proposing that an individual's immune response is imprinted by previously encountered influenza viral antigens (Ranjeva et al., 2019). OAS could be beneficial and harmful in immunity against influenza. The elderly showed a lower infection rate compared to young people during the 2009 pandemic (swine flu, A/H1N1). It is believed that previous exposure to H1N1 viruses that were circulated in the late 1970s in older people conferred the protection by inducing the cross-immunity to conserved antigens. However, the increased susceptibility of the elderly to influenza virus infection in the 2013-2014 H1N1 season was observed while the young population with immunity to nonmutated antigens displayed enhanced protection (Tanner et al., 2021). In research investigated the effect of repeated vaccination (RV) in ferrets (Music et al., 2019) after A/Hong Kong H3N2 challenge, the ferrets immunized once (current season only) showed lower

viral shedding, less body weight loss and relatively faster recovery of body weight compared to another group of ferrets repeatedly immunized with commercial vaccines twice (10 months apart). One explanation for reduced vaccine effectiveness in the RV group can be related to OAS. Suboptimal antibodies (to the conserved domains between two vaccines) for protection are amplified by repeated vaccination.

To overcome the reduced vaccine effectiveness in the elderly, high-dose or adjuvanted vaccines have been developed to elicit more robust immunogenicity.

1.7 Hypothesis and Research Objectives

Chapter1: Broad cross-protection by recombinant live attenuated influenza H3N2 seasonal virus expressing conserved M2 extracellular domain in a chimeric hemagglutinin.

The objective of project 1 was to determine whether intranasal immunization with live recombinant H3N2 virus expressing chimeric 4xM2e-HA would induce broadly cross-protective immunity against different subtypes of influenza A viruses in a mouse model. In recent years, antigenic drifts have severely limited the effectiveness of the H3N2 component of seasonal influenza vaccines. Here, using the reverse genetic (rg) technique, we generated reassortant seasonal influenza rgH3N2 4xM2e virus containing chimeric 4xM2e-HA in which the HA and NA genes were derived from A/Switzerland/9715293/2013 (H3N2) and the remaining 6 genes from the A/PR8 backbone. Reassortant rgH3N2 4xM2e virus containing chimeric 4xM2e-HA was found to retain comparable growth properties but displayed highly attenuated phenotypes in mice. Cross-protective efficacy against different subtypes (H1N1, H3N2, H5N1, H7N9, H9N2) of influenza A virus was tested in intranasally immunized BALB/c mice with rgH3N2 4xM2e. This

study implicates a strategy of improving cross-protection by utilizing currently licensed recombinant influenza vaccine platforms.

Chapter2: Enhanced cross-protection by hetero prime-boost vaccination with recombinant influenza viruses containing chimeric hemagglutinin-M2e epitopes.

The goal of project 2 was to test whether a strategy of hetero prime-boost vaccination with recombinant influenza viruses expressing chimeric 4xM2e-HA would induce more effective cross-protection than homologous prime-boost vaccination. The impact of heterosubtypic vaccination with recombinant 4xM2e-HA influenza virus vaccines and pre-existing immunity on cross-protection against influenza viruses remains unknown. In this study, I investigated the efficacy of cross-protection by heterosubtypic prime-boost vaccination with live recombinant 4xM2e-HA H1N1 and H3N2 influenza virus vaccines in C57BL/6 mice known to be a low responder to immune-subdominant conserved epitopes. The experimental outcomes of project 2 demonstrated that hetero prime-boost strategies using recombinant 4xM2e-HA influenza virus vaccination induced more effective cross-protection against antigenically different viruses than homologous repeat vaccination in C57BL/6 mice. The roles of M2e and stalk immunity in conferring cross-protection were explored and discussed in this study.

Chapter 3: Hemagglutinin virus-like particle is immunogenic and provides heterologous protection against influenza virus in young adult and aged mice.

The goal of project 3 was to investigate immune responses and homo and cross-protective efficacy in aged mice after vaccination with a platform of virus-like particles (VLP) presenting H1 HA with and without molecularly anchored cytokine adjuvants incorporated, in comparison with those in young adult mice. Vaccine effectiveness is inferior in the aged

population at high risk of severe illness from influenza virus infection. For the elderly, safe and highly immunogenic vaccines need to be developed. In project 3, host immune responses and homo and cross-protective efficacy was determined, after vaccination with a VLP vaccine platform which expresses H1 HA from A/PR8/34 (PR8 HA VLP) in young adults and aged (18-month-old) BALB/c mice. In addition, I investigated the adjuvant impact of cytokines (GM-CSF and IL-12) engineered to be incorporated into HA VLP vaccines on inducing IgG antibodies, hemagglutination inhibition titers, and homo and hetero protection in aged mice, compared to those in young adult mice. Higher doses of vaccination with H1 HA VLP and cytokines incorporated onto H1 HA VLP were found to be more effective in inducing protective immunity against homo and hetero viruses in aged mice.

2 BROAD CROSS-PROTECTION BY RECOMBINANT LIVE ATTENUATED INFLUENZA H3N2 SEASONAL VIRUS EXPRESSING CONSERVED M2 EXTRACELLULAR DOMAIN IN A CHIMERIC HEMAGGLUTININ

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2.1 Abstract

Hemagglutinin (HA)-based current vaccines provide suboptimum cross-protection. Influenza A virus contains an ion channel protein M2 conserved extracellular domain (M2e), a target for developing universal vaccines. Here we generated reassortant influenza virus rgH3N2 4xM2e virus (HA and NA from A/Switzerland/2013/62/H3N2) expressing chimeric 4xM2e-HA fusion proteins with 4xM2e epitopes inserted into the H3 HA N-terminus. Recombinant rgH3N2 4xM2e virus was found to retain equivalent growth kinetics as rgH3N2 in egg substrates. An intranasal single inoculation of mice with live rgH3N2 4xM2e virus was effective in priming the induction of M2e specific IgG antibody responses in mucosal and systemic sites as well as T cell responses. The rgH3N2 4xM2e primed mice were protected against a broad range of different influenza A virus subtypes including H1N1, H3N2, H5N1, H7N9, and H9N2. The findings support a new approach to improving current vaccine platforms' efficacy by recombinant influenza virus inducing immunity to HA and cross-protective M2e antigens.

2.2 Introduction

Influenza virus causes one of the most common respiratory diseases in humans, resulting in significant public health concerns and deaths annually worldwide (Krammer et al., 2018b, Iuliano et al., 2018). Influenza A virus is a negative-sense single-stranded RNA virus containing eight segmented genomes, belonging to the *Orthomyxoviridae* family, and has an antigenic

variety from 18 subtypes (H1-H18) of hemagglutinin (HA) and 11 subtypes (N1-N11) of neuraminidase (NA) (Tong et al., 2013). Antigenic diversity is a challenging difficulty in preventing influenza. Vaccination has been the most effective preventive measure against influenza virus infection. The most common licensed platforms are inactivated influenza and live-attenuated influenza vaccines (LAIV).

The overall vaccine effectiveness during the 2005-2018 seasons is a wide range of low efficacy between 10 and 60% as estimated in the US Flu Vaccine Effectiveness Network (CDC, 2021e). Due to the emergence of drifting mutations in circulating H3N2 strains, the vaccine effectiveness against H3N2 was estimated to be 6% during the 2014–2015 season (Zimmerman et al., 2016, Shim et al., 2016). To overcome continued antigenic changes, universal vaccination strategies have been focused on inducing immunity to conserved epitopes and domains present in all influenza A viruses, including the M2 extracellular domain epitopes (M2e) (Saelens, 2019, Kolpe et al., 2017) and the HA stalk domains (Krammer and Palese, 2019, Nachbagauer et al., 2015).

Different platforms and vaccine adjuvants have been investigated to overcome the low immunogenicity of M2e epitopes. M2e-based vaccine candidates include Hepatitis B virus core protein conjugates (M2e-HBc) with adjuvants (Neirynck et al., 1999, De Filette et al., 2006), virus-like particles (VLP) presenting M2e tandem repeats (5xM2e VLP) (Kim et al., 2013b), and flagellin conjugates (4.M2e-tFliC) (Wang et al., 2012) and fusion with oligomer stabilizing domains (M2e-tGCN4) (De Filette et al., 2008). M2e expressing viral vectored vaccines were reported using adenovirus (Zhou et al., 2010), modified vaccinia virus Ankara (Hessel et al., 2014), and a T7-bacteriophage (Hashemi et al., 2012). These previous strategies inducing M2e immunity alone

were insufficient for conferring optimum protection and incompatible with current vaccine platforms. No universal vaccine against influenza is on the market.

Vaccination of combined M2e VLP and inactivated influenza vaccines induced both cross-protective M2e and strain specific HA immunity (Song et al., 2011, Kim et al., 2014). To enhance the cross-protective efficacy by a strategy of utilizing current vaccine platforms, recombinant influenza H1N1 virus A/Puerto Rico/8/1934 (A/PR8) was engineered to express chimeric 4xM2e-HA where tandem M2e epitopes were inserted in the N-terminus HA (Kim et al., 2017a). A chimeric HA with a single M2e in the head site Ca was tested in inactivated recombinant A/PR8 virus inducing cross-protection (Sun et al., 2019). However, the protection was not tested against a wide range of different subtypes.

In recent years, antigenic drifts have severely limited the effectiveness of the H3N2 component of seasonal influenza vaccines. Here, using the reverse genetic (rg) technique, we generated reassortant seasonal influenza rgH3N2 4xM2e virus containing chimeric 4xM2e-HA in which the HA and NA genes were derived from A/Switzerland/2013/62 (H3N2) and the remaining 6 genes from the A/PR8 backbone. Reassortant rgH3N2 4xM2e virus containing chimeric 4xM2e-HA was found to retain comparable growth properties but display highly attenuated phenotypes in mice. An intranasal single inoculation of mice with rgH3N2 4xM2e virus could effectively induce a broad range of enhanced cross-protection against different influenza A virus subtypes including H1N1, H3N2, H5N1, H7N9, and H9N2. This study implicates a strategy of improving cross-protection by utilizing currently licensed recombinant influenza vaccine platforms.

2.3 Materials and Methods

2.3.1 Cells and viruses

For DNA transfection to generate reassortants, 293T cells obtained from ATCC were used and maintained in Dulbecco's Modified Eagle *Medium* media. Embryonated chicken eggs for influenza virus amplification were obtained from Hy-Line N.A. LLC (Mansfield, GA) and confirmed free of influenza virus. A/Puerto Rico/8/1934 (A/PR8, H1N1), A/Philippines/2/82 (A/Phil, H3N2), and rgH5N1 A/Vietnam virus which contains HA (polybasic residues removed) and NA from A/Vietnam/1203/2004 and six internal genes from A/PR8 were previously described (Song et al., 2011). Reverse genetics (rg) H3N2 (A/Switzerland/2013, A/SW) is a reassortant virus X-247 with A/PR8 backbone (CDC influenza resources) containing HA and NA genes from A/Switzerland /9715293/2013 (H3N2) and 6 remaining genes from A/PR8. The viruses were inactivated using formalin (37%) at 1:4000 dilutions in purified virus concentrations (1 mg/ml) (Ko et al., 2018). The rgH7N9 is a reassortant virus containing HA and NA derived from A/Shanghai/2/2013 (H7N9) and the A/PR8 backbone genes (Deng et al., 2018). The reassortant H9N2 virus is A/chicken/Hong Kong/G9/1997 x PR8-IBCDC-2 (CDC influenza resources). The rgPR8 Ms, rgPR8 Mm and rgPR8 Mg reassortant viruses containing avian M2 were generated using the A/PR8 backbone genes by replacing an M gene from A/Shanghai/2/2013 (H7N9), A/Mandarin duck/Korea/PSC24-24/2010 (H5N1) and A/Chicken/Korea/Gimje/2008 (H5N1) respectively.

2.3.2 Generation of recombinant reassortant rgH3N2 virus containing 4xM2e-HA

A full-length copy of the HA gene from A/SW (H3N2) was used to insert a cloning site for BsmBI restriction enzyme between the signal peptide (SP) sequence and N-terminus in the ectodomain of HA by polymerase chain reaction (PCR) primers. The two PCR products of SP

fragment and HA full-length with each BsmBI site were linked together by overlapping reactions of PCR products, resulting in the SP-BsmBI-HA construct with an H3 HA full length ectodomain and transmembrane domain. The 4xM2e encoding gene is composed of heterologous M2e sequences derived from human (2x hM2e), swine (1x sM2e), and avian (1x aM2e) influenza A viruses (Figure 1A) as described (Kim et al., 2017a). This 4xM2e gene was amplified using PCR primers with BsmBI sites for insertion into the SP-BsmBI-HA H3 construct in dual promoter pCI plasmid (Figure 1A). The inserted 4xM2e gene contains flexible linkers AAAGGAA or AAAPGAA between each M2e domain as well as between the 4xM2e sequence and the N-terminus H3 HA (Figure 1A). The correct insertion of 4xM2e into the full-length H3 HA was confirmed by DNA sequencing of the construct.

To generate reassortant rgH3N2 virus containing 4xM2e-HA (rgH3N2 4xM2e), 293T cells were co-transfected with plasmids encoding 4xM2e-HA (H3) and N2 NA from A/SW (H3N2) and six other plasmids encoding the A/PR8 backbone genes. Two days after transfection, the supernatants were inoculated into 10-day-old embryonated chicken eggs. The rescue of replication-competent recombinant viruses was initially screened in allantoic egg fluids by a hemagglutination activity assay and the genetic identity of the reassortant vaccine virus was confirmed by sequence analysis.

2.3.3 Characterization and pathogenicity of reassortant rgH3N2 4xM2e-HA virus

The expression of 4xM2e-HA of the rescued reassortant viruses was characterized by enzyme-linked immunosorbent assay (ELISA) using influenza A virus M2e specific monoclonal antibody (14C2 mAb) (Abcam Inc., Cambridge, MA) or polyclonal antisera specific for the H3N2

virus. Growth capability and kinetics of rescued rgH3N2 4xM2e virus were compared with rgH3N2 virus in embryonated eggs.

To verify pathogenicity of recombinant rgH3N2 4xM2e virus compared to A/PR8 pathogenic WT virus, BALB/c mice were intranasally (IN) inoculated with 1×10^5 or 1×10^6 EID₅₀ (50% egg infectious dose) of rgH3N2 4xM2e, rgH3N2, or 10^4 EID₅₀ WT A/PR8 pathogenic viruses. At three days after IN infection, nasal turbinates and lung tissue samples were collected and homogenized in DMEM. The supernatants clarified by centrifugation were used to determine virus titers by limiting dilutions in the embryonated eggs and the limit of detection was $1.2 \log_{10}$ EID₅₀/ml. Another set of mice (n=5 /group) infected with recombinant and WT viruses was monitored daily for 14 days to record body weight changes.

2.3.4 Immunizations and virus challenge of mice

BALB/c mice (n=30 /group, 6- to 8-week-old age, Jackson Laboratories) were IN inoculated with 10^5 EID₅₀ for a prime single dose of recombinant rgH3N2 4xM2e or rgH3N2 virus. Another set of primed mice (n=15 /group) was boosted with 10^6 EID₅₀ of recombinant rgH3N2 4xM2e or rgH3N2 virus at four weeks after priming. Blood samples were collected at two weeks after each inoculation for serological immune assays. After three weeks of prime or prime-boost dose inoculation, rgH3N2 4xM2e, rgH3N2 immune, or unvaccinated (mock) mice were challenged with different subtype viruses as indicated. LD₅₀ dose causing 50% lethality in mice was pre-determined. Differential challenge doses were used depending on the pathogenicity and subtype of virus and experimental conditions (prime, prime-boost, T cell depletion). All animal experiments in this study were approved by the Georgia State University IACUC review boards. Mouse animal experiments including virus infection, blood and tissue collections were performed

in accordance with the approved IACUC protocol (A21004) and regulations. The study was carried out in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

2.3.5 Antibody ELISA and hemagglutination inhibition (HAI) assays

Virus-specific antibody responses were determined by ELISA using inactivated H1N1 (A/PR8), H3N2 (A/SW, A/Phil), or rgH5N1 viruses as a coating antigen (4µg/ml). M2e-specific antibody responses were determined using human, swine, and avian M2e peptide antigens of chemically synthesized 23 amino acids (aa) polypeptides as described (Kim et al., 2013b, Kim et al., 2014). IgG isotypes and IgA antibodies were measured using horse-radish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, and IgA secondary antibodies (Southern Biotechnology), color reactions developed with tetramethylbenzidine substrates (TMB, Invitrogen). Antibody levels are presented as optical density absorbance values at 450nm (BioTek ELISA plate reader) or concentrations as calculated using standard IgG and IgA (Southern Biotech). Bronchoalveolar lavage fluids (BALFs) were obtained by infusing 1.5 ml of phosphate-buffered saline (PBS) into the lungs (Kim et al., 2013b). HAI assays were determined against homologous, heterologous, and heterosubtypic influenza viruses using chicken red blood cells (Quan et al., 2009, Kim et al., 2014). Briefly, 1:4 ratios of sera and receptor destroying enzymes (RDE, Sigma) were mixed and incubated for 18 hours at 37°C. After inactivation, the RDE-treated sera were serially diluted and mixed with a virus (8 HA units). HAI titers were determined as the highest dilution factor inhibiting the formation of buttons with 0.5% chicken red blood cells.

2.3.6 In Vivo T-Cell depletion

For *in vivo* systemic T-cell depletion prior to challenge, primed mice with rgH3N2 4xM2e or rgH3N2 received treatment with anti-CD4 (CD4 clone GK1.5) and/or anti-CD8 (CD8 clone 53.6.7) mAbs. Antibodies (BioXCell, West Lebanon, NH) were injected into the mice with intraperitoneal (IP) and IN sequential delivery at two days interval (anti-CD4 200µg /mouse (IP) and 10 µg/mouse (IN), anti-CD8, 150 µg /mouse (IP), 10 µg/mouse (IN)). The levels of CD4 and CD8 T cells were below the detection after treating with CD4 and CD8 depleting antibodies as confirmed by flow cytometry of blood (Figure 12). All groups (n=4) were challenged with a lethal dose of A/PR8 H1N1 (1.5 LD₅₀), rgPR8 Mg (3 LD₅₀), rgPR8 Ms (10 LD₅₀) or A/Phil H3N2 (17 LD₅₀) influenza virus. Mice were monitored daily to record weight changes and mortality.

2.3.7 Intracellular cytokine staining of T cells

BALF and lung cells were stimulated with M2e peptides (SLLTEVETPIRNEWGSRN) (5 µg/mL) for 5 h at 37 °C in the presence of brefeldin A (BFA) (20 µg/mL). After stimulation, lymphocytes were stained with T cell marker mAb for CD4 (CD4-PE/Cy5, BD Biosciences) and CD8 (CD8α-PE, Biolegend) by following a procedure of BD Cytofix/Cytoperm Plus Kit. Intracellular staining of the permeabilized lymphocytes was conducted with IFN-γ cytokine mAb (anti-mouse IFN-γ-APC/Cy7, BD Biosciences). All samples were analyzed using LSR-II/Fortessa flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed using the FlowJo software (FlowJo V10, Tree Star, Inc.).

2.3.8 In vivo efficacy tests of immune sera

Immune sera collected at 4 weeks after boost inoculation were heat-inactivated at 56°C for 30 min, 4 folds diluted, and then mixed with 2 LD₅₀ of influenza virus rgH5N1 (A/Vietnam) and

incubated at room temperature for 1 hour as previously described (Kim et al., 2014, Song et al., 2011). The rgH5N1 virus mixed with immune sera was used to IN infect naive mice (n = 3) and body weight and survival rates were daily monitored for 8 days.

2.3.9 In vitro IgG antibody detection and cytokine ELISA assays

Secreted IgG antibodies specific for M2e, rgH3N2 and A/Phil were determined from mediastinal lymph nodes (MLN) from BALB/c mice. The cells from MLN were isolated at day 6 after challenge and cultured for 6 days in the plate pre-coated with M2e, rgH3N2 or A/Phil. The combined levels of IgG antibodies secreted into the culture supernatants and captured on the plate were analyzed by ELISA and presented. The inflammatory cytokines such as TNF- α , IFN- γ and IL-6 from bronchoalveolar lavage fluids (BALF) and lung extracts were measured by cytokine ELISA as described in the previous study (Lee et al., 2016b). Cytokines were detected using Ready-SET-Go kits with TNF- α , IFN- γ or IL-6 specific antibodies (eBioscience, San Diego, CA).

2.3.10 Statistical analysis

Two-way or one-way ANOVA were used to determine the statistical significance when comparing two different conditions. *P*-values of less than or equal to 0.05 were considered significant. Data analysis was performed using Prism software (GraphPad Software Inc., San Diego, CA).

2.4 Results

2.4.1 Generation of reassortant influenza H3N2 virus vaccine containing 4xM2e in an HA conjugate

The current strain specific HA-based influenza vaccine is less effective in conferring cross-protection. M2e has been targeted to induce broad but weak cross-protection. To induce

immunity against both HA and highly conserved M2e epitopes, a seasonal A/Switzerland/2013 H3 HA gene conjugated with four tandem M2e (4xM2e) repeat was constructed (Figure 1A and Table 1). Rescued viruses expressing wild type (WT) HA (rgH3N2) or chimeric HA (rgH3N2 4xM2e) were amplified in eggs and harvested. Antigenic characterization by ELISA showed that rgH3N2 4xM2e virus was highly reactive to M2e specific mAb 14C2 whereas rgH3N2 and A/PR8 virus controls did not show such M2e reactivity (Figure 1B). Both rgH3N2 and rgH3N2 4xM2e viruses displayed high antigenic reactivity to mouse antisera of rgH3N2 infection and goat antisera of A/Indiana/2011 (H3N2) immunization (Figure 2). These results support that 6:2 reassortant rgH3N2 4xM2e virus displays high reactivity to M2e specific 14C2 mAb and retains similar antigenicity to antisera of H3N2 viruses as compared to rgH3N2 virus.

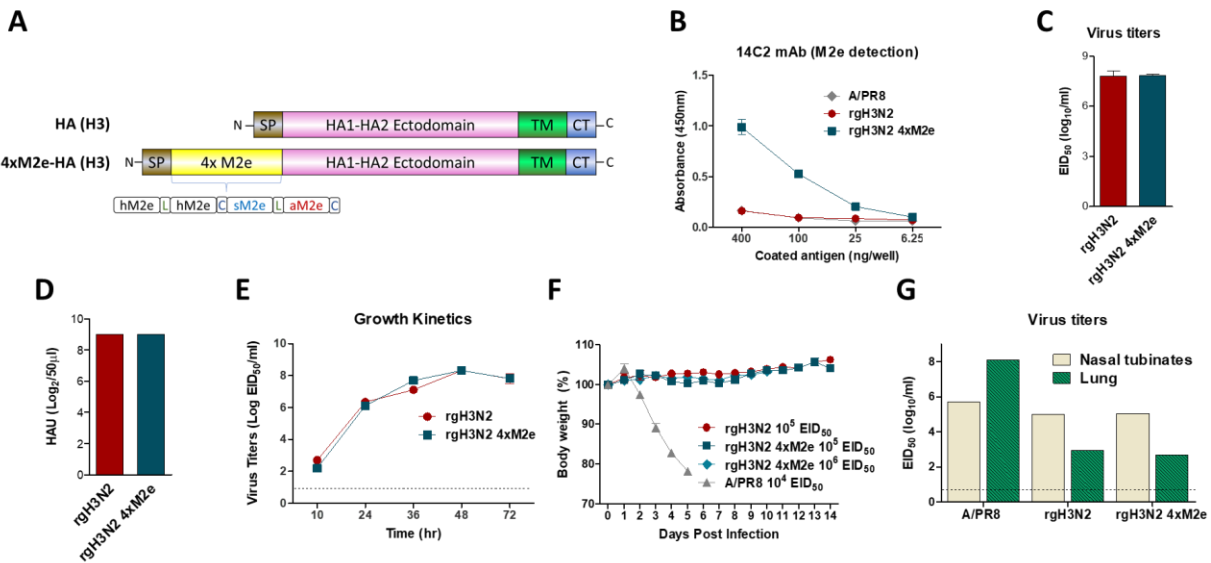


Figure 1 Reassortants rgH3N2 and rgH3N2 4xM2e virus expressing chimeric 4xM2e-HA are attenuated in vivo

(A) Diagram for chimeric 4xM2e-HA (H3) structures. The sequence H3 HA was derived from A/Switzerland/2013 (H3N2). SP: signal peptide, tandem repeat 4xM2e is composed of human, swine, and avian influenza A viruses. hM2e: SLLEVTETPIRNEWGSRSDSSD, sM2e: SLLEVTETPTRSEWESRSDSSD, aM2e: SLLEVTETPTRNEWESRSDSSD. L and C represent linker (AAAGGAA) and connector (AAPGAA) respectively. (B) 14C2 M2e specific mAb ELISA assays were performed to characterize 6:2 reassortants rgH3N2 and rgH3N2 4xM2e viruses (4 µg/ ELISA plate well) generated by reverse genetics (rg) using the

A/PR8 backbone. (C) Infectious virus titers (EID_{50}) of rgH3N2 and rgH3N2 4xM2e virus preparations in egg substrates. (D) Hemagglutination activity units (HAU, $\log_2/50 \mu\text{l}$) for rgH3N2 and rgH3N2 4xM2e viruses using chicken red blood cells. (E) Growth kinetics of reassortant viruses in embryonated chicken eggs at 33°C infected with 10^4EID_{50} of rgH3N2 and rgH3N2 4xM2e. c-e: A representative out of 2 repeats. (F) Body weight changes in BALB/c mice ($n=10$, too small variations to be shown) IN inoculated with reassortant rgH3N2 viruses (10^5 - 10^6EID_{50}) or A/PR8 (10^4EID_{50}). (G) Infectious virus titers (EID_{50}) in nasal turbinates and lung extracts from mice ($n=3$, pooled) at 3-day post infection with A/PR8 (10^4EID_{50}), rgH3N2 (10^6EID_{50}) and rgH3N2 4xM2e (10^6EID_{50}).

Vaccine virus (rgH3N2 4xM2e)

Conserved M2e	M2e sequences
Human M2e	SLLTEVET PIRNEW GSRSNDSSD
Swine M2e	SLLTEVET PTREWE ESRSDSSD
Avian M2e	SLLTEVET PTRNEW ESRSDSSD

Challenge virus

M2 gene from	M2e sequences
A/Puerto Rico/8/1934 (H1N1)	SLLTEVET PIRNEW GCRCN GSSD
A/California/07/2009 (H1N1)	SLLTEVET PTREWE ECRCSDSSD
A/Philippines/2/82 (H3N2)	SLLTEVET PIRNEW GCRCN DSSD
A/Madarin duck/Korea/PSC24-24/2010 (H5N1)	SLLTEVET PTREWE ECRCSDSSD
A/Chicken/Korea/Gimje/2008 (H5N1)	SLLTEVET PTREWE ECRCSDSSD
A/Shanghai/2/2013 (H7N9)	SLLTEVET PTRTGW ECNCSGSSE

Table 1.1 M2e sequences utilized for rgH3N2 4xM2e vaccine and reassortant challenge viruses.

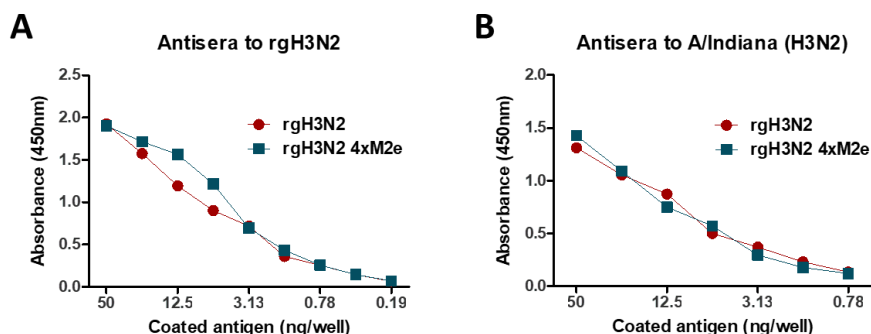


Figure 2 ELISA assays to characterize 6:2 reassortants rgH3N2 and rgH3N2 4xM2e viruses generated by reverse genetics (rg) using the A/PR8 backbone

(A) ELISA (4 μg / ELISA plate well) using anti-rgH3N2 (A/Switzerland/9715293/2013 (H3N2)) mouse sera as primary antibody. (B) ELISA using anti-A/Indiana/2011 (H3N2) goat sera as primary antibody.

2.4.2 Reassortant rgH3N2 4xM2e virus is attenuated in mice but does not compromise replication capacity in egg substrates

We determined whether rgH3N2 4xM2e virus would retain the comparable replication capacity in egg substrates, compared to WT rgH3N2. Both chimeric rgH3N2 4xM2e and WT rgH3N2 viruses displayed similarly high levels of egg infectious titers ($\text{EID}_{50}/\text{ml}$) and hemagglutination activity units (HAU) (Figure 1C, D). Next, at designated time points after incubation of embryonated chicken eggs with 10^4 EID_{50} of rgH3N2 or rgH3N2 4xM2e, *in vitro* growth kinetics of infectious titers (EID_{50}) were determined (Figure 1E). The results showed that rgH3N2 4xM2e virus exhibited approximately equivalent growth kinetics in egg substrates as WT rgH3N2 virus. The reassortant rgH3N2 4xM2e virus does not compromise replication capacity in egg substrates. Consistent, the overall trends in the growth patterns of rgH3N2 and rgH3N2 4xM2e reassortants in MDCK cells were similarly observed as those in the eggs despite some variations at different culture times and temperatures (Figure 3). Therefore, rgH3N2 4xM2e virus does not compromise replication capacity in egg substrates.

As an indicator of pathogenicity, weight changes and activity were monitored in mice after intranasal inoculation with rgH3N2 4xM2e and rgH3N2 viruses. Both mouse groups infected with rgH3N2 (10^5 EID_{50}) and rgH3N2 4xM2e (10^5 and 10^6 EID_{50}) did not show body weight loss (Figure 1F) while the mice with A/PR8 even at a 100-fold lower dose (10^4 EID_{50}) consistently displayed severe weight loss and died of infection. Consistent, mice inoculated with WT A/PR8 virus showed highest levels of replicating viral titers in the lower respiratory tracts of lungs, which is at approximately 100 folds higher than those in the upper respiratory nasal turbinates at day 3 post inoculation (10^4 EID_{50}) (Figure 1G). In contrast, rgH3N2 (10^6 EID_{50}) and rgH3N2 4xM2e (10^6

EID₅₀) viruses showed approximately 10⁵ magnitudes lower lung viral titers than WT A/PR8 virus (Figure 1G). Instead, 100 folds higher viral replications were observed in the nasal turbinates than those in the lungs after inoculation of mice with rgH3N2 and rgH3N2 4xM2e virus (Figure 1G). These results suggest that reassortant rgH3N2 and chimeric rgH3N2 4xM2e viruses are highly attenuated in mice with restricted replication in the upper respiratory tracts but retains replication capacity in egg substrates.

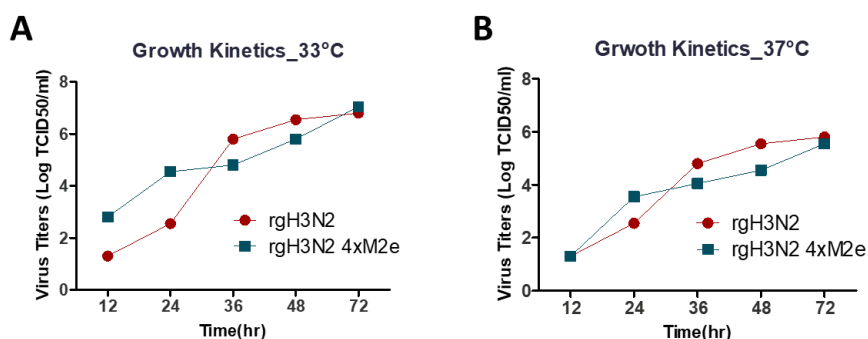


Figure 3 . Growth kinetics for rgH3N2 and rgH3N2 4xM2e in MDCK cells

Virus replications of rgH3N2 and rgH3N2 4xM2e were determined in MDCK cells at different time points at 33°C and 37°C culture temperature as indicated after inoculation. The titers were determined and presented in TCID₅₀/ml (A) at 33°C and (B) at 37°C.

2.4.3 Prime dose of live rgH3N2 4xM2e virus induces M2e specific IgG isotype antibody responses

Intranasal prime inoculation of mice with live rgH3N2 4xM2e virus (10⁵ EID₅₀) induced M2e specific IgG1 and IgG2a antibody responses at significantly higher levels than rgH3N2 (Figure 4A, B). IgG antibodies specific for human M2e were induced at higher levels in prime sera from rgH3N2 4xM2e vaccination than those specific for avian M2e or swine M2e (Figure 5A), suggesting possible contribution of the extra 2x hM2e in the rgH3N2 4xM2e construct. Both rgH3N2 and rgH3N2 4xM2e immunized groups showed similarly high levels of IgG isotype

antibodies specific for H3N2 virus (Figure 4C, D). Both sera from rgH3N2 and rgH3N2 4xM2e immune mice exhibited vaccine strain specific HAI activities against rgH3N2 (A/Switzerland) virus (Figure 4E) but not against A/PR8 (H1N1), A/Philippines (A/Phil H3N2) and A/Vietnam (A/Viet rgH5N1). Therefore, prime dose of live rgH3N2 4xM2e but not rgH3N2 virus could induce M2e specific IgG isotype antibodies in mice.

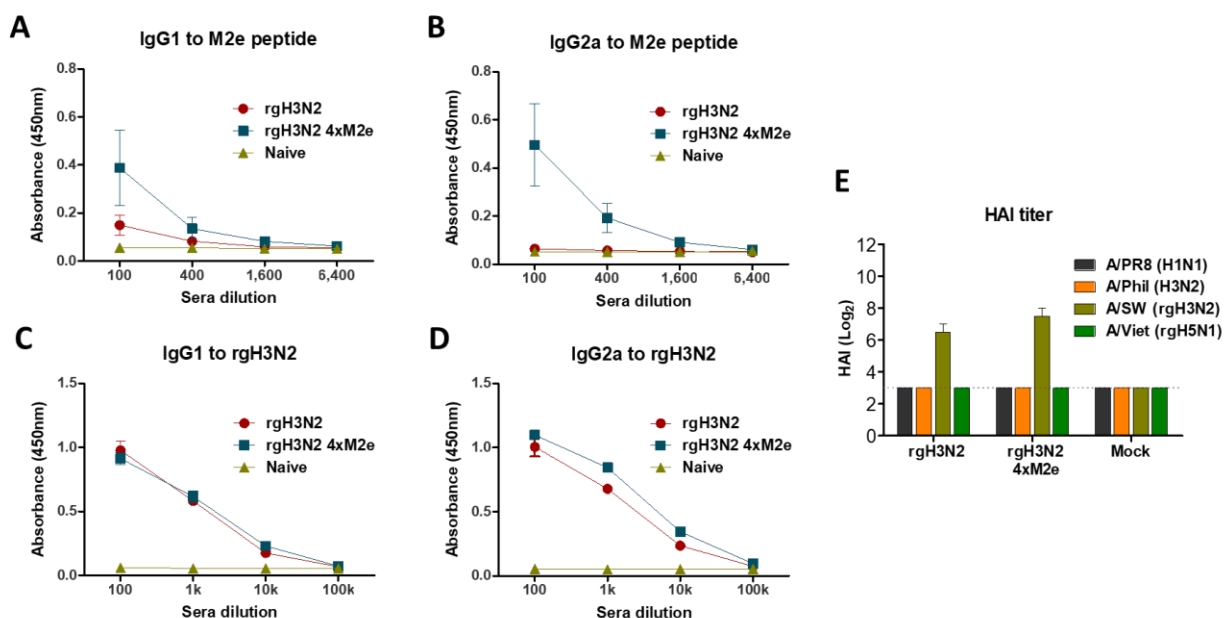


Figure 4 Intranasal prime inoculation of live rgH3N2 4xM2e virus induces M2e specific serum IgG isotype antibody responses

(A) M2e specific IgG1 antibodies. (B) M2e specific IgG2a antibodies. (C, D) Virus rgH3N2 specific IgG1 and IgG2a antibodies. Serum samples were collected at 2 weeks after prime dose intranasal inoculation of mice (n=15 /group). (E) Serum HAI titers against the viruses (A/PR8, A/Phil, rgH3N2 and A/Viet) were determined 14 days after immunization. rgH3N2: 6:2 reassortant with WT HA and NA of A/Switzerland/2013. rgH3N2 4xM2e: 6:2 reassortant with 4XM2e-HA and NA of A/Switzerland/2013. Each individual mouse animal is analyzed. Error bars indicate mean \pm SEM.

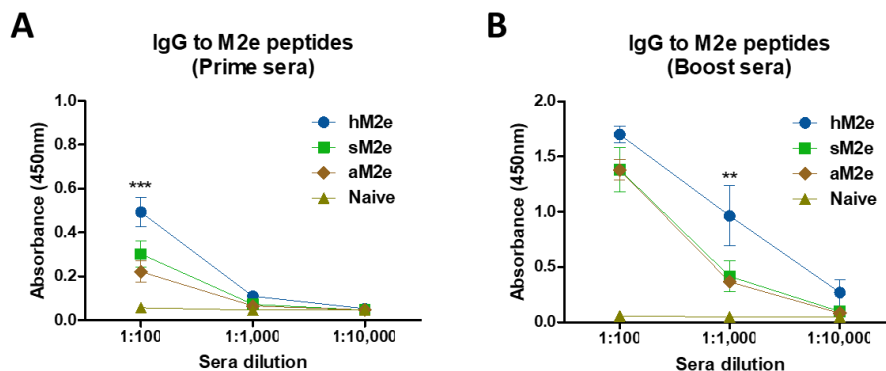


Figure 5 IgG antibody responses to humanM2e, swine M2e, and avian M2e peptides after prime or prime boost vaccination with rgH3N2 4xM2e

Mouse sera were collected 2 weeks after intranasal prime or prime boost inoculation of rgH3N2 4xM2e. Total IgG antibody specific for human M2e (hM2e), avian M2e (aM2e) and swine M2e (sM2e) were measured from (a) prime and (b) boost sera. Error bars show mean \pm SEM. The statistical significances between hM2e detection versus sM2e and aM2e were determined using two-way ANOVA and indicated in **, $P < 0.01$; ***, $P < 0.001$.

2.4.4 Prime dose of live rgH3N2 4xM2e vaccination provides broad cross-protection

Intranasally prime vaccinated mice with 10^5 EID₅₀ of rgH3N2 or rgH3N2 4xM2e were challenged with different subtype influenza viruses including H1N1 (A/PR8), rgPR8 with avian M2 (rgPR8 Mm, rgPR8 Mg), H3N2 (A/Phil), rgH5N1 (A/Viet), rgH7N9 (A/Shanghai), and rgH9N2 (A/Hong Kong). The rgH3N2 4xM2e group showed higher survival rates (100%) and a trend of less weight loss against A/PR8 challenge, compared to the rgH3N2 group with 60% survival rates (Figs. 6A, E). Next, we tested PR8 reassortants containing avian M2 (rgPR8 Mm, rgPR8 Mg, Figs. 7A, B, D, E) as a challenge virus, following single-dose vaccination with rgH3N2 or rgH3N2 4xM2e. The rgH3N2 4xM2e group tends to show less weight loss against rgPR8 Mm (M gene from A/Mandarin duck/Korea/PSC24-24/2010, H5N1) or rgPR8 Mg (M gene from A/Chicken/Korea/Gimje/2008, H5N1), compared to those in the rgH3N2 control group. When challenged with a wild type 2009 H1N1 pandemic A/California/04/09 virus containing swine M2, similar trend was observed. The

rgH3N2 4xM2e primed mice showed a pattern of less weight loss and quicker recovery than the rgH3N2 group (Figure 8). However, the difference between these two groups was not statistically significant.

To test the breadth of cross-protection, different subtypes of PR8 reassortants were used as a challenge virus. The rgH3N2 4xM2e primed mice exhibited significantly less weight loss (~5%) than rgH3N2 primed mice (~16%) at day 5 after challenge with rgH5N1 (Figs. 6B, F). Similarly, when challenged with rgH7N9 virus, the rgH3N2 4xM2e group displayed average weight loss 6% compared to 19% weight loss in rgH3N2 prime mice and the mock control with over 20% weight loss and 0% survival rates (Figs. 6C, G). After a high lethal dose (100 LD₅₀) challenge with rgH9N2 virus, rgH3N2 4xM2e primed mice showed 75% survival protection whereas rgH3N2 immune and mock control mice all died of infection (Figs. 7C, F). When challenged with rgH9N2 at 20 LD₅₀ dose, rgH3N2 4xM2e primed mice showed better protection as evidenced by significantly preventing weight loss (average 11% day 6) and enhancing survival rates than rgH3N2 immune mice displaying 25% weight loss (day 6) and 25% survival rates (Figs. 6D, H).

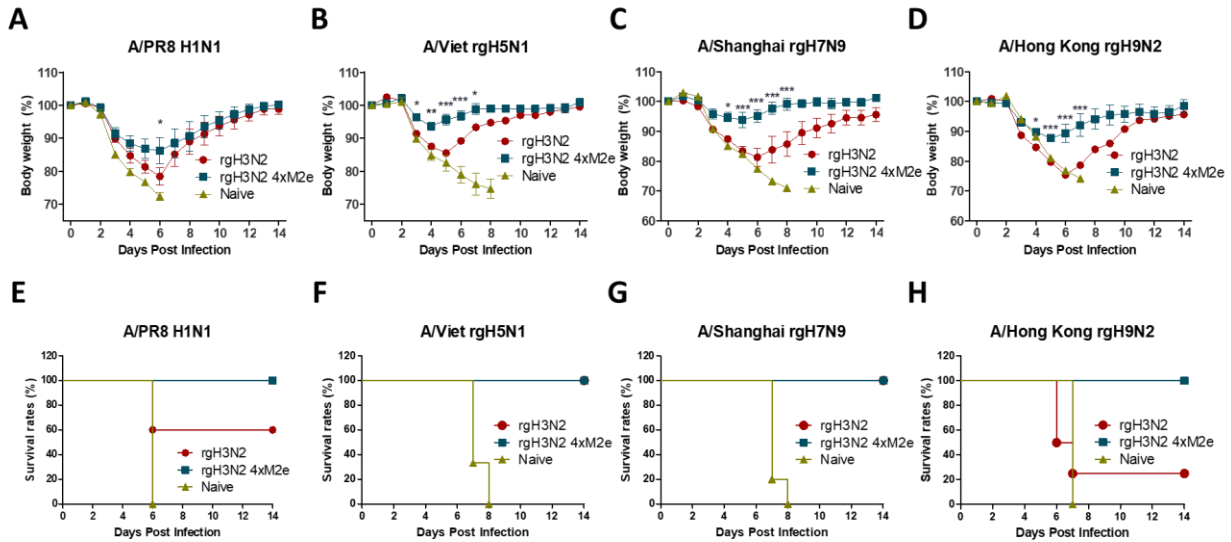


Figure 6 A single dose of live rgH3N2 4xM2e virus provides enhanced heterosubtypic cross-protection against different subtype influenza A viruses

Weight changes and survival rates in primed mice after challenge with (A, E) H1N1 A/PR8 (3 LD₅₀), (B, F) rgH5N1 A/Vietnam (5 LD₅₀), (C, G) rgH7N9 virus (reassortant A/Shanghai, 6 LD₅₀) and (D, H) rgH9N2 virus (reassortant A/Hong Kong, 20 LD₅₀). Differential challenge doses were used depending on the pathogenicity and HA phylogenetic distance of virus. Groups of mice (n=5 or 6) were intranasally primed with rgH3N2 (10⁵ EID₅₀) or rgH3N2 4xM2e (10⁵ EID₅₀) and then challenged 3 weeks later. Two independent repeat of mouse challenge experiments confirms the reproducibility of data. rgH3N2: 6:2 reassortant with WT HA and NA of A/Switzerland/2013. rgH3N2 4xM2e: 6:2 reassortant with 4xM2e-HA and NA of A/Switzerland/2013. Each individual mouse animal is analyzed. Error bars show mean ± SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using two-way ANOVA and indicated in *, P < 0.05; **, P < 0.01; ***, P < 0.001.

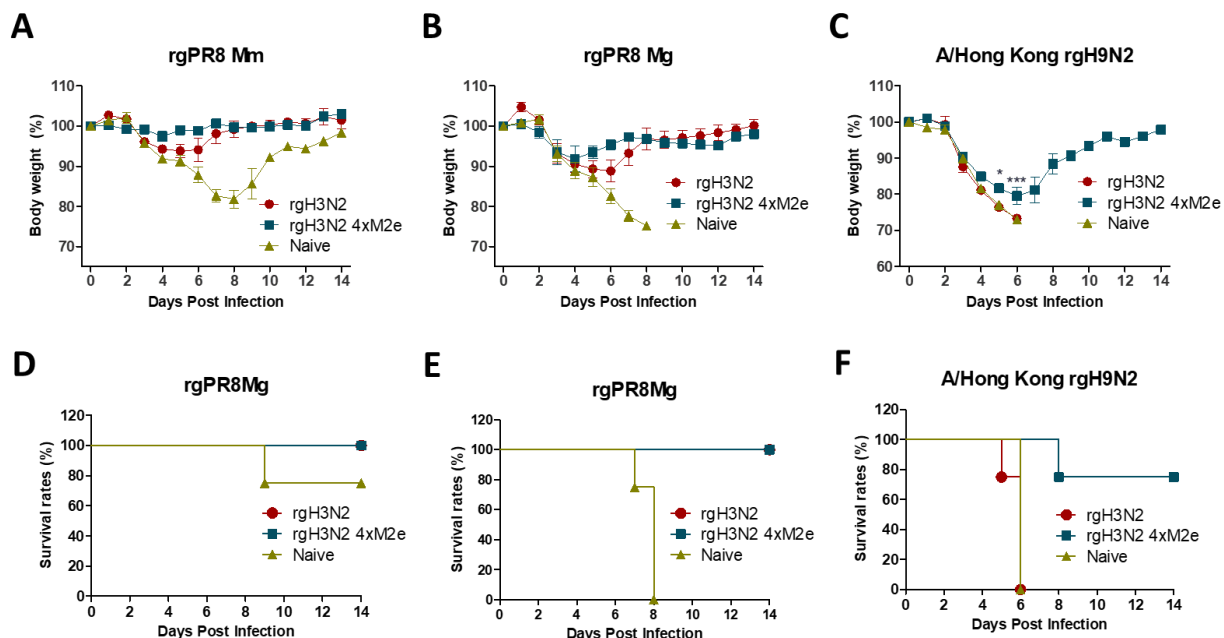


Figure 7 A single dose of live rgH3N2 4xM2e virus provides enhanced heterosubtypic cross-protection against A/PR8 (H1N1) reassortants containing avian M gene or rgH9N2 virus
 (A-C) Weight changes and (D-F) survival rates after challenge with rgPR8 Mm (M gene from A/Mandarin duck/Korea/PSC24-242010, H5N1, 1 LD₅₀), rgPR8 Mg (M gene from A/Chicken/Korea/Gimje/2008, H5N1, 3 LD₅₀) and high dose of rgH9N2 virus (reassortant A/Hong Kong, 100 LD₅₀), respectively. Groups of mice (n=3 or 4) were intranasally primed with rgH3N2 (10⁵ EID₅₀) or rgH3N2 4xM2e (10⁵ EID₅₀) and then challenged 3 weeks later. Error bars show mean ± SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using two-way ANOVA and indicated in *, P < 0.05; **, P < 0.01; ***, P < 0.001.

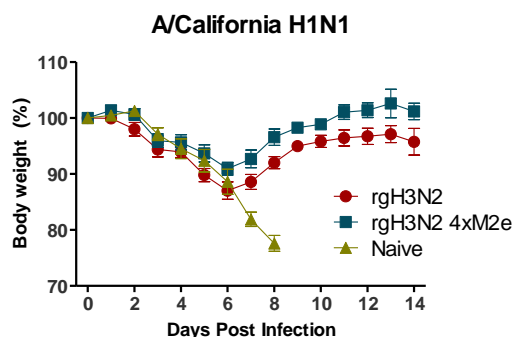


Figure 8 Body weight changes in BALB/c mice after challenge with A/California virus containing swine M2

The groups of BALB/c mice were infected with A/California/04/09 (6 LD₅₀) containing swine M2 at 3 weeks after single dose intranasal immunization with rgH3N2 or rgH3N2 4xM2e (10⁵ EID₅₀ each).

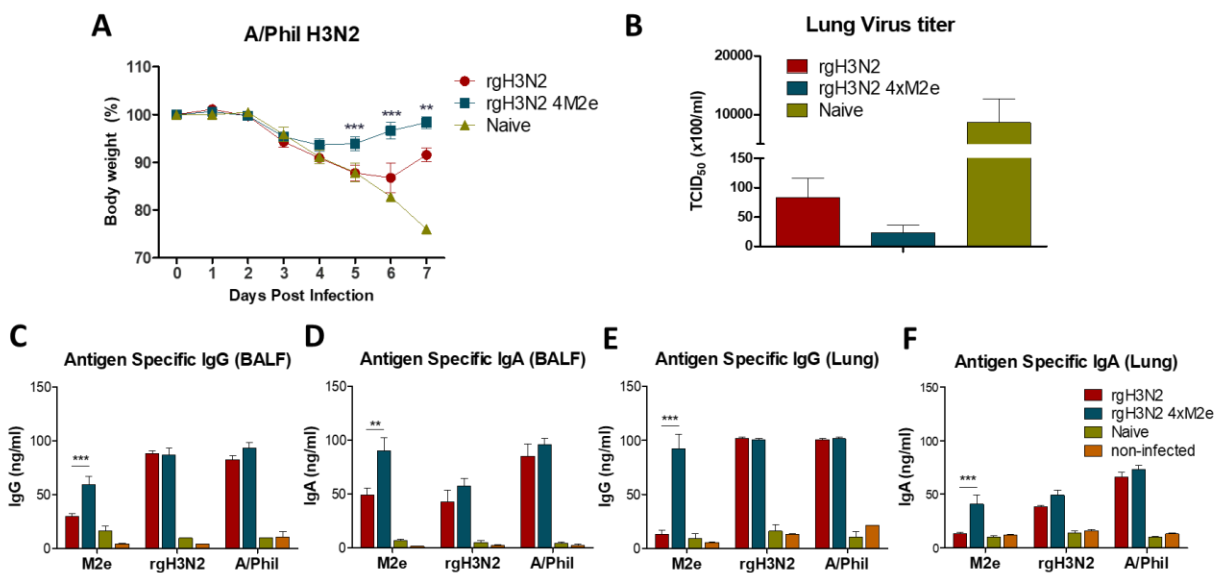


Figure 9 Prime dose rgH3N2 4xM2e provides enhanced protection against heterologous H3N2 A/Phil virus and M2e specific IgG and IgA antibodies in mucosal sites

The mice primed with rgH3N2 or rgH3N2 4xM2e were challenged with heterologous A/Phil H3N2 virus (50 LD₅₀). A relatively high challenge dose was used because of the same subtype virus. (A) Body weight changes after A/Phil challenge. Two independent repeat of mouse challenge experiments confirms the reproducibility of data. (B) Lung viral titers (TCID₅₀ x100/ml) at 5-day post challenge with A/Phil. (C-F) IgG and IgA antibody responses specific for M2e or viral (rgH3N2, A/Phil) antigens in BALF and lung extracts at 5 days after challenge. ELISA viral antigens are rgH3N2 and A/Phil. Error bars indicate mean value \pm SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using two-way ANOVA (a, c-e) or one-way ANOVA (b) and indicated in *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

After challenge with A/Phil (H3N2), 3% weight loss was observed with rgH3N2 4xM2e primed mice in contrast to 12% weight loss was observed with rgH3N2 primed mice (Figure 9A). Collectively, these results suggest that single dose intranasal priming of mice with rgH3N2 4xM2e could provide enhanced cross-protection against diverse subtypes of influenza A viruses containing human or avian M2.

2.4.5 Priming with rgH3N2 4xM2e virus results in enhanced responses of M2e specific IgG and IgA antibodies in mucosal respiratory and systemic sites upon challenge

Consistently, the mice that received a single dose (10^5 EID₅₀) of rgH3N2 4xM2e showed more effective cross-protection against A/Phil H3N2 virus as evidenced by less weight loss compared to the mice with rgH3N2 (Figure 9A). At day 5 post infection with A/Phil, naïve mice showed the highest levels of lung virus titers ($\sim 10^6$ TCID₅₀) as determined in MDCK cells (Figure 9B). Approximately 100 folds lower lung viral titers (8×10^3 TCID₅₀) were observed in the rgH3N2 group than those in naïve mice, which are approximately 3.5-fold higher titers than those (2.3×10^3 TCID₅₀) in the rgH3N2 4xM2e group (Figure 9B). There was no statistical significance in lung viral titers between the rgH3N2 and rgH3N2 4xM2e group (Figure 9B) and a similar pattern of viral titers was detected when determined by EID₅₀ in egg substrates.

Significantly higher levels of M2e specific IgG (Figs. 9C, D) and IgA (Figs. 9D, F) antibodies were induced in BALF (Figs. 9C, D) and lung extracts (Figs. 9E, F) from the rgH3N2 4xM2e group compared to the rgH3N2 group at 5 days after infection with A/Phil. Meanwhile virus (rgH3N2, A/Phil) specific IgG and IgA antibodies were similarly induced in BALF and lung extracts at high levels in the both rgH3N2 4xM2e and rgH3N2 groups (Figs. 9C-F).

We determined recall immune responses by measuring M2e and virus specific IgG at day 5 or 7 post challenge with heterologous A/Phil virus (Figure 10). The rgH3N2 4xM2e primed mice showed significantly increased M2e specific IgG levels in sera (3-fold in OD values) (Figure 10A) and draining lymph nodes MLN after challenge (Figure 10D) while the other groups did not. The increased IgG levels specific for vaccine rgH3N2 (Figure 10B) and challenge virus A/Phil (Figure 10C) were similarly observed in post-challenge sera from both groups. Intracellular cytokine

staining results indicate the induction of M2e-stimulated CD4 T cells secreting IFN- γ at high frequencies (Figs. 10E, F) and relatively low numbers of IFN- γ ⁺ CD8 T cells (Figure 11) in BALF and lung cells from the rgH3N2 4xM2e primed mice. These results suggest that the levels of M2e specific antibodies and T cell immunity induced by priming rgH3N2 4xM2e in mucosal (BALF, lungs) and systemic (sera, MLN) sites might have contributed to cross-protection.

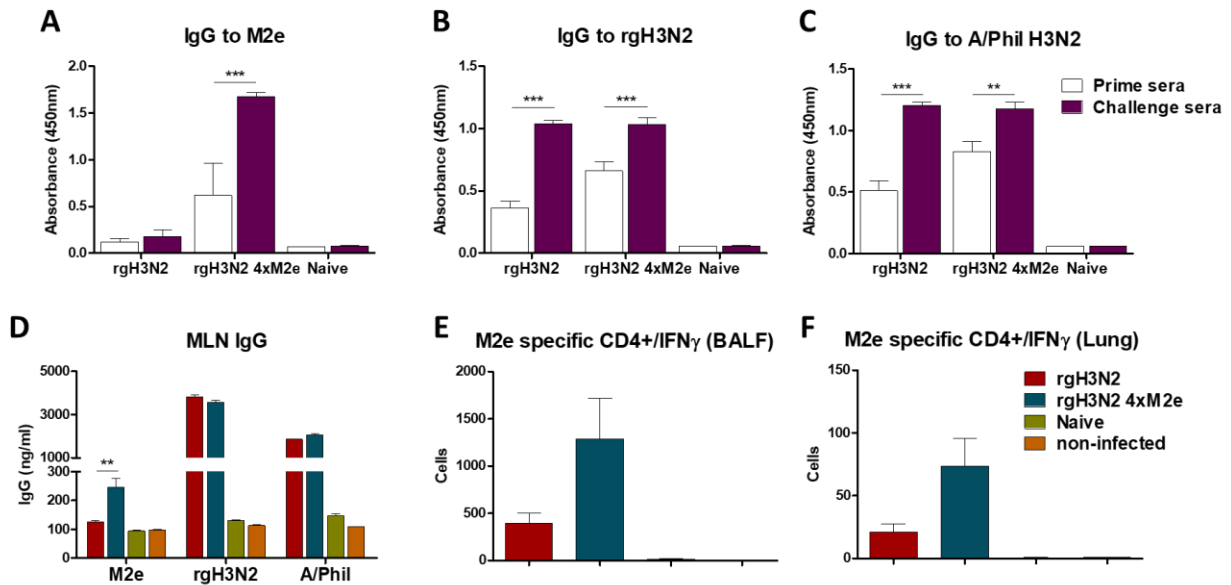


Figure 10 Single inoculation of rgH3N2 4xM2e virus effectively primes M2e and virus specific immune responses

Sera after rgH3N2 or rgH3N2 4xM2e immunization (day 14) and challenge with A/Phil H3N2 virus (50 LD₅₀, day7) were collected respectively to determine IgG antibody response specific for (A) M2e (1:100 sera), (B) rgH3N2 (1:10,000 sera), and (C) A/Phil (H3N2) (1:10,000 sera) antigens. (D) In vitro production of M2e, rgH3N2, and A/Phil specific IgG antibodies in mediastinal lymph node (MLN) cell cultures. (E-F) Flow cytometry of intracellular cytokine staining for detection of IFN- γ secreting CD4⁺ T cells specific for M2e from BALF (E) and lung cells (F) at day 5 (D-F) or 7 (A-C) following A/Phil challenge. Error bars indicate mean \pm SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using two-way ANOVA and indicated in *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

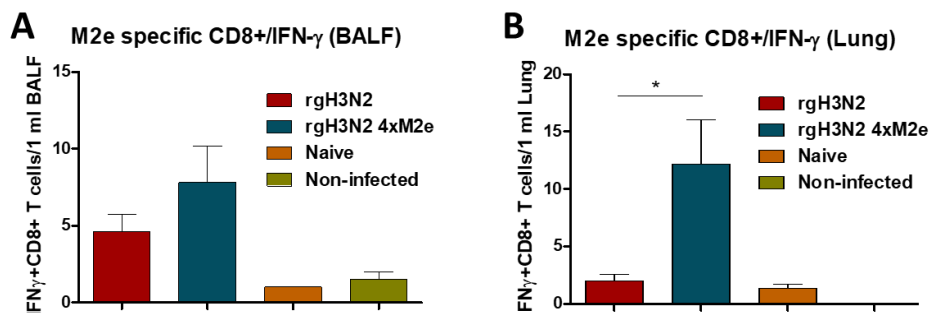


Figure 11 Measurement of M2e specific CD8⁺ cells secreting IFN-γ by flow cytometry

BALB/c mice were intranasally inoculated with rgH3N2 or rgH3N2 4xM2e (10^5 EID₅₀ each). IFN-γ secreting CD8⁺ cells specific for M2e were detected 5 days after A/Phil challenge (50 LD₅₀) in BALF (A) and lung cells (B) from immunized mice. IFN-γ⁺ CD4⁺ T cells were presented from total BALF (1 ml) and Lung (1 ml) cells from individual mouse. Error bars indicate mean ± SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using one-way ANOVA and indicated in *, $P < 0.05$.

2.4.6 Depletion of CD4 and CD8 T cells results in differential cross-protection between rgH3N2 4xM2e and rgH3N2 prime vaccination

We observed substantial cross-protection by priming rgH3N2 virus even without the induction of M2e specific antibodies and cross HAI activities, suggesting the roles of cross-protective T cells. Also, to determine whether M2e specific antibodies would significantly contribute to cross-protection, rgH3N2 or rgH3N2 4xM2e primed mice were treated with T cell depleting anti-CD4 or anti-CD8 antibodies prior to challenge with 25 LD₅₀ of A/Phil (H3N2). As shown in Figure 12, T cells in the groups immunized with rgH3N2 or rgH3N2 4xM2e were effectively depleted by delivering either anti-CD4 or anti-CD8 or both antibodies prior to infection with A/Phil (H3N2, 17 LD₅₀). Anti-CD4 (Figs. 13A, C) or anti-CD8 (Figs. 13B, D) antibody treatment resulted in significant weight loss (24% or 16% respectively) in the rgH3N2 group, lowering the survival rate (50% with anti-CD4, Figure 13C), compared to moderate weight loss (9.5 to 13%) in the rgH3N2 4xM2e group. When both CD4 and CD8 were depleted, the rgH3N2 group displayed significant weight loss and all mice reaching the humane endpoint (Figs. 14A, E). In contrast, 100%

mice from the rgH3N2 4xM2e group survived with 8% body weight loss (Figs. 14A, E). These results suggest that M2e antibodies contribute to cross-protection by a prime dose of rgH3N2 4xM2e regardless of T cells at the time of challenge whereas CD4 and CD8 T cells particularly CD4 T cells might play a significant role in cross-protection by rgH3N2.

We extended the impact of both CD4 and CD8 depletion on cross-protection against A/PR8 (H1N1) and PR8 reassortants with avian M2 (Figure 14). Treatment of rgH3N2 4xM2e primed mice with CD4 and CD8 depleting antibodies resulted in 100% survival rates and approximately 13% weight loss after A/PR8 (H1N1) challenge whereas rgH3N2 primed mice showed over 20% weight loss and 0% survival rates (Figs. 14B, F). With CD4 and CD8 T cell depletion, the significant differences in weight changes and survival rates between the groups were observed after rgPR8 Mg challenge (Figs. 14C, G). While rgH3N2 4xM2e mice lost 9% of body weight with 100% survival rate, rgH3N2 mice had 20% weight loss with 20% survival rates. In consistent, the rgH3N2 4xM2e group showed significantly enhanced survival protection with less weight loss against a high lethal dose (20 LD₅₀) of rgPR8 Ms virus (M gene from A/Shanghai/2/2013, H7N9) compared to the rgH3N2 group with 0% survival (Figs. 14D, H). The weight recovery was delayed in surviving mice with CD4 and CD8 T cell depletion after challenge, suggesting an important role of T cells in recovery or preventing severe viral pathology. These results support the differential roles of CD4 and CD8 T cells and M2e specific IgG antibodies in conferring cross-protection by rgH3N2 and rgH3N2 4xM2e prime immunization.

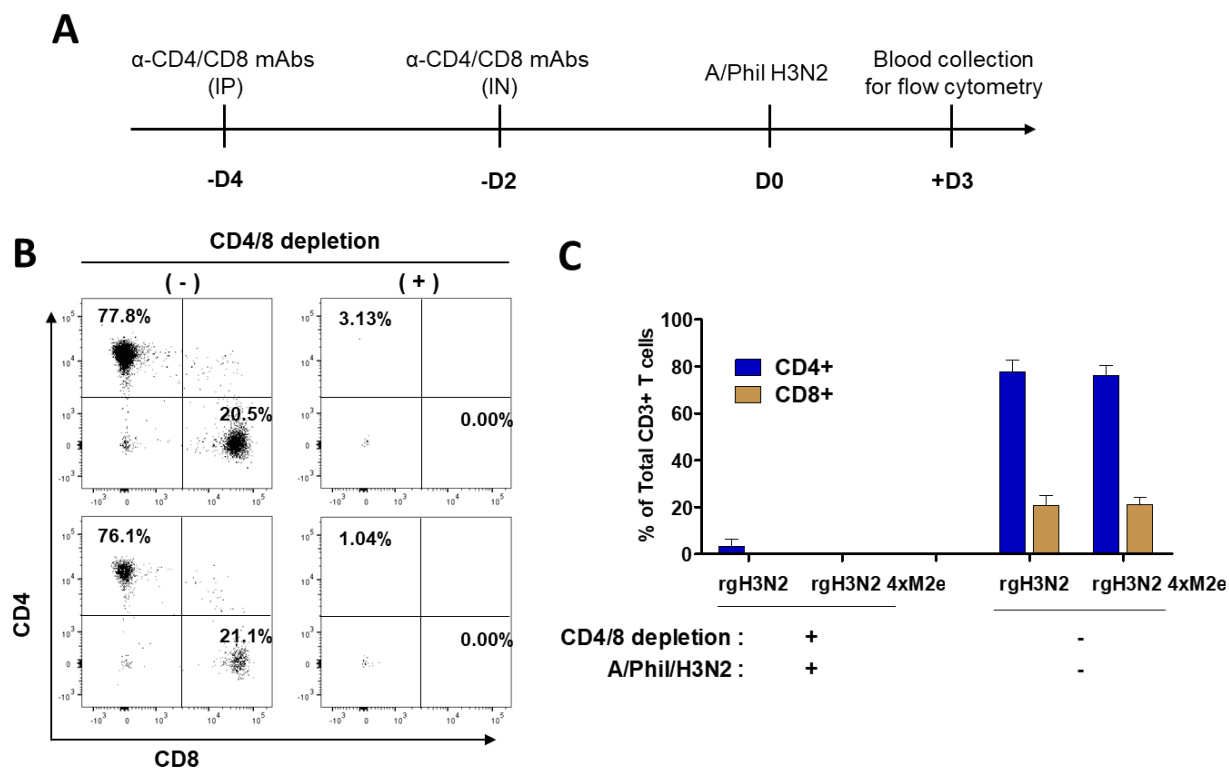


Figure 12 The efficacy of CD4 and CD8 T cell depletion in vaccinated mice after CD4 and CD8 T cell depleting antibody treatment

(A) Schematic diagram for CD4 and CD8 T cell depleting antibody treatment schedule. (B) Flow cytometry profiles of CD4⁺ and CD8⁺ T cells out of total CD3⁺ T cells in peripheral blood mononucleocytes (PBMC). (C) Percentages of CD4⁺ T cells out of CD3⁺ cells, and CD8⁺ T cells out of total CD3⁺ cells in PBMC. rgH3N2: the group of mice vaccinated with rgH3N2 (n=3). rgH3N2 4xM2e: the group of mice vaccinated with rgH3N2 4xM2e (n=3).

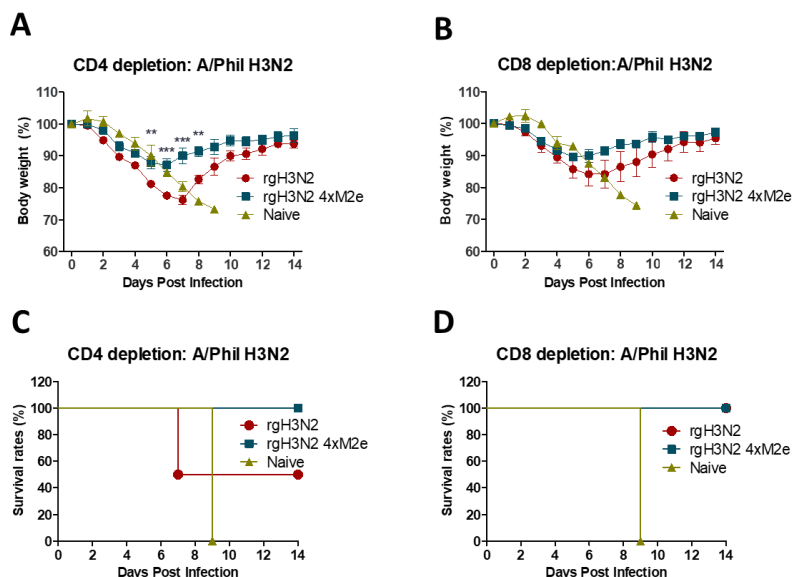


Figure 13 The roles of CD4⁺ and CD8⁺ T cells in cross-protection by reassortant rgH3N2 4xM2e prime inoculation

The rgH3N2 or rgH3N2 4xM2e primed mice (n=4 /group) with were treated with (A, C) α -CD4 or (B, D) α -CD8 antibodies for T cell depletion prior to A/Phil (H3N2) challenge (25 LD₅₀). Weight changes and survival rates were monitored after A/Phil challenge. Error bars indicate mean value \pm SEM. The statistical significances between rgH3N2 group and rgH3N2 4xM2e group were determined using two-way ANOVA and indicated in *, P < 0.05; **, P < 0.01; ***, P < 0.001.

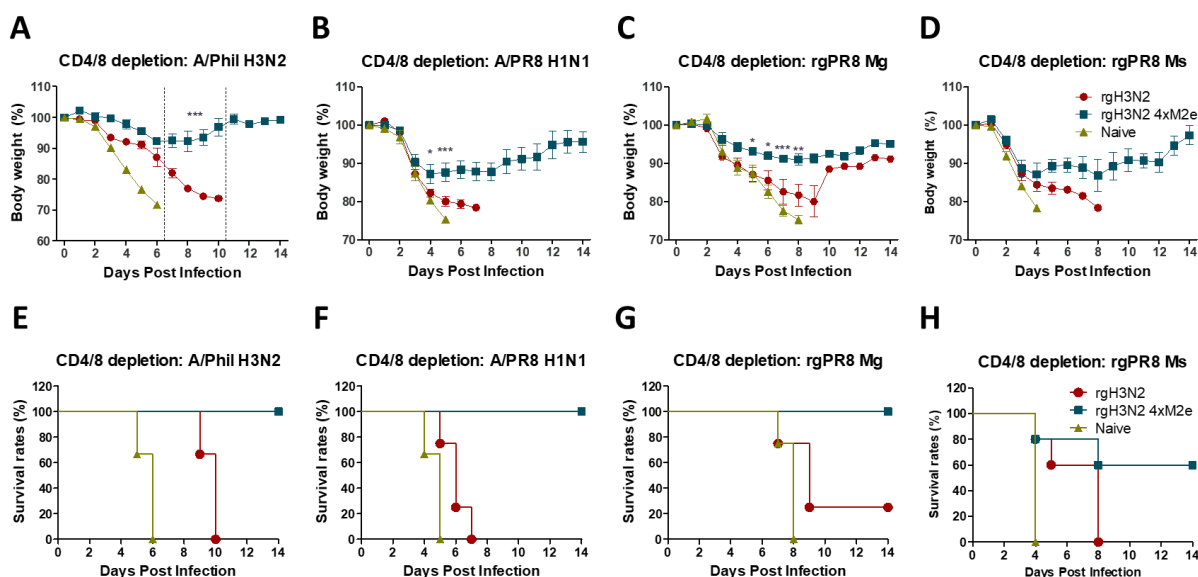


Figure 14 Depletion of CD4 and CD8 T cells results in differential cross-protection between rgH3N2 and rgH3N2 4xM2e primed mice

The rgH3N2 or rgH3N2 4xM2e primed mice (n=4 or 5/group) were treated with α -CD4/ α -CD8 antibodies for T cell depletion prior to influenza virus infection. (A-D) Weight changes and (E-H) survival rates were

monitored followed by A/Phil (H3N2) (17 LD₅₀), A/PR8 (1.5 LD₅₀), rgPR8 Mg (3 LD₅₀) and rgPR8 Ms (10 LD₅₀) challenge, respectively. Differential challenge doses were used depending on the subtype, pathogenicity, and T cell-depletion. Error bars indicate mean \pm SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using two-way ANOVA and indicated in *, $P < 0.05$; ***, $P < 0.001$.

2.4.7 Boost dose of rgH3N2 4xM2e further enhances M2e antibodies

Boost inoculation (10^6 EID₅₀ rgH3N2 4xM2e) induced significantly higher levels of M2e specific IgG1 and IgG2a isotype antibodies (Figs. 15A, B) than post prime IgG antibody levels (Figs. 4A, B). Consistent with post prime, hM2e IgG antibody levels post boost with rgH3N2 4xM2e were significantly higher than those of swine M2e or avian M2e antibodies (Figure 5B). Both rgH3N2 and rgH3N2 4xM2e groups increased IgG isotype antibodies and HAI activity against vaccine strain rgH3N2 but not against heterologous viruses including A/PR8, A/Phil, and rgH5N1 (Figs. 16A-C) compared to prime sera.

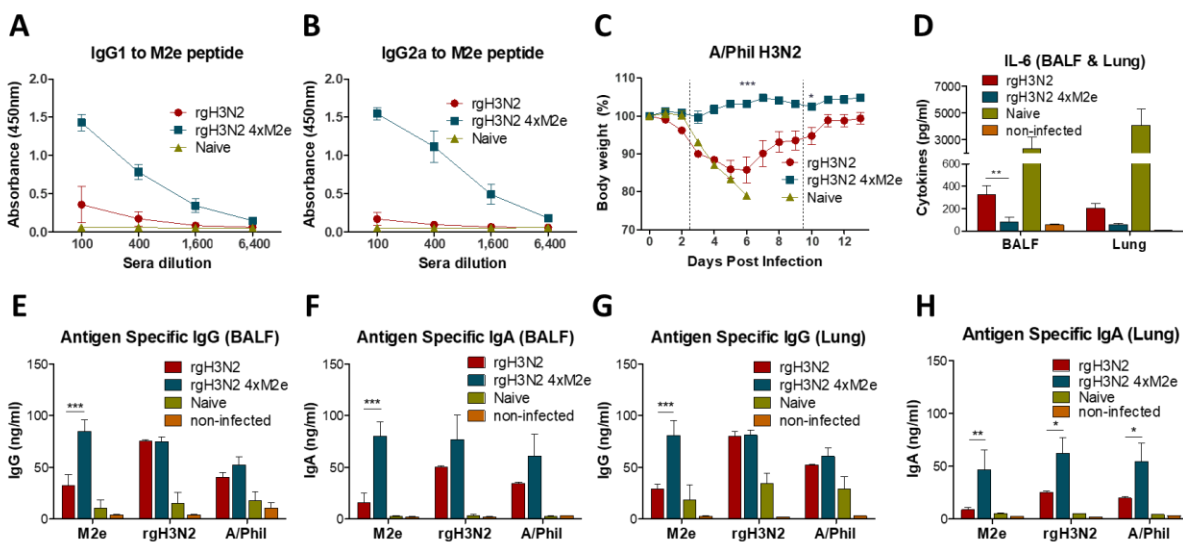


Figure 15 Boost dose of rgH3N2 4xM2e enhances cross-protection against A/Phil (H3N2) challenge, correlating with M2e specific IgG levels and lower inflammatory cytokine

Sera were collected at 2 weeks after boost immunization of primed mice with rgH3N2 or rgH3N2 4xM2e. (A) IgG1 or (B) IgG2a antibody responses to M2e peptide. The rgH3N2 or rgH3N2 4xM2e boosted mice ($n=4$ /group) were treated with α -CD4/ α -CD8 ($10\mu\text{g}/10\mu\text{g}$ per individual) antibodies for T cell depletion prior to A/Phil (H3N2) challenge. (C) Weight changes of rgH3N2 or rgH3N2 4xM2e prime-boost mice after challenge with A/Phil (200 LD₅₀). (D) IL-6 levels in BALF or lung extracts at day 5 post challenge with A/Phil. ELISA of IgG or IgA antibodies specific for M2e peptide and inactivated virus antigens (rgH3N2 or A/Phil)

in (E, F) BALF and (G, H) lung extracts at day 5 post challenge with A/Phil. Error bars indicate mean \pm SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using two-way ANOVA and indicated in *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

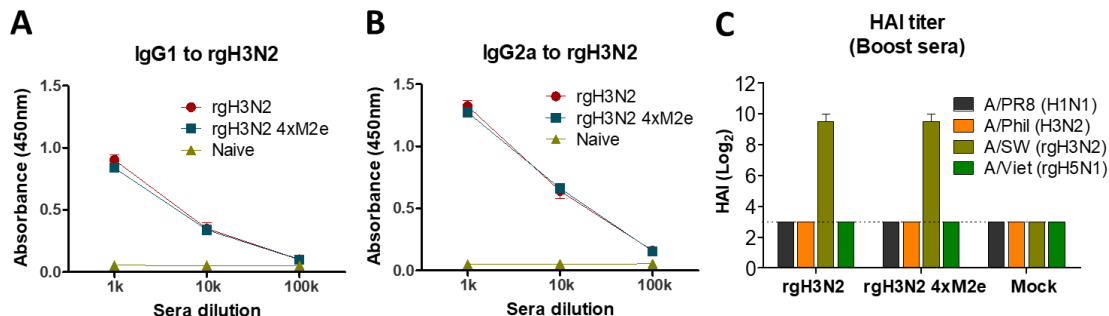


Figure 16 Intranasal boost vaccination with live rgH3N2 4xM2e virus further enhances rgH3N2 virus specific IgG and HAI antibodies

Sera were collected at 2 weeks after boost immunization of primed mice with rgH3N2 or rgH3N2 4xM2e. (A) IgG1 or (B) IgG2a antibody detection specific for rgH3N2. (C) Boost serum HAI titers against A/PR8, A/Phil, rgH3N2 and A/Viet. Error bars indicate mean \pm SEM.

2.4.8 Boost dose of rgH3N2 4xM2e enhances cross-protection and correlates with higher M2e specific IgG levels and lower inflammatory cytokines in the respiratory sites

At 3 weeks after boost dose, rgH3N2 and rgH3N2 4xM2e immunized mice were treated with T cell depleting anti-CD4 and anti-CD8 antibodies and then challenged with A/Phil H3N2 at a high dose. The rgH3N2 4xM2e group showed 100% protection without weight loss whereas the rgH3N2 group displayed substantial weight loss (15%) after A/Phil challenge (Figure 15C). Consistent with efficacy of cross-protection, inflammatory cytokine IL-6 levels were low in BALF and lung extracts from the rgH3N2 4xM2e group (Figure 15D). Significantly higher levels of M2e specific IgG and IgA antibodies in BALF and lung extracts were induced in the rgH3N2 4xM2e group than those in the rgH3N2 group after A/Phil challenge (Figs. 15E-H). Also, the levels of IgA antibodies specific for rgH3N2 and A/Phil viruses were higher in lung extracts from the rgH3N2 4xM2e group than those from the rgH3N2 group (Figure 15H).

Consistently, boost dose of rgH3N2 4xM2e also enhanced cross-protection against heterosubtypic rgH5N1 virus, as shown by less weight loss (~4% versus 13% in rgH3N2 control) and undetectable lung viral loads (Figs. 17A, B). Particularly IgG levels specific for M2e were induced at significantly higher levels in BALF and lung extracts from the rgH3N2 4xM2e group than those in the rgH3N2 group or naïve group (Figure 17C). IgG levels to vaccine (rgH3N2) and challenge virus (rgH5N1) were similar in both groups. As expected, the rgH3N2 and rgH3N2 4xM2e groups presented significantly lower amounts of TNF- α , IFN- γ and IL-6 than the naïve group after infection with rgH5N1 (Figure 17D). Moreover, rgH3N2 4xM2e vaccine group exhibited lower levels of proinflammatory cytokines (IFN- γ) compared to the rgH3N2 group.

To determine the role and capability of immune sera in inducing cross-protection against heterosubtypic rgH5N1 virus, naïve mice were intranasally infected with A/Vietnam virus (2 LD₅₀) after mixing with boost immune sera from the rgH3N2 and rgH3N2 4xM2e group, and naïve sera respectively (Figure 17E). The group with rgH3N2 4xM2e immune sera displayed significantly less weight loss (~3%) compared to the rgH3N2 serum group with substantial weight loss (~15%) similar to the naïve serum group (Figure 17E). At day 8 post challenge with rgH5N1 virus, the rgH3N2 group started to recover (Figure 17E). Taken together, these results indicate that M2e specific antibodies in mucosal and systemic sites provide cross-protection by restricting viral replication and preventing inflammation.

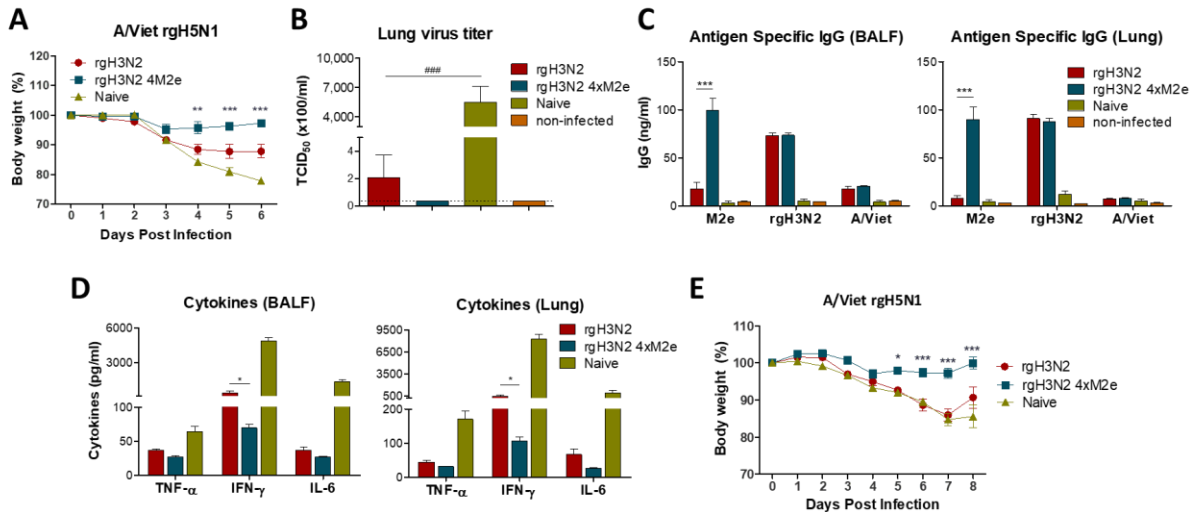


Figure 17 Boost with rgH3N2 4xM2e enhances cross-protection against heterosubtypic A/Viet rgH5N1 virus by vaccination and immune sera

(A, B) Heterosubtypic cross-protective efficacy of boost immune mice after challenge with rgH5N1 (A/Vietnam, 50 LD₅₀). (A) Weight changes of boost immunized mice (n=5 /group) after rgH5N1 challenge. (B) Lung viral titers (TCID₅₀ x100/ml) at 6-day post challenge with rgH5N1. (C) ELISA of IgG antibodies specific for M2e, rgH3N2, or rgH5N1 in BALF and lung extracts at day 6 post challenge with A/Viet rgH5N1. ELISA viral antigens are M2e peptide, rgH3N2 or A/Viet. (D) Inflammatory cytokines in BALF and lung extracts at day 6 post challenge with A/Viet rgH5N1. (E) Roles of boost immune sera in cross-protection against rgH5N1 virus as determined by monitoring weight changes. Naïve mice (n=3 /group) were intranasally inoculated with rgH5N1 (2 LD₅₀) virus mixed with boost immune sera (rgH3N2, rgH3N2 4xM2e) or unvaccinated naïve sera. Error bars indicate mean ± SEM. The statistical significances between rgH3N2 group versus rgH3N2 4xM2e group were determined using two-way ANOVA (A, C-E) or one-way ANOVA (B) and indicated in *, P < 0.05; **, P < 0.01; ***, P < 0.001; ###, P < 0.0005.

2.5 Discussion

The induction of strain specific neutralizing antibodies is the main immunity by current influenza vaccination, which is suboptimal for providing cross-protection. M2 is incorporated into influenza virions at a very low level (Zebedee and Lamb, 1988) and poorly immunogenic after vaccination or even with live virus infection (Zhong et al., 2014, Feng et al., 2006). Several strategies were reported in an attempt to overcome the poor immunogenicity of M2e, including fusion of M2e peptides to immunogenic carrier proteins and use of adjuvants (Neirynck et al., 1999, Fan et al., 2004, De Filette et al., 2005) or delivery of VLPs containing M2e epitopes (Bessa

et al., 2008, Hashemi et al., 2012, Kim et al., 2013b). M2e fusion protein vaccines (M2e-HBc, M2e-flagellins) were tested in Phase I/II clinical trials, resulting in high seroconversion inducing M2e specific antibody responses (Mezhenskaya et al., 2019, Deng et al., 2015). Intramuscular vaccination with M2e fusion protein vaccines was well tolerated except for the high doses (3 µg or 10 µg) of M2e-flagellin conjugates in healthy individuals (Turley et al., 2011). However, there has been no further clinical advancement of M2e based vaccines probably due to low efficacy of M2e immunity alone (Subbarao and Matsuoka, 2013, Jegerlehner et al., 2004, Song et al., 2011). One drawback might be short-lived M2e antibodies. Another disadvantage is the non-neutralizing nature of M2e immunity conferring low efficacy. An approach to overcome these drawbacks of M2e fusion protein vaccines would be to develop chimeric influenza virus vaccines where multi M2e epitopes are fused to the HA molecules in a way retaining HA functional and immunogenic integrity. It is expected that the tolerability and protective efficacy of chimeric influenza virus vaccines would be compatible with seasonal vaccines in addition to providing extra M2e immunity. In this approach, we generated recombinant seasonal H3N2 influenza A virus expressing chimeric H3 HA molecules with heterologous tandem repeat 4xM2e epitopes. This recombinant H3N2 virus with chimeric 4xM2e-HA was found to be highly attenuated, effective in inducing both M2e and HA immunity, conferring cross-protection against different viruses H1N1, reassortants with avian M2, H3N2, H5N1, H7N9, and H9N2 subtypes in mice with a single dose.

The comparable *in vitro* growth kinetics of rgH3N2 4xM2e viruses suggest the integrity of HA functions in recombinant H3N2 virus containing chimeric H3 4xM2e-HA. The pathogenicity of rgH3N2 reassortants (up to 10⁶ EID₅₀) with the PR8 backbone was highly attenuated in mice by

1,000 to 10,000 folds in the aspects of weight changes and viral replication (10^3 versus 10^8 EID₅₀ titers) in the lungs compared to the WT A/PR8 (H1N1) virus (10^4 EID₅₀). The attenuated rgH3N2 phenotypes might serve as an appropriate platform to test vaccine candidates in mice. Owing to the attenuated phenotypes, a single prime dose of rgH3N2 4xM2e within a typical range (10^5 – 10^6 EID₅₀) for LAIV vaccination (Kotomina et al., 2019, Isakova-Sivak et al., 2017) could mimic vaccination without displaying pathogenic phenotypes. Chimeric rgH3N2 4xM2e was not defective in inducing immunity to virus.

It is significant to observe substantial levels of IgG isotype antibodies specific for M2e after prime with recombinant seasonal rgH3N2 4xM2e virus compared to WT rgH3N2 virus. The rgH3N2 4xM2e primed mice were protected against H1N1 A/PR8 and rgH9N2 virus as shown by 100% survival rates and relatively quicker recovery than the rgH3N2 group with 0 to 30% survival rates. Also, prime dose of rgH3N2 4xM2e provided higher protection with minimum weight loss against lethal challenge with H3N2 A/Phil, rgH5N1, and rgH7N9 virus although rgH3N2 primed mice survived in the absence of inducing cross reactive HAI activity. The efficacy of cross-protection by priming with rgH3N2 4xM2e appears to be correlated with enhanced levels of M2e IgG antibodies in sera and mucosal (BALF, lungs) sites as well as IFN- γ secreting CD4 T cells (Figs. 10E, F).

The rgH3N2 control group showed substantial protection against H1N1, rgH5N1, rgH7N9, and rgH9N2 virus and A/Phil (H3N2) virus in the absence of cross-reactive HAI titers, lowering 100 folds in lung viral titers compared to mock control mice after infection. With CD4 or CD8 T cell depletion, the differences in cross-protection against H3N2 A/Phil and H1N1 A/PR8 viruses were more evident between the rgH3N2 and rgH3N2 4xM2e primed mice. Also, the survival rates

were lower particularly in the rgH3N2 group and recovery was delayed under a condition of both CD4 and CD8 T cell depletion even after low challenge doses with group 1 H1N1 virus A/PR8 and reassortants. These results support the significant roles of cross protective T cells particularly during a recovery phase. It is also possible that HA stalk specific antibodies induced in both chimeric 4xM2e-HA and WT rgH3N2 virus groups would contribute to cross-protection against group 2 viruses with relatively high doses. This cross-protection observed in the control rgH3N2 group, despite significantly lower efficacy than rgH3N2 4xM2e, indicates a caveat in testing vaccine efficacy of live recombinant influenza viruses in mouse models. Consistent, the mice surviving pathogenic influenza virus infection were reported to confer heterosubtypic cross-protection (Kim et al., 2017a, Guo et al., 2011, O'Neill et al., 2000).

The levels of M2e specific IgG antibodies were significantly increased post boost with live rgH3N2 4xM2e virus, indicating effective priming of B cells by the first dose. In line with this outcome, enhanced cross-protection against A/Phil H3N2 virus was observed in the rgH3N2 4xM2e group displaying no apparent weight loss after boost compared to the rgH3N2 control. Use of higher dose (50 LD₅₀) challenge is because of prime-boost vaccination and the same subtype as the rgH3N2 vaccine strain. When challenged with heterosubtypic rgH5N1 virus, the increases in M2e specific IgG responses in BALF and lungs but not IgG responses to rgH5N1 were higher than those with homosubtypic A/Phil H3N2 challenge. Consistently, BALF and lungs showed highly enhanced levels of IgG and IgA antibodies specific for M2e, compared to the control group.

A recent study reported that inactivated chimeric influenza viruses containing an M2e epitope in the immunodominant head site of HA could induce IgG antibodies to M2e and stalk

domains after intramuscular vaccination, conferring cross-protection (Sun et al., 2019, Kim et al., 2020). The size of foreign epitopes or fragments to be inserted into the N-terminus of HA appears to be highly flexible as large as 246 residues while maintaining HA functional (Hatzioannou et al., 1999). Whereas recombinant influenza viruses containing the only a limited length of foreign epitopes less than 18 residues in the HA head domain could be rescued to generate replication-competent viruses (Lee et al., 2016a, Garcia-Sastre and Palese, 1995). The location of inserting foreign epitopes in HA molecules should be considered in the size of epitopes and routes of vaccination.

The non-neutralizing immune mechanisms of protection by M2e antibodies were reviewed (Deng et al., 2015, Mezhenskaya et al., 2019, Lee et al., 2015a) in addition to M2e T cell contributions. Previous studies reported that M2e vaccine immune sera did not exhibit neutralization activity by plaque reduction or tissue culture infectivity assays (Deng et al., 2018, Jegerlehner et al., 2004). Mechanisms of protection by passive transfer of M2e antibodies include antibody dependent cell-mediated cytotoxicity and antibody dependent cell-mediated phagocytosis, which involves Fc receptors, complements, natural killer (NK) cells, and macrophages. Passive transfer of M2e vaccine immune sera prior to virus infection provides survival advantages (Wang et al., 2014). The infection of naïve mice with a mix of challenge virus and sera required smaller amounts of sera than the passive transfer of sera prior to infection. Our current and previous studies indicate that both simultaneous mix and prior-to-infection passive transfer approaches produced similar outcomes. We previously reported that the efficacy of M2e immune sera was significantly reduced or abrogated in Fcγ receptor knockout mice, suggesting a critical role of Fcγ receptors in mediating M2e antibody mediated protection (Kim

et al., 2013a, Kim et al., 2017b, Lee et al., 2014a). M2e antibody-dependent NK cell activity was reported to be important for M2e immune mediated protection (Jegerlehner et al., 2004).

In summary, recombinant seasonal influenza rgH3N2 4xM2e virus containing tandem repeat 4xM2e epitopes in the N-terminus of HA molecules retains comparable growth properties *in vitro* and LAIV-like attenuation phenotypes *in vivo* in mice. Intranasal prime vaccination with rgH3N2 4xM2e virus could provide broad and enhanced cross-protection against different subtypes H1N1, H3N2, rgH5N1, rgH7N9, and rgH9N2 as well as reassortant viruses with avian M2. *In vivo* limited replication of recombinant influenza virus containing foreign epitopes in the N-terminus HA appears to be an attractive strategy to induce systemic and mucosal immune responses to the inserted epitopes. This study provides insight into developing broad cross-protective recombinant influenza virus vaccines. This approach of recombinant influenza virus vaccine platforms with a licensed master backbone should be further tested in ferrets, a more relevant animal model in future studies.

2.6 Acknowledgement

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3 ENHANCED CROSS-PROTECTION BY HETERO PRIME-BOOST VACCINATION WITH RECOMBINANT INFLUENZA VIRUSES CONTAINING CHIMERIC HEMAGGLUTININ-M2e EPITOPES

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3.1 Abstract

Annual repeat influenza vaccination raises concerns about protective efficacy against mismatched viruses. We investigated the impact of heterologous prime-boost vaccination on inducing cross-protection by designing recombinant influenza viruses with chimeric hemagglutinin (HA) carrying M2 extracellular domains (M2e-HA). Heterologous prime-boost vaccination of C57BL/6 mice with M2e-HA chimeric virus more effectively induced M2e and HA stalk specific IgG antibodies correlating with cross protection than homologous prime-boost vaccination. Induction of M2e and HA stalk specific IgG antibodies was compromised in 1-year old mice, indicating significant aging effects on priming subdominant M2e and HA stalk IgG antibody responses. This study demonstrates that a heterologous prime-boost strategy with recombinant influenza virus expressing extra M2e epitopes provides more effective cross-protection than homologous vaccination.

3.2 Introduction

Influenza A virus belongs to the *Orthomyxoviridae* family, a negative-sense single-stranded RNA virus containing eight segmented genomes. It has a wide variety, originating from 18 hemagglutinin (HA) subtypes (H1-H18) and 11 neuraminidase (NA) subtypes (N1-N11), with antigenically diverse strains isolated in each subtype (Tong et al., 2013). Hundreds of millions of people are infected yearly with influenza viruses, which leads to 290,000 to 646,000 deaths

globally, with young children and the elderly being the most vulnerable (Iuliano et al., 2018, Lee et al., 2018, Thompson et al., 2003).

Due to the emergence of drifting mutations and pandemics, overall vaccine effectiveness is in a wide range of low efficacy between 10 % and 60 % (CDC, 2021e). Low vaccine effectiveness comes from multiple factors such as aging, health and pre-existing immune status, antigenic mismatches, and poor immunogenicity of vaccines. Vaccine strains are annually updated to reflect better the circulating influenza strains and vaccination is recommended every year. While annually repeated influenza vaccination was effective with variable efficacy (Casado et al., 2018, Mastalerz-Migas et al., 2015a, Beyer et al., 1999, Smith et al., 1999, de Bruijn et al., 1999, Keitel et al., 1997), several recent studies have indicated that repeated annual vaccination did not result in improving vaccine effectiveness particularly when circulating strains are mismatched (McLean et al., 2014, Leung et al., 2017, Morimoto and Takeishi, 2018, Song et al., 2020). It has been a high priority to enhance the vaccine efficacy and develop broadly cross-protective vaccines.

Influenza virus HA proteins consist of the immune-dominant but highly variable head domain, which provides a strain-specific neutralizing target, as well as the relatively conserved stalk domain, which mediates the viral membrane fusion (Krammer et al., 2018a). To overcome the immune-subdominant nature of HA stalk domains, influenza viruses were reverse-genetically engineered to contain chimeric HA where the variable head domain was replaced with a corresponding domain from the antigenically far distant strains without changing the stalk domain (Nachbagauer et al., 2021, Liao et al., 2020). Recombinant chimeric HA influenza virus vaccines were reported to induce high levels of stalk specific IgG responses leading to enhanced cross-protection in animal models (Krammer et al., 2013). In phase I clinical studies, AS03-

adjuvanted chimeric HA-based influenza virus vaccine induced durable IgG responses to the HA stalk domain (Nachbagauer et al., 2021, Bernstein et al., 2020). While these results provide a proof-of-concept for developing stalk-based cross-protective vaccines, they would not confer sufficient protection against currently circulating strains due to their antigenically unrelated HA head domain.

Influenza A virus contains ion channel protein M2 extracellular epitopes (M2e) which are highly conserved but poorly immunogenic, despite being a promising universal antigenic target (Saelens, 2019). To induce immunity to both M2e and circulating HA, replication-competent influenza viruses were genetically modified to retain and express chimeric HA molecules with tandem repeat 4xM2e in the N-terminus HA (4xM2e-HA) from H1N1 (Kim et al., 2017a), H3N2 (Park et al., 2021), and H7N9 virus (Mezhenskaya et al., 2021a). The live recombinant 4xM2e-HA influenza virus vaccines were immunogenic in inducing strain specific neutralizing antibodies and M2e immunity, conferring differential cross protection in BALB/c mice. However, the efficacy of homologous prime-boost vaccination would likely be limited due to pre-existing immunity.

The impact of heterologous prime-boost vaccination with recombinant 4xM2e-HA influenza virus vaccines and pre-existing immunity on inducing cross-protection against influenza viruses remains unknown. In this study, we investigated the efficacy of cross-protection by heterosubtypic prime-boost vaccination with live recombinant 4xM2e-HA H1N1 and H3N2 influenza virus vaccines in C57BL/6 mice that are known to be a less responder to low immunogenic conserved epitopes. Heterologous prime-boost strategies using recombinant 4xM2e-HA influenza virus vaccines were found to be more effective in inducing cross-protection

against antigenically different viruses in C57BL/6 mice compared to homologous prime-boost vaccination strategies. M2e and HA stalk immunity might have played a role in cross-protection.

3.3 Materials and Methods

3.3.1 Cells and viruses

293T cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium media (DMEM) and used for plasmid DNA transfection. Influenza viruses were amplified in embryonated chicken eggs (Hy-Line N.A., Mansfield, GA) and confirmed to be free of the influenza virus. A/Puerto Rico/8/34 (A/PR8, H1N1), A/California/04/09 (A/Cal, H1N1), A/Philippines/2/82 (A/Phil, H3N2), and A/Vietnam rgH5N1 (A/Viet, rgH5N1) virus were used, which possesses HA (polybasic residues removed) and NA from A/Vietnam/1203/2004 and 6 other backbone genes of A/PR8 as previously described (Song et al., 2011). H3N2 recombinant virus was a reassortant virus with A/PR8 backbone (X-247 rgH3N2, International Reagent Resource, FR-1366) carrying HA and NA genes from A/Switzerland/9715293/2013. The influenza viruses were propagated in 10-day-old embryonated chicken eggs and inactivated by treatment of 37% formalin at 1:4,000 (v/v) dilutions as described previously (Quan et al., 2008).

3.3.2 Construction of attenuated A/PR8 backbone H1N1 and H3N2 viruses expressing chimeric 4xM2e-HA

Recombinant influenza viruses carrying wild type or chimeric hemagglutinin (HA) were generated using reverse genetics with PR8 backbone genes within the pHW2000 plasmids (kindly provided by Dr. Robert G. Webster, St. Jude Children's Research Hospital). The attenuated PR8 backbone (attPR8) was generated by introducing mutations in the PB1 (K391E, E581G, A661T)

and PB2 (N265S) polymerase genes granting temperature-sensitive (ts) attenuation as described (Jung et al., 2020). Reassorted H3N2 virus contained HA and NA genes derived from A/Switzerland/9715293/2013 (H3N2). The chimeric 4xM2e-HA genes encoded for four tandem repeats of M2e (4xM2e composed of 2x human M2e, swine M2e and avian M2e) inserted into the N-terminus of A/PR8 H1 HA (4xM2e-HA H1) (Kim et al., 2017a) or A/Switzerland H3 HA (4xM2e-HA H3, Figure 18A) as previously described (Park et al., 2021). The 4xM2e-HA_{Cal head} construct contained A/Cal HA head domain (Cys₅₂-Cys₂₇₇) in the 4xM2e-HA H1 context replacing the A/PR8 HA head domain (Figs. 18A, B). Eight plasmids encoding A/PR8 backbone plus each gene segment, including the desired subtype 4xM2e-HA construct and NA were co-transfected into 293T cells and incubated for 2 days. Chicken embryonated eggs were inoculated with transfection supernatants to rescue different subtype chimeric 4xM2e-HA recombinant viruses. The rescue of recombinant influenza virus was initially confirmed by measuring hemagglutination activity of egg allantoic fluids.

3.3.3 Characterization and pathogenicity assessment of recombinant viruses

Recombinant viruses rescued were characterized by measuring replication titers in embryonated chicken eggs (50% egg infectious dose, EID₅₀) and hemagglutination activity using 0.5% chicken red blood cells (RBC, Lampire Biological Laboratories). The existence of 4xM2e in HA was confirmed with enzyme-linked immunosorbent assay (ELISA) using M2e specific monoclonal antibody (14C2 mAb) (Abcam Inc., Cambridge, MA). To assess the pathogenicity of recombinant viruses, C57BL/6J mice were intranasally infected with 10⁶ EID₅₀ of attPR8, attPR8 with 4xM2e-HA H1 (attPR8 4xM2e) or 5x10⁵ of EID₅₀ of H3N2 or H3N2 with 4xM2e-HA H3 (H3N2 4xM2e), and body weight changes were monitored. Moreover, nasal turbinates and lung tissue

samples were collected and homogenized in RPMI media at 3 days after intranasal infection with 10^4 EID₅₀ of A/PR8, PR8 with 4xM2e-HA_{Cal} head (Cal 4xM2e) or 10^6 EID₅₀ of attPR8, attPR8 4xM2e H3N2 or H3N2 4xM2e. Serially diluted supernatants were inoculated in the embryonated eggs to determine virus titers.

3.3.4 Immunization and influenza virus challenge in mice

C57BL/6J mice received sequential prime-boost intranasal immunizations with recombinant live viruses in an interval of 3 weeks. The first set of mice groups (n = 20 /group, 6-8 weeks old, Jackson Laboratories) were intranasally primed with 10^6 EID₅₀ of attPR8 or attPR8 4xM2e H1N1 viruses and boosted with 10^6 EID₅₀ of attPR8, attPR8 4xM2e, H3N2, H3N2 4xM2e or 10^5 EID₅₀ of Cal 4xM2e H1N1 viruses. One-year-old C57BL/6J mice (n =5) were primed with attPR8 4xM2e (10^6 EID₅₀) and boosted with H3N2 4xM2e (10^6 EID₅₀). For reverse order immunization, mice (n = 20 /group, 6-8 weeks old, Jackson Laboratories) were intranasally vaccinated with 5×10^5 EID₅₀ of H3N2 or H3N2 4xM2e and boosted with 10^6 EID₅₀ of H3N2, attPR8 or attPR8 4xM2e H1N1 viruses. Mouse blood samples were collected through retro-orbital bleeding 2 weeks after each immunization. Mouse groups, including naïve group (unvaccinated mice) from the H1N1 primed set and H3N2 primed another set of hetero prime-boost immunizations, were challenged with A/Viet rgH5N1 (10 LD₅₀, 1.6×10^6 EID₅₀) and A/Phil (100 LD₅₀, 1.4×10^4 EID₅₀) respectively. All animal experiments in this study were approved by the Georgia State University Institutional Animal Care and Use Committee (IACUC) review boards. Mouse animal experiments including virus infection, blood collection, and tissue collections were performed in accordance with the approved IACUC protocol (A21004) and regulations.

3.3.5 Enzyme-Linked Immunosorbent Assay (ELISA)

IgG antibodies specific for the viruses were measured by ELISA with coating antigens (4 µg/ml) from inactivated A/PR8 H1N1, A/Cal H1N1, A/SW H3N2, A/Phil H3N2, and A/Viet rgH5N1 viruses. M2e-specific IgG antibody responses were determined using M2e peptide antigen (23 amino acids) as described previously (Kim et al., 2013b, Kim et al., 2014). Construction and preparation of the consensus group 1 stalk and group 2 stalk proteins were previously reported (Chae et al., 2019) and used as coating antigens for measuring HA stalk specific IgG antibodies. To determine IgG antibodies to both M2e and HA stalk domains, chimeric M2e-H1 HA stalk protein (aa1-117 of HA2 domain from A/PR8 virus) and M2e-H3 stalk protein (aa1-117 of HA2 domain from A/Aichi/1968 H3N2 virus) were expressed in *E. Coli* and purified (Jeeva et al., unpublished data) and used as an ELISA coating antigen. HRP (Horseradish Peroxidase) conjugated goat anti-mouse IgG (Southern Biotechnology) was used as a secondary antibody. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Invitrogen™) was utilized for color development. IgG antibody levels were read by BioTek ELISA plate reader at 450nm.

3.3.6 Hemagglutination Inhibition (HAI) Titers

Mouse sera were treated with receptor destroying enzymes (RDE, Sigma) with a 1:4 ratio, incubated at 37 °C overnight, and inactivated at 56°C for 30 minutes. RDE-treated serum samples were serially diluted (two-fold) and incubated with an equal volume of viruses (4 hemagglutination activity units). HAI titers were measured with 0.5% RBC.

3.3.7 Preparation of lung samples

Lung lysates and bronchoalveolar lavage fluids (BALFs) were collected on day 5 after the challenge. Lung extracts were obtained from the lung homogenates in 1.5ml of RPMI media after

centrifugation and used to determine virus titers in embryonated chicken eggs, IgG antibody responses specific for M2e, M2e-H1 stalk and M2e-H3 stalk were measured. BALFs were harvested by infusing 1ml of phosphate-buffered saline (PBS) into the trachea. IL-6 cytokine levels from BALFs and lung extracts were determined by Ready-SET-Go kits with IL-6 specific antibodies (eBioscience, San Diego, CA) according to the manufacturer's instructions.

3.3.8 Antibody-dependent cell-mediated cytotoxicity (ADCC) analysis

ADCC in immune sera was quantified by ADCC Reporter Bioassay Kit (Promega Life Sciences). The target MDCK cells (4×10^4 /100ul/well) on the 96-well Optical-Bottom plates (Thermo Scientific™) were infected with 100x TCID₅₀ of A/Viet rgH5N1 or A/Phil H3N2 virus one day prior to assay. Infected cells were washed with 200 µL RPMI medium (Thermo Fisher Scientific) and supplemented with 25 µL assay buffer (containing 4% low IgG serum in RPMI medium provided by assay kit). Heat-inactivated immune sera (25 µL) diluted in assay buffer were added to the cells and then further incubated for 6 h at 37 °C following the treatment of mouse effector Jurkat cells (Promega Life Sciences, 75,000 cells/well). Fluorescence was read by a Cytation 5 imaging reader (BioTek) after 5-min incubation at room temperature with 75 µL /well of Bio-Glo luciferase assay substrate (Promega Life Sciences). The fold induction was calculated after subtracting the culture medium background as suggested in the technical manual. The measurements were conducted in triplicate using three replicate wells.

3.3.9 Statistical analysis

Statistically significant differences were determined among groups using two- or one-way ANOVA. A p-values that were less than or equal to 0.05 were considered statically significant. Data were analyzed using Prism software (GraphPad Software Inc., San Diego, CA).

3.4 Results

3.4.1 In vitro and in vivo virological characterization of recombinant influenza viruses containing chimeric 4xM2e-HA

The rescued recombinant influenza viruses containing chimeric HA where foreign gene fragments were genetically linked to the N-terminus of HA were reported to be highly stable even after 10 passages (Kim et al., 2017a, Park et al., 2021, Lee et al., 2015b, Mezhenkaya et al., 2021a). The rescued recombinant live attenuated influenza viruses H1N1 (A/South Africa/3626/2013), H3N2 (A/Switzerland/9715293/2013), and H7N9 (A/Anhui/1/2013) containing 4xM2e-HA were confirmed by sequencing the full-length HA in previous studies (Kotomina et al., 2020, Mezhenkaya et al., 2021a). To determine the impact of heterologous prime-boost vaccination on cross-protection, we generated three different recombinant influenza A viruses containing tandem repeat 4xM2e-HA (Figure 18A). The attPR8 4xM2e is an A/PR8 reassortant virus with an attenuated backbone and chimeric 4xM2e-HA H1 of A/PR8. The Cal 4xM2e virus contains the A/PR8 wild type backbone and 4xM2e-HA where the head domain (C₅₂-C₂₇₇) of A/PR8 H1 HA was replaced with that of A/California/2019 (A/Cal). The reassortant H3N2 and H3N2 4xM2e viruses contain HA and 4xM2e-HA respectively, H3 HA and N2 NA genes from A/Switzerland/2013 (A/SW, H3N2) and the internal and A/PR8 wild type backbone as previously described (Park et al., 2021). The prediction of structural modeling of 4xM2e-HA chimera indicates similarity in displaying the globular head domain, stalk domain, and tandem repeat 4xM2e linked to the N-terminus of HA and juxtaposed to the bottom of the stalk (Figure 18B). These recombinant viruses propagated in embryonated chicken eggs retained high infectious titers (Figure 18C). The Cal 4xM2e, A/SW H3N2, and H3N2 4xM2e viruses showed

approximately 4 folds lower hemagglutination activity units (HAU), compared to those of A/PR8, attPR8, and attPR8 4xM2e viruses respectively (Figure 18D). M2e epitopes in attPR8 4xM2e, H3N2 4xM2e, and Cal 4xM2e viruses were detected at significantly higher levels compared to those of corresponding viruses with unmodified HA (Figure 18E).

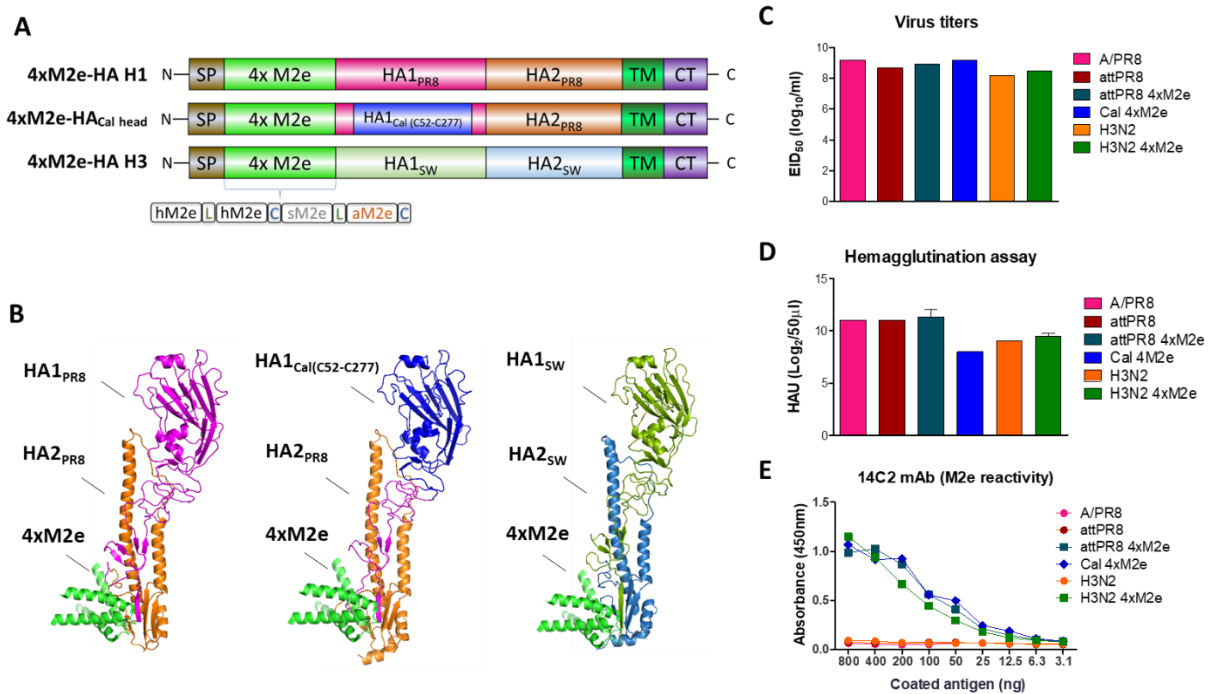


Figure 18 *In vitro* characterization of recombinant influenza viruses containing M2e epitopes (4xM2e) in chimeric HA conjugates

(A) Constructs of chimeric hemagglutinin (HA) carrying 4xM2e in its N-terminus. 4xM2e-HA H1: A/PR8 H1 HA containing 4xM2e, 4xM2e-HA_{Cal} head: A/PR8 4xM2e-HA head domain (Cys₅₂-Cys₂₇₇) was replaced with A/Cal HA head domain, 4xM2e-HA H3: A/SW H3 HA carrying 4xM2e. SP: signal peptide, tandem repeat 4xM2e is composed of human M2e (SLLTEVETPIRNEWGSRSSDSSD), swine M2e (SLLTEVETPTRSEWESRSDSSD), and avian M2e (SLLTEVETPTRNEWESRSDSSD). L and C represent linker (AAAGGAA) and connector (AAAPGAA). (B) Predictive three-dimensional structure of the chimeric HA monomers for 4xM2e-HA H1, 4xM2e-HA_{Cal} head and 4xM2e-HA H3 respectively, utilizing PyMol program. (C) Titration of infectious viruses in allantoic fluids of embryonated eggs inoculated with A/PR8, attPR8, attPR8 4xM2e, Cal 4xM2e, H3N2, or H3N2 4xM2e virus. (D) Hemagglutination activity in recombinant virus stocks using chicken RBC. (E) Reactivity of recombinant virus to M2e specific mAb 14C2. Error bars indicate mean \pm SEM.

To test the pathogenicity of recombinant viruses, body weight changes were monitored in C57BL/6J mice after intranasal inoculation with attPR8 (10^6 EID₅₀), attPR8 4xM2e (10^6 EID₅₀), H3N2 (5×10^5 EID₅₀) or H3N2 4xM2e (5×10^5 EID₅₀) at the doses used as prime vaccination. No body weight loss was detected in all groups (Figure 19A). In addition, no weight loss was observed in the mice intranasally primed with 10^6 EID₅₀ of attPR8 or attPR8 4xM2e and homo- or heterologous boost with 10^6 EID₅₀ of attPR8, H3N2, attPR8 4xM2e, or H3N2 4xM2e, or 10^5 EID₅₀ of Cal 4xM2e (Figure 19B).

Infectious titers of virus replication in upper (nose) and lower (lung) respiratory tracts were determined at 3 days after intranasal inoculation of mice with A/PR8, attPR8, attPR8 4xM2e, Cal 4xM2e, H3N2 or H3N2 4xM2e (Figure 19C). The wild type A/PR8 and Cal 4xM2e viruses replicated at higher titers in lung lysates than in nasal turbinates by 10^2 and 10^6 folds, respectively. In contrast, attPR8, attPR8 4xM2e, H3N2, and H3N2 4xM2e viruses were detected at higher virus titers in the nasal turbinates than in the lung by approximately 10^4 , 10^2 , 10 and 10^2 folds respectively (Figure 19C), confirming their attenuated phenotypes.

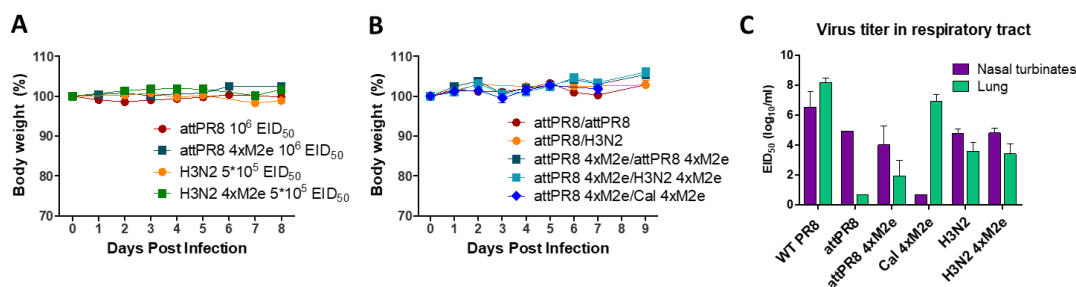


Figure 19 Pathogenicity of recombinant influenza viruses in mice

(A) Body weight changes of C57BL/6J mice ($n = 8-24$ /group) after intranasal inoculation with live attPR8 (10^6 EID₅₀), attPR8 4xM2e (10^6 EID₅₀), H3N2 (5×10^5 EID₅₀) or H3N2 4xM2e (5×10^5 EID₅₀). (B) Body weight changes in C57BL/6J mice ($n = 5-9$ /group) prime boost immunized with attPR8/attPR8, attPR8/H3N2, attPR8 4xM2e/attPR8 4xM2e, attPR8 4xM2e/H3N2 4xM2e and attPR8 4xM2e/Cal 4xM2e. 10^6 EID₅₀ of these viruses were inoculated except for Cal 4xM2e (10^5 EID₅₀). (C) Virus replications in mouse lung and nasal turbinate ($n = 4-5$) at day3 post challenge with A/PR8 (10^4 EID₅₀), attPR8 (10^6 EID₅₀), attPR8 4xM2e

(10^6 EID₅₀), Cal 4xM2e (10^4 EID₅₀), H3N2 (10^6 EID₅₀) and H3N2 4xM2e (10^6 EID₅₀). Each individual animal is analyzed. Error bars indicate mean \pm SEM.

3.4.2 H1N1 virus prime and heterologous boost with recombinant viruses carrying

4xM2e-HA induces enhanced M2e specific IgG and differential levels of HA stalk specific IgG antibodies

We determined the impact of heterologous boost in mice primed with H1N1 virus (attPR8+/-M2e) on inducing a profile of IgG antibodies for homo and hetero viruses, M2e, and HA stalk domains, in a set of five groups. The groups consisted of attPR8/attPR8, attPR8/H3N2, attPR8 M2e/attPR8 M2e, attPR8 M2e/H3N2 M2e and attPR8 M2e/Cal M2e where M2e indicates 4xM2e in the vaccine groups (Figure 20A). Intranasal prime inoculation of C57BL/6J mice with attPR8 or attPR8 M2e induced IgG antibodies to homologous A/PR8 at substantial levels and cross-reactive IgG antibodies to A/SW H3N2 and rgH5N1 reassortant viruses at lower levels (Figure 21). M2e and group 1 stalk specific IgG antibodies were induced at very low levels after prime dose attPR8 M2e, suggesting that M2e and HA stalk domains are immuno-subdominant (Figure 21). Heterosubtypic boost (attPR8 M2e/H3N2 M2e) induced highest levels of IgG antibodies specific for M2e followed by the heterologous boost group (attPR8 M2e/Cal M2e) (Figure 20B). Homo- and heterologous boost groups showed similar levels of IgG antibodies to A/PR8, A/Cal H1N1, and A/Viet rgH5N1 viruses (Figs. 20C, E, F). The attPR8/H3N2 and attPR8 M2e/H3N2 M2e vaccinated groups induced an increased level of IgG antibodies to A/SW H3N2 virus than other groups (Figure 20D). Meanwhile, the heterologous attPR8 M2e/Cal M2e group induced the highest levels of consensus group 1 stalk IgG responses (Figure 20G). Also, the group boosted with H3N2 or H3N2 M2e showed the highest IgG antibody reactivities to consensus group 2 stalk domain as expected (Figure 20H). These results suggest that a strategy of hetero

boost vaccination is more effective in inducing IgG antibodies specific for M2e and HA stalk immuno-subdominant epitopes.

The immunogenicity of hetero boost attPR8 M2e/H3N2 M2e was tested in one-year-old C57BL/6J mice after intranasal vaccination (Figure 22). Prime dose of attPR8 M2e induced substantial levels of IgG antibodies for A/PR8 H1N1, H3N2 (A/SW), and rgH5N1, but M2e and HA stalk IgG antibodies were detected at very low levels (Figure 22). After H3N2 M2e boost in aged C57BL/6J mice (attPR8 M2e/H3N2 M2e, Figure 23), IgG antibodies to M2e, A/PR8, H3N2, and rgH5N1 viruses but not to HA stalk domain were induced at increased levels despite lower levels being induced compared to those in young adult mice (Figure 20). These data implicate that priming vaccination at young ages might be required for effective induction of HA stalk IgG antibodies.

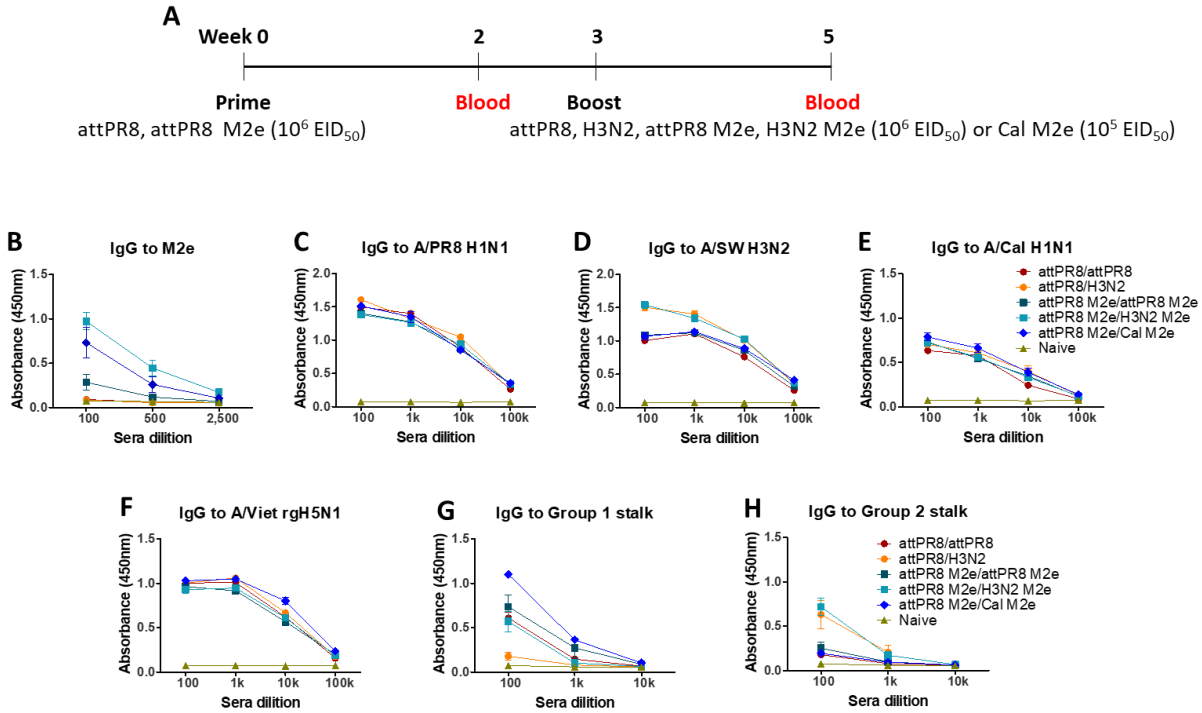


Figure 20 IgG antibodies specific for M2e, different viruses, and HA stalk domains after hetero boost with recombinant viruses in H1N1 primed

(A) Timeline strategy of hetero prime-boost intranasal immunization with recombinant viruses. C57BL/6J mice received prime vaccination with 10^6 EID₅₀ of attPR8 H1N1 or attPR8 M2e H1N1 were inoculated with 10^6 EID₅₀ of attPR8, attPR8 M2e, H3N2 M2e, or 10^5 EID₅₀ of Cal M2e in a 3-week interval ($n = 8$ /each group). M2e indicates chimeric 4xM2e-HA. Serum IgG antibody responses specific for M2e peptide (B), A/PR8 virus (C), H3N2 virus (D), A/Cal virus (E), A/Viet virus (F), consensus group 1 stalk (G), and consensus group 2 stalk (H). Mouse sera were collected at 2 weeks after prime-boost immunization. attPR8/attPR8: homologous prime-boost attPR8 H1N1, attPR8/H3N2: heterosubtypic attPR8 prime and A/SW H3N2 boost, attPR8 M2e/attPR8 M2e: heterosubtypic prime-boost with chimeric 4xM2e-HA viruses, and attPR8 M2e/Cal M2e: heterologous H1N1 prime-boost with chimeric 4xM2e-HA viruses. M2e in the vaccine groups indicates tandem repeat 4xM2e. Each individual animal is analyzed. Error bars indicate mean \pm SEM.

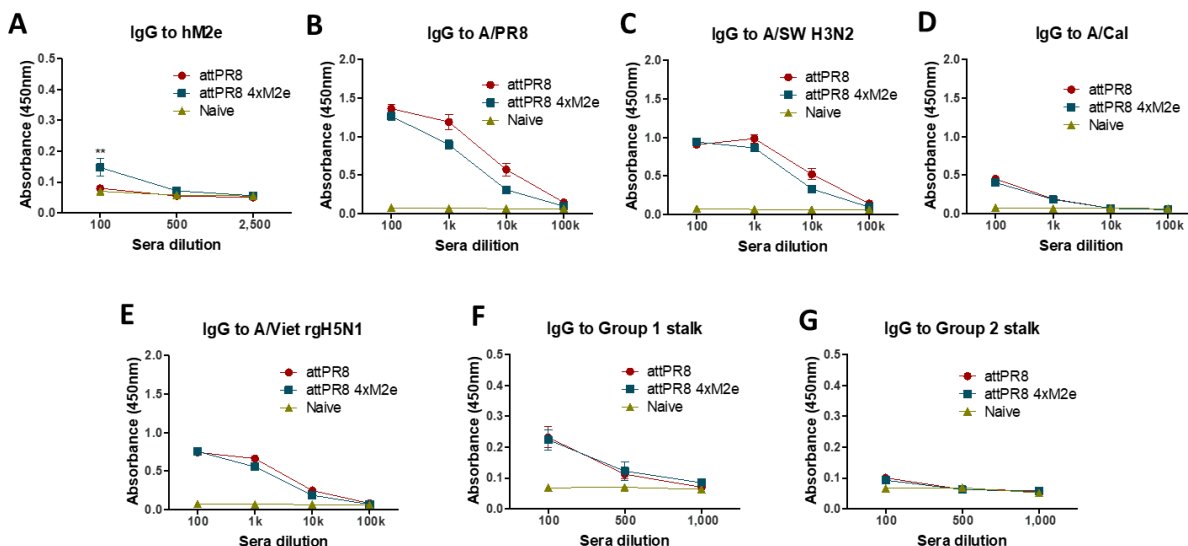


Figure 21 IgG antibodies specific for M2e, viruses, and HA stalk domains after prime dose immunization with attPR8 or attPR8 M2e

C57BL/6J mice (6-8 weeks, $n = 13-19$) were intranasally inoculated with 10^6 EID₅₀ of attPR8 or attPR8 M2e. Serum IgG antibody responses at 2 weeks after prime specific for M2e (A), A/PR8 Virus (B), H3N2 virus (C), A/Cal virus (D), A/Viet virus (E), consensus group 1 stalk protein (F), and consensus group 2 stalk protein (G). Each individual mouse sera were analyzed. Error bars indicate mean \pm SEM. The statistical significances were determined using two-way ANOVA and indicated in **, $P < 0.01$.

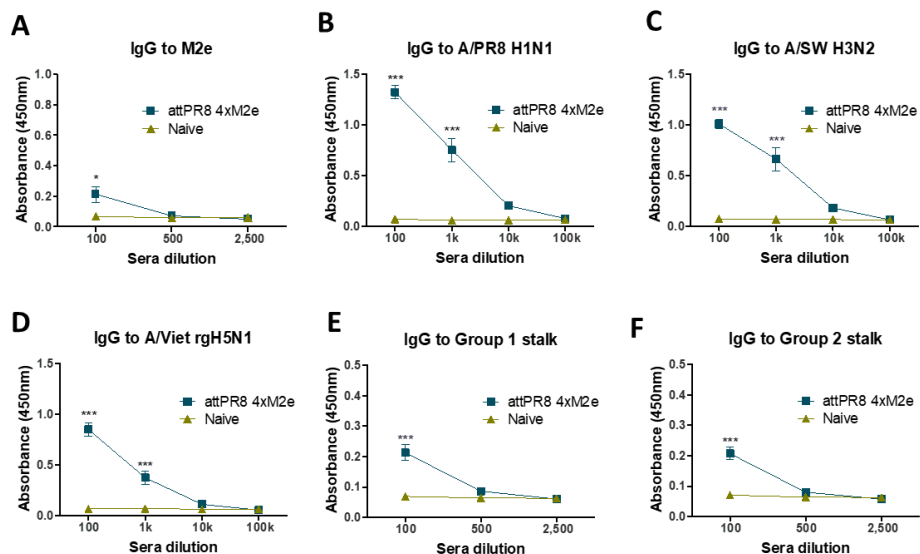


Figure 22 IgG antibody responses in 1-year-old mice after attPR8 or attPR8 M2e vaccination

Sera samples were collected at 2 weeks after attPR8 or attPR8 M2e immunization in 1-year-old C57BL/6J mice ($n = 5$). IgG antibody detection to M2e (A), A/PR8 virus (B), H3N2 virus (C), A/Viet virus (D), consensus

group 1 stalk protein (E) and consensus group 2 stalk protein (F). Each individual mouse sera were analyzed. Error bars indicate mean \pm SEM. The statistical significances were determined using two-way ANOVA and indicated in *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

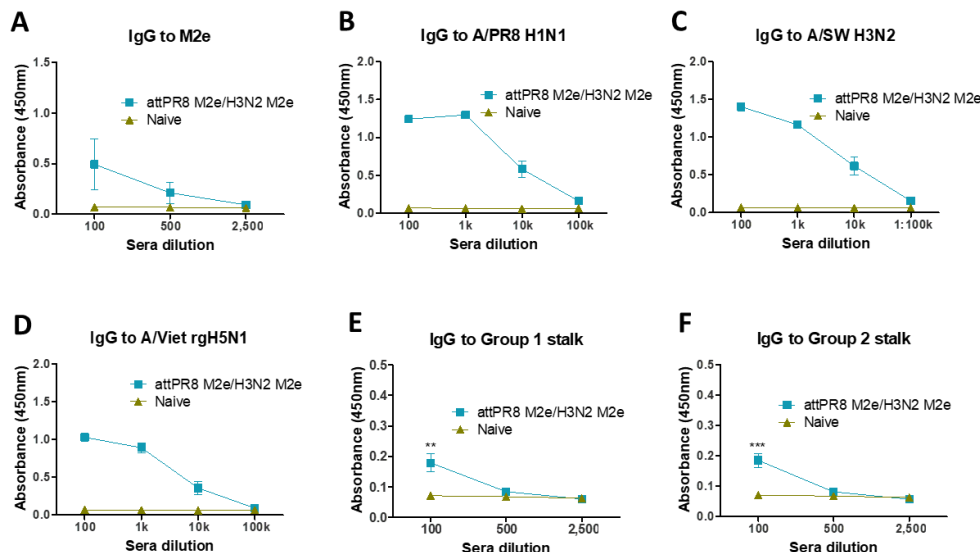


Figure 23 IgG antibodies specific for M2e, different viruses, and HA stalk domains in 1 year old C57BL/6J mice after attPR8 M2e prime and H3N2 M2e boost

IgG antibody immune responses to M2e peptide (A), A/PR8 virus (B), H3N2 virus (C), A/Viet virus (D), consensus group 1 stalk protein (E), and consensus group 2 stalk protein (F). One year old C57BL/6J mice ($n = 4$) were inoculated with 10^6 EID₅₀ of attPR8 M2e and H3N2 M2e in a 3-week interval and bled at 2 weeks after boost. Each individual animal is analyzed. Error bars indicate mean \pm SEM.

3.4.3 Strain specific HAI titers were induced by heterosubtypic prime boost chimeric recombinant influenza virus vaccination

Prime dose of attPR8 M2e induced A/PR8 specific hemagglutination inhibition (HAI) titers at approximately 2 folds lower than attPR8 in young adult mice and 4 folds higher than that in 1-year old mice (Figure 24A). Homologous boost with attPR8 and attPR8 M2e increased HAI titers against A/PR8 virus by 4 folds in young adult mice whereas hetero boost with H3N2 M2e (attPR8 M2e/H3N2 M2e) induced 2 folds increases in A/PR8 HAI titers, but not by Cal M2e boost or in 1-year old mice (Figs. 24A, B). High HAI titers against H3N2 (A/SW) were induced by hetero boost

with H3N2 or H3N2 M2e in young adult mice, which were approximately 20 folds higher than in 1-year-old mice with H3N2 M2e boost. No cross HAI titers against rgH5N1 virus were detected in young adult and aged mice after vaccination (Figure 24B).

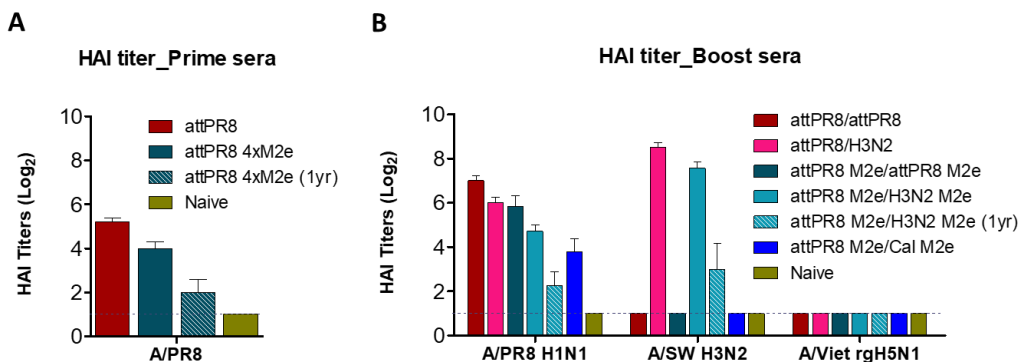


Figure 24 HAI titers against hetero prime and boost viruses

(A) HAI titers against A/PR8 virus in young adult and 1-year old mouse sera after prime dose (10^6 EID₅₀) of attPR8 or attPR8 M2e. (B) HAI titers against A/PR8 H1N1, A/SW H3N2, and A/Viet rgH5N1 viruses after hetero boost in young adult and 1-year-old mice. Mouse sera were collected at day 14 following immunization. Group labels are the same as in the Figure 20 legend. Each individual mouse sera were analyzed ($n = 8$ /group). Error bars indicate mean \pm SEM.

3.4.4 Hetero boost with recombinants 4xM2e-HA viruses in H1N1 M2e primed mice improves cross protection against rgH5N1 virus

To investigate cross protective efficacy, mice were challenged with A/Viet rgH5N1 virus at a lethal dose 3 weeks after boost immunization (Figure 25A). The hetero boost groups (attPR8 M2e/H3N2 M2e and attPR8 M2e/Cal M2e) presented similarly least weight loss (~4.2%) whereas the homo boost groups (attPR8/attPR8 and attPR8 M2e/attPR8 M2e) displayed substantial weight loss, 10% and 12% respectively (Figure 25A). In an additional experimental set to determine lung viral titers, the hetero boost attPR8 M2e/H3N2 M2e group showed slight weight loss (~4%) by day 3 post challenge, they began to recover by day 4 when lung viral loads were reduced to a minimum of 10^5 folds lower than the titer in naive mice (10^7 EID₅₀/ml) after infection with rgH5N1 virus (Figs. 25B, C). Meanwhile, the homo boost attPR8 M2e/attPR8 M2e group

manifested moderate weight loss (~9%) and lung viral loads ($10^{3.8}$ EID₅₀/ml), which was 100 folds higher than hetero boost with H3N2 M2e. The attPR8/attPR8 and attPR8/H3N2 groups did not induce M2e specific IgG antibodies, and they showed more weight loss and higher lung viral titers, $10^{5.2}$ EID₅₀/ml and $10^{4.3}$ EID₅₀/ml respectively than 4xM2e-HA chimeric viruses (Figs. 25B, C), correlating the protective efficacy with M2e immunity. Hetero vaccination with attPR8 M2e/H3N2 M2e prevented severe weight loss and reduced viral loads in 14 months old mice by the time of challenge with rgH5N1 virus (Figure 26).

Consistent with the levels of lung viral titers, hetero prime boost attPR8 M2e/H3N2 M2e vaccination protected against lung inflammation as shown by the lowest levels of inflammatory cytokine IL-6 in bronchoalveolar lavage fluid (BALF) and lung at day 4 after rgH5N1 infection (Figure 25D). The homologous prime-boost attPR8 M2e/attPR8 M2e and attPR8/H3N2 groups showed high levels of inflammatory IL-6 in lung while low levels in BALF (Figure 25D). The homo prime boost attPR8/attPR8 group with least protection showed higher IL-6 levels in BALF and lung than other more protective vaccine groups.

We also determined whether rapid induction of mucosal IgG antibodies specific for M2e and HA stalk domains would correlate with heterosubtypic cross protection against rgH5N1 virus (Figure 25E). The attPR8 M2e/H3N2 M2e group showed the highest levels of lung IgG antibodies specific for M2e and M2e-H1 stalk domains at day 4 after rgH5N1 challenge in young adult mice (Figure 25E). which was 2 and 7 folds higher respectively than those in 14 months old mice (Figure 26C). In contrast, attPR8/attPR8 and attPR8/H3N2 groups induced M2e and HA stalk IgG antibodies at non-detectable levels, similar to naïve mice after infection (Figure 25E). Three to four folds lower levels of lung IgG antibodies for M2e and HA stalk antibodies were observed in

the homo attPR8 4xM2e/attPR8 4xM2e group than those in the hetero prime boost attPR8 M2e/H3N2 M2e group (Figure 25E). Taken together, these results indicate the effectiveness of heterologous prime-boost vaccination in conferring cross protection against rgH5N1 virus.

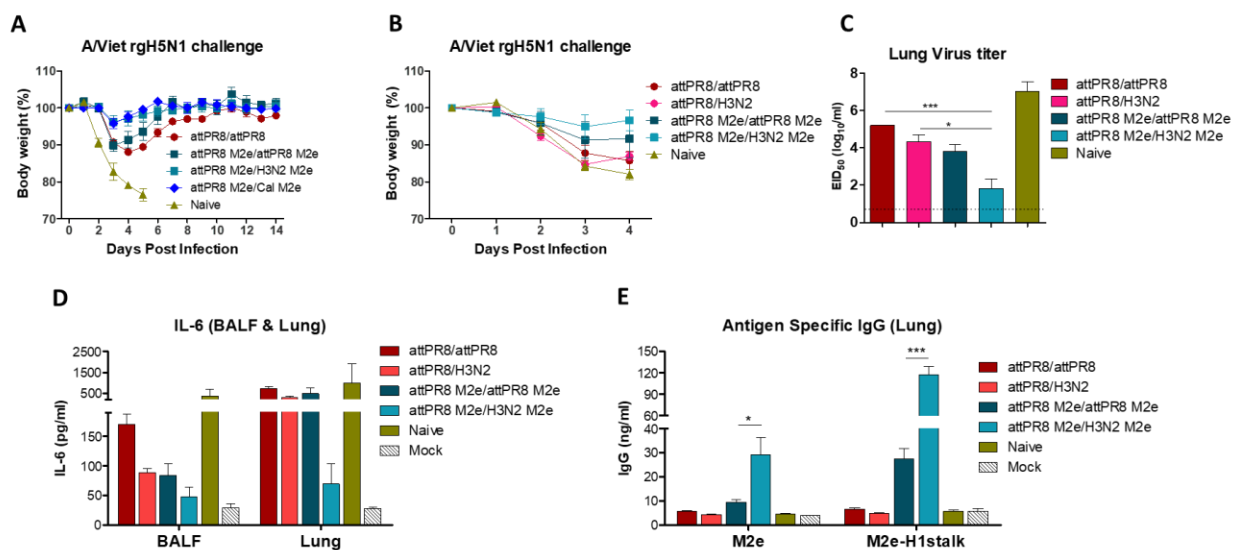


Figure 25 Hetero boost immunization with 4xM2e-HA chimeric virus enhances cross protection against A/Viet rgH5N1 virus

C57BL/6J mice were challenged with A/Viet rgH5N1 virus (reassortant A/PR8-A/Vietnam/1203/2004, 10 LD₅₀, 1.6 x 10⁶ EID₅₀). (A) Body weight changes after challenge in young adult mice (n = 5/group). Group labels are the same as in the Figure 20 legend. (B-E) Young adult mice (n = 4/group) were intranasally prime boost immunized with attPR8/attPR8, attPR8/H3N2, attPR8 M2e/attPR8 M2e, and attPR8 M2e/H3N2 M2e (n = 5/group) and then challenged with A/Viet rgH5N1 virus. (B) Body weight changes for 4 days after challenge until mice were sacrificed. (C) Virus titers in lung lysates at 4 days after A/Viet challenge. (D) Inflammatory cytokine IL-6 levels in BALF or lung lysates day 4 post challenge with rgH5N1. (E) IgG antibodies specific for M2e and M2e-H1 stalk (HA2 aa1-117 from A/PR8) in lung lysates day 4 post challenge. Error bars indicate mean ± SEM. The statistical significances were determined using one-way (C) or two-way (D, E) ANOVA and indicated in **, P < 0.01; ***, P < 0.001.

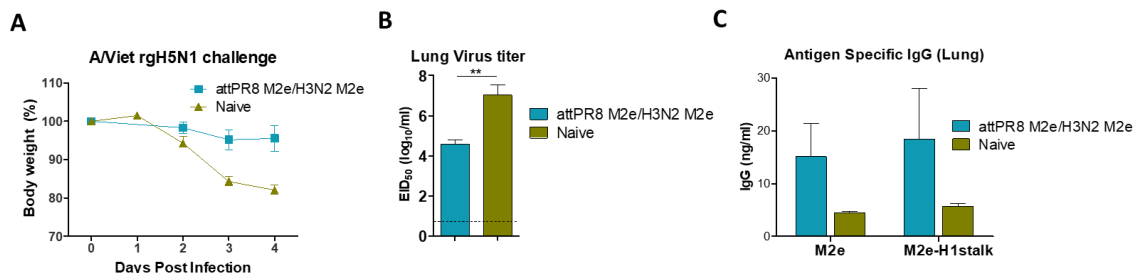


Figure 26 Heterosubtypic attPR8 M2e prime H3N2 M2 boost intranasal vaccination protects one-year-old C57BL/6J mice against A/Viet rgH5N1 virus challenge

Mice (n = 5, 1-year-old) were sequentially immunized with attPR8 M2e (5×10^5 EID₅₀, 1.6×10^6 EID₅₀) and H3N2 M2e (10^6 EID₅₀) with 3 weeks interval prior to A/Viet rgH5N1 challenge (10 LD₅₀). (A) Body weight changes after A/Viet rgH5N1 virus challenge for 4 days until mice were sacrificed. (B) Lung virus titers at 4 days after challenge. (C) Lung IgG antibodies specific for M2e or chimeric M2e-H1 stalk (HA2 aa1-117 from A/PR8) at day 4 after A/Viet rgH5N1 virus challenge. Naïve mouse group infected with A/Viet rgH5N1 virus is from Figure 25 young naïve mice. Each individual mouse was analyzed. Error bars indicate mean \pm SEM. The statistical significances were determined using one-way ANOVA and indicated in **, $P < 0.01$.

3.4.5 Hetero boost in H3N2-primed mice with attPR8 M2e virus effectively induce M2e

IgG antibodies

Conversely, we determine the impact of hetero boost in H3N2-primed mice by comparing the following three prime-boost combinations, H3N2/H3N2, H3N2/attPR8, and H3N2 M2e/attPR8 M2e (Figure 27A). Significantly enhanced levels of IgG antibodies specific for M2e were induced in the hetero boost H3N2 M2e/attPR8 M2e group (Figure 27B) compared to prime dose of H3N2 M2e (Figure 28). Similar levels of IgG antibodies specific to homologous and heterosubtypic viruses such as A/PR8 (H1N1), A/SW (H3N2), and A/Phil (H3N2) were detected in all prime-boost immunized mouse groups (Figs. 27C-E).

Consensus group 1 stalk specific IgG antibodies were induced to a moderate level in the H3N2 M2e/attPR8 M2e group but not in the H3N2/attPR8 group (Figure 27F). IgG antibodies to consensus group 2 stalk were induced at the highest levels in the homo prime-boost H3N2/H3N2

group and substantial levels of consensus group 2 stalk IgG antibodies were similarly observed in the hetero prime-boost H3N2/attPR8 and H3N2 M2e/attPR8 M2e groups (Figure 27G).

Homologous strain (H3N2) specific HAI titers were induced at protective levels (~ 128) by prime dose of H3N2 or H3N2 4xM2e (Figure 27H). HAI titers against H3N2 virus were further enhanced by 4 folds by homo boost H3N2/H3N2 vaccination (Figs. 27H, I). Heterosubtypic attPR8 or attPR8 M2e boost immunization (H3N2/attPR8 and H3N2 M2e/attPR8 M2e) retained HAI titers induced by prime H3N2 dose (Figure 27H, I). Induction of HAI titers against A/PR8 might have been suppressed in H3N2 primed mice after attPR8 boost (Figure 27I). No HAI responses were observed against A/Phil from all groups (Figure 27I).

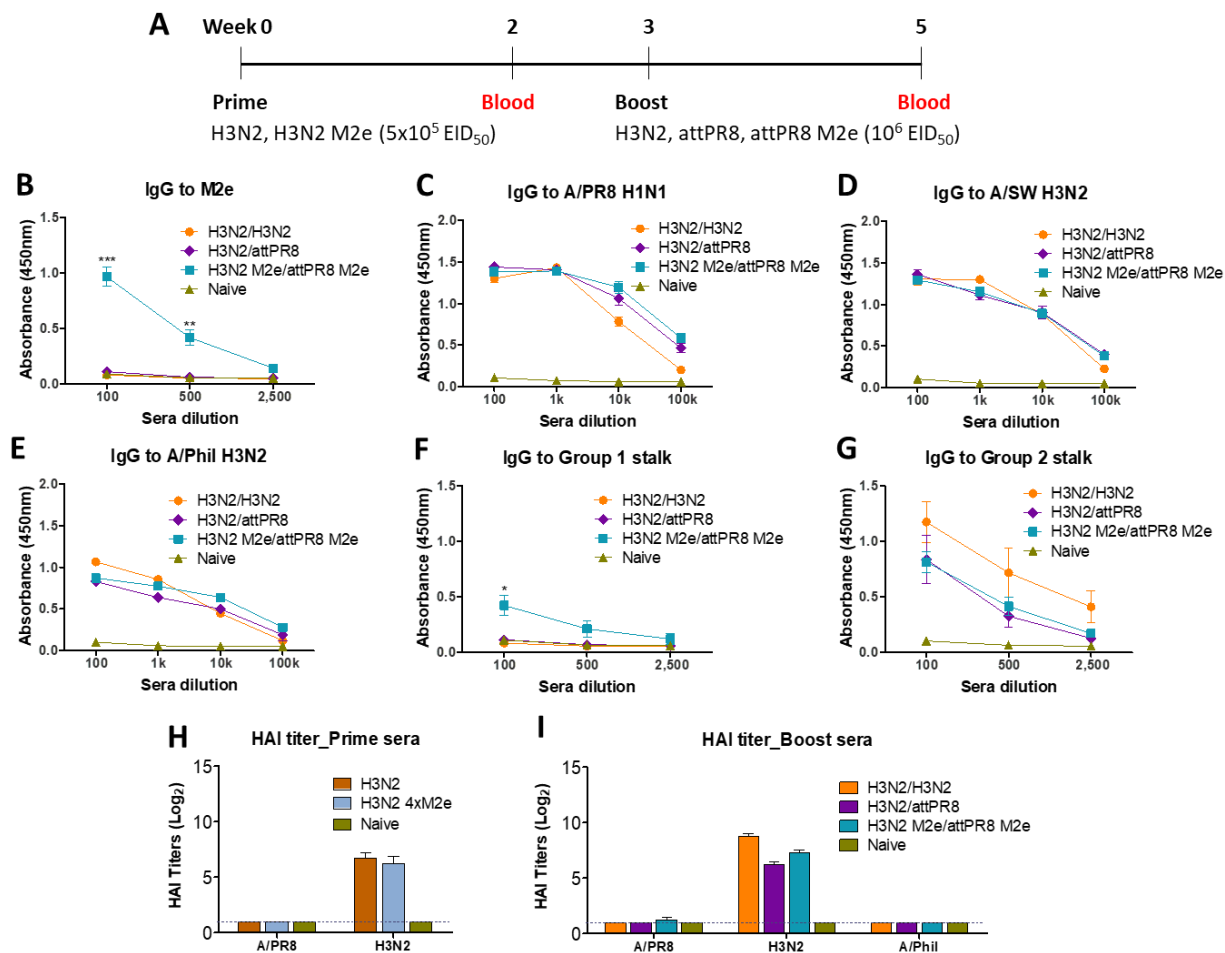


Figure 27 Intranasal heterosubtypic boost with chimeric 4xM2e-HA A/PR8 virus enhances M2e IgG responses in H3N2 primed mice

(A) Timeline for a reverse order of H3N2 prime and H1N1 boost vaccination strategy. Mice ($n = 5/\text{group}$) were intranasally inoculated in prime-boost regimens with H3N2/H3N2, H3N2/attPR8 or H3N2 M2e/attPR8 M2e (prime dose: 5×10^5 EID₅₀, boost dose: 10^6 EID₅₀) and immune sera were collected at 3 weeks after boost. IgG antibody responses to M2e (B), A/PR8 virus (C), A/SW H3N2 virus (D), A/Phil H3N2 virus (E), consensus group 1 stalk protein (F) and consensus group 2 stalk protein (G). HAI titers against influenza viruses were measured from immune sera after prime (H) and prime-boost (I) immunization. Each individual animal was analyzed. Error bars indicate mean \pm SEM.

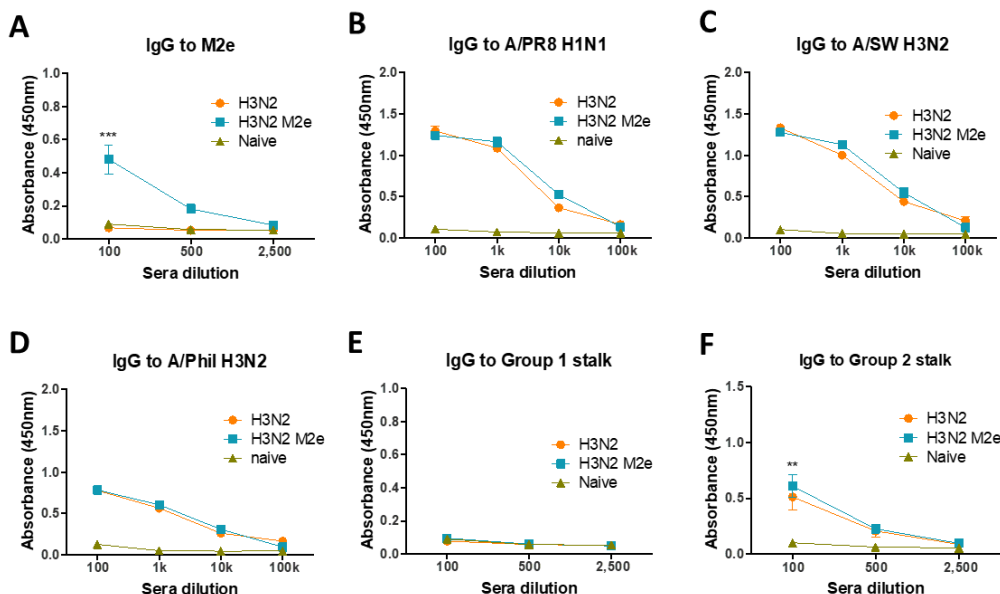


Figure 28 IgG antibodies specific for M2e, viruses, and HA stalk domains after prime vaccination with H3N2 or H3N2 M2e

Mouse sera were collected 2 weeks after intranasal single dose (5×10^5 EID₅₀) inoculation of H3N2 or H3N2 M2e. Total IgG antibodies specific for M2e (A), A/PR8 virus (B), H3N2 virus (C), A/Phil virus (D), consensus group 1 stalk protein (E) and consensus group 2 stalk protein (F). Each individual mouse sera were analyzed. Error bars indicate mean \pm SEM. The statistical significances were determined using two-way ANOVA and indicated in **, $P < 0.01$; ***, $P < 0.001$.

3.4.6 Hetero boost vaccination in H3N2-primed mice induces effective protection against heterologous H3N2 virus

We evaluated whether heterosubtypic boost in H3N2 primed mice would be more effective in inducing cross-protection against A/Phil H3N2 virus challenge (Figure 29). The hetero prime-boost H3N2/attPR8 and H3N2 M2e/attPR8 M2e groups displayed only $\sim 4\%$ weight loss and then all mice recovered (Figure 29A). In contrast, the homo prime-boost H3N2/H3N2 group showed approximately 10% weight loss at day 2 after A/Phil infection before recovering weight to a normal level (Figure 29A). Naïve mice continued to display weight loss after A/Phil virus infection until day 5; at this point all mice were sacrificed to collect lung tissues for viral titration. Reduced lung virus titers were detected in the hetero prime-boost H3N2 M2e/attPR8 M2e group

after A/Phil challenge, which were ~10 folds lower than those of the homo or hetero prime-boost H3N2/H3N2 and H3N2/attPR8 groups and approximately 10^5 folds lower than those in naïve mice with infection (Figure 29B). Significantly higher levels of IgG antibodies specific for M2e, M2e-H1 stalk, and M2e-H3 stalk were measured in lung lysates from the hetero prime-boost H3N2 M2e/attPR8 M2e group (Figure 29C), likely correlating with lowest lung viral titers (Figure 29B). Despite the highest levels of group 2 stalk specific IgG responses (Figure 27G), the H3N2/H3N2 group did not show enhanced cross-protection against A/Phil virus (H3N2) (Figs. 29A, B).

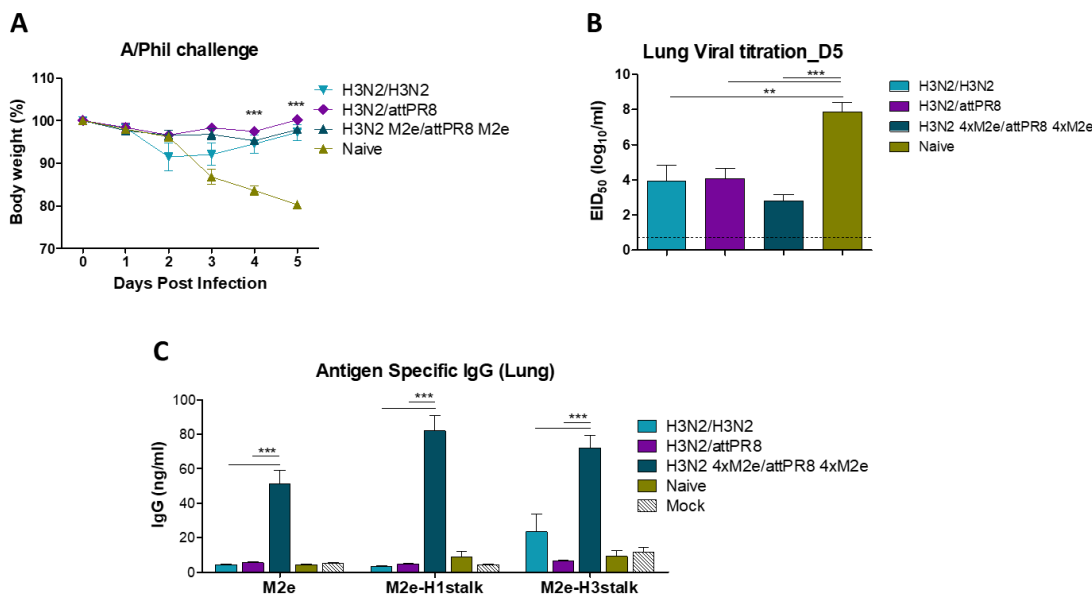


Figure 29 H3N2 M2e/attPR8 M2e prime-boost vaccination provides effective lung viral control after heterologous A/Phil H3N2 virus challenge

The groups (n = 5/group) of mice immunized with H3N2/H3N2, H3N2/attPR8 or H3N2 M2e/attPR8 M2e were challenged with A/Phil virus (100x LD₅₀, 1.4 x10⁴ EID₅₀). (A) Body weight changes after A/Phil H3N2 virus challenge for 5 days until mice were sacrificed. (B) Lung virus titers at day 5 after A/Phil virus challenge. (C) Lung IgG antibodies specific for M2e, chimeric M2e-H1 stalk (HA2 aa1-117 from A/PR8), and chimeric M2e-H3 stalk (HA2 aa1-117 from A/Aichi/1968 H3N2). Each individual animal is analyzed. Error bars indicate mean ± SEM. The statistical significances were determined using one-way (B) or two-way (C) ANOVA and indicated in **, P < 0.01; ***, P < 0.001.

3.5 Discussion

Clinical data suggest that repeat seasonal influenza vaccination might not be highly effective in inducing protective antibody responses (McLean et al., 2014, Thompson et al., 2016, Leung et al., 2017, Morimoto and Takeishi, 2018, Song et al., 2020) or lead to substantial heterogeneity in vaccine effectiveness (Belongia et al., 2017). Our previous studies showed that homologous prime-boost with A/PR8 4xM2e-HA or prime dose of attenuated rgH3N2 4xM2e-HA virus vaccination could be more effective in conferring broader cross-protection in BALB/c mice than the wild type HA counterpart virus (Kim et al., 2017a, Park et al., 2021). . Accordingly, we determine whether heterologous prime-boost vaccination with recombinant influenza viruses containing chimeric 4xM2e-HA would induce more effective cross-protection in different genetic background C57BL/6 mice known to be less responsive to influenza vaccination than BALB/c mice (Petrovic et al., 2018). We found that heterosubtypic prime-boost provided more effective in conferring cross-protection than homologous vaccination. Hetero prime-boost with recombinant influenza viruses containing 4xM2e-HA induced high levels of IgG antibodies specific for M2e and reduced lung viral loads more effectively after heterosubtypic or heterologous virus challenge.

Prime dose vaccination induced low levels of IgG antibodies for M2e and HA stalk epitopes in 4xM2e-HA chimeric virus, suggesting their immune-subdominance (Figure 21). Boost exposure to antigenically more distant chimeric virus H3N2 4xM2e-HA effectively generated M2e specific IgG antibodies. In the attenuated A/PR8 H1N1 (4xM2e-HA) prime and antigenically different virus boost groups, the levels of M2e antibodies were correlated with lowering lung viral titers and inflammatory cytokine (IL-6) against rgH5N1 virus challenge. The contribution of M2e antibodies to lowering lung viral loads was also observed in the attenuated H3N2 4xM2e-HA/PR8 4xM2e-

HA group after A/Phil (H3N2) challenge. Importantly, Cal-head 4xM2e-HA boost induced higher levels of IgG binding to group 1 stalk proteins; the group 1 stalk IgG antibody levels were likely correlated with prevention against severe weight loss against rgH5N1 virus challenge as shown by the PR8-4xM2e/Cal-head 4xM2e group. These immunologic outcomes were consistent with the chimeric HA based vaccination strategy where multiple vaccinations were administered with heterosubtypic modified HA-head domains (Krammer et al., 2013, Hai et al., 2012). Consistently, a prior study demonstrating heterosubtypic immunity by sequential vaccination with live attenuated chimeric HA head-switched viruses in ferrets (Liu et al., 2019). The correlative contribution of M2e specific IgG antibodies to lowering lung viral loads was also observed in the attenuated H3N2 4xM2e-HA/PR8 4xM2e-HA group after A/Phil (H3N2) challenge. Thus, in the attenuated A/PR8 H1N1 prime and antigenically different boost groups, the combined M2e and HA stalk specific IgG levels might have contributed to cross-protection against weight loss and to clearing lung viral loads. Fc receptors (FcR) are known to be required for mediating protection by M2e and HA stalk specific antibodies (El Bakkouri et al., 2011, Kim et al., 2013a, DiLillo et al., 2016, DiLillo et al., 2014), suggesting a significant role of antibody-dependent cell-mediated cytotoxicity (ADCC) in conferring protection. Not only natural killer cells from human donors (FcγRIIIA+primary NK) but also FcγRIIIA engineered NK-92 cells and FcγRIIIA/NFAT-RE/luc2 engineered Jurkat T cells have been used as effector cells in bioassay evaluation of ADCC (Hsieh et al., 2017). We demonstrated that immune sera from the H3N2 4xM2e-HA/PR8 4xM2e-HA group were found to activate FcR in Jurkat cells via the target MDCK cells infected with A/Phil H3N2 or A/Viet rgH5N1 viruses, supporting a possible role of ADCC in mediating protection (Figure 30).

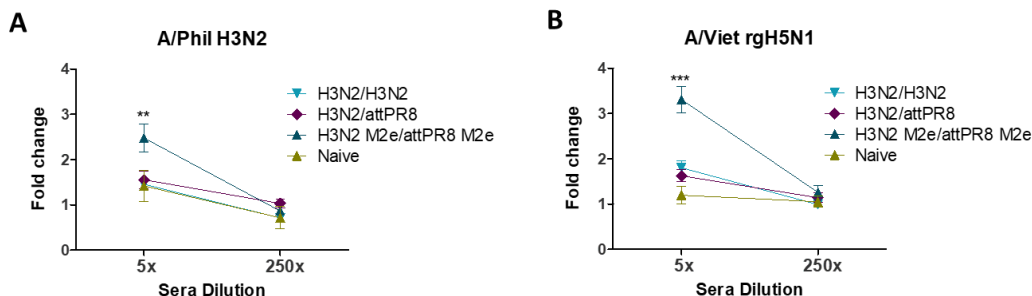


Figure 30 Heterosubtypic sequential immunization with recombinant influenza A viruses carrying 4xM2e-HA induces ADCC activity

Mouse immune sera ($n = 5$) were collected 2 weeks after prime-boost immunization with H3N2/H3N2, H3N2/attPR8 and H3N2 M2e/attPR8 M2e and used to incubate on the MDCK cells infected with different influenza viruses before co-culturing with effector Jurkat cells. Luminescence as indicative of ADCC activities in each immune sera stimulating effector Jurkat cells via target MDCK cells infected with A/Phil H3N2 (A) and A/Viet rgH5N1 (B) virus; presented as fold changes. Pooled mouse sera in triplicates were analyzed. Error bars indicate mean \pm SEM. The statistical significances were determined using two-way ANOVA and indicated in **, $P < 0.01$; ***, $P < 0.001$.

Repeat vaccination was shown to induce lower antibody titers than current seasonal vaccination only (Thompson et al., 2016, Sanyal et al., 2019, Leung et al., 2017) and might lead to reduced antibody-affinity maturation (Khurana et al., 2019). In addition, repeated homologous vaccination was less effective in protection against H3N2 virus than single vaccination in ferrets despite no significant differences in antibody levels (Music et al., 2019). It should be noted that compared to the heterosubtypic H3N2/H1N1 (A/PR8) vaccination, prime-boost with H3N2 virus (2013 isolate) resulted in more weight loss against A/Phil virus (1982 isolate) at days 2 to 3 post challenge, while it induced higher levels of homologous HAI titers and IgG antibodies for group 2 stalk protein and similar outcomes in lung viral loads. Similarly, higher levels of group 1 stalk IgG antibodies induced by attenuated PR8/PR8 prime-boost did not improve cross-protection against rgH5N1 virus compared to the PR8/H3N2 group, which induced near background levels of group 1 stalk specific antibodies. High levels of anti-HA stalk antibodies were reported in the elderly individuals (Nachbagauer et al., 2016), in which population influenza-related morbidity and

mortality are mostly associated. Thus, caution should be given in correlating HA stalk specific antibody levels with cross-protection. Therefore, inducing extra M2e immunity might provide a safeguard, protecting from severe disease and mortality during a pandemic to which individuals are naïve.

The elderly populations display poor responses to vaccination because of immunosenescence, limited naïve B cells and IgG repertoire (Crooke et al., 2019), reduced capacity to target antigenically distinct epitopes, and less somatic hypermutations (Henry et al., 2019). One year old C57BL/6 mice were less effective compared to young adult mice in inducing HAI titers, IgG antibodies to M2e and particularly to HA stalk domain. They were also poorly responsive to boost dose in the PR8 4xM2e/H3N2 4xM2e group. To overcome poor responses to low immunogenic but conserved epitopes, immunizing younger age populations with vaccines targeting M2e and HA stalk proteins would be an approach for future studies.

A strategy of heterosubtypic prime-boost with vaccines inducing strain specific HAI titers and IgG antibody responses to conserved M2e and HA stalk domains would improve the breadth of cross-protection. In the attenuated A/PR8 prime set with A/SW H3N2 boost, HAI titers against both vaccine strains were induced without significant compromise. However, in the reverse A/SW H3N2 prime and attenuated A/PR8 boost, HAI responses to A/PR8 were compromised although both strains are antigenically unrelated. This might be because attenuated A/PR8 viruses would not replicate at sufficient levels under pre-existing immunity against A/SW H3N2 virus whereas A/SW H3N2 virus could replicate both in the upper and lower respiratory tracts (Figure 19C). Hence, further studies will be needed to better understand heterosubtypic prime-boost strategies. One caveat of this study is that live attenuated influenza vaccines are not

generally recommended in adults and elderly populations probably due to pre-existing immunity negatively affecting vaccine effectiveness. Live attenuated vaccine platforms of head-domain switched chimeric HA-based universal influenza virus vaccine candidates were not effective inducing anti-H1 stalk IgG responses whereas AS03-adjuvanted inactivated virus vaccines were immunogenic in raising durable HA stalk specific antibodies at several folds higher levels in phase 1 studies (Bernstein et al., 2020, Nachbagauer et al., 2021). Inactivated virus platforms of recombinant virus vaccines with 4xM2e-HA were not effective in inducing M2e specific IgG antibody responses after intramuscular immunization of mice (data not shown). In contrast, previous studies reported alternative strategies of recombinant influenza viruses where the foreign epitopes (M2e, conserved NA or neutralizing epitopes of respiratory syncytial virus fusion protein) were inserted in the immunodominant HA head domain, which induced epitope specific antibodies after vaccination of mice with inactivated vaccine platforms and conferred improved protection against challenge with antigenically different viruses (Kim et al., 2020, Lee et al., 2016). A universal vaccine that effectively induces both protective M2e and HA stalk immune responses remains to be developed.

3.6 Acknowledgements

This study was supported by NIH/NIAID grants AI093772 (S.M.K.), AI154656 (S.M.K.), and AI147042 (S.M.K). The following reagent was obtained from International Reagent Resource/CDC: Reassortant A/Switzerland/9715293/2013 virus (X-247 rgH3N2, FR-1366) which carries the A/PR8 backbone genes, and H3 HA and N2 NA genes from A/Switzerland/9715293/2013.

4 HEMAGGLUTININ VIRUS-LIKE PARTICLE IS IMMUNOGENIC AND PROVIDES HETEROLOGOUS PROTECTION AGAINST INFLUENZA VIRUS IN YOUNG ADULT AND AGED MICE

4.1 Abstract

Immunosenescence and weak responses to vaccination result in high mortality and morbidity of influenza in elderly population. In this study, we investigated the immunogenicity and cross-protection of virus-like particle containing hemagglutinin from A/Puerto Rico/8/1934 H1N1 (PR8 HA VLP) in 18 months old (aged) mice in comparison with those in young adult mice. PR8 HA VLP vaccination induced homologous and cross-reactive IgG antibodies to A/PR8, A/WSN/1933 (H1N1), and A/California/2009 (H1N1) in young adult and aged mice. Lower IgG responses specific for heterologous virus and HA stalk protein antigens and hemagglutination inhibition titers were induced in aged mice in a dose dependent pattern. Young adult mice with PR8 HA VLP vaccination conferred higher efficacy of homologous protection against A/PR8 and heterologous protection against A/WSN than old age mice. Also, cross-protection against antigenically distinct A/California/2009 was observed in young adult mice but not in aged mice after PR8 HA VLP immunization and challenge. Higher doses of PR8 HA VLP were required for protection in aged mice. Efficacy of controlling lung viral replication against A/PR8 and A/WSN was significantly enhanced in aged mice by incorporation of cytokine-adjuvants (GM-CSF and IL-12) into PR8 HA VLP via protein transfer *in vitro*. Effects of cytokines incorporated onto HA VLP were substantial on inducing IFN- γ ⁺ CD4 and CD8 T cell responses in young adult and aged mice. Induction of IgG antibodies and cross-protection by PR8 HA VLP vaccination was comparable in male and female mice. These results suggest that VLP-based influenza vaccine is immunogenic in

young and old age mice and a strategy of cytokine-adjuvanted HA VLP vaccination might provide more protective benefits in elderly population.

4.2 Introduction

Influenza viruses belong to the Orthomyxoviridae and consist of eight segmented negative-sense single-stranded RNAs (Krammer et al., 2018b). Influenza A viruses are a highly transmissible agent responsible for human respiratory illness annually and causing pandemics (Harrington et al., 2021). CDC estimates that 70-85 percent of seasonal flu-related deaths occur among the people 65 years and older. Effective vaccination has been proven to be the most effective measure to prevent infectious diseases. However, vaccine effectiveness in the elderly population is lower compared to young adult age groups (Rondy et al., 2017). This phenomenon of low responses to vaccination is mainly due to their weakened immune system which is described as immunosenescence and partially due to prior influenza exposure and original antigenic sin (Tanner et al., 2021, Hodgins et al., 2019). Currently, a high dose influenza vaccine and an adjuvanted vaccine were approved in the U.S. for the people 65 years and older (CDC, 2021d, CDC, 2021a). These enhanced influenza vaccines induce elevated hemagglutination inhibition (HAI) titers by improved immunogenicity of vaccines (CDC, 2021c).

Virus-like particles (VLPs) are self-assembled nanoparticles (20-200 μm) that closely resembles the structure of virus in sizes and shapes but lacking viral genomes (Kang et al., 2012). The nanosized VLPs are allowed to traffic into lymph nodes and enable uptake by antigen-presenting cells (APCs) (Cubas et al., 2009). VLPs are immunogenic and induce both humoral and cellular anti-viral immune responses (Crisci et al., 2012). VLPs can be generated to display diverse antigenic epitopes (Frietze et al., 2016). Vaccination of ferrets with VLPs containing influenza

hemagglutinin (HA) from A/California/2009 was shown to induce more protective immunity than conventional inactivated virus vaccination (Hossain et al., 2011). These features represent VLP as a potent and safe vaccine platform especially for older adults and immunocompromised people. Expressing immunostimulatory molecules (ISMs) in VLPs would be an effective strategy to improve the immunogenicity of influenza VLPs, thus developing broader cross-protection (Kang et al., 2012).

Granulocyte macrophage colony-stimulating factor (GM-CSF) and Interleukin-12 (IL-12) as endogenous immune activators were reported to enhance the immunogenicity of vaccines for Ebola, SARS-CoV-2, influenza viruses and tumor in a mouse model (Eager and Nemunaitis, 2005, Herbert et al., 2009, Suschak et al., 2018, Vernet et al., 2021). To deliver these immune activators, VLPs can be genetically modified to express GM-CSF and IL-12. However, this method of genetic approaches applied multiple recombinant baculovirus (rBV) infection to display diverse molecules and was not highly effective in enhancing immune responses to vaccine antigens (Skountzou et al., 2007).

Previous studies have shown *in vitro* incorporation into the VLP lipid membranes of ISMs such as B7-1, IL-12, ICAM-1 or GM-CSF via membrane-anchoring phosphatidylinositol (GPI) conjugates using protein transfer technology (McHugh et al., 1999, Nagarajan and Selvaraj, 2002, Poloso et al., 2002, Patel et al., 2015b), which resulted in enhancing antitumor immune responses. Also, incorporation of GPI anchored GM-CSF to VLPs was reported to improve anti-viral immunity after vaccination of young adult mice (Patel et al., 2015a).

Here, influenza VLP vaccines that express HA from A/PR8 (PR8 HA VLP) were modified to display GPI anchored GM-CSF and GPI-IL-12 on the VLP surface via protein transfer. The goal of

this study was to determine whether influenza VLP vaccines with cytokines incorporated would enhance protective humoral and cellular immunity in aged mice, compared to those in young adult mice. Aged mice required higher doses of influenza VLP vaccine to induce protective immune responses than young adult mice. PR8 HA VLP (PR8 HA-Cy VLP) displaying cytokines (GM-CSF and IL-12) was conferred improved protection against homologous and heterologous influenza viruses in aged mice. PR8 HA-Cy VLP was more effective in inducing cellular immune responses although the impact of cytokine incorporation into VLP on protection was not significant in young adult mice. This study demonstrates a strategy of improving protection by adjuvantation of influenza VLP vaccines with GM-CSF and IL-12 immune activators in young adult and aged mouse models.

4.3 Materials and Methods

4.3.1 Preparation of influenza virus-like particles (VLPs)

VLP expressing HA protein from A/PR/8/1934 H1N1 virus (PR8 HA VLP) was prepared by Medigen, Inc. (Frederick, MD) and incorporation of cytokines including GM-CSF and IL-12 on to PR8 HA VLP (PR8 HA-Cy VLP) was performed by Metacclipse Therapeutics Corporation (Atlanta, GA). In brief, a gene encoding HA (A/PR/8/1934) was codon-optimized for expression in insect cells and cloned into pFastbac plasmid vector with influenza M1 gene for dual expression (M1+HA). Recombinant baculovirus (rBV) expressing M1 and HA was generated in insect cells by using the Bac-to-Bac expression system and transfection with M1+HA pFastbac bacmid DNA as previously described (Pushko et al., 2017, Tretyakova et al., 2016, Tretyakova et al., 2013). PR8 HA VLP was produced in Sf9 insect cells after infection with rBV expressing HA and M1. Soluble cytokines were expressed and purified from CHO-K1 cells after transfection with GM-CSF or IL-

12 expressing vectors that also contains GPI-membrane-anchoring sequence derived from CD59. The GPI-IL-12 construct was placed in a pUB6blast vector (Invitrogen), whereas GPI-GM-CSF construct was placed in a pcDNA3Neo vector (Invitrogen). Purified GPI-cytokines (GM-CSF and IL-12) were incorporated onto PR8 HA VLP by protein transfer (HA-Cy VLP) as previously described (Patel et al., 2015a). Characterization of PR8 HA VLP was performed by Coomassie blue staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and incorporation of GM-CSF or IL-12 was determined by western blot analysis using anti-mouse GM-CSF or anti-mouse IL-12 p40 antibodies (BioXCell) respectively.

4.3.2 Immunization and virus challenges

Young adult female, male (6-8 weeks old, Jackson Laboratories) and aged female (18-month-old, NIH/NIA) BALB/c mice received sequential prime-boost intramuscular vaccination with PR8 HA VLP (HA VLP) or PR8 HA-Cy VLP (HA-Cy VLP with GM-CSF and IL-12 incorporated) in an interval of 3 weeks. Before homologous A/Puerto Rico/8/1934/H1N1 (A/PR8/34) virus challenge (2 LD_{50} , $10^{3.36} \text{ EID}_{50}$), young adult female mice ($n= 6/\text{group}$) were prime-boost immunized with $1 \mu\text{g}$ of HA VLP and aged female mice ($n= 5/\text{group}$) were prime-boost immunized with $1 \mu\text{g}$ or $3 \mu\text{g}$ of HA VLP or HA-Cy VLP (Figure 36). Young female mice ($n= 4/\text{group}$) were prime-boost immunized with $1 \mu\text{g}$ or $3 \mu\text{g}$ of VLP or HA-Cy VLP prior to A/WSN/1933/H1N1 (A/WSN/33) virus infection (2 LD_{50} , $10^{1.06} \text{ EID}_{50}$ or 8 LD_{50} , $10^{1.66} \text{ EID}_{50}$ respectively, Figure 37). Aged female mice ($n= 3-6/\text{group}$) were prime-boost immunized with $1 \mu\text{g}$ or $6 \mu\text{g}$ of VLP or HA-Cy VLP before 1.5 LD_{50} (8.7 EID_{50}) of A/WSN/33 virus challenge (Figure 37). Young female mice ($n= 3/\text{group}$) were prime-boost immunized with $6 \mu\text{g}$ of VLP or HA-Cy VLP prior to A/California/04/2009/H1N1 (A/Cal/09) virus infection (Figure 39). Young male mice ($n= 3/\text{group}$)

were prime-boost immunized with 1 µg of VLP or HA-Cy VLP prior to infection by 8 LD₅₀ (8.7 EID₅₀) of A/WSN/33 virus or 2 LD₅₀ (10^{3.6} EID₅₀) of A/Cal/09 virus (Figure 41). All animal experiments in this study were approved by the Georgia State University Institutional Animal Care and Use Committee (IACUC) review boards. Mouse animal experiments including virus infection, blood and tissue collections were performed in accordance with the approved IACUC protocol (A21004) and regulations.

4.3.3 Enzyme linked immunosorbent assay (ELISA)

IgG antibodies specific for the viruses were measured by ELISA with coating antigens (4 µg/ml) from inactivated A/PR8/34, A/WSN/33, A/Cal/09 H1N1 and A/Viet rgH5N1 viruses. A/PR8/34 specific IgG isotypes (IgG1 and IgG2a) were measured. HA stalk specific IgG antibodies were determined using consensus group 1 HA stalk protein as coating antigens constructed and prepared as previously reported (Chae et al., 2019). HRP (Horseradish Peroxidase) conjugated goat anti-mouse IgG, IgG1, and IgG2a (Southern Biotechnology) were used as a secondary antibody. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Invitrogen™) was utilized for color development. IgG antibody levels were read by BioTek ELISA plate reader at 450nm.

4.3.4 Hemagglutination inhibition (HAI) titers

Mouse prime-boost immunized sera were treated with receptor destroying enzymes (RDE, Sigma) with 1:3 ratio and incubated at 37 °C overnight and inactivated at 56°C for 30 minutes. RDE-treated serum samples were serially diluted (two-fold) and treated with the equal volume of viruses (4 hemagglutination activity units). HAI titers were measured as the highest dilution factor inhibiting the formation of buttons with 0.5% chicken red blood cells (RBC).

4.3.5 Assays of lung viral titers and inflammatory cytokines

Lung lysates were obtained day 5 - 9 post infection from the lung homogenates in 1.5ml of RPMI media after centrifugation. Virus titers were determined by calculating 50% egg infectious dose (EID₅₀) after incubation at 37°C for 3 days in the 10-day-old embryonated chicken eggs. Cytokine interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- α), and interferon gamma (IFN- γ) ELISA was performed as described previously using Ready-Set-Go kits (eBioscience, San Diego, CA).

4.3.6 Intracellular cytokine staining of T cells

Lung cells were stimulated with inactivated A/PR8/33 (4 μ g/ml) or A/PR8 HA peptides (CD4⁺ epitopes (2.5 μ g/ml each peptide): SFERFEIFPKE, HNTNGVTAACSH, CPKYVRS AKLRM, KLKNSYVNKKGK, NAYVSVVTSNYNRRF, CD8⁺ epitopes (5 μ g/ml each peptide): LYEKVKSQL, IYSTVASSL)) for 5 h at 37 °C in the presence of brefeldin A (BFA) (20 μ g/mL). After stimulation, lymphocytes were stained with T cell marker antibodies for CD4 (CD4-PE/Cy5, BD Biosciences) and CD8 (CD8 α -PE, Biolegend) by following a procedure of BD Cytofix/Cytoperm Plus Kit. Intracellular staining of the permeabilized lymphocytes was conducted with IFN- γ cytokine mAb (anti-mouse IFN- γ -APC/Cy7, BD Biosciences). All samples were analyzed by using LSR-II/Fortessa flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed using the FlowJo software (FlowJo V10, Tree Star, Inc.).

4.3.7 Statistical analysis

Two-way or one-way ANOVA were used to determine the statistical significance when comparing two different conditions. P-values of less than or equal to 0.05 were considered

significant. Data analysis was performed using Prism software (GraphPad software Inc., San Diego, CA).

4.4 Results

4.4.1 PR8 HA VLP and PR8 HA-Cy vaccines are immunogenic in aged mice at a higher dose compared to young adult mice

To enhance the immunogenicity, endogenous immune activators (cytokines) such as granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-12 (IL-12) were incorporated into PR8 HA VLP via a glycosylated phosphatidylinositol (GPI) anchor (Prepared by Medigen, Inc. (Frederick, MD) and Metacclipse Therapeutics Corporation (Atlanta, GA) as in described in materials and methods section, Figure 31A) (Patel et al., 2015a). A/PR8 hemagglutinin (HA) H1 and matrix M1 proteins from PR8 HA VLP expressed and purified from insect cells were characterized by SDS-PAGE and Coomassie blue staining (Figure 31B), indicating the high purity and major components HA and M1. The incorporated cytokines were confirmed by western blot analysis with anti-GM-CSF and anti-IL-12 antibodies (Figure 31C).

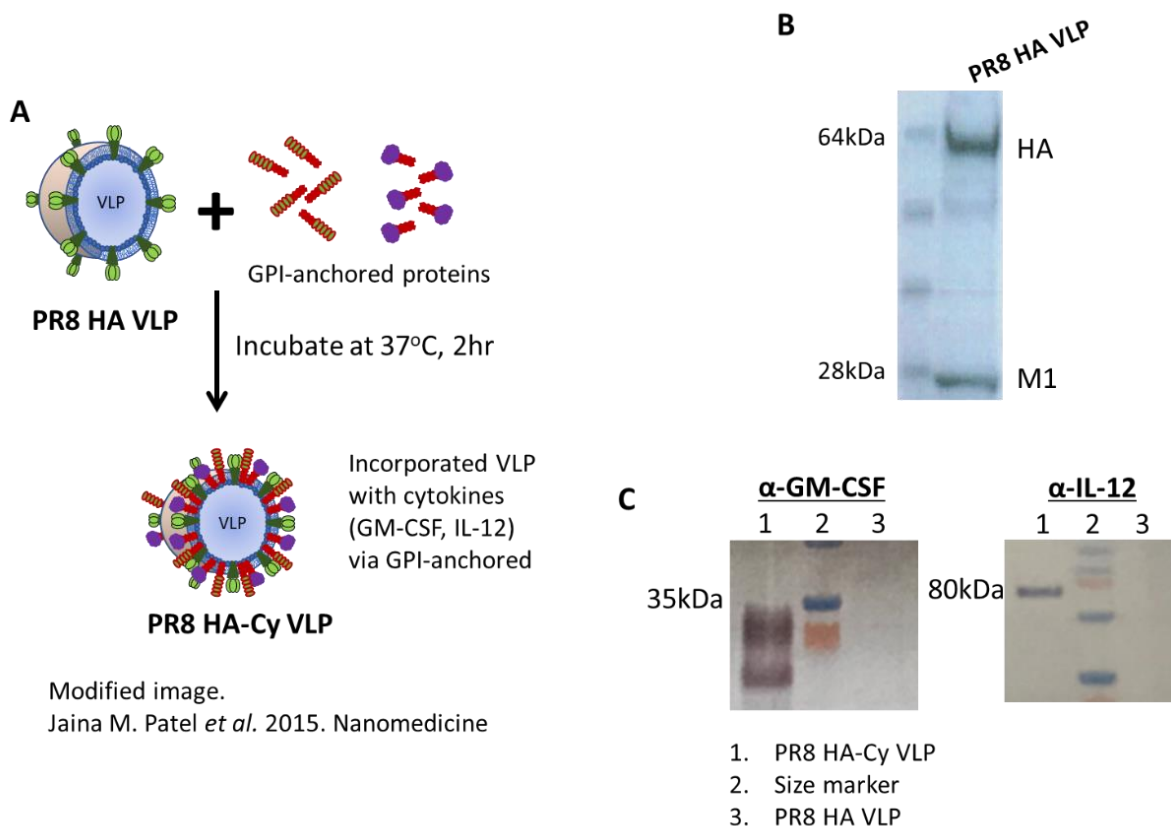


Figure 31 Preparation of PR8 HA VLP and PR8 HA-Cy VLP vaccines

(A) Schematic diagram of protein transfer of GPI-anchored cytokines (GM-CSF and IL-12) onto PR8 HA VLP. (B) SDS-PAGE gel staining of PR8 HA VLP expressed in insect cells after purification, displaying HA and M1 proteins as major components. (C) Western blot analysis of incorporated GM-CSF or IL-12 in PR8 HA VLPs using anti-mouse GM-CSF antibody and anti-mouse IL-12 p40 antibody respectively. [Define PR8 HA VLP and PR8 HA-Cy VLP here] PR8 HA VLP: Virus-like particle (VLP) that expresses HA from A/PR8/34, PR8 HA-Cy VLP: PR8 HA VLP incorporated with GM-CSF and IL-12.

Here we focused on investigating the homologous immunogenicity and efficacy of A/PR8/1934 (H1N1) HA VLP (HA VLP) and PR8 HA VLP incorporated with GM-CSF and IL-12 (HA-Cy VLP) in old aged BALB/c mice (16-18 months old age) in comparison with those in young adult BALB/c mice. Young adult (6-8 weeks old, Figs. 32A-C) and aged (16 – 18 months old, Figs. 32D-F) BALB/c mice were intramuscularly immunized with HA VLP or HA-Cy VLP at different doses (1, 3 or 6 μ g, respectively in Figure 32). After low dose (1 μ g) prime vaccination of each VLP, young adult mice induced higher levels of A/PR8 specific IgG antibody response compared to those in

aged mice (Figs. 32A, D). With increasing the HA VLP and HA-Cy VLP vaccine dose to a moderate (3 μ g) to high (6 μ g) level, the aged mouse groups after prime vaccination induced substantially higher responses of IgG antibodies specific for homologous A/PR8 virus despite slightly lower than those in young adult mice (Figs. 32B, C, E, F). Boost immunization effects on enhancing IgG antibody responses were most prominent with 1 μ g of HA VLP or HA-Cy VLP primed young adult age and elderly aged mice (Figs. 32A, D). Boost immunization with 3 μ g or 6 μ g of both HA VLP and HA-Cy VLP resulted in moderate increases in IgG levels specific for A/PR8 virus, where IgG levels in young adult mice (Figs. 32B, C) were still significantly higher than those in aged mice (Figs. 32E, F). There were more prominently lower levels of IgG2a isotypes in aged mice (Figs. 33E, H) than those in young adult mice (Figs. 33B, D) although both IgG1 and IgG2a antibodies were lower in aged mice (Figs. 33E-H). Moderate to high doses of HA-Cy VLP (3 or 6 μ g) immunization induced slightly increased IgG1 antibody responses to A/PR8 compared to HA VLP inoculation in both young and aged mice (Figs. 33A, C, E, G). These results suggest that HA VLP and HA-Cy VLP effectively primed the induction of A/PR8 specific IgG antibodies in aged mice in a dose dependent manner. The capacity to induce IgG antibodies to low dose HA VLP or HA-Cy VLP prime and boost vaccination was significantly compromised in aged mice compared to young adult mice.

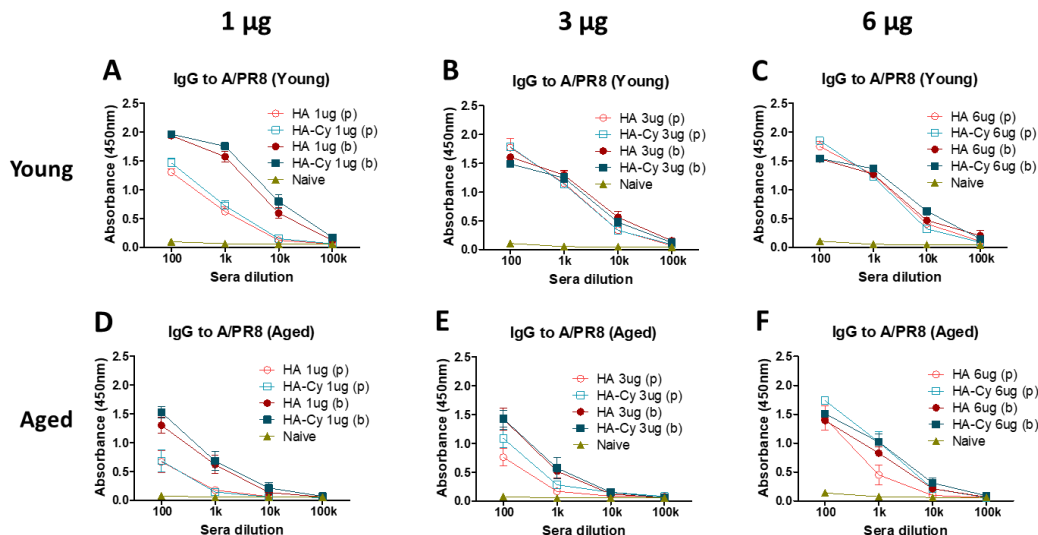


Figure 32 Intramuscular immunization of PR8 HA VLP and PR8 HA-Cy VLP induces IgG antibodies specific for homologous A/PR8 virus in young and aged mice

Young adult and aged BALB/c mice (n= 6/group) were immunized with 1 µg (A, D), 3 µg (B, E) or 6 µg (C, F) of HA VLP (HA) or HA-Cy VLP (HA-Cy). Mouse sera were collected at 2 weeks after prime or boost immunization. IgG antibody responses to A/PR8 virus were measured in prime (p) or boost (b) immune sera. Each individual mouse is analyzed. Error bars indicate mean ± SEM.

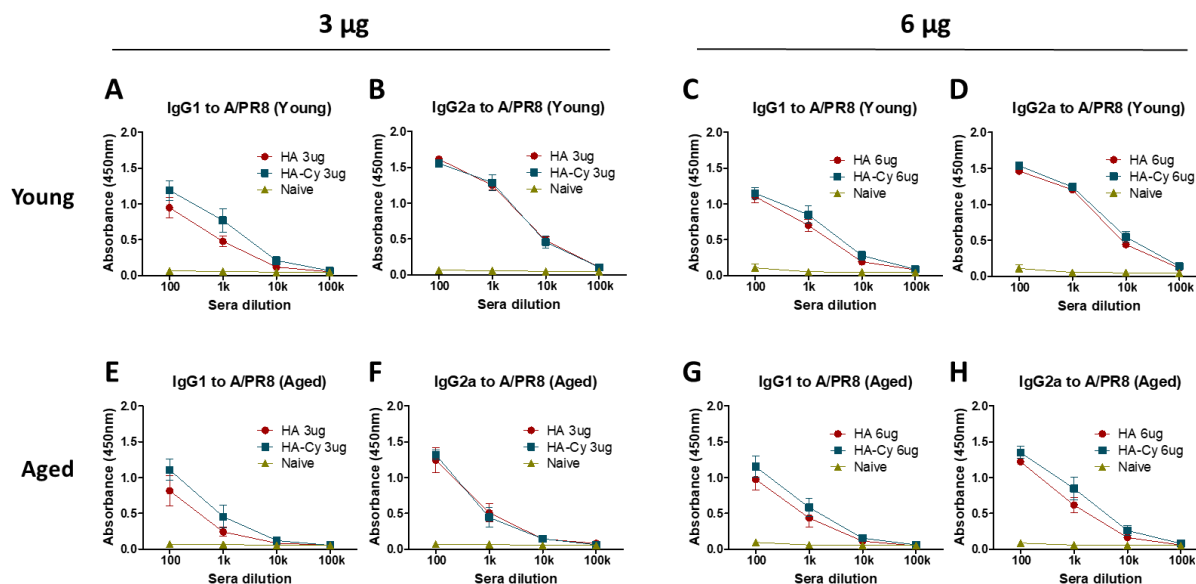


Figure 33 A/PR8 specific serum IgG isotype antibody responses after boost dose of PR8 HA VLP or PR8 HA-Cy VLP

Young adult (A-D) and aged (E-H) BALB/c mice (n= 6/group) were received boost dose of HA VLP (HA) or HA-Cy VLP (HA-Cy). IgG1 and IgG2a antibody responses to A/PR8 2 weeks after 3 µg (A, B, E, F) or 6 µg (C, D, G, H) of each VLP inoculation. Each individual mouse is analyzed. Error bars indicate mean ± SEM.

4.4.2 Aged mice induce low levels of cross-reactive IgG antibody responses after

HA VLP or HA-Cy VLP vaccination

Sera of boost immunization with HA VLP or HA-Cy VLP were analyzed for cross reactivity against heterologous viruses, A/WSN/1934 (H1N1) and A/California/2009 (H1N1) in young (Figure 34A-G) and aged mice (Figure 34H-M). Boost sera from aged mice displayed significantly lower levels (~3 folds) of IgG antibodies specific for A/WSN/1934 (H1N1) (Figure 34H, K) and A/California/2009 (H1N1) (Figure 34I, L) than those from young adult mice after immunization with 3 µg (Figure 34A-C, H-J) or 6 µg (Figure 34E-G, K-M) of each VLP (Figure 34A, B and Figure 34E, F respectively). With a higher dose (6 µg) of HA VLP or HA-Cy VLP boost sera from young adult and aged mice showed increased levels of IgG antibody reactivity to A/WSN (Figure 34E, K) than with a 3 µg each VLP (Figure 34A, H). We observed a similar pattern of cross-reactive IgG antibodies recognizing A/California/2009 (H1N1) but at significantly lower levels than those recognizing A/WSN in both young adult and elderly aged mice, probably due to antigenic distance (Figure 34B, F, I, L). Approximately 2-fold higher IgG antibodies to group 1 stalk were induced from young adult mice than aged mice after boost dose (both 3 and 6 µg) of HA VLP or HA-Cy VLP (Figure 34C, G compared to Figure 34J, M). These results suggest that elderly aged mice have more limited capacity to induce cross reactive IgG antibody responses to heterologous viruses than young adult mice.

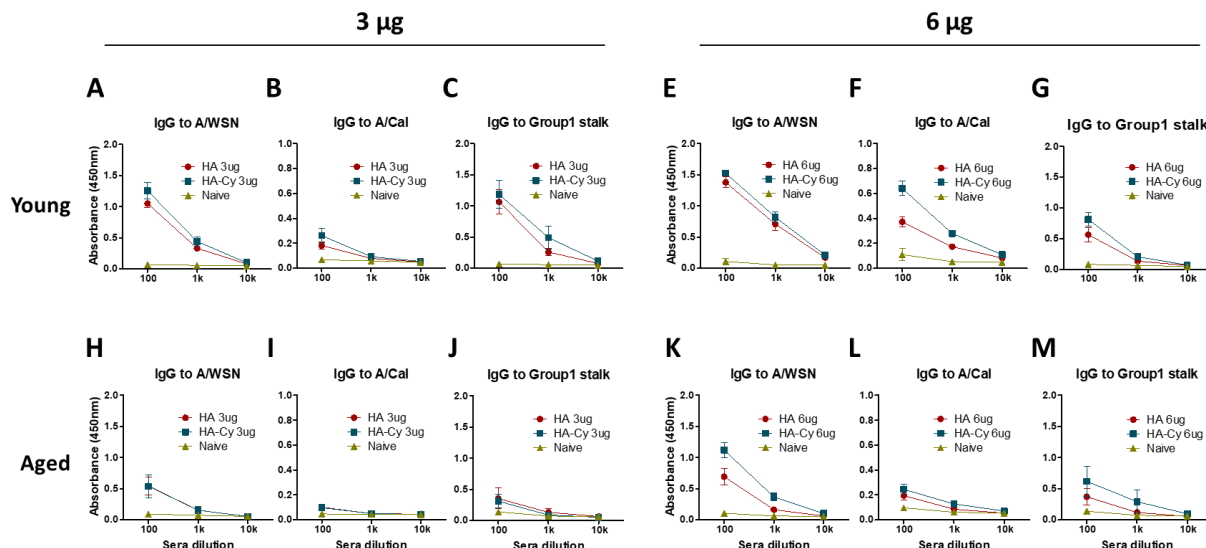


Figure 34 IgG antibody reactivities to A/WSN, A/Cal and group 1 stalk domain induced by vaccination with PR8 HA VLP or PR8 HA-Cy VLP in young and aged mice

Immune sera were collected at 2 weeks after boost immunization with HA VLP or HA-Cy VLP from young adult (A-G) and aged (H-M) Balb/c mice ($n = 6/\text{group}$). HA VLP (HA) or HA-Cy VLP (HA-Cy) with vaccine dose 3 μg (A-C, H-J) or 6 μg (E-G, K-M) were inoculated intramuscularly in two doses at an interval of 3 weeks. IgG antibody responses to A/WSN (A, E, H, K), A/Cal (B, F, I, L), and group 1 stalk domain (C, G, J, M). Each individual mouse is analyzed. Error bars indicate mean \pm SEM.

4.4.3 HA VLP and HA-Cy VLP induces hemagglutination inhibition activity against homologous at higher levels in young adult than those in aged mice

To determine hemagglutination inhibition (HAI) titers, sera from young adult and aged mice were collected after boost immunization with 1, 3 or 6 μg of HA VLP or HA-Cy VLP (Figure 35). HAI activity was observed in all doses of immune sera against homologous A/PR8 but not against heterologous viruses including A/WSN and A/Cal. Although both age groups with HA VLP or HA-Cy VLP immunization showed significantly increased HAI activity than the naive group, young adult mice showed 20-fold higher HAI titers than aged mice after 1 μg of HA VLP or HA-Cy VLP immunization (Figure 35A). With higher doses of immunization (3 or 6 μg), 8-16 folds increased HAI titer was observed from aged mice (Figs. 35B, C). These results support that high dose vaccination is required for aged mice.

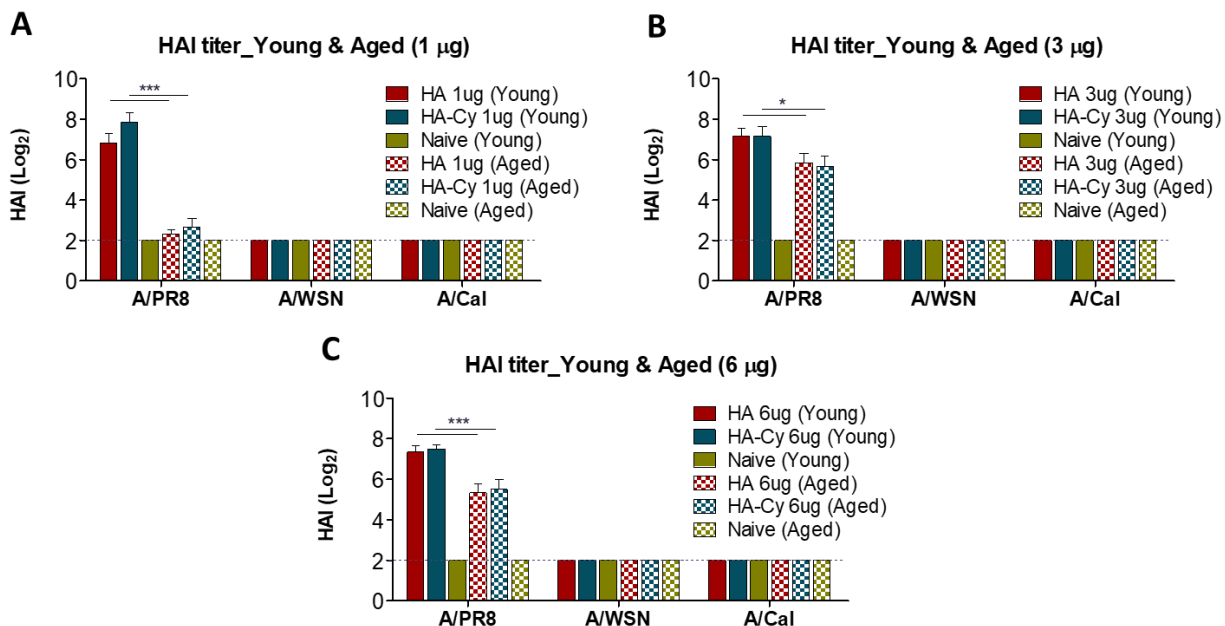


Figure 35 Serum hemagglutination inhibition (HAI) titers against homologous and heterologous viruses

HAI titers against the viruses (A/PR8, A/WSN, and A/Cal) were determined in sera from young adult and aged mice ($n = 6/\text{group}$) at 2 weeks after boost immunization with HA VLP or HA-Cy VLP. (A-C) 1 µg, 3 µg or 6 µg of each VLP were administered respectively. Each individual mouse is analyzed. The statistical significances were determined using two-way ANOVA and indicated in *, $P < 0.1$; ***, $P < 0.001$. Error bars indicate mean \pm SEM.

4.4.4 A low dose HA VLP confers homologous protection in young adult mice whereas higher dose HA VLP is required for protection in aged mice

To determine the protection efficacy, young adult BALB/c mice were challenged with a lethal dose of A/PR8 at 3 or 4 weeks after HA VLP low dose (1 µg) boost immunization. Vaccinated mice did not lose body weight meanwhile the naïve group reached humane end point (20%) after A/PR8 challenge (Figure 36A). Also, at day 5 after A/PR8 challenge, lung virus titers were determined by measuring 50 % egg infectious dose (EID_{50}) from the HA VLP vaccinated and naïve young adult groups (Figure 36B). No virus was detected from HA VLP young adult group in contrast to the naïve group displaying high lung virus titers ($7.4 \log_{10}\text{EID}_{50}/\text{ml}$). Similarly, the aged

BALB/c mice immunized with low dose HA VLP or HA-Cy VLP (1 μ g) were challenged with a lethal dose of A/PR8 at 4 weeks after boost. The aged mouse group with low dose of HA VLP vaccination showed severe weight loss ($\sim 20\%$) with 80% survival rates whereas HA-Cy vaccinated group lost less body weight ($\sim 10\%$) and survived at 100%. Unvaccinated mice did not survive after infection (Figure 36C, D). For further assessment, the aged BALB/c mouse groups that were vaccinated with 3-fold higher dose (3 μ g) of HA VLP or HA-Cy VLP were challenged with a lethal dose of A/PR8. No body weight loss was observed in both HA VLP and HA-Cy groups (Figure 36E). HA VLP vaccination displayed lower lung virus titers (2.4 \log_{10} EID₅₀/ml, Figure 36F) compared to the unvaccinated naïve group showing 1,000 folds higher titers (7.4 \log_{10} EID₅₀/ml). Notably, no virus was detected from the HA-Cy VLP aged mice group (Figure 36F). All together, these results suggest that a lower dose HA VLP can confer homologous protection in young mice whereas higher dose HA VLP is required for protection in aged mice. Also, HA-Cy VLP can improve the protection by more effectively controlling the viral replication in the lung.

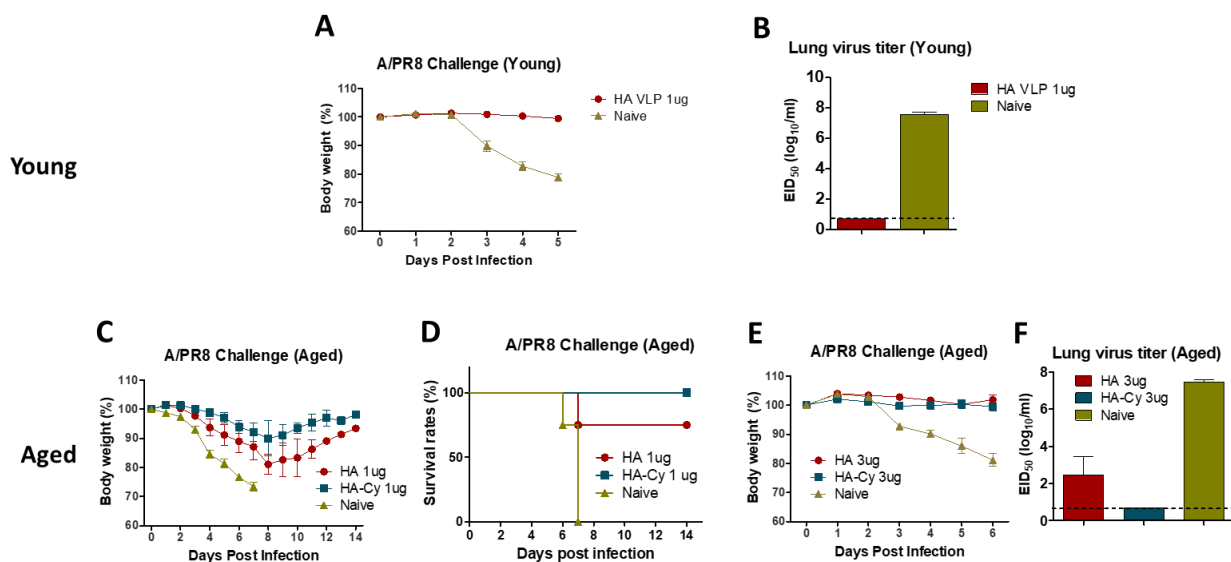


Figure 36 Aged mice need a higher dose of PR8 HA-Cy VLP to induce protection against homologous A/PR8 virus than young adult mice

Young adult mice (n= 6/group, A, B) were prime-boost immunized with a low dose (1 µg) of HA VLP prior to A/PR8 virus infection (2 LD₅₀, 10^{3.36} EID₅₀). (A) Body weight changes and (B) lung virus titer at day 5 after A/PR8 challenge. Aged BALB/c mice (n= 5/group) were prime-boost immunized with 1 µg (C, D) or 3 µg (E, F) of HA VLP or HA-Cy VLP. (C, E) Weight changes, (D) survival rate and (F) lung virus titer at day 6 after A/PR8 virus (2 LD₅₀) challenge. Naïve: Naïve mice with infection. Each individual mouse is analyzed. Error bars indicate mean ± SEM.

4.4.5 HA VLP or HA-Cy VLP vaccine even at low dose provides heterologous cross-protection against A/WSN in young adult mice

We determined whether HA VLP or HA-Cy VLP would induce cross-protection against A/WSN/1933. At 3 weeks after low dose (1 µg HA VLP prime and boost vaccination), young adult BALB/c mice were challenged with a lethal dose of A/WSN (Figure 37A). HA VLP or HA-Cy VLP vaccinated mice did not lose body weight after infection while the naïve control group showed severe body weight loss and did not survive (Figure 37A). In an additional set to further test cross-protection, the mice vaccinated with 3 µg of prime-boost HA VLP or HA-Cy VLP were challenged with 4 folds higher dose of A/WSN. The HA VLP or HA-Cy VLP immune mice were well cross protected against A/WSN without displaying weight loss and lung virus titers were below the limit of detection (Figure 37B) at day 8 after challenge. In addition, lower inflammatory cytokine such as IL-6 was detected from the lung of vaccinated groups with HA VLP or HA-Cy VLP (3 µg) compared to naïve mice after A/WSN infection (Figure 38A). These results suggest that HA VLP or HA-Cy VLP vaccine even at low dose can provide heterologous cross-protection against A/WSN in young adult mice.

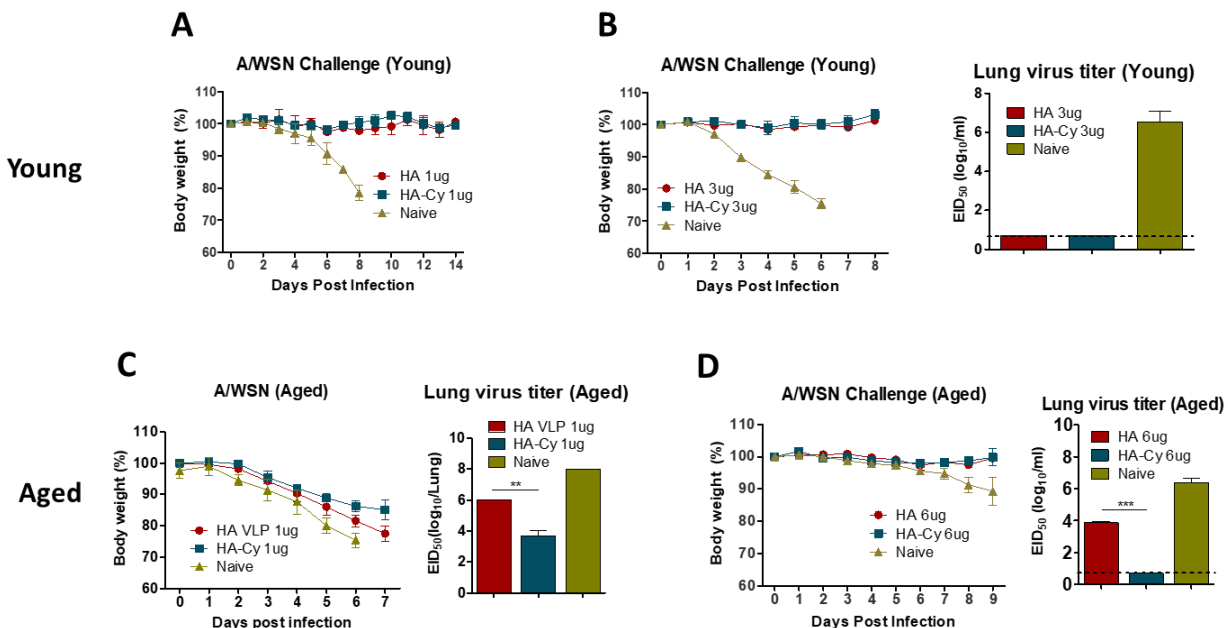


Figure 37 Aged mice require a higher dose of PR8 HA-Cy VLP to provide cross-protection against A/WSN virus than young adult mice

Young female adult BALB/c mice (n = 4/group) were prime-boost immunized with 1 µg (A) and 3 µg (B) of HA VLP or HA-Cy VLP before heterologous virus challenge. (A) Body weight changes after A/WSN infection (2 LD₅₀, 10^{1.06} EID₅₀) in a low dose (1 µg) of each VLP vaccine groups. (B) Weight changes and lung virus titer at day 8 after A/WSN (8 LD₅₀, 10^{1.66} EID₅₀) challenge in higher dose (3 µg) of each VLP vaccine groups. (C, D) Aged BALB/c (n = 3-6/group) mice received prime-boost immunization with 1 µg (C) or 6 µg (D) of HA VLP or HA-Cy VLP before A/WSN infection (1.5 LD₅₀, 8.7 EID₅₀). Body weight changes and lung virus titers at day 9 (D) after A/WSN (1.5 LD₅₀, 8.7 EID₅₀) challenge. Naïve: Naïve mice with infection. Each individual mouse is analyzed. Error bars indicate mean ± SEM. The statistical significances were determined using One-way ANOVA and Turkey's multiple comparison test and indicated in **, P < 0.01; ***, P < 0.001.

4.4.6 Aged mice require a higher dose HA VLP or HA-Cy VLP vaccine to induce heterologous cross-protection

To determine the efficacy of cross-protection in aged BALB/c mice, HA VLP low dose (1 µg) boosted mice were challenged with A/WSN (Figure 37C). At day 7 after challenge, all mouse groups including HA VLP, HA-Cy VLP and naïve lost body weight (18%, 14% and 25% loss respectively, Figure 37C). PR8 HA-Cy VLP immunized mice displayed approximately 100-fold lower lung virus titer (3.7 log₁₀EID₅₀/ml) than PR8 HA VLP group (6 log₁₀EID₅₀/ml). Both

immunized groups showed reduced lung virus titer compared to naïve control group ($8 \log_{10}\text{EID}_{50}/\text{ml}$) at day 7 after challenge (Figure 37C). To determine the effect of higher dose vaccines, aged BALB/c mice prime-boost immunized with 6 μg of HA VLP or HA-Cy VLP were challenged with a lethal dose of A/WSN. Weight loss was not observed from the vaccinated groups with 6 μg of HA VLP or HA-Cy VLP while naïve mice lost 11% body weight by day 9 after infection (Figure 37D). Since HA VLP or HA-Cy immunized aged mice did not show weight loss, we determined lung viral titers. Approximately 300-fold lower lung virus titer was detected from the HA VLP immunized mice ($3.9 \log_{10}\text{EID}_{50}/\text{ml}$) than the naïve group ($6.4 \log_{10}\text{EID}_{50}/\text{ml}$) and no virus was detectable in HA-Cy VLP group (Figure 37D). As in young mice (Figure 38A), reduced inflammatory cytokines including TNF- α , IFN- γ and IL-6 were detected from the lung of vaccinated groups with HA VLP or HA-Cy VLP (3 μg) compared to naïve mice after A/WSN infection (Figure 38B). Intracellular cytokine staining data displayed significantly increased levels of IFN- γ secreting CD4 T cells (Figure 38C) and CD8 T cells (Figure 38D) upon stimulation with HA peptides or inactivated A/PR8 virus in lung cells from HA VLP or HA-Cy VLP (3 μg) boosted both young and aged mice compared to unvaccinated naïve mice. Moreover, elevated IFN- γ secreting CD4 T (Figure 38C) cells and CD8 T cells (Figure 38D) were observed in HA-Cy VLP boosted group compared to HA VLP vaccine group in all cases. Taken together, these results indicate that T cell immunity induced by HA VLP and HA-Cy VLP. Also, aged mice require a higher dose HA VLP vaccine to induce heterologous cross-protection against A/WSN virus and HA-Cy VLP might contribute on enhanced cross-protection in aged mice.

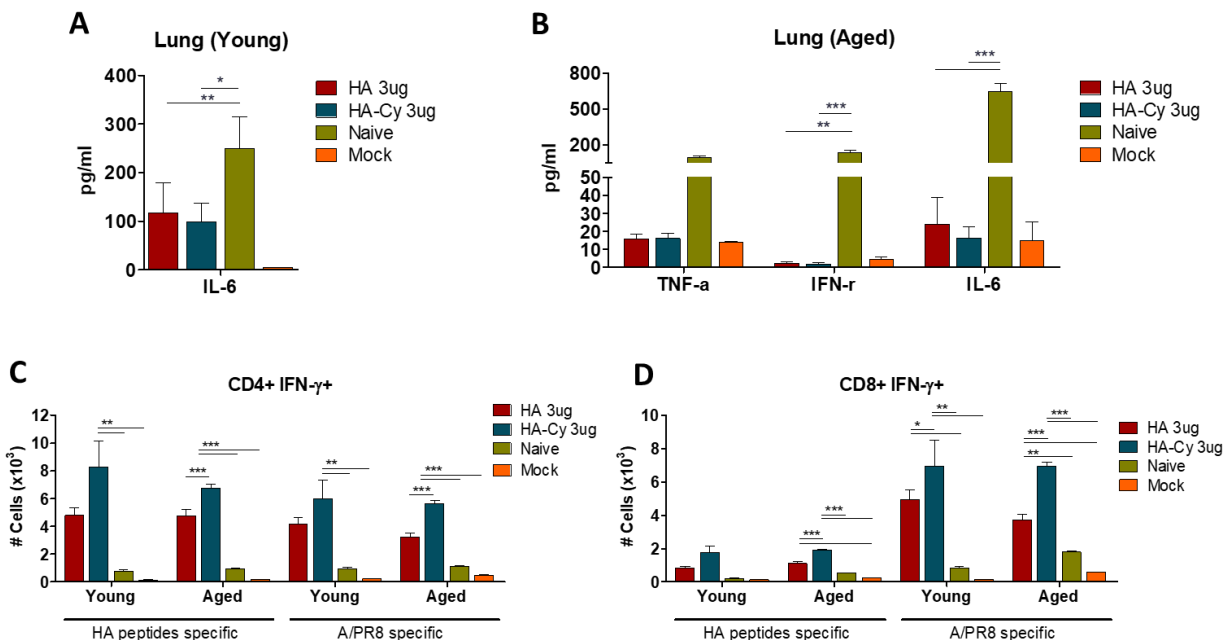


Figure 38 PR8 HA VLP or PR8 HA-Cy VLP vaccination reduced inflammatory cytokines and induced T cell responses in young and aged mice

Inflammatory cytokines and T cell responses were determined in lung tissues collected day 5 post challenge from young adult and aged BALB/c mice ($n = 6/\text{group}$) that were prime-boost immunized with 3 μg of HA VLP or HA-Cy VLP. (A) Inflammatory IL-6 in lung lysates from young mice after A/WSN challenge (8 LD_{50} , $10^{1.66} \text{ EID}_{50}$). (B) Inflammatory cytokines TNF- α , IFN- γ and IL-6 in lung lysate from aged mice after A/WSN challenge (1.5 LD_{50} , 8.7 EID_{50}). IFN- γ -producing CD4+ T cells (C) or CD8+ T cells (D) specific for HA peptides and A/PR8 after intracellular staining were gated and quantified by flow cytometry from lung cells at day 5 after challenge by A/WSN (young adult mice: $10^{1.66} \text{ EID}_{50}$, aged mice: 8.7 EID_{50}). Each individual mouse is analyzed. Error bars indicate mean \pm SEM. The statistical significances were determined using One-way ANOVA and Turkey's multiple comparison test and indicated in *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

4.4.7 PR8 HA VLP vaccine provides heterologous cross-protection against antigenically distant A/California/2009 virus in young adult mice

To test cross-protection against an antigenically distant virus, young adult mice boosted with HA VLP or HA-Cy VLP (6 μg each) were challenged with a lethal dose of A/California/2009 at 3 weeks after boost (Figure 39). The young adult HA VLP or HA-Cy VLP immune mice displayed 100% survival rate and less weight loss (15%) than the naïve group (>25%) that did not survive

after A/Cal challenge. Collectively, these data suggest that HA VLP or HA-Cy VLP can provide survival protection against antigenically distant A/Cal in young adult mice.

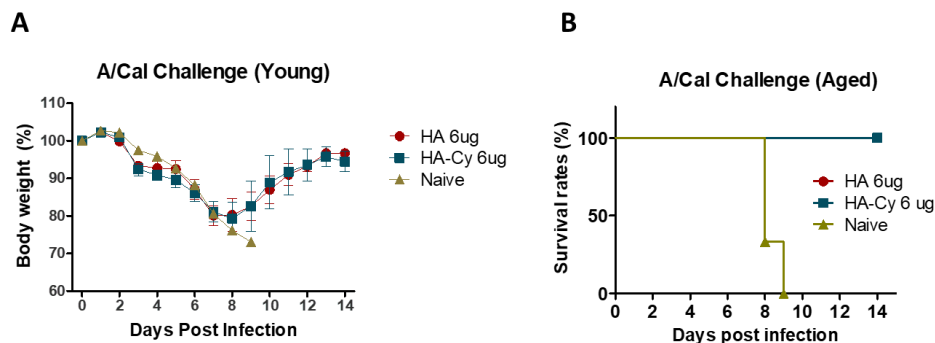


Figure 39 PR8 HA VLP vaccination induces survival protection against heterologous A/Cal virus in young mice

(A) Body weight changes and (B) survival rate after challenge with A/Cal virus (2 LD₅₀, 10^{3.6} EID₅₀) from young adult mice (n= 3/group) immunized with 6 µg of HA VLP or HA-Cy VLP. Each individual mouse is analyzed. Error bars indicate mean ± SEM.

4.4.8 Male BALB/c mice induce comparable immune responses and cross-protection after PR8 HA VLP vaccination

Gender is an important biological parameter to be considered in determining the vaccine effectiveness. Virus specific IgG antibody responses in immune sera from young adult male BALB/c mice (n= 6/group) prime-boost immunized with HA VLP or HA-Cy VLP (1 µg each) were determined in comparison with those from young adult female BALB/c mice (Figure 40). The levels of A/PR8 specific IgG antibodies at 2 weeks after prime immunization (Figure 40A) and A/PR8 specific IgG, IgG1, or IgG2a antibody responses after boost vaccination (Figs. 40B-D respectively) of male mice were comparable with those of female mice. As induced in female mice, a similar pattern in the levels of IgG antibody responses specific for A/WSN (Figure 40E), A/Cal (Figure 40F) or group 1 HA stalk domain (Figure 40G) were observed in young adult male mice.

HA VLP or HA-Cy VLP (1 μ g each) boosted male mice were protected against A/WSN while the naïve group did not survive (Figure 41A). When the mice groups were infected with A/Cal (Figure 41B), significantly enhanced protection was observed in the immunized mice with 1 μ g of each VLP (11% body weight loss) compared to the naïve group (17% body weight loss). Taken together, these results suggest that male BALB/c mice induced comparable immune responses and cross-protection after HA VLP or HA-Cy VLP vaccination as observed in female mice.

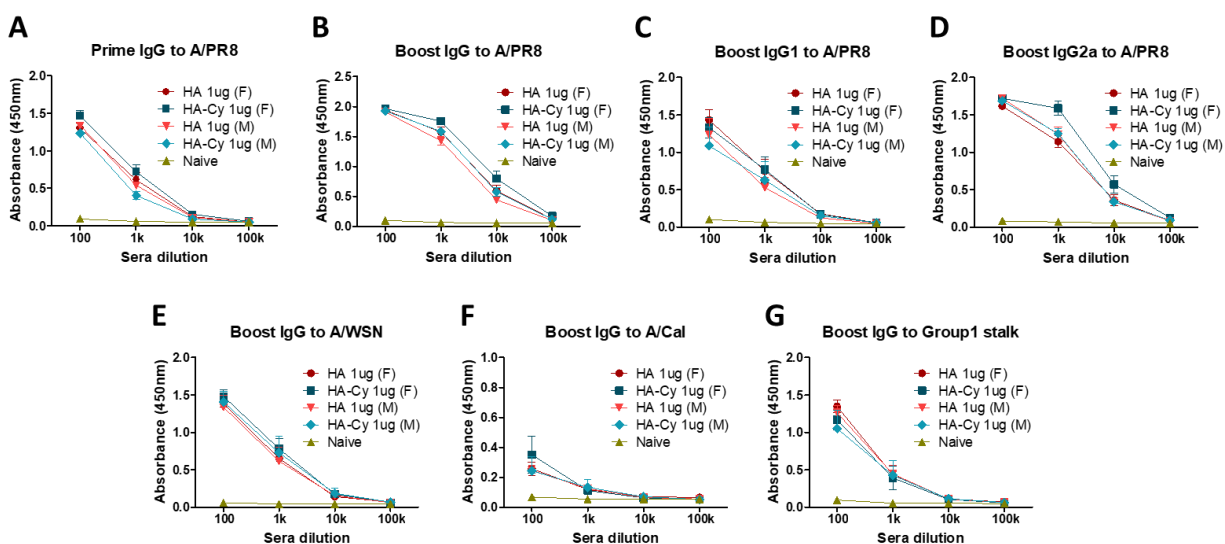


Figure 40 Comparable levels of serum IgG antibody responses are induced in female and male young adult mice after PR8 HA VLP immunization

BALB/C mice (n= 6/group) of young female (f) and male (m) were immunized with prime-boost doses (1 μ g) of HA VLP or HA-Cy VLP at an interval of 3 weeks. IgG antibody responses in mouse prime (A) and boost sera (B-G) were measured by ELISA at 2 weeks after each immunization. IgG (A, B), IgG1 (C), and IgG2a (D) antibody responses to A/PR8. IgG antibodies to A/WSN (E), A/Cal (F) and group 1 stalk domain (G). Each individual mouse is analyzed. Error bars indicate mean \pm SEM.

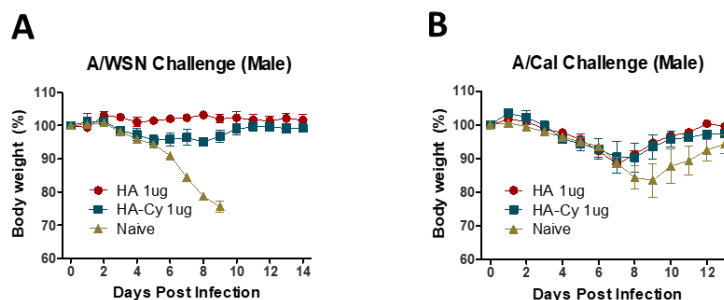


Figure 41 Male young adult mice vaccinated with PR8 HA VLP or PR8 HA-Cy VLP are protected against heterologous A/WSN or A/Cal virus challenge

Body weight changes after challenge with (A) A/WSN (8 LD₅₀, 8.7 EID₅₀) or (B) A/Cal virus (2 LD₅₀, 10^{3.6} EID₅₀) in young adult male mice (n= 3/group) immunized with 1 μ g of HA VLP or HA-Cy VLP. Each individual mouse is analyzed. Error bars indicate mean \pm SEM.

4.5 Discussion

Prior clinical trials in healthy adults reported acceptable safety and immunogenicity results of influenza HA VLP vaccines (2009 H1N1, H5N1, H7N9) produced in insect cells, comparable to conventional influenza vaccines (Pillet et al., 2019, Lopez-Macias et al., 2011, Landry et al., 2010, Fries et al., 2013), supporting VLP as a potential vaccine platform. In this study to better understand immune responses to vaccination in elderly, we compared dose-dependent immunogenicity and efficacy against homologous and heterologous influenza viruses in young adult and aged mouse models. The aged mice required higher doses to induce IgG antibodies for homologous viral antigens but displayed low capacity to generate IgG antibodies for heterologous viruses and HA stalk proteins, compared to young adult mice. The immunogenicity and efficacy of HA VLP were comparable in female and male mice. To enhance vaccine efficacy in aged mice, the adjuvant effects of cytokines (GM-CSF, IL-12) incorporated onto HA VLP (HA-Cy VLP) were investigated. Cytokine adjuvant effects of HA-Cy VLP were prominent on inducing T

cell responses in young and aged mice, and on lowering lung viral titers in aged mice after vaccination and challenge.

Intramuscular vaccination with HA VLP (A/Cal/09, 10 µg) was previously shown to confer protection against homologous A/Cal virus and partial protection against heterologous A/WSN/33 in young adult female BALB/c mice (Quan et al., 2010). High dose (10 or 40 µg) of PR8 HA VLP repetitive intranasal immunization induced protection against homologous (A/PR8/34) and heterologous (A/WSN) viruses (Quan et al., 2007). These previous reports are consistent with the results from this study demonstrating high IgG antibodies and HAI titers by low dose (1 µg) PR8 HA VLP in young adult mice. In contrast, higher doses (3 or 6 µg) of PR8 HA VLP were required to induce substantial HAI titers in aged mice, which were still lower than those in young adult mice. This result supports the current vaccine strategy that high dose influenza vaccines (4-fold higher dose than standard vaccine) are recommended for adults ages 65 and older (Li et al., 2021). A/WSN/33 is antigenically closer to A/PR8/34 (87.1 % amino acid sequence homology in HA1) compared to A/Cal/09 (76.5% amino acid sequence homology), suggesting that antigenic distance is correlating with the levels of cross-reactive IgG antibodies. Immunological impairment in aged mice was more prominent in generating IgG antibodies for heterologous viral antigens (A/WSN, A/Cal) and immune-subdominant HA stalk proteins compared to young adult mice. This is in line with the aging immunosenescence and waning immune system, severely limiting the B cell repertoire diversity in the elderly population at greater risk of influenza infection compared to young individuals (Nikolich-Zugich, 2018, Reber et al., 2012). Moreover, reduced affinity maturation of antibody via somatic hypermutation (SHM) caused by the defects in germinal

centers from elderly population has been described (Dunn-Walters et al., 2003, Frasca and Blomberg, 2009).

With a low dose (1 μ g) of PR8 HA VLP immunization, young adult mice induced protection preventing weight loss against homologous A/PR8/34 virus challenge, while aged mice displayed severe weight loss (~18%). For enhanced protection without weight loss, aged mice required a higher dose (3 μ g) HA VLP which was still not capable of clearing lung viral titers. These results of homologous protection appear to be strongly correlated with HAI activity titers in immunized mouse sera (Figure 35). The young adult group showed high HAI responses to homologous A/PR8/34 virus even with low dose (1 μ g) of PR8 HA VLP whereas higher doses (3 μ g or 6 μ g) were required to elicit sufficient HAI titers since 1 μ g PR8 HA VLP vaccination in aged mice HAI titers induced very low HAI titers. Vaccinated young adult mice with PR8 HA VLP (1 or 3 μ g) showed 100% protection against A/WSN/33 virus and survival protection against A/Cal/09 virus. Aged mice with 1 μ g HA VLP were not protected against A/WSN/33 virus and protection of lowering lung viral titers by several hundred-folds was observed in aged mice with a higher dose (6 μ g). Aged mice were not protected against A/Cal/09 virus (data not shown). Both young adult and aged mice had negative HAI titers to heterologous A/WSN/33 and A/Cal/09 viruses, suggesting that heterologous protection was mediated by non-neutralizing immunity. Consistent, seroconversion rates post vaccination for elderly were less than 30% while young adults showed 50-75% seroconversion rates (Crooke et al., 2019). The declined vaccine-induced humoral responses are attributed to the reduction of neutralizing and HAI activities of antibodies in elderly individuals (Sasaki et al., 2011).

Use of GM-CSF as adjuvant such as DNA vaccine and antigen targeting was reported to promote antibody avidity maturation (Lai et al., 2007) and cross-priming (Chiodoni et al., 1999, Skountzou et al., 2007). IL-12 activates dendritic cells, macrophages, T lymphocytes and natural killer (NK) cells, and cytotoxic CD8 T cell responses (Fallon et al., 2014, Jaffee, 1999, Trinchieri and Scott, 1999, Vagliani et al., 1996, Trinchieri, 1994). We wanted to test a hypothesis that incorporation of GM-CSF and IL-12 onto PR8 HA VLP (HA-Cy VLP) would overcome the weak immune system in an aged mouse model. In young adult mice, the impact of different doses of HA-Cy VLP vaccination was not significant on inducing humoral responses such as IgG and HAI titers, and on cross-protection against A/Cal virus although the HA-Cy VLP group induced higher levels of IgG antibodies for A/WSN and IFN- γ ⁺ CD4 and CD8 T cell responses. PR8 HA VLP exhibited sufficient immunogenicity and protection, clearing lung viral replication against A/PR8 virus and A/WSN virus in young adult mice. In contrast the aged mice with HA-Cy VLP vaccination were more effective in preventing weight loss against homologous A/PR8 virus challenge, and in lowering lung viral titers after homo (A/PR8) and hetero (A/WSN) virus challenge as well as in inducing higher levels of IFN- γ ⁺ CD4 and CD8 T cell responses. Nonetheless, there were no significant increases in IgG antibodies and HAI titers in the HA-Cy VLP group compared to the HA VLP group in aged mice, suggesting that cytokines incorporated onto HA VLP exhibit adjuvant effects on enhancing cellular T cell responses. Induction of IFN- γ ⁺ CD4 T cells was critical in lung protection and host survival (Green et al., 2013), supporting a possible role of T cell immunity in cross-protection. In addition, HA stalk antibodies and cytotoxic CD8 T cells induced by VLP vaccination might have contributed to protection against influenza virus (Hemann et al., 2013, Jang and Ross, 2021). Particularly, there have been several studies to develop universal influenza

vaccines by targeting the conserved HA stalk domains (Nachbagauer et al., 2021, Margine et al., 2013, Mullarkey et al., 2016). However, HA stalk specific antibodies are rarely induced by inactivated influenza virus vaccination (Krammer and Palese, 2013). We found that HA VLP vaccination was effective in inducing HA stalk specific IgG antibodies in young adult mice but not in aged mice. Thus, it is also possible that HA stalk specific IgG antibodies induced in young adult mice might have contributed to more effective cross-protection in your adult mice than aged mice. Taken altogether, a strategy of cytokine-adjuvanted HA VLP vaccination might provide a potential vaccine platform for elderly population.

5 DISCUSSION AND FUTURE DIRECTION

5.1 Enhanced cross-protection by a single intranasal dose of recombinant influenza virus

vaccine inducing M2e and HA immunity

Highly conserved M2e is a promising target as universal influenza vaccine and M2e-based protective immunity has been reported in numerous previous studies (Lee et al., 2015a, Deng et al., 2015). However, M2e is a poor immunogen and M2e immunity standalone provides relatively weak broad cross-protection due to non-neutralizing immune mechanisms (Lee et al., 2016b). To enhance the immunogenicity of M2e, multiple strategies have been introduced, which include M2e presenting VLP (Kim et al., 2013b, Kim et al., 2018), toll-like receptor (TLR) (Mastalerz-Migas et al., 2015b, Miller et al., 2020), gold nanoparticles, liposomes and immune stimulating complexes including cholera toxin subunit (Calzas and Chevalier, 2019). Clinical trials for M2e-based vaccine safety and efficacy were carried out. An M2e-flagellin fusion vaccine was immunogenic in 96% vaccine recipients in phase I trials after low prime-boost dose but showed adverse effects with high doses that might be due to the toxicity of the flagellin adjuvant (Lee et

al., 2014b, Turley et al., 2011, Taylor et al., 2011). Another clinical trial utilized ACAM-FLU-A (M2e epitopes fused to hepatitis B core protein) and reported the induction of M2e-specific antibodies in a phase I clinical trial (ClinicalTrials.gov Identifier No. NCT00819013) (reviewed in (Zheng et al., 2014)). The major objective of my dissertation projects was to test whether extra M2e immunity in addition to homologous virus neutralizing antibodies would enhance broad cross-protection in a mouse model after single dose intranasal immunization with recombinant influenza virus vaccines expressing chimeric 4xM2e-HA molecules.

Previous studies investigated M2e-induced protective immunity and secondary long term immune impacts using a VLP vaccine platform (M2e-VLP) in comparison with HA-based vaccines (Lee et al., 2016b, Schotsaert et al., 2013, Schotsaert et al., 2016). M2e vaccine-induced immunity provides infection-permissive protection against sublethal doses of heterosubtypic influenza viruses whereas HA-based trimeric soluble HA protein (Schotsaert et al., 2016), whole inactive virus (Schotsaert et al., 2013), and split vaccines (Lee et al., 2016b) were highly effective in inducing strain-specific homologous protection but not antigenically different influenza viruses. These studies clearly demonstrated the immunological benefits of M2e-based vaccines conferring survival protection and often preventing severe morbidity against heterologous viruses compared to HA-based seasonal vaccination ineffective in inducing cross-protection. M2e-based immunity was reported to induce cross-reactive CD8⁺ T cells contributing to cross-protection (Lee et al., 2016b). The M2e immune mice that were protected against primary lethal challenge infections developed strong long-term immunity against heterosubtypic viruses during secondary infections (Lee et al., 2016b, Schotsaert et al., 2013, Schotsaert et al., 2016). In contrast, the HA-based vaccinated mice that were completely protected against homologous

virus were highly susceptible to secondary heterosubtypic virus infections, mimicking pandemics (Lee et al., 2016b, Schotsaert et al., 2013, Schotsaert et al., 2016). As an alternative vaccination strategy, VLP expressing five tandem repeat M2e (M2e5x VLP) showed prominently enhanced cross-protection against a lethal dose of heterosubtypic influenza virus when it is supplemented to licensed split influenza vaccine compared to split vaccine only (Kim et al., 2014). Split vaccine induced strain specific but highly effective homologous protection while M2e4x VLP provided cross-protection against different subtypes of influenza viruses, despite weak protection accompanied with body weight loss in a mouse model (Lee et al., 2016b). Therefore, M2e immunity has limitation as a sole vaccine antigen although M2e-based vaccination confers relatively weak cross-protection.

Moreover, homologous serum hemagglutination inhibition (HAI) titers and neutralizing antibodies induced by HA-based vaccines against homologous influenza virus should be considered in developing universal vaccines and effective vaccination strategies translatable to humans. Vaccination strategies inducing both M2e immunity and neutralizing HAI antibodies provided scientific rationales for my projects 1 and 2 of testing recombinant 4xM2e-HA influenza virus vaccines.

In chapter 1, I have investigated the enhanced efficacy of cross-protection by single intranasal immunization with recombinant live influenza virus vaccine that expresses chimeric HA with an insertion of four tandem repeats of M2e domains in its N-terminus (4xM2e-HA). Sufficient M2e specific antibodies were induced in BALB/c mice even with an intranasal single dose of recombinant H3N2 subtype influenza virus that carries 4xM2e-HA (rgH3N2 4xM2e). The impact of M2e protective immunity on inducing enhanced cross-protection was prominent after

challenge with different subtypes of influenza viruses including H1N1, H3N2, H5N1, H7N9 and H9N2. These results are consistent with other animal studies (mice or ferrets) describing that M2e can induce broad permissive cross-protection against different subtypes of influenza viruses (Tao et al., 2017, Ingrole et al., 2021, Deng et al., 2015, Mezhenkaya et al., 2019).

5.2 Improvement of cross-protection by inducing both HA and M2e immunities with

heterologous prime-boost immunization with recombinant influenza virus vaccines

As seasonal influenza vaccinations are annually recommended in 6 months ages and older individuals, the effects of repeated vaccination need to be investigated further. A study performed in 2014 (McLean et al., 2014) described a reduced vaccine effectiveness in the group where had vaccinated over the prior five years compared to those individuals not receiving vaccine previously or those who received only the previous year's vaccine. This result implies that vaccination history might affect vaccine effectiveness. Similar observations were reported in another study (Smith et al., 1999) and this phenomenon was explained as "an antigenic distant theory". When the vaccine components have small antigenic differences between current and last season's vaccines, current seasonal vaccination may augment and stimulate preexisting memory B cells induced by previous season vaccination or infection. As a result, the immune response to current vaccine antigens can be interfered (antibody interference) (Murray, 2015). Consistent with these previous studies, in the chapter 2 of my project 2, I found that enhanced cross-protection was induced by heterologous prime-boost immunization with recombinant live influenza H1N1 and H3N2 subtype viruses expressing 4xM2e in their chimeric HA molecules. Enhanced cross-protection by heterologous prime-boost with recombinant influenza virus

vaccines was found to be correlated with increased cross-protective IgG antibodies specific for M2e and HA stalk domains compared to homologous prime-boost immunization in C57BL/6 mice.

In both project 1 and project 2, M2e epitopes were conveyed in live attenuated influenza virus (LAIV) platform to overcome the drawbacks of M2e alone weak immunogenicity and efficacy. LAIV mimicking natural infection is one of primary influenza vaccine types and believed to have a greater potential to induce cross-protective humoral and cellular immune responses in animals and in young children (Ambrose et al., 2011, Carter and Curran, 2011). I also observed cross-reactive IgG antibodies after rgH3N2 (4xM2e) or attPR8 (4xM2e) live influenza virus immunization. The cross-reactive antibodies have been reported to contribute to protection against heterologous virus challenges in animal models (Dong et al., 2018, Nachbagauer et al., 2017a) and in clinical investigations (Mandelboim et al., 2014, Chen et al., 2018).

Although vaccination is particularly important for vulnerable older adults, the influenza vaccine efficacy is significantly less in the elderly due to immunosenescence than in younger adults. In project 2, C57BL/6 mice at 1-year-old age received heterosubtypic prime-boost immunization with live attenuated influenza vaccines (LAIV) including attPR8 4xM2e and H3N2 4xM2e showed partial protection against A/Viet rgH5N1 virus infection and displayed reduced IgG antibody responses to vaccine antigens compared to young adult mice. Since LAIV is still a live virus, it is recommended only for healthy people 2-49 years of age (CDC, 2021c). Also, LAIV has some limitations with variable vaccine efficacy in individuals who already have been exposed to influenza virus partially due to their preexisting antibodies (Lopez and Legge, 2020). Clinical study has investigated the effectiveness of the LAIV during 2014-2015 influenza season (Matrajt et al., 2020) that is wide discrepancies in vaccine effectiveness (VE) between studies. The

research group explained the causes of different VE using mathematical models and suggested antigenic distances (AD) between the vaccina strain, pre-existing immunity, and the challenge strains as the main factors. In brief, VE would be low if the antigenic distances are small between the HA of vaccine strains and prior HA strains responsible for pre-existing immunity. This is consistent with the Original Antigenic Sin (OAS) theory (Zhang et al., 2019, Adalja and Henderson, 2010). Replication of LAIV in the upper respiratory tracts for inducing immune response would be limited by pre-existing immunity if the vaccine strain and pre-immunity are antigenically similar. Generally, VE of LAIV is higher in younger population who is relatively naïve individuals and in naive animal models. The efficacy of LAIV have shown discrepancies between in animal models and clinical studies. LAIV elicits superior efficacy compared to inactivated influenza vaccine (IIV) in animal model while clinical reports showed less efficacy in LAIV (Chung et al., 2019, Chen et al., 2010, Roy et al., 2020b, Roy et al., 2020a). It is explained by the impact of pre-existing immunity that reduce LAIV efficacy (Roy et al., 2020b). Therefore, LAIV vaccination need to be improved to induce enhanced effectiveness of vaccines. A ferret study (Nachbagauer et al., 2017b) reported heterologous vaccination strategy using chimeric HA vaccines, demonstrating higher protective immunity against pandemic H1N1 virus challenge from LAIV-spilt sequential immunizations compared to IIV-IIV immunization, which was not replicated in phase 1 clinical studies using similar vaccination strategies (Bernstein et al., 2020).

Intramuscular injection of inactivated vaccine is commonly used for influenza seasonal vaccination. In project 1, intranasal inoculation of single dose live H3N2 4xM2e induced a significant level of M2e specific IgG antibodies that contributed to enhanced cross-protection in BALB/c mice. However, inactivated H3N2 4xM2e failed to induce detectable M2e specific IgG

antibody responses after single dose intramuscular immunization and did not provide improved cross-protection even after second boost compared to the control inactivated virus with wild type HA (data not shown). No improved cross-protection might be due to the low levels of IgG antibodies for M2e and the induction of other non-M2e immunity such as conserved HA stalk and NA. A strategy of recombinant influenza viruses expressing conserved M2e epitopes at the N-terminus of HA would not be effective in inactivated vaccine platforms.

In contrast, previous studies reported alternative strategies of recombinant influenza viruses where the foreign epitopes (M2e, conserved NA or neutralizing epitopes of respiratory syncytial virus fusion protein) inserted in the immunodominant HA head domain of influenza viruses that induced epitope specific antibodies in inactivated vaccine platforms and conferred improved protection against challenge with antigenically different viruses (Kim et al., 2020, Lee et al., 2016a). Locations of foreign epitopes inserted into the chimeric HA might be attributed to inducing immune responses to the inserted region of HA in either LAIV or inactivated vaccine platforms. Structurally, the HA head domain is highly immunogenic whereas the N-terminus is more flexible that could be conjugated as large as 246 amino acids with retaining the HA functionality. However, the HA head domain only allows less than 18 amino acids to maintain the HA functionality. Therefore, different strategies should be considered in engineering recombinant influenza viruses to improve the vaccine efficacy, depending on the employment of vaccine platforms.

For future studies to induce cross-protective immune responses, another conserved domain, supplementation of HA stem immunity can be considered. While HA head domain is highly immunogenic but hypervariable, HA stem domain is immune subdominant but conserved

within the same HA group (Yassine et al., 2018, Ekiert et al., 2011). The HA stem possesses neutralizing epitopes that allow to induce cross-protective IgG antibodies against different subtypes of influenza virus (Thrane et al., 2020). In a previous study using nanotechnology (Deng et al., 2018) have described vaccine efficacy of double-layered protein nanoparticles incorporated with both HA stalk domains (from A/PR8 H1 or A/Aichi H3) and M2e (Uni4C13) in BALB/c mice. Enhanced cross-protection mainly induced by ADCC (antibody-dependent cellular cytotoxicity) and ADCP (antibody-dependent cellular phagocytosis) and similar findings were described using chimeric conjugated protein vaccine of thermostable H1 HA stem and M2e repeat (M2e-H1stem) (Jeeva *et al.*, in preparation). In addition, improved cross-protective vaccine efficacy was observed in young and aged BALB/c mice immunized with VLPs displaying multi- consensus epitopes of NA subtypes and 5xM2e (Kim *et al.*, under review).

5.3 Influenza vaccination for the elderly

In chapter 3, virus-like particle (VLP) was used as vaccine platform to induce protective immune responses in old aged (18-month-old) BALB/c mice. The safety and immunogenic features of VLP represent attractive applicability particularly for immunocompromised or elderly people. Currently, multiple VLP-based vaccines are available for clinical trials or commercial uses. VLP-based vaccines for human papillomavirus, hepatitis B virus, and malaria have been approved by U.S. food and drug administration (FDA). And other VLP-based vaccines are under clinical trials against variety of viruses including seasonal influenza (phase 3, trial No.: NCT03301051, plant-derived quadrivalent VLP and NCT04120194, protein nanoparticle), pandemic influenza (phase 2), RSV (phase 3), human immunodeficiency virus, Ebola, and COVID-19 (phase 1) as reviewed in (Nooraei et al., 2021). From the project 3 studies to investigate protective immune responses to

HA VLP vaccination in aged mice, I found that aged mice required higher doses of PR8 HA VLP vaccine than young adult mice to provide protective responses to homologous and heterologous viruses. This is consistent with a current influenza vaccine strategy that older people ≥ 65 years are suggested to receive high-dose vaccine such as Fluzone® High-Dose Quadrivalent licensed inactivated influenza vaccine that contains influenza A and B strains (60 μg of each hemagglutinin). Adjuvanted inactivated influenza vaccine, FLUAD Quadrivalent is approved only for 65 years and older in U.S. in November 2015 at 18 years later of the approval in Italy (CDC, 2021a). This vaccine contains MF59 which is an oil-in-water emulsion of squalene oil. The mechanism of this adjuvant is not fully understood but it triggers immunostimulation and significantly enhance the immunogenicity (Li et al., 2021). However, there is still some gaps to be improved due to the shortcomings during the vaccine preparation that utilizes eggs. Egg adaptation mutation might affect the vaccine effectiveness and egg proteins in the vaccine ingredients can cause allergic responses. HA VLP contains lipid bilayer membranes as in the enveloped influenza viruses and thus was reported to be easily amenable for incorporating GPI-anchored cytokine adjuvants (Patel et al., 2015b, Patel et al., 2015a). Therefore, we selected a VLP vaccine platform and tested the impacts of cytokine molecular adjuvants (GM-CSF and IL-12) incorporated onto PR8 HA VLP aged (PR8 HA-Cy VLP) in BALB/c mice. PR8 HA-Cy VLP was able to enhance protective efficacy by reducing lung virus titers in aged mice. These results from the project 3 studies on HA VLP with cytokine adjuvants provide new insights into developing effective vaccination in aged population.

5.4 A new platform of mRNA-based next generation influenza vaccines

As a future direction for developing effective vaccination strategies, recently, the success of mRNA vaccines against COVID-19 has shown the potential as a next generation influenza vaccine platform. One of advantages of mRNA vaccine over current vaccines is that it can be produced rapidly (6-8 weeks) compared to current influenza vaccines which takes 6 to 9 months. This allows rapid update of genome sequence for effective vaccine formulations (Meurens, 2020). Also, its cost-effective synthetic format provide safety which does not require complications of cell- or egg-based production. The mRNA vaccine is an inherently unstable molecule and easily degraded by nucleases (Wadhwa et al., 2020). Lipid nanoparticles (LNPs) provide a promising vehicle that enables delivery of nucleic acids of mRNA into cells effectively by receptor-mediated endocytosis as it is worked well and proven for COVID-19 mRNA vaccines (Hou et al., 2021, Schoenmaker et al., 2021). Numerous relevant preclinical data have been reported. In 2012, Kramps' group (Petsch et al., 2012) firstly demonstrated protective efficacy of mRNA vaccine against infectious disease (influenza) using animal models. In addition, multiple clinical trials have been initiated for influenza mRNA vaccine development. Currently, influenza HA-based mRNA vaccines (phase 1 or 2, Moderna, mRNA-1010, Sanofi/Translate Bio, MRT-5400, MRT-5401, and Pfizer, PF-07252220) are in clinical trials (ClinicalTrials.gov) and the clinical findings of SARS-CoV-2 mRNA vaccine that induced protective immune response in the elderly (>80 years) (Collier et al., 2021) suggest the potential of mRNA vaccine for influenza virus.

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7 VITAE

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Positions and Employment

- 2017-2021 **Georgia State University, Atlanta, Georgia, U.S.**
 Research Assistant
- 2013-2017 **Emory University, Atlanta, Georgia, U.S.**
 Research Assistant, Department of Physics
- 2007-2013 **Yonsei University Health System, College of Dentistry, Seoul, KOREA**
 Research and Teaching Assistant
- 2012-2013 **International Journal of Oral Biology (IJOB), Seoul, KOREA**
 Editorial Assistant
- 2005-2007 **Ab Frontier Co., Ltd (Lab Frontier), Seoul, KOREA**
 Research Scientist, Antibody QC team
- 2003-2005 **Ewha Women's University, Seoul, KOREA**
 Teaching Assistant, Department of Science Education

Education

- 2017-2021 **Georgia State University, Atlanta, Georgia, U.S.**
 PhD, Translational Biomedical Sciences
- 2003-2005 **Ewha Women's University, Seoul, KOREA**
 MS, Department of Genetics in Life Sciences
- 1999-2003 **Ewha Women's University, Seoul, KOREA**
 BS, BBA, Double major in Biological Sciences and Business Administration

Honors and Awards

- 2018 Student Travel Award: 37th Annual Meeting of the American Society for Virology
- 2010 Best Employee Award: Yonsei University Health System, College of Dentistry
- 2010 Research Poster Presentation Award: Yonsei University, College of Dentistry
- 2003-2005 Student Assistant Scholarships from Ewha Women's University

Presentation

- 2021 Poster Presentation at the American Society for Virology, Online
- 2020 Poster Presentation at the Georgia Bio, Online
- 2019 Poster Presentation at the American Society for Virology, Minnesota, MI, U.S.A
- 2018 Poster Presentation at the International Society for Vaccines, Atlanta, GA, U.S.A
Poster Presentation at the American Society for Virology, College Park, MD, U.S.A
- 2017 Invited Oral Presentation at the Yonsei University, College of Dentistry, Seoul, Korea
- 2010 Poster Presentation at the Gordon Research Conference, Denver, U.S.A.
- 2010 Poster Presentation at the Yonsei Dental Symposium, Seoul, Korea

Professional Memberships

- 2018-2021 American Society For Virology (ASV)
- 2007-2013 The Korean Academy of Oral Biology (membership)
- 2003 Women Into Science and Engineering (WISE)

Publications

1. **Park BR**, Kim KH, Kotomina T, Kim MC, Kwon YM, Jeeva S, Jung YJ, Bhatnagar N, Isakova-Sivak I, Mezhenkaya D, Rudenko L, Wang BZ, Kang SM. 2021. Broad cross protection by recombinant live attenuated influenza H3N2 seasonal virus expressing conserved M2 extracellular domain in a chimeric hemagglutinin. *Sci Rep* 11:4151.
 2. **Park BR**, Jeeva S, Kim KH, Kwon YM, Oh J, Kim MC, Shin CH, Seong BL, Kang SM. 2021. Enhanced cross protection by hetero prime boost vaccination with recombinant influenza viruses containing chimeric hemagglutinin-M2e epitopes. (2021, Under revision).
 3. **Park BR**, Chung H, Kim KH, Jeeva S, Bhatnagar N, Kang SM. 2021. Virus-like particles carrying influenza hemagglutinin is immunogenic and provide cross protection in young and aged mice (in preparation for submission)
- (Papers published prior to PhD studies)
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