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Adjuvant Effects on Enhancing Influenza Vaccine Effectiveness and Heterologous Prime-Boost
Influenza Vaccination Strategy

by

Noopur Bhatnagar

Under the Direction of Sang-Moo Kang, PhD

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

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2022

ABSTRACT

Seasonal influenza vaccination is ineffective in conferring cross-protection against antigenically different influenza viruses, particularly in the elderly population. The overarching goal of my dissertation research projects was to develop influenza vaccination strategies for inducing more effective cross-protection against influenza viruses.

Adjuvants are used to enhance vaccine-specific immune responses, but the efficacy comparison of different adjuvants remains to be determined. In chapter one, I investigated the comparative effects of adjuvants approved for human use on enhancing the immunogenicity and cross-protective efficacy of inactivated split influenza virus vaccination. The adjuvants studied include QS-21 (a saponin derived from the soap bark tree, *Quillaja saponaria*) plus monophosphoryl lipid (MPL) [QS-21+MPL], oligonucleotide CpG plus MPL (CpG+MPL), and Bacillus Calmette–Guérin Cell Wall Skeleton (BCG-CWS) adjuvant. The experimental outcomes demonstrated that QS-21+MPL adjuvant combination was most effective in inducing T helper type 1 (Th1) IgG antibody responses, whereas both CpG+MPL and QS-21+MPL combination adjuvants exhibited similar potency in enhancing vaccination responses leading to increased protection against influenza virus challenge in adult C57BL/6 and aged BALB/c mice.

In chapter two, I investigated whether VSA-1, an analog of licensed saponin QS-21, exhibits adjuvant properties on enhancing the immunogenicity and cross-protection by influenza split virus vaccination in C57BL/6 mice. The experimental outcomes demonstrated that a single dose of VSA-1 adjuvanted vaccination conferred higher efficacy of protection against challenge with homologous H1N1 virus than QS-21 and alum adjuvants. Prime-boost VSA-1 adjuvanted vaccination induced humoral and cellular immune responses and cross-protection against H5N1

virus. These findings provide evidence warranting further development of VSA-1 as a promising alternative adjuvant for QS-21 replacement.

Recent clinical studies have reported that repeat annual vaccination diminishes vaccine efficacy. In chapter three, I investigated the efficacy of cross-protection by heterologous prime-boost vaccination with inactivated influenza virus vaccines in BALB/c mice. The experimental outcomes of this study demonstrated that the heterologous prime-boost vaccination strategy induced cross-reactive virus- and hemagglutinin (HA) stalk-specific IgG antibodies and more effective cross-protection against antigenically different viruses than homologous vaccination with the same antigen in BALB/c mice. These research outcomes support the positive impacts of a heterologous prime-boost strategy on conferring more effective cross-protection.

INDEX WORDS: Influenza virus, Vaccine, Adjuvants, Inactivated split influenza virus, Inactivated influenza virus, Cross-protection

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Influenza Vaccination Strategy

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May 2022

DEDICATION

I dedicate this dissertation to my parents – Anil and Renu Bhatnagar, my brother – Nikhil, my sister-in-law – Pratibha, my nephew – Vihaan, and my best friend – Manish, who have been my pillars of strength throughout my journey as a Ph.D. student. They have always motivated me, supported me, and have made innumerable sacrifices to help me achieve my goals. I would like to additionally dedicate this dissertation to my grandparents and extended family, who have always showered their blessings on me and have prayed for my success. Although my family has been miles away from me in India, their love, support, patience, and encouragement have never wavered.

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LIST OF ABBREVIATIONS

aa	amino acid
aDCs	activated dendritic cells
Alum	aluminum hydroxide
ANOVA	Analysis of variance
APC	antigen-presenting cell
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BCG-CWS	Bacillus Calmette-Guérin- Cell Wall Skeleton
BSL-2+	Biosafety level 2
CpG	cytosine and guanine with phosphodiester backbone
DC	dendritic cell
DMSO	dimethyl sulfoxide
DTP	Diphtheria, tetanus, and pertussis
EID ₅₀	50% egg infectious dose
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked Immunospot assay
FBS	fetal bovine serum
GC	germinal center
HA	Hemagglutinin
HAI	Hemagglutination inhibition
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRP	horse-radish peroxidase
IFN	Interferon
IgG	Immunoglobulin G
IgG1	Immunoglobulin G 1
IgG2a	Immunoglobulin G 2a
IgG2b	Immunoglobulin G 2b
IgG2c	Immunoglobulin G 2c
IL	Interleukin
IN	intranasal
IP	intraperitoneal
KC	Keratinocyte-derived chemokine
LD ₅₀	Median lethal dose
LPS	Lipopolysaccharide
LRT	lower respiratory tract
M1	Matrix protein
M2	Membrane protein

MC	<i>Momordica cochinchinensis</i>
MCP-1	Monocyte chemoattractant protein 1
MLN	Mediastinal lymph node
MMR	Measles, Mumps, and Rubella
MPL	monophosphoryl Lipid A
mRNA	messenger ribonucleic acid
NA	Neuraminidase
NEP	Nuclear export protein
NLRP3	NOD-like receptor 3
NP	Nucleoprotein
NS2	Non-structural protein 2
NSP1	Non-structural protein 1
OAS	original antigenic sin
OD	optical density
ODN	oligonucleotide
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB1-F2	Polymerase basic protein 1-F2
PB2	Polymerase basic protein 2
PBS	Phosphate-buffered saline
pDCs	plasmacytoid dendritic cells
PRR	pathogen recognition receptor
QS	<i>Quillaja Saponaria</i> Molina
R ²	Coefficient of correlation
RDE	receptor destroying enzyme
rg	reverse genetics
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SA	sialic acid
SAR	structure-activity relationship
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SBA	saponin-based adjuvant
sCal	inactivated split virus vaccine derived from A/California/H1N1 strain
Th1	T helper 1
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR9	Toll-like receptor 9
TMB	tetramethyl benzidine
TNF	tumor necrosis factor
URT	upper respiratory tract
VE	vaccine effectiveness

VLP	Virus-like particle
VSA-1	Derivative of <i>Momordica saponin</i> 1

1 INTRODUCTION

1.1 Influenza virus

Influenza virus is the cause of seasonal and pandemic flu. It infects up to 15% of the global population, causing frequent hospitalization and over 650,000 deaths annually (Iuliano et al., 2018). It belongs to the *Orthomyxoviridae* family, and there are four *genera*, A, B, C, and D, based on the expression of surface proteins, namely, matrix protein (M1), membrane protein (M2), and nucleoprotein (NP). Influenza A, B, and C viruses mainly infect humans, and influenza D infects cattle and pigs. In addition to infecting humans, influenza A viruses can infect a variety of species (including many bird and animal species). Type A and B cause the annual influenza epidemics that have up to 20% of the population sniffing, aching, coughing, and running high fevers. Type C also causes flu; however, type C flu symptoms are much less severe. Usually, the virus outbreak is epidemic and seasonal, but sometimes it causes pandemics and shows severe symptoms and relatively high mortality (Taubenberger & Kash, 2010; Webster et al., 1992). Influenza A is responsible for a pandemic that occurs due to the emergence of novel strains of viruses with its varied animal species reservoirs (Long et al., 2019). Some influenza viruses may give rise to pandemic strains in humans, as in the case of the most recent 2009 H1N1 Swine flu pandemic, which claimed about 284,000 deaths worldwide (Nickol & Kindrachuk, 2019). Another pandemic caused by the 1918 H1N1 Spanish flu virus was a serious cause of concern and is estimated to have caused about 50 million deaths worldwide, while the 1957 H2N2 Asia flu and the 1968 H3N2 Hong Kong flu pandemics each were reported to claim approximately one million lives (Taubenberger et al., 2019). Such pandemic influenza outbreaks occur when a new influenza strain emerges and gets the ability to transmit to humans (Taubenberger & Kash, 2010).

Influenza virus has a negative-sense, single-stranded, and segmented RNA genome. It contains 7 (*genera C*) or 8 (*genera A* and *B*) segmented negative-sense RNA, and each RNA encodes 1 or 2 genes. The Influenza A viruses have eight segments that encode for the 11 viral genes: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2; also known as nuclear export protein, NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1 – F2 (PB1-F2) (Collins et al., 2001). Influenza virus is enveloped and is spherical in shape with an approximate diameter of 80 to 120 nm (Jalilian et al., 2013).

The subtypes of the influenza A virus are based on the surface expression of major glycoproteins, hemagglutinin (HA), and neuraminidase (NA). Currently, 18 subtypes of HA and 9 subtypes of NA have been found. HA is a lectin and plays a critical role in the binding and entry of the virus into the host cells. On the other hand, NA activity is vital for the release of the virus from the infected cell (Iwasaki & Pillai, 2014; Russell et al., 2006; Tong et al., 2013). The influenza A viruses can be further divided into two groups based on the phylogenetic differences in the 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).

Influenza virus can be transmitted via direct contact and also via airborne droplet dissemination. Generally, the incubation period for the virus to cause infection is 1 to 3 days, after which common symptoms such as headaches, runny nose, sore throat, fever, cough, and chills may start to occur. Flu symptoms are mild in healthy populations, and such individuals usually recover within 7 to 10 days; however, the symptoms can be more severe and may even lead to higher mortality in children, the elderly, and immune-compromised patients (Bridges et al., 2003; Finelli

et al., 2008; Lin & Nichol, 2001; Taubenberger & Morens, 2008), which might be because the CD4⁺ T cell-mediated adaptive immune responses are not fully developed or are reduced in such populations, and they may fail to develop strong innate and adaptive immune responses.

Influenza viruses evolve by the action of error-prone RNA-dependent RNA polymerase via a complex process known as reassortment which involves the accumulation of mutations over time and the rearrangement of viral RNA segments in cells infected with two or more different viruses. The reassortment of influenza genes from different strains causes a genetic shift and can lead to the emergence of entirely new influenza strains. These new strains may result in pandemic influenza outbreaks and cause severe symptoms, high hospitalization rates, and mortality because most populations do not have pre-existing immune responses. Minor mutations in the HA or NA genes (genetic drift) cause virus antigenicity alteration. This genetic drift of the influenza virus is responsible for the outbreaks of severe seasonal flu. Because of the antigenic diversity of the influenza virus and the antigenic specificity of HA and NA, the world health organization (WHO) predicts seasonal flu strains and recommends annual vaccination (Jalilian et al., 2013; Treanor, 2004).

Currently, trivalent (2 influenza A and 1 influenza B strains) or quadrivalent (2 influenza A and 2 influenza B strains) inactivated whole influenza virus vaccines are approved for use in populations with a wide age range. In addition, live attenuated influenza virus vaccines can also be administered to individuals between the ages of 2-49 years (Jalilian et al., 2013).

1.2 Influenza virus life cycle

Influenza virus infects the respiratory tract and mainly replicates in the epithelial cells of the respiratory tract. Virus infection starts with binding of the virus to the host epithelial cells in

the upper respiratory tract. HA plays a role in this attachment by recognizing the sialic acid receptors on the cell surface with host specificity. HA proteins from human-adapted influenza viruses preferentially bind to α -2,6 sialic acid (SA) that is abundantly present in the human upper respiratory tract (URT), whereas the avian influenza viruses bind to α -2,3 SA that is found on epithelial cells of the human lower respiratory tract (LRT) and the intestinal tract of birds (Krammer et al., 2018; Shao et al., 2017). Viruses are internalized into the cells by receptor-mediated endocytosis. The conformational change in HA induced by acidification leads to the exposure of fusion peptide that results in the fusion of the viral envelop with the endosomal membrane, followed by the release of the viral RNA into the cytoplasm. The RNAs (containing eight viral genetic materials: PB2, PB1, PA, HA, NP, NA, M, and NS) are further imported into the nucleus of the host cells for transcription and replication. The RNA polymerase complex, which is composed of three subunits (PB2, PB1, and PA), is responsible for transcription and replication. The eighth RNA gene segment, NS, encodes two proteins, including NS1 and NS2, and the seventh RNA segment (M) encodes M1 and M2 proteins. The budding of new virions from the host plasma membrane is facilitated by NA that is involved in the viral release from the cells via the cleavage of SA following the hydrolysis of the glycosidic linkage (Dou et al., 2018).

1.3 Vaccines

Vaccination is one of the most effective methods for preventing influenza virus infections and complications. Since the first attempt at cowpox immunization by Edward Jenner, many types of vaccines have been tried and tested experimentally and clinically. Successful vaccinations can elicit antigen-specific isotype-switched antibodies and long-term cellular memory responses (Taylor et al., 2012). The goal of vaccination is to stimulate the activation of a primary adaptive

immune response and promote the production of memory cells capable of combating a specific pathogen. An effective vaccine must be capable of inducing protective immunity without causing harm to the population being treated. Furthermore, since pathogens are recognized by different effector lymphocytes, vaccination must induce a primary immune response that promotes activation of the exact lymphocytes needed to combat the actual pathogen. Following are the different types of currently available vaccines used successfully for protection against a variety of pathogens.

1.3.1 Inactivated vaccines

Inactivated pathogens are the safest and most widely used vaccine platform. Pathogens are amplified and inactivated either by chemicals (including formalin treatment), heat, or irradiation. Since inactivating pathogens via these methods still maintains many of the antigens that are important in promoting a primary immune response, inactivated vaccines provide a simple means of inducing this response using a largely intact pathogen that cannot cause disease. Inactivated vaccines generally require a second dose as a booster because the first dose does not elicit an immune response that provides a high level of protection. The whole inactivated pathogen has strong immunogenicity and is cost-effective compared to other vaccine platforms. However, it sometimes has adverse effects such as inflammation and pain at the site of injection due to high immunogenicity (Vajo et al., 2007).

1.3.2 Live attenuated vaccines

Live attenuated pathogens are used in measles, mumps, and rubella (MMR), chickenpox, and rotavirus vaccines. The pathogens are attenuated via passages in foreign hosts or gene modification. Thus, the attenuated pathogens are still live but have reduced virulence in the host. These vaccines can elicit strong cellular and antibody responses because of their live

characteristics. However, a significant drawback is the possibility of reversion of the virus to its virulent form that can cause diseases. In addition, this type of vaccine incurs high costs because refrigeration is necessary to preserve the potency of the living organisms (Izurieta et al., 2005).

1.3.3 Subunit vaccines

Recently, many subunit vaccines have been developed to reduce the potential adverse effects of the whole pathogen-derived vaccines and elicit an appropriate type of immune response. These subunit vaccines include only antigenic epitopes. Subunit vaccines represent a strategy used to raise a proper primary immune response blocking cellular receptors responsible for adhesion and entry of pathogens into the target cells. Since the adhesion molecules of viruses are not directly responsible for generating disease conditions and symptoms, they can be employed to generate a primary adaptive immune response. Subunit vaccines are currently being used in the human hepatitis B virus, human papilloma virus, and Shingrix vaccines. These subunit vaccines are considered safer than other types of vaccines and show lower immunogenicity than the whole pathogen-derived vaccines (Hovden et al., 2005; Mbow et al., 2010; Tripp & Tompkins, 2014).

1.3.4 Conjugate vaccines

A conjugate vaccine is a protective strategy based on coupling a weak antigen with a stronger carrier antigen. These vaccines have multiple epitopes and thus are also considered multivalent vaccines. Conjugate vaccines commonly require boosters to promote full protection against the pathogen. The conjugate vaccine used against *Haemophilus influenzae* type b, a causative agent of bacterial meningitis, is an example of a conjugate vaccine that requires more than one dose.

1.3.5 Recombinant vector vaccines

Recombinant vector vaccines are a type of vaccine currently under development and undergoing clinical trials for infections caused by human immunodeficiency virus (HIV), rabies virus, and measles virus. The strategy underlying these vaccines is utilizing harmless attenuated viruses to express a pathogenic antigen capable of eliciting an adaptive immune response. These vaccines take advantage of the ability to isolate a gene capable of encoding an antigen from a pathogen and placing the gene into a plasmid or into a harmless or attenuated virus. The Janssen COVID-19 vaccine from Johnson and Johnson is an example of a recombinant Adenovirus vector vaccine that has been modified to express a surface protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus.

1.3.6 DNA vaccines

DNA vaccines utilize a similar strategy to that of recombinant vector vaccines. However, instead of using a virus as a carrier of the gene, the DNA vaccine is placed directly into the host in hopes of having host cells pick up the DNA encoding protein antigens. The DNA is typically mixed with microscopic gold particles, and the coated gold particles are then introduced with highly pressurized gas. Alternatively, the DNA vaccine can be mixed with a chemical that is easily taken up by the host cells. Vaccines currently under development attempt to introduce DNA into target cells to express antigens within those host cells and activate CD8 T cells. DNA vaccines are currently being tested for influenza and herpes virus infections.

1.3.7 Messenger RNA vaccines

Messenger RNA (mRNA) vaccines rely on strategies similar to their DNA and recombinant vector vaccine counterparts. These vaccines are based on the central dogma of molecular biology, whereby mRNA serves as the code for protein translation. An mRNA molecule

that encodes an antigen is delivered into a target cell. The target cell takes up the mRNA to translate into the protein antigen and induce an adaptive immune response. The mRNA vaccine can be mixed with cationic liposomes for delivery into the target cells. Pfizer/BioNTech and Moderna rapidly developed and licensed mRNA vaccines in the market to combat the COVID-19 pandemic.

1.3.8 Virus-like particles (VLP) vaccines

Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome, yielding safer and cheaper vaccine candidates. VLP vaccines enable strong immunity due to dense epitope, induce cross-immunity, are stable in quality and have longer half-life in serum. They are very effective against diseases such as human papillomavirus (HPV), hepatitis B, and malaria. However, their generation and production require high cost and technology.

Inactivated virus vaccines have been the preferred choice for vaccination against influenza viruses. Based on the immune status of the populations and the antigenic and genetic information about the circulating viruses, vaccine strains for preparing inactivated vaccines are recommended each year by the WHO. Since this decision has to be made more than 6 months prior to the influenza season, the selected vaccine strains occasionally differ antigenically from the viruses circulating during the subsequent influenza season. Limited antigenic match between the selected vaccine strains and the actually circulating strains may result in low efficacy. According to CDC, the influenza vaccine effectiveness was estimated between 10% and 60% during 2004-2020 season (CDC, 2021c). This shortcoming of current influenza virus vaccines necessitates the development of more effective vaccines.

1.4 Aging-related immunosenescence

Influenza results in approximately 650,000 deaths in the global population annually, and people aged 65 years or above are at an overwhelmingly higher risk, occupying 70-85% of the seasonal flu-related deaths (based on CDC estimation in the United States) (CDC, 2021b). Annual vaccination has been the most effective strategy to reduce the risk of influenza from 6% to 2.4% in the elderly (Tanner et al., 2021). However, the vaccine effectiveness (VE) in people above 65 years of age (17-53%) has been dropping disproportionately compared to young adults (70-90%) (Goodwin et al., 2006). The conventional influenza vaccine is less immunogenic in the elderly (only 30-40%), and this results in the failure of induction of hemagglutination inhibition (HAI) antibodies in at least 20% of the elderly recipients (Keren et al., 1988) which are known to be correlated with protection (Dunning et al., 2016). The low overall effectiveness is mainly due to an altered immune response which is different from children and young adults, who remain to be the primary targets for designing influenza vaccines (Ciabattini et al., 2018). Age-associated deleterious changes in the immune system, including immunosenescence and inflammaging are responsible for low vaccine effectiveness (Tanner et al., 2021). Immunosenescence refers to an age-related immunological dysfunction accompanied by the gradual deterioration in the generation of innate and adaptive immune responses. For example, the downregulation of primary lymphoid organs causes decreased production of B and T cells progenitors, reduced phagocytosis and chemotaxis, and increased production of pro-inflammatory cytokines and dysfunctional memory cells (Ciabattini et al., 2018). Inflammaging is a state of chronic hyperinflammation. The constantly increased levels of inflammation interfere with the detection and generation of truly protective inflammatory responses to pathogens. Therefore, inflammaging substantially contributes to increased susceptibility to infection in the elderly (Lambert et al., 2012).

The history of influenza virus infection and vaccination of recipients needs to be considered to determine vaccine effectiveness in the elderly. This can be described by the original antigenic sin (OAS) concept, which explains that an individual's immune response is imprinted by influenza viral antigens that the individual has previously encountered (Ranjeva et al., 2019). OAS could prove to be both beneficial as well as harmful in the immunity against influenza. The elderly showed a lower infection rate than young people during the 2009 pandemic (swine flu, A/H1N1). It is believed that previous exposure of older people to H1N1 viruses that circulated in the late 1970s conferred protection by inducing cross-immunity to conserved antigens. However, an increased susceptibility of the elderly to influenza virus infection in the 2013-2014 H1N1 season was observed, while the young population with immunity to non-mutated antigens displayed enhanced protection (Tanner et al., 2021).

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

1.5 Adjuvants

Humans are immunized with many kinds of vaccines throughout life to prevent infectious diseases. The antigen-specific immune responses can be increased by effective vaccination with adjuvants, which correlates with the efficacy in preventing infectious diseases (Rappuoli et al., 2011). Adjuvants are substances that can enhance the immune system's response to a vaccine antigen and are used to improve the effectiveness of a vaccine. Adjuvants are injected along with vaccine antigens to help the immune system generate antibodies specific for a pathogen. Adjuvants enhance the magnitude, longevity, and breadth of specific immune responses to antigens, and direct the quality of the immune response. They should have minimal toxicity while enhancing the

immune effects of vaccination (COLER et al., 2009; Reed et al., 2009). To increase immune responses and efficacy of the vaccination, vaccine adjuvants have been developed and used with the subunit vaccines (Mbow et al., 2010; McKee et al., 2010). Generally, the adjuvants are known to stimulate innate immune cells at the site of injection, induce rapid responses to pathogens, amplify the immune responses, modulate T helper cell responses, and finally enhance long-lasting B and T cell immunity. Moreover, vaccine adjuvants can reduce the number of immunizations and vaccine dose (antigen dose sparing) (Coffman et al., 2010). Strong innate immune responses are expected to generate better antigen-specific adaptive immune responses.

1.5.1 Bacillus Calmette-Guérin (BCG) Cell Wall Skeleton (CWS)

Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, has commonly been used worldwide as a prophylactic vaccine against tuberculosis. BCG strongly enhances the non-specific immune response, thus augmenting specific protection, and has beneficial clinical effects. In the 1970s, Azuma et al isolated the bioactive component of BCG from its cell wall and termed it the BCG cell wall skeleton (BCG-CWS) (Azuma et al., 1971; Azuma et al., 1974). *Bacillus Calmette-Guerin* (BCG) vaccination is associated with better clinical outcomes in COVID-19 patients due to immune training rather than specific immune memory (Escobar et al., 2020). Clinical benefits of BCG cell-wall skeleton (CWS) as an innate immune therapy have been demonstrated in cancer patients (Kodama et al., 2009). CWS is known to mediate adjuvant effects via Toll-like receptor 2 (TLR2) and TLR4 signaling pathways (Tsuji et al., 2000). CWS-adjuvanted influenza vaccination significantly enhances the immunogenicity and protective efficacy in infant, adult, and older age BALB/c mouse models (Kim et al., 2021).

1.5.2 *Quillaja Saponaria-21 (QS-21)*

QS-21, a key component of GlaxoSmithKline's AS series of combination adjuvants including the FDA-approved AS01b, is arguably the most potent immunostimulant that can induce strong and balanced humoral and cellular immune responses (Leroux-Roels, 2010; Leroux-Roels et al., 2015; Polhemus et al., 2007; Stewart et al., 2006; Tielemans et al., 2011; Vandepapeliere et al., 2008; Vandepapeliere et al., 2005). QS-21 is a combination of two isomeric bidesmosidic saponins isolated from the bark of *Quillaja Saponaria* Molina (QS), which is an evergreen tree native to temperate central Chile. However, the current shortage of QS-21, along with its dose-limiting toxicity, laborious and low-yielding purification, and chemical instability, limits its application and highlights the imperative need for its more accessible alternatives (Harandi et al., 2010; Martin & Briones, 1999; Ragupathi et al., 2011).

1.5.3 *CpG ODN (synthetic oligonucleotides)*

Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs trigger cells that express Toll-like receptor (TLR) 9 (including human dendritic cells and B cells) to mount an innate immune response which is characterized by the production of T helper type 1 (Th1) and proinflammatory cytokines. When used as vaccine adjuvants, CpG ODNs improve the function of professional antigen-presenting cells and boost the generation of humoral and cellular vaccine-specific immune responses. Preclinical studies have indicated that CpG ODNs improve the activity of vaccines targeting infectious diseases and cancer. Clinical trials have demonstrated that CpG ODNs have a good safety profile and increase the immunogenicity of co-administered vaccines (Bode et al., 2011).

1.5.4 Monophosphoryl Lipid A (MPL)

Monophosphoryl lipid A (MPLA) is a derivative of the lipid A region of lipopolysaccharide (LPS) and has lower toxicity than LPS. MPLA retains the immunologically active lipid A portion of the parent molecule, LPS (Okemoto et al., 2006). Despite the toxicity associated with LPS which prohibits its potential clinical use, MPLA has been developed as a vaccine adjuvant (Casella & Mitchell, 2008). Both LPS and MPLA are known TLR4 agonists, but signal through different adaptors, LPS signals via MyD88 and MPLA signals via TRIF. The decreased toxicity of MPLA is attributed to the recruitment of TRIF upon TLR4 activation, resulting in decreased induction of inflammatory cytokines (Mata-Haro et al., 2007). MPLA has been extensively tested as an adjuvant in mice and has been reported to induce a strong Th1 response (Didierlaurent et al., 2009; Fransen et al., 2007; Rhee et al., 2010). The mechanism of action of MPLA still needs to be completely understood, but reports have suggested that it improves vaccine immunogenicity by enhancing antigen presenting cell maturation (Rhee et al., 2010). MPL is a component in the licensed AS01, AS02 and AS04 adjuvant combinations.

1.5.5 Derivative of *Momordica Saponin 1* (VSA-1)

Extensive structure-activity-relationship (SAR) studies of QS-21 led to the recent discovery of promising new saponin adjuvants that can be derived from more accessible natural sources. Semisynthetic saponin adjuvant VSA-1 is prepared in one step from naturally occurring *Momordica Saponin I* (MS I), which can be easily isolated from the inexpensive seeds of *Momordica cochinchinensis* SPRENG (MC), a widely available perennial vine (Iwamoto et al., 1985). In earlier SAR studies of QS saponin derivatives, a QS derivative with a structure closely resembling that of VSA-1 showed significant adjuvant activity (P. Wang, X. Ding, et al., 2019). Also, VSA-1 potentiates antigen-specific IgG1 and IgG2a immune responses in BALB/c mice,

indicating a mixed Th1/Th2 immune response. VSA-1 showed lower acute toxicity than Quil A which is a QS-21-containing mixture with a toxicity profile similar to that of QS-21 (P. Wang, X. Ding, et al., 2019).

1.5.6 Aluminum hydroxide (Alum)

Alum is one of the most widely used adjuvants and has been in use for more than 70 years in veterinary and human vaccines. It adsorbs the vaccine antigens and makes antigen depot at the site of injection. These antigen depots can release the vaccine antigen slowly, so that the immune system remains stimulated by the antigen for a longer time. Also, the alum can stimulate innate immune cells like neutrophils and macrophages through NOD-like receptor P3 (NLRP3) inflammasome signaling pathway. The inflammasome-activated immune cells secrete pro-inflammatory cytokines such as IL-1 β and IL-18 (Eisenbarth et al., 2008; Kool et al., 2008; Sharp et al., 2009).

Alum is the most common adjuvant in human subunit vaccines such as the diphtheria, tetanus, and pertussis (DTP) toxoid, human papillomavirus (HPV), haemophilus influenza type B, and pneumococcal conjugate vaccines. It has relatively weaker adjuvant effects compared to other adjuvants, but it is one of the very few approved adjuvants for human use in the USA because of its high safety record (Mbow et al., 2010).

Table 1.1 Adjuvants used in the study

Adjuvant or adjuvant combination	Receptors or signaling pathway	Vaccines
Alum (Aluminium Hydroxide)	NLRP3 inflammasome	Various (HBV, HPV, Diphtheria, tetanus, pneumococcus)
QS-21 (Saponin from American barks)	Stimulate immune cells, deliver Ag to cytoplasm	Clinical trials
VSA-1 (analog of QS-21)	Potentially stimulate immune cells, deliver Ag to cytoplasm	Pre-clinical trials
CpG ODN (synthetic oligonucleotides)	TLR9 signaling stimulation	HBV
MPL (Monophosphoryl Lipid A)	TLR4 signaling stimulation	Clinical trials
QS-21 + MPL combination (Components of AS01 and AS02)	Combination TLR4 signaling stimulation	Malaria, TB, Cancer
CpG + MPL combination	TLR4+TLR9 signaling stimulation	Pre-clinical trials
BCG CWS (<i>Mycobacterium bovis</i> Bacillus Calmette-Guérin Cell Wall Skeleton)	TLR2+TLR4 signaling stimulation	Cancer

1.6 Possible adjuvant mechanisms

Many adjuvants are known to increase the vaccine efficacy by stimulation of the innate immune system, especially, antigen presenting cells (APCs). The APCs include dendritic cells (DCs) and macrophages. Adjuvants help in APC activation when the APCs uptake antigens. The activated APCs migrate to the secondary lymphoid organs, present the antigenic information to the immune cells and initiate antigen-specific immune responses. To increase efficacy of antigen-specific antibodies, isotype-switching and somatic hypermutation of immunoglobulin genes in B cells are required. These antibody maturation processes are known to be germinal center (GC) reaction because the reaction occurs in the GC of secondary lymphoid organs. GCs are formed as a result of cognate interactions of CD4⁺ T cells and B cells as well as the cytokines produced by

activated CD4⁺ T cells. Therefore, CD4⁺ T cells have been considered to be a critical cell type for adjuvants to induce strong antigen-specific immune responses (McKee et al., 2010; Pashine et al., 2005).

For vaccines aiming to induce cell-mediated immunity such as cancer vaccines, it is important they stimulate both antigen cross-presentation by DCs and DC maturation to initiate an optimal CD8⁺ T cell response. The “ideal” adjuvant thus combines both these characteristics and can prolong antigen exposure to the immune system. Saponin-based adjuvants (SBAs) stand out to enhance DC cross-presentation but are relatively poor in immune activation. Therefore, additional DC activation by e.g., TLR ligands is crucial. Moreover, combination of multiple pathogen recognition receptor (PRR) agonists can induce synergistic effects on DC activation (Trinchieri & Sher, 2007). Activating both the vacuolar and cytosolic pathway might be beneficial to enhance DC cross-presentation (Ho et al., 2018). To achieve prolonged antigen exposure, a new type of adjuvant formulation might be required. Based on pre-clinical and clinical data, a picture is emerging that an optimal vaccine adjuvant may require a combination of adjuvants rather than a single adjuvant entity. The clinically approved vaccine adjuvants AS01, AS02, and AS04 demonstrate that a combination of different adjuvants, especially TLR ligands combined with other adjuvant(s) such as saponins or alum, can be both potent and safe to use for human vaccination.

1.7 Homologous repeated influenza vaccination versus heterologous prime-boost influenza vaccination strategy

There is a high antigenic diversity in influenza A viruses, originating from 18 HA subtypes (H1-H18) and 11 NA subtypes (N1-N11). Influenza A virus HA subtypes are divided into two main phylogenetic groups, Group 1, and Group 2 (Pica & Palese, 2013). Influenza B virus has

evolved into antigenically distinct Victoria and Yamagata lineage strains. Current influenza vaccines contain either 3 or 4 strains of inactivated viruses. The vaccine components are from the same strains that were used in previous years or some vaccine strains are annually updated to better reflect the circulating influenza strains. Annual repeat vaccinations are recommended as a preventive measure, resulting in variable effectiveness against influenza (de Bruijn et al., 1999; Petrie & Monto, 2017; Sasaki et al., 2011; Smith et al., 1999). The overall effectiveness after seasonal vaccination has been in a wide low range between 10% and 60% during last decades. There are controversial studies reporting heterogeneity and reduced vaccine effectiveness in the current annual repeat influenza vaccination strategy (Belongia et al., 2017; Khurana et al., 2019; Leung et al., 2017; McLean et al., 2014; Smith et al., 1999; Song et al., 2020; Thompson et al., 2016). In a study investigating the effects of repeated vaccination (RV) in ferrets (Music et al., 2019), the ferrets that were immunized once (current season only) showed lower viral shedding, reduced body weight loss, and relatively faster recovery of weight after A/Hong Kong/H3N2 challenge, compared to another group of ferrets that were repeatedly immunized with commercial vaccines twice (10 months apart). An explanation for reduced vaccine effectiveness in the RV group can be related to the concept of Original Antigenic Sin wherein suboptimal antibodies (to the conserved domains between two vaccines) for protection are amplified by repeated vaccination.

To overcome the shortcoming of repeated vaccination, a strategy of heterologous prime-boost vaccination has been explored. A homologous prime-boost regimen is used in traditional vaccines, but better preventive effects have been reported for infectious diseases by a heterologous prime-boost strategy, which consists of priming with a specific antigen followed by boosting with another distinct antigen that is different from prime. The heterologous prime-boost vaccination

strategy can induce strong humoral and cellular immune responses (Kardani et al., 2016; Lu, 2009; Pan et al., 2020). A clinical study demonstrated that higher levels of antibody responses were induced by vaccines containing new strains as compared to the vaccines containing strains that were used in the previous year (Nunzi et al., 2017). It was also reported that original antigenic sin was not observed in humans and ferrets with prior influenza virus infection after exposure to the 2009 H1N1 pandemic virus (O'Donnell et al., 2014). Thus, it would be interesting to understand if a heterologous prime-boost vaccination strategy using inactivated influenza viruses would prove to be better than homologous repeated vaccination strategy in terms of conferring cross-protection against antigenically distinct influenza viruses.

1.8 Hypothesis and research objectives

1.8.1 Chapter 1: Comparison of the effects of different potent adjuvants on enhancing the immunogenicity and cross-protection by influenza virus vaccination in young and aged mice

The objective of this study was to compare the effects of three potent adjuvants or combination adjuvants (CWS, QS-21+MPL, CpG+MPL) on enhancing the immunogenicity and homologous and heterosubtypic protection of influenza vaccination in adult C57BL/6 and aged BALB/c mouse models. Few vaccine adjuvants have been licensed and used for human vaccines. In addition, the efficacy comparison of different adjuvants and the action mechanisms of the adjuvants have not been fully understood. It is highly significant to compare different adjuvant effects and understand the action mechanisms of adjuvants to improve the efficacy of vaccination. Despite many studies reporting superior effects of new adjuvants over Alum, parallel comparison to determine the superiority of these potent adjuvants in terms of immunogenicity and protection

from challenge remains unknown. In this study, I investigated the effects of adjuvants or adjuvant combinations on enhancing the humoral and cellular immunogenicity and homologous and cross-protective efficacy of inactivated split virus prepared from the 2009 H1N1 pandemic influenza virus strain. The experimental outcomes of this study demonstrated that QS-21+MPL adjuvant combination was most effective in inducing Th1 type IgG antibody responses, whereas both CpG+MPL and QS-21+MPL combination adjuvants exhibited similar potency in enhancing vaccination responses leading to increased protection by influenza challenge in adult C57BL/6 and aged BALB/c mouse models.

1.8.2 Chapter 2: Adjuvant effects of saponin analog VSA-1 on enhancing homologous and heterosubtypic protection by influenza virus vaccination

The goal of this study was to evaluate whether a newly synthesized semisynthetic saponin analog compound, VSA-1 has vaccine adjuvant effects. Alum remained the only licensed adjuvant for about 70 years until 1990s. Few other new potent adjuvants have been licensed for use in human vaccines since the 1990s. QS-21, a mixture of saponin compounds, has been included in the AS01-adjuvanted Shingrix vaccine. In this study, I investigated whether VSA-1, which is an analog of QS-21, exhibits adjuvant properties on enhancing the immunogenicity and homologous and cross-protection by influenza split virus vaccination in C57BL/6 mice. Development of a new type of adjuvant is an approach to provide more effective and safer vaccination and protection against infectious pathogens. Young children, the elderly and human immunodeficiency virus (HIV) infected patients have a high risk of infection by pathogens such as influenza virus, so they are required to get vaccinations. However, most of the commercial vaccines are for the healthy population, and vaccine efficacy is low in the immuno-compromised individuals. More effective new adjuvants need to be developed for improving the vaccine efficacy for this population

(Rappuoli et al., 2011; Siegrist & Aspinall, 2009; Weinberger et al., 2008). The experimental outcomes of this study demonstrated that a single dose of VSA-1 adjuvanted vaccination conferred superior protection against challenge with homologous H1N1 virus than QS-21 and alum adjuvants. VSA-1 exhibited adjuvant effects by inducing humoral and cellular immune responses as well as cross-protection against H5N1 virus after two doses of adjuvanted vaccination. Overall, this study provides supportive evidence warranting further development of VSA-1 as an alternative promising candidate for QS-21 replacement.

1.8.3 Chapter 3: Heterologous prime-boost vaccination with inactivated viruses induces more effective cross-protection than homologous repeat vaccination

The goal of this study was to test whether a strategy of heterologous prime-boost vaccination with inactivated influenza viruses would induce more effective cross-protection than homologous prime-boost vaccination. The impact of heterosubtypic vaccination with inactivated influenza virus as vaccines and pre-existing immunity on cross-protection against different subtypes of influenza viruses remains unknown. In this study, I investigated the efficacy of cross-protection by heterosubtypic prime-boost vaccination with inactivated H1N1 and H7N9 influenza virus vaccines in BALB/c mice. The experimental outcomes of this study demonstrated that the heterologous prime-boost vaccination strategy using inactivated virus vaccines induced more effective cross-protection against antigenically different viruses such as H3N2, H5N1, and H9N2 than homologous repeat vaccination with the same antigen in BALB/c mice. Boost immunization with antigenically different viruses from the strain used for prime immunization was found to induce hemagglutination inhibition activities against the prime virus strain as well as heterologous boost virus and higher efficacy of cross-protection. Possible correlates of group-specific HA stalk IgG antibody responses and roles of humoral and cellular immunity were explored.

2 CHAPTER 1. COMPARISON OF THE EFFECTS OF DIFFERENT POTENT ADJUVANTS ON ENHANCING THE IMMUNOGENICITY AND CROSS- PROTECTION BY INFLUENZA VIRUS VACCINATION IN YOUNG AND AGED MICE

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2.1 Abstract

Vaccination against influenza viruses suffers from low efficacy in conferring homologous and cross-protection, particularly in older adults. Here, we compared the effects of three different adjuvant types (QS-21+MPL, CpG+MPL and bacterial cell wall CWS) on enhancing the immunogenicity and homologous and heterosubtypic protection of influenza vaccination in young adult and aged mouse models. A combination of saponin QS-21 and monophosphoryl lipid A (QS-21+MPL) was most effective in inducing T helper type 1 (Th1) T cell and cross-reactive IgG as well as hemagglutination inhibiting antibody responses to influenza vaccination. Both combination adjuvants (QS-21+MPL and CpG+MPL) exhibited high potency by preventing weight loss and reducing viral loads and enhanced homologous and cross-protection by influenza vaccination in adult and aged mouse models. Bacillus Calmette-Guerin cell-wall skeleton (CWS) displayed substantial adjuvant effects on immune responses to influenza vaccination but lower adjuvant efficacy in inducing Th1 IgG responses, cross-protection in adult mice, and in conferring

homologous protection in aged mice. This study has significance in comparing the effects of potent adjuvants on enhancing humoral and cellular immune responses to influenza virus vaccination, inducing homologous and cross-protection in adult and aged populations.

2.2 Introduction

It is estimated that the influenza epidemic viruses infect 3 to 5 million individuals, causing approximately 250,000–500,000 annual deaths and an enormous economic burden worldwide (Viboud et al., 2010). Annually updated vaccination is recommended to reflect the new antigenic changes due to the high mutation frequencies of influenza viruses. Vaccine effectiveness has been variably low, between 10% and 60%, from 2005 to 2018 seasons (CDC, 2021c). Older individuals are known to have further lower influenza vaccination efficacy (12–13%), accounting for the majority (90%) of influenza-related deaths (Iuliano et al., 2018; Thompson et al., 2003). Aging-related immunosenescence explains the reduced capacity to generate protective de novo B and T cells to vaccination (Frasca & Blomberg, 2020; Kim et al., 2021). Potent vaccine adjuvants would enable enhancing the efficacy of influenza vaccination in older adults.

Aluminum salts (alum) have been the most commonly used adjuvant for licensed vaccine products, promoting T helper type 2 (Th2) antibody responses but with limited efficacy (Young et al., 2015). Since the 1990s, more potent adjuvants have been developed and approved for use in human vaccines that include Squalene oil-in-water emulsion MF59, AS04 (Alum+MPL), AS01 (Saponin QS-21+MPL in liposome), and CpG oligodeoxynucleotides (O'Hagan et al., 2020). MF59 in influenza vaccination significantly enhances vaccine immunogenicity in humans (O'Hagan et al., 2013), although cross-reactive antibodies are relatively limited (Bihari et al., 2012). The discovery of natural and synthetic agonists of pathogen recognition receptors such as

Toll-like receptors (TLRs) has contributed to advancing the adjuvant field and understanding the mechanism of adjuvant actions. Particularly, monophosphoryl lipid A (MPL in AS04) and CpG, TLR4, and TLR9 agonists, respectively, are approved for use in licensed vaccines, including Cervarix and Heplisav (O'Hagan et al., 2020). AS01 containing MPL and QS-21 from *Quillaja Saponaria* is included in Shingles vaccination for older individuals (≥ 50 years old) (James et al., 2018; Lal et al., 2015). Bacillus Calmette-Guerin (BCG) vaccination is associated with better clinical outcomes in COVID-19 patients due to immune training rather than specific immune memory (Escobar et al., 2020). Clinical benefits of BCG cell-wall skeleton (CWS) as an innate immune therapy have been demonstrated in cancer patients (Kodama et al., 2009). CWS is known to mediate adjuvant effects via TLR2 and TLR4 signaling pathways (Tsuji et al., 2000). CWS-adjuvanted influenza vaccination significantly enhances the immunogenicity and protective efficacy in infant, adult, and older age BALB/c mouse models (Kim et al., 2021). CpG plus MPL adjuvanted influenza vaccination has been shown to induce enhanced protection against homologous and heterosubtypic viruses in mice (Ko et al., 2018). Despite many studies reporting superior effects of new adjuvants over Alum, parallel comparison to determine the superiority of these potent adjuvants in terms of immunogenicity and protection from challenge remains unknown. In this study, we compared the effects of three potent adjuvants or combination adjuvants (CWS, QS-21+MPL, CpG+MPL) on enhancing the immunogenicity and homologous and heterosubtypic protection of influenza vaccination in adult C57BL/6 and aged BALB/c mouse models. QS-21+MPL was found to be most effective in inducing Th1 type IgG antibody responses, whereas both CpG+MPL and QS-21+MPL combination adjuvants exhibited similar potency in enhancing vaccination responses leading to increased protection by influenza challenge in adult C57BL/6 and aged BALB/c mouse models.

2.3 Materials and Methods

2.3.1 *Animals, reagents, and viruses*

Adult C57BL/6 mice (6- to 8-week-old, female) were purchased from Jackson laboratory (Bar Harbor, ME). Aged BALB/c mice (17- to 20- month-old, female) were provided by the National Institutes of Health (NIH). BALB/c mice at ages of 8–10 months, purchased from Taconic Biosciences (Rensselaer, NY), were aged to become 17- to 20-month-old in the animal facility at Georgia State University to supplement the limited supply of aged mice from NIH. All mouse experiments were performed under the guidelines of the approved IACUC protocol (A21004). Pure *Quillaja* saponin, QS-21, and monophosphoryl lipid A (MPL) adjuvants were purchased from Desert King International (San Diego, CA, USA) and Sigma Aldrich (St. Louis, MO), respectively, and dissolved in dimethyl sulfoxide (DMSO) following the manufacturer's protocol. Mouse-specific oligodeoxynucleotide (ODN) with CpG motifs (ODN1826, 5'-TCC ATG ACG TTC CTG ACG TT-3') was synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The lyophilized CpG was resuspended in ultra-pure water. Cell wall skeleton (CWS) from *Mycobacterium bovis* BCG (Pasteur 1173P2) was generously provided by Dr. Jo (Chungnam National University, Korea) and prepared as described (Kim et al., 2021). All the adjuvants were aliquoted and saved at - 80 °C until use. A/California/04/2009 (A/Cal) H1N1 virus was used as the split vaccine strain and for homologous challenge. Reverse genetics (rg) reassortant H5N1 (rgH5N1) virus, containing HA and NA derived from A/Vietnam/1203/2004 and six internal genes from A/Puerto Rico/8/ 1934, as described previously (Song et al., 2011), was used as a heterologous challenge virus. A/Cal H1N1 and rgH5N1 viruses were propagated using embryonated chicken eggs. Inactivated viruses used as coating antigens for Enzyme-linked

immunosorbent assay (ELISA) were prepared as described previously (Ko et al., 2018). Briefly, A/Cal H1N1 or rgH5N1 influenza viruses were inactivated with 1% formalin and concentrated by ultracentrifugation (SW32 Ti rotor, 123,760×g, 1 h). The inactivated virus pellet was resuspended in phosphate-buffered saline (PBS) and was used as the ELISA coating antigen. For influenza split vaccine (sCal) preparation, inactivated A/Cal H1N1 virus was treated with 1% Triton X-100 to disrupt virion particles. The disrupted virus particles were dialyzed against PBS 3 times overnight. The total protein concentration of the inactivated viruses was determined by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.3.2 Immunization and virus challenge of mice

To determine the protective efficacy of combination adjuvants against homologous virus (A/Cal H1N1), groups of 6-week-old C57BL/6 mice (n = 7) were intramuscularly immunized with sCal (3 µg/mouse) alone or sCal (3 µg/mouse) plus CWS (25 µg/mouse), QS-21 (10 µg) + MPL (1 µg)/mouse, or CpG (4 µg) + MPL (1 µg)/mouse. Young adult (6 weeks old) BALB/c mice (n = 4) were prime immunized with sCal (0.6 µg/mouse) alone vaccine for comparison with C57BL/6 mice. Blood samples were collected at 2 weeks after immunization, and sera were separated. Three weeks after prime immunization, the mice were challenged with a lethal dose of homologous A/Cal H1N1 virus ($2 \times \text{LD}_{50}$, equivalent to $\text{Log}10^{3.7} \times \text{EID}_{50}$). For cross-protection studies, C57BL/6 mice (n = 6) were intramuscularly immunized (prime and boost) with sCal alone or sCal plus CWS, QS-21+MPL, or CpG+MPL at a 3-week interval. Three weeks after boost, the mice were challenged with a lethal dose of heterosubtypic rgH5N1 virus ($2 \times \text{LD}_{50}$, equivalent to $\text{Log}10^{3.4} \times \text{EID}_{50}$) in the BSL-2+ designated animal facility at Georgia State University. Considering the availability and further aging disadvantages in response to vaccination, BALB/c strain was used for the aged mouse model studies. BALB/c mice (n = 12), at 17–20 months of age, were

intramuscularly immunized (prime and boost) with sCal (1 µg/ mouse) alone or sCal plus CWS, QS-21+MPL, or CpG+MPL at a 3-week interval. Four weeks after boost, the immunized mice were challenged with a lethal dose of homologous A/Cal H1N1 virus ($2 \times \text{LD}_{50}$). After challenge, body weight changes and survival rates were monitored for 14 days, and lung viral titers and detailed immunological profiles were determined in bronchoalveolar lavage (BAL), mediastinal lymph nodes (MLN), lung and spleen tissues collected at day 5 post infection.

2.3.3 Antibody enzyme-linked immunosorbent assay (ELISA) and in vitro IgG

antibody detection

To measure antigen-specific antibody levels, inactivated A/Cal H1N1 or rgH5N1 viruses (200 ng/well) were coated onto ELISA plates and then incubated with diluted immune sera as detailed previously (Ko et al., 2018; Ko et al., 2016). IgG isotypes were measured using anti-mouse immunoglobulin IgG, IgG1 and IgG2a (or IgG2c), horse-radish peroxidase (HRP)-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL), and tetramethylbenzidine (TMB) substrate (Invitrogen, Waltham, MA). Antibody levels are presented as optical density (OD) absorbance values at 450 nm (BioTek ELISA plate reader) or as concentrations calculated using standard IgG (Southern Biotech). Additionally, consensus group I hemagglutinin (HA) stalk protein (50 ng/well), prepared as described (Chae et al., 2019), and N1 neuraminidase (NA) protein (20 ng/well, BEI Resources, NR-19234) derived from A/California/04/2009 (H1N1) virus were used to determine HA stalk and NA-specific IgG antibodies. Bronchoalveolar lavage fluid (BALF) was obtained by infusing 1.5 mL of PBS into the lungs. Lung extracts were prepared in 1.5 mL of Roswell Park Memorial Institute (RPMI) 1640 without fetal bovine serum (FBS) by mechanical grinding of lung tissues harvested at day 5 after challenge. BALF and lung extracts were used to determine vaccine antigen (A/Cal)-specific

antibody levels. Secreted IgG antibodies specific for A/Cal H1N1 were determined from mediastinal lymph nodes (MLN, 5×10^5 cells/well) and spleen tissues (5×10^5 cells/well) from C57BL/6 and BALB/c mice. The cells from MLN and spleen were isolated at day 5 post infection and cultured for 1 day and 5 days in 96-well cell culture plates (Corning, Kennebunk, ME) pre-coated with inactivated A/Cal H1N1 virus (200 ng/well). The combined levels of IgG antibodies secreted into the culture supernatants and those captured on the plate were analyzed by ELISA.

2.3.4 Hemagglutination inhibition (HAI) assay

HAI titers in immune sera treated with receptor destroying enzymes (RDE, Sigma-Aldrich) and inactivated (at 56 °C for 30 min) were determined by using 4 HA units of A/Cal H1N1 or rgH5N1 virus and 0.5% chicken red blood cells (RBC, Lampire Biological Laboratories, Pipersville, VA) as previously described (Ko et al., 2018; Ko et al., 2016).

2.3.5 Lung viral titration

Lung extracts prepared in 1.5 mL of RPMI 1640 without FBS by mechanical grinding of lung tissues harvested at day 5 after challenge were used to determine viral titers in embryonated chicken eggs (Hy-Line North America, LLC, Mansfield, GA), as described (Kim et al., 2019). Virus titers as 50% egg infection dose (EID₅₀)/mL were evaluated according to the Reed and Muench method (Reed & Muench, 1938).

2.3.6 Enzyme-linked immunospot (ELISpot) assay

Interferon (IFN)- γ secreting cell spots were determined by culturing splenocytes (5×10^5 or 10^6 cells/well) and lung cells (5×10^5 or 3×10^5 cells/well) for 72 h on multi-screen 96-well plates (MilliporeSigma, St. Louis, MO) coated with cytokine-specific capture antibodies as described (Song et al., 2011). Inactivated influenza A/Cal H1N1 was included as an antigenic

stimulator (4 µg/mL) and IFN-γ secreting T cells were counted using an ELISpot reader (Biosys, Miami, FL).

2.3.7 Flow cytometry analysis

Lung cells were harvested from the layer of percoll gradients between 44% and 67% and stimulated with 4 µg/mL inactivated A/Cal H1N1 virus in the presence of Brefeldin A (20 µg/mL) for 5 h at 37 °C as described (Lee et al., 2018, 2019). *In vitro* cultured cells (lung and BAL cells) were stained with anti-CD3-PacificBlue (Clone 17A2, Biolegend, San Diego, CA), anti-CD4-PE/Cy5 (Clone RM405, BD Biosciences, San Jose, CA) antibodies, and then fixed and permeabilized using BD Cytofix/Cytoperm™ Plus Kit (BD Biosciences). After staining the cells with anti-IFN-γ-APC/Cy7 (Clone XMG1.2, BD), anti-TNF-α-PE/Cy7 (Clone MP6-XT22, Biolegend), and anti-Granzyme B-FITC (Clone NGZB, eBioscience) antibodies, live lymphocytes were first gated by forward versus side scatter strategic gating, followed by the gating of CD3⁺ T cells and then CD4 T cells secreting cytokines. The number of effector T cells in BAL and lung were expressed by reflecting the frequency gated out of the total cells from each mouse. Cells positive for intracellular cytokines were revealed through acquisition on a Becton-Dickinson LSR-II/Fortessa flow cytometer (BD, San Diego, CA) and analyzed by Flowjo software (Tree Star Inc., Ashland, OR).

2.3.8 Analysis of acute innate immune responses to intraperitoneal adjuvant treatment

Naïve C57BL/6 mice were intraperitoneally injected with 200 µL of PBS, QS-21 (10 µg) + MPL (1 µg), or CpG (4 µg) + MPL (1 µg). Sera were collected from the mice at 2 h and 20 h after adjuvant injection to analyze systemic cytokine and chemokine levels using Ready-SET-Go kits with IL-6 or MCP-1 specific antibodies (eBioscience, San Diego, CA). Immune cells in peritoneal exudates from the mice were collected in 2 mL of PBS at 20 h after adjuvant treatment

and cellular phenotypes were determined by cell-specific phenotypic markers as described (Ko et al., 2018).

2.3.9 Statistical analyses

All results are presented as mean \pm standard errors of the mean (SEM). The statistical significance for all the experiments was calculated by one-way or two-way analysis of variance (ANOVA). P-values ≤ 0.05 were considered significant. Data analysis was performed using Prism software (GraphPad Software Inc., San Diego, CA).

2.4 Results

2.4.1 Adjuvants enhance the protective efficacy of split vaccination despite differential

IgG responses in C57BL/6 mice

C57BL/6 strain is a relatively low responder mouse model in humoral immune responses to experimental vaccination (Chen et al., 1999; Ko et al., 2018; Mislson et al., 2010). To compare the efficacy of different adjuvants, groups of C57BL/6 mice were intramuscularly primed with split sCal (H1N1) vaccine (3 μ g) plus CWS, QS-21+MPL, or CpG+MPL (Fig. 2.1A). To directly compare the levels of IgG responses between young adult BALB/c and C57BL/6 mice, a group of BALB/c mice was intramuscularly primed with split sCal (H1N1) vaccine (Fig. 2.1B and C). The BALB/c mice with low dose sCal (0.6 μ g) vaccination induced higher levels of IgG and IgG1 antibodies specific for the virus antigen, compared to those in C57BL/6 mice immunized with sCal (3 μ g), as shown by ELISA OD values (Fig. 2.1D–F). Therefore, we reasoned that C57BL/6 mouse strain would be an appropriate model to compare the potency of different vaccine adjuvants. CWS was not combined with MPL because CWS exhibits adjuvant effects via TLR2 and TLR4 signaling pathways (Tsuji et al., 2000; Uehori et al., 2003). The QS-21+MPL adjuvanted sCal group showed

the highest levels of IgG1 and IgG2c isotype antibodies for A/Cal H1N1 after prime vaccination in C57BL/6 mice, followed by the CpG+MPL group, whereas the CWS group induced the lowest levels of IgG2c (Fig. 2.1D–F). The inclusion of adjuvants significantly enhanced IgG responses specific for HA stalk domain and HAI activities against homologous A/Cal H1N1 virus at similar levels (Fig. 2.1G and H). All adjuvanted vaccine groups were protected against lethal challenge with homologous A/Cal H1N1 virus, displaying 100% survival rates without severe weight loss, in contrast to the sCal alone and naïve groups that showed severe weight loss and no survival rates (Fig. 1I). At day 5 post challenge, approximately 100-fold lower levels of lung viral titers were detected in all adjuvanted vaccine groups than those in sCal alone and naïve infected groups (Fig. 1J).

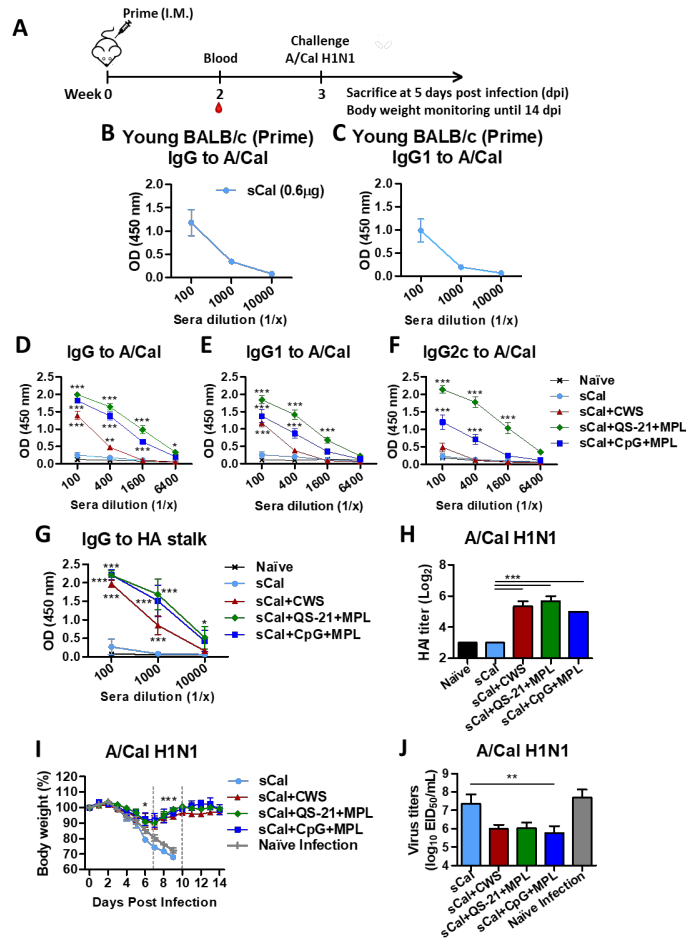


Figure 2.1 A single dose of adjuvanted influenza vaccination confers protection against lethal challenge with homologous A/Cal H1N1 virus in C57BL/6 mice. (A) Immunization scheme (n = 7). C57BL/6 mice were intramuscularly immunized with sCal (3 µg) split vaccine alone or sCal plus CWS (25 µg), QS-21 (10 µg) + MPL (1 µg) or CpG (4 µg) + MPL (1 µg). A timeline for collection of blood samples and challenge is indicated. (B and C) Antibodies to A/Cal H1N1 virus were determined in prime sera from young adult BALB/c mice (n = 4) intramuscularly immunized with low dose sCal (0.6 µg). (D–F) A/Cal H1N1 virus-specific IgG and isotype antibody levels were determined in prime sera. (G) Antibody responses specific for consensus group I full-length HA stalk in prime sera. (H) HAI titers against A/Cal H1N1 virus in prime sera. Three weeks post immunization, the mice were challenged with a lethal dose of A/Cal H1N1 virus ($2 \times \text{LD}_{50}$). (I) Body weight changes were monitored for 14 days. (J) Lung viral titers as 50% egg infectious titers (EID_{50}) at day 5 post infection using embryonated chicken eggs. Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to sCal group

2.4.2 Adjuvanted vaccination enhances IgG levels at respiratory sites, IgG secreting plasma and cytokine-producing T cells at early time post challenge in C57BL/6 mice

Adjuvant effects on inducing vaccine antigen-specific B and T cell responses were compared at day 5 post challenge with A/Cal H1N1 virus (Fig. 2.2). Higher levels of A/Cal-specific IgG antibodies were observed in BALF and lung extracts from the adjuvanted vaccine groups (Fig. 2.2A and B). Also, after *in vitro* culturing MLN and spleen cells for day 1 or 5, A/Cal-specific IgG antibodies from the adjuvanted vaccine groups were produced at higher levels than those from sCal and naïve mice at day 5 post infection (Fig. 2.2C and D). IFN- γ producing spots from lung and spleen samples and IFN- γ^+ CD4 $^+$ T cells from BALF were induced at higher levels in the adjuvanted sCal vaccinated C57BL/6 mice than those in sCal vaccinated and naïve mice at early time post challenge (Fig. 2.2E–G).

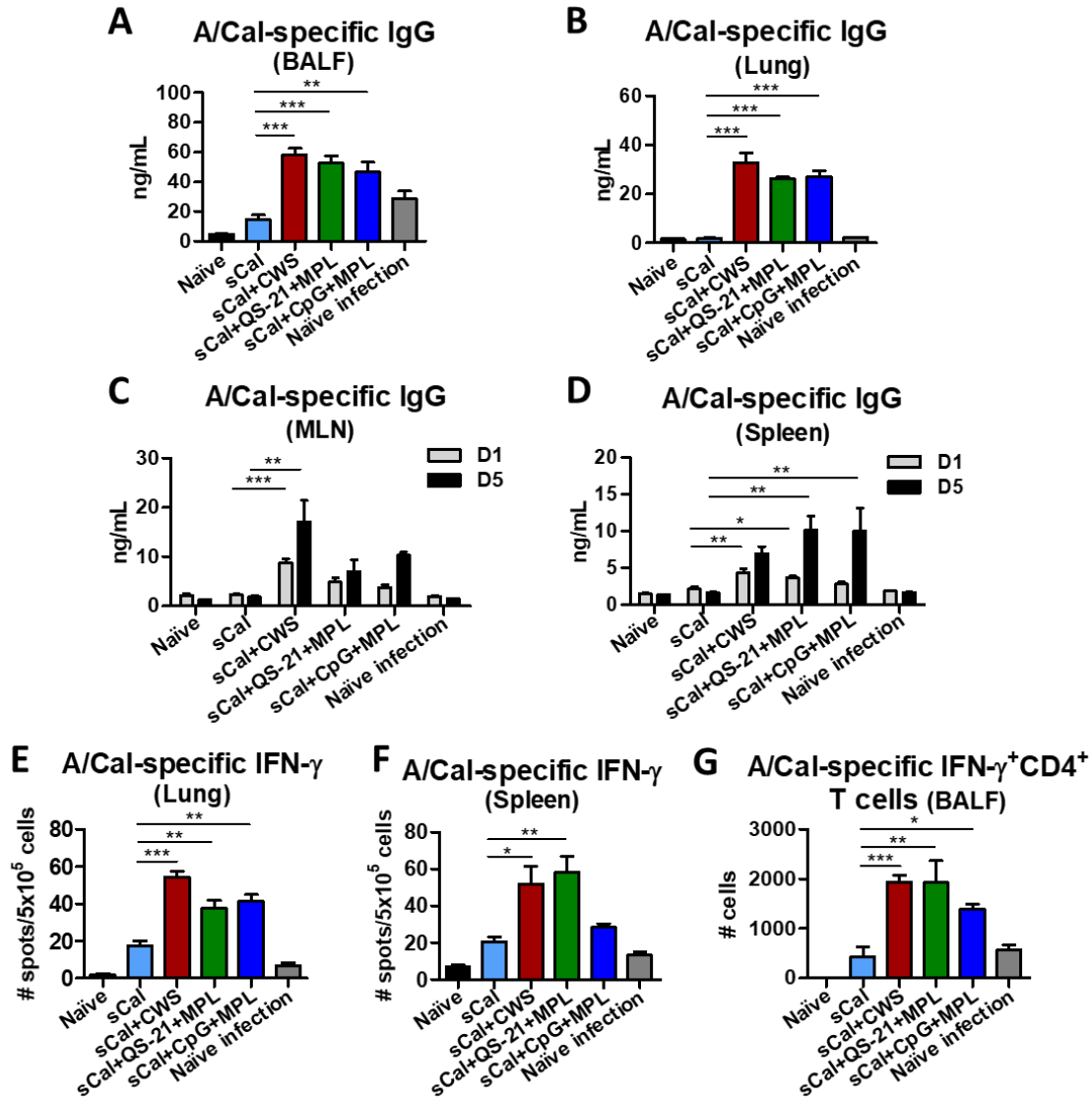


Figure 2.2 Adjuvanted vaccination enhances antibody-secreting cells and effector T cells upon influenza virus infection in young C57BL/6 mice. (A and B) A/ Cal virus-specific IgG levels in BALF and lung samples collected at day 5 post challenge (n = 4). (C and D) A/Cal virus-specific IgG production from in vitro cultures of MLN cells and splenocytes harvested on day 5 post infection, for 1 day and for 5 days in the presence of inactivated A/Cal H1N1 virus (200 ng/well). (E and F) Cytokine ELISpot of lung cells and splenocytes at day 5 post infection after in vitro stimulation with inactivated A/Cal (4 μ g/mL per well). (G) Flow cytometry data of IFN- γ -secreting CD4⁺ T cells in BALF airway samples. Statistical significance was calculated by using one-way ANOVA and Dunnett's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, p < 0.05, **, p < 0.01, ***, p < 0.001 compared to sCal group

2.4.3 Prime-boost adjuvanted vaccination induces humoral and cellular immune responses contributing to heterosubtypic cross-protection in C57BL/6 mice

A regimen of prime boost vaccination and heterosubtypic rgH5N1 virus challenge is presented (Fig. 2.3A). The highest levels of IgG and IgG2c isotype antibodies specific for A/Cal H1N1 virus were induced in the QS-21+MPL adjuvanted sCal group after booster dose, followed by the CpG+MPL and CWS groups (Fig. 2.3B–D). Substantial levels of IgG and IgG1 (Fig. 2.3B and C) but not IgG2c isotype antibodies (Fig. 2.3D) were induced after boosting with sCal vaccine alone. IgG antibodies binding to heterosubtypic rgH5N1 virus (Fig. 2.3E), HA stalk domain (Fig. 2.3F), and N1 NA (Fig. 2.3G) were induced at the highest levels in the QS-21+MPL adjuvanted sCal group after boost, followed by the CpG+MPL and CWS groups. Consistent with the IgG levels, the highest titers of HAI were induced in the QS-21+MPL adjuvanted sCal group, followed by the CWS and CpG+MPL groups at 2 weeks and 3 months after boost vaccination (Fig. 2.3H and I). We determined whether prime boost adjuvanted vaccination would induce cross-protection in C57BL/6 mice after lethal challenge with rgH5N1 virus. The CpG+MPL and QS-21+MPL adjuvanted sCal groups displayed the least weight loss and rapidly recovered by day 8 post challenge (<5%), whereas the average peak weight loss in the CWS group was approximately 11% (Fig. 3J). The sCal only and naïve groups showed severe weight loss, and all mice died of infection in contrast to 100% protection in the adjuvanted sCal vaccine groups (Fig. 2.3J and K). The CWS adjuvant group showed the lowest levels of HA stalk and N1 NA antibodies (Fig. 2.3F and G) after vaccination and the highest T cell responses at recall compared to the other groups (Fig. 2.2E–G).

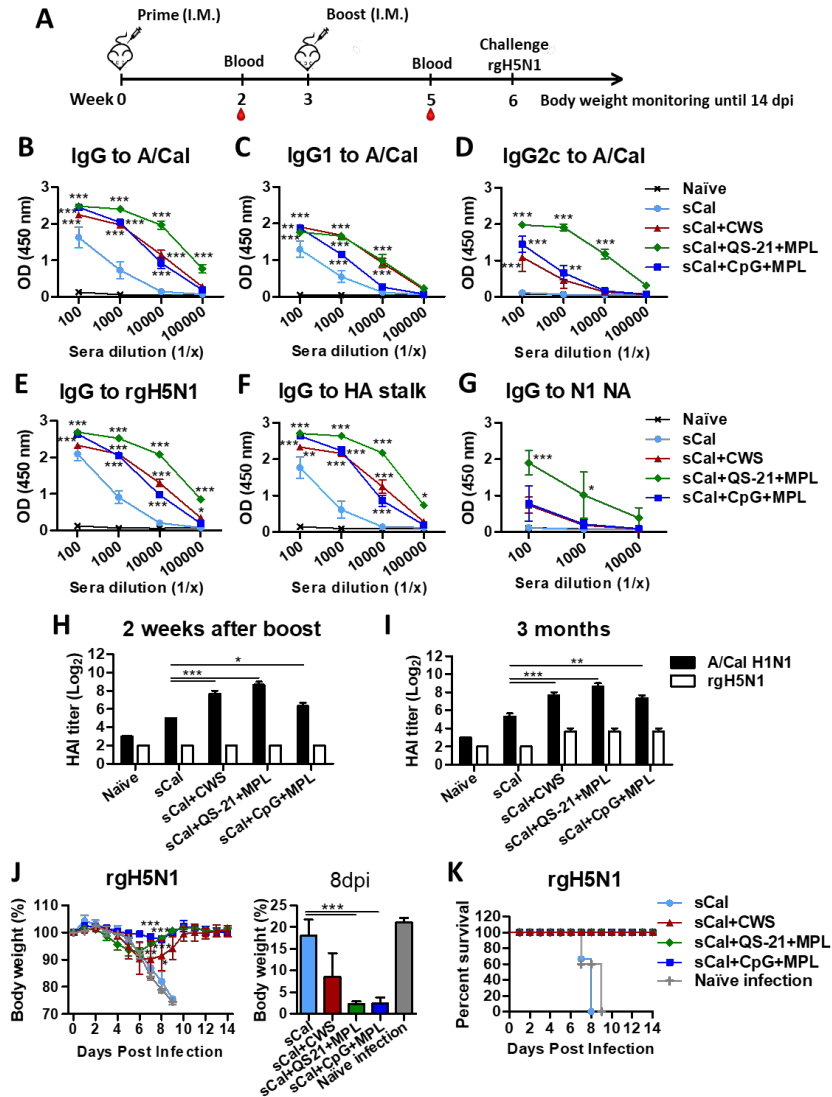


Figure 2.3 Adjuvanted prime-boost vaccination confers protection against lethal infection with heterosubtypic rgH5N1 virus in young C57BL/6 mice. (A) Immunization scheme (n = 6). C57BL/6 mice were intramuscularly prime and boost immunized at a 3-week interval with sCal alone or sCal plus CWS, QS-21+MPL, or CpG+ MPL. (B–D) A/Cal H1N1 virus-specific IgG and isotype antibody levels in boost sera. (E) rgH5N1 virus-specific IgG antibody levels in boost sera. (F) Consensus group I full-length HA stalk-specific IgG antibody levels in boost sera. (G) N1 NA-specific IgG antibody levels in boost sera. (H and I) HAI titers against A/Cal H1N1 virus at two weeks (H) and three months (I) after boost. (J) Body weight changes and (K) Survival rates after lethal infection with rgH5N1 virus ($2 \times LD_{50}$). Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to sCal group

To determine the roles of T cells in conferring cross-protection, both CD4 and CD8 T cells were depleted before and after challenge, and body weight changes and survival rates were monitored (Fig. 2.4). The CWS adjuvanted sCal group with CD4/8 depletion was not protected (Fig. 2.4A), suggesting dependency on T cell immunity. Meanwhile, the QS-21+MPL and CpG+MPL groups (Fig. 2.4B and C) with CD4/8 depletion displayed severe weight loss in a wide range of 12–18% against lethal challenge with rgH5N1 virus, suggesting that both humoral and cellular immunity contribute to cross-protection.

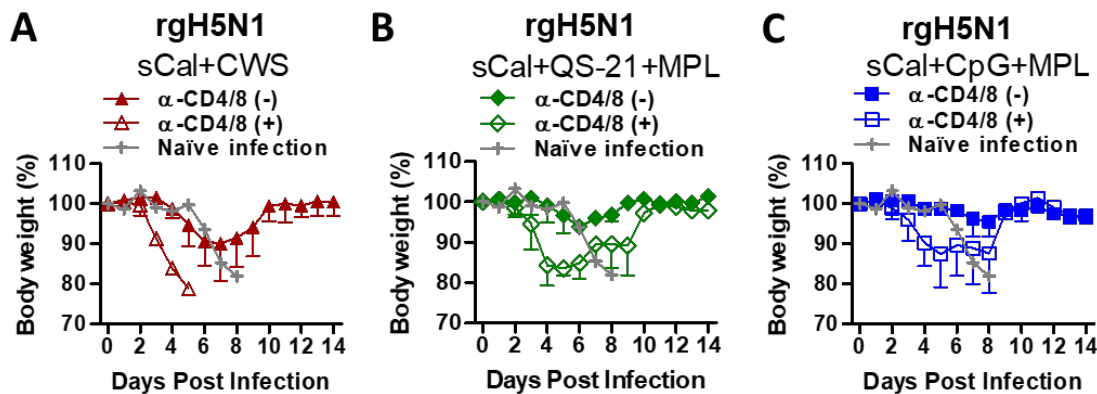


Figure 2.4 CD4 and CD8 T cells play an important role in conferring cross-protection in the adjuvanted vaccination groups. For in vivo systemic T cell depletion prior to and post challenge, prime-boost immunized C57BL/6 mice ($n = 3$) received treatment with anti-CD4 (CD4 clone GK1.5) and anti-CD8 (CD8 clone 53.6.7) monoclonal antibodies (mAbs). Antibodies (BioXCell, West Lebanon, NH) were injected into the mice with intraperitoneal (IP; 1 day before challenge) and intranasal (IN; 1 day after challenge) sequential delivery at a 2-day interval (anti-CD4 200 μ g and anti-CD8 150 μ g /mouse for IP injection, 10 μ g anti-CD4/8/mouse for IN inoculation). All groups ($n = 3$) were challenged with a lethal dose of rgH5N1 influenza virus ($2 \times LD_{50}$). (A-C) Body weight changes were monitored for 14 days after challenge. Error bars indicate the mean \pm standard errors of the mean (SEM)

2.4.4 Adjuvanted vaccination enhances IgG antibodies, HAI titers, and protection in aged BALB/c mice

A regimen of prime boost vaccination and homologous A/Cal H1N1 virus challenge in aged BALB/c mice is presented (Fig. 2.5A). QS-21+MPL adjuvanted sCal prime vaccination of aged BALB/c mice induced a measurable level of IgG2a isotype, in contrast to CWS adjuvanted sCal prime dose promoting IgG1 antibody production, but the overall IgG and isotype levels were significantly lower than those in young adult C57BL/6 mice (Fig. 2.6A–C). Adjuvanted sCal boost vaccination substantially enhanced the induction of IgG, IgG1, and IgG2a isotype antibodies, with the QS-21+MPL group displaying the highest IgG2a levels in aged BALB/c mice (Fig. 2.5B–D). IgG antibodies binding to the HA stalk domain (Fig. 2.5E) and N1 NA (Fig. 2.5F) were induced at the highest levels in the QS-21+MPL adjuvanted sCal group after boost, followed by the CWS and CpG+MPL groups. The HAI functional antibodies against homologous A/Cal H1N1 virus were induced at higher levels in the CWS and QS-21+MPL groups than those in the CpG+MPL group, while the sCal vaccine alone group could not induce detectable levels of HAI titers in aged BALB/c mice (Fig. 2.5G). Both CpG+MPL and QS-21+MPL adjuvanted sCal groups were 100% protected against lethal challenge with A/Cal H1N1 virus, with the CpG+MPL group displaying a trend of lower weight loss (~4%) compared to the QS-21+MPL vaccine group (~6%) and quicker recovery, but there were no statistical differences (Fig. 2.5H). The CWS group also provided 100% protection against A/Cal H1N1 virus challenge, despite a moderate weight loss (~9%) without significant differences among the adjuvanted groups. In contrast, heat-killed lactic acid bacterium (LAB) shown to be an effective adjuvant for influenza vaccination in C57BL/6 mice (Jung et al., 2020) did not improve the vaccine efficacy, resulting in severe weight loss in aged BALB/c mice (Fig. 2.7). The sCal alone and naïve aged BALB/c mice experienced severe weight loss and did

not survive A/Cal virus challenge (Fig. 2.5H). Approximately 100-fold lower lung viral titers were detected in the adjuvanted sCal groups at day 5 after challenge, compared to those in naïve infected aged BALB/c mice (Fig. 2.5I).

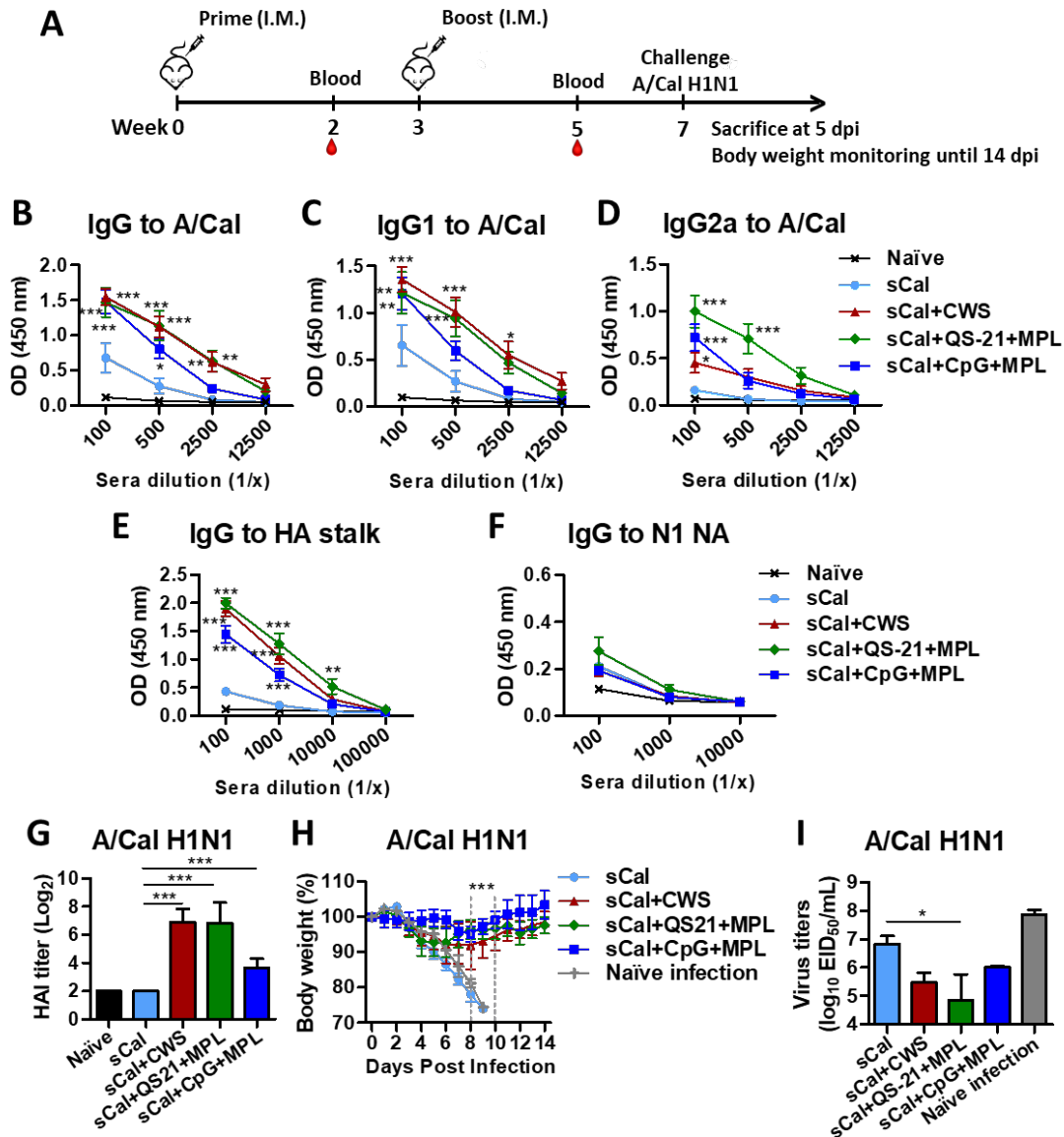


Figure 2.5 Adjuvanted boost vaccination confers protection against lethal challenge with homologous

A/Cal H1N1 virus in aged BALB/c mice. (A) Immunization scheme. Aged BALB/c mice ($n = 12$) were intramuscularly prime and boost immunized with sCal ($1 \mu\text{g}$) alone or sCal plus CWS, QS-21+MPL, or CpG+MPL. (B-D) A/Cal H1N1 virus-specific IgG and isotype antibody levels in boost sera. (E and F) IgG antibody specific for HA stalk and N1 NA proteins in boost sera. (G) HAI titers against A/Cal H1N1 virus in boost sera. (H) Body weight changes after challenge with a lethal dose of A/Cal H1N1 virus ($2 \times \text{LD}_{50}$). (I) Lung viral titers (EID_{50}) at day 5 post infection. Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to sCal group

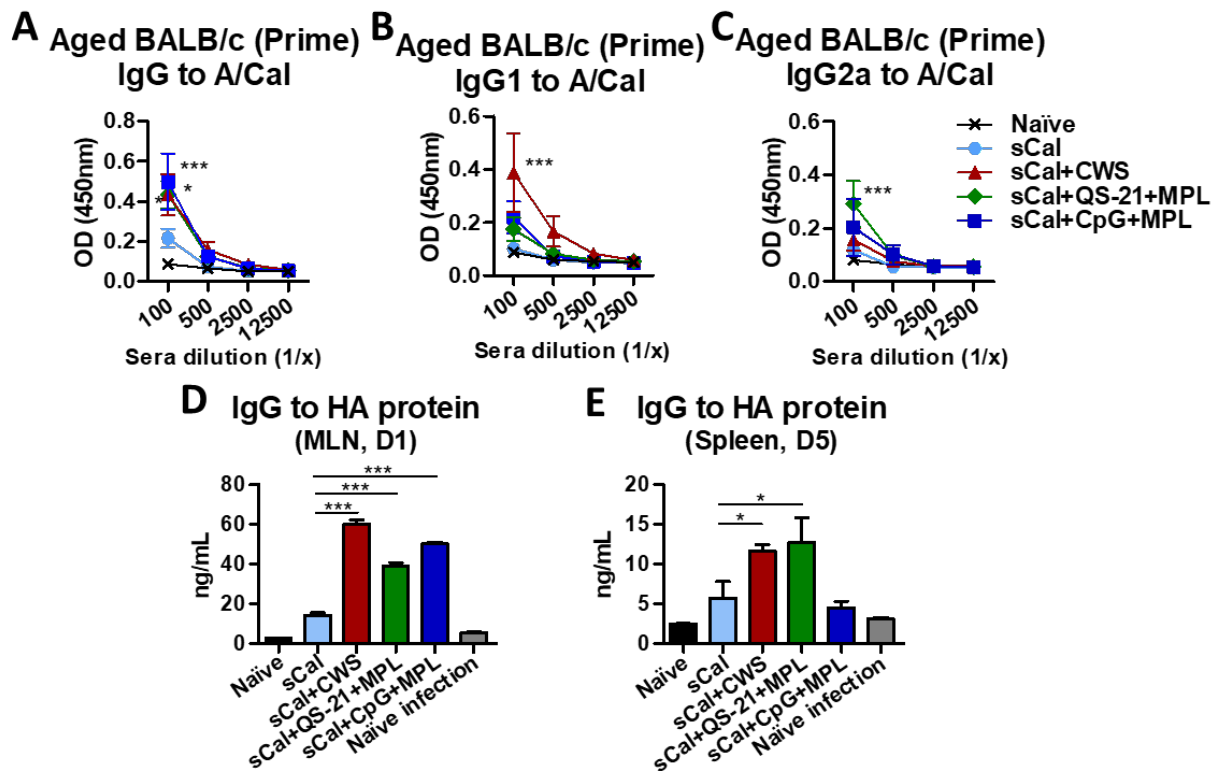


Figure 2.6 Adjuvanted vaccination enhances IgG antibodies after prime immunization, and HA protein-specific antibody-secreting cells and effector T cells upon influenza virus infection in aged BALB/c mice. Aged BALB/c mice ($n = 7$) were intramuscularly prime, and boost immunized with sCal (1 μ g) alone or sCal plus CWS, QS-21+MPL, or CpG+MPL. Blood samples were collected at 2 weeks after prime immunization. Four weeks after boost, the immunized mice were challenged with a lethal dose of homologous A/Cal H1N1 virus ($2 \times LD_{50}$). (A-C) A/Cal H1N1 virus-specific IgG and isotype antibody levels were determined in prime sera. (D and E) H1 HA (A/Cal)-specific IgG production from MLN cells and splenocytes harvested on day 5 post infection after in vitro culture for 1 day (MLN) or for 5 days (spleen) with H1 HA protein (BEI Resources, NR-15749). IgG antibody levels secreted into the culture and captured on the plates were determined by ELISA. Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, ***, $p < 0.001$ compared to sCal group

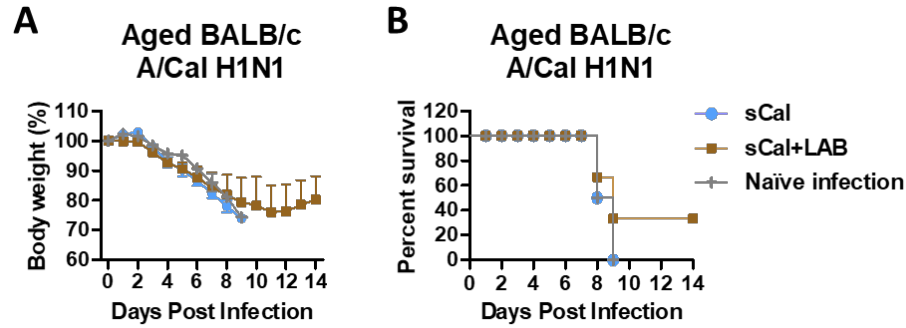


Figure 2.7 Heat-killed lactic acid bacterium (LAB) displays weak adjuvant effects on the efficacy of influenza vaccination in aged BALB/c mice. Aged BALB/c mice ($n = 3$) were intramuscularly prime-boost immunized twice at a 3-week interval with sCal (1 μ g) or sCal plus heat-killed LAB (10^8 PFU). Three weeks post immunization, the mice were challenged with a lethal dose of A/Cal H1N1 virus ($2 \times LD_{50}$). (A) Body weights of the immunized mice were monitored for 14 days. (B) Survival rates of the immunized mice after lethal infection with A/Cal H1N1 virus were determined. Error bars indicate the mean \pm standard errors of the mean (SEM)

2.4.5 Aged BALB/c mice with adjuvanted vaccination induce IgG levels at respiratory sites, IgG secreting plasma and cytokine-producing T cells at early time post challenge

Correlating with protection, higher levels of A/Cal-specific IgG antibodies were induced in BALF and lung extracts at day 5 post challenge from the adjuvanted vaccine groups, compared to the significantly low levels in the sCal vaccine only and naïve aged BALB/c mice (Fig. 2.8A). Also, one- or five-day *in vitro* culture of MLN cells and spleen cells produced higher levels of A/Cal virus-specific IgG antibodies from the adjuvanted vaccine groups than those in sCal alone and naïve aged BALB/c mice (Figs. 2.8B and C). These IgG antibodies produced *in vitro* recognized HA proteins (Figs. 2.6D and E) in a similar pattern as the binding to A/Cal H1N1 virus. Intracellular cytokine staining results showed that higher levels of CD4⁺ T cells secreting IFN- γ (in all three adjuvants), TNF- α (highest in CWS), or granzyme B (highest in QS-21+MPL) were

detected in lung tissue samples from the adjuvanted sCal groups at day 5 post infection than those in sCal alone and naïve groups (Fig. 2.8D–F). In the cytokine ELISpot data (Fig. 2.8G and H), the QS-21+MPL adjuvanted sCal group showed the highest levels of IFN- γ^+ spots in lung and spleen tissues, followed by the CWS group. The CpG+MPL group also showed substantial levels of IFN- γ^+ spots in lung and spleen tissues, but at a slightly lower level than the QS-21+MPL and CWS adjuvant groups.

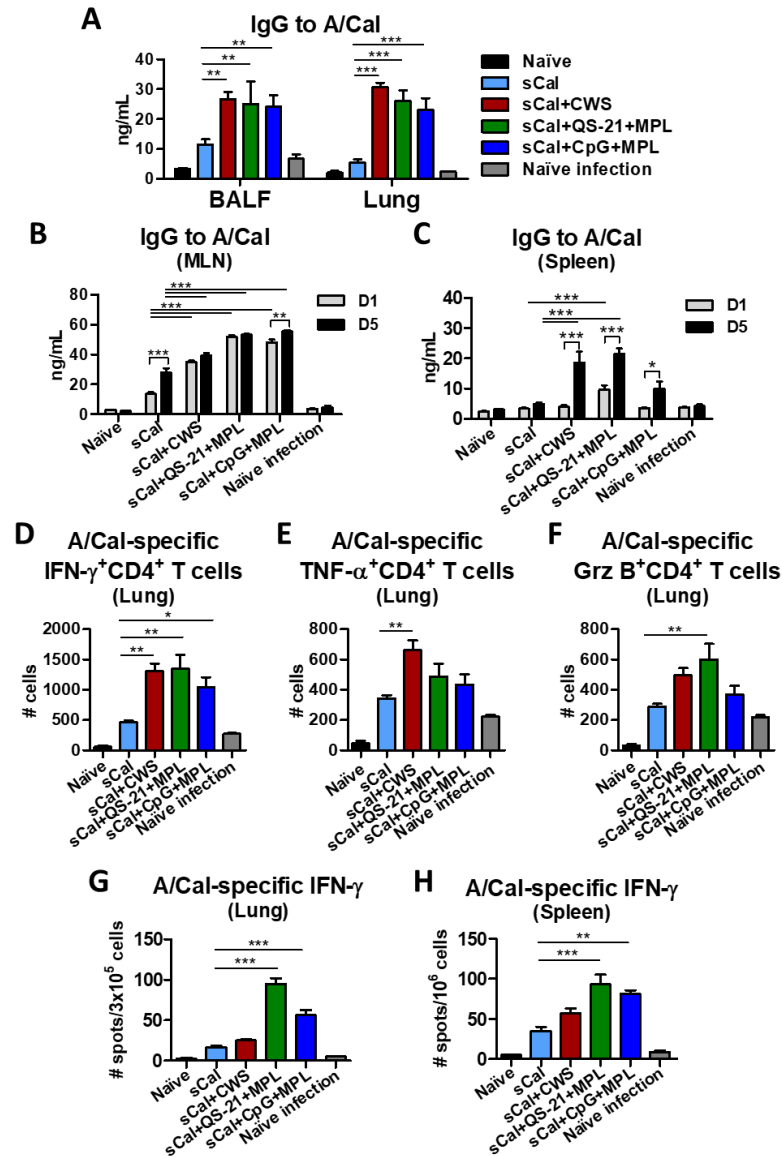


Figure 2.8 Adjuvanted boost vaccination enhances antibody-secreting cells and effector T cells upon influenza virus infection in aged BALB/c mice. (A) A/Cal virus-specific IgG levels in BALF and lung samples harvested on day 5 post infection (n = 4). (B and C) A/Cal virus-specific IgG production from MLN cells and splenocytes harvested on day 5 post infection after in vitro culture for 1 day or 5 days with inactivated A/Cal H1N1 virus antigen. (D-F) Flow cytometry data of IFN- γ ⁺CD4⁺ T cells, TNF- α ⁺CD4⁺ T cells, and Granzyme B⁺CD4⁺ T cells in lung cells after intracellular cytokine staining. (G and H) Cytokine ELISpot of lung cells and splenocytes at day 5 post infection after in vitro stimulation with inactivated A/Cal H1N1 virus (4 μ g/mL per well). Statistical significance was calculated by using one-way ANOVA and Dunnett's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, p < 0.05, **, p < 0.01, ***, p < 0.001 compared to sCal group

2.4.6 Acute innate immune responses by QS-21+MPL combination adjuvant and unique features of enhancing adaptive immune responses to vaccination

To better understand the QS-21+MPL combination adjuvant effects, we analyzed acute innate immune responses after intraperitoneal injection of QS-21+MPL in C57BL/6 mice (Fig. 2.9). QS-21+MPL acutely induced inflammatory cytokine IL-6 and chemokine MCP-1 at higher levels in sera within 2 h than CpG+MPL, and then returned to near-basal levels, whereas both combination adjuvants acutely recruited monocytes, neutrophils, and dendritic cells at the site of injection (Fig. 2.9A-E). Also, the addition of MPL to QS-21 (QS-21+MPL) resulted in over 4-fold higher increases in IgG2b and IgG2c isotype antibody levels than QS-21 adjuvant alone suggesting immunological advantages of the combination adjuvant (Fig. 2.10). Interestingly, QS-21 equivalent Quil-A adjuvanted influenza vaccination could induce virus-specific IgG antibody responses in CD4-deficient mice at a significant level but not CpG adjuvant (Fig. 2.11), suggesting less dependence of Quil-A on CD4 T cells in exhibiting adjuvant effects.

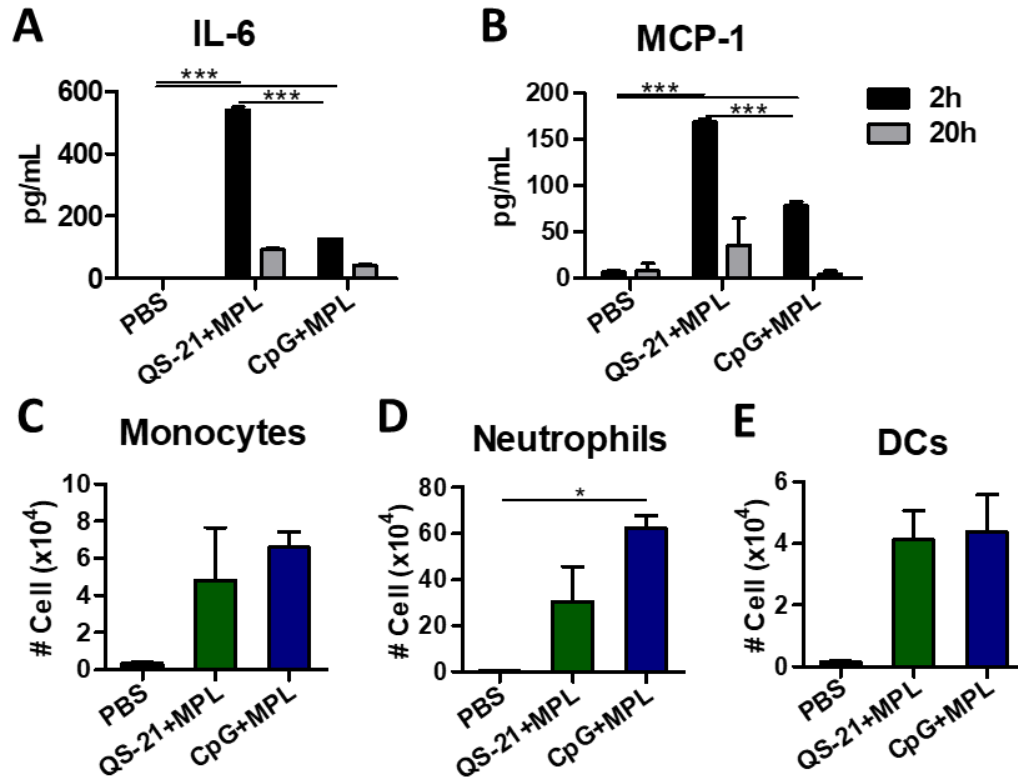


Figure 2.9 Acute induction of innate immune responses to intraperitoneal injection with adjuvants.

Naïve C57BL/6 mice ($n = 3$) were intraperitoneally injected with PBS, QS-21+MPL, or CpG+MPL. (A and B) Cytokine (IL-6) and chemokine (MCP-1) levels. (C-E) Cellular phenotypes in peritoneal exudates at 20 h after injection. Cell numbers of each cell population per mouse were calculated by multiplying cell percentages with total cell numbers. (C) Monocytes ($CD11b^+CD11c^+F4/80^+Ly6c^{hi}$), (D) Neutrophils ($CD11b^+CD11c^+F4/80^+Ly6c^+$), (E) Dendritic cells (DCs, $CD11b^+CD45^+F4/80^+CD11c^+$). Statistical significance was calculated by using one-way ANOVA and Dunnett's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, ***, $p < 0.001$

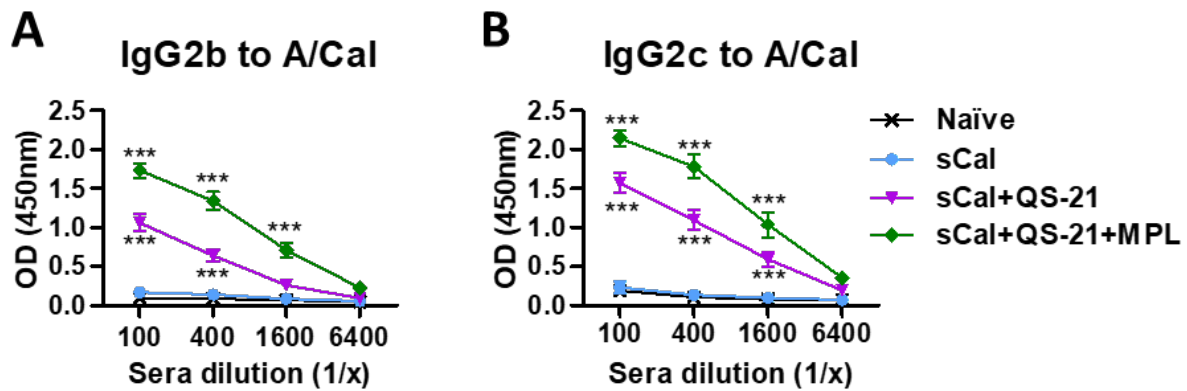


Figure 2.10 Addition of MPL to QS-21 in sCal vaccination results in increased virus-specific IgG isotype antibodies after prime immunization in young C57BL/6 mice. C57BL/6 mice (n=5) were intramuscularly prime-boost immunized with sCal (3 μ g) alone or sCal plus QS-21 (10 μ g) or QS-21 (10 μ g)+MPL (1 μ g). Blood samples were collected at 2 weeks after boost immunization. (A and B) A/Cal virus-specific IgG2b and IgG2c antibody levels were determined in boost sera. Statistical significance was calculated by using two-way ANOVA and Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). ***; $p < 0.001$ compared to sCal group

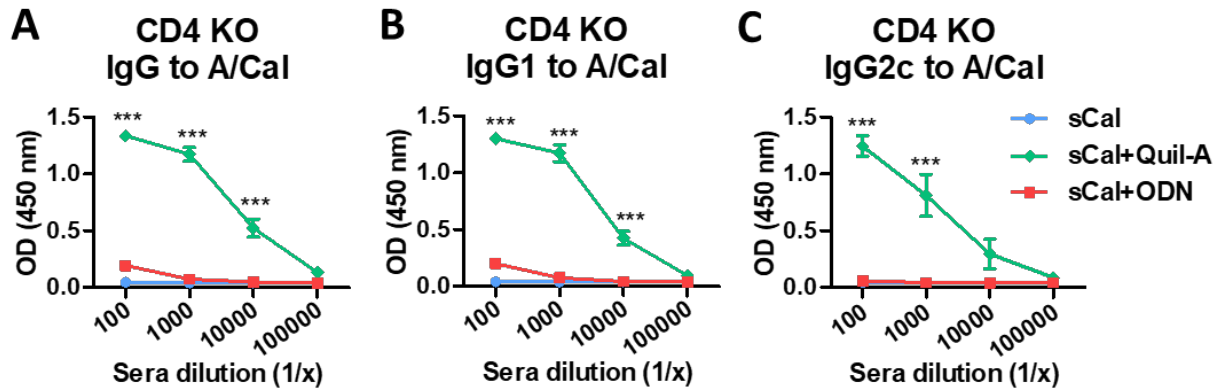


Figure 2.11 Quil-A–adjuvanted vaccination induces isotype-switched IgG Abs in CD4⁺ T cell-deficient mice. CD4 knockout C57BL/6 mice (n=5) were intramuscularly prime-boost immunized with sCal (3 μ g) alone or sCal plus Quil-A (a saponin adjuvant purified from bark extract of the *Quillaja Saponaria Molina* tree, 5 μ g), or CpG oligodeoxynucleotide (ODN2395, 4 μ g). Blood samples were collected at 2 weeks post immunization. (A-C) A/Cal H1N1 virus-specific IgG and isotype antibody levels were determined in boost immune sera. Statistical significance was calculated by using two-way ANOVA and Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). ***, $p < 0.001$ compared to sCal group

2.5 Discussion

QS-21 and MPL are the active components of a liposome-based AS01 adjuvant included in the highly effective Shingrix vaccine product for the elderly population and immunocompromised patients (Cunningham et al., 2016; Lal et al., 2015; Vink et al., 2020), progressively replacing an old live attenuated vaccine, Zostavax. Our previous studies reported potent adjuvant effects of CWS (Kim et al., 2021) and CpG+MPL combination adjuvant (Ko et al., 2018) on influenza vaccination. Here, we compared these potent adjuvant effects on enhancing the immunogenicity of influenza vaccination and efficacy of protection against homologous and heterosubtypic viruses in adult C57BL/6 and aged BALB/c mouse models. All three different adjuvants were able to increase IgG antibodies specific for virus and HA stalk domain, and HAI titers as well as confer homologous protection after prime dose in an adult C57BL/6 model, heterosubtypic protection after adjuvanted boost vaccination in adult C57BL/6 mice, and homologous protection after prime-boost vaccination in an aged BALB/c mouse model with differential efficacies. Both humoral and cellular immunity in mucosal and systemic sites induced by adjuvanted vaccination might have contributed to cross-protection. A head-to-head comparison of the effects of potent adjuvants on vaccine immunogenicity and efficacy is rare, although there is a significant focus on developing and licensing combination adjuvants such as AS04 (Alum+MPL), AS03 (α -tocopherol in oil-in-water emulsion), and AS01. Beyond the use of AS01 in Shingrix, malaria and tuberculosis vaccines containing AS01 were reported to be protective in clinical trials (Rts, 2014; Van Der Meeren et al., 2018), adding to the value of AS01. Consistent with the results in this study, a synergy of QS-21 and MPL actions was shown to recruit neutrophils and monocytes (Dendouga et al., 2012) and induce IFN- γ by stimulating macrophages and inflammatory cytokines (Coccia et al., 2017). Recently, CpG adjuvant was approved for use in

licensed hepatitis B virus vaccine (O'Hagan et al., 2020). Our previous studies demonstrated that MF59 oil-in-water emulsion, AS04, and MPL, but not alum adjuvant, could enhance vaccine immunogenicity and protection in CD4-deficient mice as in wild-type mice (Ko et al., 2016). BCG-CWS was shown to exhibit adjuvant effects on vaccination mainly via TLR2 and partly TLR4 signaling pathways (Tsuji et al., 2000) and even in CD4-deficient mice (data not shown). These three potent adjuvants appear to share a common TLR4 signaling pathway and stimulate innate immune responses leading to enhanced immunogenicity and protective efficacy of sCal vaccination. Although all adjuvanted single-dose vaccinations induced comparably enhanced protection against homologous virus in C57BL/6 mice as evidenced by lung viral clearance and lower weight loss compared to the naïve infected mice, the effects of CWS, QS-21+MPL, and CpG+MPL adjuvants were differential in inducing Th1 type IgG2c (or IgG2a) isotype antibodies specific for the virus. QS-21+MPL adjuvant in influenza vaccination was found to be more effective in promoting T helper type 1 (Th1) IgG2c (in C57BL/6 mice), IgG2a antibodies (in BALB/c mice), and protection in both mouse strains, whereas CpG+MPL was a potent adjuvant in enhancing the protection by influenza vaccination in both adult C57BL/6 and aged BALB/c mouse models. Th1 IFN- γ cytokines promote B cells to produce IgG2c isotype antibodies in C57BL/6 mice (IgG2a in BALB/c mice), whereas Th2 cytokine responses promote IgG1 isotype antibodies (Stevens et al., 1988). Murine IgG2a isotype is known to be the most effective in inducing antibody-dependent cell-mediated cytotoxicity and protection via Fc receptor-mediated mechanisms (Kipps et al., 1985; Oishi et al., 1992; Wiedinger et al., 2020), suggesting an immunological advantage in promoting Th1 type IgG antibody responses by QS-21+MPL adjuvanted vaccination. Prime boost vaccination of C57BL/6 mice with sCal alone was shown to induce HAI antibodies lowering lung viral titers by 100 folds against homologous A/Cal H1N1

virus after challenge, which was dependent on CD4 T cell help (Ko et al., 2017). MF59 and AS04 were demonstrated to overcome CD4 T cell deficiency defect in exhibiting adjuvant effects on enhancing homologous protection of sCal vaccination (Ko et al., 2016; Ko et al., 2017). Here, we extended potent adjuvant effects on heterosubtypic protection after boost dose in an adult C57BL/6 mouse model and homologous protection in an aged BALB/c mouse model. CWS was also effective in inducing mucosal IgG and IFN- γ ⁺ CD4 T cell responses at early time post challenge, as observed in the QS-21+MPL and CpG+MPL groups. The least weight loss against rgH5N1 virus was observed in the CpG+MPL group, followed by the QS-21+MPL group, although QS-21+MPL adjuvanted sCal (H1N1) vaccination was most effective in inducing humoral IgG2c and HAI titers against A/Cal H1N1 virus. Particularly, the adjuvant effects on enhancing IgG antibodies to stalk and NA were prominent. Under a condition without cross-reactive HAI antibodies against rgH5N1, this study suggested that adjuvants enhanced T cell immunity, IgG antibodies for stalk domain, and binding IgG antibodies to rgH5N1 and N1 NA, contributing to cross-protection. Notably, CpG+MPL adjuvant effect on enhancing cross-protection was comparable to QS-21+MPL, preventing weight loss more effectively than the CWS adjuvanted vaccination, although there were no significant differences. Older aged mice were found to be very poor in inducing protective immune responses to influenza vaccination. Our previous study reported that CWS has potent adjuvant effects on increasing the immunogenicity and protective efficacy of influenza vaccination in young infant, adult, and older aged BALB/c mice (Kim et al., 2021). MF59-adjuvanted sCal vaccination shown to be protective even in CD4-knockout mice (Ko et al., 2016) could not prevent severe weight loss against homologous challenge in aged BALB/c mice despite enhanced IgG antibody responses (data not shown). Heat-killed LAB, which also exhibited adjuvant effects on improving influenza vaccine efficacy even in CD4 knockout mice

(Jung et al., 2020) displayed weak adjuvant effects on influenza vaccination, resulting in severe weight loss and partial survival after lethal challenge with homologous virus in aged BALB/c mice. QS-21+MPL adjuvant in sCal vaccination was more effective than CWS and CpG+MPL in enhancing IgG2a antibodies in addition to comparable HAI titers and IFN- γ ⁺ CD4 T cell responses in aged BALB/c mice. The CpG+MPL adjuvanted sCal vaccination of aged BALB/c mice was most effective in preventing weight loss after challenge, although there were no statistically significant differences among the adjuvants compared in this study.

2.6 Conclusion

This study has significance in providing a comparative assessment of the effects of three different potent adjuvants or combination adjuvants on enhancing the humoral and cellular immunogenicity and protection against homologous and heterosubtypic viruses in both adult C57BL/6 and aged BALB/c mouse models. Combination adjuvant, QS-21+MPL, was found to be the most effective in enhancing the immunogenicity of the sCal vaccine, whereas CpG+MPL was highly effective in improving the vaccine efficacy of cross-protection in adult mice and homologous protection in aged mice despite no statistically significant differences between these two potent combination adjuvants. Considering the differential effects of different adjuvants on inducing stalk antibodies, it will be informative to assess the efficacy of cross-protection in naïve mice by passive transfer of immune antisera from the mice with different adjuvanted vaccinations.

2.7 Acknowledgements

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through BEI Resources, NIAID, NIH: H1 Hemagglutinin (HA) Protein with C-Terminal Histidine Tag from Influenza Virus, A/California/04/2009 (H1N1) pdm09, Recombinant from Baculovirus, NR-15749; and N1 Neuraminidase (NA) Protein with N-Terminal Histidine Tag from Influenza Virus, A/California/04/ 2009 (H1N1) pdm09, Recombinant from Baculovirus, NR-19234.

3 CHAPTER 2. ADJUVANT EFFECTS OF SAPONIN ANALOG VSA-1 ON ENHANCING HOMOLOGOUS AND HETEROSUBTYPIC PROTECTION BY INFLUENZA VIRUS VACCINATION

3.1 Abstract

Adjuvants are vaccine components that enhance the magnitude, breadth, and durability of the immune response generated by the vaccine antigen. Alum remained the main adjuvant licensed for human use until the 1990s. A few other adjuvants have been licensed for use in human vaccines since the 1990s. QS-21, a mixture of saponin compounds, has been included in the AS01-adjuvanted Shingrix vaccine. Here, we investigated the adjuvant effects of VSA-1, a newly developed semisynthetic analog of QS-21, on promoting protection in mice after vaccination with inactivated split virus vaccine. The adjuvant effects of VSA-1 on improving vaccine efficacy after prime immunization were evident, as shown by significantly higher levels of hemagglutination inhibiting antibody titers and IFN- γ ⁺ T cell responses and enhanced homologous protection, compared to those by QS-21 and alum adjuvants. VSA-1 adjuvant effects on enhancing heterosubtypic protection after two doses of adjuvanted vaccination were comparable to those of

QS-21. T cell immunity played an important role in conferring cross-protection by VSA-1 adjuvanted vaccination. Overall, the findings in this study suggest that VSA-1 exhibits desirable adjuvant properties and a unique pattern of innate and adaptive immune responses, contributing to improved homologous and heterosubtypic protection by inactivated split influenza vaccination in mice.

3.2 Introduction

Influenza viruses infect between 5% and 15% of the global population, causing frequent hospitalization and up to 650,000 deaths annually (Iuliano et al., 2018). Seasonal influenza vaccines can provide an effective intervention to limit the spread of the influenza virus. Yet continual influenza virus mutation and its ability to evade immunity pose a constant threat. Annual vaccination is ineffective in conferring cross-protection against antigenically different influenza viruses. Therefore, it is of high priority to improve the cross-protective efficacy of influenza vaccines. Effective vaccination for increased efficacy of homologous and cross-protection could be achieved by using potent adjuvants added along with a vaccine antigen to boost immune responses.

Adjuvants have become an indispensable component of successful subunit vaccines (Banday et al., 2015; Bastola et al., 2017; Di Pasquale et al., 2015; Leroux-Roels, 2010; Temizoz et al., 2016; Wang & Singh, 2011). Adjuvants (a) enhance the ability of a vaccine to elicit strong and durable immune responses, including in immunologically compromised individuals such as immunologically immature neonates, the aged, and immune-suppressed individuals (Bonam et al., 2017), (b) reduce antigen dose and the number of immunizations, and (c) modulate the nature of the immune response. With the increasingly important role of adjuvants, several new adjuvants

have been approved for human use (Garçon et al., 2011; Giudice et al., 2018; O'Hagan et al., 2017; Shi et al., 2019). Nonetheless, aluminum salts (Alum) are the most widely used adjuvants for licensed vaccine products. However, Alum has been shown to exhibit relatively weaker adjuvant effects than other adjuvants. Alum is efficient in eliciting antibody responses with a T helper (Th) type 2 profile but with limited efficacy (Young et al., 2015).

Another adjuvant, QS-21, which is a key component of GlaxoSmithKline's adjuvant system (AS) series of combination adjuvants, including the FDA-approved AS01b, is arguably the most potent immunostimulant that can induce strong and balanced humoral and cellular immune responses (Leroux-Roels et al., 2015; Tielemans et al., 2011; Vandepapeliere et al., 2008; Vandepapeliere et al., 2005). QS-21 is a mixture of two isomeric bidesmosidic saponins isolated from the tree bark of *Quillaja Saponaria* Molina (QS), an evergreen tree native to temperate central Chile. However, the current shortage of QS-21, along with its dose-limiting toxicity, laborious and low-yielding purification, and chemical instability, limits its application and highlights the imperative need for its more accessible alternatives (Harandi et al., 2010; Martin & Briones, 1999; Ragupathi et al., 2011).

Extensive structure-activity-relationship (SAR) studies of QS-21 led to the recent discovery of promising new saponin adjuvants that can be derived from more accessible natural sources (Wang et al., 2013; Wang et al., 2016; P. Wang, X. Ding, et al., 2019; Pengfei Wang et al., 2019; P. Wang, Ð Škalamera, et al., 2019). Semisynthetic saponin adjuvant VSA-1 was prepared in one step from naturally occurring *Momordica* Saponin I (MS I) (P. Wang, X. Ding, et al., 2019), which can be easily isolated from the inexpensive seeds of *Momordica cochinchinensis* SPRENG (MC), a widely available perennial vine (Iwamoto et al., 1985). VSA-1 saponin analog was reported to exhibit significant adjuvant activity (P. Wang, X. Ding, et al., 2019). Also, VSA-

1 potentiates antigen-specific IgG1 and IgG2a immune responses in BALB/c mice, indicating a mixed Th1/Th2 immune response. VSA-1 showed lower acute toxicity than Quil A, which is a QS-21-like saponin adjuvant (P. Wang, X. Ding, et al., 2019).

In this study, we investigated the adjuvant effects of VSA-1 on promoting homologous and heterosubtypic protection after prime or prime-boost vaccination of C57BL/6 mice with inactivated split 2009 influenza H1N1 pandemic virus vaccine. VSA-1 was found to exhibit desirable adjuvant properties by promoting humoral and cellular adaptive immune responses and contributing to improved homologous and heterosubtypic protection by inactivated split influenza vaccination in mice.

3.3 Materials and Methods

3.3.1 Animals, reagents, and viruses

Adult C57BL/6 and BALB/c mice (6- to 8-week-old, female) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility at Georgia State University. All mouse studies were approved by Georgia State University (GSU) Institutional Animal Care and Use Committee (IACUC, A21004) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the NIH.

Preparation of VSA-1 was described in detail previously (P. Wang, X. Ding, et al., 2019), where VSA-1 is a modified derivative of Momordica Saponin I (MS I) isolated from the inexpensive seeds of *Momordica cochinchinensis* (Lour.) (Fig. 3.1) (Iwamoto et al., 1985). VSA-1 was prepared as a white solid, which was then dissolved in pure autoclaved distilled water. Pure Quillaja saponin, QS-21, was purchased from Desert King International (San Diego, CA, USA) and was dissolved in dimethyl sulfoxide (DMSO) following the manufacturer's protocol. VSA-1

and QS-21 were aliquoted and stored at -80°C until use. Aluminum hydroxide (Alum) was purchased from Sigma Aldrich (St. Louis, MO) and stored at 4°C until use.

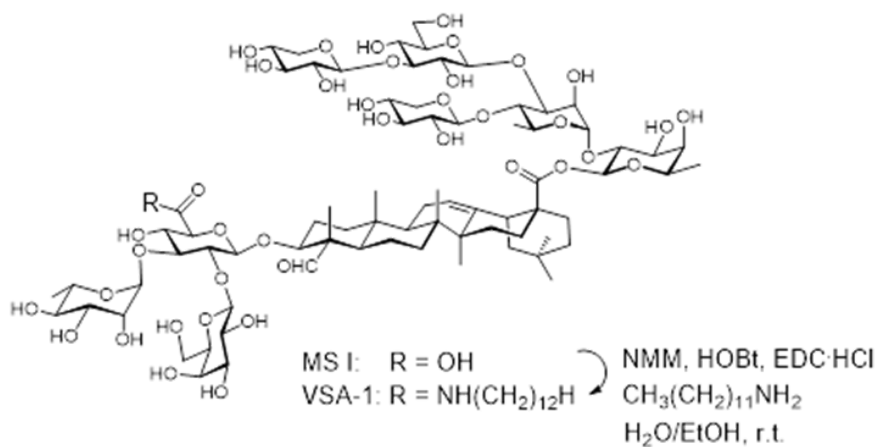


Figure 3.1 The structure of VSA-1 derived from *Momordica saponin I* which was isolated from the seeds of *Momordica cochinchinensis* Spreng (P. Wang, X. Ding, et al., 2019)

A/California/04/2009 (A/Cal) H1N1 virus was used as the split vaccine strain and for homologous challenge. Reverse genetics (rg) reassortant H5N1 (rgH5N1) virus, containing HA and NA derived from A/Vietnam/1203/2004 and six internal genes from A/Puerto Rico/8/1934, as described previously (Song et al., 2011), was used as a heterologous challenge virus. A/Cal H1N1 and rgH5N1 viruses were propagated using embryonated chicken eggs. Inactivated viruses used as coating antigens for Enzyme-linked immunosorbent assay (ELISA) were prepared as described previously (Ko et al., 2018). Briefly, A/Cal H1N1 or rgH5N1 influenza viruses were inactivated with 1% formalin and concentrated by ultracentrifugation (SW32 Ti rotor, $123,760\times g$, 1 h). The inactivated virus pellet was resuspended in phosphate-buffered saline (PBS) and was used as the ELISA coating antigen. For influenza split vaccine (sCal) preparation, inactivated A/Cal H1N1 virus was treated with 1% Triton X-100 to disrupt virion particles. The disrupted virus particles were dialyzed against PBS 3 times overnight. The total protein concentration of the inactivated

viruses was determined by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Adult C57BL/6 and BALB/c mice (6- to 8-week-old, female) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility at Georgia State University. All mouse studies were approved by Georgia State University (GSU) Institutional Animal Care and Use Committee (IACUC, A21004) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the NIH.

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3.3.2 Immunization and virus challenge of mice

To determine the protective efficacy of VSA-1 against homologous virus (A/Cal H1N1), groups of 6-week-old C57BL/6 mice ($n = 5$ per group) were intramuscularly immunized with sCal ($3 \mu\text{g}/\text{mouse}$) alone or sCal ($3 \mu\text{g}/\text{mouse}$) plus VSA-1 ($50 \mu\text{g}/\text{mouse}$), QS-21 ($10 \mu\text{g}/\text{mouse}$), or Alum ($50 \mu\text{g}/\text{mouse}$). Blood samples were collected at 2 weeks after immunization, and sera were separated. Three to four weeks after prime immunization, the mice were challenged with a lethal dose of homologous A/Cal H1N1 virus ($3 \times \text{LD}_{50}$, equivalent to $\text{Log}10^{3.7} \times \text{EID}_{50}$ per mouse). For cross-protection studies, C57BL/6 mice ($n = 7$ per group) were intramuscularly immunized (prime and boost) with sCal ($3 \mu\text{g}/\text{mouse}$) alone or sCal ($3 \mu\text{g}/\text{mouse}$) plus VSA-1 ($50 \mu\text{g}/\text{mouse}$ prime; $25 \mu\text{g}/\text{mouse}$ boost), QS-21 ($10 \mu\text{g}/\text{mouse}$), or alum ($50 \mu\text{g}/\text{mouse}$) at a 3-week interval. Three to four weeks after boost, the mice were challenged with a lethal dose of heterosubtypic rgH5N1 virus ($3 \times \text{LD}_{50}$, equivalent to $\text{Log}10^{3.4} \times \text{EID}_{50}$ per mouse). After challenge, body weight changes and survival rates were monitored for 14 days, and lung viral titers and immunological profiles were determined in bronchoalveolar lavage (BAL), mediastinal lymph nodes (MLN), lung and spleen tissues collected at day 5 (homologous challenge with A/Cal H1N1 virus) or day 6 (heterosubtypic challenge with rgH5N1 virus) post infection.

3.3.3 Antibody Enzyme-linked immunosorbent assay (ELISA)

To measure antigen-specific antibody levels, inactivated A/Cal H1N1 or rgH5N1 viruses (200 ng/well) were coated onto ELISA plates and then incubated with diluted immune sera as detailed previously (Ko et al., 2018; Ko et al., 2016). IgG isotypes were measured using anti-mouse immunoglobulin IgG, IgG1 and IgG2a (or IgG2c), horse-radish peroxidase (HRP)-conjugated secondary antibodies (Southern Biotechnology, Birmingham, AL), and tetramethylbenzidine (TMB) substrate (Invitrogen, Waltham, MA). Antibody levels are presented as optical density absorbance values at 450 nm (BioTek ELISA plate reader). Additionally, consensus group I hemagglutinin (HA) stalk protein (50 ng/well), prepared as described (Chae et al., 2019), and N1 neuraminidase (NA) protein (20 ng/well, BEI Resources, NR-19234) were used to determine HA stalk and NA-specific IgG antibodies.

3.3.4 Hemagglutination inhibition (HAI) assay

HAI titers in immune sera treated with receptor destroying enzymes (RDE, Sigma-Aldrich, St. Louis, MO) and inactivated (56 °C, 30 min) were determined by using 4 HA units of A/Cal H1N1 or rgH5N1 virus and 0.5% chicken red blood cells (RBC, Lampire Biological Laboratories, Pipersville, VA) as previously described (Ko et al., 2018).

3.3.5 Lung viral titration

Lung extracts prepared in 1.5 mL of Roswell Park Memorial Institute (RPMI) 1640 without fetal bovine serum (FBS) by mechanical grinding of lung tissues harvested at day 5 or 6 after challenge were used to determine viral titers in embryonated chicken eggs (Hy-Line North America, LLC., Mansfield, GA), as described (Kim et al., 2019). Virus titers as 50% egg infection dose (EID₅₀)/mL were evaluated according to the Reed and Muench method (Reed & Muench, 1938).

3.3.6 Cytokine ELISA and in vitro IgG antibody detection

The inflammatory cytokine, tumor necrosis factor (TNF)- α , from bronchoalveolar lavage fluids (BALF) and lung extracts was measured by cytokine ELISA as described (Lee et al., 2016). Cytokines were detected using Ready-SET-Go kit with TNF- α specific antibodies (eBioscience, San Diego, CA).

BALF was obtained by infusing 1.5 mL of PBS into the lungs. Lung extracts were prepared in 1.5 mL of RPMI 1640 without FBS by mechanical grinding of lung tissues harvested at day 5 after challenge. BALF and lung extracts were used to determine vaccine antigen (A/Cal)-specific and rgH5N1-specific antibody levels.

Secreted IgG antibodies specific for A/Cal H1N1 or rgH5N1 were determined from mediastinal lymph nodes (MLN, 5×10^5 cells/well) and spleen tissues (5×10^5 cells/well) from C57BL/6 mice. The cells from MLN and spleen were isolated at day 5 or 6 post infection and cultured for 1 day and 5 days in plates pre-coated with inactivated A/Cal H1N1 virus or inactivated rgH5N1 virus. The combined levels of IgG antibodies secreted into the culture supernatants and those captured on the plate were analyzed by ELISA.

3.3.7 Enzyme-linked Immunospot (ELISpot) assay

Interferon (IFN)- γ secreting cell spots were determined by culturing splenocytes (5×10^5 or 10^6 cells/well) and lung cells (5×10^5 or 3×10^5 cells/well) for 72 h on multi-screen 96-well plates (MilliporeSigma, St. Louis, MO) coated with cytokine-specific capture antibodies as described (Song et al., 2011). Inactivated influenza A/Cal H1N1 was included as an antigenic stimulator (4 μ g/mL) and IFN- γ -secreting T cells were counted using an ELISpot reader (BioSys, Miami, FL).

3.3.8 *Flow cytometry analysis*

Lung and spleen tissues were harvested on day 5 or 6 after challenge. Lung cells harvested from the layer of percoll gradients between 44% and 67%, and spleen cells processed from spleen tissues were stimulated with 4 µg/mL inactivated A/Cal H1N1 virus in presence of Brefeldin A (20 µg/mL) for 5 hours at 37 °C as described (Lee et al., 2019; Lee et al., 2018). *In vitro* cultured cells (lung and spleen cells) were stained with anti-CD3-PacificBlue (Clone 17A2, Biolegend, San Diego, CA), anti-CD4-PE/Cy5 (Clone RM405, BD Biosciences, San Jose, CA) antibodies, and then fixed and permeabilized using BD Cyto-fix/Cytoperm™ Plus Kit (BD Biosciences). After staining the cells with anti-IFN-γ-APC/Cy7 (Clone XMG1.2, BD), anti-TNF-α-PE/Cy7 (Clone MP6-XT22, Biolegend), and anti-Granzyme B-FITC (Clone NGZB, eBioscience) antibodies, live lymphocytes were first gated by forward versus side scatter strategic gating, followed by gating CD3⁺ T cells and then CD4 T cells secreting cytokines. The number of effector T cells in BAL and lung were expressed by reflecting the frequency gated out of the total cells from each mouse. Cells positive for intracellular cytokines were revealed through acquisition on a Becton-Dickinson LSR-II/Fortessa flow cytometer (BD, San Diego, CA) and analyzed by Flowjo software (Tree Star Inc., Ashland, OR).

3.3.9 *In vivo protection efficacy test of immune sera*

Immune sera collected at two weeks after boost immunization were diluted 50 folds, heat-inactivated at 56 °C for 30 min, followed by mixing with the same volume of 2.5×LD₅₀ A/Cal H1N1 virus and incubating at room temperature for 30 min as described (Kim et al., 2013). The mixture of A/Cal H1N1 virus and sera was intranasally administered to naïve BALB/c mice (n = 3 per group), and body weight changes and survival rates were monitored daily for 14 days.

3.3.10 In vivo depletion of T cells

For *in vivo* systemic T cell depletion before and post challenge, prime-boost immunized C57BL/6 mice (n = 3) received treatment with anti-CD4 (CD4 clone GK1.5) and anti-CD8 (CD8 clone 53.6.7) mAbs. Antibodies (BioXCell, West Lebanon, NH) were injected into the mice with intraperitoneal (IP; 1 day before challenge) and intranasal (IN; 1 day after challenge) sequential delivery at a 2-day interval (anti-CD4 200 µg and anti-CD8 150 µg /mouse for IP injection, 10 µg anti-CD4/8/mouse for IN inoculation). All groups (n = 3 per group) were challenged with a lethal dose of rgH5N1 influenza virus ($2 \times \text{LD}_{50}$), and body weight changes and survival rates were monitored daily for 14 days after challenge.

3.3.11 Intraperitoneal injection of adjuvants

Naïve C57BL/6 mice (n = 3 per group) were intraperitoneally injected with 200 µl of PBS, VSA-1 (50 µg), QS-21 (10 µg), or Alum (50 µg). The inflammatory cytokine, interleukin (IL)-6, and chemokines, keratinocytes-derived chemokine (KC), and monocyte chemoattractant protein 1 (MCP-1) were measured in blood samples collected at 2 h and 20 h after injection by cytokine and chemokine ELISA using Ready-SET-Go kits (eBioscience, San Diego, CA). Immune cells in peritoneal exudates from the mice were collected in 2 mL of PBS at 20 h after injection of adjuvants, and cellular phenotypes were determined by flow cytometry using cell-specific phenotypic markers as described (Ko et al., 2018).

3.3.12 Statistical analyses

All results are presented as mean \pm standard errors of the mean (SEM). The statistical significance for all the experiments was calculated by one-way or two-way analysis of variance (ANOVA) and Dunnett's or Bonferroni's post-multiple comparison tests. P-values ≤ 0.05 were

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

3.4 Results

3.4.1 VSA-1 in influenza vaccination is effective in enhancing virus-specific IgG antibodies and HAI titers after a single dose

The recommended dose of QS-21 in mice is no more than 20 μg due to its dose-limiting toxicity, and its typical dose is 10 μg (Ng et al., 2016). A previous study reported that VSA-1 exhibited much lower acute toxicity than the natural QS saponins and mice could tolerate up to 2,000 μg of VSA-1 (P. Wang, X. Ding, et al., 2019). Thus, we used VSA-1 at 25 to 50 μg dose in this study.

To determine whether VSA-1 adjuvant would improve the efficacy of a single dose influenza vaccination, groups of C57BL/6 mice were primed with sCal vaccine (3 $\mu\text{g}/\text{mouse}$) alone or together with the comparing adjuvants: VSA-1, QS-21, or Alum. VSA-1 only without vaccine was used as a mock control (Fig. 3.2A). The sCal + VSA-1 and sCal + QS-21 groups induced higher levels of virus-specific IgG after a single dose of immunization than other comparing groups, including sCal + Alum and vaccine only groups (Fig. 3.2B). The sCal + VSA-1 group also induced higher levels of virus-specific IgG1 and IgG2b than QS-21 adjuvanted influenza vaccination. Similar levels of IgG2c isotype antibody were induced by VSA-1 or QS-21 adjuvanted vaccination. In contrast, alum adjuvanted vaccination-induced IgG1 dominant isotype and lowest levels of IgG2c antibodies (Figs. 3.2C-E). The adjuvanted sCal vaccine groups also induced higher levels of HA stalk-specific IgG antibodies (Fig. 3.2F).

Since hemagglutination inhibition (HAI) titer is known to be a protective immune correlate, we measured HAI titers in sera collected at 2 weeks after prime immunization. Both the VSA-1 and QS-21 adjuvanted groups showed higher levels of A/Cal H1N1-specific HAI titers than the Alum adjuvanted and vaccine-only groups. The sCal + VSA-1 group showed a 2-fold higher titer than the sCal + QS-21 group (Fig. 3.2G). These data suggest that a new adjuvant VSA-1 in influenza vaccination can be more effective in enhancing IgG1 and IgG2b isotypes and HAI titers compared to QS-21 and IgG2c isotype antibodies than Alum adjuvanted vaccination.

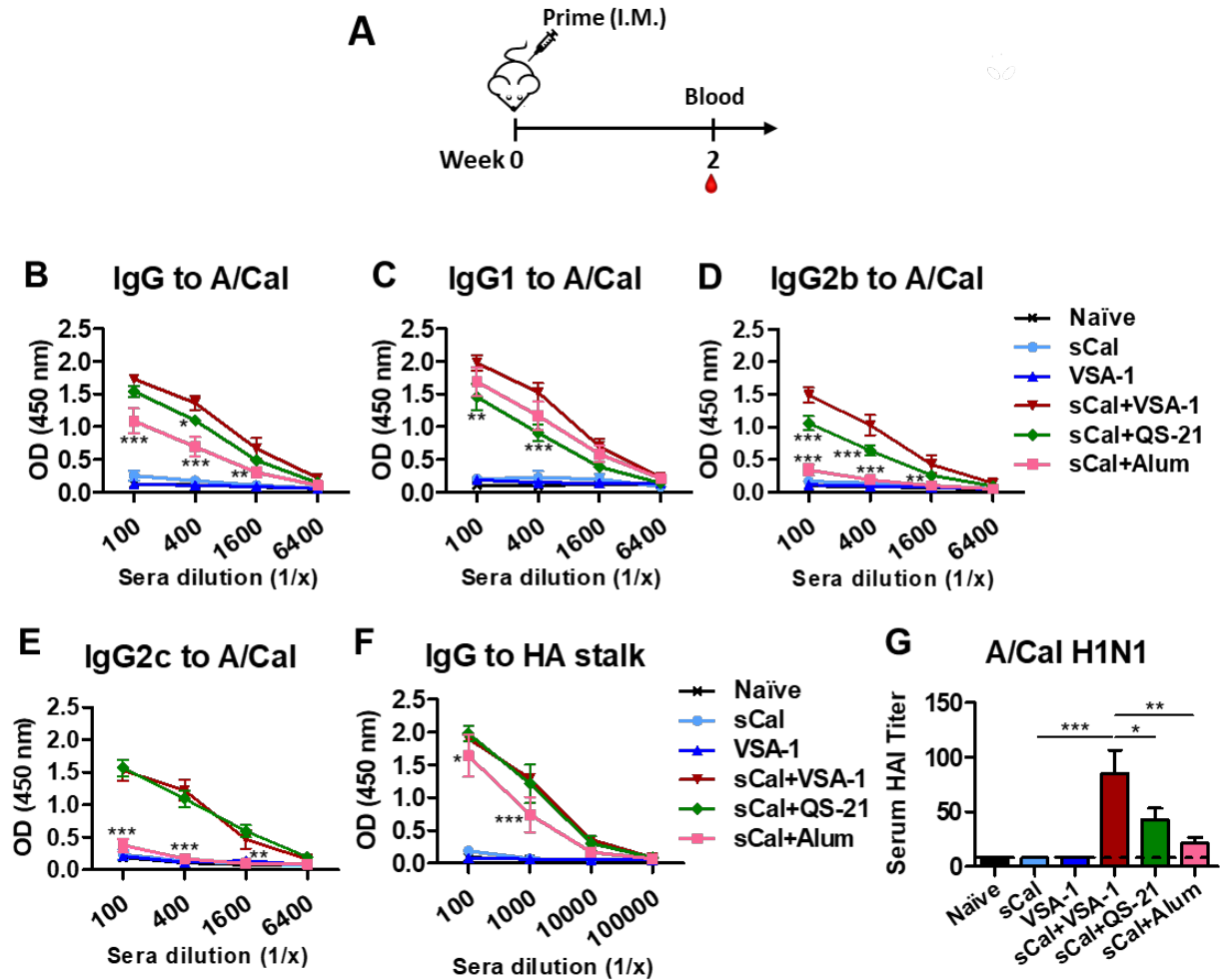


Figure 3.2 A single dose of VSA-1 adjuvanted influenza vaccination enhances virus-specific IgG antibodies and HAI titers in C57BL/6 mice. (A) Immunization scheme (n = 5 per group). C57BL/6 mice were intramuscularly immunized with sCal (3 µg) split vaccine alone or sCal plus VSA-1 (50 µg), QS-21 (10 µg) or Alum (50 µg). Mock: VSA-1 (50 µg) only, Naïve: PBS control. The timeline for collection of blood samples is indicated. (B-E) A/Cal H1N1 virus-specific IgG and isotype antibody levels were determined in prime sera. (F) Antibody responses specific for consensus group I full-length HA stalk in prime sera. (G) HAI titers against A/Cal H1N1 virus in prime sera. The dotted line represents the limit of detection. Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to sCal+VSA-1 group

3.4.2 Single dose of VSA-1 adjuvanted influenza vaccination induces enhanced protection against homologous A/Cal H1N1 virus

To determine the adjuvant effects on improving homologous protection, the immunized C57BL/6 mice were challenged with A/Cal H1N1 virus at a lethal dose at 3 weeks after prime immunization. Body weight changes were monitored for 14 days (Fig. 3.3A). By day 6-7 post challenge, mice in the sCal vaccine only group displayed severe weight loss (over 20%) and died of infection similar to those observed in naïve or adjuvant only mock control groups. Also, the QS-21 and alum adjuvanted vaccine groups exhibited substantial weight loss (~18-20%) with 80% survival rates. Notably, the sCal + VSA-1 group displayed least weight loss (~10%) among the adjuvanted groups and 100% survival rates, quickly recovering normal weight (Figs. 3.3B, C). Lung samples were collected at day 5 after challenge to determine virus titers in embryonated chicken eggs (Fig. 3.3D). The sCal + VSA-1 and sCal + QS-21 groups showed a significantly lower level of virus titers by 100 folds compared to those in Alum adjuvanted, sCal vaccine only, and mock control groups. These data suggest that VSA-1 would be more effective than QS-21 or alum adjuvant in enhancing influenza vaccine efficacy by preventing severe disease even after prime vaccination only.

The levels of inflammatory cytokines provide an additional barometer for assessing the protective efficacy of vaccination. The naïve infection control group showed the highest levels of TNF- α , IL-6, IFN- γ and IL-1 β cytokines in airway BALF and lung samples at 5 days after infection. In contrast, the sCal + VSA-1 adjuvanted group more effectively prevented the induction of inflammatory cytokine, TNF- α , than other adjuvanted groups (Figs. 3.3E, F). Adjuvanted vaccine groups showed reduced levels of inflammatory cytokines (IL-6, IFN- γ , IL-1 β) compared to naïve mice after lethal infection (Figs. 3.4A-F).

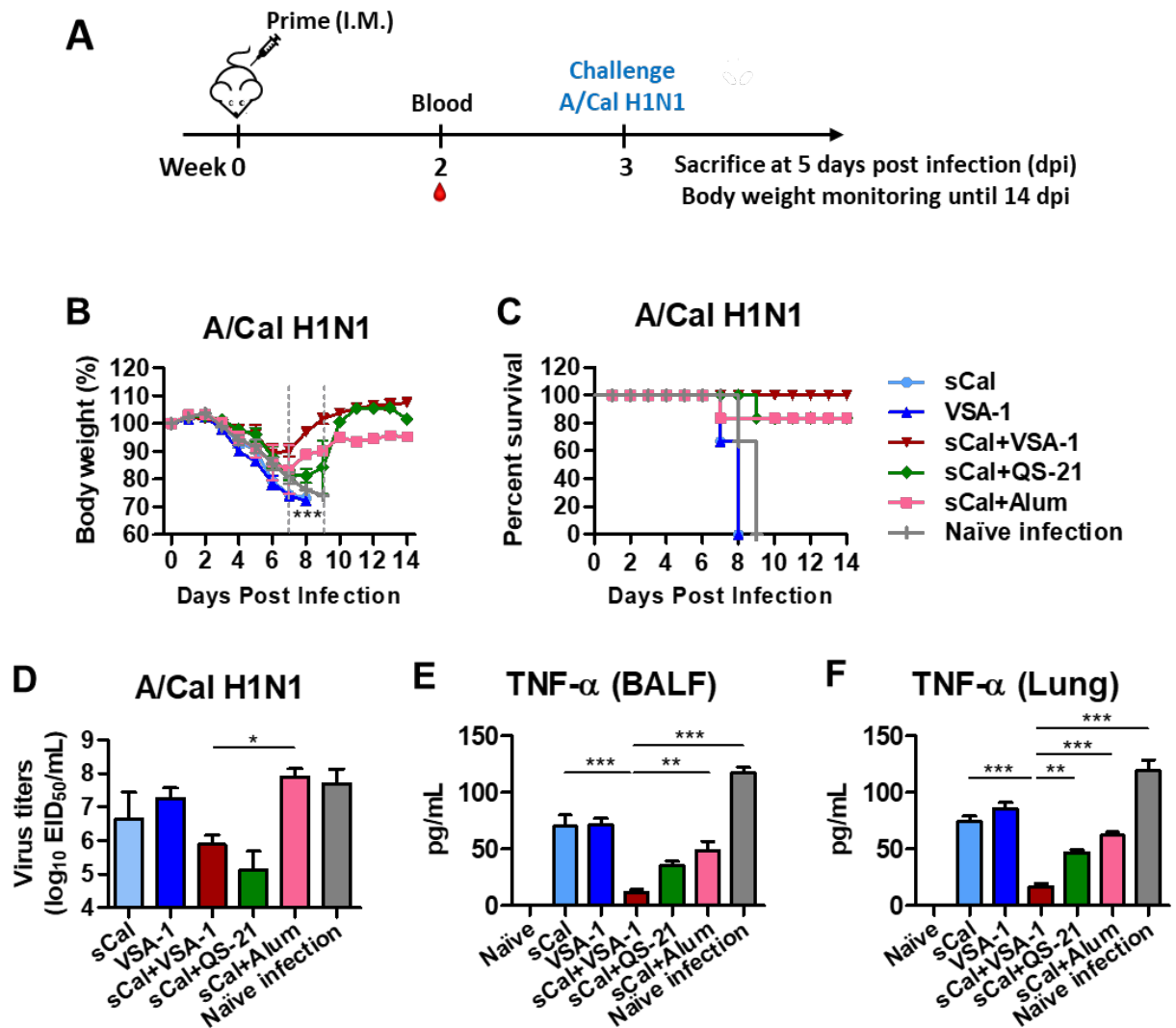


Figure 3.3 A single dose of VSA-1 adjuvanted influenza vaccination confers protection against lethal challenge with homologous A/Cal H1N1 virus in C57BL/6 mice. (A) Immunization and virus challenge scheme ($n = 5$ per group) with the same vaccine groups as in Fig. 3.2. At three to four weeks post immunization, the mice were challenged with a lethal dose of A/Cal H1N1 virus ($3 \times \text{LD}_{50}$). (B) Body weight changes and (C) survival rates were monitored for 14 days. (D) Lung viral titers as 50% egg infectious titers (EID₅₀) at day 5 post infection using embryonated chicken eggs. (E and F) TNF- α cytokine levels in BALF and lung extracts as an indicator of inflammation. Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the sCal+VSA-1 group

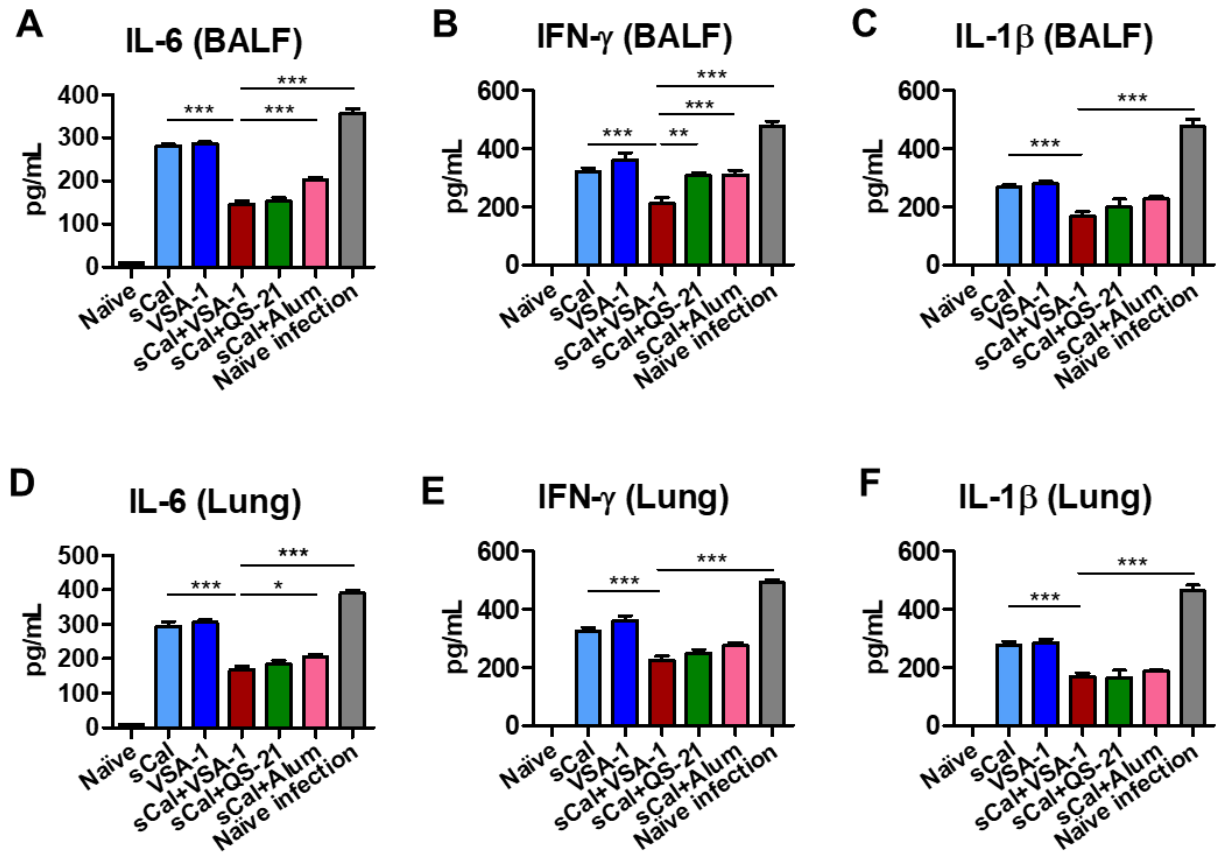


Figure 3.4 Cytokines in BALF and lungs of mice with prime vaccination after homologous virus challenge. C57BL/6 mice ($n = 3$ per group) were intramuscularly immunized with sCal ($3 \mu\text{g}$) split vaccine alone or sCal plus VSA-1 ($50 \mu\text{g}$), QS-21 ($10 \mu\text{g}$) or Alum ($50 \mu\text{g}$). VSA-1 ($50 \mu\text{g}$) only immunized mice were used as mock control. Three to four weeks post immunization, the mice were challenged with a lethal dose of A/Cal H1N1 virus ($3 \times \text{LD}_{50}$). BALF and lung samples of the immunized C57BL/6 mice ($n = 3$ per group) were harvested at day 5 post A/Cal H1N1virus infection. Cytokine levels of BALF (A-C) and lung extracts (D-F) were measured by ELISA. Statistical significance was calculated by using one-way ANOVA and Dunnett's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the sCal+VSA-1 group

3.4.3 *VSA-1 adjuvanted split virus vaccination enhances antibody-secreting cell and IFN- γ -producing T cell responses*

An important goal of vaccination is to induce long-lived antibody-secreting cell (ASC) responses. Vaccinated mice were challenged with A/Cal H1N1 virus after 3 weeks of prime vaccination, and spleen and MLN cells were harvested on day 5 after challenge for analysis of IgG antibodies secreted from ASCs in *in vitro* culture supernatants by ELISA (Figs. 3.5A, B). The groups immunized with sCal vaccine only and the split vaccine plus QS-21 or Alum showed low levels of IgG antibodies after 1-day culture of spleen and MLN cells. Importantly, the sCal+VSA-1 group showed significantly higher levels of IgG antibodies in cultures of spleen and MLN cells.

The VSA-1 and QS-21 adjuvanted vaccine groups induced significantly higher levels of mucosal IgG antibodies in BALF and lung extracts than the control groups (Fig. 3.5C, D). Mucosal IgG antibodies in BALF and lung extracts were detected at low or background levels in the alum adjuvanted and vaccine-only control groups. Enhanced levels of HA stalk domain-specific IgG antibodies were observed in the VSA-1, QS-21, and Alum adjuvanted sCal groups but not in other control groups (Fig. 3.5E). The VSA-1 and QS-21 adjuvanted sCal groups showed significantly enhanced levels of A/Cal H1N1-specific HAI titers by over 6 folds at day 5 after challenge (Fig. 3.5F) compared to those before challenge (Fig. 3.3G). The alum adjuvant, vaccine alone, and mock control groups did not show increased HAI titers at day 5 after challenge. These data suggest that B cells can be effectively primed for rapid recall to generate virus-specific IgG responses in mucosal and systemic sites upon challenge even after a single dose of VSA-1 adjuvanted vaccination.

To determine adjuvant effects on eliciting cellular immune responses, lung cells and splenocytes harvested at day 5 after infection, were stimulated with inactivated A/Cal H1N1 virus.

The number of IFN- γ producing cells in the VSA-1 adjuvanted vaccination group was higher than those in the Alum and QS-21 adjuvanted vaccination groups in spleen cells and higher than those in naïve infected mice in lung cells (Fig. 3.5G, H). Intracellular cytokine staining flow cytometry data showed that IFN- γ^+ CD4 $^+$ T and IFN- γ^+ CD8 $^+$ T cells from lungs were induced at higher levels in the VSA-1 adjuvanted sCal vaccinated C57BL/6 mice than those in sCal vaccinated, naïve mice and other comparing groups at early time post challenge (Figs. 3.5I, J).

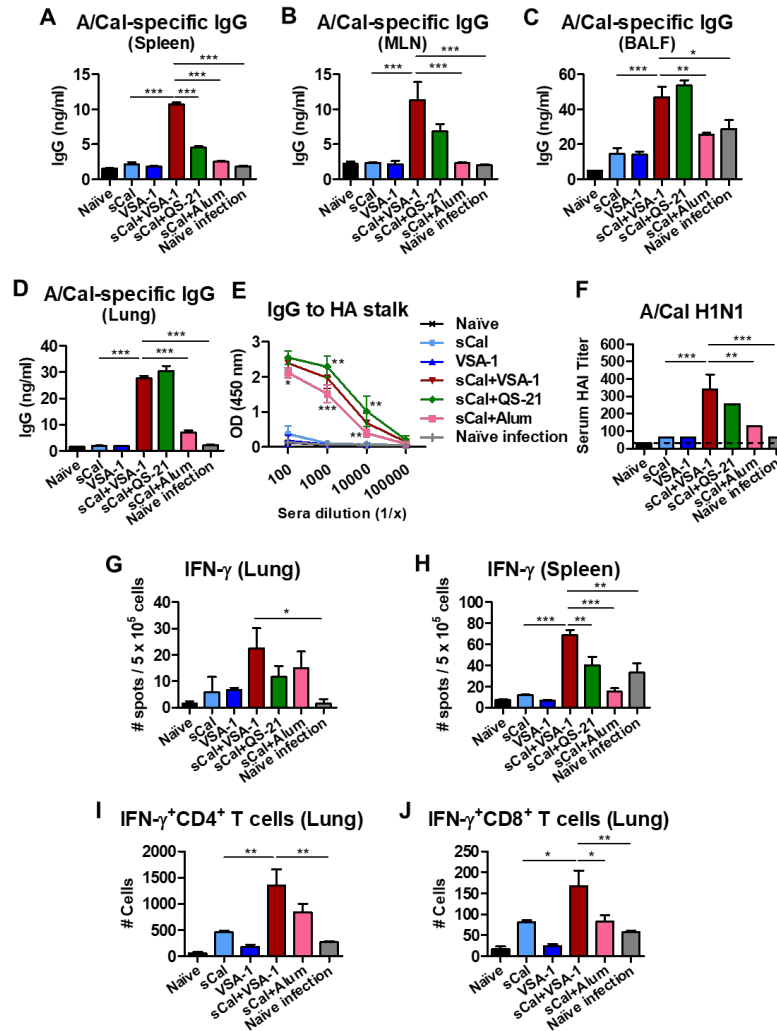


Figure 3.5 VSA-1 adjuvanted vaccination enhances antibody-secreting cells and effector T cells upon influenza virus infection in C57BL/6 mice. (A and B) A/Cal virus-specific IgG production from splenocytes and MLN cells harvested on day 5 post infection, cultured *in vitro* for 1 day in the presence of inactivated A/Cal H1N1 virus (n = 3 per group). (C and D) A/Cal virus-specific IgG levels in BALF and lung samples collected at day 5 post challenge. (E) Antibody responses specific for group I full-length HA stalk in sera after homologous challenge. (F) HAI titers against A/Cal H1N1 virus after challenge. The dotted line represents the limit of detection. (G and H) Cytokine ELISpot of lung cells and splenocytes at day 5 post infection after *in vitro* stimulation with inactivated A/Cal virus. (I and J) Flow cytometry data of IFN- γ -secreting CD4⁺ and CD8⁺ T cells in lung samples. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to sCal+VSA-1 group

3.4.4 Boost immunization with VSA-1 adjuvanted influenza vaccine further enhances virus-specific IgG antibodies, HAI titers, and homologous protection

To determine whether VSA-1 adjuvant included in the split influenza virus vaccination would improve the efficacy of boost vaccination, groups of C57BL/6 mice were primed and then boosted with sCal vaccine (3 ug/mouse) alone or together with adjuvants. VSA-1 only was used as a mock control (Fig. 3.6A). The sCal + VSA-1 and sCal + QS-21 groups induced higher levels of virus-specific IgG, IgG1, IgG2b and IgG2c after boost immunization than other groups (Fig. 3.6B, Figs. 3.7A-C). IgG antibodies binding to N1 NA (Fig. 3.6C) were induced at the highest levels in the VSA-1 and QS-21 adjuvanted sCal group after boost. Consistent with the IgG levels, the highest titers of HAI were induced in the VSA-1 adjuvanted sCal group, followed by the QS-21, alum, and vaccine only groups at 2 weeks after boost (Fig. 3.6D). To determine the effects of humoral responses in immune sera on homologous protection, naïve BALB/c mice were intranasally inoculated with a mixture of A/Cal H1N1 virus and immune sera collected from VSA-1, QS-21 or alum adjuvanted sCal-immunized mice, or naïve sera. Naïve sera did not provide protection against A/Cal H1N1 virus as evidenced by severe weight loss (> 25%) and 0% survival rates in naïve mice (Figs. 3.6E and F). In contrast, immune sera from VSA-1 adjuvanted sCal group conferred protection in naïve mice with no weight loss and 100% survival rates which were comparable to the QS-21 adjuvanted group, meanwhile, immune sera from the alum adjuvanted group provided protection to naïve mice with more severe weight loss (~18%) (Figs. 3.6E and F). These data suggest that boost immunization with split virus together with VSA-1 adjuvant induces higher levels of virus-specific IgG isotype antibodies and HAI titers, and protection against A/Cal virus, than sCal only and alum adjuvanted vaccination.

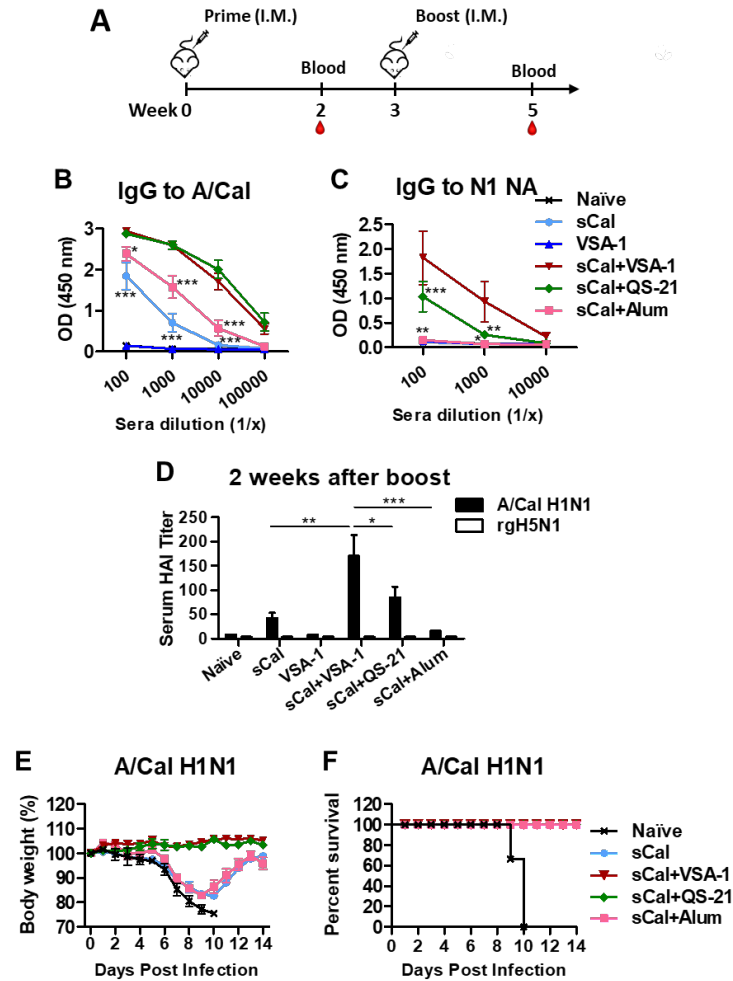


Figure 3.6 VSA-1 adjuvanted prime-boost influenza vaccination enhances IgG antibodies, HAI titers, and homologous protection. (A) Immunization scheme ($n = 7$ per group). The adjuvanted prime-boost vaccine and control groups are the same as described in Figure 3.2 legend. (B) A/Cal H1N1 virus-specific IgG and isotype antibody levels in boost sera. (C) N1 NA-specific IgG antibody levels in boost sera. (D) HAI titers against A/Cal H1N1 and rgH5N1 viruses in boost sera. The dotted line represents the limit of detection. (E and F) The role of boost antisera in homologous protection in naïve mice. Naïve BALB/c mice ($n = 3$ per group) were intranasally infected with a mixture of a lethal dose of A/Cal H1N1 virus ($3 \times LD_{50}$) and boost antisera. Body weights and survival rates were monitored for 14 days. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the sCal+VSA-1 group

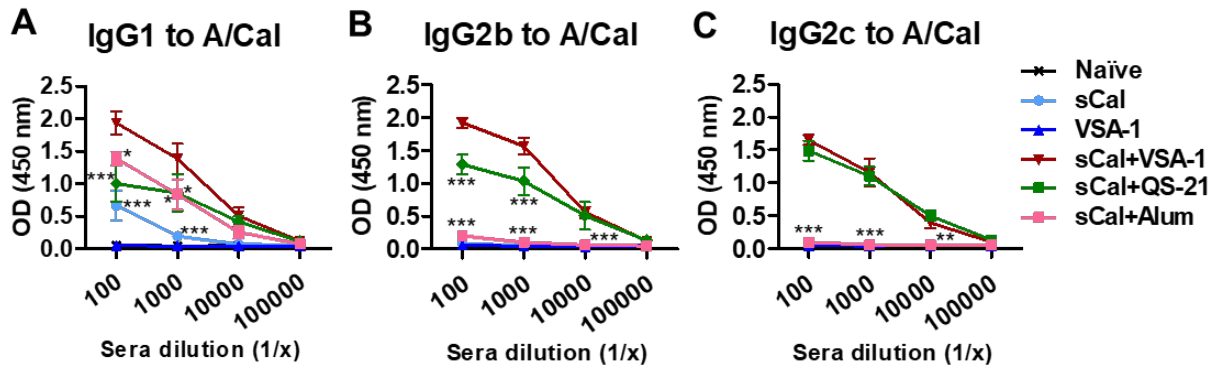


Figure 3.7 VSA-1 adjuvanted prime-boost influenza vaccination enhances virus-specific IgG isotype switched antibodies in C57BL/6 mice. C57BL/6 mice ($n = 3$ per group) were intramuscularly prime-boost immunized with sCal (3 μ g) split vaccine alone or sCal plus VSA-1 (50 μ g prime, 25 μ g boost), QS-21 (10 μ g) or Alum (50 μ g). VSA-1 (50 μ g prime, 25 μ g boost) only immunized mice were used as mock control. (A-C) A/Cal H1N1 virus-specific IgG isotype antibody levels in boost sera. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the sCal+VSA-1 group

3.4.5 VSA-1 adjuvanted prime-boost influenza vaccination induces cross-protection against heterosubtypic rgH5N1 virus

A regimen of prime-boost vaccination and heterosubtypic rgH5N1 virus challenge is presented (Fig. 3.8A). IgG antibodies binding to heterosubtypic rgH5N1 virus (Fig. 3.8B) and HA stalk domain (Fig. 3.8C) were induced at the highest levels in the VSA-1 and QS-21 adjuvanted sCal groups after boost. To determine the adjuvant effects of VSA-1 on improving heterosubtypic protection, the immunized C57BL/6 mice were challenged with rgH5N1 virus at a lethal dose at 3 weeks after boost. From day 3 post challenge, all the groups started showing body weight loss (Fig. 3.8D). By day 6-7 post challenge, the sCal vaccine only group displayed ~20% weight loss similar to that observed in naïve or adjuvant only treated and infected mice. Notably, the sCal + VSA-1 group displayed least weight loss (~5%) with 100% survival rate and quickly recovered

body weight by day 8 post challenge compared to either adjuvant or vaccine alone groups (Figs. 3.8D, E). On day 6 after infection, the sCal + VSA-1 and sCal + QS-21 groups showed a 100-fold lower level of virus titers compared to the naïve infection control group (Fig. 3.8F). High levels of TNF- α , IL-6, IFN- γ and IL-1 β cytokines were induced in lung samples from naïve mice (3.9A-D) at 6 days after infection. In contrast, the adjuvanted (VSA-1, QS-21, alum) sCal vaccination suppressed the induction of inflammatory cytokines (TNF- α , IL-6, IFN- γ , IL-1 β) in lung, compared to vaccine alone group. These data suggest that VSA-1 adjuvanted split virus vaccination confers higher efficacy of protection against heterosubtypic virus than split virus only and alum adjuvanted vaccination.

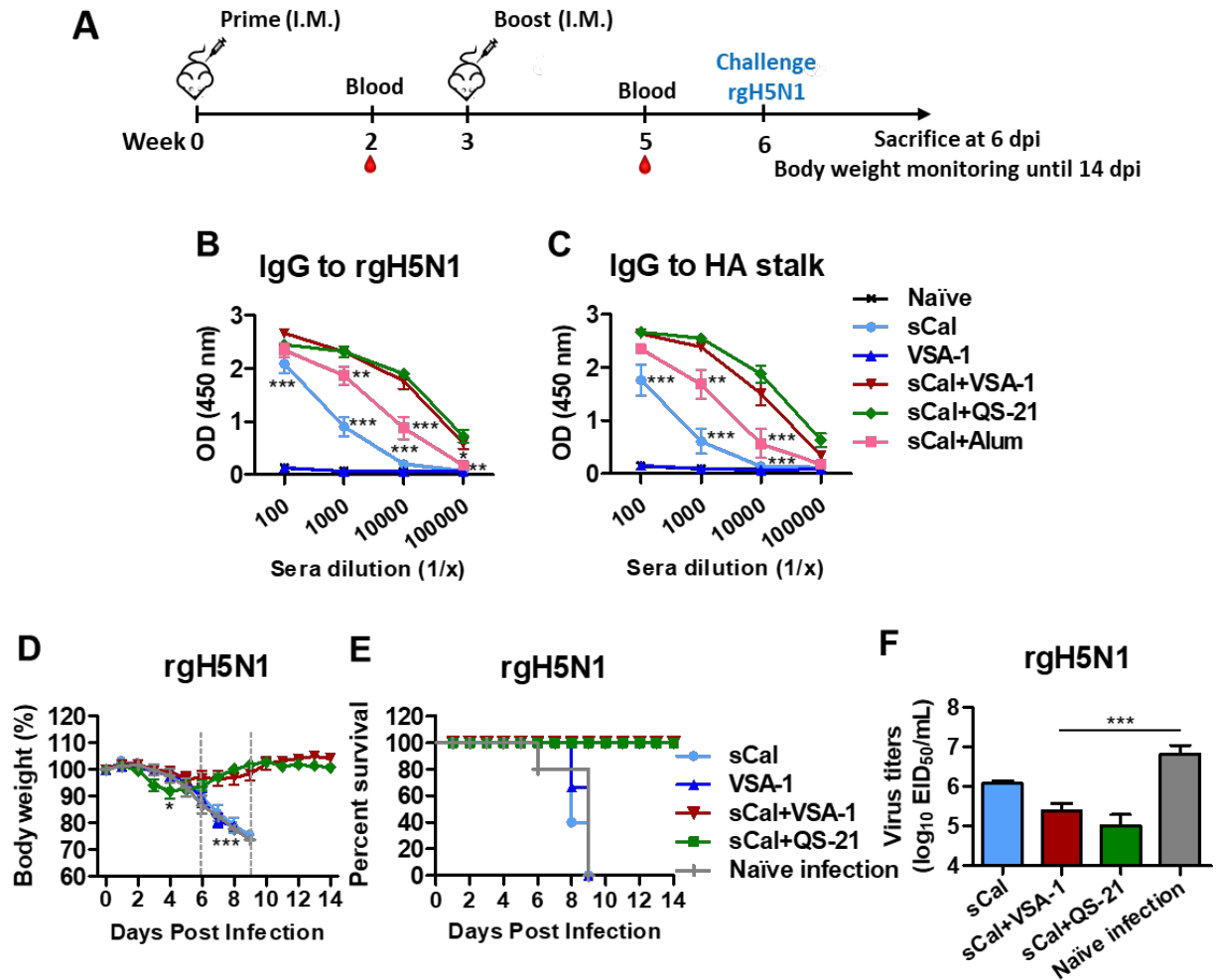


Figure 3.8 VSA-1 adjuvanted prime-boost vaccination confers protection against lethal infection with heterosubtypic rgH5N1 virus in C57BL/6 mice. (A) Immunization and virus challenge scheme ($n = 4$ per group) with the same vaccine groups as in Fig. 3.6. (B) rgA/Viet H5N1 virus-specific IgG antibody levels in boost sera. (C) Consensus group I full-length HA stalk-specific IgG antibody levels in boost sera. (D) Body weight changes and (E) survival rates after challenge with a lethal dose of rgH5N1 virus ($2 \times \text{LD}_{50}$). (F) Lung viral titers (EID_{50}) at day 6 post infection. Statistical significance was calculated by using one- or two-way ANOVA and Dunnett's or Bonferroni's post-multiple comparison tests. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, ***, $p < 0.001$ compared to sCal+VSA-1 group

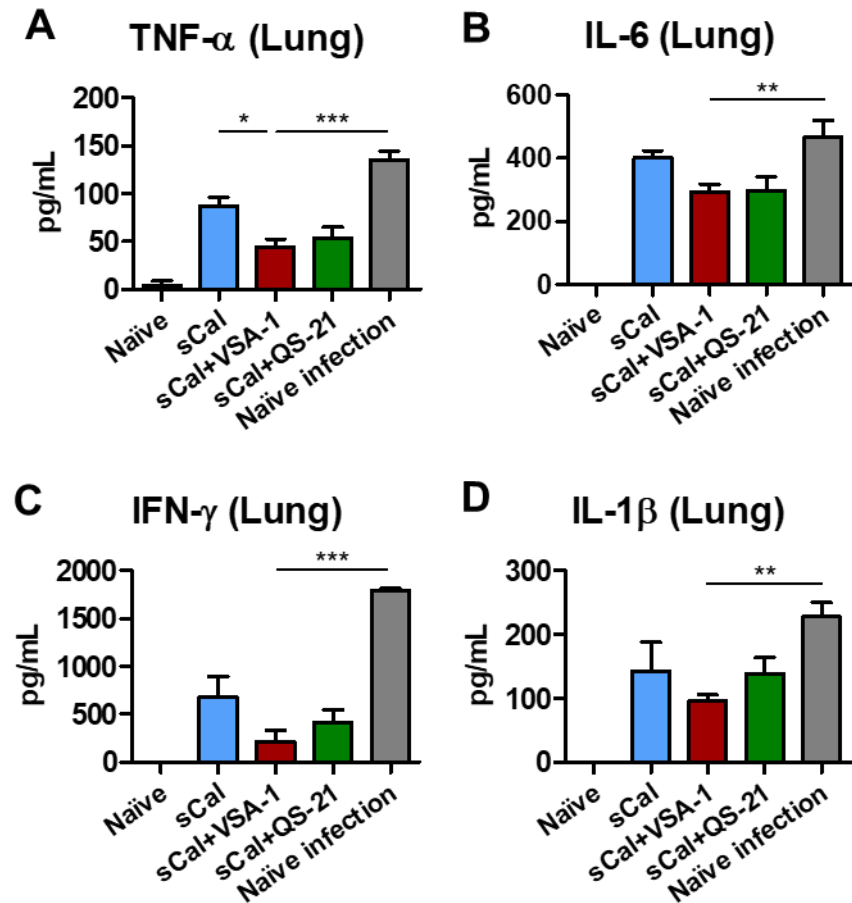


Figure 3.9 VSA-1 adjuvanted prime-boost influenza vaccination prevents the induction of inflammatory cytokines after lethal challenge with heterosubtypic rgH5N1 virus in C57BL/6 mice. Vaccination regimen is the same as described in Figure 3.7. Three to four weeks post boost immunization, the mice were challenged with a lethal dose of rgH5N1 virus ($3 \times \text{LD}_{50}$). Lung samples of the immunized C57BL/6 mice were harvested at day 6 post rgH5N1 virus infection. (A) TNF- α , (B) IL-6, (C) IFN- γ , and (D) IL-1 β cytokine levels of lung extracts were measured by ELISA. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the sCal+VSA-1 group

3.4.6 VSA-1 adjuvanted vaccination induces effector T cells and T-cell dependent protection against heterosubtypic rgH5N1 virus

IFN- γ ⁺ and TNF- α ⁺-CD4⁺ T and CD8⁺ T cells from lung and spleen tissues were induced at higher levels in the VSA-1 and QS-21 adjuvanted sCal vaccinated C57BL/6 mice than those in sCal and naïve groups day 6 post challenge with rgH5N1 virus as shown by intracellular cytokine staining flow cytometry assay (Figs. 3.10A-D).

To investigate whether T cell immunity would contribute to cross-protection, CD4 and CD8 T cells were depleted from the VSA-1 adjuvanted sCal prime-boost immunized mice prior to and after challenge with rgH5N1 virus (Fig. 3.10E). Severe weight loss (>25%) with 0% survival rate were observed in the VSA-1 adjuvanted sCal vaccinated mice after CD4 and CD8 T cell depletion whereas non-depleted VSA-1 adjuvanted sCal vaccinated mice were protected against weight loss after rgH5N1 virus challenge with the survival rate of 100% (Figs. 3.10E, F). These results suggest that protection against heterosubtypic rgH5N1 virus is dependent on CD4 and CD8 T cells in the mice vaccinated with sCal + VSA-1.

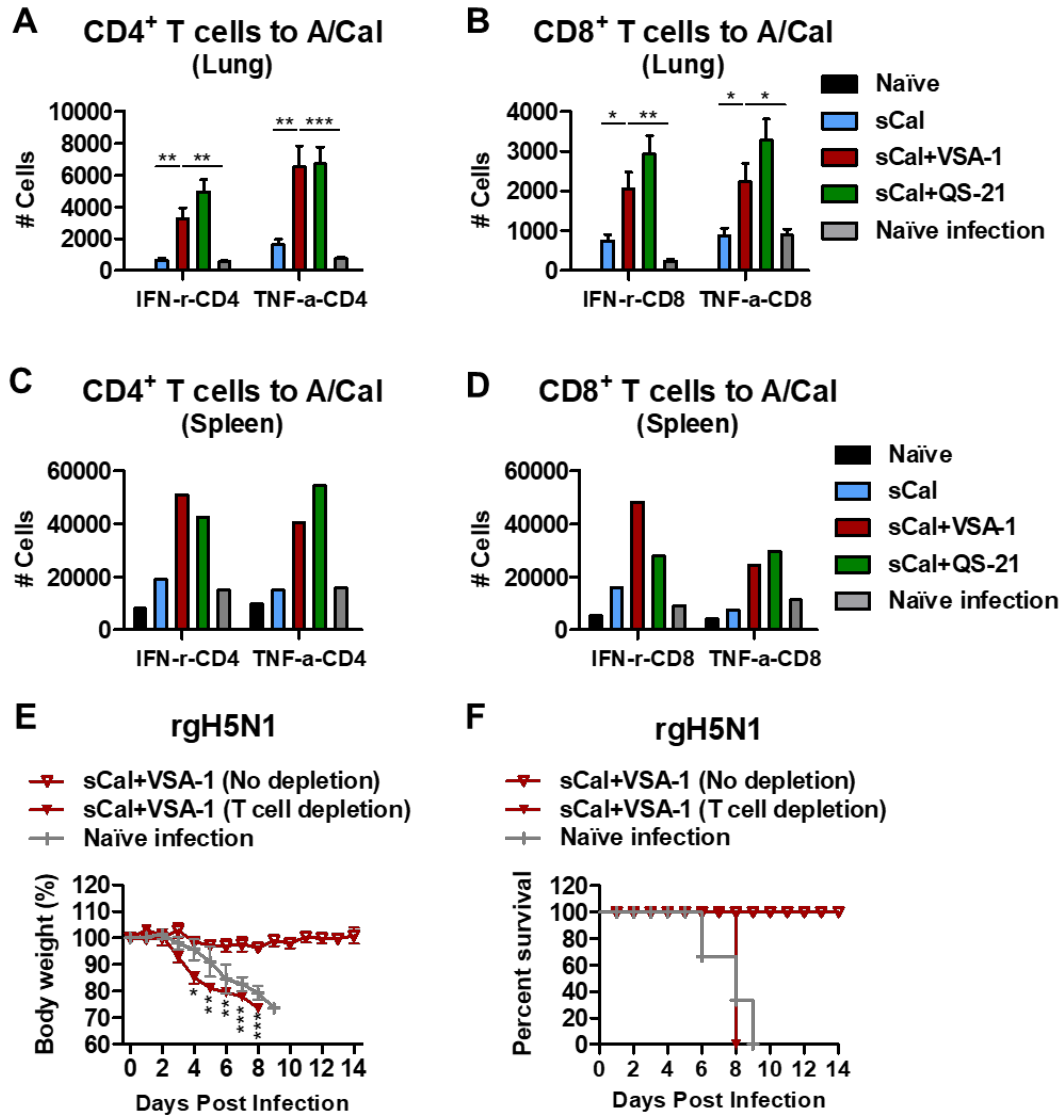


Figure 3.10 VSA-1 adjuvanted vaccination enhances effector T cells and confers T-cell mediated protection against heterosubtypic rgH5N1 virus. (A-D) Flow cytometry data of IFN- γ -secreting CD4⁺ and CD8⁺ T cells in lung (A and B) and spleen (C and D) samples day 6 post infection. (E) Body weight changes and (F) survival rates in VSA-1 adjuvanted prime-boost immunized C57BL/6 mice (n = 3 per group) with CD4 and CD8 T cells depleted and rgH5N1 virus challenge. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the sCal+VSA-1 group

3.4.7 VSA-1 adjuvanted prime-boost vaccination enhances antibody-secreting cell and IFN- γ -producing T cell responses

Spleen and MLN cells were harvested at day 6 after rgH5N1 challenge for analysis of IgG antibodies secreted from ASCs in *in vitro* culture supernatants by ELISA (Figs. 3.11A-D). The groups immunized with the split vaccine only showed low levels of A/Cal H1N1 and rgH5N1-specific IgG antibodies after 1-day and 5-day culture of spleen and MLN cells, whereas the VSA-1 and QS-21 adjuvanted split virus vaccinated groups showed higher levels of antibodies in cultures of spleen and MLN cells. The VSA-1 and QS-21 adjuvanted vaccine groups induced significantly higher levels of A/Cal H1N1 and rgH5N1 virus-specific mucosal IgG antibodies in lung extracts than the control groups (Fig. 3.11E, F).

To determine adjuvant effects of VSA-1 on eliciting cellular immune responses, lung cells and splenocytes harvested at day 6 after infection, were stimulated with inactivated A/Cal H1N1 virus or inactivated rgH5N1 virus. The number of A/Cal H1N1-specific and rgH5N1-specific IFN- γ producing cells was higher in the VSA-1 and QS-21 adjuvanted vaccination group than those in the split only vaccination and naïve infection groups in lung and spleen cells (Figs. 3.12A-D).

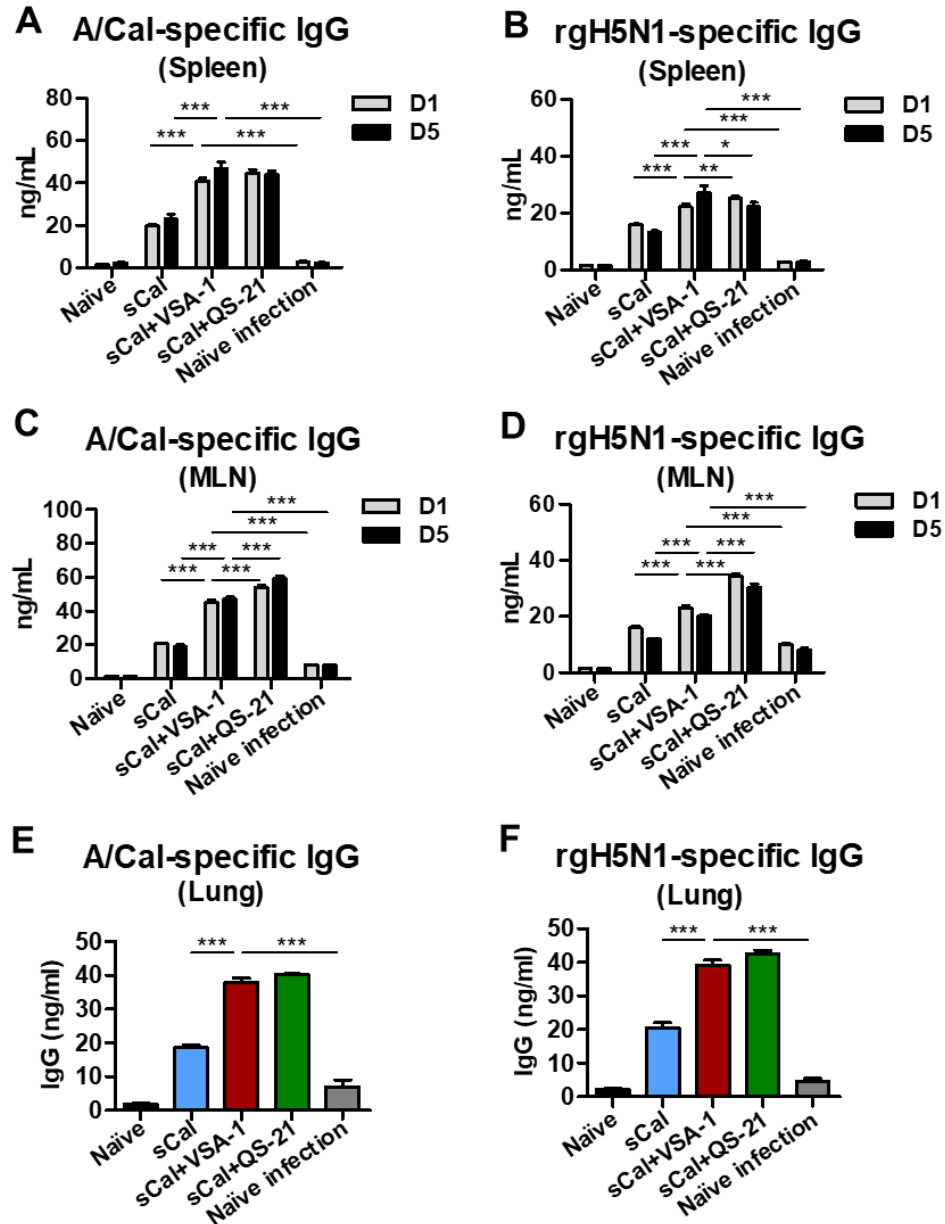


Figure 3.11 VSA-1 adjuvanted vaccination enhances antibody-secreting cell responses. (A-D) A/Cal virus-specific and rgH5N1 virus-specific IgG production from splenocytes (A and B) and MLN cells (C and D) harvested on day 6 post heterosubtypic rgH5N1 virus infection, cultured *in vitro* for 1 day or 5 days in the presence of inactivated A/Cal H1N1 virus or inactivated rgH5N1 virus (n = 4 per group). (E) A/Cal virus-specific and (F) rgH5N1 virus-specific IgG levels in lung samples collected at day 6 post challenge. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to the sCal+VSA-1 group

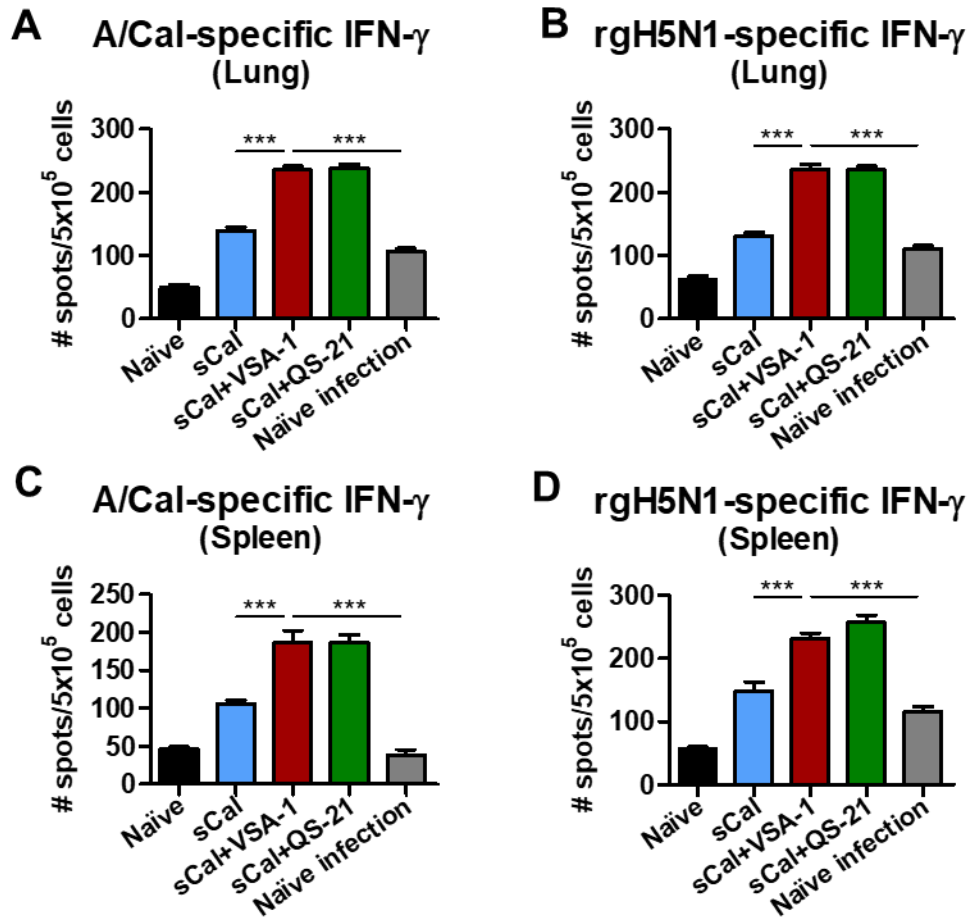


Figure 3.12 VSA-1 adjuvanted prime-boost influenza vaccination enhances effector T cells after lethal challenge with homologous A/Cal H1N1 virus in C57BL/6 mice. Vaccination regimen is the same as described in Figure 3.7. Three to four weeks post boost immunization, the mice were challenged with a lethal dose of rgH5N1 virus ($3 \times \text{LD}_{50}$). Lung and spleen samples of the immunized C57BL/6 mice were harvested at day 6 post rgH5N1 virus infection. (A-D) Cytokine ELISpot of lung cells (A and B) and splenocytes (C and D) at day 6 post infection after in vitro stimulation with inactivated A/Cal H1N1 virus or rgH5N1 virus. Error bars indicate the mean \pm standard errors of the mean (SEM). ***, $p < 0.001$ compared to the sCal+VSA-1 group

3.4.8 Acute innate immune responses are differentially modulated by VSA-1 and other comparing adjuvants, QS-21, and alum

To evaluate the acute innate immune effects of VSA-1 on inducing cytokines, chemokines, and cell recruitment at the site of injection, we injected naïve C57BL/6 mice with PBS, VSA-1, QS-21, or Alum intraperitoneally and determined cytokines and chemokines in sera and cell phenotypes in the peritoneal cavity. VSA-1 and QS-21 were more potent in acutely inducing cytokine (IL-6) and chemokines (KC, MCP-1) in sera compared to alum. VSA-1 and QS-21 induced moderate levels of these cytokines and chemokines within 2 h after injection (Figs. 3.13A-C), whereas after 20 h, moderate to low levels of chemokines (KC, MCP-1) were detected in sera in these groups.

The phenotypes of innate cells infiltrated into the peritoneal cavity at 20 h after injection with adjuvants were determined by flow cytometry (Figs. 3.13D-K). VSA-1 injection recruited monocytes, neutrophils, eosinophils, and dendritic cell (DC) subsets (aDCs, CD11b⁺ DCs, pDCs) in the peritoneal cavity at high levels, which were similar to alum, whereas QS-21 induced these cells at low levels at the site of injection. Overall, VSA-1 modulates acute immune responses of cytokines, chemokines, and innate immune cells at the site of injection in a unique pattern different from other comparing adjuvants.

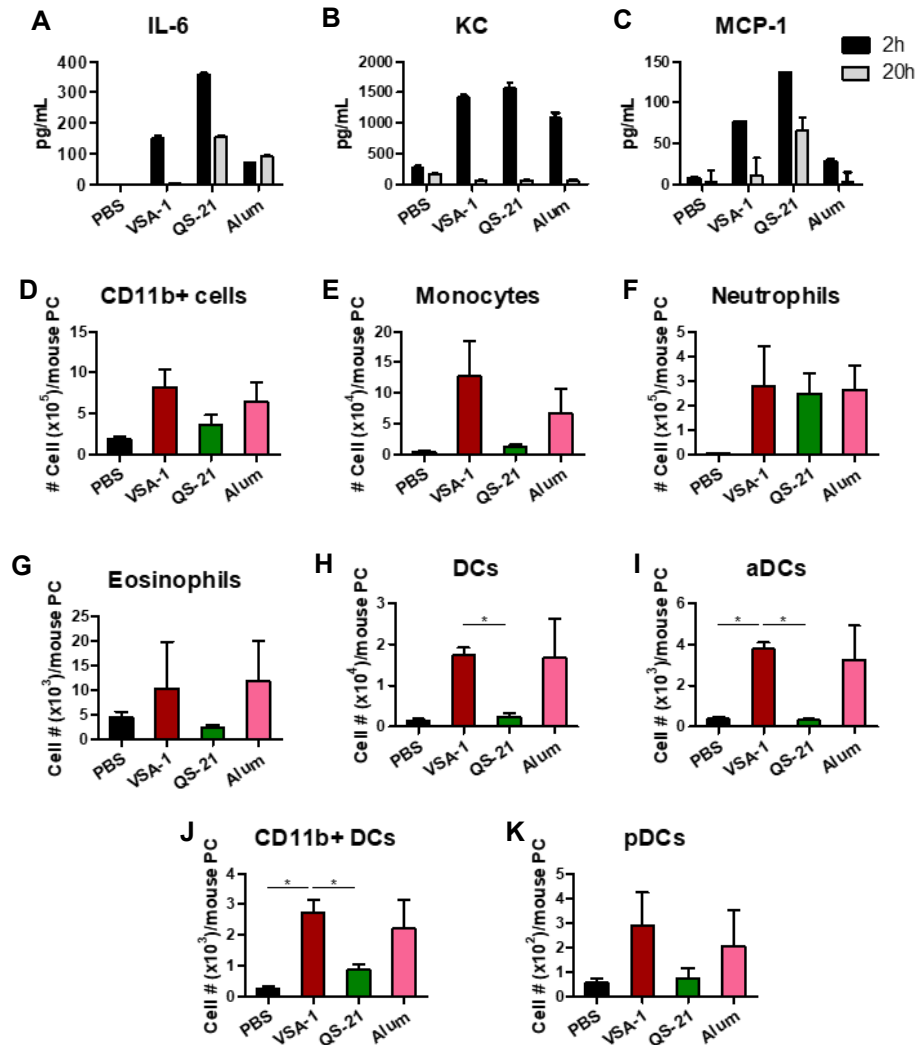


Figure 3.13 Acute innate immune induction of cytokines and chemokines in sera and innate cellular infiltrates in the peritoneal cavity after intraperitoneal injection of adjuvants. Naïve C57BL/6 mice ($n = 3$ per group) were intraperitoneally injected with PBS, VSA-1 (50 μ g), QS-21 (10 μ g) or Alum (50 μ g). (A-C) Cytokine and chemokine ELISA levels in sera at 2 h and 20 h after injection. (D-K) Cellular phenotypes in peritoneal exudates at 20 h after injection. Cell numbers of each cell population per mouse peritoneal cavity (PC) were calculated by multiplying cell percentages with total cell numbers. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$ compared to the VSA-1 group

3.5 Discussion

VSA-1 is a newly developed semisynthetic saponin adjuvant, based on extensive structure-activity-relationship studies of QS-21 which is widely used as an adjuvant component in licensed human vaccines. AS01 is a combination of immunostimulants QS-21 and MPL with liposomes and AS02 is a combination of QS-21 and MPL with an oil-in-water emulsion (Garçon & Di Pasquale, 2017). AS01b is included in human Shingles vaccine (Shingrix) and malaria vaccine (Mosquirix) and AS02 in developmental Tuberculosis, melanoma, and malaria vaccines (Cunningham et al., 2016; Garçon et al., 2011; Kumarasamy et al., 2016; Lal et al., 2015). Alum is a gold standard adjuvant commonly used in licensed human vaccines. In this study, we investigated the adjuvant effects of VSA-1 on enhancing the immunogenicity and efficacy of sCal (2009 H1N1 pandemic virus) influenza vaccination in C57BL/6 mice, in comparison with the effects of QS-21 and Alum adjuvants. VSA-1 adjuvant effects could be more potent in inducing IgG1 and IgG2b isotypes as well as HAI functional antibodies, IFN- γ ⁺ T cell responses, and homologous protection with single-dose sCal vaccination compared to those by QS-21 and Alum adjuvanted sCal vaccinations. Particularly, VSA-1 adjuvanted sCal prime-boost vaccination, but not sCal alone vaccination, significantly induced both CD4 and CD8 T cell responses that play a critical role in conferring effective cross-protection.

Alum adjuvant is biased in skewing the immune responses to Th2 type IgG1 antibodies. Consistent with this, in our study, alum potentiated Th2 type immune responses inducing IgG1 antibodies and exhibited weak adjuvant effects on inducing HA functional antibodies, correlating with high lung viral titers and low efficacy of protection after challenge. In contrast, VSA-1 adjuvant was effective in promoting the induction of IgG1, IgG2b, and IgG2c isotype antibodies and HAI titers at highest levels, suggesting that it can be developed as a more potent and suitable

vaccine adjuvant than QS-21. The action mechanisms of VSA-1 remain unknown. Previous studies on QS-21 would provide some insights into the mechanisms of saponin carbohydrate adjuvants. One hypothesis is that QS-21 might facilitate vaccine antigen uptake by antigen presenting cells by interacting with lectin receptors through carbohydrate domains, stimulating certain cytokines that activate T cell and B cell responses (Marciani, 2018). An alternative mechanism is cholesterol-dependent endocytosis of vaccine antigens and QS-21 into dendritic cells (Lorent et al., 2014). The high affinity of QS-21 to membrane cholesterol may lead to pore formation by destabilizing the membrane structure and facilitate the delivery of vaccine antigens into the cytosol of antigen presenting cells for further processing into peptides for T cell activation. QS-21 is proposed to stimulate T cells via the mitogen-activated protein kinase through CD2 molecules, resulting in the production of Th1 cytokines (Marciani, 2018). Studies on QS-21 in mouse antigen presenting cells reported that QS-21 in combination with TLR4 agonist MPL A activated NOD-like receptor P3 (NLRP3) inflammasome, a multi-protein complex, inducing subsequent release of proinflammatory cytokines and potentially contributing to INF- γ -mediated Th1 responses (Marty-Roix et al., 2016). Alum (at a dose of 100 μ g) and QS-21 (at a dose of 5 μ g) have been known to induce necrotic cell death where QS-21 induced macrophage and dendritic cell death in a caspase-1-, ASC-, and NLRP3-independent manner (Marty-Roix et al., 2016). This necrotic cell death mechanism of QS-21 might have led to a decrease in the total cell numbers observed in the peritoneal exudates 24 h after mice were intraperitoneally injected with QS-21 in this study (Figs. 13.3D-K). To abrogate potential cell lytic activity and toxicity of QS-21, a delivery platform of cholesterol-based liposomes was utilized in the AS01 formulation (Garçon & Di Pasquale, 2017). Since cholesterol-quenched QS-21 retained adjuvant potency as free QS-21, the linking of cell lytic activity and an immune-stimulant effect of QS-21 is questionable. There are several

drawbacks inherent to QS-21 as a natural product, including chemical instability and heterogeneity, scarcity, and dose-limiting toxicity. Therefore, VSA-1 which is a semisynthetic compound would be a more homogeneous and potent vaccine adjuvant as supported in this study, would be non-toxic even at high doses, and safer than QS-21 (P. Wang, X. Ding, et al., 2019).

In AS01 formulation, MPL and QS-21 were shown to synergistically stimulate the production of immune mediators such as IFN- γ , IL-12, and IL-18 as well as recruit neutrophils and monocytes and activate natural killer and innate lymphoid cells (Coccia et al., 2017; Dendouga et al., 2012; O'Hagan et al., 2020). VSA-1 was found to be a more effective stimulator for recruiting monocytes and diverse dendritic cell populations to the site of injection than QS-21. In contrast, QS-21 induced the production of IL-6 inflammatory cytokine and MCP-1 chemokine in sera within 2 hours post injection transiently at higher levels than VSA-1. It remains to be determined whether combination with MPL and VSA-1 will exhibit synergistic adjuvant effects as AS01 in future studies.

It is highly significant to enhance the effectiveness of seasonal vaccination by formulating vaccines containing safe adjuvants. Induction of T cell immunity and HA stalk and NA IgG antibodies was previously reported to be independently correlated with cross-protection (Aydillo et al., 2020). Both VSA-1 and QS-21 adjuvanted sCal vaccinations induced cross-protection against rgH5N1 virus even in the absence of cross-reactive HAI antibodies. HA stalk-specific IgG antibodies, as well as IFN- γ^+ and TNF- α^+ CD4 and CD8 T cell responses, were observed with the VSA-1 and QS-21 adjuvanted sCal groups at significantly higher levels, which appears to be correlating with cross-protection. Particularly, depletion of CD4 and CD8 T cells led to abrogation of cross-protection, supporting a critical role of cross-protective T cell immunity induced by VSA-1 adjuvanted vaccination.

In summary, VSA-1 as a new semisynthetic saponin-based adjuvant played a significant role in enhancing IgG isotype and HAI functional antibodies after influenza split prime vaccination, conferring superior homologous protection over split vaccine, and Alum or QS-21 adjuvanted vaccination in C57BL/6 mice. Both VSA-1 and QS-21 adjuvanted sCal boost vaccinations were highly effective in inducing humoral and cellular immune responses as well as cross-protection against rgH5N1 virus even in the absence of cross-reactive HAI antibodies. This study provides supportive evidence warranting further development of VSA-1 as an alternative promising candidate for QS-21 replacement.

3.6 Conclusion

This study has significance in evaluating whether a newly synthesized semisynthetic compound, VSA-1, which is an analog of licensed saponin adjuvant QS-21, exhibits adjuvant effects on enhancing the humoral and cellular immunogenicity and protection against homologous and heterosubtypic viruses in an adult C57BL/6 mouse model. The adjuvant effects of VSA-1 on improving vaccine efficacy after prime immunization were evident as shown by significantly higher levels of hemagglutination inhibiting antibody titers and IFN- γ ⁺ T cell responses, and enhanced homologous protection, compared to QS-21 and alum adjuvants. VSA-1 adjuvant effects on enhancing heterosubtypic protection after two doses of adjuvanted vaccination were found to be comparable to those of QS-21. T cell immunity was found to play an important role in conferring cross-protection by VSA-1 adjuvanted vaccination. Overall, the findings in this study suggest that VSA-1 exhibits desirable adjuvant properties and a unique pattern of innate and adaptive immune responses, contributing to improved homologous and heterosubtypic protection by inactivated split influenza vaccination in mice.

3.7 Acknowledgements

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4 CHAPTER 3. HETEROLOGOUS PRIME-BOOST VACCINATION WITH INACTIVATED INFLUENZA VIRUSES INDUCES MORE EFFECTIVE CROSS-PROTECTION THAN HOMOLOGOUS REPEAT VACCINATION

4.1 Abstract

With concerns about the efficacy of repeat annual influenza vaccination, it is important to better understand the impact of prior vaccine immunity and to develop an effective vaccination strategy. Here, we determined the impact of heterologous prime-boost vaccination on inducing broader protective immunity compared to repeat vaccination with the same antigen. The primed mice that were intramuscularly boosted with a heterologous inactivated virus (H1N1, H3N2, H5N1, H7N9, H9N2) vaccine showed increased strain-specific hemagglutination inhibition (HAI)

titers against prime and boost vaccine strains by up to eight folds. Heterologous prime-boost vaccination of mice with inactivated viruses more effectively induced high levels of IgG antibodies for group 1 and 2 HA stalk domains, as well as cross-protection and lung viral clearance, compared to homologous vaccination. Both humoral and T cell immunity were found to play a critical role in conferring cross-protection by heterologous prime-boost vaccination. These results support a strategy to enhance the cross-protective efficacy by heterologous prime-boost influenza vaccination.

4.2 Introduction

Influenza virus causes 290,000 to 640,000 deaths globally (Iuliano et al., 2018; Thompson et al., 2003). Influenza A virus has high antigenic diversity, originating from 18 hemagglutinin (HA) subtypes (H1-H18) and 11 neuraminidase (NA) subtypes (N1-N11). Influenza A virus HA subtypes are divided into two main phylogenetic groups, group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, and H17) and group 2 (H3, H4, H7, H10, H14, and H15) (Pica & Palese, 2013). Influenza B virus has evolved into antigenically distinct Victoria and Yamagata lineage strains. Current influenza vaccines are either trivalent (H1N1, H3N2, one influenza B virus) or quadrivalent containing influenza A H1N1 and H3N2 as well as both lineages of influenza B virus strains. The vaccine components are from the same strains used in previous years or some vaccine strains are updated annually to better reflect the circulating influenza strains. Annual repeat vaccinations are recommended as a preventive measure, resulting in variable effectiveness against influenza (de Bruijn et al., 1999; Petrie & Monto, 2017; Sasaki et al., 2011; Smith et al., 1999). Because of the continuing emergence of drifting mutations and pandemics, the overall effectiveness after seasonal vaccination has been in a wide low range between 10 % and 60 %

during the last decades (CDC; Keitel et al., 1997). During the 2014–2015 season, the efficacy of the H3N2 vaccine component was estimated to be as low as 6%, partially due to drifting mutations in the circulating H3N2 strains (Zimmerman et al., 2016).

There are controversial studies reporting that annual repeat influenza vaccination did not improve protective immune responses (Leung et al., 2017; McLean et al., 2014), lowered vaccine efficacy against mismatch strains (Morimoto & Takeishi, 2018), and failed to increase antibody affinity maturation in humans (Khurana et al., 2019; Leung et al., 2017; Sanyal et al., 2019). Pre-existing immunity shapes the immune responses toward earlier influenza antigens or conserved domains (Cobey & Hensley, 2017; Guthmiller & Wilson, 2018). It has been a high priority to better understand how prior immune responses modulate cross-protective immune responses to vaccination and enhance vaccine efficacy toward broader protection.

In this study, we tested a hypothesis that a strategy of heterologous prime-boost vaccination with antigenically diverse inactivated influenza viruses would induce broader protective immune responses than repeat vaccination with the same antigen in a mouse model. Boost immunization with antigenically different viruses from the strain used for prime was found to enhance HAI activities against the prime and heterologous boost viruses as well as cross-protection. Possible correlates of group-specific HA stalk IgG antibody responses and roles of humoral and cellular immunity were explored.

4.3 Materials and Methods

4.3.1 Animals, reagents, and viruses

Adult BALB/c mice (6- to 8-week-old, female) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility at Georgia State University (GSU). All

mouse studies were approved by GSU Institutional Animal Care and Use Committee (IACUC, A21004) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals of the NIH.

Pure *Quillaja* saponin, QS-21, and monophosphoryl lipid A (MPL) adjuvants were purchased from Desert King International (San Diego, CA, USA) and Sigma Aldrich (St. Louis, MO), respectively, and dissolved in dimethyl sulfoxide (DMSO) following the manufacturer's protocol. Mouse-specific oligodeoxynucleotide (ODN) with CpG motifs (ODN1826, 5'-TCC ATG ACG TTC CTG ACG TT-3') was synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The lyophilized CpG was resuspended in ultra-pure water.

Group 1 and 2 inactivated influenza viruses were used for immunizations and as coating antigens for Enzyme-linked immunosorbent assay (ELISA). Group 1 inactivated influenza A viruses were as follows: A/Puerto Rico/8/1934 H1N1 (iPR8/H1N1), A/California/04/2009 H1N1 (iCal/H1N1), A/Indonesia/5/2005 H5N1 (iIndo/H5N1), and A/Hong Kong/1073/1999 H9N2 (iHK/H9N2). Group 2 viruses used include A/Hong Kong/1/1968 H3N2 (iHK/H3N2), and reassortant A/Shanghai/11/2013 H7N9 with A/Puerto Rico/8/1934 H1N1 backbone (iSH/H7N9) (Deng et al., 2018; Park et al., 2021). The inactivated viruses were prepared as described previously (Ko et al., 2018). Briefly, influenza viruses were inactivated with 1% formalin and concentrated by ultracentrifugation (SW32 Ti rotor, 123,760×g, 1 h). The inactivated virus pellet was resuspended in phosphate-buffered saline (PBS), aliquoted and stored at -80 °C until use. The total protein concentration of the inactivated viruses was determined by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Challenge viruses included group 2 - A/Philippine/2/1982 H3N2 (Phil/H3N2) virus, and group 1 - A/Hong Kong/1073/1999 H9N2 (HK/H9N2) virus, and reverse genetics (rg) reassortant H5N1 (rgH5N1) virus containing HA and NA derived from

A/Vietnam/1203/2004 and six internal genes from A/Puerto Rico/8/1934 H1N1, as described previously (Song et al., 2011). Phil/H3N2, HK/H9N2 and rgH5N1 viruses were propagated using embryonated chicken eggs.

4.3.2 Immunization and virus challenge of mice

Groups of 6-week-old BALB/c mice ($n = 5$) were intramuscularly immunized with 5 μ g of a specific strain of inactivated influenza virus and then boosted with 5 μ g of a homologous, heterologous or heterosubtypic inactivated virus (H1N1, H3N2, H5N1, H7N9, H9N2)/mouse. The groups with prime – boost vaccine strains consisted of iPR8/H1N1 - iPR8/H1N1, iPR8/H1N1 - iCal/H1N1, iCal/H1N1 - iPR8/H1N1, iPR8/H1N1 - iHK/H3N2, iHK/H3N2 - iPR8/H1N1, iPR8/H1N1 - iIndo/H5N1, iIndo/H5N1 - iPR8/H1N1, iPR8/H1N1 - iSH/H7N9, iSH/H7N9 - iPR8/H1N1, iPR8/H1N1 - iHK/H9N2, iHK/H9N2 - iPR8/H1N1 for prime-boost vaccinations without adjuvant unless specified. Blood samples were collected at 2 weeks after immunizations, and sera were separated to analyze virus- and HA stalk-specific IgG antibody levels as well as HAI titers. Six to eight weeks after boost, the mice were challenged with a lethal dose of Phil/H3N2 virus ($6.7 \times LD_{50}$), or HK/H9N2 virus ($6.7 \times LD_{50}$) or rgH5N1 virus ($5 \times LD_{50}$). After challenge, body weight changes and survival rates were monitored for 14 days, and lung viral titers and detailed immunological profiles were determined in mediastinal lymph nodes (MLN), and spleen tissues collected at day 6 post infection.

4.3.3 Antibody Enzyme-linked immunosorbent assay (ELISA)

To measure antigen-specific antibody levels, inactivated viruses (200 ng/well) were coated onto ELISA plates and then incubated with diluted immune sera as detailed previously (Ko et al., 2018; Ko et al., 2016). IgG isotypes were measured using anti-mouse immunoglobulin IgG, IgG1 and IgG2a, horse-radish peroxidase (HRP)-conjugated secondary antibodies (Southern

Biotechnology, Birmingham, AL), and tetramethylbenzidine (TMB) substrate (Invitrogen, Waltham, MA). Antibody levels are presented as optical density absorbance values at 450 nm (BioTek ELISA plate reader). Additionally, consensus group 1 and group 2 hemagglutinin (HA) stalk proteins (50 ng/well), prepared as described (Chae et al., 2019), were used to determine HA stalk-specific IgG antibodies.

4.3.4 Hemagglutination inhibition (HAI) assay

HAI titers in immune sera treated with receptor destroying enzymes (RDE, Sigma-Aldrich, St. Louis, MO) and inactivated (56 °C, 30 min) were determined by using 4 HA units of PR8/H1N1, Cal/H1N1, HK/H3N2, Indo/H5N1, SH/H7N9, HK/H9N2, and Phil/H3N2 viruses and 0.5% chicken red blood cells (RBC, Lampire Biological Laboratories, Pipersville, VA) as previously described (Ko et al., 2018; Ko et al., 2016).

4.3.5 Lung viral titration

Lung extracts, prepared in 1.5 mL of Roswell Park Memorial Institute (RPMI) 1640 without fetal bovine serum (FBS) by mechanical grinding of lung tissues, harvested at day 6 after challenge were used to determine viral titers in embryonated chicken eggs (Hy-Line North America, LLC., Mansfield, GA), as described (Kim et al., 2019; Ko et al., 2016). Virus titers as 50% egg infection dose (EID₅₀)/mL were evaluated according to the Reed and Muench method (Reed & Muench, 1938).

4.3.6 Cytokine ELISA and in vitro IgG antibody detection

Lung extracts were prepared in 1.5 mL of RPMI 1640 without FBS by mechanical grinding of lung tissues harvested at day 6 after challenge. Lung extracts were used to determine G1 and G2 HA stalk-specific antibody levels. The inflammatory cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-6, interferon (IFN)- γ and IL-1 β from lung extracts were measured by cytokine

ELISA as described (Lee et al., 2016). Cytokines were detected using Ready-SET-Go kit with TNF- α , IL-6, IFN- γ or IL-1 β specific antibodies (eBioscience, San Diego, CA).

Secreted IgG antibodies specific for G1 and G2 HA stalk were determined from mediastinal lymph nodes (MLN, 5×10^5 cells/well) from mice. The cells from MLN were isolated at day 6 post infection and cultured for 5 days in plates pre-coated with G1 and G2 HA stalk proteins. The combined levels of IgG antibodies secreted into the culture supernatants and those captured on the plate were analyzed by ELISA.

4.3.7 Enzyme-linked Immunospot (ELISpot) assay

Interferon (IFN)- γ secreting cell spots were determined by culturing lung cells (2×10^5 cells/well) for 72 h on multi-screen 96-well plates (MilliporeSigma, St. Louis, MO) coated with cytokine-specific capture antibodies as described (Song et al., 2011). Inactivated influenza viruses (iPR8/H1N1 and iSH/H7N9) were included as antigenic stimulators (4 μ g/mL) and IFN- γ -secreting cell spots were counted using an ELISpot reader (BioSys, Miami, FL).

4.3.8 In vivo protection efficacy test of immune sera

Immune sera collected at two weeks after boost immunization were diluted 4 folds, heat-inactivated at 56 °C for 30 min, followed by mixing with the same volume of Phil/H3N2 virus ($1.5 \times \text{LD}_{50}$) and incubating at room temperature for 30 min as described (Kim et al., 2013). The mixture of Phil/H3N2 virus and sera was intranasally administered to naïve BALB/c mice ($n = 4$ per group), and body weight changes and survival rates were monitored daily for 14 days.

4.3.9 In vivo depletion of T cells

For *in vivo* systemic T cell depletion before and post challenge, prime-boost immunized BALB/c mice ($n = 4$) received treatment with anti-CD4 (CD4 clone GK1.5) or anti-CD8 (CD8 clone 53.6.7) mAbs as previously described (Ko et al., 2017). Antibodies (BioXCell, West

Lebanon, NH) were injected into the mice with intraperitoneal (IP; 2 days before challenge), and intraperitoneal (IP; 2 days after challenge) plus intranasal (IN; 2 days after challenge) sequential delivery at a 4-day interval (anti-CD4 200 µg and anti-CD8 150 µg/mouse for IP injection, 10 µg anti-CD4/8/mouse for IN inoculation). All groups were challenged with a lethal dose of Phil/H3N2 influenza virus ($1.5 \times LD_{50}$), and body weight changes and survival rates were monitored daily for 14 days after challenge.

4.3.10 Statistical analyses

All results are presented as mean \pm standard errors of the mean (SEM). The statistical significance for all the experiments was calculated by one-way analysis of variance (ANOVA). P-values ≤ 0.05 were considered significant. Data analysis was performed using Prism software (GraphPad Software Inc., San Diego, CA).

4.4 Results

4.4.1 Heterologous and heterosubtypic prime-boost vaccinations induce cross-reactive virus-specific IgG and group-specific HA stalk antibodies

To study the impact of pre-existing immunity, groups of BALB/c mice were intramuscularly primed with a specific strain of inactivated influenza virus and then boosted with a homologous, heterologous or heterosubtypic inactivated virus (H1N1, H3N2, H5N1, H7N9, H9N2) (Fig. 4.1A). Prime immunization with a strain of inactivated virus induced IgG responses specific for that prime strain, for example, iPR8/H1N1-primed mice induced highest levels of iPR8/H1N1 virus-specific IgG antibodies (Fig. 4.1B), iCal/H1N1-primed mice induced highest levels of iCal/H1N1 virus-specific IgG antibodies and same was the case with all other primed groups (Table 4.1). Also, all the groups induced prime virus vaccine strain-specific group 1 or 2

stalk-specific IgG antibodies; iPR8/H1N1-, iCal/H1N1-, iIndo/H5N1-, and iHK/H9N2-primed groups induced high to moderate levels of group 1 stalk-specific IgG antibodies, whereas iHK/H3N2-, and iSH/H7N9-primed groups induced moderate levels of group 2 stalk-specific IgG antibodies. Boost immunizations showed highly cross-reactive and heterogeneous patterns of IgG antibodies (Fig. 4.1C and Table 4.2). Also, group 1 and 2 stalk-specific IgG levels were highly boosted after prime-boost vaccinations. Specifically, the iPR8/H1N1 - iSH/H7N9 and iSH/H7N9 - iPR8/H1N1 heterologous prime-boost vaccination groups induced highest levels of IgG antibodies for all homologous, heterologous and heterosubtypic viruses, and group 1 and 2 HA stalk domains. These results suggest that heterosubtypic prime-boost vaccination induces cross-reactive IgG antibodies to different virus subtypes and both group 1 and 2 HA stalk domains.

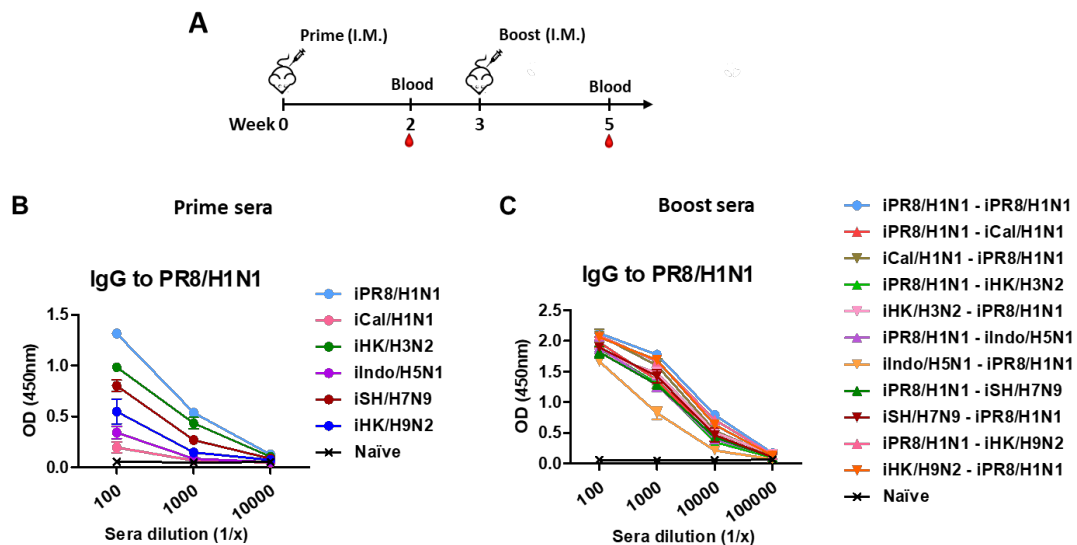


Figure 4.1 Virus- and HA stalk-specific IgG antibody responses in primed or prime-boost immune sera. (A) Immunization scheme (n = 5 per group). BALB/c mice were intramuscularly primed with 5 µg of a specific strain of inactivated influenza virus and then boosted with 5 µg of a homologous, heterologous or heterosubtypic inactivated virus (H1N1, H3N2, H5N1, H7N9, H9N2)/mouse. (B) PR8/H1N1 virus-specific ELISA IgG antibody levels in prime sera. (C) PR8/H1N1 virus-specific ELISA IgG antibody levels in boost sera. Virus-specific IgG antibody levels were presented as optical density (OD) values of ELISA readings

Table 4.1 Virus- and HA stalk-specific IgG antibody responses (ELISA OD values) in primed immune sera collected 2 weeks after prime vaccination. The prime vaccine groups are the same as described in Figure 4.1 legend and vaccination scheme. IgG antibody levels in prime sera were determined as represented in Figure 4.1B data.

	ELISA prime sera – Coating antigen						Stalk-specific IgG	
	iPR8/ H1N1	iCal/ H1N1	iHK/ H3N2	iIndo/ H5N1	iSH/ H7N9	iHK/ H9N2	G1 stalk	G2 stalk
Prime	100x sera dilution						100x sera dilution	
iPR8/H1N1	1.32	0.26	1.01	0.80	0.65	0.58	0.92	0.06
iCal/H1N1	0.20	1.00	0.24	0.23	0.17	0.41	0.54	0.06
iHK/H3N2	0.98	0.42	1.95	0.90	0.91	0.59	0.08	0.25
iIndo/H5N1	0.34	0.11	0.47	1.55	0.41	0.24	0.19	0.07
iSH/H7N9	0.80	0.25	1.30	0.92	1.24	0.18	0.08	0.33
iHK/H9N2	0.55	0.12	0.74	0.73	0.61	1.10	0.19	0.10
Naïve	0.05	0.05	0.06	0.05	0.05	0.05	0.08	0.10

Table 4.2 Different virus- and HA stalk-specific IgG antibody responses (ELISA OD values) in immune sera collected after homologous and heterologous prime-boost vaccinations. The boost vaccination groups are the same as described in Figure 4.1 legend and vaccination scheme. IgG antibody levels in boost sera were determined as represented in Figure 4.1C data.

		ELISA boost sera – Coating antigen						Stalk-specific IgG	
		iPR8/ H1N1	iCal/ H1N1	iHK/ H3N2	iIndo/ H5N1	iSH/ H7N9	iHK/ H9N2	G1 stalk	G2 stalk
Prime	Boost	1000x sera dilution						100x sera dilution	
iPR8/H1N1	iPR8/H1N1	1.77	0.58	1.84	1.54	1.35	1.32	1.91	0.64
iPR8/H1N1	iCal/H1N1	1.36	0.78	1.61	1.51	1.30	1.12	2.15	0.38
iCal/H1N1	iPR8/H1N1	0.82	0.83	1.04	1.00	0.67	0.54	2.18	0.46
iPR8/H1N1	iHK/H3N2	1.60	0.54	2.20	1.94	1.65	1.06	0.85	1.64
iHK/H3N2	iPR8/H1N1	1.28	0.42	2.02	1.58	1.25	0.82	1.89	1.66
iPR8/H1N1	iIndo/H5N1	1.31	0.32	1.72	1.97	1.46	0.65	2.26	0.85
iIndo/H5N1	iPR8/H1N1	1.42	0.25	1.67	1.82	1.39	0.69	1.79	0.27
iPR8/H1N1	iSH/H7N9	1.48	0.49	2.08	2.03	1.75	0.66	1.54	1.65
iSH/H7N9	iPR8/H1N1	1.67	0.39	2.08	1.76	1.62	0.71	1.98	1.05
iPR8/H1N1	iHK/H9N2	1.27	0.42	2.16	1.74	1.32	1.10	1.01	0.99
iHK/H9N2	iPR8/H1N1	1.69	0.31	2.08	1.95	1.71	0.89	1.98	1.06
Naïve		0.05	0.04	0.05	0.05	0.05	0.04	0.08	0.07

4.4.2 Pre-existing immunity enhances the induction of HAI titers against prior prime and current boost viruses

We measured HAI titers as a protective immune correlate in sera collected at 2 weeks after prime immunization as well as sera collected 2 weeks after boost immunization with inactivated viruses (Fig. 4.2A). Prime dose of inactivated viruses induced strain-specific HAI titers (Fig. 4.2B and Table 4.3), and no cross-reactive HAI titers were detected after prime immunization. After prime-boost vaccinations, 8-fold-increased HAI titers against PR8/H1N1 virus were induced in

the iPR8/H1N1 - iPR8/H1N1 homologous group, whereas heterologous or heterosubtypic boost induced HAI titers against both prime and boost strains at higher levels by 2-8 folds (Fig. 4.2C and Table 4.4). Also, no cross-reactive HAI titers were induced after boost immunization. These results suggest that heterosubtypic prime-boost vaccination can effectively induce HAI titers against the heterologous strains used for prime and boost and that pre-existing immunity promotes the induction of HAI antibodies against both strains.

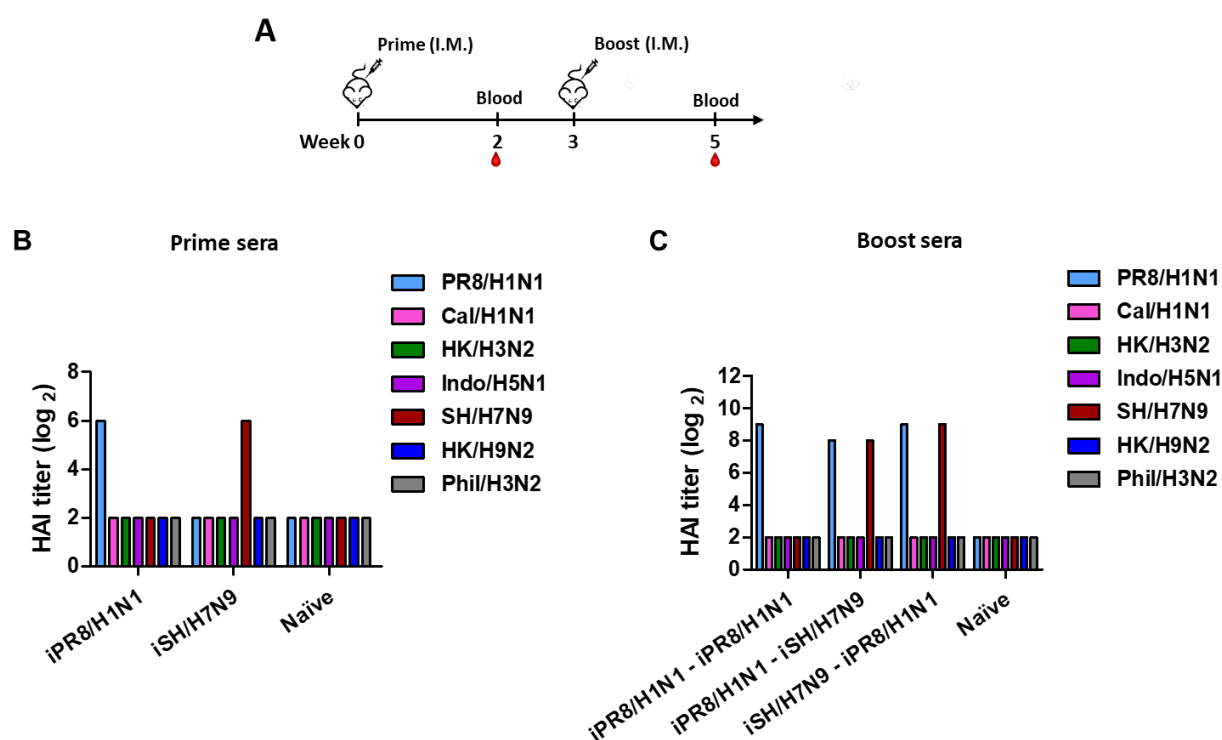


Figure 4.2 HAI titers in immune sera collected after prime vaccination or homologous and heterologous prime-boost vaccinations. (A) Immunization scheme ($n = 5$ per group) was the same as in Fig. 1. Blood samples were collected 2 weeks after prime and after boost immunizations, and sera were separated to analyze HAI titers. (B) HAI titers against different viruses as indicated in prime sera. (C) HAI titers against different viruses as indicated in boost sera from representative vaccine groups

Table 4.3 HAI titers against homologous and heterosubtypic viruses in primed immune sera collected 2 weeks after prime vaccination. HAI titers in prime sera were determined as represented in Figure 4.2B data.

HAI titers in prime sera							
Prime	PR8/H1N1	Cal/H1N1	HK/H3N2	Indo/H5N1	SH/H7N9	HK/H9N2	Phil/H3N2
iPR8/H1N1	1:64	-	-	-	-	-	-
iCal/H1N1	-	1:64	-	-	-	-	-
iHK/H3N2	-	-	1:256	-	-	-	-
IIndo/H5N1	-	-	-	1:32	-	-	-
iSH/H7N9	-	-	-	-	1:64	-	-
iHK/H9N2	-	-	-	-	-	1:256	-
Naïve	-	-	-	-	-	-	-

Table 4.4 HAI titers in immune sera collected after homologous and heterologous prime-boost vaccinations. HAI titers in boost sera were determined as represented in Figure 4.2C data.

Prime	Boost	HAI titers in boost sera						
		PR8/ H1N1	Cal/ H1N1	HK/ H3N2	Indo/ H5N1	SH/ H7N9	HK/ H9N2	Phil/ H3N2
iPR8/H1N1	iPR8/H1N1	1:512	-	-	-	-	-	-
iPR8/H1N1	iCal/H1N1	1:256	1.64	-	-	-	-	-
iCal/H1N1	iPR8/H1N1	1:256	1:256	-	-	-	-	-
iPR8/H1N1	iHK/H3N2	1:256	-	1:1024	-	-	-	-
iHK/H3N2	iPR8/H1N1	1:256	-	1:512	-	-	-	-
iPR8/H1N1	iIndo/H5N1	1:256	-	-	1:64	-	-	-
iIndo/H5N1	iPR8/H1N1	1:512	-	-	1:64	-	-	-
iPR8/H1N1	iSH/H7N9	1:256	-	-	-	1:256	-	-
iSH/H7N9	iPR8/H1N1	1:512	-	-	-	1:512	-	-
iPR8/H1N1	iHK/H9N2	1:256	-	-	-	-	1:256	-
iHK/H9N2	iPR8/H1N1	1:512	-	-	-	-	1:512	-
Naïve		-	-	-	-	-	-	-

4.4.3 Heterologous and heterosubtypic prime-boost vaccination induces cross-protection

To investigate the cross-protective efficacy, mice were challenged with Phil/H3N2 virus at 3 weeks after boost immunization (Fig. 4.3A). Naïve mice after infection with Phil/H3N2 virus showed severe weight loss of over 21% (Fig. 4.3B). The H1N1 homologous prime-boost groups, iCal/H1N1 - iCal/H1N1 and iPR8/H1N1 - iPR8/H1N1 did not survive or displayed high weight loss of 16.2%. The iPR8/H1N1 - iCal/H1N1 group exhibited similarly high weight loss (~16%) but the reverse order iCal/H1N1 - iPR8/H1N1 group displayed less weight loss of 10%, compared to the iCal/H1N1 - iCal/H1N1 group which did not survive the challenge. The heterosubtypic

H1N1 and H3N2 prime-boost groups (iPR8/H1N1 - iHK/H3N2 and iHK/H3N2 - iPR8/H1N1) displayed weight loss of ~13% and 9%, respectively, and the homologous iHK/H3N2 prime-boost group a weight loss ~10% (Fig. 4.3C). The low weight loss in the iHK/H3N2- iHK/H3N2 group might be because both the inactivated virus used for prime-boost immunization and the challenge virus belong to the same H3N2 subtype virus. The heterosubtypic H1N1 and H5N1 prime-boost groups (iPR8/H1N1 - iIndo/H5N1 and iIndo/H5N1 - iPR8/H1N1) showed a weight loss of ~14% and 13%, respectively (Fig. 4.3D) and the heterosubtypic H1N1 and H9N2 prime-boost groups (iPR8/H1N1 - iHK/H9N2 and iHK/H9N2 - iPR8/H1N1) a weight loss of ~9% and 10%, respectively (Figs. 4.3E). These results suggest that phylogenetically distant heterologous prime-boost vaccinations could be more effective in conferring cross-protection against Phil/H3N2 virus than homologous vaccination.

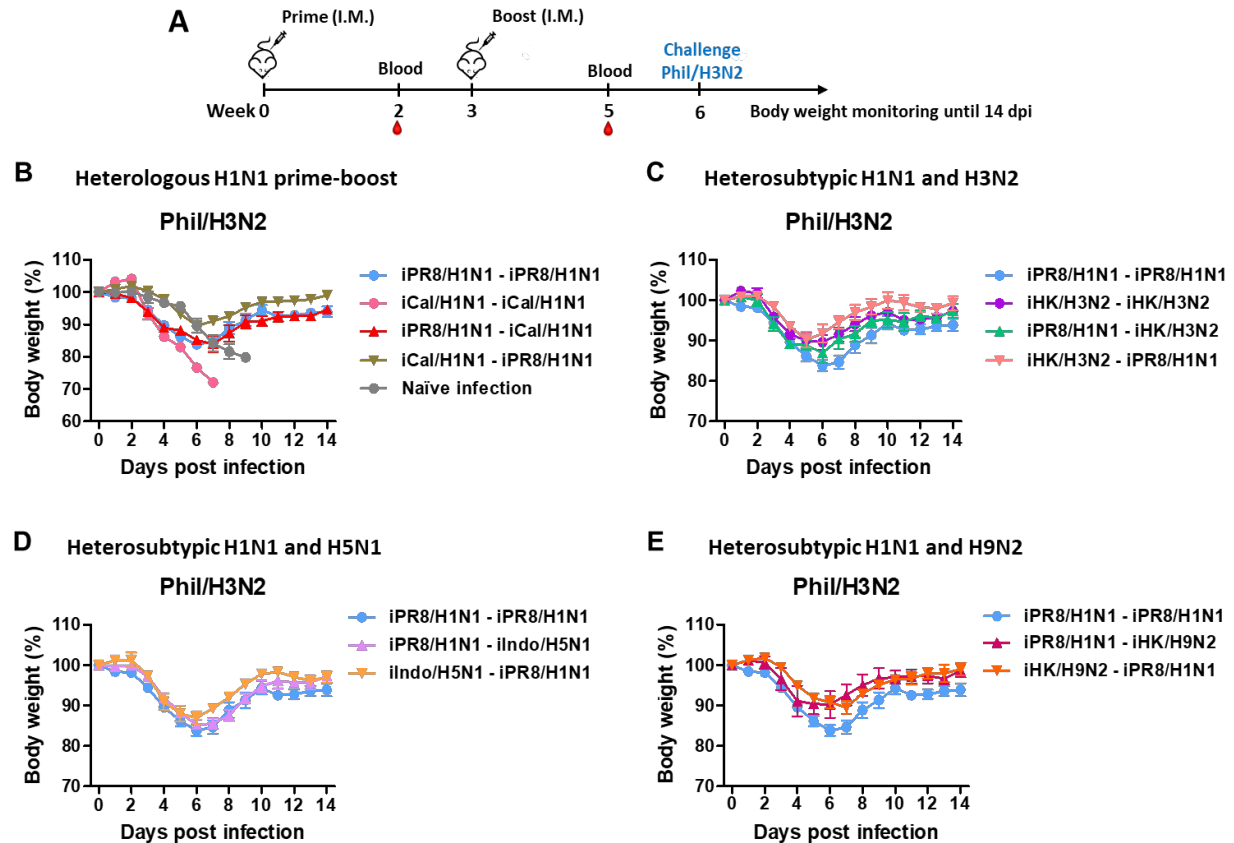


Figure 4.3 Body weight changes in the heterologous prime-boost groups after challenge with Phil/H3N2 virus. (A) Timeline for immunization and challenge ($n = 5$ per group) with the same vaccine groups as in Fig. 1. Eight weeks post boost immunization, the mice were challenged with a lethal dose of Phil/H3N2 virus ($6.7 \times LD_{50}$). (B-E) Body weight changes were monitored for 14 days. Error bars indicate the mean \pm standard errors of the mean (SEM)

4.4.4 Heterosubtypic H1N1 and H7N9 prime-boost vaccination induces enhanced cross-protection against different subtypes of influenza viruses

The heterosubtypic iPR8/H1N1 - iSH/H7N9 and iSH/H7N9 - iPR8/H1N1 groups displayed only ~10% and 8% weight loss, respectively with 100% survival (Fig. 4.4A, B). In contrast, the homologous iSH/H7N9 - iSH/H7N9 and iPR8/H1N1 - iPR8/H1N1 groups displayed severe weight loss (~17%). These results suggest that the heterosubtypic H1N1 and H7N9 prime-boost

vaccinations are more effective in inducing cross-protection against Phil/H3N2 virus than homologous repeat vaccination.

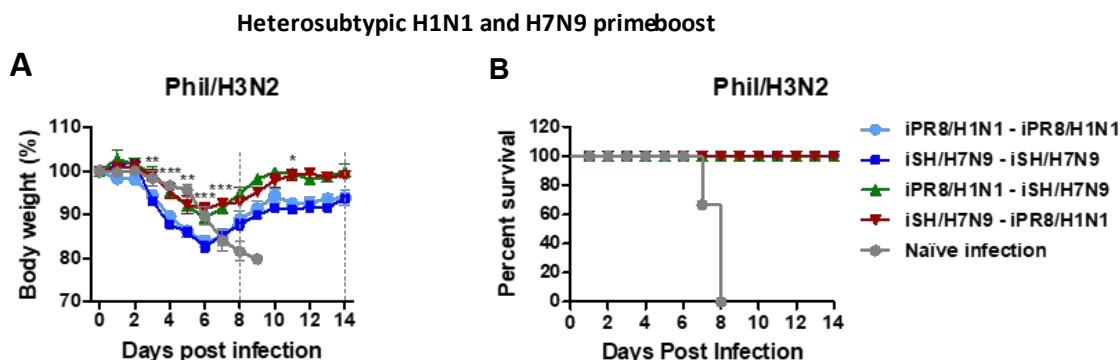


Figure 4.4 Heterosubtypic iSH/H7N9 – iPR8/H1N1 prime-boost vaccination induces more effective cross-protection against Phil/H3N2 virus challenge than homologous vaccination. Immunization (n = 3 per group) with the same vaccine groups as in Fig. 1 in addition to iCal/H1N1 – iCal/H1N1, iHK/H3N2 – iHK/H3N2 and iSH/H7N9 – iSH/H7N9 prime-boost vaccine groups. Eight weeks post boost immunization, the mice were challenged with the same dose of Phil/H3N2 virus ($6.7 \times LD_{50}$) as in Figure 3. (A and B) Body weight changes were monitored for 14 days. Statistical significance was calculated by using two-way ANOVA and Bonferroni's post-multiple comparison test. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, comparison between iSH/H7N9 – iSH/H7N9 and iSH/H7N9 – iPR8/H1N1 groups

We also evaluated the possibility of a correlation between HA stalk-specific antibodies and protection against Phil/H3N2 virus challenge. There was a moderate correlation between the group 2 (but not group 1) HA stalk-specific IgG levels and the efficacy of protection against Phil/H3N2 virus with a correlation coefficient (R^2) value of 0.4 (Figs. 4.5A, B).

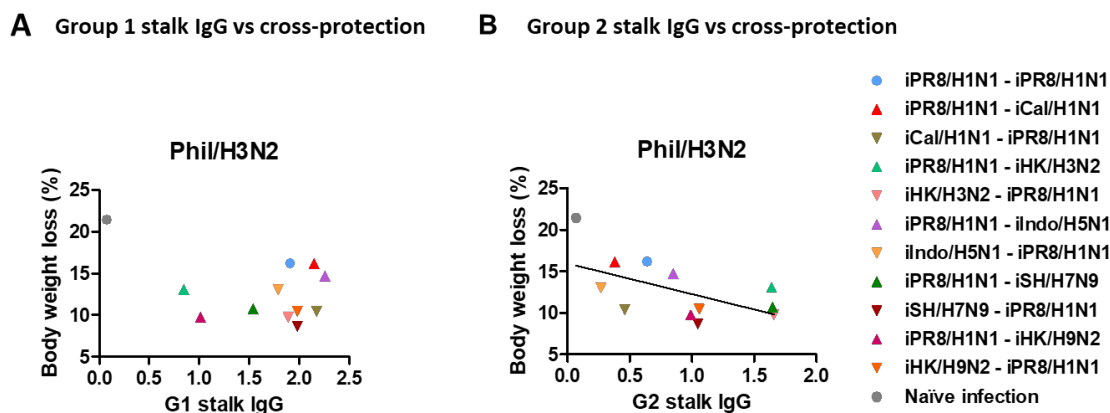


Figure 4.5 Data plots to implicate a possible correlation between HA stalk-specific IgG antibody levels and changes in weight loss after Phil/H3N2 virus challenge. (A) Group 1 stalk-specific and (B) Group 2 stalk-specific IgG antibodies were plotted against the body weight loss percentages in the different vaccine groups after challenge with Phil/H3N2 virus ($6.7 \times LD_{50}$). A line represents the correlation with a correlation coefficient of 0.4

To investigate the role of adjuvants in enhancing cross-protection, QS-21+MPL and CpG+MPL adjuvant combinations were included in prime as well as boost vaccination in the heterologous iPR8/H1N1 – iSH/H7N9 group. A regimen of prime-boost vaccination is presented (Fig. 4.6A). Both adjuvant combinations slightly increased group 1 and group 2 HA stalk IgG antibodies as well as HAI titers against PR8/H1N1 and SH/H7N9 viruses as compared to the groups without adjuvants (Fig. 4.6B-D). To investigate whether heterosubtypic H1N1 and H7N9 prime-boost would induce broader cross-protection against different subtypes of viruses, mice were challenged with group 1 HA - either HK/H9N2 or rgH5N1 virus at 6 weeks after boost immunization (Fig. 4.6A). The heterosubtypic iPR8/H1N1 - iSH/H7N9 prime-boost group displayed lower weight loss (~18%) and 100% survival rate against HK/H9N2 virus challenge as compared to the homologous iPR8/H1N1 - iPR8/H1N1 and naïve infection groups that displayed weight loss of over 25% and 0% survival rates (Figs. 4.6E). The inclusion of QS-21+MPL adjuvant combination in the prime and boost immunizations along with iPR8/H1N1 - iSH/H7N9 showed

slightly lesser body weight loss (17.8%) and 100% survival rate but there were no significant differences between the vaccination groups with and without adjuvants.

Similarly, iPR8/H1N1 - iSH/H7N9 prime-boost group displayed slightly lower weight loss (~20%) with ~67% survival rate against challenge with rgH5N1 virus ($5 \times \text{LD}_{50}$) as compared to the naïve infection group that displayed weight loss of over 25% with 0% survival rates (Figs. 4.6F). The inclusion of QS-21+MPL or CpG+MPL adjuvant combinations in the iPR8/H1N1 – iSH/H7N9 vaccination showed lesser body weight loss (18.63% and 16.01% respectively) and 100% survival rates, indicating that the addition of CpG+MPL adjuvant in the heterosubtypic prime-boost group slightly enhanced survival against rgH5N1 virus challenge but there were no significant differences in weight loss changes. Taken together, these results further support the effectiveness of heterologous prime-boost vaccination in conferring broader cross-protection against different subtypes of influenza viruses.

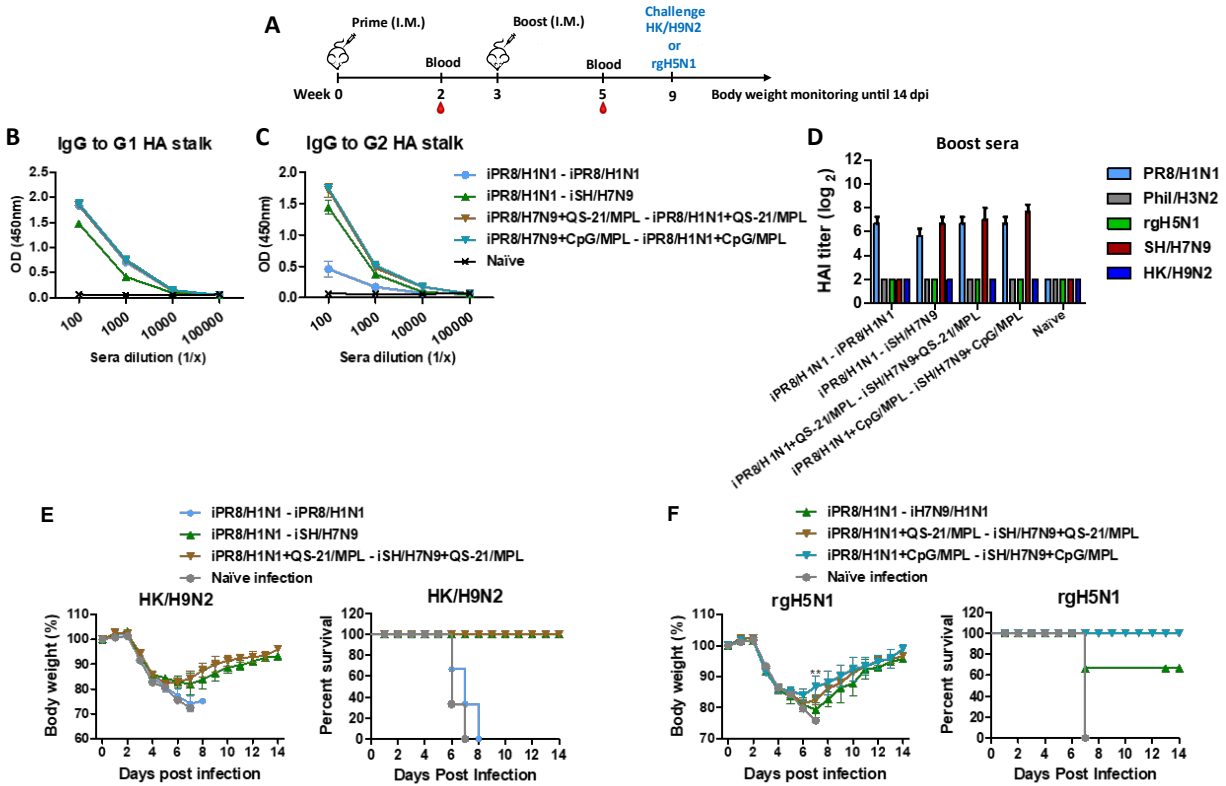


Figure 4.6 Heterosubtypic prime-boost vaccination induce cross-protection against different subtypes of viruses. (A) Immunization scheme (n = 3 per group). BALB/c mice were intramuscularly primed with 5 μ g of a specific strain of inactivated influenza virus and then boosted with 5 μ g of a homologous, or heterosubtypic inactivated virus (H1N1, or H7N9) with or without adjuvants QS-21 (10 μ g)/MPL (1 μ g) or CpG (4 μ g)/MPL (1 μ g)/mouse. (B) Group 1 and (C) Group 2 HA stalk-specific IgG antibody levels in boost sera. (D) HAI titers against different viruses as indicated in boost sera. (E and F) Body weight changes and survival rates were monitored for 14 days after HK/H9N2 virus ($6.7 \times LD_{50}$) challenge (E) and rgH5N1 virus ($5 \times LD_{50}$) challenge (F). Statistical significance was calculated by using two-way ANOVA and Bonferroni's post-multiple comparison test. Error bars indicate the mean \pm standard errors of the mean (SEM). **, $p < 0.01$ compared to the iPR8/H1N1 – iSH/H7N9 group

4.4.5 Heterosubtypic prime-boost vaccination controls lung viral loads and inflammatory cytokines

To further investigate the cross-protective efficacy, lung samples were collected at day 6 after Phil/H3N2 challenge to determine virus titers in embryonated chicken eggs (Fig. 4.7A). The

iPR8/H1N1 - iSH/H7N9 and iSH/H7N9 - iPR8/H1N1 groups showed significantly lower levels of virus titers by ~100 folds compared to those in the naïve infection group and several fold lower viral titers than homologous vaccination (Fig. 4.7B). The levels of inflammatory cytokines provide an additional barometer for assessing the protective efficacy of vaccination. The naïve infection control group showed the highest levels of TNF- α , IL-6, IFN- γ and IL-1 β cytokines in lung samples at 6 days after infection. In contrast, the iSH/H7N9 - iPR8/H1N1 group more effectively prevented the induction of inflammatory cytokines than the homologous groups (Figs. 4.7C-F). These data suggest that heterosubtypic prime-boost vaccination would be effective in controlling viral loads and preventing severe inflammation.

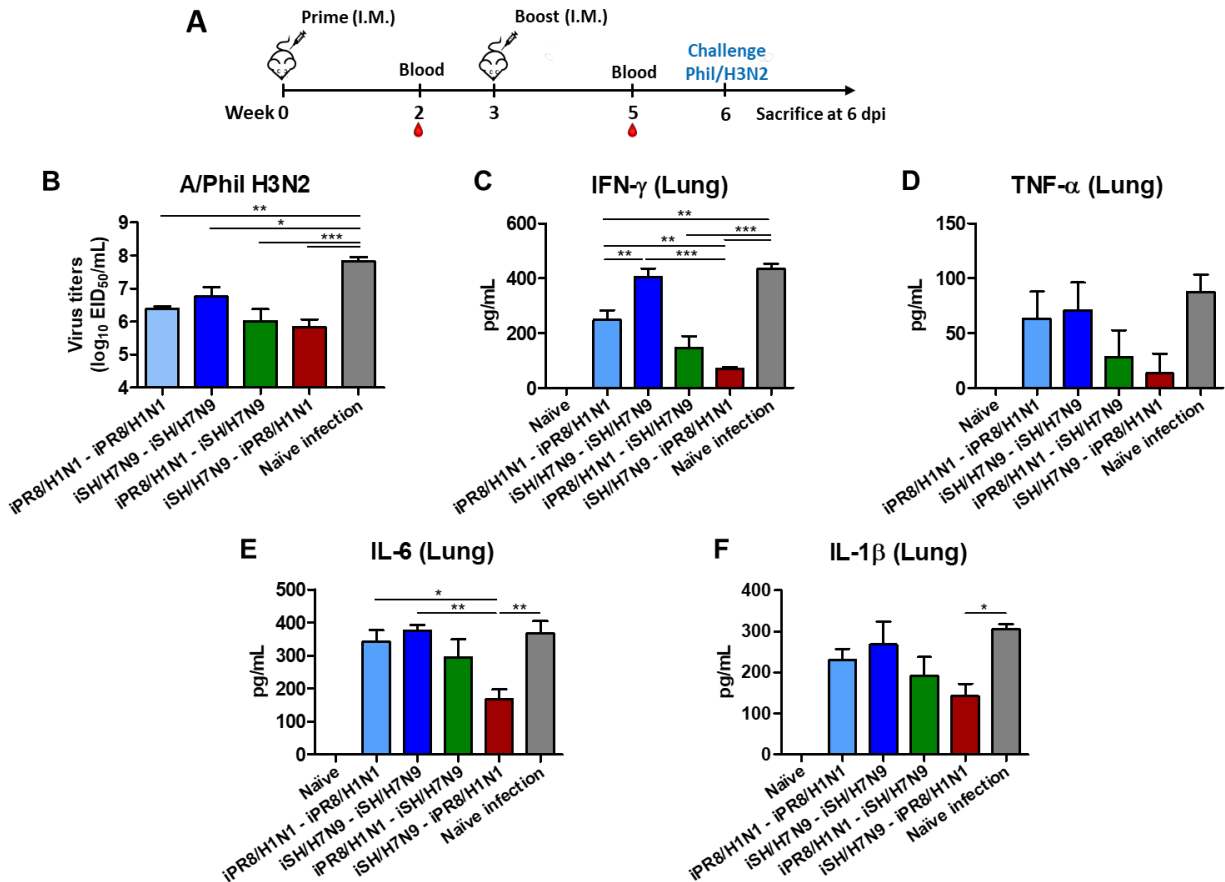


Figure 4.7 Heterosubtypic prime-boost vaccination controls lung viral loads and inflammatory cytokines more effectively than homologous vaccination. (A) Immunization scheme (n = 6 per group). Five to six weeks post boost immunization, the mice were challenged with a lethal dose of Phil/H3N2 virus ($6.7 \times \text{LD}_{50}$). (B) Lung viral titers as 50% egg infectious titers (EID₅₀) at day 5 post infection using embryonated chicken eggs. (C) IFN- γ , (D) TNF- α , (E) IL-6 and (F) IL-1 β cytokine levels in lung extracts. Statistical significance was calculated by using one-way ANOVA and Tukey's post-multiple comparison test. Error bars indicate the mean \pm standard errors of the mean (SEM). Lines under * symbols mark the comparing groups. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$

4.4.6 Heterosubtypic prime-boost vaccination enhances IFN- γ -producing T cell and HA stalk-specific antibody-secreting lung cellular responses after Phil/H3N2 virus challenge

To determine the early recall cellular immune responses, lung cells harvested at day 6 after Phil/H3N2 virus infection, were stimulated with iPR8/H1N1 or iSH/H7N9 virus. The numbers of IFN- γ producing cell spots in the iSH/H7N9 - iPR8/H1N1 or iPR8/H1N1 - iSH/H7N9 vaccination groups were higher than those in the homologous iPR8/H1N1 - iPR8/H1N1 and iSH/H7N9 - iSH/H7N9 vaccination groups, and much higher than naïve infected mice (Fig. 4.8A).

The heterosubtypic prime-boost vaccination groups induced significantly higher levels of group 1 and group 2 HA stalk domain-specific IgG antibodies in lung extracts than the naïve infected mice (Fig. 4.8B, C). As expected, the homologous prime-boost groups showed only group 1 or 2 stalk-specific HA stalk binding antibodies. Consistent with a pattern in lung extracts, group 1 stalk-specific IgG antibodies after 5-day culture of MLN cells were induced at a moderate level in the heterosubtypic prime-boost groups but not in the iSH/H7N9 - iSH/H7N9 group (Figs. 4.8D, E). IgG antibodies to consensus group 2 stalk were induced at moderate levels in the heterosubtypic prime-boost groups but not in the iPR8/H1N1 - iPR8/H1N1 group. These data suggest that B cells can be effectively primed for rapid recall to generate HA stalk domain-specific IgG responses in systemic MLN and mucosal lung sites upon challenge after heterosubtypic prime-boost vaccination.

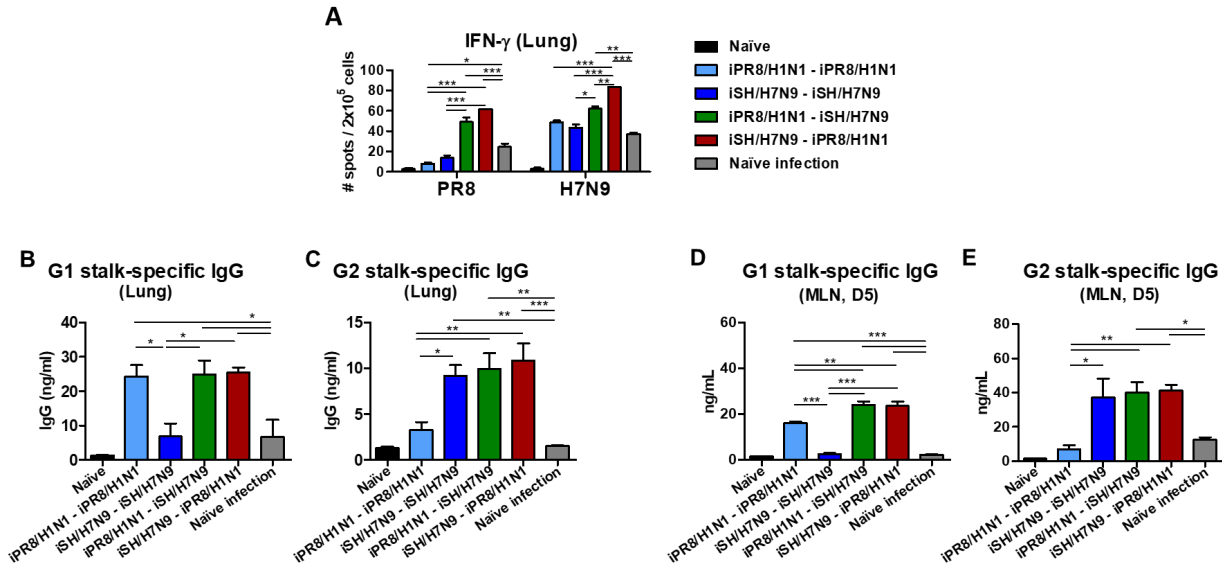


Figure 4.8 Cytokine producing and stalk-specific IgG secreting lung cellular responses after heterosubtypic prime-boost vaccination and Phil/H3N2 virus challenge. Immunization scheme ($n = 4$ per group) with the same vaccine groups as in Fig. 6. (A) Cytokine ELISpot of lung cells at day 6 post infection after *in vitro* stimulation with inactivated PR8/H1N1 or SH/H7N9 virus. (B) Group 1 and (C) Group 2 full-length HA stalk-specific IgG levels in lung samples collected at day 6 post challenge. (D) Group 1 and (E) Group 2 HA stalk-specific IgG production from MLN cells harvested on day 6 post infection, cultured *in vitro* for 5 days in the presence of Group 1 or 2 HA stalk protein. Statistical significance was calculated by using one-way ANOVA and Tukey's post-multiple comparison test. Error bars indicate the mean \pm standard errors of the mean (SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

4.4.7 Immune sera and T cells induced after heterosubtypic prime-boost vaccination contribute to protection against Phil/H3N2 virus

To determine the roles of humoral immune antisera in conferring heterosubtypic protection, naïve BALB/c mice were intranasally inoculated with a mixture of Phil/H3N2 virus at a lethal dose and antisera collected from vaccinated or naïve mice. Naïve sera did not provide protection against Phil/H3N2 virus as evidenced by severe weight loss ($> 25\%$) and 0% survival

rates in naïve mice (Figs. 4.9A, B). In contrast, immune sera from the heterosubtypic iSH/H7N9 – iPR8/H1N1 group conferred protection in naïve mice with moderate weight loss (~11%) and 100% survival rates, meanwhile, antisera from the iPR8/H1N1 - iPR8/H1N1 group did not provide protection to naïve mice that displayed severe weight loss (>25%). These data suggest that antisera from heterosubtypic iSH/H7N9 – iPR8/H1N1 vaccination can provide more effective protection against Phil/H3N2 virus than homologous iPR8/H1N1 vaccination.

To investigate whether T cell immunity would contribute to cross-protection, CD4 and CD8 T cells were depleted from the heterosubtypic prime-boost immunized mice prior to and after challenge with Phil/H3N2 virus. Severe weight loss (>25%) with 0% survival rate were observed in the either CD4 or CD8 T cell depleted groups whereas non-depleted vaccinated mice were 100% protected against weight loss after Phil/H3N2 virus challenge (Figs. 4.9C, D). These results suggest that protection against heterosubtypic Phil/H3N2 virus is dependent on both CD4 and CD8 T cells in the mice vaccinated with iSH/H7N9 – iPR8/H1N1.

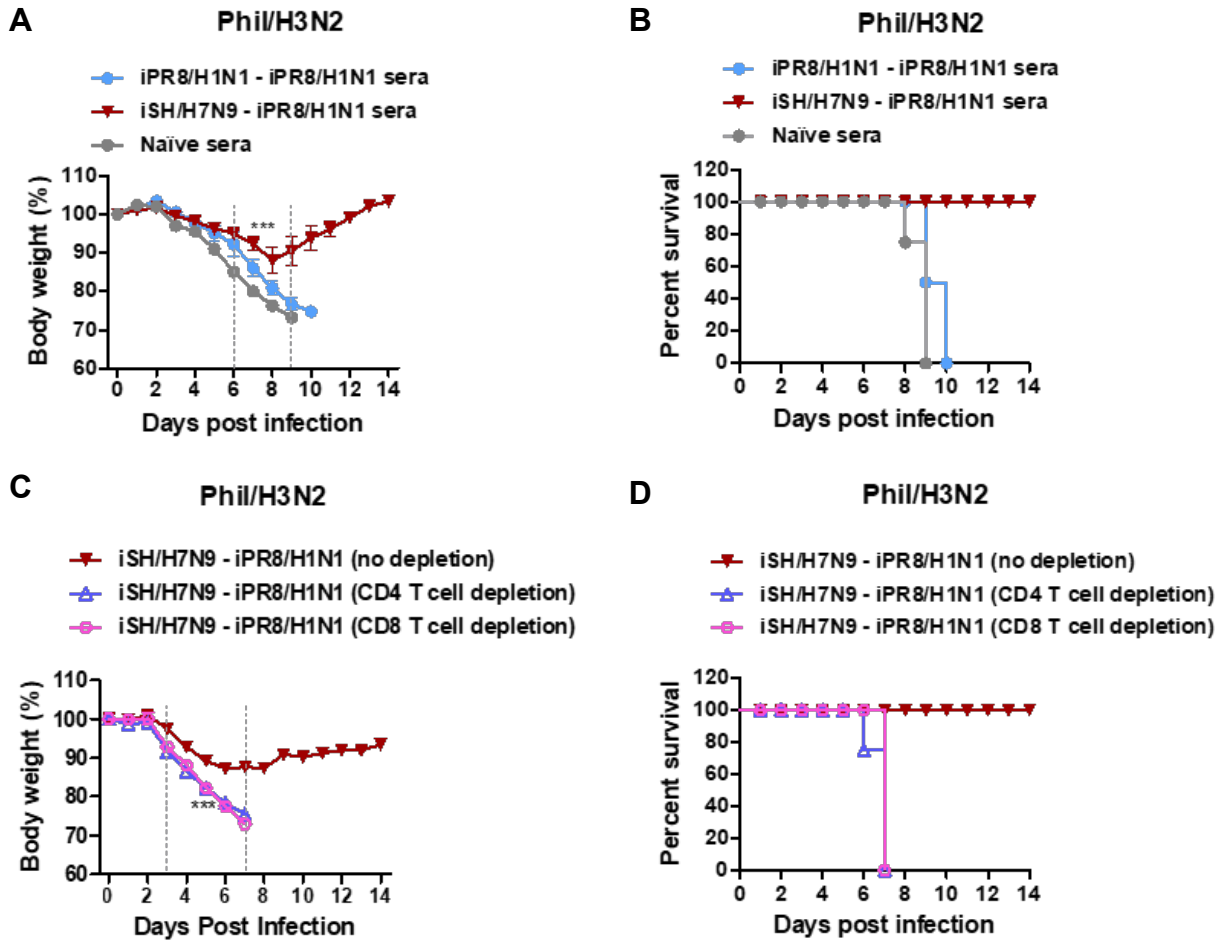


Figure 4.9 Immune sera and T cells induced after heterosubtypic prime boost vaccination contribute to protection against Phil/H3N2 virus. (A) Body weight changes and (B) survival rates in naïve mice ($n = 4$ per group) after inoculation with a mixture of a Phil/H3N2 virus ($2.5 \times LD_{50}$) and immune sera. (C) Body weight changes and (D) survival rates in the heterosubtypic prime-boost immunized BALB/c mice ($n = 4$) with CD4 and CD8 T cells depleted and Phil/H3N2 virus challenge. Statistical significance was calculated by using two-way ANOVA and Bonferroni's post-multiple comparison test. Error bars indicate the mean \pm standard errors of the mean (SEM). ***, $p < 0.001$ compared to the naïve sera group in (A) and no depletion group in (C)

4.5 Discussion

There is an important controversial issue in the heterogeneity and reduced vaccine effectiveness in the current annual repeat influenza vaccination strategies (Belongia et al., 2017;

Khurana et al., 2019; Leung et al., 2017; McLean et al., 2014; Morimoto & Takeishi, 2018; Smith et al., 1999; Song et al., 2020; Thompson et al., 2016). In this study, we investigated whether heterologous prime-boost vaccination would induce more effective cross-protection compared to homologous repeat vaccination. Prime vaccination induced vaccine strain-specific HAI titers. A strategy of heterologous prime – boost vaccine combinations induced several fold increased HAI activities against the prime virus strain as well as the heterologous boost virus and group-specific HA stalk IgG antibody responses at higher levels compared to those in prime sera. Heterologous prime-boost vaccination more effectively prevented severe weight loss after challenge with Phil/H3N2 virus than homologous repeat vaccination in the absence of serum HAI activity against the challenge virus. Boost vaccine strains different from the primed or pre-existing immune virus could be a more effective strategy of vaccination inducing broader protection.

It is critical to better understand how prior immunity affects the quality of antibody responses to vaccination with the same or different influenza strains, particularly in the aspect of improving the efficacy of current influenza vaccination and preparing for future pandemics. In contrast to vaccine strain-specific IgG antibody responses after the prime dose, boost vaccination induced broadly cross-reactive IgG antibodies binding to different virus strains, regardless of the strain used in boost vaccination. It was noted that A/Cal/H1N1 showed the least antigenicity against immune sera from different groups, which might be an advantage in avoiding immunity and becoming a pandemic strain. The pattern of HAI titers was highly strain-specific and predictive, correlating with the different antigenic strains used for prime and boost vaccinations, compared to the binding IgG antibodies. HAI titers against A/PR8/H1N1 were significantly increased after heterologous prime-boost vaccinations, when used either in prime or boost, comparable to prime vaccination with PR8/H1N1. In addition, the HAI titers against the different

strains used as either prime or boost strains (iCal/H1N1 - iPR8/H1N1, iHK/H3N2 - iPR8/H1N1, iSH/H7N9 - iPR8/H1N1) were similar or higher than those of prime vaccination with the same strain, regardless of the phylogenetic and antigenic distances (Fig. 4.10A-C). The results of heterologous prime-boost vaccination suggest weak evidence of displaying a phenomenon of original antigenic sin, which is to induce antibody responses toward previously exposed influenza antigens (Zhang et al., 2019). That is, prior priming with iPR8/H1N1 did not interfere with inducing HAI titers against a booster strain, while inducing higher HAI titers against PR8/H1N1. It was also reported that original antigenic sin was not observed in humans and ferrets with prior influenza virus infection after exposure to the 2009 H1N1 pandemic virus (O'Donnell et al., 2014). Consistent with our study, a clinical study demonstrated that higher levels of antibody responses were induced by vaccines containing new strains as compared to the vaccines containing the same strains that were used in the previous year (Nunzi et al., 2017). Current recommendations for seasonal vaccination might be improved, especially when the vaccine antigenic composition is the same as that of the previous winter season.

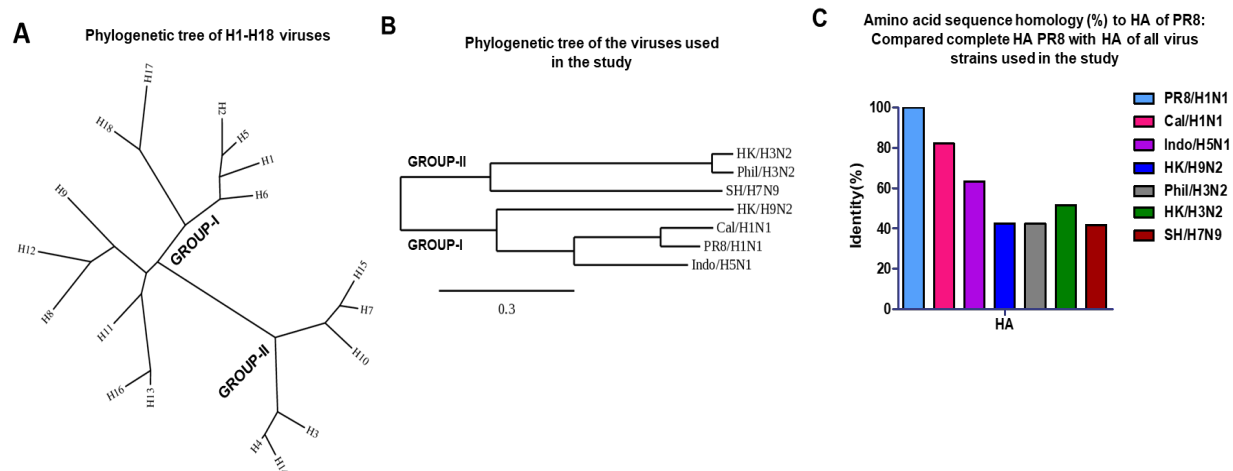


Figure 4.10 Phylogenetic tree and amino acid (aa) homology between the HA of A/PR8/H1N1 and HA of other influenza virus strains. (A) A phylogenetic tree was constructed based on the aa sequences of the HA genes in H1-H18 influenza viruses obtained from GenBank. (B) Phylogenetic tree based on the aa sequences of the HA genes in the influenza viruses used in the study obtained from GenBank. Influenza A (H1-H18) HA sequences were obtained from GenBank: NC_002017 H1 HA (YP_163736) for A/Puerto Rico/8/1934 (H1N1), NC_026433A H1 HA (YP_009118626) for A/California/07/2009 (H1N1), L11125 for H2 HA of A/Berkeley/1968 (AAA43089), CY080523 H3 HA (ADV76673) for A/Hong Kong/1-10-MA21-1/1968 (H3N2), IVU08858 H3 HA (AAA18781) for A/Philippines/2/82 (H3N2), MT421019 for H4 HA (QJ155045), CY116646 for H5 HA of A/Indonesia/5/2005 (H5N1) (AFM78567), CY166897 for H6 HA (AHL82551), KC853228 for H7 HA of A/Shanghai/2013 (H7N9) (AGI60292), CY097534 for H8 HA (AEM75966), KF188366 for H9 HA of A/chicken/Hong Kong/G9/1997 (H9N2) (AGO17823), MF613851 for H10 HA (ASV60666), CY191275 for H11 HA (AKF35393), CY133357 for H12 HA (AGE03167), CY054300 for H13 HA (ADB46159), MK327694 for H14 HA (AZQ09016), KP087869 for H15 HA (AIY68624), CY177441 for H16 HA (AHM98288), CY103892 for H17 HA (AFC35438), CY125945 for H18 HA (AGX84934). The complete HA and HA2 sequence were used to construct the phylogenetic trees by using Clone Manager program and online tools (<http://www.phylogeny.fr>). (C) The aa sequence homology between the HA of A/PR8/H1N1 and HA of other influenza virus strains. The HA sequences of the viruses used in this study were obtained from GenBank to analyze the amino acid (aa) sequence identity. The percentages of the aa homology of the HA sequence of A/PR8/H1N1 virus with the HA sequences of other virus strains were calculated using Needle (EMBOSS; EMBL-EBI)

Pre-existing immune history shapes the profile of anti-influenza virus immune responses (Zhang et al., 2019) but its impact on the protective efficacy of influenza vaccination is not well understood. Heterologous prime-boost vaccinations, regardless of vaccine strains used, were found to provide similar or more effective cross-protection than the homologous prime-boost groups. For example, higher efficacies of cross-protection against Phil/H3N2 virus were observed in the heterologous prime-boost groups of iCal/H1N1 - iPR8/H1N1, iHK/H3N2 - iPR8/H1N1, iSH/H7N9 - iPR8/H1N1, and iPR8/H1N1 - iHK/H9N2. None of these heterologous groups showed cross-reactive HAI activity against Phil/H3N2 virus, suggesting non-neutralizing immune-mediated protection. HA stalk-specific IgG antibodies were reported to be independently correlated with protection against different subtype viruses (Ng et al., 2019). Group 2 HA stalk-specific IgG levels were substantially induced in these heterologous vaccinations except for iCal/H1N1 - iPR8/H1N1, which induced high group 1 HA-specific stalk antibodies. It is notable that heterologous iHK/H9N2 - iPR8/H1N1 vaccination induced both group 1 and 2 stalk-specific IgG antibodies even though both H1 and H9 are phylogenetically categorized into group 1 HA. Preparing stabilized recombinant stalk protein vaccines has been challenging, and the efficacy of group 2 stalk vaccines has been low, particularly with heterologous viruses (Corbett et al., 2019; Impagliazzo et al., 2015; Sutton et al., 2017). An alternative strategy to develop a universal influenza vaccine inducing HA stalk-specific antibodies was extensively demonstrated by using reassortant viruses with chimeric HA of different subtype head but the same strain HA stalk domain (Hai et al., 2012; Krammer et al., 2014; Nachbagauer et al., 2017). There are some correlations between stalk antibodies and cross-protection (Fig. 4.5B) and antisera of iSH/H7N9 - iPR8/H1N1 vaccination conferred protection against Phil/H3N2 virus in naïve mice. However, the levels of stalk-specific IgG antibodies alone would not be fully supportive for cross-protection as

observed in the iCal/H1N1 – iPR8/H1N1 group inducing cross protection against Phil/H3N2, preventing severe weight loss despite a low level of group 2 stalk antibodies. Depletion of CD4 or CD8 T cells in mice vaccinated with iSH/H7N9 - iPR8/H1N1 resulted in abrogation of cross-protection, suggesting an important role of T cell immunity.

Clinical studies on repeat annual influenza vaccination indicate some concerns such as diminished B-cell responses, reduction in antibody affinity maturation, and lower vaccine efficacy against mismatch strains (Khurana et al., 2019; Leung et al., 2017; Morimoto & Takeishi, 2018; Nunzi et al., 2017; Sanyal et al., 2019). In summary, heterologous vaccinations could induce enhanced and broader antibody responses such as HAI titers against different prime and boost strains as well as group 1 and 2 stalk IgG responses. Improved cross-protection was observed with various heterologous prime-boost vaccinations compared to the homologous prime-boost. Both humoral and cellular immunity were independently important and contributed to cross-protection by heterologous prime-boost vaccinations. Vaccination with antigenically different vaccine strains rather than repeat vaccination with the same antigen might provide a strategy for improving the efficacy and breadth of influenza vaccination. There are limitations in this study, not fully reflecting complex history of pre-existing immunity in humans. Comparing multi-valent vaccine components versus sequential heterologous influenza vaccination will be important for future studies.

4.6 Conclusion

This study has significance in evaluating whether a strategy of heterologous prime-boost influenza vaccination would confer better cross-protective effects than homologous vaccination. It was found that heterologous prime-boost vaccination more effectively prevented severe weight

loss after challenge with A/Phil/H3N2 virus than homologous repeat vaccination. Boost vaccine strains different from the primed or pre-existing immune virus could be a more effective strategy of vaccination inducing broader protection. These results support a strategy to enhance the cross-protective efficacy by heterologous prime-boost influenza vaccination.

4.7 Acknowledgements

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5 DISCUSSION AND FUTURE DIRECTIONS

5.1 Diseases prevented by adjuvanted vaccinations

Many different kinds of vaccines are used for immunizations to prevent infectious diseases. Adjuvants have been used to increase antigen-specific immune responses and effectively prevent infectious diseases (Rappuoli et al., 2011). Adjuvants can play a key role in developing successful vaccines by enhancing immunogenicity, antigen dose sparing, fewer immunizations, long-lasting B and T cell immunity. More effective and safer adjuvants need to be developed for improving the vaccine efficacy for the immune-compromised individuals and the elderly (Rappuoli et al., 2011; Siegrist & Aspinall, 2009; Weinberger et al., 2008). Aluminum salts (alum) are still the most common type of adjuvants used in human subunit vaccines such as diphtheria, tetanus, and pertussis (DTP) toxoid, human papillomavirus (HPV), Haemophilus influenza type B, and pneumococcal conjugate vaccines. Few other vaccine adjuvants have been licensed and used for human vaccines. QS-21 and MPL, the active components of a liposome-based AS01 adjuvant, are

included in the highly effective Shingrix vaccine product for the elderly population and immunocompromised patients (Cunningham et al., 2016; Lal et al., 2015; Vink et al., 2020), progressively replacing an old live attenuated vaccine, Zostavax. Our previous studies reported potent adjuvant effects of BCG-CWS (Kim et al., 2021) and CpG+MPL combination adjuvant (Ko et al., 2018) on influenza vaccination. The efficacy comparison of these adjuvants and the action mechanisms of adjuvants have not been fully understood. It is highly significant to compare the effects of different adjuvants and understand their action mechanisms to improve the efficacy of vaccination. In project 1, I compared the effects of different types of adjuvants (QS-21 + MPL, CpG + MPL, and BCG-CWS) on inducing immunogenicity and cross-protection in young and aged mouse models. It was found that individual and combination adjuvants at low doses were effective in enhancing the protective efficacy of inactivated split influenza vaccine. In chapter 2, I investigated whether a newly synthesized semisynthetic compound, VSA-1, an analog of licensed saponin adjuvant QS-21, exhibits adjuvant effects by enhancing the protective efficacy of inactivated split influenza vaccine. It was found that VSA-1 induced higher vaccine antigen-specific IgG responses and better homologous protection than QS-21 after a single dose of immunization. Prime-boost immunization with VSA-1 adjuvanted vaccine effectively induced heterosubtypic protection which was comparable to QS-21.

5.2 Action mechanisms of vaccine adjuvants

Adjuvants are innate immune stimulators and form pro-inflammatory microenvironments at the site of injection. Conventionally, adjuvants are known to increase the production of inflammatory cytokines and chemokines and recruit various innate immune cells such as monocytes and neutrophils at the injection site (Calabro et al., 2011). The activation of T cells

depends on antigen presenting cells (APCs), such as dendritic cells (DCs) of the innate immune system. It has been well established that $CD4^{+}$ T cells provide critical help for inducing the production of long-lived protective antibody by B cells (MacLennan et al., 1997) and for generating effective $CD8^{+}$ memory T cells (Khanolkar et al., 2007). Thus, it is believed that adjuvant effects on enhancing antibody responses to T cell-dependent vaccine antigens are mediated by $CD4^{+}$ T helper cells through adjuvant-activated innate immune components as demonstrated in many studies (Galli et al., 2009; Gaspal et al., 2005; Kamath et al., 2009; McAleer & Vella, 2010; McKee et al., 2007; Serre et al., 2011; Sokolovska et al., 2007). A conventional concept is that adjuvants activate innate immune components, which subsequently determine a specific type of T helper cells in orchestrating the quantity and quality of protective antibodies (Coffman et al., 2010; McAleer & Vella, 2010; O'Hagan et al., 2012).

The objective of project 1 was to compare the effects of three potent adjuvants or combination adjuvants (CWS, QS-21+MPL, CpG+MPL) on enhancing the immunogenicity and cross-protection of influenza vaccination in young adult C57BL/6 and aged BALB/c mouse models. Consistent with the results of previous studies (Coccia et al., 2017; Dendouga et al., 2012), a synergy of QS-21 and MPL actions was shown to recruit neutrophils and monocytes and induce $IFN-\gamma$ by stimulating macrophages and inflammatory cytokines. These three potent adjuvants appear to share a common TLR4 signaling pathway and stimulate innate immune responses leading to enhanced immunogenicity and protective efficacy of inactivated split influenza vaccination. Although all adjuvanted single-dose vaccinations induced enhanced protection against homologous virus in C57BL/6 mice as evidenced by lung viral clearance and lower weight loss compared to the naïve infected mice, the effects of CWS, QS-21+MPL, and CpG+MPL adjuvants were differential in inducing Th1 type IgG2c (or IgG2a) isotype antibodies specific for

the virus. QS-21+MPL adjuvant in influenza vaccination was found to be more effective in promoting T helper type 1 (Th1) IgG2c (in C57BL/6 mice), IgG2a antibodies (in BALB/c mice), and protection in both mouse strains, whereas CpG+MPL was a potent adjuvant in enhancing the protection by influenza vaccination in both adult C57BL/6 and aged BALB/c mouse models. Th1 IFN- γ cytokines promote B cells to produce IgG2c isotype antibodies in C57BL/6 mice (IgG2a in BALB/c mice), whereas Th2 cytokine responses promote IgG1 isotype antibodies (Stevens et al., 1988). Murine IgG2a isotype is known to be effective in inducing antibody-dependent cell-mediated cytotoxicity and protection via Fc receptor-mediated mechanisms (Kipps et al., 1985; Oishi et al., 1992; Wiedinger et al., 2020), suggesting an immunological advantage in promoting Th1 type IgG antibody responses by QS-21+MPL adjuvanted vaccination. To better understand the QS-21+MPL combination adjuvant effects, acute innate immune responses after intraperitoneal injection of QS-21+MPL were analyzed. QS-21+MPL acutely induced inflammatory cytokine IL-6 and chemokine MCP-1 at higher levels in sera within 2 h than CpG+MPL, and then returned to near-basal levels, whereas both combination adjuvants acutely recruited monocytes, neutrophils, and dendritic cells at the site of injection.

In project 2, I investigated whether a newly synthesized semisynthetic compound, VSA-1, exhibits adjuvant properties by enhancing the efficacy of inactivated split influenza vaccination in C57BL/6 mice. The adjuvant effects of VSA-1 on improving vaccine efficacy after immunization were evident when compared to licensed adjuvants, QS-21, and alum. VSA-1 adjuvant effects were more potent in inducing IgG1 and IgG2b isotypes as well as HAI functional antibodies, IFN- γ^+ T cell responses, and homologous protection with single-dose split vaccination compared to those by QS-21 and Alum adjuvanted split vaccinations. Particularly, VSA-1 adjuvanted split prime-boost vaccination, but not split alone vaccination, significantly induced both CD4 and CD8

T cell responses that play a critical role in conferring effective cross-protection. Alum adjuvant is biased in skewing the immune responses to Th2 type IgG1 antibodies. Consistent with this, in our study, alum potentiated Th2 type immune responses inducing IgG1 antibodies and exhibited weak adjuvant effects on inducing HA functional antibodies, correlating with high lung viral titers and low efficacy of protection after challenge. In contrast, VSA-1 adjuvant was effective in promoting the induction of IgG1, IgG2b, and Ig2c isotype antibodies and HAI titers at highest levels compared to QS-21 and alum, suggesting that it can be developed as a more potent and suitable vaccine adjuvant than these licensed adjuvants. Previous studies on QS-21 would provide some insights into the mechanisms of saponin carbohydrate adjuvants. One hypothesis is that QS-21 might facilitate vaccine antigen uptake by antigen presenting cells by interacting with lectin receptors through carbohydrate domains, stimulating certain cytokines that activate T cell and B cell responses (Marciani, 2018). An alternative mechanism is cholesterol-dependent endocytosis of vaccine antigens and QS-21 into dendritic cells (Lorent et al., 2014). The high affinity of QS-21 to membrane cholesterol may lead to pore formation by destabilizing the membrane structure and facilitate the delivery of vaccine antigens into the cytosol of antigen presenting cells for further processing into peptides for T cell activation. QS-21 is proposed to stimulate T cells via the mitogen-activated protein kinase through CD2 molecules, resulting in the production of Th1 cytokines (Marciani, 2018). Studies on QS-21 in mouse antigen presenting cells reported that QS-21 in combination with TLR4 agonist MPL A activated NOD-like receptor P3 (NLRP3) inflammasome, a multi-protein complex, inducing subsequent release of proinflammatory cytokines and potentially contributing to INF- γ -mediated Th1 responses (Marty-Roix et al., 2016). Alum (at a dose of 100 μ g) and QS-21 (at a dose of 5 μ g) have been known to induce necrotic cell death where QS-21 induced macrophage and dendritic cell death in a caspase-1-, ASC-, and

NLRP3-independent manner (Ko et al., 2016; Marty-Roix et al., 2016). To abrogate potential cell lytic activity and toxicity of QS-21, a delivery platform of cholesterol-based liposomes was utilized in the AS01 formulation (Garçon & Di Pasquale, 2017). Since cholesterol-quenched QS-21 retained adjuvant potency as free QS-21, the linking of cell lytic activity and an immune-stimulant effect of QS-21 is questionable. There are several drawbacks inherent to QS-21 as a natural product, including chemical instability and heterogeneity, scarcity, and dose-limiting toxicity. Therefore, VSA-1 which is a semisynthetic compound would be a more homogeneous and potent vaccine adjuvant as supported in this study, would be non-toxic even at high doses, and safer than QS-21 (Wang, 2021; Pengfei Wang et al., 2019).

5.3 Comparison of the effects of adjuvants on influenza vaccination in young population and the elderly

Influenza viruses affect people aged 65 years or above at an overwhelmingly higher rate, which is evident as they occupy 70-85% of the total seasonal flu-related deaths (based on CDC estimation in the United States) (CDC, 2021b). Influenza vaccine effectiveness (VE) in the elderly (17-53%) has been dropping disproportionately compared to young adults (70-90%) (Goodwin et al., 2006). The conventional influenza vaccine is less immunogenic in the elderly (only 30-40%), and this results in the failure of induction of hemagglutination inhibition (HAI) antibodies in at least 20% of the elderly recipients (Keren et al., 1988) which are known to be correlated with protection (Dunning et al., 2016). An altered immune response is the main cause of the low overall effectiveness. Age-associated deleterious changes in the immune system, including immunosenescence and inflammaging are responsible for low vaccine effectiveness (Tanner et al., 2021). Adjuvanted vaccines have been developed to overcome reduced vaccine effectiveness in

the elderly and elicit more robust immunogenicity. Adjuvanted inactivated influenza vaccine, FLUAD Quadrivalent was approved for people who are 65 years and older in U.S. in November 2015, which was 18 years after its first 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). It is highly significant to enhance the effectiveness of seasonal vaccination by formulating vaccines containing safe adjuvants.

Combination adjuvant, QS-21+MPL, was found to be the most effective in enhancing the immunogenicity of the split vaccine, whereas CpG+MPL was highly effective in improving the vaccine efficacy of cross-protection in adult mice and homologous protection in aged mice despite no statistically significant differences between these two potent combination adjuvants. This study has significance in providing a comparative assessment of the effects of three different potent adjuvants or combination adjuvants and provides a future direction in formulating vaccines that are designed for adult population as well as the elderly to confer better protection.

5.4 Development of VSA-1 as a new type of adjuvant in influenza vaccines

Development of a new type of adjuvant is an approach to provide more effective and safer vaccination and protection against infectious pathogens. Alum (aluminum salts) remained the sole adjuvant used in licensed human vaccines for over 70 years until the 1990s. It adsorbs the vaccine antigens and makes antigen depot at the site of injection. The antigen depots can release vaccine antigen slowly, so the immune system is stimulated by the antigen for a longer time. Also, alum can stimulate innate immune cells like neutrophils and macrophages through NLRP3 inflammasome signaling pathway. The inflammasome-activated immune cells secrete pro-

inflammatory cytokines such as IL-1 β and IL-18 (Eisenbarth et al., 2008; Kool et al., 2008; Sharp et al., 2009). Alum has relatively weak adjuvant effects compared to other adjuvants. It is efficient in eliciting a high antibody response with a Th2 profile instead of eliciting a protective Th1 response. A Th1 response is necessary for vaccines against cancers and intracellular pathogens such as HIV, TB, and malaria (Dorfmeier et al., 2012; Plotkin, 2010; Sha et al., 2005).

Since the 1990s, few other adjuvants have been approved for use in defined human vaccines, including oil-in-water emulsions (MF59 and AS03) used in influenza vaccines and a combination adjuvant (i.e., AS04, composed of monophosphoryl lipid A (MPL) adsorbed to alum) used in HBV and HPV vaccines in Europe and the United States. Despite the progress, developing subunit vaccines is still bottle-necked by the lack of safe and effective adjuvants. Because of their urgent need, the discovery and development of novel adjuvants has emerged as a critical frontline effort in vaccine research. Having promising lead compounds is the critical first step toward successful development of synthetic vaccine adjuvants. Among various vaccine adjuvants studied, QS-21, a saponin adjuvant obtained from the bark of *Quillaja Saponaria* (QS) Molina, stimulates mixed Th1 and Th2 responses. It significantly outperformed other classes of adjuvants (including glucan formulations, peptidoglycans, amphiphilic block copolymers, bacterial nucleosides, and bacterial lipopolysaccharide) (Kensil et al., 2004; Ragupathi et al., 2011) and has been evaluated in over 100 clinical trials of vaccines against cancer and infectious diseases (Ragupathi et al., 2011). Although it is the immunostimulant of choice in many clinical trials of vaccines, QS-21 has its own drawbacks. For example, the content of QS-21 in QS tree bark extracts is low (Rappuoli et al., 2011). Overexploitation of QS bark has resulted in ecological damage and shortage of available supplies even under the current demand, leading to stricter environmental regulations and increased price (Ragupathi et al., 2011). It has been estimated that the current global supply

of naturally available QS-21 is only enough for about 6 million doses (100 µg/dose for human use), which is not sufficient for widespread clinical use (Plotkin, 2010). The limited supply of QS-21, along with the chemical instability, dose-limiting toxicity, and laborious and low-yielding purification, hinder its wider use (Plotkin, 2010; Ragupathi et al., 2011).

VSA-1, as a potential new adjuvant, is an analog of a licensed adjuvant, QS-21 developed on the basis of extensive structure-activity relationship studies of QS-21 (Wang et al., 2016). The advantages of using VSA-1 as an adjuvant are that it induces a potential mixed Th1/Th2 immune response, has similar adjuvant activity as that of QS-21, has higher structural stability than QS-21, is non-toxic and its purification procedure is cost-effective. Project 2 was a new study to investigate the efficacy and action mechanisms of VSA-1 in comparison with other licensed vaccine adjuvants, QS-21 and alum. VSA-1 as a new semisynthetic saponin-based adjuvant played a significant role in enhancing IgG isotype and HAI functional antibodies after influenza split prime vaccination, conferring superior homologous protection over split vaccine alone, and Alum or QS-21 adjuvanted split vaccination in C57BL/6 mice. Both VSA-1 and QS-21 adjuvanted split boost vaccinations were highly effective in inducing humoral and cellular immune responses as well as cross-protection against rgH5N1 virus even in the absence of cross-reactive HAI antibodies.

This study provides supportive evidence warranting further development of VSA-1 as an alternative promising candidate for QS-21 replacement. It remains to be determined whether combination with MPL and VSA-1 will exhibit synergistic adjuvant effects as AS01 in future studies. Also, since AS01 containing MPL and QS-21 from *Quillaja Saponaria* is included in Shingles vaccination for older individuals (≥ 50 years old) (James et al., 2018; Lal et al., 2015), as a future direction for developing effective vaccines for the elderly, it might be interesting to study

the adjuvant effects of VSA-1 in the elderly to develop vaccine formulations including VSA-1 adjuvant for these target populations.

5.5 Heterologous prime-boost vaccination strategy to induce effective cross-protection

Influenza vaccine efficacy is low and highly variable depending on the seasonal variant outbreaks and subtypes. There are controversial questions about repeat vaccination leading to reduced IgG antibody affinity maturation and B cell responses, thereby reducing influenza vaccine efficacy against mismatch influenza virus strains in humans (Belongia et al., 2017; Khurana et al., 2019; Leung et al., 2017; McLean et al., 2014; Morimoto & Takeishi, 2018; Smith et al., 1999; Song et al., 2020; Thompson et al., 2016). Pre-existing immunity from infection early in life and prior vaccinations might shape the immune responses toward earlier influenza antigens, a concept known as ‘original antigenic sin’ (OAS) (Zhang et al., 2019). Current recommendations for seasonal vaccination need to be improved, especially when the vaccine antigenic composition is the same as that of the previous winter season. Project 3 is innovative in developing a strategy of heterologous prime-boost vaccination which would be more effective in inducing cross-protection against influenza viruses and less likely to induce OAS responses in comparison with repeat vaccination. I investigated whether heterologous prime-boost vaccination would induce more effective cross-protection compared to repeat vaccination. Prime vaccination induced vaccine strain-specific HAI titers. Boosting with a heterologous strain induced HAI activities against the prime virus strain as well as the heterologous boost virus and group-specific HA stalk IgG antibody responses at higher levels in most combinations compared to those in prime sera. Heterologous prime-boost vaccination more effectively prevented severe weight loss after challenge with A/Phil/H3N2 virus than homologous repeat vaccination in the absence of serum HAI activity

against the challenge virus. Overall, these results suggest that boost vaccine strains different from the primed or pre-existing immune virus could be a more effective strategy of vaccination inducing broader protection. Consistent with our study, a clinical study demonstrated that higher levels of antibody responses were induced by vaccines containing new strains as compared to the vaccines containing strains that were used in the previous year (Nunzi et al., 2017). A previous study demonstrated that OAS could be prevented by administering certain vaccine adjuvants during the first or the second exposure (Kim et al., 2012). In our study, the inclusion of QS-21+MPL or CpG+MPL adjuvant combinations in the prime and boost immunizations along with inactivated viruses showed lesser body weight loss against heterosubtypic viruses, indicating that their addition in the heterosubtypic prime-boost group slightly enhanced cross-protection but there were no significant differences in weight loss changes.

The scope of heterologous prime-boost vaccination might be extended beyond influenza vaccines. A study explored the utility of employing a heterologous prime-boost strategy in which different combinations of the four types of leading COVID-19 vaccine candidates that are undergoing clinical trials in China were tested in a mouse model (He et al., 2021). The results showed that sequential immunization with adenovirus vectored vaccine followed by inactivated/recombinant subunit/mRNA vaccine administration specifically increased levels of neutralizing antibodies and promoted the modulation of antibody responses to predominantly neutralizing antibodies. Moreover, a heterologous prime-boost regimen with an adenovirus vector vaccine also improved Th1-biased T cell responses. As a future direction, it would be interesting to test the heterologous prime-boost strategy for the development and application of effective COVID-19 vaccines to control the SARS-CoV-2 pandemic. Vaccination with antigenically

different vaccine strains rather than the repeat vaccination with the same antigen could provide a strategy for improving the overall efficacy and breadth of vaccination.

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7 APPENDICES

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Comparison of the effects of different potent adjuvants on enhancing the immunogenicity and cross-protection by influenza virus vaccination in young and aged mice
Author: Noopur Bhatnagar, Ki-Hye Kim, Jeeva Subbiah, Bo Ryoung Park, Eun-Ju Ko, Baik-Lin Seong, Sang-Moo Kang
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Instructor name	Dr. Sang-Moo Kang		

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8 VITAE

NOOPUR BHATNAGAR

Georgia State University
Department of Biology, Institute for Biomedical Sciences
Email: nbhatnagar1@student.gsu.edu

EDUCATION

Ph.D. in Biology August 2014 - Present
Georgia State University, College of Arts and Science (Expected graduation date: May 3, 2022)

Master of Science in Biology January 2013 - December 2014
Georgia State University, College of Arts and Science

Master of Science in Bioanalytical Sciences July 2010 - June 2012
The University of Mumbai, Guru Nanak Khalsa College of Arts, Science & Commerce

Bachelor of Science in Biotechnology July 2007 - May 2010
The University of Mumbai, Vivekanand Education Society's College of Arts, Science & Commerce

RESEARCH EXPERIENCE

Institute for Biomedical Sciences, and Department of Biology, Georgia State University, Atlanta, USA

Graduate Research Assistant October 2013 - Present
Current Projects:

- Studying the effect of novel and licensed adjuvants on protection against pandemic influenza virus infections
- Studying a strategy of heterologous prime-boost vaccination that can effectively induce cross-reactive HAI titers and cross-protection better than repeat annual vaccination

Worked on mouse models induced with colitis-associated cancer and studied the tumor-suppressive role of epithelial derived-matrix metalloproteinase (MMP9)

AYUSH [Ayurveda, Yoga, Unani, Siddha, and Homoeopathy (Traditional Systems of Medicine)], Mumbai, India

Project Trainee November 2011
Ministry of Health and Family Welfare, Government of India, Ramnarain Ruia College of Arts and Science, Mumbai

Prepared Ayurvedic formulations for the treatment of eye, ear, and brain disorders, developed an anti-emetic preparation, and tested them for compliance to AYUSH standards by checking the saponification value, acid value and by performing High-Performance Thin Layer Chromatography (HPTLC)

Bhabha Atomic Research Center, Mumbai, India**Research Intern - Bio-Organic Division**

May 2011- August 2011

Thesis title: "Evaluation of the healing potential of reduced Dihydroxyselenol (DHS_{red}) against Indomethacin-induced gastropathy: An insight into the mechanism of action"

- Studied the healing activities of novel compounds against Indomethacin-induced stomach ulceration in a mouse model
- Studied the mechanisms involved in mucosal defense during Indomethacin-induced inflammation and modulation of the mucosal defense pathways by a novel compound

Shreedhar Bhat Laboratories, Bangalore, India**Trainee**

May 2009 - September 2009

Comprehensive training in advanced microbiological and molecular biology techniques

Projects:

- Recombinant DNA Technology
- Genetic Engineering
- Plant Tissue Culture
- Immunological Studies

TEACHING EXPERIENCE**Teaching Assistant, Department of Biology, Georgia State University, Atlanta**

June 2013 – December 2018

Responsibilities included teaching students, conducting, and supervising lab experiments, grading exams, and maintaining student records

Teaching Assistant, The University of Mumbai, India

October 2010 – December 2011

Conducted laboratory experiments for first-year master's students in Bioanalytical Sciences course

- Trained students on physical testing of tablet dosage forms
- Conducted lab courses on gel electrophoresis

AWARDS AND HONORS

- Awarded first prize in the 'Three Minute Thesis Competition' in the Doctoral category, Georgia State University, Atlanta 2022
- Student Travel Award: 41st Annual Meeting of the American Society for Virology 2022
- Student Registration Award: 40th Annual Meeting of the American Society for Virology 2021
- Student Travel Award: 39th Annual Meeting of the American Society for Virology 2020
- Biology Graduate Student Travel Award: Annual Meeting of the American Gastroenterological Association 2014
- Volunteer for the Georgia Science Olympiad held at Georgia State University, Atlanta, USA 2014 & 2017

- Organizer for the Technical Festival PARADIGM, G.N. Khalsa College, Mumbai, India
2010 & 2011
- Awarded first prize in ‘Science Poster Competition’ organized by the Department of Chemistry, V.E.S. College, Mumbai, India
2008-2009
- Organizer for the Technical Festival BIOEFFERVESCENCE, V.E.S. College, Mumbai, India
2008

ORAL AND POSTER CONFERENCE PRESENTATIONS/ABSTRACT PUBLICATIONS

- **Noopur Bhatnagar**, Ki-Hye Kim, Jeeva Subbiah, Bo Ryoung Park, Pengfei Wang, Sang-Moo Kang. Adjuvant effects of saponin analog VSA-1 on influenza vaccination inducing homologous and heterosubtypic protection. ASV 2021
- **Noopur Bhatnagar**, Ki-Hye Kim, Jeeva Subbiah, Sang-Moo Kang. Heterologous prime-boost influenza vaccination provides a strategy to induce more effective cross-protection than repeat vaccination. Georgia Bio 2020
- **Noopur Bhatnagar**, Ki-Hye Kim, Jeeva Subbiah, Sang-Moo Kang. A strategy of heterologous prime-boost influenza vaccination is more effective in inducing cross-reactive HAI titers and cross-protection than repeat vaccination. ASV 2020
- **Noopur Bhatnagar**, Lewins Walter, Hamed Laroui, Pallavi Garg. Epithelial derived MMP9 exhibits tumor-suppressive role in colitis-associated cancer. AGA 2014

Abstracts published in Gastroenterology Journal, Volume 146, Issue 5, Supplement 1, S-1-S-1099, May 2014

- **Noopur Bhatnagar**, Lewins Walter, Hamed Laroui, Pallavi Garg. “Epithelial derived MMP9 exhibits tumor-suppressive role in colitis-associated cancer”
- Lewins Walter, Anupama Ravi, Christopher Harper, **Noopur Bhatnagar**, Yutao Yan, Pallavi Garg. “MMP9 mediated activation of Notch1 is the plausible mechanism for its tumor-suppressive role in colitis-associated cancer as exhibited by Notch-Flox/FloxCre mice”

PROFESSIONAL MEMBERSHIPS

- The American Association of Immunologists (AAI) 2021-2022
- American Society for Virology (ASV) 2020-2022
- American Gastroenterological Association (AGA) 2014

JOURNAL PUBLICATIONS

1. **Bhatnagar N**, Kim KH, Subbiah J, Park BR, Ko EJ, Seong BL, Kang SM. Comparison of the effects of different potent adjuvants on enhancing the immunogenicity and cross-protection by influenza virus vaccination in young and aged mice. Antiviral Res. 2022; 197:105229. doi: 10.1016/j.antiviral.2021.105229

2. **Bhatnagar N**, Kim KH, Subbiah J, Park BR, Wang P, Wang BZ, Kang SM. Adjuvant effects of a new saponin analog VSA-1 on enhancing homologous and heterosubtypic protection of influenza virus vaccination (in preparation for submission)
3. **Bhatnagar N**, Kim KH, Subbiah J, Park BR, Worsham SM, Liu R, Oh J, Shin CH, Garcia KR, Kang SM. Heterologous prime-boost vaccination with inactivated influenza viruses induces more effective cross-protection than homologous repeat vaccination (in preparation for submission)
4. Kim KH*, **Bhatnagar N***, Jeeva S, Oh J, Park BR, Shin CS, Wang BZ, Kang SM. Immunogenicity and Neutralizing Activity Comparison of SARS-CoV-2 Spike Full-Length and Subunit Domain Proteins in Young Adult and Old-Aged Mice. *Vaccines* (Basel). 2021; 9(4):316. doi:10.3390/vaccines9040316 (*equal contribution)
5. Li Z*, Kim KH*, **Bhatnagar N***, Park BR, Subbiah J, Jung YJ, Raha J, Kang SM, Chen X. Physical radiofrequency adjuvant enhances immune responses to influenza H5N1 vaccination. *FASEB J.* 2022; 36(3):e22182. doi:10.1096/fj.202101703R (*equal contribution)
6. Bommireddy R, Stone S, **Bhatnagar N**, Kumari P, Munoz LE, Oh J, Kim KH, Berry JTL, Jacobsen KM, Jaafar L, Naing SH, Blackerby AN, Van der Gaag T, Wright CN, Lai L, Pack CD, Ramachandiran S, Suthar MS, Kang SM, Kumar M, Reddy SJC, Selvaraj P. Influenza virus-like particle-based hybrid vaccine containing RBD induces immunity against influenza and SARS-CoV-2 viruses. *bioRxiv preprints* 2022. doi: 10.1101/2022.02.01.478657
7. Rubino I, Han S, Oh E, Kumaran S, Lawson M, Jung YJ, Kim KH, **Bhatnagar N**, Lee SH, Kang HJ, Lee DH, Chu KB, Kang SM, Quan FS, Choi HJ. Study of the Pathogen Inactivation Mechanism in Salt-Coated Filters. *ACS Applied Materials & Interfaces* 2021; 13 (14), 16084-16096. doi: 10.1021/acsami.1c01837
8. 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. Broad cross-protection by recombinant live attenuated influenza H3N2 seasonal virus expressing conserved M2 extracellular domain in a chimeric hemagglutinin. *Sci. Rep.* 2021; 11:4151. doi: 10.1038/s41598-021-83704-0
9. Mezhenkaya D, Isakova-Sivak I, Kotomina T, Matyushenko V, Kim MC, **Bhatnagar N**, Kim KH, Kang SM, Rudenko L. A Strategy to Elicit M2e-Specific Antibodies Using a Recombinant H7N9 Live Attenuated Influenza Vaccine Expressing Multiple M2e Tandem Repeats. *Biomedicines*. 2021; 9(2):133. doi:10.3390/biomedicines9020133
10. Walter L, Pujada A, **Bhatnagar N**, Bialkowska AB, Yang VW, Laroui H, Garg P. Epithelial derived-matrix metalloproteinase (MMP9) exhibits a novel defensive role of tumor suppressor in colitis-associated cancer by activating MMP9-Notch1-ARF-p53 axis. *Oncotarget*. 2017; 8(1):364-378. doi:10.18632/oncotarget.13406