ScholarWorks@GSU

Vaccination Approaches to Enhance Cross-Protection against Influenza by Inducing Immunity to Neuraminidase and M2e

Authors	Raha, Jannatul
Citation	Raha, Jannatul. "Vaccination Approaches to Enhance Cross-Protection against Influenza by Inducing Immunity to Neuraminidase and M2e." 2024. Dissertation, Georgia State University. https://doi.org/10.57709/37939950
DOI	https://doi.org/10.57709/37939950
Download date	2026-03-13 18:04:17
Link to Item	https://hdl.handle.net/20.500.14694/2368

Vaccination Approaches to Enhance Cross-Protection against Influenza by Inducing Immunity to Neuraminidase and M2e

by

Jannatul Ruhan Raha

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

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

Doctor of Philosophy

at the Institute for Biomedical Sciences

Georgia State University

2024

ABSTRACT

Conventional hemagglutinin (HA) based inactivated influenza vaccines provide insufficient cross-protection against antigenically distant influenza strains, especially in older adults. The major goal of my dissertation research projects was to study influenza vaccination strategies to improve cross-protection by inducing extra immunity to neuraminidase (NA) and M2 ion channel ectodomain (M2e) proteins.

In chapter one, to address the issue of poor influenza vaccine efficacy in the elderly population, I employed a supplementation vaccination strategy, where I combined bivalent inactivated split vaccines (H1N1+H3N2) with a virus-like particle (VLP) expressing consensus NA (N1+ N2 + B NA) plus M2e repeat (5xM2e of human, swine and avian M2e sequences), referred as NA-M2e here, in 17-month-old-mice by a 2-dose immunication regimen. Vaccination with split plus NA-M2e induced protective IgG antibodies towards T-helper type 1 and effector T cell responses in aged mice, conferring enhanced protection against homologous and heterologous viruses compared to split vaccine . These findings suggest a promising strategy to overcome aging-related declines in vaccine efficacy and improve immune responses and vaccine efficacy in the elderly.

In chapter two, I investigated the protection efficacy of split plus NA-M2e vaccination in young adult mice and the adjuvant effects of supplementing NA-M2e on improving the immunogenicity and efficacy of split vaccine. Vaccination with combined split and NA-M2e was more effective in conferring enhanced cross-protection than either vaccine alone in young adult mice. Injection of NA-M2e VLP and combined split and NA-M2e VLP vaccines resulted in recruiting more activated monocytes, macrophages, and dendritic cell subsets in the lymphoid tissues within a day. Young adult mice, when co-immunized with split plus NA-M2e vaccines,

could induce stronger humoral and cellular immune responses compared to their aged counterparts. These findings suggest the combination of split plus NA-M2e vaccines is more effective in conferring the high efficacy of homo and cross-protection, probably via activating both innate and adaptive immune responses.

Most approved vaccines provide robust systemic immunity via intramuscular immunization (IM) but not effective mucosal immunity in protecting against respiratory pathogens. In Chapter 3, I investigated the immunogenicity and efficacy of NA-M2e VLP vaccine after delivery via the intranasal route versus intramuscular route. Intranasal immunization with NA-M2e induced comparable systemic IgG antibodies, elevated IgA antibodies, and increased populations of effector T cells, memory B cells, and antigen-presenting cells in the mucosal site compared to intramuscular immunization in mice. Intranasal delivery of NA-M2e VLP vaccine induced more effective cross-protection against antigenically variant influenza strains than intramuscular injection. These findings suggest that intranasal delivery of NA-M2e vaccine can be an effective route in inducing humoral and cellular immune responses at local mucosal sites, contributing to cross-protection.

INDEX WORDS: Influenza virus, Universal Influenza Vaccine, Inactivated Split Influenza Vaccines, Virus-Like Particle (VLP), Cross-Protection, Mucosal Immunity

Copyright by
Jannatul Ruhan Raha
2024

Vaccination Approaches to Enhance Cross-Protection Against Influenza by Inducing Immunity to Neuraminidase and M2e

by

Jannatul Ruhan Raha

Committee Chair: Sang-Moo Kang, Ph.D.

Committee: Mukesh Kumar, Ph.D.

Baozhong Wang, Ph.D.

Office of Academic Assistance – Graduate Programs

Institute for Biomedical Sciences

Georgia State University

December 2024

DEDICATION

I dedicate this dissertation to my parents – Mahbuba Sultana & Md. Rohul Amin Siddique, my husband Dr. Khalid Hossain, my sister Fairoz Wasima Hridi, my grandparents, aunts Nasima Ferdousi & Kazi Susmita Lazuk, and the Hossain family.

ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest gratitude to my Ph.D. advisor and dissertation committee chair, Dr. Sang-Moo Kang, for his unwavering support, guidance, patience, effort, and encouragement throughout my doctoral journey which have been instrumental in shaping me as an independent researcher. The skills and experiences I have gained under his mentorship will remain with me as cornerstones of my career and life.

I would also like to extend my heartfelt thanks to my committee members, Dr. Baozhong Wang, and Dr. Mukesh Kumar for their continuous guidance and support. Their profound knowledge, insights, and perspectives in the fields of infectious diseases and viral immunology have been invaluable, greatly enriching my research and development as a scientist.

I sincerely thank the Kang lab's current and former lab members for their support, collaboration, and fostering of a motivating environment. Their teamwork and camaraderie have played a significant role in my professional development during my Ph.D. journey.

I wholeheartedly thank my parents for dedicating their lives to giving me the best education, and upbringing and instilling the best values in me. I sincerely believe that all my achievements are a result of their prayers. I would like to thank my aunts and the Hossain family for their love, support and encouragement. Special thanks to my sister and cousins for all the good times and being a constant source of happiness in my life.

It is an honor for me to express gratitude to my husband, Khalid, for standing by my side since the beginning of my Ph.D. journey. I genuinely appreciate his patience and the sacrifices he made so I could pursue my career aspirations. He has brought out the best in me, and it has been my greatest pleasure and blessing to share this life with him.

Table of Contents

ACKNOWLEDGEMENTS .... **v**

LIST OF FIGURES .... **xiii**

LIST OF ABBREVIATIONS .... **xvi**

1. INTRODUCTION **1**

1.1 Influenza Virus **1**

1.2 Influenza Virus Life Cycle **3**

1.3 Current Influenza Vaccines **4**

1.4 Vaccine Platforms **5**

1.4.1 Inactivated Virus Vaccines ***5***

1.4.2 Live Attenuated Influenza Vaccine (LAIV) ***6***

1.4.3 Recombinant Protein Vaccines ***6***

1.4.4 Viral Vector Vaccines ***6***

1.4.5 Virus-like Particle (VLP) Vaccines ***7***

1.4.6 Nucleic Acid Vaccines ***7***

1.5 Antigenic Targets for Universal Influenza Vaccines (UIVs) **8**

1.5.1 HA-Based UIVs ***8***

1.5.2 NA-Based UIVs ***9***

1.5.3 M2e-Based UIVs ***10***

1.5.4 NP and M1 Based UIVs ***11***

1.6 Immunosenescence 12

1.7 Intranasal Vaccination and Mucosal Immunity 13

1.8 Hypothesis and Research Objectives 14

1.8.1 Chapter 1: Supplementation of Seasonal Vaccine with Multi-Subtype Neuraminidase and M2 Ectodomain Virus-Like Particle Improves Protection Against Homologous and Heterologous Influenza Viruses in Aged Mice 15

1.8.2 A Strategy of Enhancing the Protective Efficacy of Seasonal Vaccines by Providing Additional Immunity to Neuraminidase and M2e 16

1.8.3 Intranasal Vaccination with Multi Neuraminidase and M2 Ectodomain Virus-Like Particle Enhances Broad Protection Against Influenza 16

2. Chapter 1: Supplementation of Seasonal Vaccine with Multi-Subtype Neuraminidase and M2 Ectodomain Virus-Like Particle Improves Protection Against Homologous and Heterologous Influenza Viruses in Aged Mice 18

2.1 Abstract 18

2.2 Introduction 18

2.3 Methods 20

2.3.1 Viruses and Vaccines 20

2.3.2 Immunization and Challenge 21

2.3.3 Antibody Responses and Hemagglutination Inhibition (HAI) Assay 22

2.3.4 Lung Viral Titration 22

2.3.6 Cytokine Responses 23

2.3.7 Flow Cytometry *23*

2.3.8 In Vivo Protection Experiment with Antisera *23*

2.3.9 In Vitro BMDC Experiment *24*

2.3.10 Statistical Analysis *24*

2.4 Results **24**

2.4.1 Addition of NA-M2e to Split Vaccine Enhances Immune Responses in Aged Mice *24*

2.4.2 S+NA-M2e Vaccines Increase HAI Activities, IgG O Isotypes, and IgG Levels Reactive to Viral Antigens *27*

2.4.3 S+NA-M2e Vaccination Promotes the Efficacy of Homologous and Heterologous Protection in Aged Mice *32*

2.4.4 NA-M2e Supplemented Split Vaccination Promotes the Induction of Cellular Immune Responses *35*

2.4.5 Antisera of NA-M2e Supplemented Vaccination Confer Homologous and Heterologous Protection *39*

2.5 Discussion **41**

2.6 Conclusion **47**

2.7 Acknowledgements **47**

3. CHAPTER 2: A STRATEGY OF ENHANCING THE PROTECTIVE EFFICACY OF SEASONAL VACCINES BY PROVIDING ADDITIONAL IMMUNITY TO NEURAMINIDASE AND M2e **48**

3.1 Abstract **48**

3.2 Introduction 48

3.3 Methods 50

3.3.1 Viruses and Vaccines 50

3.3.2 Immunization and Challenge 51

3.3.3 Antibody Responses and Hemagglutination Inhibition (HAI) Assay 52

3.3.4 Lung Viral Titration 52

3.3.5 Cytokine Responses 53

3.3.6 Flow Cytometry 53

3.3.7 In Vivo Protection Experiment with Antisera 53

3.8.8 Statistical Analysis 54

3.4 Results 54

3.4.1 NA-M2e Enhances the Immunogenicity of Split Vaccine by Stimulating Innate Immune Responses After Prime Vaccination 54

3.4.2 S+NA-M2e Boost Immunization Induces High Antibody Responses to HA, NA and M2e Vaccine Antigens 58

3.4.3 Supplemented S+NA-M2e Vaccination Induces Enhanced Homologous and Heterologous Protection 60

3.4.4 NA-M2e Supplementation Protects Against Inflammatory Cytokine Induction Levels and Promotes IgG-Secreting Cell Responses Post-Challenge 62

3.4.5 NA-M2e Promotes Cellular Immune Responses After Co-Vaccination and Challenge 64

3.4.6 S+NA-M2e Antisera Contribute to Effective Protection Against Homologous and Heterologous Influenza Viruses *66*

3.4.7 Vaccination with Split and NA-M2e in Primed Mice Induces Increased IgG Levels and Improves Protection Against Heterologous Virus *68*

3.5 Discussion **71**

3.6 Conclusion **76**

3.7 Acknowledgements **77**

4. Chapter 3: Intranasal Vaccination with Multi Neuraminidase and M2 Ectodomain Virus-Like Particle Enhances Broad Protection Against Influenza **78**

4.1 Abstract **78**

4.2 Introduction **78**

4.3 Methods **80**

4.3.1 Viruses and Vaccines *80*

4.3.2 Immunization and Challenge *81*

4.3.3 Antibody Responses *81*

4.3.4 Lung Viral Titration *82*

4.3.5 Cytokine Responses *82*

4.3.6 Flow Cytometry *82*

4.3.7 Enzyme-Linked Lectin Assay (ELLA) *83*

4.3.8 Passive Sera Transfer *83*

4.3.9 Statistical Analysis 84

4.4 Results 84

4.4.1 Intranasal Delivery of NA-M2e Vaccine Induces IgG Antibodies at Comparable or Lower Levels as Intramuscular Route in Boost Sera 84

4.4.2 Intranasal Delivery of NA-M2e Vaccine Provides Higher Efficacy of Broad Protection than IM Route 86

4.4.3 Intranasal Immunization with NA-M2e Vaccine enhances IgA Antibody Responses 88

4.4.4 Intranasal Delivery of NA-M2e Vaccine Efficiently Induces Effector T Cell Responses in the Lung Tissues 90

4.4.5 NA-M2e Intranasal Delivery Increases B Cell Responses in the Lung 92

4.4.6 IN and IM Delivery of NA-M2e Vaccine Differentially Mobilizes Innate Immune Cells in the Lung and Spleen Tissues Within a Day 94

4.4.7 Roles of Antisera in Conferring Protection in Naïve Mice 96

4.5 Discussion 97

4.6 Conclusion 102

4.7 Acknowledgements 103

5. DISCUSSION AND FUTURE DIRECTIONS 104

5.1 Enhancing Cross-Protective Efficacy of Inactivated Split Vaccines by Inducing NA and M2e Immunity 104

5.2 Bridging the Immunity Gap: Enhancing Influenza Vaccine Efficacy in the Elderly 106

5.3 The Role of Pre-Existing Immunity in Shaping Influenza Vaccine Responses.......... 109

5.4 Nasal Vaccines for Induction of Mucosal Immunity 111

5.5 Limitations of Current Vaccines and Next-Generation Influenza Vaccines 113

6. REFERENCES.. 115

7. VITAE 153

LIST OF FIGURES

Figure 1. Supplementation with NA-M2e Enhances Antibody Responses to Vaccine Antigens. 26

Figure 2. IgG Responses to Heterologous Viral Antigens. 27

Figure 3. Immunization with S + NA-M2e Vaccines Elevates HAI Titers and IgG2a Levels. 29

Figure 4. Higher Levels of IgG Antibody Responses to HA Protein Antigens in Split Immunized Young Mice Compared to Aged Mice. 30

Figure 5. Sera from S + NA-M2e Vaccinated Group Show High Reactivity to Group 1 NA and Group 2 NA Viral Antigens Expressed on Infected Cells. 31

Figure 6. Supplemented S + NA-M2e Vaccine Confers Enhanced Protection Against Homologous A/Cal/H1N1 Virus in Aged Mice. 34

Figure 7. Improved Protection Against Heterologous A/Phil/H3N2 Virus is Conferred by S + NA-M2e Vaccine in Aged Mice. 35

Figure 8. Humoral and Cellular Responses After Challenge. 37

Figure 9. Effector Humoral Responses After Heterologous Challenge (9 dpi). 38

Figure 10. Significantly Enhanced IFN-γ+ Splenic T Cell Responses in Aged Mice with S+NA-M2e Vaccination After A/Cal/H1N1 Challenge. 38

Figure 11. Young Adult Mice Show Higher Fold Increases in IFN-γ+ CD4 T Cell Responses Than Aged Mice. 39

Figure 12. Roles of Antisera from Vaccinated Aged Mice in Conferring Protection Against Homologous and Heterologous Influenza Viruses in Naïve Adult Mice. 41

Figure 13. Comparison of IFN-γ + T Cell Responses in NA-M2e Vaccinated Mice Before and After Challenge. ... 46

Figure 14. NA-M2e Vaccine Stimulates Bone Marrow Dendritic Cells (BMDC) In Vitro to Secrete Cytokines. ... 46

Figure 15. S+NA-M2e Vaccination Induces Significant Antibody Responses and Innate Immune Cell Responses After Prime Immunization. ... 56

Figure 16. Activation of Dendritic Cell (DC) Subsets by S+NA-M2e Vaccination 1 Day Post Prime Immunization. ... 57

Figure 17. Elevated Levels of IgG Antibody Levels to HA, NA and M2e Antigens in S+NA-M2e Vaccinated Boost Sera. ... 59

Figure 18. Reduced Body Weight Loss and Lung Viral Titers in S+NA-M2e Vaccinated Mice Following Both Homologous and Heterologous Virus Challenges. ... 61

Figure 19. Diminished Pro-Inflammatory Cytokine Levels and Increased Antibody-Secreting cell Responses Demonstrated by S+NA-M2e Boost Vaccinated Mice After Lethal Virus Challenge. ... 63

Figure 20. Induction of Robust T Cell Responses in S+NA-M2e Immunized Mice. ... 65

Figure 21. Boost Sera from S+NA-M2e Vaccinated Mice Protect Against Lethal Influenza Virus Challenges. ... 67

Figure 22. High Antibody Responses Elicited by Sp+NA-M2e Group in Sequential Vaccination Strategy. ... 70

Figure 23. Improved Protection Against Heterologous A/Phil H3N2 Challenge by Split+m-cNA-M2e Sequentially Vaccinated Mice. ... 71

Figure 24. S+NA-M2e Vaccination Strategy Induces Higher IgG2a/IgG1 Ratios Indicating Th1 Type Immune Response. 76

Figure 25. Higher IgG Antibody Levels to Vaccine Antigens in IN Immunized Boost Sera than in Prime Sera. 86

Figure 26. IN Immunized Mice Demonstrate Higher Protective Efficacy Against Influenza A Viruses Compared to IM Immunized Mice in Lethal Virus Challenge. 88

Figure 27. Elevated Levels of IgA Antibodies Induced in NA-M2e Intranasally Immunized Mice. 90

Figure 28. IN Immunized Mice Elicit Significant T Cell Responses in the Lungs. 92

Figure 29. Higher Induction of B Cells in IN Immunized Mice. 93

Figure 30. Activation of Innate Immune Cells in the Lungs of IN Immunized Mice 1 Day Post Prime Immunization. 95

Figure 31. IM and IN boost (2nd dose) Immunized Sera Similarly Inhibit Viruses in NAI Assays and Confer Protection Against Influenza Viruses in Passive Sera Transfer Experiments. 97

Figure 32. Significant Induction of Activated Alveolar Macrophages in IN Immunized Group. 102

LIST OF ABBREVIATIONS

aAM	Activated Alveolar Macrophage
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ANOVA	Analysis of covariance
APC	Antigen-presenting cell
ASC	Antibody Secreting Cell
BM	Bone marrow
BMDC	Bone marrow dendritic cell
Brm	Tissue resident memory B cell
CCl-2	C-C motif chemokine ligand 2
CDC	Complement dependent cytotoxicity
cHA	Chimeric HA
COBRA	Computationally optimized broadly reactive antigens
DC	Dendritic cell
dLN	Draining lymph node
EID_{50}	Egg Infectious Dose 50%
ELISA	Enzyme-linked immunosorbent assay
ELLA	Enzyme-linked lectin assay
FDA	Food and Drug Administration
GC	Germinal Center
GM-CSF	Granulocyte macrophage colony-stimulating factor
HA	Hemagglutinin
HAI	Hemagglutinin Inhibition
HAU	Hemagglutination activity units
HBV	Hepatitis B virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus

HRP	Horse radish peroxidase
IAV	Influenza A virus
iBNY	inactivated virus derived from B/New York (Yamagata) strain
IBV	Influenza B virus
iCal	Inactivated virus vaccine derived from A/California/H1N1 strain
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G 1
IgG2a	Immunoglobulin G 2a
IIV	Inactivated influenza vaccines
IL	Interleukin
IM	Intramuscular
IN	Intranasal
iPhil	Inactivated virus vaccine derived from A/Philippines/H3N2 strain
iSwz	Inactivated virus vaccine derived from A/Switzerland/H3N2 strain
iViet	Inactivated virus vaccine derived from rgA/Vietnam/H5N1 strain
LAIV	Live attenuated influenza vaccine
M	Microfold
M1	Matrix protein 1
M2	Matrix protein 2
M2e	Matrix 2 ectodomain
mAb	Monoclonal antibodies
MBC	Memory B cell
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
mLN	Mediastinal lymph node
MPL-A	Monophosphoryl Lipid A
NA	Neuraminidase

NALT	Nasal-associated lymphoid tissues
NEP	Nuclear export protein
NK cells	Natural Killer cell
NP	Nucleoprotein
NS1	Non-structural protein 1
OAS	Original Antigenic Sin
OD	Optical Density
PA	Polymeric acidic protein
PB1	Polymeric basic protein 1
PB2	Polymeric basic protein 2
PC	Plasma cell
pDC	Plamacytoid dendritic cell
rBV	Recombinant baculovirus
RDE	Receptor-destroying enzymes
rg	Reverse genetics
S+NA-M2e	Split+NA-M2e
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
sCal	Inactivated split virus vaccine derived from A/California/H1N1 strain
SEM	Standard Error of the Mean
sIgA	Secretory immunoglobulin A
sSwz	Inactivated split virus vaccine derived from A/Switzerland/H3N2 strain
$TCID_{50}$	Tissue Culture Infectious Dose 50%
Tfh	T follicular helpter cell
Th1	T-helper type 1
Th2	T-helper type 2
TNF-α	Tumor necrosis factor alpha
UIV	Universal influenza vaccines
VLP	Virus-like particle
vRNA	Viral RNA

WHO	World Health Organization

1. INTRODUCTION

1.1 Influenza Virus

The influenza virus is the causative agent of seasonal and pandemic flu diseases that affect the respiratory tract of those infected. The flu is a highly contagious respiratory illness that causes an estimated 290,000 to 650,000 deaths globally each year (WHO, 2018). The clinical manifestations of influenza are fever, sore throat, cough, fatigue, and body aches. Key factors in its spread include the emergence of new virus strains, high transmissibility, and increased susceptibility in immunocompromised individuals. Influenza pandemics arise when a novel influenza virus emerges, causing widespread transmission because of limited pre-existing immunity in the population. The 1918 Spanish flu, the deadliest pandemic recorded in history, infected approximately 500 million people and caused approximately 50 million deaths worldwide. Other significant pandemics include the 1957 Asian flu (H2N2), the 1968 Hong Kong flu (H3N2), and 2009 H1N1 swine flu (H1N1) (Taubenberger & Morens, 2006). Vaccination is considered the best measure to prevent flu disease, while antiviral treatments alleviate symptoms and complications.

The influenza, a filamentous or spherical-shaped virus, belongs to the *Orthomyxoviridae* family, which consists of enveloped viruses with segmented, negative, single-stranded RNA genomes (Skelton & Huber, 2022). The spherical forms are approximately 100 nm in diameter, while the filamentous forms are around 300 nm in length. The *Orthomyxoviridae* family consists of the *genera,* A, B, C, and D, classified on the expression of surface proteins, including matrix protein (M1), matrix protein (M2), and nucleoprotein (NP). Both types A and B are responsible for annual flu epidemics in humans, whereas type C typically causes milder symptoms and type D does not infect humans (Hutchinson & Yamauchi, 2018). Influenza A viruses (IAVs) are the most aggressive and notorious as they can infect a broad range of hosts, cause severe illnesses, and are

associated with the highest morbidity and mortality. IAVs are responsible for causing both seasonal epidemics and all past influenza pandemics (Krammer et al., 2018). Each of its 8 negative-sense RNA segments encodes for 1 or 2 genes, all together encoding 11 viral proteins. The host cell-derived lipid membrane is studded with membrane proteins such as hemagglutinin (HA), neuraminidase (NA), and matrix protein (M2) which are overlayed on matrix protein (M1) that encapsulates the virion core. Internal proteins consist of the nuclear export protein (NEP, also called NS2) and the ribonucleoprotein complex. This complex includes the viral RNA segments coated with nucleoprotein (NP) and the RNA-dependent RNA polymerase, which is made up of the polymeric basic (PB1, PB2) and polymeric acidic (PA) subunits (Bouvier & Palese, 2008). While the non-structural protein 1 (NS1) is involved in viral replication and modulating the host cell response, the PB1-F2 protein contributes to viral pathogenicity by promoting cell death and immune evasion (Varga et al., 2011). Influenza B shares the same surface proteins, hemagglutinin (HA) and neuraminidase (NA), as Influenza A. However, instead of the M2 protein, Influenza B contains two unique proteins: BM2, an ion channel, and NB (Bouvier & Palese, 2008).

The IAVs are categorized based on the expression of the surface glycoproteins HA and NA, and 18 subtypes of HA and 11 subtypes of NA have been discovered so far (Wang et al., 2019). The influenza uses hemagglutinin (HA), a lectin that binds to sialic acid linkages on host cell surfaces, to facilitate viral entry. After replication, neuraminidase (NA), a receptor-destroying enzyme, cleaves these sialic acid linkages to release newly formed virions from the host cell, allowing the virus to spread and infect other cells (Russell et al., 2006). Influenza viruses are further classified based on phylogenetic differences in their HA proteins. Group 1 includes HA subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18, while Group 2 consists of H3, H4, H7, H10, H14, and H15. On the other hand, group 1 NA consists of N1, N4, N5, and N8

and group 2 NA comprises of N2, N3, N6, N7, and N9. Bat-derived N10 and N11 do not belong to either group (Wu et al., 2014).

Influenza viruses are transmitted through the respiratory route in humans and spread due to direct contact via fecal-oral, fecal-fecal, and fecal respiratory routes in birds (Krammer et al., 2018). Viral replication at the respiratory or intestinal tract peaks at 48 hours post-infection, and declines thereafter, with negligible shedding after a week (Taubenberger & Morens, 2008). While mild infections impact the upper respiratory tract, severe infections, such as those caused by avian H5N1 in humans, are caused due to infections in the lower respiratory tract. Cytokines and chemokines, such as IL-6, IL-1β, TNF-α, and CCL-2 help recruit inflammatory cells. Children, the elderly, and immunocompromised individuals are more vulnerable to severe outcomes due to their impaired or underdeveloped immune systems, which may result in inadequate CD4+ T cell-mediated adaptive immune responses (Lin & Nichol, 2001; Taubenberger & Morens, 2008; Weinstein et al., 2003). This reduced immune function can lead to failure in mounting effective innate and adaptive immune responses.

Influenza viruses undergo mutations via antigenic drift and shift mechanisms. Antigenic drift involves gradual mutations in the viral genome over time, leading to changes in viral antigenicity. In contrast, antigenic shift occurs when two or more antigenically different influenza viruses co-infect a cell, resulting in the reassortment of their genomes and leading to the emergence of a novel strain with a rearrangement of genetic material from both viruses (Hay et al., 2001). Therefore, constant surveillance and annual vaccine updates are crucial to preventing flu. Some of the approved flu vaccines on the market are Afluria, Fluarix, Flublok, Fluzone, Flulaval and FluMist (FDA, 2024).

1.2 Influenza Virus Life Cycle

The influenza virus primarily infects the respiratory tract, where it replicates mainly in the epithelial cells of the upper respiratory tract. The viruses use hemagglutinin protein to recognize and bind to terminal N-acetylneuraminic (sialic) acid with α-2,6 linkage on human tracheal epithelial cells. Receptor-mediated endocytosis occurs, and the virus is internalized. The acidity of the endosomal compartment causes hydrogen ions to be pumped into the virion particle via the M2 ion channel. Consequently, there is a disruption of protein-protein interactions and conformational changes in HA that result in viral RNP release from the viral matrix into the cell cytoplasm (Martin & Helenius, 1991). The RNA is transported into the nucleus for replication to generate more viral RNA and for transcription of mRNA, which is then translated into viral proteins by the ribosomes. To be fully infectious, the virions must contain a full genome including all 8 segments. Evidence suggests that packaging is a selective process, where discrete packaging signals on all vRNAs segments ensure that a full genome is incorporated into most viral particles (Fujii et al., 2003). Virus budding occurs at the cell membrane and is probably initiated by the accumulation of M1 matrix protein on the cytoplasmic side of the lipid bilayer. Even after completion of budding, HA continues to bind to sialic acids on the cell surface. NA releases viral particles by cleaving the sialic acid residues from glycoproteins to release progeny viruses from host cells (Bouvier & Palese, 2008; Dou et al., 2018).

1.3 Current Influenza Vaccines

Vaccination is one of the most effective methods of preventing influenza infections and its associated complications. Several flu vaccines currently on the market rely on inactivated virus vaccines and live attenuated influenza vaccine platforms, with a focus on HA-based immunity. The adjusted influenza vaccine efficacy was estimated to be between 10%-56% on average during the 2004-2024 seasons (CDC, 2024). Poor efficacy in HA-based vaccines can be attributed to

factors such as frequent mutations in the HA head domain, strain-specificity, vaccine mismatch, inability to induce cross-protection, and limited long-term immune responses. As a result, researchers are increasingly focused on developing safe, effective universal influenza vaccines that can generate robust antibody responses to conserved viral epitopes and induce long-lasting cellular memory.

1.4 Vaccine Platforms

1.4.1 Inactivated Virus Vaccines

Whole inactivated virus vaccines are produced by growing vaccine seed virus in chicken embryonated eggs and chemically inactivated with formaldehyde or β-propiolactone. This is an established technology with a long history of safety and can be produced rapidly. However, these vaccines are standardized for HA content only and are strain-specific. Consequently, they often require annual updates due to the hypervariability of HA, tend to provide low efficacy in older individuals, and cannot induce long-term immunity (Sakai-Tagawa et al., 2017).

In the split inactivated vaccines, the viral envelope of the whole virion is chemically disrupted using detergents or diethyl ether. These vaccines have fewer side effects than whole inactivated viruses and are effective in inducing immune responses to viral proteins. They confer limited protection to newly emergent viruses. Fluzone and Fluarix are two commonly approved vaccines that use this platform.

Subunit vaccines are further purified to contain HA and NA antigens in the majority. Since it lacks genetic material and causes a targeted immune response, it is safe to use and induces fewer side effects. However, it often requires adjuvants due to low immunogenicity.

1.4.2 Live Attenuated Influenza Vaccine (LAIV)

Live attenuated vaccines are available in North America and some European countries. These vaccines are derived from cold-adapted and temperature-sensitive master donor viruses which are propagated in eggs (Ghendon et al., 1984; Jin et al., 2003; Yamayoshi & Kawaoka, 2019). LAIVs mimic natural infection by inducing both mucosal and systemic immunity, producing IgA and IgG antibodies (Abs), and eliciting cross-reactive immune responses at the initial site of viral replication. However, they are not recommended for children under 2 years of age, pregnant women, and individuals with certain underlying conditions due to potential side effects. FluMist, a LAIV by AstraZeneca, is available for self-administration in the US.

1.4.3 Recombinant Protein Vaccines

Recombinant protein vaccines, such as the FluBlok, are produced by inserting genes for expressing flu proteins, such as HA, into a recombinant-protein-expressing system using yeast or insect cells and baculovirus (Cox & Hollister, 2009). It is safe, has a faster production process, and can be tailored to the specific virus strains but has low immunogenicity, relatively higher production costs than inactivated vaccines, and limited protection against divergent flu strains. While the mechanism of action of this vaccine is analogous to that of inactivated vaccines, the commercial formulation of the recombinant HA vaccine has three times the amount of HA compared to inactivated influenza vaccines, allowing it to generate antibody titers that are comparable to those elicited by conventional inactivated vaccines (Cox et al., 2008).

1.4.4 Viral Vector Vaccines

Vector-based vaccines use genetic engineering technology to deliver genetic material that codes for influenza antigens in a usually harmless virus such as the adenovirus. The host cells produce antigens that trigger strong humoral and cellular immune responses and can confer

protection against diverse influenza strains (Chen et al., 2021). They have complex production processes and induce pre-existing immunity or cause potential side effects to the vector virus. Currently, there are no approved influenza viral vector vaccines.

1.4.5 Virus-like Particle (VLP) Vaccines

The virus-like particle (VLP) vaccines mimic the organization and conformation of the virus but do not contain the viral genome (Mohsen & Bachmann, 2022). They induce strong immune responses by expressing viral proteins on a membrane and simulating the actual virus. Since VLPs lack genetic material, they cannot replicate and are generally considered safe; however, since VLPs mimic the virus's in structure, they can induce strong immune responses. Flexibility in designing these vaccines can help develop universal vaccines containing conserved proteins of the virus that may lead to improved cross-protection. Furthermore, they are stable and have a longer half-life in the serum. However, they are complex and costly to produce. Although approved VLP vaccines include human papillomavirus (HPV), hepatitis B, and malaria, there are no approved influenza VLP on the market. Novavax biotech company has developed an influenza VLP candidate which has been tested in clinical trials (Novavax, 2020).

1.4.6 Nucleic Acid Vaccines

Nucleic acid vaccines, which include DNA and RNA vaccines, generate immune responses against the virus by providing genetic instructions. The DNA vaccine contains a plasmid that encodes for viral or bacterial protein. It is delivered into the cells via a needle or electroporation to facilitate host cell uptake. Inside the nucleus, the plasmid DNA is transcribed into mRNA which is then translated to produce antigens. mRNA vaccines, however, bypass the transcription step, as the mRNA is directly translated into protein in the cytoplasm. Once the antigens are produced and processed, they activate immune responses to fight pathogens. mRNA vaccines are mixed with

cationic liposomes for delivery into target cells. Nucleic acid vaccines are safe as they lack infectious materials, has a simple and fast manufacturing process, and can be rapidly scaled up (Wang & Yuan, 2024). DNA vaccines are stable, cheap to produce, and do not require refrigeration, but efficient delivery into host cells has been a challenge, limiting their effectiveness. On the other hand, mRNA vaccines are faster to develop and produce, and the risk of integration into the host genome is minimal as the mRNA remains in the cytoplasm during translation. Pfizer/BioNTech and Moderna developed mRNA vaccines against SARS-CoV-2, which have been successfully licensed for preventing COVID-19 infections. Moderna's seasonal influenza vaccine, mRNA-1010, has met its primary endpoint in Phase 3 clinical trial (Moderna, 2023).

1.5 Antigenic Targets for Universal Influenza Vaccines (UIVs)

Universal influenza vaccines (UIVs) are aimed at providing at least 75% effectiveness against symptomatic flu against groups 1 and 2 influenza viruses along with long-lasting protection for at least 12 months in all populations (Paules et al., 2017). Vaccine platforms such as recombinant protein-based vaccines, virus-vectored vaccines, virus-like particle vaccines, nanoparticle-based vaccines, and nucleic acids, are being used to develop these universal vaccines. Conserved antigenic targets for universal vaccine development include the HA stalk, NA, M2e, and internal proteins such as M1 and NP (Nguyen & Choi, 2021). Since these proteins induce non-neutralizing immunity and are immune-subdominant, they have the potential to generate broad cross-protection but induce weak immune responses.

1.5.1 HA-Based UIVs

The HA has two distinct domains, a globular head and a stalk region. Conventional influenza vaccines target the HA global head domain which is highly variable and undergoes frequent mutations. This variability limits the strain-specific effectiveness of the vaccine,

necessitating annual updates. Current strategies that target the less variable HA stalk regions include the use of headless HA, chimeric HA, mosaic HA, computationally optimized broadly reactive antigens (COBRA), and *breathing* HA (Nguyen & Choi, 2021). In the headless HA approach, the HA head domain is removed via chemical or genetic treatments. It is crucial to develop stable headless HA, keeping the original conformation to generate broadly cross-reactive anti-stalk HA. These antibodies confer protection via non-neutralizing Fc-dependent antibody-dependent cellular cytotoxicity (ADCC) mechanisms (Impagliazzo et al., 2015; Yassine et al., 2015). The chimeric HA (cHA) approach involves sequential vaccination with cHAs comprising of the same stalk domain but different head domains to induce anti-stalk antibodies. Sequential vaccinations with different vaccine formulations have been shown to induce highly stalk-reactive antibodies and consequently protect mice against antigenically distant IAVs (Krammer et al., 2013; Ryder et al., 2015), however, multiple immunizations are required in this strategy which is not convenient. Next, in the mosaic HA approach, the stalk and the epitopes of the HA head domain outside the major antigenic sites are used to develop vaccines. While the computationally optimized broadly reactive antigen (COBRA) utilizes computational methods to generate consensus sequences of HA or NA to design vaccines, the "breathing HA" concept involves exposing the hidden, more conserved regions of the head domain to induce broadly reactive antibodies. HA-based UIVs have shown potential in pre-clinical studies, but further research is needed to induce robust protection against divergent IAVs.

1.5.2 NA-Based UIVs

The NA undergoes slower antigenic changes compared to HA and is a crucial target for antiviral drugs such as Oseltamivir and Zanamivir. Antibodies to NA provide permissive immunity as they inhibit budding of new viruses rather than blocking cellular entry. Additionally, these

antibodies induce cross-protection within the same subtype and are not very effective in conferring protection to heterosubtypic viruses. Human monoclonal antibodies (mAbs) targeting neuraminidase (NA) could neutralize influenza A virus, inhibit NA activity, and confer broad in vivo protection in mice against virus challenges from group 1 (including human N1 and avian N1, N4, N5, and N8), group 2 (including human N2, swine N3, and avian N2, N6, N7, and N9), as well as an influenza B virus from the B/Victoria/2/87 lineage (Stadlbauer et al., 2019). A prior study conducted in guinea pigs showed that NA-based vaccination prevented transmission of the virus (McMahon et al., 2019) . Highly conserved NA epitope (NA amino acids 222-230, situated near the enzymatic site) is a promising cross-reactive target for vaccine design against influenza A subtypes and B viruses (Gravel et al., 2010). Conventional inactivated vaccines are not standardized for NA and induce anti-NA responses with around 30% seroconversion (Couch et al., 2012; Laguio-Vila et al., 2015). It is essential to supplement the inactivated vaccines on market with NA-based vaccines to improve NA-based immunity.

1.5.3 M2e-Based UIVs

M2 protein, considered a proton-selective ion channel, is responsible for the internal acidification of the virus during uncoating and membrane fusion. The extracellular N-terminal domain (M2e) is smaller and has low immunogenicity. To address this, researchers have attempted to enhance M2e immunogenicity by incorporating carrier proteins, adding adjuvants, using VLP platforms and using nanoparticle formulations (Schepens et al., 2018). While anti-M2e antibodies cannot neutralize viruses, they can reduce virus replication via complement-dependent cytotoxicity, ADCC and antibody-dependent cellular phagocytosis (ADCP) mechanisms (Lee et al., 2015). A previous study demonstrated that a VLP vaccine consisting of human, swine, and avian M2e sequences induced the recruitment of innate immune cells and the production of

cytokines and chemokines at infection sites, conferring protection against different subtypes of influenza viruses (Kim et al., 2018b; M.-C. Kim et al., 2013). Another study demonstrated that 24 hours after an H3N2 virus challenge, healthy volunteers who were intravenously administered a monoclonal antibody targeting M2e (TCN-032) (NCT01719874) demonstrated reduced symptom scores, and viral replication compared to those receiving a placebo (Ramos et al., 2015). Despite showing potential in clinical studies, M2e-based vaccines have not been commercialized yet. Due to being less immunogenic, they need to be paired with carrier proteins or adjuvants which may result in unintended side-effects (Turley et al., 2011). Furthermore, M2 in IAVs is structurally different from M2 in IBVs, therefore, anti-M2e mediated protection is insufficient against IBVs (Pielak & Chou, 2011). Lastly, clinical trials in humans reveal a decrease in antibody titer over time, therefore, M2-based vaccines need to be supplemented with other influenza vaccine antigens or adjuvants to induce a robust, long-lasting immunity.

1.5.4 NP and M1 Based UIVs

Internal proteins like NP and M1 are produced within infected cells, where they are processed and presented by major histocompatibility complex (MHC) molecules for recognition by T cells (Doucet et al., 2011). Therefore, incorporating internal proteins in vaccines has the potential to improve T cell responses. A study showed that using a sequential vaccination strategy mice primed with M1 based DNA vaccine and boosted with recombinant M1 protein from H9N2 provided complete homologous protection and partial heterologous protection in virus challenges (Zheng et al., 2014). Several NP based vaccines have shown improved efficacy in pre-clinical studies (Cookenham et al., 2020; Del Campo et al., 2019; Yang et al., 2014). Modified vaccinia virus Ankara (MVA) vector encoding NP and M1 (MVA-NP+N1) acted as an adjuvant to enhance antibody and T cell responses in animal models. This vaccine has been shown to provide safety,

reduce flu symptoms, the length of viral shedding, boost memory T cell responses, and induce antigen-specific T cell responses and multifunctional cytokines (Antrobus et al., 2012; Berthoud et al., 2011; Lillie et al., 2012; Powell et al., 2013). Despite showing promise, vaccines targeting the internal proteins have not been successful yet, probably due to the lack of humoral immunity, and, in addition, their efficacy is impacted by HLA diversity, warranting further research to resolve these issues.

1.6 Immunosenescence

Elderly individuals (≥ 65 years) represent a key demographic requiring protection from influenza viruses. Age-related dysregulation of innate and adaptive immune responses puts them at a significant disadvantage, leading to an increased risk of severe illness, hospitalizations, and deaths (Iuliano et al., 2018). Influenza vaccine efficacy rates vary from 17% to 53% in elderly individuals (Katherine Goodwin et al., 2006). The increased susceptibility and weakened immune response in older adults, caused by the progressive dysregulation of the immune system, is referred to as immunosenescence.

Aged phagocytes have reduced potential to exert their actions and engulf infected cells. Furthermore, natural killer (NK) cells demonstrate dysfunctional migration and cytotoxicity, and dendritic cells (DCs) show altered potential for antigen-presenting capabilities. Decreased activation, suboptimal antigen uptake, and presentation, along with lower expression of co-stimulatory molecules, collectively diminish their overall functionality (Allen et al., 2020; Chan et al., 2021). Age-related involution of bone marrow and thymus results in reduced production of naïve B and T cells and decreased generation of de novo antibody responses to novel antigens (Aspinall & Andrew, 2000). B cells exhibit limited capacity for somatic hypermutation and class switch recombination, leading to reduced affinity maturation and impaired effector functions

(Frasca et al., 2008). Therefore, they have weakened ability to neutralize viruses. Additionally, in elderly individuals T cells exhibit several aging related defects. The ratio of $CD4^+/CD8^+$ cells increases along with an increase in differentiated memory T cells (Herndler-Brandstetter et al., 2011). However, there is a reduction in IL-2 production, cytolytic activity of CD8 T cells, and activation of T follicular helper cells, leading to impaired clonal expansion and altered differentiation in response to antigen stimulation (Kovaiou & Grubeck-Loebenstein, 2006; Sage et al., 2015; Scheuring et al., 2002). Antibodies in older individuals are short-lived, take longer to reach peak levels, and decline more rapidly (Henry et al., 2019). Moreover, aging individuals often have comorbidities that require long-term use of medication (Agarwal et al., 2018). A prior study demonstrated that older individuals who have been taking metformin long-term had significantly lower induction of antibody response upon influenza vaccination and the virus-neutralizing activity of their sera 28 days post vaccination was considerably reduced than those not taking metformin. Strategies to improve vaccine efficacy in aged individuals are imperative to reduce influenza-related illnesses and deaths.

1.7 Intranasal Vaccination and Mucosal Immunity

Intramuscular immunization is a common vaccination route because it is easy to administer, well-tolerated, and poses relatively fewer side effects. On the other hand, intranasal vaccination offers the advantage of being non-invasive, and needle-free, and demonstrates protection in both local mucosal sites and systemic milieu. In contrast, while intramuscular immunization triggers strong systemic responses by inducing and secreting IgG and IgA antibodies in the bloodstream, it does not generate robust immune responses at mucosal sites. When administered intramuscularly, dendritic cells (DCs) and macrophages capture the antigens and migrate to local lymph nodes, where they prime naïve T and B cells, leading to their differentiation

into effector cells, such as CD4 helper T cells and CD8 cytotoxic T lymphocytes. These effector cells then migrate to the site of injection via the bloodstream. Furthermore, naïve B cells differentiate into plasma cells, which secrete antigen-specific antibodies. Systemic IgG antibodies, along with mucosal IgG antibodies that diffuse from the bloodstream, are essential in providing protection following intramuscular administration of inactivated influenza vaccines (IIV) (Sridhar et al., 2015). In the case of intranasal immunization, DCs pick up antigens and travel to the nasopharyngeal tonsils/adenoids and palatine tonsils (Waldeyer's ring) sites rich in nasal-associated lymphoid tissues (NALT). The NALT is equipped with antigen-presenting cells (APCs), B and T cells and a layer of epithelial cells containing microfold (M) cells that are specialized for antigen uptake (Corr et al., 2008). APCs beneath the epithelial layer containing M cells phagocytose the absorbed antigens and present them to naïve T cells. The stimulated T cells secrete cytokines to activate B cells. The activated B cells then differentiate into antigen-specific IgA^+ B cells, and along with the antigen-specific T cells, migrate to the effector sites via the thoracic duct and blood circulation. At the effector sites, IgA-inducing cytokines such as IL-6, IL-10, and IL-21 cause terminal differentiation of the IgA^+ B cells to IgA-producing plasma cells (Rika Nakahashi-Ouchida et al., 2023). VLPs are a promising platform for intranasal (IN) immunization due to the absence of viral genetic material, making them safe for use. Their small structure closely resembles that of a natural virus, enhancing their ability to bypass the mucosal barrier, interact with NALT structures, and trigger immune responses effectively. IN immunization with VLPs expressing structural proteins of the 1918 H1N1 virus induced cross-reactive IgG and IgA antibodies and protected mice against the replication-competent homologous virus as well as a lethal challenge (L. A. Perrone et al., 2009).

1.8 Hypothesis and Research Objectives

1.8.1 Chapter 1: Supplementation of Seasonal Vaccine with Multi-Subtype Neuraminidase and M2 Ectodomain Virus-Like Particle Improves Protection Against Homologous and Heterologous Influenza Viruses in Aged Mice

Protection due to seasonal influenza vaccination relies on neutralizing antibodies directed against HA, whereas universal vaccines targeting conserved surface proteins, NA and M2e, induce non-neutralizing antibodies that mediate various effector functions. HA-based vaccines are strain-specific, whereas NA and M2e-based vaccines provide broad cross-protection although weak. Moreover, vaccine efficacy in the elderly is poor due to aging-related immune dysfunctions. The goal of this study was to compare the cross-protective efficacy of split vaccines (H1N1 + H3N2)with a novel NA-M2e VLP vaccine, comprising of consensus sequences of N1, N2, flu B NA and tandem repeats of M2e (5xM2e) in aged mice. Furthermore, to address the limitations of the strain-specificity of seasonal vaccines, we supplemented split vaccines with NA-M2e and administered them in aged mice in a prime-boost regimen. Evident from our studies, S+NA-M2e vaccine combination conferred better protection in homologous and heterologous challenges experiments by preventing body weight loss and lowering lung viral loads. Vaccine-specific responses due to S+NA-M2e vaccination were skewed towards T-helper type 1 immune response, and vaccine-specific antibodies raised in aged mice were effective in providing complete protection to young mice challenged in passive sera transfer experiments. The findings in this study present a promising strategy for broadening protection against antigenically distant influenza viruses in the elderly population.

1.8.2 A Strategy of Enhancing the Protective Efficacy of Seasonal Vaccines by Providing Additional Immunity to Neuraminidase and M2e

Developing a truly universal vaccine candidate is challenging due to the vast genetic and antigenic diversity among influenza viruses and the poor immunogenicity of conserved antigenic targets. The objective of this study was to investigate whether supplementing split vaccines (H1N1 + H3N2) with NA-M2e VLP vaccine (S+NA-M2e) enhances cross-protection against antigenically variant influenza viruses in healthy, young adult mice, as well as the innate and adaptive immune responses elicited due to this vaccination strategy. The S+NA-M2e vaccination strategy induced vaccine-specific antibodies to all 3 HA, NA, and M2e antigens and hemagglutination inhibition titers against homologous viruses. The S+NA-M2e vaccinated group showed a balanced Th1/Th2 immune response, inducing higher IgG2a antibodies compared to the split-only group. Furthermore, this group stimulated antigen-presenting innate immune cells 1 day after 1st dose of immunization and induced effector T cell responses after boost and challenge experiments. The S+NA-M2e vaccination strategy could aid in pandemic preparedness by providing enhanced protection in the event of strain mismatch.

1.8.3 Intranasal Vaccination with Multi Neuraminidase and M2 Ectodomain Virus-Like Particle Enhances Broad Protection Against Influenza

Nasal vaccines are known to induce immunity by inducing humoral and cellular responses at local mucosal sites as well as systemically. The NA-M2e VLP provides broad cross-protection against antigenically different influenza viruses in the intramucular route, but its efficacy in the intranasal route has not been explored. In Chapter 3, the goal was to evaluate whether intranasal immunization with NA-M2e VLP could elicit vaccine-specific immune responses and enhance protection against heterologous strains of the influenza virus. Intranasally immunized mice

demonstrated broad cross-protection against A/Cal/H1N1, A/PR8/H1N1, rgA/Viet/H5N1, and A/Phil/H3N2 viruses with significantly lower body weight loss and lung viral titers after challenge. Furthermore, this group demonstrated significantly increased IgA and IgG antibodies both locally and systemically and induced effector T cell responses and memory B cell responses at mucosal sites. Overall, intranasal immunization with NA-M2e enhances mucosal immunity and confers greater efficacy against influenza vaccine strains compared to intramuscular immunization.

2. Chapter 1: Supplementation of Seasonal Vaccine with Multi-Subtype Neuraminidase and M2 Ectodomain Virus-Like Particle Improves Protection Against Homologous and Heterologous Influenza Viruses in Aged Mice

Copyright © 2024, Antiviral Research, DOI: 10.1016/j.antiviral.2024.105877

2.1 Abstract

The conventional inactivated split seasonal influenza vaccine offers low efficacy, particularly in the elderly and against antigenic variants. Here, to improve the efficacy of seasonal vaccination for the elderly population, we tested whether supplementing seasonal bivalent (H1N1 + H3N2) split (S) vaccine with M2 ectodomain repeat and multi-subtype consensus neuraminidase (NA) proteins (N1 NA + N2 NA + flu B NA) on a virus-like particle (NA-M2e) would induce enhanced cross-protection against different influenza viruses in aged mice. Immunization with split vaccine plus NA-M2e (S+NA-M2e) increased vaccine-specific IgG antibodies towards T-helper type 1 responses and hemagglutination inhibition titers. Aged mice with NA-M2e supplemented vaccination were protected against homologous and heterologous viruses at higher efficacies, as evidenced by preventing weight loss, lowering lung viral loads, inducing broadly cross-protective humoral immunity, and IFN-γ^{+} CD4 and CD8 T cell responses than those with seasonal vaccine. Overall, this study supports a new strategy of NA-M2e supplemented vaccination to enhance protection against homologous and antigenically different viruses in the elderly.

2.2 Introduction

According to the World Health Organization (WHO), influenza epidemics cause 3-5 million cases of severe illness and 290,000 to 650,000 deaths each year worldwide (WHO, 2023). Hemagglutinin (HA) is the immunodominant surface glycoprotein, while neuraminidase (NA) is

the second major glycoprotein on the influenza surface. The current vaccines on the market target the HA variable global head domain, inducing HA strain-specific immunity (Wiersma et al., 2015). While vaccination is the most effective prevention measure, its efficacy is estimated to be 40-60% even when the circulating strain is well-matched with the vaccine strain (Hollingsworth et al., 2021) and further down to 10% when variants emerge (Zimmerman et al., 2016). Additionally, seasonal vaccines are less effective in the elderly over 65 years old (K. Goodwin et al., 2006; Sasaki et al., 2011; Simonsen et al., 2005). The aging population is most susceptible to seasonal flu-related deaths, with an increased risk of secondary infection (Keilich et al., 2019). The poor influenza vaccine efficacy in the elderly is the consequence of immunosenescence (Bowdish et al., 2015; Dorrington & Bowdish, 2013; Smetana et al., 2018). With aging, changes in the immune system increase susceptibility to influenza infections (Dorrington & Bowdish, 2013). The Centers for Disease Control (CDC) recommends fluzone high-dose quadrivalent vaccine, having four times the antigen amount or FLUAD quadrivalent adjuvanted flu vaccine, for individuals over 65 years of age in the US (CDC, 2023).

Universal influenza vaccine candidates are mostly focused on the conserved regions of the virus. The ectodomain of M2 ion channel protein (M2e) is highly conserved and often included in universal vaccine constructs such as heterologous tandem 5xM2e repeat presented on virus-like particles (VLP) (M. C. Kim, J. S. Lee, et al., 2013; M. C. Kim, J. M. Song, et al., 2013). NA undergoes slower antigenic changes than HA, and NA immunity can inhibit the release of viral progenies from infected cells and provide an independent correlate of protection (Memoli et al., 2016; Monto et al., 2015). Seasonal vaccination induces sub-optimal immune responses to M2e and NA antibodies due to immunodominant HA effects. To overcome these limitations, we previously reported multi-subtype consensus NA proteins (N1 NA + N2 NA + flu B NA) and

5xM2e repeat containing VLP (NA-M2e, Fig 1A) which could provide broad cross-protection (Kim et al., 2022). However, the comparison with seasonal vaccine for the immunologic benefits or weaknesses of NA-M2e vaccination remains to be determined when conferring homologous and heterologous protection particularly in an aging population model.

In this study to address the poor efficacy of seasonal influenza vaccines in the aging population and sub-optimal protection against variants, we hypothesized that supplementing an inactivated split seasonal vaccine with a cross-protective NA-M2e vaccine would confer enhanced protection against homologous and antigenically distant influenza strains in aged mice by inducing additional immunity to NA and M2e. We investigated the strengths and weaknesses of NA-M2e vaccination, in comparison with seasonal split (H1N1+H3N2) vaccine, as well as the impact of supplementing split vaccine with NA-M2e VLP on enhancing the immunogenicity and protection efficacy against homologous and heterologous virus challenges in aged mice.

2.3 Methods

2.3.1 Viruses and Vaccines

Live viruses A/California/04/2009 H1N1 (A/Cal/H1N1), A/Philippines/2/1982 H3N2 (A/Phil/H3N2), reassortant rgA/Viet/H5N1 (containing HA and NA from A/Vietnam/1203/2004 H5N1 and a A/Puerto Rico/8/1937 (A/PR8 H1N1) backbone) (Song et al., 2011), B/Florida/4/2006 (B/FL), and A/Switzerland/9715293/2013 (A/Swz/H3N2) were used for virus challenges. Viruses were amplified by propagation in 10-day-old embryonated chicken eggs. Harvested allantoic fluid was centrifuged (2,000 rpm, 20 minutes) and stored at -80 °C. Inactivated bivalent split vaccine strains used to immunize mice were A/Cal/H1N1 and A/Swz/H3N2 viruses, which were produced from 1% formalin-inactivated viruses and then treated with Triton X-100 for virion particle disruption as described (Bhatnagar et al., 2022). The quality of the split vaccines was determined

by hemagglutination activity units (HAU) assays, which provides information on the integrity of HA functionality and antigenic sites. H1N1 sCal split vaccine exhibited 255 HAU /µg sCal whereas H3N2 sSWz split vaccine 128 HAU/ µg sSWz. NA-M2e vaccine (Fig. 1A) was expressed in insect cells and prepared as previously described (Kim et al., 2022).

2.3.2 Immunization and Challenge

Aged BALB/c mice (n=10, 17–18-month-old female) obtained from Charles River (via NIH/NIA support) or young BALB/c mice (n=10, 5-6 weeks old female) were intramuscularly immunized with bivalent split vaccine (S) [total protein 1.2 µg inactivated split A/Cal/H1N1 (sCal) plus 0.6 µg inactivated split A/Swz/H3N2 (sSwz)], 10 µg NA-M2e, or S+NA-M2e combined vaccines in a prime-boost regimen at a 3-week interval. The sCal split vaccine 1.2 µg dose was adjusted to be similarly immunogenic in aged mice as 0.6 µg sSwz vaccine. HA content was estimated to be approximately 30% of total protein of inactivated virus vaccines (Lucy A. Perrone et al., 2009; Tarasov et al., 2020). NA-M2e was dosed at 10 µg to be consistent with previous studies on NA-M2e VLP (Kim et al., 2022) and 5xM2e VLP (M. C. Kim, J. S. Lee, et al., 2013). NA content incorporated on monomeric N1 NA VLP was estimated to be approximately 2% of total N1 VLP protein (K. H. Kim et al., 2019). Immunized mice along with naïve controls were then challenged with a lethal dose of A/Cal/H1N1 (5.3xLD_{50}, 2x10^3 EID_{50},) or A/Phil/H3N2 (3xLD_{50}, 5.6x10^2 $TCID_{50}$) 4 weeks after boost vaccination. The body weight changes, and survival were monitored daily for 14 days. All animal studies presented in this manuscript were approved by the Georgia State University (GSU) Institutional Animal Care and Use Committee review boards.

2.3.3 Antibody Responses and Hemagglutination Inhibition (HAI) Assay

Antibody responses were determined by ELISA as previously described (Bhatnagar et al., 2022; Ko et al., 2018). Coating antigens included 1 µg/ml N2 NA (A/Brisbane/10/2007 H3N2), N1 NA (A/Brisbane/59/2007 H1N1), influenza B NA (B/Florida/4/2006); 0.6 µg/ml H1 HA protein (A/California/04/2009 H1N1), H3 HA protein (A/Brisbane/10/2007 H1N1); and 4 µg/ml human M2e (hM2e, SLLTEVETPIRNEWGSRSN) peptide and inactivated influenza viruses A/California/04/2009 H1N1 (iCal H1N1), A/Switzerland/9715293/2013 H3N2 (iSwz H3N2), A/Philippines/2/1982 H3N2 (iPhil H3N2) and rgA/Vietnam/1203/2004 H5N1 (Viet H5N1). For *in vitro* IgG ELISA to determine IgG antibody-secreting cell responses of mediastinal lymph node (mLN) and spleen, harvested cells were applied onto pre-coated plates with respective antigens and cultured for 1 day (D1) or 5 days (D5). The IgG and IgG isotypes were measured using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a secondary antibodies as described (Bhatnagar et al., 2022). The quantitative concentrations of IgG were obtained by converting the respective OD values using the standard curve equations of purified IgG antibodies (Southern Biotech, Birmingham, AL). For the HAI assay, heat-inactivated sera treated with receptor-destroying enzymes (RDE, Sigma-Aldrich) were mixed with 4 HA units of viruses. 0.5% chicken red blood cells were added to the mixture to determine HAI titers as described (Bhatnagar et al., 2022).

2.3.4 Lung Viral Titration

10-day-old-embryonated chicken eggs were inoculated with diluted lung lysate samples, and allantoic fluid was tested for lung viral titers by determining their hemagglutination activity as described (Bhatnagar et al., 2022; Wen & Wang, 1959). For lung viral titration in MDCK cells, the cells were seeded at $4x10^4$ cells per well, and diluted lung lysates from infected mice were

added to the cells following a 3-day incubation. HAI assay was performed with harvested supernatant to determine viral titers.

2.3.6 Cytokine Responses

Ready-Set-Go IL-6, TNF-α kits (eBioscience, San Diego, CA), and Mouse IFN-γ Uncoated ELISA kit from Invitrogen were used to determine cytokine levels in lung lysate samples and bone-marrow-derived dendritic cell (BMDC) cultures (Ko et al., 2018).

2.3.7 Flow Cytometry

Spleen, mLN or lung tissues harvested from aged mice on day 7 or day 9 post challenge were homogenized and prepared as single-cell suspensions. Single cells were *in vitro* stimulated with 5 μg/ml of the following antigens N1 NA (A/Cal/04/2009 H1N1), N2 NA (A/Brisbane/10/2007 H3N2) and hM2e peptide in the presence of Brefeldin A (20 μg/ml) at 37°C for 5 hours. Staining of lymphocytes was done with anti-mouse CD3 (clone 17A2, BD, San Diego, CA), CD4 (clone 553051, BD), CD8 (clone 25-0081-82, eBiosciences, San Diego, CA), and IFN-γ (clone XMG1.2, BD) mAb. For intracellular cytokine staining, the BD Cytofix/Cytoperm Plus kit was used (Ko et al., 2018). The Becton-Dickinson LSR-II/Fortessa flow cytometer was applied to acquire the cytokine-expressing cells, which were then analyzed by Flowjo software (Tree Star, Inc., Ashland, OR). The data were converted to absolute cell numbers from the cell count obtained from harvested cells.

2.3.8 In Vivo Protection Experiment with Antisera

In passive serum protection experiments, sera from aged naïve or immunized mice were heat-inactivated at 56°C, mixed with a lethal dose of viruses, A/California/04/2009 H1N1 (2xLD_{50}, 1.6x10^4 EID_{50}) in BALB/c mice, A/Switzerland/9715293/2013 H3N2 (7xLD_{50}) in DBA/2J mice, rgA/Vietnam/1203/2004 H5N1 (4xLD_{50}, 2.6x10^4 EID_{50}) in C57BL/6 mice, B/Florida/04/2006

($2xLD_{50}$, $5.3x10^4$ EID_{50}) in BALB/c mice, and incubated at room temperature for 30 minutes as described (Getie-Kebtie et al., 2013). The mixture of virus and sera was administered intranasally to young naïve mice (5-7 weeks old), and body weight change patterns were monitored daily for 2 weeks.

2.3.9 In Vitro BMDC Experiment

The bone marrow (BM) cells were harvested from aged (17 months old) mice's femur and tibia regions and cultured in the presence of recombinant mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF) to enrich dendritic cells (DCs) as described (Le et al., 2021). After culturing for 6 days, harvested immature BMDCs were treated with split (1.2 µg), NA-M2e (1 µg, 10 µg), or split (1.2 µg) +NA-M2e (1 µg, 10 µg) vaccines. Supernatants from BMDC cultured for 2 days were used to determine cytokines.

2.3.10 Statistical Analysis

All data in this study are represented as mean ± standard errors of the mean (SEM). To determine statistical significance for all experiments, unpaired two-tailed Student's *t* tests, one-way or two-way analysis of covariance (ANOVA) was performed, followed by Bonferroni or Tukey's multiple comparison posttests. P values that were less than 0.05 ($p<0.05$) were considered statistically significant. The prism software (GraphPad Software, Inc., San Diego, CA) was used for data analysis.

2.4 Results

2.4.1 Addition of NA-M2e to Split Vaccine Enhances Immune Responses in Aged Mice

To test whether NA-M2e supplemented split (sCal H1N1 + sSwz H3N2) (Fig. 1A) vaccination would enhance the efficacy of seasonal vaccine, groups of aged mice were intramuscularly vaccinated with split, NA-M2e, or S (split) +NA-M2e vaccines, and IgG levels

analyzed at 2 weeks after each immunization. IgG antibodies specific for iCal H1N1 were higher after 1st dose (prime) with S+NA-M2e than other vaccines without statistical significance (Fig. 1B). Significant increases in IgG levels specific to iSwz H3N2 and M2e were observed after 1st dose in the S+NA-M2e group compared to split (Figs. 1C-D). S+NA-M2e 2nd dose (boost) immunization-induced IgG antibodies for iCal H1N1, iSwz H3N2, M2e, N2 NA, N1 NA, and flu B NA at the highest level (Figs. 1E-J). The S+NA-M2e group also showed high IgG responses to inactivated A/Phil/H3N2 and inactivated rgA/Viet/H5N1 virus strains (Figs. 2A, B). Overall, substantial increases and the highest levels of vaccine-specific IgG responses were observed in the supplemented split group compared to split alone after 1st dose and 2nd dose vaccinations in aged mice.

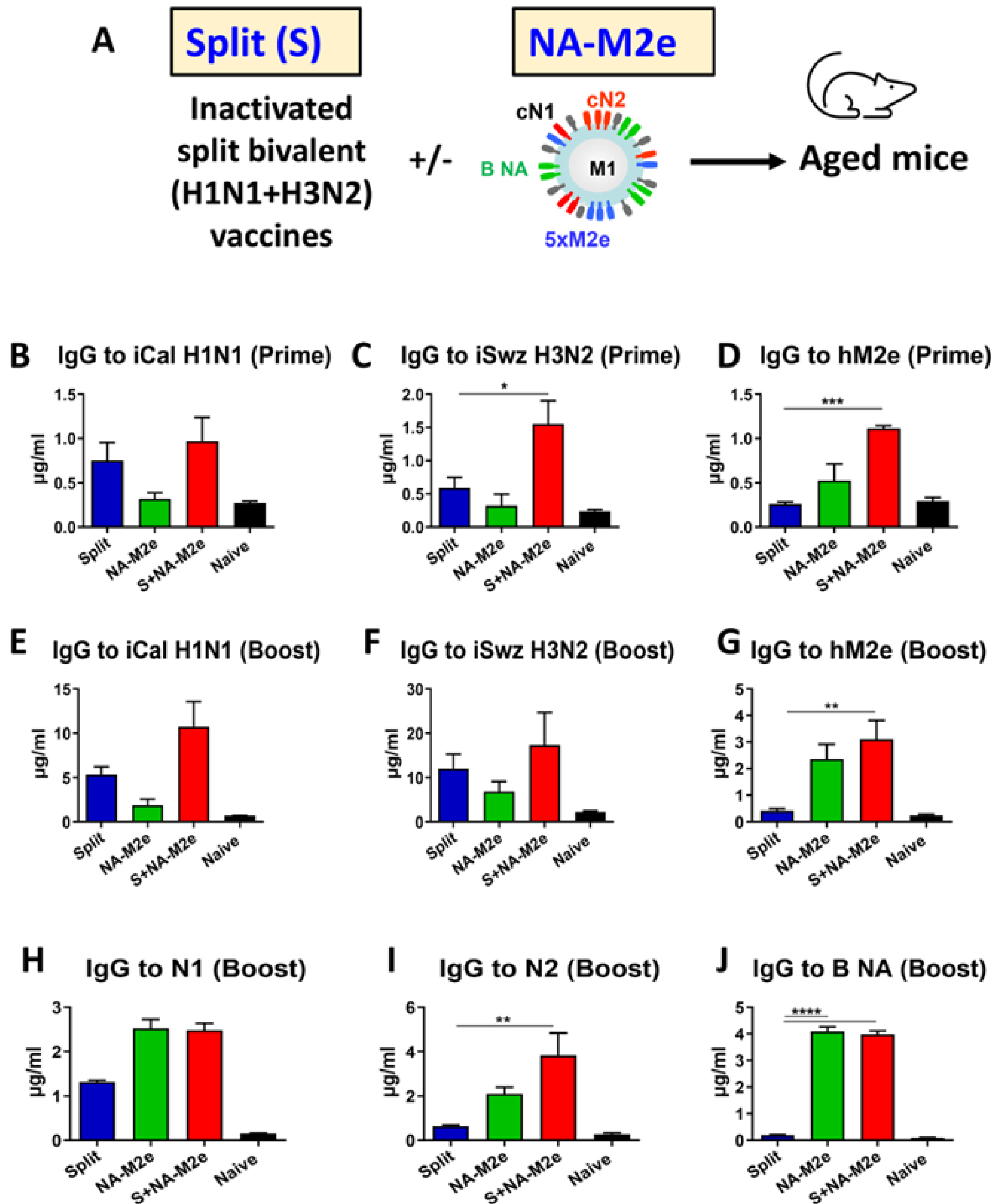


Figure 1. Supplementation with NA-M2e Enhances Antibody Responses to Vaccine Antigens. (A) Diagram showing split (S) ± NA-M2e vaccines. Groups of 17 months-old, aged mice were primed (1st dose) and boosted (2nd dose) with either inactivated bivalent split vaccine containing sCal (A/California) H1N1 + sSwz (A/ Switzerland) H3N2, NA-M2e (VLP containing N1 NA + N2 NA + flu B NA and 5xM2e) or S + NA-M2e combination vaccines. Prime IgG antibody responses specific for (B) iCal H1N1, (C) iSwz H3N2, (D) hM2e and boost IgG antibody responses specific for (E) iCal H1N1, (F) iSwz H3N2, (G) hM2e, (H) N1 NA, (I) N2 NA, and (J) flu B NA were determined by ELISA and presented in concentrations (µg/ml). Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with other groups and performed by (B–J) using two-way ANOVA with Dunnett's posttest and indicated as *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, and ****; $p < 0.0001$.

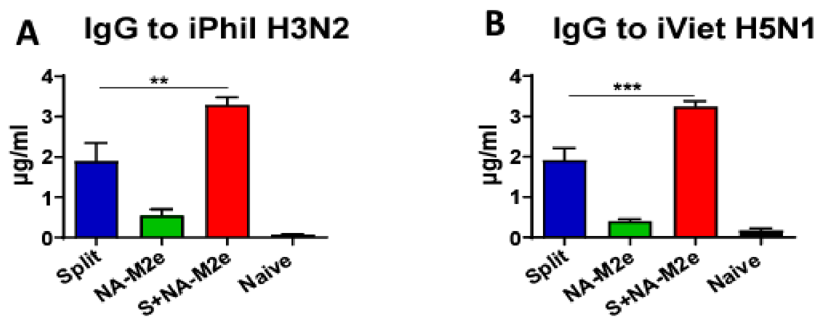


Figure 2. IgG Responses to Heterologous Viral Antigens. Groups (n=5) of 17 months-old, aged mice were primed (1st dose) and boosted (2nd dose) with either inactivated split (bivalent vaccine containing sCal H1N1 + sSwz H3N2), NA-M2e (VLP containing N1 NA + N2 NA + flu B NA and 5xM2e) or S+NA-M2e combination vaccines. IgG antibody responses to viral antigens (μg/ml) in boost sera from vaccinated aged mice. (A) IgG response to inactivated A/Philippines/02/1982 H3N2. (B) IgG response to inactivated rgA/Vietnam/1203/2004 H5N1. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with other groups and performed by using one-way ANOVA with Dunnett's comparison test and indicated as **; p<0.01, ***; p<0.001.

2.4.2 S+NA-M2e Vaccines Increase HAI Activities, IgG Isotypes, and IgG Levels Reactive to Viral Antigens

The 2nd dose serum IgG antibody responses to H1 HA and H3 HA antigens (Figs. 3A-B) in the S+NA-M2e group revealed significantly higher IgG levels by 3-5-fold-changes than the split group, consistent with NA-M2e adjuvant effect. Immuno-senescence is evident as split vaccination of aged mice induced significantly lower levels of IgG antibodies to H1 HA and H3 HA protein antigens than young adult mice (Figs. 4A-B) when administered with the same bivalent vaccine (sCal + sSwz). The S (sCal + sSwz) +NA-M2e group increased HAI titers in boost sera against the homologous A/Cal/H1N1 by 2 fold-changes (Fig. 3C) and A/Swz/H3N2 by 4 fold-changes (Fig. 3D) compared to the split group (~20 HAI titers). No groups showed cross-reactive HAI activity against A/Phil/ H3N2 virus (Fig. 3E) as this strain is antigenically far distant to A/Swz/H3N2 vaccine (A/Phil/1982 versus A/Swz/2013 year of virus isolation).

Split vaccination induced IgG1 but not IgG2a isotype antibodies (Figs. 3F-G, I-J). In contrast, S+NA-M2e vaccinated mice raised higher levels of IgG2a specific for iCal and iSwz than the split and NA-M2e groups (Figs. 3G, 3J). The NA-M2e group induced substantial levels of IgG2a for iSwz H3N2 (Figs. 3J), indicating the contribution of IgG specific for N2 NA. Accordingly, the S+NA-M2e group had the highest IgG2a/IgG1 ratios for all antigens (Figs. 3H, 3K). Interestingly, the split group showed higher IgG1 levels to iCal H1N1 antigen than S+NA-M2e group but the pattern is reversed with iSwz H3N2 antigen, indicating differential immunogenicity between sCal H1N1 and sSwz H3N2 vaccines. Importantly, the overall IgG2a/IgG1 ratio remained higher for S+NA-M2e group for both iCal H1N1 and iSwz H3N2 antigens. These results reveal that the addition of NA-M2e to split vaccines enhanced HAI titers and IgG2a isotype antibodies, indicating type I T-helper (Th1) responses.

We found that 2nd dose sera from S+NA-M2e vaccinated mice recognized homologous (A/Cal/H1N1) (Fig. 5A) and heterologous N1 NA virus antigens expressed on the infected cells (A/WSN/H1N1, A/PR8/H1N1, A/Viet/H5N1; Figs. 5B-D) at higher levels than the split and NA-M2e groups. Second dose sera from the S+NA-M2e group also showed significant levels of IgG antibodies reactive to viral antigens expressed on the cell surfaces by infecting with N2 and N9 NA viruses (A/HK/H9N2, A/NC/H3N2, A/HK/H3N2, A/SH/H7N9; Figs. 5E-H). Split and NA-M2e groups' antisera showed higher levels of IgG reactivity to cell surface viral antigens compared to naïve sera.

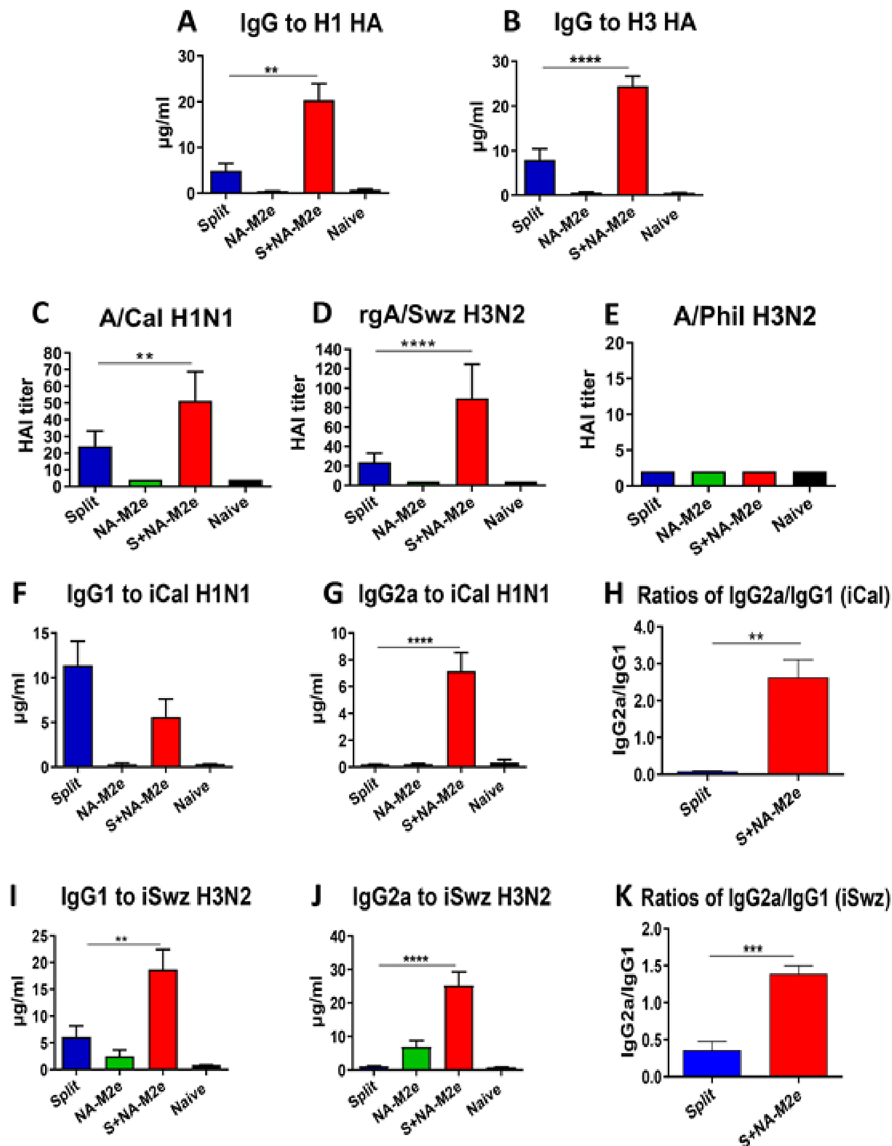


Figure 3. Immunization with S + NA-M2e Vaccines Elevates HAI Titers and IgG2a Levels. (A–B) Boost (2nd dose) IgG responses (in µ g/ml) to H1 HA (derived from A/California/2009) and H3 HA (from A/Brisbane/2007) antigens. HAI titers in boost sera against (C) A/California/H1N1, (D) A/Switzerland/H3N2, and (E) A/Philippines/H3N2 viruses. IgG isotype responses in boost sera to iCal H1N1 (F) IgG1, (G) IgG2a, (H) Ratio of IgG2a/IgG1. Responses to iSwz H3N2 (I) IgG1, (J) IgG2a, (K) Ratio of IgG2a/IgG1. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with S + NA-M2e group and performed by using one way ANOVA (A-E, F-G, I-J) with Dunnett's multiple comparison test or unpaired two-tailed Student's t-test (H, K) and indicated as **; $p < 0.01$, ***; $p < 0.001$ and ****; $p < 0.0001$.

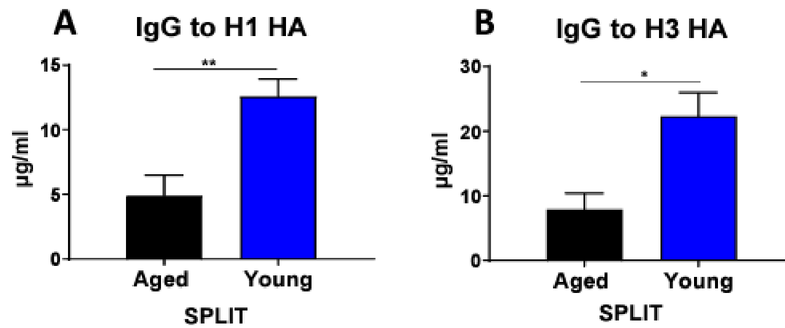


Figure 4. Higher Levels of IgG Antibody Responses to HA Protein Antigens in Split Immunized Young Mice Compared to Aged Mice. Groups of young (6 – 8 weeks old) and aged (17 months old) mice were boost (2nd dose) vaccinated with the same inactivated split vaccines (1.2 µg sCal H1N1 + 0.6 µg sSwz H3N2) and IgG antibody responses to H1 HA (derived from A/California/2009) and H3 HA (from A/Brisbane/2007) protein antigens in boost sera were determined by ELISA. (A) IgG responses to H1 HA. (B) IgG responses to H3 HA. Error bars are represented as mean ± SEM. Statistical significance was determined by using unpaired, two-tailed Student's t test and indicated as *; $p<0.05$, **; $p<0.01$.

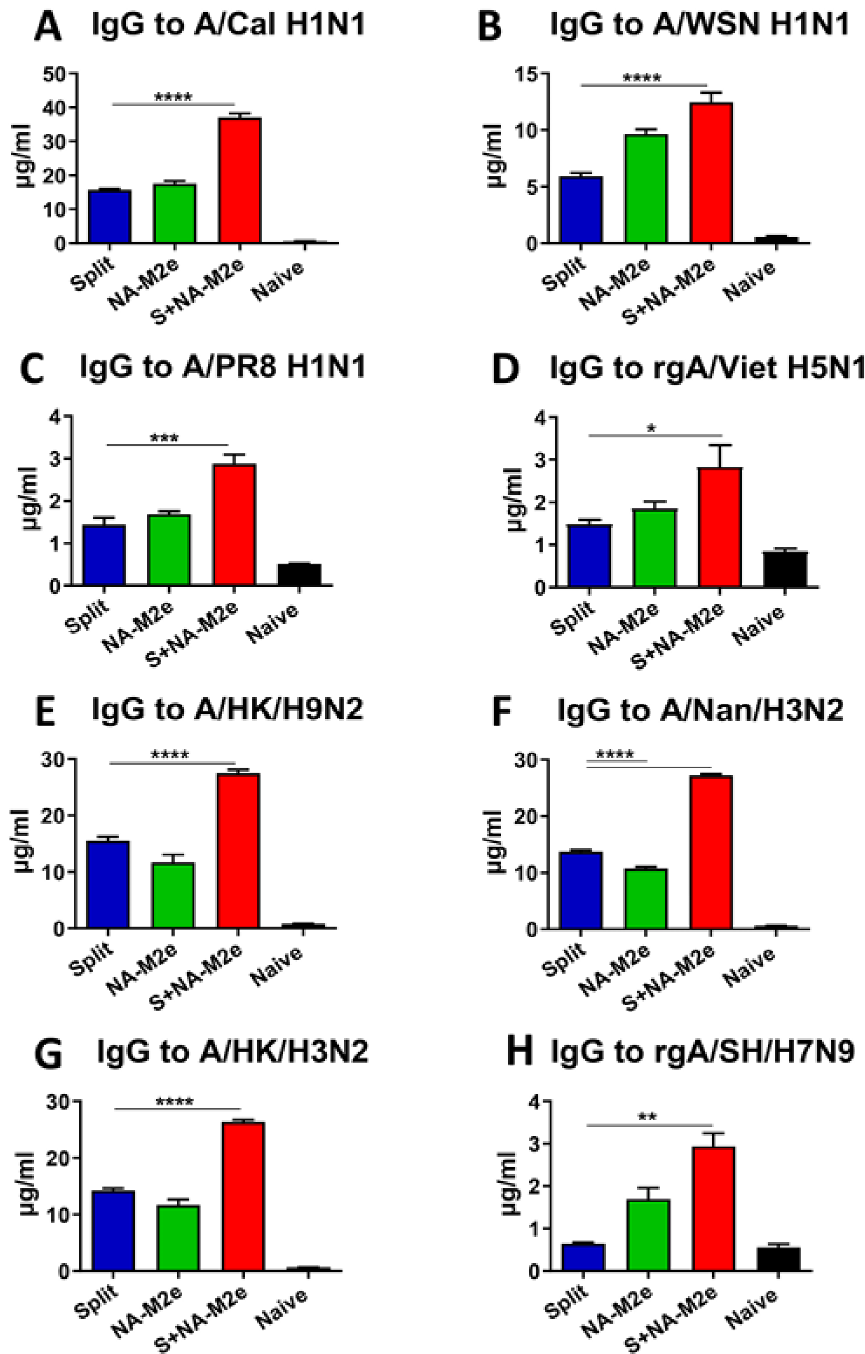


Figure 5. Sera from S + NA-M2e Vaccinated Group Show High Reactivity to Group 1 NA and Group 2 NA Viral Antigens Expressed on Infected Cells. Antibody levels in 2nd dose (boost) sera (g/ml) recognizing viral antigens expressed on MDCK cells presented after infection with each virus. Reactivity to N1 NA viruses (A) A/California/04/2009 H1N1 (B) A/WSN/1933 H1N1 (C) A/Puerto Rico/8/1934 H1N1 and (D) reassortant rgA/Vietnam/1203/2004 H5N1. Reactivity to N2 and N9 NA viruses (E) A/Hong Kong/1073/99 H9N2 (F) A/Nanchang/933/1995 H3N2 (G) A/Hong Kong/1/1968 H3N2 and (H) rgA/Shanghai/02/2013 H7N9 (A/PR8 backbone). Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with other groups and performed by using two-way ANOVA with Dunnett's multiple comparison test and indicated as **; $p < 0.01$, ***; $p < 0.001$ and ****; $p < 0.0001$.

2.4.3 S+NA-M2e Vaccination Promotes the Efficacy of Homologous and Heterologous Protection in Aged Mice

To determine the protective efficacy of NA-M2e supplemented split seasonal vaccination (S+NA-M2e), the mice were challenged with A/Cal/H1N1 virus. The S+NA-M2e group lost approximately 7% of body weight, whereas naïve mice displayed over 20% weight loss and had to get euthanized by day 9 (Figs. 6A-B). The NA-M2e (monitored for 9 days before sacrifice) and split groups showed about 11% and 13% weight loss, respectively. All vaccinated mice recovered by day 14 and were 100% protected against lethal challenge. The split alone vaccinated group still retained substantial levels of viral titers on day 9 post-challenge in aged mice, which were lower than those in naïve mice with infection. The NA-M2e group displayed lower EID_{50} lung viral titers (Fig. 6C) or similar $TCID_{50}$ titers (Fig. 6D) as split without significant differences. On the other hand, the S+NA-M2e group was highly effective in reducing viral loads by over 10,000 and 500 fold-changes, compared to the naïve and split vaccine groups, respectively in both egg and MDCK substrates. Inflammatory cytokines were not detected (for IL-6) or observed at a low level for IFN-γ in lungs from the S+NA-M2e group, which were produced at the highest level in naïve mice after infection (Figs. 6E-F). The split and NA-M2e groups had similar IL-6 levels, which were lower by 1.6 fold-changes than those of naïve infected mice. Overall, the results indicate that supplementing split vaccines with NA-M2e is effective in providing protection against homologous A/Cal/H1N1 challenge compared to either split or NA-M2e vaccine alone in aged mice.

To assess the efficacy of heterologous protection, we challenged mice with A/Phil/H3N2 virus. Unvaccinated mice experienced severe weight loss and did not survive lethal challenge. Both S+NA-M2e and NA-M2e groups (monitored for 9 days before sacrifice) showed moderate

(10-12%) weight loss and 100% protection (Figs. 7A-B). The split group showed substantial weight loss and 50% survival rates. S+NA-M2e vaccinated mice effectively controlled viral loads by showing a 100-fold reduction in lung viral titers in egg substrates and lowering $TCID_{50}$ titers by 1,000 fold-changes, compared to viral titers in naïve mice (Figs. 7C-D). The NA-M2e group showed a similar pattern in lung viral titers as the S+NA-M2e group and was 10-fold less than that of split vaccine alone (Fig. 7C). The S+NA-M2e group had the lowest IL-6, IFN-γ, and negligible TNF-α levels, preventing cytokine-mediated lung inflammation due to viral infection (Figs. 7E-G). The naïve group had the highest levels of pro-inflammatory cytokines, followed by the split and then NA-M2e groups. Altogether, these results provide evidence that supplementation of split vaccines with NA-M2e promotes the efficacy of homologous and heterologous protection in aged mice.

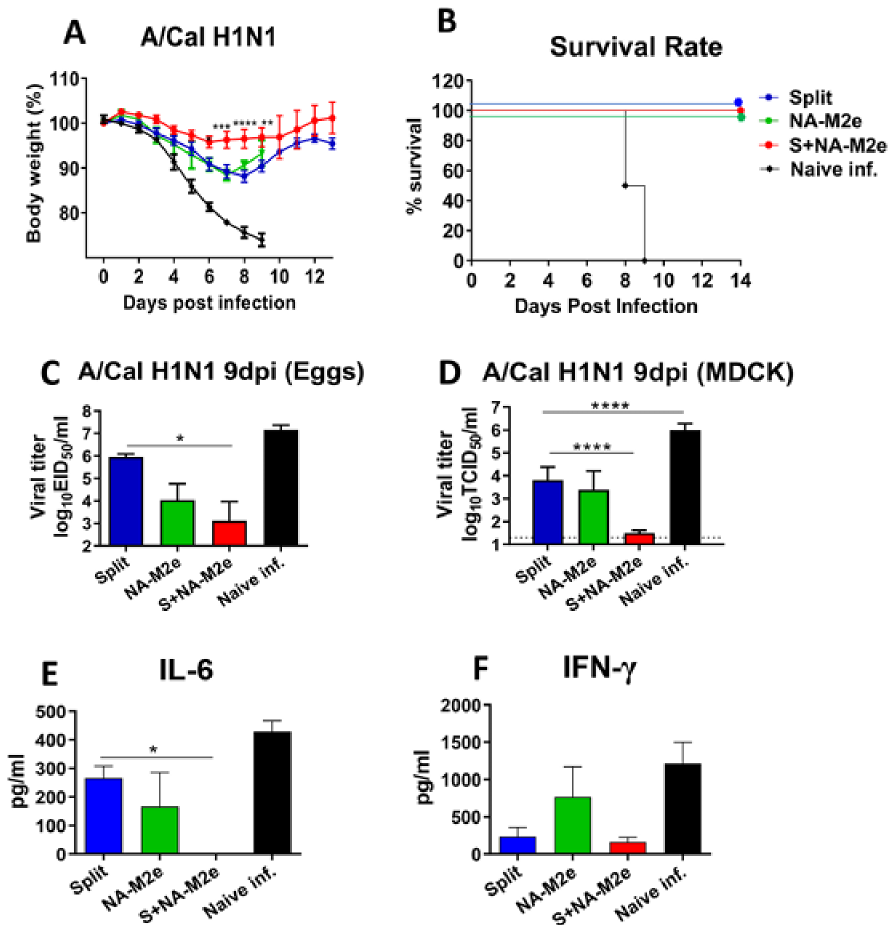


Figure 6. Supplemented S + NA-M2e Vaccine Confers Enhanced Protection Against Homologous A/Cal/H1N1 Virus in Aged Mice. (A) Body weight changes for 14 days (B) Survival rate. (C–F) Mice were sacrificed on day 9 post challenge (9 dpi) to harvest tissues. Lung viral titers in eggs (C) and in MDCK cells (D). (E–F) Cytokine responses in lung lysates (E) IL-6 (F) IFN-γ. Error bars are represented as mean ± SEM. The statistical significance was determined by using one (C–F) or two-way ANOVA (A) with Tukey's multiple comparison test and indicated as *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, and ****; $p < 0.0001$.

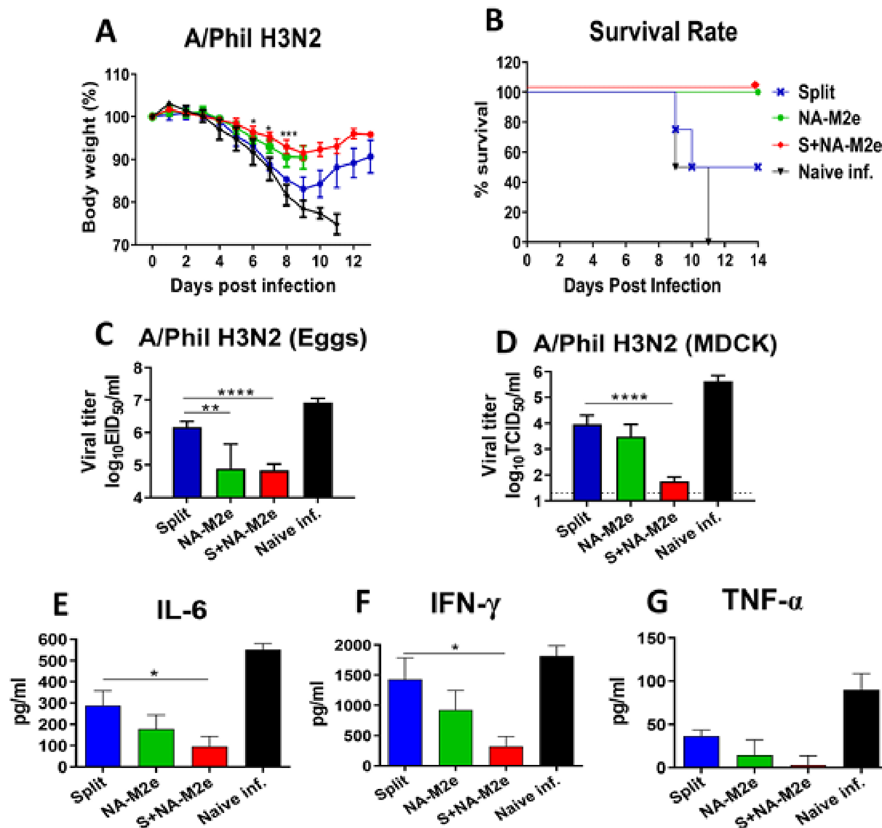


Figure 7. Improved Protection Against Heterologous A/Phil/H3N2 Virus is Conferred by S + NA-M2e Vaccine in Aged Mice. (A) Body weight changes and (B) Survival rate for 14 days. (C–G) Data at day 9 post challenge. Lung viral titers in eggs (C) and in MDCK cells (D). Cytokine responses in lung lysates (E) IL-6 (F) IFN-γ (G) TNF. Error bars are represented as mean ± SEM. The statistical significance was determined by using one (C–G) or two-way ANOVA (A) with Tukey's multiple comparison test and indicated as *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, and ****; $p < 0.0001$.

2.4.4 NA-M2e Supplemented Split Vaccination Promotes the Induction of Cellular Immune Responses

Antibody-secreting cell (ASC) responses *in vitro* were determined after culturing cells from spleen and mLN harvested on day 9 post heterologous challenge (Figs. 8A-D). The S+NA-M2e group produced higher levels of IgG antibodies to virus and NA antigens in both spleen and mLN cells after culturing than those in other groups (Figs. 8A-C, 9A, 9C). Also, higher levels of M2e-specific IgG antibodies were secreted in both NA-M2e and S+NA-M2e groups (Figs. 8D and

9B). The split group showed IgG antibodies to iCal H1N1 and iSwz H3N2 antigens at a moderate to high level, but low or background levels of IgG antibodies to NA and M2e antigens.

Flow cytometry results of intracellular cytokine staining showed that both NA-M2e and supplemented S+NA-M2e groups induced significantly higher numbers of IFN-γ^+CD4 and IFN-γ^+CD8 T cells upon stimulation with M2e and N1 NA antigens (Figs. 8E-F) than those in the split alone group. When stimulated with N2 NA, mLN cells from the NA-M2e group showed the highest number of IFN-γ^+CD4 and IFN-γ^+CD8 T cells. Compared to the split and naïve infection control groups, the S+NA-M2e group displayed significantly enhanced levels (4 to 5 fold-changes) of IFN-γ+ splenic CD4 and CD8 T cells upon stimulation with hM2e, N2, or N1 vaccine antigens day 7 post A/Cal/H1N1 challenge (Fig. 10A-B). The aged mice with S+NA-M2e vaccination induced increased IFN-γ^+ CD4 T cells in mLN upon stimulation with hM2e, N1, or N2 antigens by approximately 2 fold-changes (Fig. 11) when compared to naïve infected group. The S+NA-M2e immunized young adult mice could elicit highly enhanced levels (3 to 4-fold-changes) of IFN-γ^+ CD4 T cells in mLN (Fig. 11), suggesting that young adult mice are more effective in inducing CD4 T cell responses than aged mice.

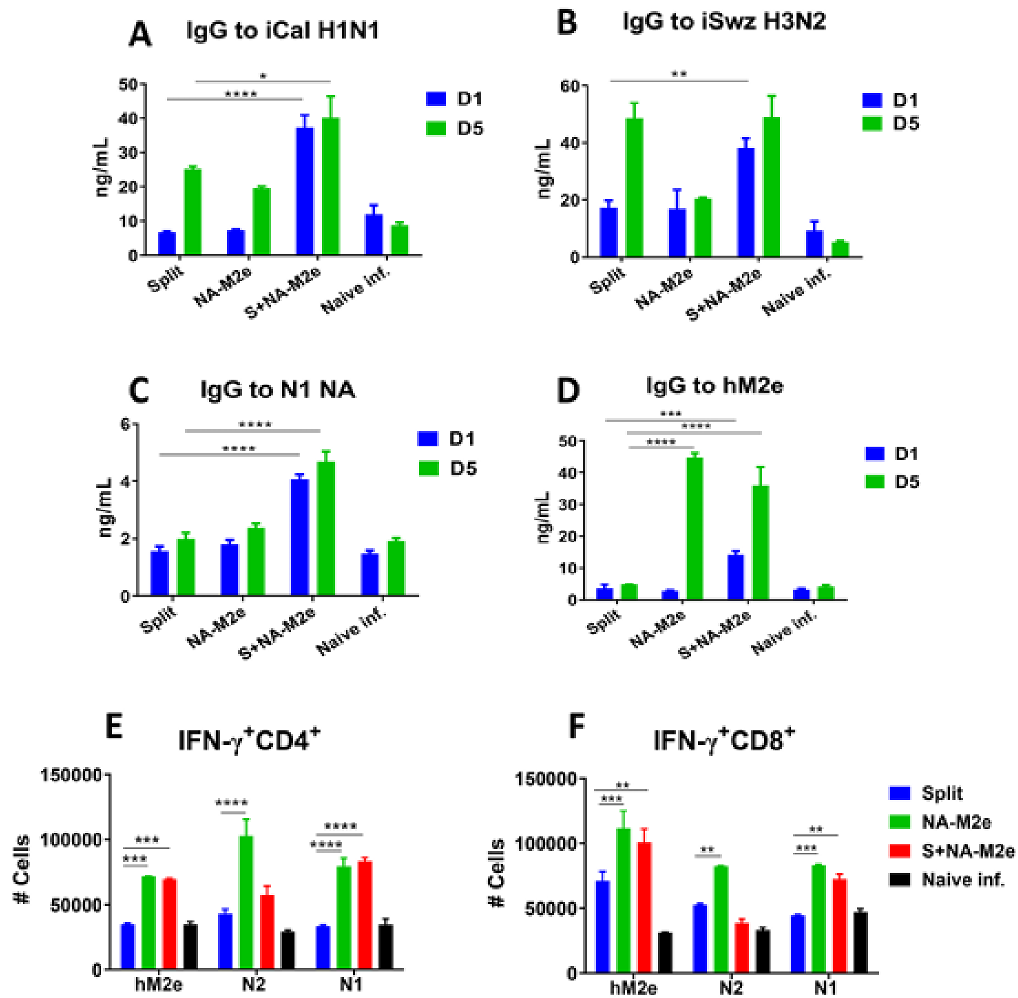


Figure 8. Humoral and Cellular Responses After Challenge. (A–D) IgG antibody levels secreted during in vitro cultures for 1-day (D1) and 5 days (D5) in spleen (A–C) and mLN (D) cells harvested on day 9 post challenge with A/Phil/H3N2. Intracellular cytokine staining to measure (E) IFN-γ + CD4 + and (F) IFN-γ + CD8 + T cell responses in mLN cells. Error bars are represented as mean ± SEM. The statistical significances were determined by comparing split group with other groups and performed using two-way ANOVA (C–G) with Dunnett's multiple comparison test and indicated as *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, and ****; $p < 0.0001$.

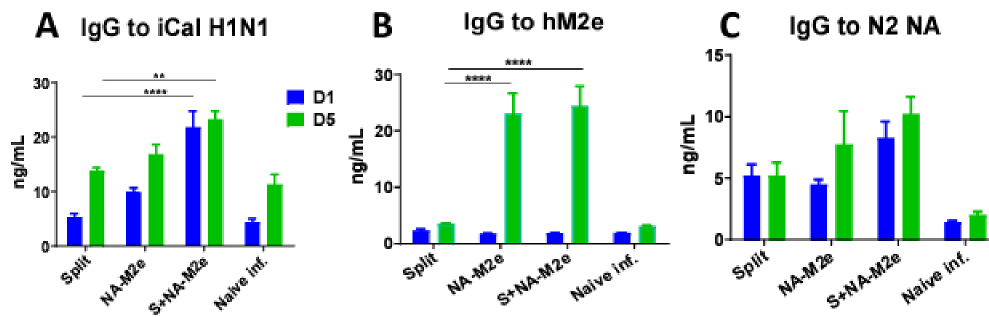


Figure 9. Effector Humoral Responses After Heterologous Challenge (9 dpi). Antibody levels secreted during in vitro cultures for 1 day (D1) and 5 days (D5) were determined in tissues harvested day 9 post challenge with A/Phil/H3N2 4 weeks after boost immunization. IgG antibody response to (A) iCal H1N1 in mLN cells (B) hM2e in spleen cells (C) N2 NA in spleen cells. Error bars are represented as mean ± SEM. The statistical significances were determined by comparing split group with other groups and performed using two-way ANOVA (C-G) with Dunnett's multiple comparison test and indicated as **; p<0.01, and ****; p<0.0001.

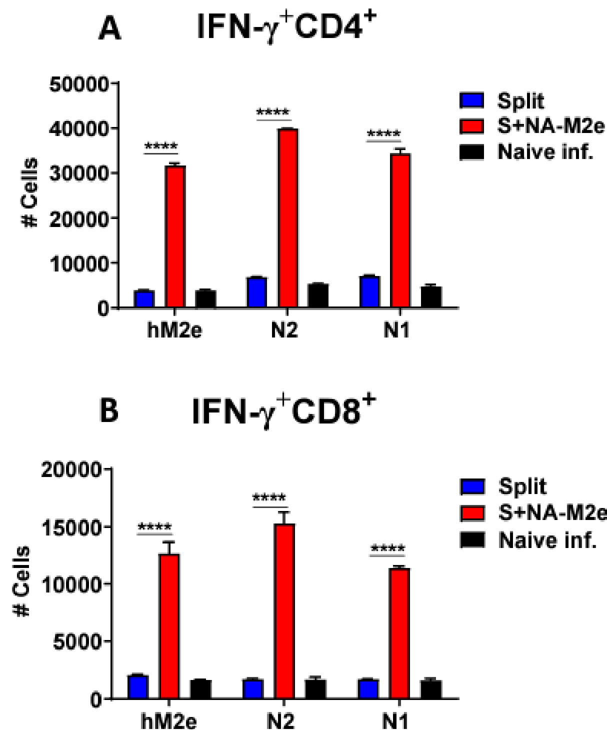


Figure 10. Significantly Enhanced IFN-γ+ Splenic T Cell Responses in Aged Mice with S+NA-M2e Vaccination After A/Cal/H1N1 Challenge. Groups of 17-months old aged mice (n=5) were boost (2nd dose) immunized with split or S+NA-M2e vaccines and sacrificed at day 7 after homologous A/Cal/H1N1 challenge. The mice were 19 months old when intracellular cytokine staining was done with splenocytes. (A) IFN-γ+CD4+ T cells (B) IFN-γ+CD8+ T cells upon hM2e, N1 NA, N2 NA in vitro stimulation. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with other groups and performed by using two-way ANOVA with Dunnett's comparison test and indicated as ****; p<0.0001.

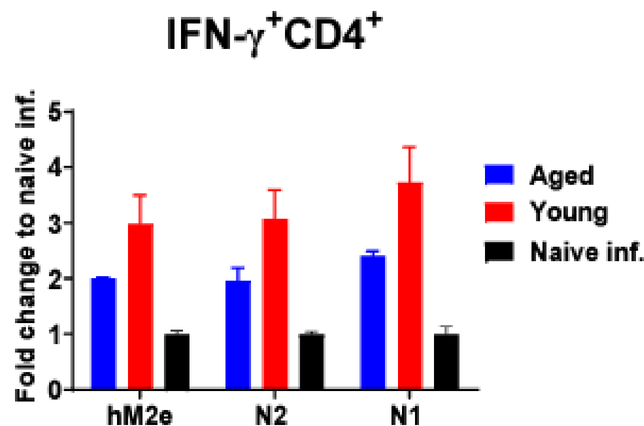


Figure 11. Young Adult Mice Show Higher Fold Increases in IFN-γ+ CD4 T Cell Responses Than Aged Mice. Groups (n=5) of young (6-8 weeks old) and aged (17 months old) mice were boosted (2nd dose) with the same dose of S+NA-M2e vaccines as described in the Methods section and challenged with A/Phil/H3N2. Young mice were 3-4 months old and aged mice 19 months old at the time of sacrifice and analysis of intracellular cytokine staining (mLN). Fold changes in IFN-γ+ T cell responses from vaccinated mice are presented in comparison with those from naïve infected mouse controls. Error bars are represented as mean ± SEM. The statistical significances were determined by using two-way ANOVA with Tukey's multiple comparison test and indicated as *; $p<0.05$, **; $p<0.01$.

2.4.5 Antisera of NA-M2e Supplemented Vaccination Confer Homologous and Heterologous Protection

To ascertain the protective roles of antisera from vaccination of aged mice, naïve mice from different backgrounds were intranasally infected with influenza virus and antisera (Fig. 12). Antisera from split, NA-M2e, or S+NA-M2e vaccination conferred 100% protection, preventing weight loss against A/Cal/H1N1 in naïve BALB/c (Figs. 12A-B). Protected young mice (5 weeks old in the growth phase) kept increasing their weights despite infection. As DBA/2J mice have natural killer (NK) cell dysregulation, enhanced lethality to the A/Swz/H3N2 virus was reported (Frank & Paust, 2020; McMahon et al., 2022). In a set with antisera and a high lethal dose (7x LD_{50}) of A/Swz/H3N2 (Fig. 12C-D), antisera from S+NA-M2e vaccination (but not other vaccines) provided resistance against severe weight loss in naïve DBA/2J mice. In a lethal

heterologous rgA/Viet/H5N1 infection (Figs. 12E-F), NA-M2e and S+NA-M2e antiserum recipients of naïve C57BL/6 mice were 100% protected despite weight loss, whereas both groups that were inoculated with virus and split or naïve sera did not survive. Lastly, NA-M2e or S+NA-M2e antisera conferred 100% protection (<4% body weight loss) against the lethal B/Florida virus in naïve BALB/c mice (Figs. 12G-H). These data support evidence that antibodies induced by NA-M2e supplemented seasonal vaccination in aged mice are effective in conferring homologous and heterologous protection against influenza viruses.

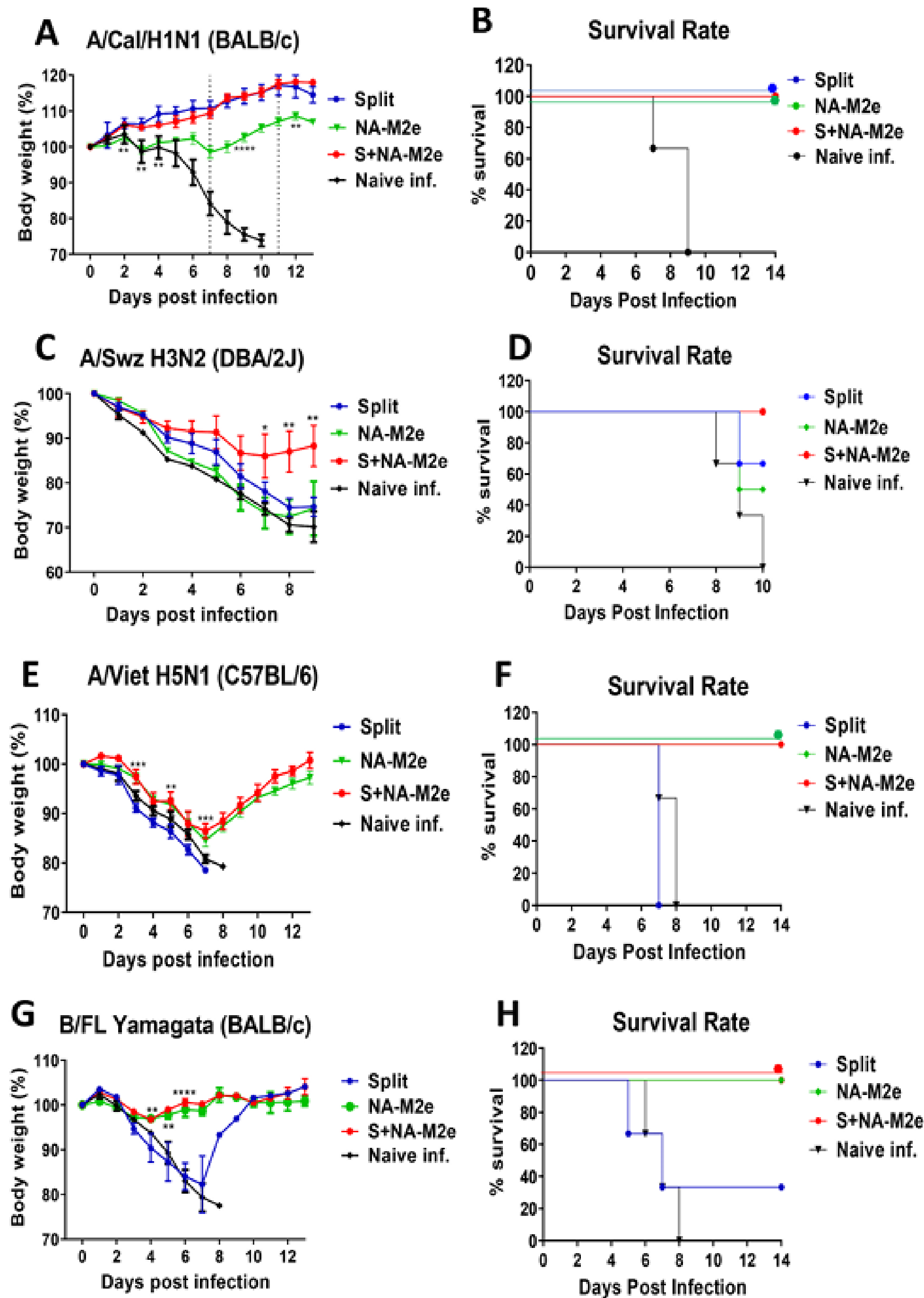


Figure 12. Roles of Antisera from Vaccinated Aged Mice in Conferring Protection Against Homologous and Heterologous Influenza Viruses in Naïve Adult Mice. Naïve mice were intranasally inoculated with a mix of each virus and boost antisera and monitored for weight changes and survival rates. (A–B) A/Cal/H1N1 infection with antisera in BALB/c. (C–D) A/Swz/H3N2 infection with antisera in DBA/2J. (E–F) rgA/Viet/H5N1 infection with antisera in C57BL/6. (G–H) B/FL infection with antisera in BALB/c. Error bars are represented as mean ± SEM. The statistical significances were determined by comparing split group with NA-M2e and S + NA-M2e groups and performed using two-way ANOVA (C–G) with Tukey's multiple comparison test and indicated as *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, and ****; $p < 0.0001$.

2.5 Discussion

It is highly significant to develop effective vaccinations in the elderly population projected to increase up to 25% of the world population by 2050 (Rehman, 2012). Studies using aged mouse models and comparison of seasonal and universal vaccines are rare due to low immune responses and difficulty of getting protected by subunit or split vaccines. Here, supplemented split vaccinations induced higher levels of M2e and NA immunity than split alone, in addition to higher IgG and HAI responses to HA and viral antigens, contributing to improved homologous and heterologous protection in aged mice. The results in this study support a strategy of supplemented seasonal vaccination inducing HA, NA, and M2e immunity to provide enhanced protection in the elderly.

Adjuvanted NA protein vaccination was reported to provide homologous protection (Wohlbold et al., 2015). Previous studies have demonstrated the protective effects of supplementing HA vaccines with N2 NA protein (10 µg), inducing NA-specific IgG responses, and providing enhanced protection against heterologous H3N2 virus (B. E. Johansson et al., 1998; Johansson et al., 2002). Addition of NA protein to inactivated influenza vaccination was reported to be less effective in inducing NA-specific antibody responses compared to separate injections, probably due to suppression by immunodominant HA (Strohmeier et al., 2021). Limited breadth of cross- protection by NA alone immunity could be partially overcome by NA-M2e vaccine composed of 5xM2e and multi-subtype NA proteins on VLP (Kim et al., 2022).

For the first time in this study, we investigated whether NA-M2e and supplemented seasonal bivalent split vaccine would confer higher efficacy of homologous and heterologous protection than split vaccine alone in an aged mouse model. The bivalent split vaccine (with 1.2 µg sCal) was not effective for inducing protection against homologous virus (A/Cal/H1N1) as substantial weight loss and high lung viral titers in aged mice. Consistently, previous studies

reported moderate to poor efficacy of split vaccines against homologous A/Cal/H1N1 virus (Bhatnagar et al., 2022). In aged mice, we found that NA-M2e vaccine was comparable to or better than split alone for conferring homologous protection. In previous studies, M2e alone immunity was cross-protective, but the efficacy was lower in the aspect of preventing weight loss disease against HA-matched virus challenge than inactivated virus (Jegerlehner et al., 2004) or split vaccine (Lee et al., 2016) in young adult mice. Antibodies from S+NA-M2e vaccinated mice were able to recognize a broad range of group 1 NA and group 2 NA viral antigens expressed on the infected cells. Accordingly, antisera from S+NA-M2e vaccination in aged mice conferred protection against homologous and heterologous influenza viruses in naïve mice. Our previous study (via T cell depletion before challenge) with monomeric split H1N1+5xM2e VLP vaccination in young adult mice demonstrated that CD4 T cell contributed more than CD8 T cell in conferring cross protection (Kim et al., 2014). Although IFN-γ^{+} T cell responses were analyzed after challenge, our data in young adult mice indicated that the overall pattern of IFN-γ^{+} CD4 and CD8 T cell responses before challenge was retained after challenge and induced at significantly higher levels in the NA-M2e vaccinated mice, compared to those in unvaccinated naïve mice after challenge (Fig. 13). However, the fold increase was higher in IFN-γ^{+} CD8 T cell responses to M2e after challenge than that before challenge, whereas the reverse pattern was observed for IFN-γ^{+} CD4 T cell responses (Fig. 13). Therefore, the T cell responses before challenge remain to be determined in aged mice with S+NA-M2e vaccination.

Furthermore, we found that NA-M2e supplemented bivalent split vaccination in aged mice induced higher levels of IgG2a antibodies to H1N1 and H3N2 viral antigens and HAI titers against vaccine viruses, suggesting adjuvant effects on enhancing the immunogenicity of co-administered

vaccines. Unlike IgG1, IgG2a can bind to F_c receptors with high affinity (Gessner et al., 1998) activating antibody-dependent cell-mediated cytotoxicity (Kipps et al., 1985) and macrophage-mediated opsonophagocytosis (Takai et al., 1994), which might have contributed to effective clearance of lung viral loads in the S+NA-M2e group, inducing enhanced levels of antibodies recognizing cell surface-expressed viral antigens from different strains. VLP was shown to be a superior platform enabling 5xM2e protein to be highly immunogenic in priming protective humoral and cellular immune responses compared to soluble 5xM2e protein (Kim et al., 2018a). Maturation and activation of immature monocyte-derived dendritic cells (DCs) were reported with rBV-expressed VLP presenting envelope proteins of human immunodeficiency virus type 1 (HIV) (Buonaguro et al., 2006). The adjuvant effects of VLP might have been attributed to its particulate nature capable of activating DCs as professional antigen presenting cells.

We tested *in vitro* stimulation of BMDCs to better understand the potential adjuvant effects of NA-M2e on enhancing Th1-type IgG2a antibodies to iCal H1N1 and iSwz H3N2 antigens in the supplemented split vaccination (Fig. 14A). BMDCs from aged mice were effectively stimulated to secrete inflammatory cytokines (IL-6, TNF-α) with 1 μg or 10 μg NA-M2e VLP of NA-M2e or supplemented split vaccines (Figs. 14B-C). Accordingly, NA-M2e and supplemented split vaccines effectively stimulated BMDCs *in vitro* to secrete inflammatory cytokines (IL-6, TNF-α). In line with our findings, previous studies demonstrated that HIV VLP can activate peripheral blood mononuclear cells to produce inflammatory cytokines (IL-6, TNF-α), anti-inflammatory IL-10, and Th1-polarizing IFN-γ (Speth, 2008). DC-derived TNF-α production was also reported to be responsible for developing CD4 T cells secreting IL-10 and Th1 cytokines (Hirata et al., 2010; Psarras et al., 2021). In a knockout mouse model, MyD88, an innate adaptor signaling molecule, was reported to be required for the induction of IgG2a isotype antibodies,

memory B and plasma cells as well as IFN-γ T cell responses, after vaccination with influenza HA VLP vaccine (Kang et al., 2011). IL-6 is a cytokine promoting T follicular helper (Tfh) cell differentiation (Crotty, 2014) and required for Tfh cell responses after immunization with mRNA lipid nanoparticle vaccine (Alameh et al., 2021). We observed higher levels of IFN-γ^+ CD4 and CD8 T cells, enhanced IgG2a antibodies, and HAI titers in the NA-M2e supplemented split group than split alone. The underlying mechanisms of NA-M2e VLP immune-enhancing effects in aged mice remain to be further investigated *in vivo*.

Aging effects on bivalent split vaccine-induced immune responses showed that young adult mice could generate significantly higher levels of IgG antibody responses to HA protein antigens after bivalent split vaccination than the aged mice (Fig. 4). The young mice induced higher levels of IFN-γ^+ T cell responses to NA antigens compared to the aged mouse counterpart after 2nd dose vaccination with S+NA-M2e and virus challenge (Fig. 11). Most human population have pre-existing immunity to influenza virus due to natural infection or vaccination by the age of 65. Therefore, it would be more translational to study the impact of pre-existing immunity on S+NA-M2e vaccination strategy by immunizing the aged mice with pre-existing immunity. Furthermore, we observed significantly higher levels of antibody secreting cell responses from *in vitro* cell cultures of draining lymph nodes and spleens than those in unvaccinated naïve mice at 1 month after 2nd dose and challenge. Future studies will need to determine long-lived antibody secreting plasma cells in bone marrow and memory B cells in aged mice by using phenotypic markers.

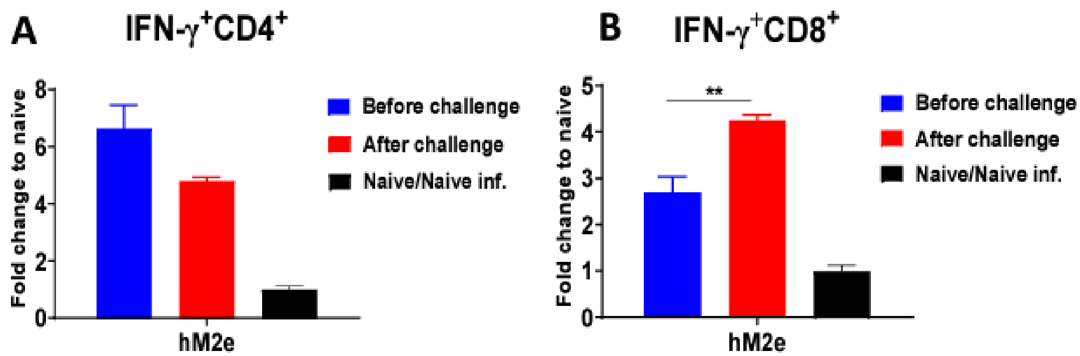


Figure 13. Comparison of IFN-γ + T Cell Responses in NA-M2e Vaccinated Mice Before and After Challenge. Groups of 6-8 weeks old young mice were boosted (2nd dose) with 10 µg of NA-M2e. Young mice were 3-4 months old at the time of sacrifice and analysis of intracellular cytokine staining in lung samples. Fold changes in T cell responses from the vaccinated mice compared to naïve (before challenge) or naïve infected (after challenge) mice (A) IFN-γ+CD4+ (B) IFN-γ+CD8+ T cells. Error bars are represented as mean ± SEM. Statistical significance was determined by using one-way ANOVA and Dunnett's multiple comparison test and indicated as **; p<0.01.

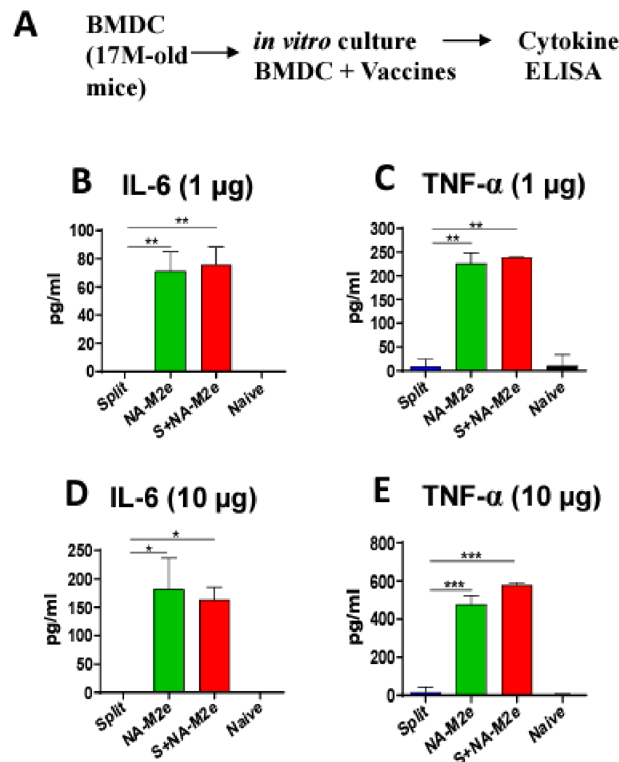


Figure 14. NA-M2e Vaccine Stimulates Bone Marrow Dendritic Cells (BMDC) In Vitro to Secrete Cytokines. (A) BMDCs were treated with 1 µg or 10 µg dose of NA-M2e vaccine, and cytokine levels were determined in culture supernatants. Cytokines levels (B-C) IL-6 and TNF-α at 1 µg dose and (D-E) IL-6 and TNF-α at 10 µg dose NA-M2e. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with other groups using one-way ANOVA and Dunnett's multiple comparison test and indicated as *; p<0.05, **; p<0.01, ***; p<0.001.

2.6 Conclusion

This study evaluates the strengths and weaknesses of the NA-M2e vaccine in comparison to the seasonal split vaccine, demonstrating that supplementation of inactivated split influenza vaccine with NA-M2e vaccine provides enhanced protection against homologous and heterologous viruses in an aged mouse model. The findings in the study could facilitate pandemic preparedness and prevent severe morbidity and mortality during an outbreak of new influenza variants.

2.7 Acknowledgements

This study was supported by NIH/NIAID grants AI154656 (S.-M.K.). The funders had no contribution in experimental design, collection of data, analysis, decision to publish, or manuscript preparation. A/Switzerland/9715293/2013 (H3N2) virus was generously gifted by Dr. Florian Krammer. The following reagents were obtained through BEI, NIAID, NIH. H1 HA protein (NR-15749), H3 HA protein (NR- 19238), B/Florida/4/2006 (NR-9696), N1 NA protein (A/California/4/2009, NR-19234), N2 NA protein (A/Brisbane/10/2007, NR-43784), and influenza B NA proteins (B/Florida/4/2006, NR-19236). A/Hong Kong/1073/99 H9N2 influenza virus (FR- 732) was obtained from International Reagent Resource.

3. CHAPTER 2: A STRATEGY OF ENHANCING THE PROTECTIVE EFFICACY OF SEASONAL VACCINES BY PROVIDING ADDITIONAL IMMUNITY TO NEURAMINIDASE AND M2e

3.1 Abstract

It is high priority to enhance the efficacy of influenza seasonal vaccines based on hemagglutinin (HA) strain-specific neutralizing immunity. Here we investigated a vaccination strategy of supplementing inactivated split seasonal vaccines with virus-like particle vaccine containing multi-subtype neuraminidase (NA) and M2 ectodomain (M2e) repeat (NA-M2e) in mice. NA-M2e and split combined vaccine (S+NA-M2e) stimulated a unique pattern of innate immune responses within a day of intramuscular injection of mice, induced hemagglutination inhibiting activities, and humoral and cellular immune responses to NA and M2e. The addition of NA-M2e to split vaccination provided higher efficacy of protection against homologous and heterologous viruses, compared to split alone, where NA-M2e significantly contributed to enhancing protection under naïve and primed mouse models. Taken together this study supports a vaccination strategy to improve the efficacy of seasonal vaccines by providing additional immunity to NA and M2e.

3.2 Introduction

Even after a century of the 1918 Spanish flu, the influenza virus claims thousands of lives each year, necessitating ongoing surveillance and annual vaccine updates. Over the years, the influenza virus has evolved into various strain variants, and it can be categorized into different subtypes based on its hemagglutinin (HA) and neuraminidase (NA) surface proteins (Bouvier & Palese, 2008). The HA head domain is highly variable, the target of most vaccines, and undergoes frequent mutations due to selection pressure from the existing immunity from vaccination and

infection (Guthmiller et al., 2022). The protection conferred by inactivated split influenza vaccines on the market primarily relies on strain-specific HA immunity; however, it is rendered less effective in the event of a strain-mismatch (Janssens et al., 2022; Tricco et al., 2013). For instance, in the 2014-2015 flu season, over 80% of the circulating H3N2 viruses had drifted antigenically from the recommended vaccine strain causing the adjusted vaccine efficacy for H3N2 viruses across all ages to drop to 13% (CDC, 2015).

Researchers have been focusing on developing universal influenza vaccines that target more conserved surface proteins, such as NA and M2 proteins, which have the potential to offer cross-protection against multiple strains (Jazayeri & Poh, 2019). Several studies have reported the effectiveness of NA and M2-based vaccines (Lei Deng et al., 2015; K.-H. Kim et al., 2019; Smith et al., 2017; Wohlbold Teddy et al., 2015). Virus-like particles (VLPs) offer the advantage of being non-infectious, highly immunogenic, and effectively stimulate the adaptive immune system (Nooraei et al., 2021) . The multi-subtype NA and M2 ectodomain VLPs (referred to as NA-M2e) provided broad cross-protection against various influenza A and B strains in animal models in prior studies (Kim, 2024; Kim et al., 2022). The M2 ectodomain (M2e) is highly conserved, and mutations in NA are less frequent; however, immune responses against these proteins exhibit low efficacy and cannot provide sterilizing immunity (Mezhenskaya et al., 2019; Schotsaert et al., 2015). Furthermore, a growing body of evidence indicates that heterologous sequential immunization strategies can enhance the breadth of protection by generating differential immune responses (Dong et al., 2024; He et al., 2021).

To address the concerns of strain-specificity of split vaccines and the absence of neutralizing immunity by NA and M2e protein-based vaccines, we previously reported a split+NA-M2e vaccination strategy that combines bivalent split inactivated (A/Cal/H1N1 + A/Swz/H3N2)

vaccines with NA-M2e VLP vaccine containing consensus NA proteins (N1 NA + N2 NA + flu B NA) and 5xM2e repeat (Raha et al., 2024) in aged mice. This vaccination strategy proved to be effective in conferring enhanced protection in aged mice against homologous and heterologous influenza strains by inducing additional immunity to NA and M2e. The underlying immune mechanisms have not been well understood. In the present study, we investigated the impact of the split+NA-M2e vaccination on regulating innate and adaptive immune responses in young BALB/c mice, representing a healthy, young population. Innate immune responses were uniquely triggered at an early time point by NA-M2e and combination vaccines that could effectively generate the adaptive immune responses. Lastly, we assessed whether a heterologous sequential vaccination strategy with split and NA-M2e would have an impact on the cross-protection. Results presented in this study corroborate the findings from prior studies on the efficacy of NA-M2e alone and split+NA-M2e vaccination strategy. This study provides new mechanistic insights into the enhanced efficacy of protection observed in split+NA-M2e vaccinated mice and exploring new vaccination strategies.

3.3 Methods

3.3.1 Viruses and Vaccines

Lethal doses of live viruses A/California/04/2009 H1N1 (A/Cal/H1N1), A/Philippines/2/1982 H3N2 (A/Phil/H3N2), rgA/Viet/H5N1 ((containing HA and NA from A/Vietnam/1203/2004 H5N1 and A/Puerto Rico/8/1937 (A/PR8 H1N1) backbone)) (Song et al., 2011), and A/Switzerland/9715293/2013 (A/Swz/H3N2) were used for challenge experiments. Additionally, for cell ELISA, A/Puerto Rico/8/1934 H1N1, A/Nanchang/933/1995 H3N2, A/Hong Kong/1073/99 H9N2 and rgA/Shanghai/02/2013 H7N9 (A/PR8 backbone) were used. The viruses were amplified and later propagated in 10-days-old embryonated chicken eggs. The harvested

allantoic fluid was centrifuged at 2000 rpm for 20 minutes and stored at -80°C. Inactivated bivalent split vaccine strains used to immunize mice were A/Cal/H1N1 and A/Swz/H3N2 viruses, and they were treated with 1% formalin for inactivation, followed by Triton X-100 for virion particle disruption as described (Bhatnagar et al., 2022). Hemagglutination activity units (HAU) assays were used to confirm the integrity of functionality and antigenic sites. H1N1 sCal split vaccine exhibited 255 HAU/µg sCal and H3N2 sSWz split vaccine 128 HAU/µg sSWz. NA-M2e vaccine (Fig. 1A) was expressed in insect cells and prepared as described (Kim et al., 2022).

3.3.2 Immunization and Challenge

Young adult BALB/c mice (n =10, 5-8-weeks-old female) obtained from Jackson laboratory (Bar Harbor, ME) were intramuscularly immunized with bivalent split vaccine (S) [total protein 1.2 µg inactivated split A/Cal/H1N1 (sCal) plus 0.6 µg inactivated split A/Swz/H3N2 (sSwz)], 10 µg NA-M2e, or S +NA-M2e combined vaccines in a prime-boost regimen at 3-week intervals. The sCal split vaccine 1.2 µg dose was adjusted to be similarly immunogenic in aged mice as 0.6 µg sSwz vaccine. HA content was estimated to be approximately 30% of total protein of inactivated virus vaccines (Lucy A. Perrone et al., 2009; Tarasov et al., 2020). NA-M2e vaccine was dosed at 10 µg to be consistent with previous studies on NA-M2e VLP (Kim et al., 2022; Raha et al., 2024) and 5xM2e VLP (M. C. Kim, J. S. Lee, et al., 2013). NA content incorporated on monomeric N1 NA VLP was estimated to be approximately 2% of total N1 VLP protein (K.-H. Kim et al., 2019). Immunized mice and naïve controls were then challenged with a lethal dose of A/Cal/H1N1 (5.3xLD_{50}, 2 ×10^3 EID_{50}), rgA/Viet/H5N1 (3xLD_{50}, 2.6x10^4 EID_{50}) or A/Phil/H3N2 (3xLD50, 2.3x10^2 EID_{50}) 4 weeks after boost vaccination. The body weight changes, and survival rates were monitored daily for 2 weeks. The animal studies presented were approved by the Georgia State University (GSU) Institutional Animal Care and Use Committee review boards.

3.3.3 Antibody Responses and Hemagglutination Inhibition (HAI) Assay

IgG antibody responses were determined using ELISA assay as described (Ko et al., 2018). The coating antigens were 0.6 μg/ml H1 HA protein (A/California/04/2009 H1N1), H3 HA protein (A/Brisbane/10/2007 H1N1), 1 μg/ml N2 NA (A/Brisbane/10/2007 H3N2), N1 NA (A/Brisbane/59/2007 H1N1), influenza B NA (B/Florida/4/2006), 4 μg/ml human M2e (M2e, SLLTE VETPIRNEWGSRSN) peptide, and inactivated influenza viruses A/California/04/2009 H1N1 (iCal H1N1), A/Switzerland/9715293/2013 H3N2 (iSwz H3N2), A/Philippines/2/1982 H3N2 (iPhil H3N2) and rgA/Vietnam/1203/2004 H5N1 (iViet H5N1). Antibody-secreting cell responses were determined via *in vitro* IgG ELISA in spleen samples as they were applied onto pre-coated plates with respective antigens and cultured for 1 day (D1) or 5 days (D5). The IgG and IgG isotypes were measured with horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1 or IgG2a secondary antibodies as described (Bhatnagar et al., 2022). HAI assay was performed with heat-inactivated sera treated with receptor-destroying enzymes (RDE, Sigma-Aldrich). The inactivated sera were mixed with 4 HA units of viruses. 0.5% chicken red blood cells were added to the mixture to determine HAI titers as described (Bhatnagar et al., 2022).

3.3.4 Lung Viral Titration

Diluted lung lysate samples from infected mice were inoculated in 10-day-old embryonated chicken eggs. The allantoic fluid was tested for lung viral titers by assessing their hemagglutination activity as described (Wen & Wang, 1959). For lung viral titration in MDCK cells, the cells were first seeded at 4 x 10^4 cells per well, and diluted lung lysates from infected mice were added to the cells following a 3-day incubation. Harvested supernatant were used to determine lung viral titers via HAI assays.

3.3.5 Cytokine Responses

Ready-Set IL-6, TNF-α (eBioscience, San Diego, CA) and mouse IFN-γ uncoated ELISA kit purchased from Invitrogen were used to determine cytokine levels in lung lysate or samples.

3.3.6 Flow Cytometry

Single cell suspensions were prepared from harvested draining lymph node and spleen samples day 1 day post prime with A/Cal/H1N1, rgA/Viet/H5N1 or A/Phil/H3N2 viruses. Cells were stained with i) anti-CD45 (clone 30-F11), anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-CD103 (clone 2E7), anti-F4/80 (clone BM8), anti-MHCII (clone I-A/I-E), anti-B220 (clone RA3-6B2), anti-Ly6C (clone AL-21), anti-CD86 (clone PO3), anti-CD8α (clone 53-6.7), SiglecF (E40-2440); ii) anti-CD45, anti-CD3, anti-CD49b (clone DX5), and anti-CD69 to analyze innate immune cells and NK cell populations. Fc receptor blocker anti-CD16/32 mAb was used prior to staining. For intracellular cytokine staining, mediastinal lymph node tissues harvested on day 7 post-challenge were homogenized and prepared as single-cell suspensions. The cells were then stimulated in vitro with 5 μg/ml of the following antigens: M2e peptide, N1 NA (A/Cal/04/2009 H1N1), and N2 NA (A/Brisbane/10/2007 H3N2), in the presence of Brefeldin A (20 μg/ml) at 37°C for 5 hours. Lymphocytes were stained using anti-mouse CD3 (clone 17A2), CD4 (clone 553051), CD8 (clone 25-0081-82), and IFN-γ (clone XMG1.2) monoclonal antibodies. Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm Plus kit (Ko et al., 2018).

3.3.7 In Vivo Protection Experiment with Antisera

For passive sera transfer experiments, sera from naïve or immunized were heat-inactivated at 56°C, and mixed with lethal doses of A/California/04/2009 H1N1 (2xLD_{50}, 1.6 ×10^4 EID50) in BALB/c mice, A/Switzerland/9715293/2013 H3N2 (4xLD_{50}) in DBA/ 2J mice, and

rgA/Vietnam/1203/2004 H5N1 (4xLD_{50}, 2.6 ×10 EID_{50}) in BALB/c and incubated for 30 minutes at room temperature as described (Getie-Kebtie et al., 2013). The mixture was intranasally administered to 5-8 weeks aged young adult mice. The body weights and survival rates for these mice were monitored for 2 weeks.

3.8.8 Statistical Analysis

All data in this study are presented as mean ± standard error of the mean (SEM). Statistical significance across all experiments was assessed by comparing the split and NA-M2e, S+NA-M2e or Sp+NA-M2e groups using one-way or two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison post-tests. A p-value of less than 0.05 ($p<0.05$) was considered statistically significant. Data analysis was performed using Prism software (GraphPad Software, Inc., San Diego, CA).

3.4 Results

3.4.1 NA-M2e Enhances the Immunogenicity of Split Vaccine by Stimulating Innate Immune Responses After Prime Vaccination

To determine the induction of IgG antibodies after prime (1st dose) vaccination, BALB/c mice (5-6 weeks old) were intramuscularly immunized with split, NA-M2e, or Split (S) + NA-M2e vaccines. 2 weeks after vaccination, sera were harvested and tested for immunogenicity. Prime serum responses in S+NA-M2e group revealed over 2-fold higher IgG antibody levels compared to split alone group for iCal H1N1 and iSwz H3N2 antigens (Figs. 15A, B). The Split group exhibited negligible levels of IgG antibodies against M2e, while both the NA-M2e and S+NA-M2e groups showed significant levels of M2e specific IgG responses (Fig. 15C).

In a different set of experiments, we determined early innate immune responses after prime vaccination. Sera were collected 6 hours after vaccination to determine cytokine and chemokine

responses and spleen and draining lymph node (dLN) tissues were harvested 24 hours post vaccination to determine innate cell populations (Figs. 15D-H). Mice immunized with NA-M2e and S+NA-M2e but not split alone exhibited elevated levels of the cytokine IL-6 and chemokine monocyte chemoattractant protein-1 (MCP-1) (Figs. 15D-E). Compared to split and naïve groups, NA-M2e and S+NA-M2e both groups showed significantly higher cell numbers of activated innate cell populations; $CD69^{+}$ NK ($CD45^{+}CD3^{-}CD49b^{+}$) cells in spleen (Fig. 15F), $CD86^{+}$ monocyte ($CD45^{+}CD11b^{+}F4/80^{low}Ly6C^{high}$, Fig. 15G), and $CD86^{+}$ macrophages ($CD45^{+}CD11b^{+}F4/80^{+}$, Fig. 15H) in spleen and dLN. Different subsets of activated dendritic cells (DC) in spleens and dLN were observed as well after prime immunization (Fig. 16). NA-M2e and S+NA-M2e similarly induced higher populations of DC subsets compared to split and naïve groups; however, some populations were more significantly elevated compared to split group than others. For instance, $CD86^{+}$ activated migratory $CD103^{+}$mDCs and plasmacytoid pDCs but not $CD11b^{+}$mDCs (Figs. A-C) were significantly elevated in NA-M2e and S+NA-M2e groups in both spleens and dLNs; however, resident $CD11b^{+}$rDCs and $CD8\alpha^{+}$rDCs (Figs. 16D-E) were significantly elevated in spleen samples. The data indicates that S+NA-M2e vaccination effectively induces IgG antibodies against viral vaccine and M2e antigens following prime immunization. Additionally, both NA-M2e and S+NA-M2e vaccines can activate innate immune cell populations at early time points post-vaccination.

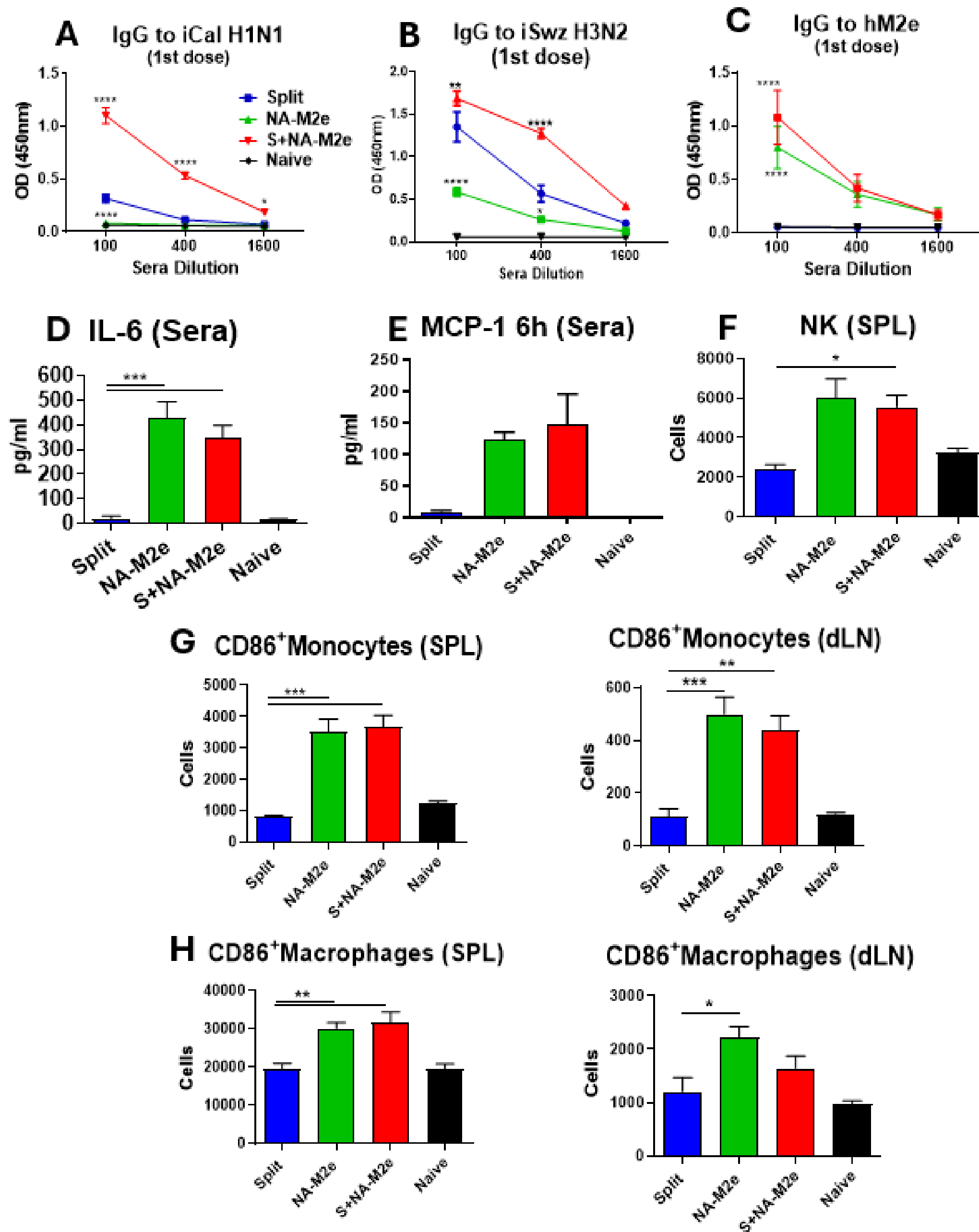


Figure 15. S+NA-M2e Vaccination Induces Significant Antibody Responses and Innate Immune Cell Responses After Prime Immunization. Groups of 5-8 weeks old young, adult BALB/c mice (N=5) were prime immunized (1st dose) with either inactivated bivalent split (S) vaccine containing sCal (A/California) H1N1 + sSwz (A/Switzerland) H3N2, NA-M2e VLP (N1 NA + N2 NA + flu B NA and 5xM2e incorporated in VLP) or S+NA-M2e combined vaccines. IgG responses in prime sera (harvested 14 days post immunization) specific for (A) iCal H1N1, (B) iSwz H3N2, (C) M2e were determined by ELISA and expressed as optical density (O.D value). To determine early timepoint immune responses, groups of mice were prime immunized and sacrificed 1 day later, and their tissues were harvested. (D) IL-6 cytokine and (E) MCP-1 chemokine levels 6 hrs post prime in sera. Activated innate immune cells (F) NK cells: $CD45^+CD3^-CD49b^+CD69^+$ in spleen; (G) Monocytes: $CD45^+CD11b^+F4/80^{low}Ly6C^{high}$ and (H) Macrophages: $CD45^+CD11b^+F4/80^+$ in spleen and dLN were determined by flow cytometry. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with NA-M2e and S+NA-M2e

groups and (A-C) using two-way ANOVA and (D-H) one-way ANOVA with Dunnett's posttest and indicated as *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, ****; $p < 0.0001$.

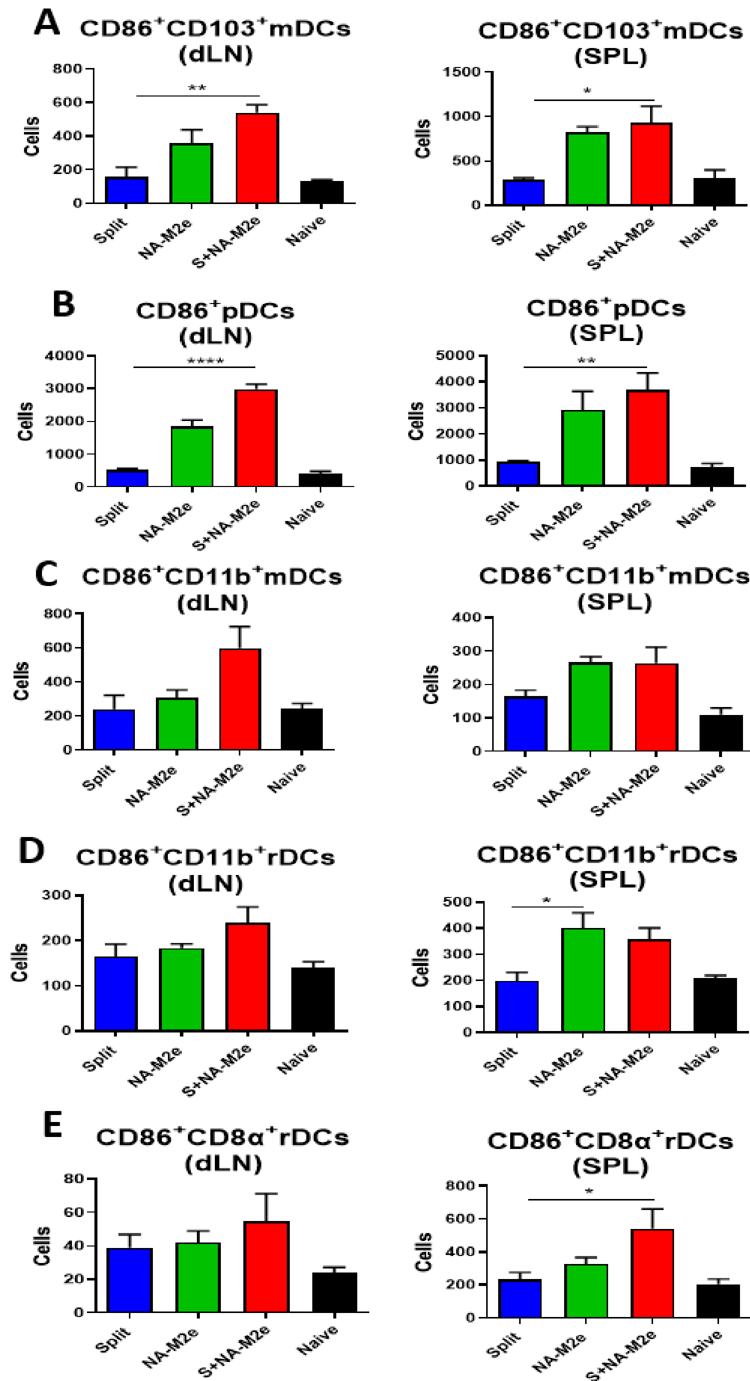


Figure 16. Activation of Dendritic Cell (DC) Subsets by S+NA-M2e Vaccination 1 Day Post Prime Immunization. Groups of prime immunized mice were sacrificed 24 hrs post vaccination and their tissues were harvested. Flow cytometry analysis of activated DC subsets in dLN and spleen (A) $CD103^{+}$mDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{high}CD103^{+}CD11b^{-}CD86^{+}$; (B) Plasmacytoid DC: $CD11b^{+}$mDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{high}CD103^{-}CD11b^{+}CD86^{+}$; (C) CD11b+mDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{high}CD103^{-}CD11b^{+}CD86^{+}$; (E) $CD11b^{+}$rDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{low}CD11b^{+}CD86^{+}$ and; (F) $CD8\alpha^{+}$rDC: $CD45^{+}F4/80^{-}$

$CD11c^{+}MHCII^{low}CD8\alpha^{+}CD86^{+}$. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with S+NA-M2e group using one-way ANOVA with Dunnett's posttest and indicated as *; $p< 0.05$, **; $p< 0.01$, ****; $p<0.0001$.

3.4.2 S+NA-M2e Boost Immunization Induces High Antibody Responses to HA, NA and M2e Vaccine Antigens

At 3 weeks after prime, mice were boost immunized (2nd dose) with the same vaccines (Fig. 17). IgG antibodies specific for iCal H1N1, iSwz H3N2 antigens (Figs. 17A-D) were observed at similarly high levels in both split and S+NA-M2e sera where there was a significant difference in H1 HA and H3 HA specific IgG levels between the groups when compared at 1000x serum dilutions (Figs. 17C-D). Both NA-M2e and S+NA-M2e groups showed significantly elevated IgG levels to M2e, N1, and B NA antigens (Figs. 17E-F, H) compared to the split group, whereas S+NA-M2e group showed highest IgG antibody levels for N2 NA (Fig. 17G). S+NA-M2e group displayed higher HAI titers against A/Cal/H1N1 and similar HAI titers A/Swz/H3N2 viruses as compared to split vaccine without significant differences.

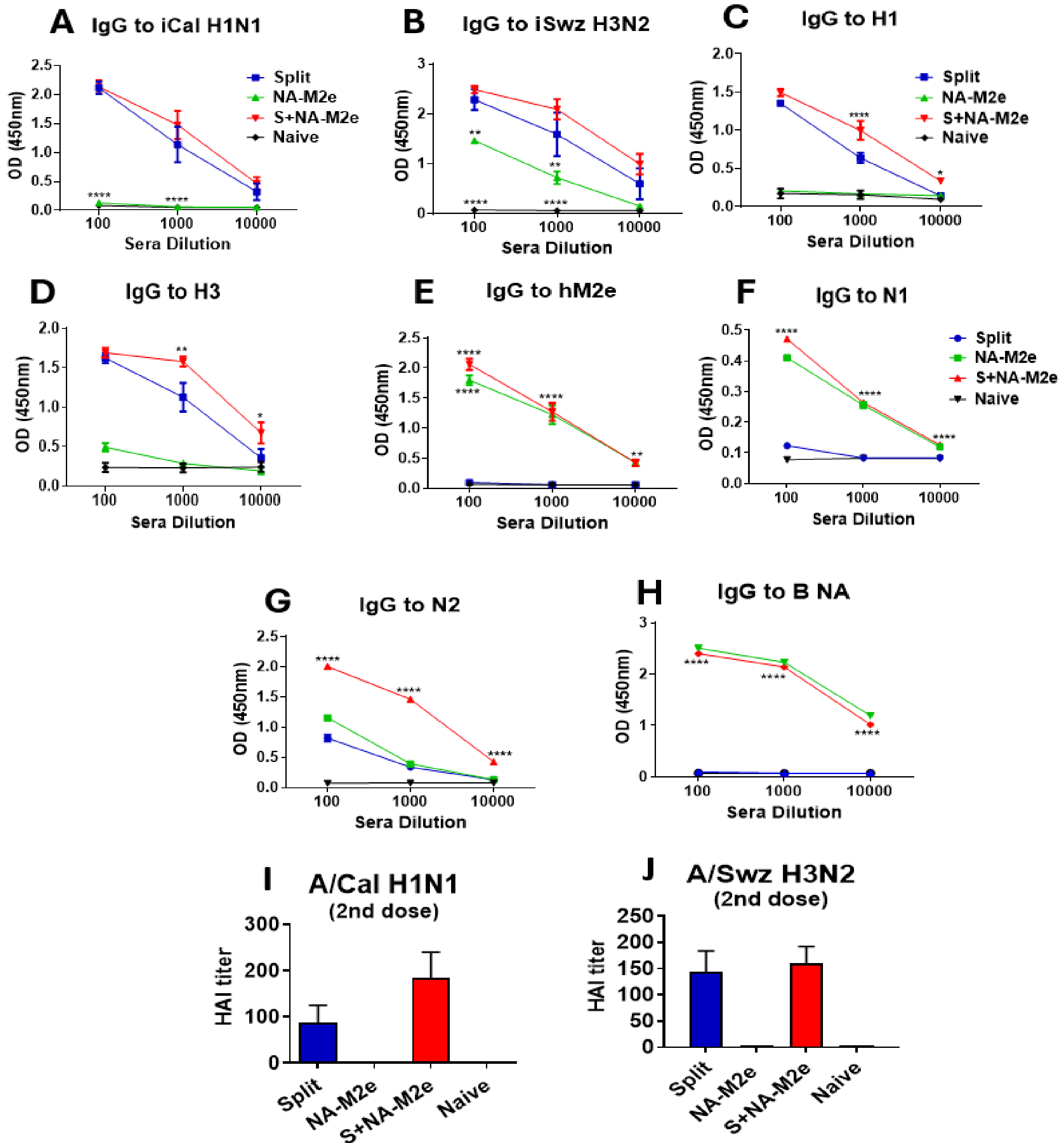


Figure 17. Elevated Levels of IgG Antibody Levels to HA, NA and M2e Antigens in S+NA-M2e Vaccinated Boost Sera. 3 weeks after prime, Split, NA-M2e and S+NA-M2e immunized mice were boosted with the same vaccines and dosages as in prime. IgG responses in boost sera specific to (A) iCal H1N1, (B) iSwz H3N2, (C) H1 HA, (D) H3 HA, (E) M2e, (F) N1, (G) N2, and (H) B NA. HAI titers in boost sera to (A) A/Cal/H1N1 and (B) A/Swz/H3N2. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with NA-M2e and S+NA-M2e groups and (A-H) using two-way ANOVA and (I-J) one-way ANOVA with Dunnett's posttest and indicated as *; $p < 0.05$, **; $p < 0.01$, ****; $p < 0.0001$.

3.4.3 Supplemented S+NA-M2e Vaccination Induces Enhanced Homologous and Heterologous Protection

Lethal doses of homologous A/Cal/H1N1 (5xLD_{50}), and heterologous rgA/Viet/H5N1 (3xLD_{50}) and A/Phil/H3N2 (3xLD_{50}) viruses were infected intranasally in mice at 3 weeks post boost to determine the protective efficacy of the vaccines (Fig. 18). The split and NA-M2e alone groups lost approximately 8% body weight while the S+NA-M2e group did not display substantial changes in body weight after A/Cal/ H1N1 challenge (Figs. 18A-B). Mice from all groups with vaccines survived this challenge. Additionally, both NA-M2e and S+NA-M2e groups showed 10%-12% loss in body weight after heterologous rgA/Viet/H5N1 and A/Phil/H3N2 challenges (Figs. 18C, E). In contrast the split group exhibited severe body weight loss of 18% and over 20% after heterologous rgA/Viet/H5N1 and A/Phil/H3N2 challenges respectively (Figs. 18C, E). NA-M2e and S+NA-M2e vaccinated mice survived 100% in heterologous challenges, whereas only 75% of split vaccinated mice survived lethal rgA/Viet/H5N1 challenge and all split vaccinated mice succumbed to death after A/Phil/H3N2 challenge (Figs. 18D, F). Naïve infected mice lost over 20% body weight and did not survive any of these lethal challenges. Furthermore, another set of mice was sacrificed 7 days post infection, and their lung samples were harvested. S+NA-M2e group displayed significantly lower lung viral titers in homo A/Cal/H1N1 challenge and reduced lung viral loads in hetero rgA/Viet/H5N1 challenge (Figs. 18G-H). NA-M2e immunized mice lowered lung viral loads in both challenges better than split despite no significant differences in the heterologous rgA/Viet/H5N1 challenge. Tied together, S+NA-M2e showed improved protection efficacy in both homologous and heterologous challenges as evidenced by lower body weight loss, lung viral titers and higher survival rates compared to split vaccinated mice.

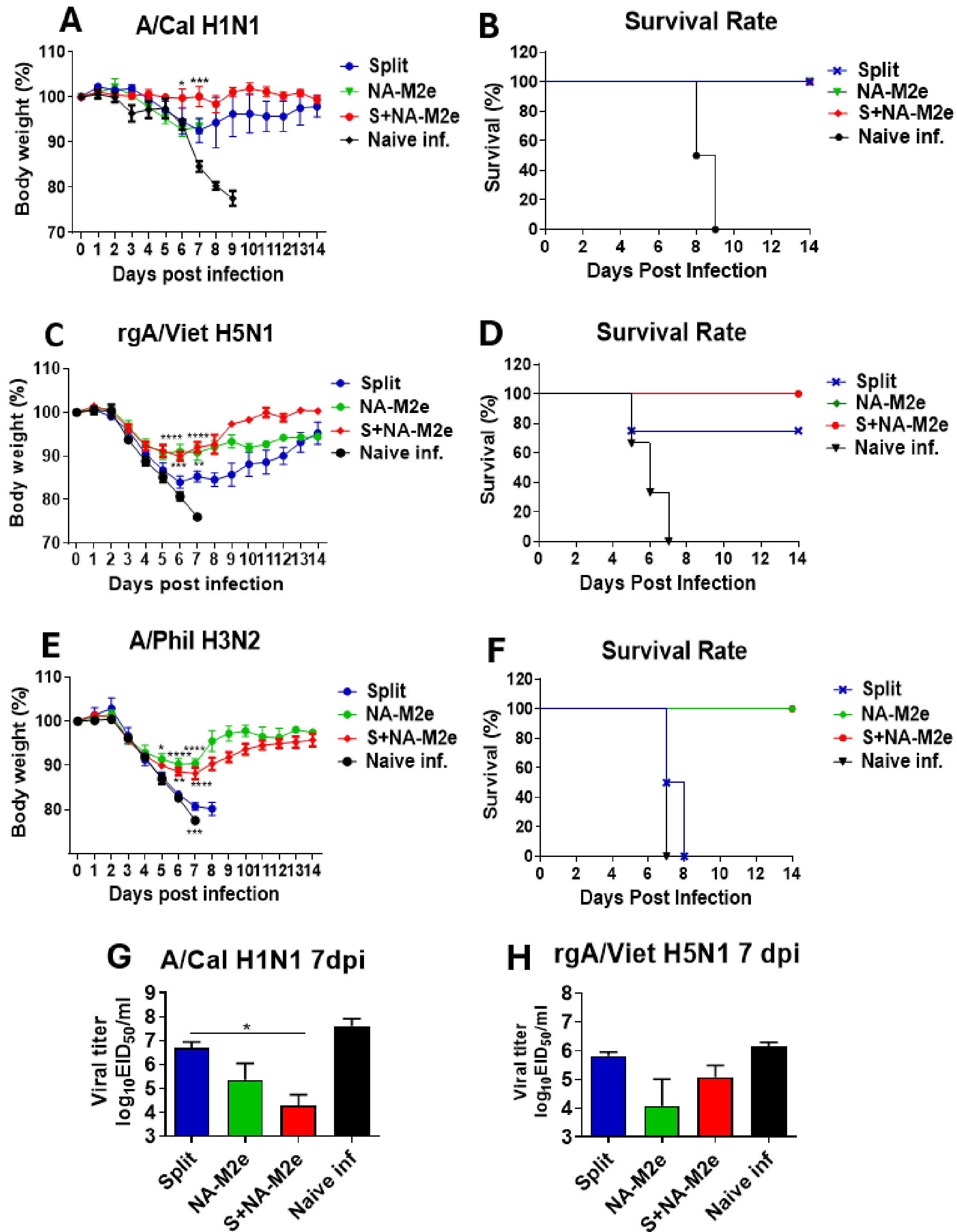


Figure 18. Reduced Body Weight Loss and Lung Viral Titers in S+NA-M2e Vaccinated Mice Following Both Homologous and Heterologous Virus Challenges. Boost immunized mice were challenged with lethal doses of influenza A viruses 4 weeks after boost. (A-B) Body weight loss and survival rates for 14 days after A/Cal/H1N1 challenge; (C-D) body weight loss and survival rates for 14 days after rgA/Viet/H5N1 challenge; and (E-F) body weight loss and survival rates for 14 days after A/Phil/H3N2 challenge. In another set of experiments, boost immunized mice were challenged and sacrificed 7 dpi and their tissues were harvested. Lung viral titers after (G) A/Cal/H1N1 and (H) rgA/Viet/H5N1 challenges. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with NA-M2e and S+NA-M2e groups and (A-F) using two-way ANOVA followed by Tukey's posttest and (D-H) one-way ANOVA followed by Dunnett's posttest and indicated as *; $p< 0.05$, **; $p< 0.01$, ***; $p< 0.001$, ****; $p<0.0001$.

3.4.4 NA-M2e Supplementation Protects Against Inflammatory Cytokine Induction Levels and Promotes IgG-Secreting Cell Responses Post-Challenge

Excessive secretion of cytokines in influenza infection can cause hypercytokinemia resulting in lung injury. Proinflammatory cytokine levels were determined in harvested lung lysate samples 7 days after challenge. Cytokine analysis revealed that the split group showed higher IL-6 and IFN-γ cytokine levels in both homologous A/Cal/H1N1 and heterologous rgA/Viet/H5N1 challenges (Figs. 19A-D). NA-M2e group displayed lower IL-6 and similar IFN-γ cytokine levels and S+NA-M2e group showed no detectable IL-6 and FN-γ cytokines after A/Cal/H1N1 challenge compared to the split group. Upon hetero rgA/Viet/H5N1 challenge, IL-6 and IFN-γ cytokine levels were lower in the NA-M2e and S+NA-M2e groups compared to the split group. The naïve group demonstrated the highest cytokine levels in both challenges.

IgG-secreting cells in spleen samples (harvested from challenged mice) were determined after *in vitro* cultures for 1 (D1) and 5 (D5) days. Splenocytes from the S+NA-M2e group produced higher levels of IgG antibodies for iCal H1N1, iSwz H3N2, iPhil H3N2, iViet H5N1, M2e and N2 antigens (Figs. 19E-J), whereas the split and NA-M2e groups showed moderate levels of antibodies to the respective antigens.

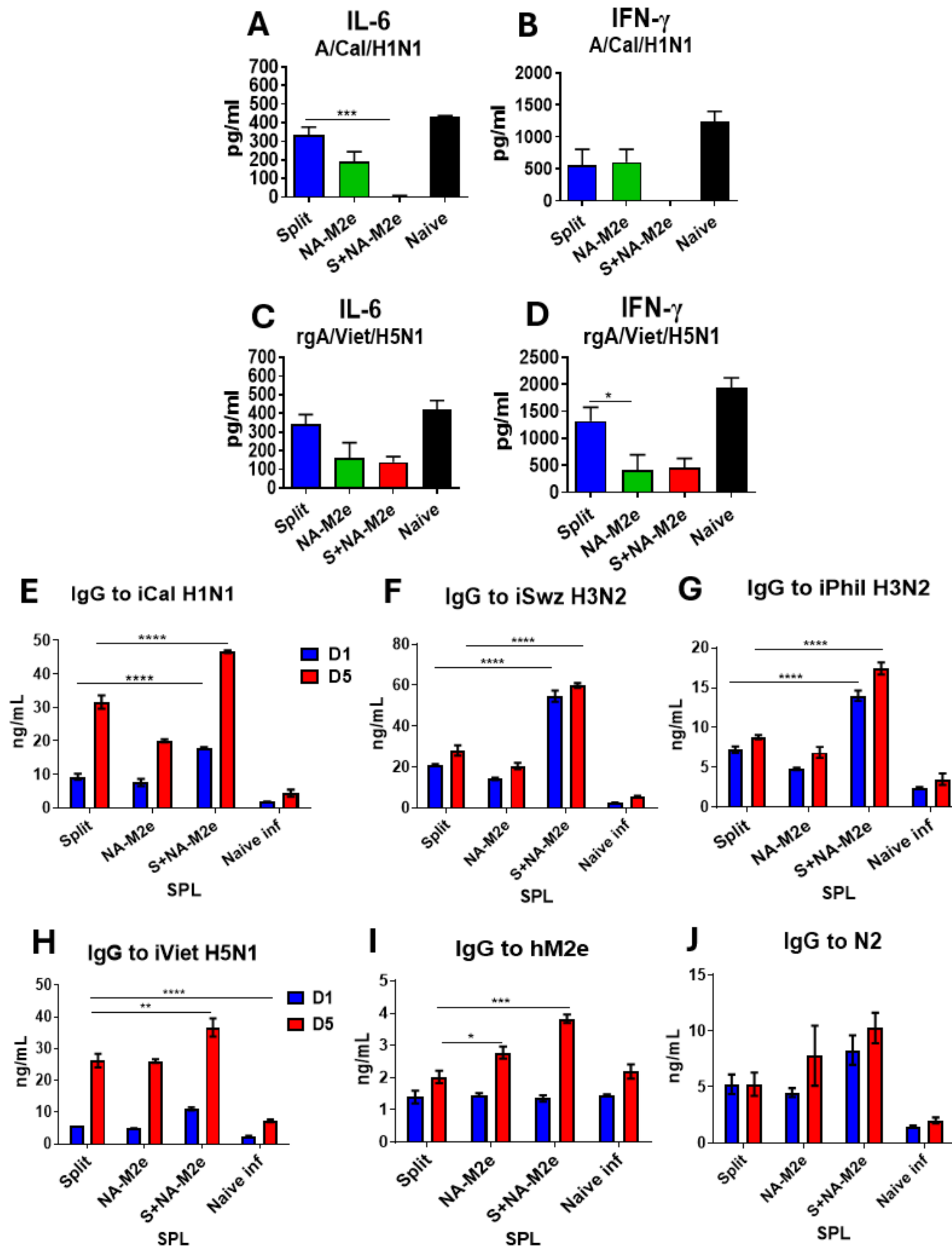


Figure 19. Diminished Pro-Inflammatory Cytokine Levels and Increased Antibody-Secreting cell Responses Demonstrated by S+NA-M2e Boost Vaccinated Mice After Lethal Virus Challenge. Mice were sacrificed and tissues were harvested 7dpi following boost immunization and challenge. Cytokine levels IL-6 and IFN-γ in (A-B) A/Cal/H1N1 and (C-D) rgA/Viet/H5N1 challenges. IgG antibody levels secreted from antibody-secreting cells during in vitro cultures for 1-day (D1) and 5 days (D5) in spleen to (E) iCal H1N1 (A/Cal/H1N1 challenge) (F) iSwz H3N2 (A/Phil/H3N2 challenge) (G) iPhil H3N2 (A/Phil/H3N2 challenge) (H) iViet H5N1(rgA/Viet/H5N1 challenge) (I) M2e (rgA/Viet/H5N1 challenge) and (J) N2 (A/Phil/H3N2 challenge). Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with S+NA-M2e groups and (A-D) using one-way ANOVA followed by Dunnett's posttest and (E-J) two-way ANOVA followed by Tukey's posttest and indicated as *; p< 0.05, **; p< 0.01, ***; p< 0.001, ****; p<0.0001.

3.4.5 NA-M2e Promotes Cellular Immune Responses After Co-Vaccination and Challenge

Intracellular cytokine staining was performed with mLN cells at 7 days after challenge with homologous A/Cal/H1N1 and heterologous rgA/Viet/H5N1 and A/Phil/H3N2 viruses (Fig. 20). S+NA-M2e co-vaccinated mice elicited significantly higher levels of IFN-γ^+ CD4 and CD8 T cell responses to N1, N2, and M2e in A/Cal/H1N1 challenge except for IFN-γ^+ CD8 T cells upon N1 NA stimulation (Figs. 20A-C). NA-M2e group induced moderate levels of IFN-γ^+ T cell responses whereas the split group was not effective in inducing cellular responses to respective NA and M2e stimulations. After A/Phil/H3N2 challenge, S+NA-M2e group induced similar or higher IFN-γ^+ T cell responses as the NA-M2e group (Figs. 20D-F) to N1, N2, and M2e antigens. Lastly, in rgA/Viet/H5N1, S+NA-M2e group induced the highest levels of IFN-γ^+ CD4 and CD8 T cell responses to N1 and M2e stimulators (Fig. 20G-H). Data from all 3 challenges confirms that S+NA-M2e induces robust IFN-γ^+ effector T cell responses to NA and M2e antigens.

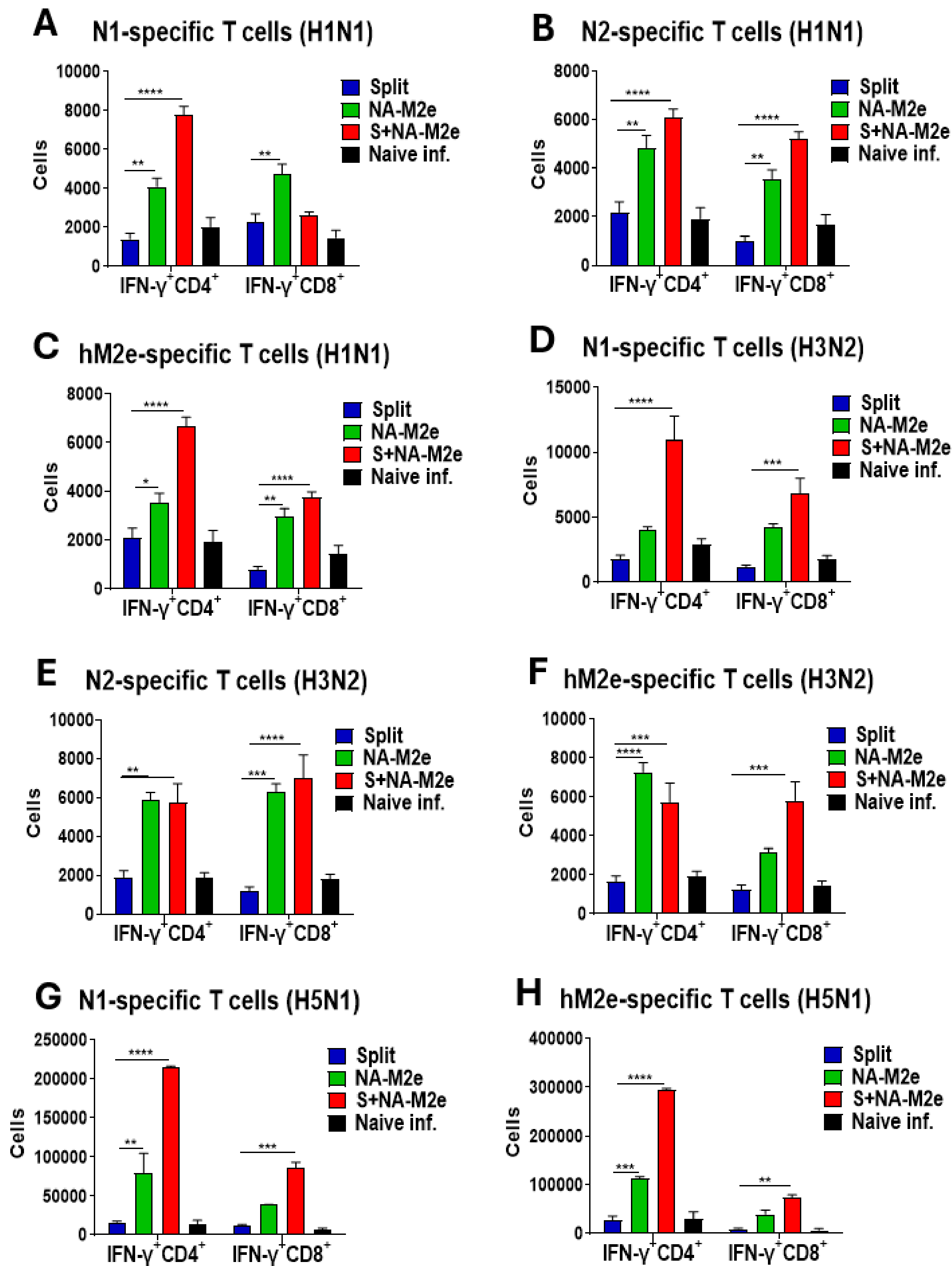


Figure 20. Induction of Robust T Cell Responses in S+NA-M2e Immunized Mice. Intracellular cytokine staining to determine IFN-γ^{+}CD4 and CD8 T cell responses to N1, N2 and M2e in (A-C) A/Cal/H1N1 challenge; (D-F) A/Phil/H3N2 challenge, and to N1 and M2e in (G-H) rgA/Viet/H5N1 challenge. Error bars are represented as mean ± SEM. Statistical significance was determined by using two-way ANOVA with Tukey's posttest and indicated as *; $p< 0.05$, **; $p< 0.01$, ***; $p< 0.001$, ****; $p<0.0001$.

3.4.6 S+NA-M2e Antisera Contribute to Effective Protection Against Homologous and Heterologous Influenza Viruses

To determine the role of antisera in conferring protection, sera from boost-immunized mice were mixed with a lethal dose of influenza viruses and administered in naïve mice intranasally (Fig. 21). In heterologous rgA/Vietnam/H5N1 co-inoculation, NA-M2e and S+NA-M2e serum recipient naive BALB/c mice lost 5% body weights whereas split antiserum recipient mice showed 10% loss in body weight changes (Fig. 21A-B). After homologous A/Cal/H1N1 co-inoculation, split and S+NA-M2e antiserum recipient naive BALB/c mice were fully protected without loss in body weights, whereas the naïve mice with NA-M2e antisera lost approximately 4% body weight (Figs. 21C-D). Lastly, DBA/2J mice were used for A/Swz/H3N2 challenge due to their susceptibility to this virus strain. After A/Swz/H3N2 co-inoculation in DBA/2J mice, S+NA-M2e serum recipients had 100% survival with no body weight loss, split serum recipients fully survived with 5% weight loss and NA-M2e sera recipients had approximately 13% body weight loss (Fig. 21E-F). Evidently, in all passive sera transfer experiments across all mice strains, S+NA-M2e sera recipients displayed no or least weight loss. Naïve serum recipient mice succumbed to death with over 20% body weight losses.

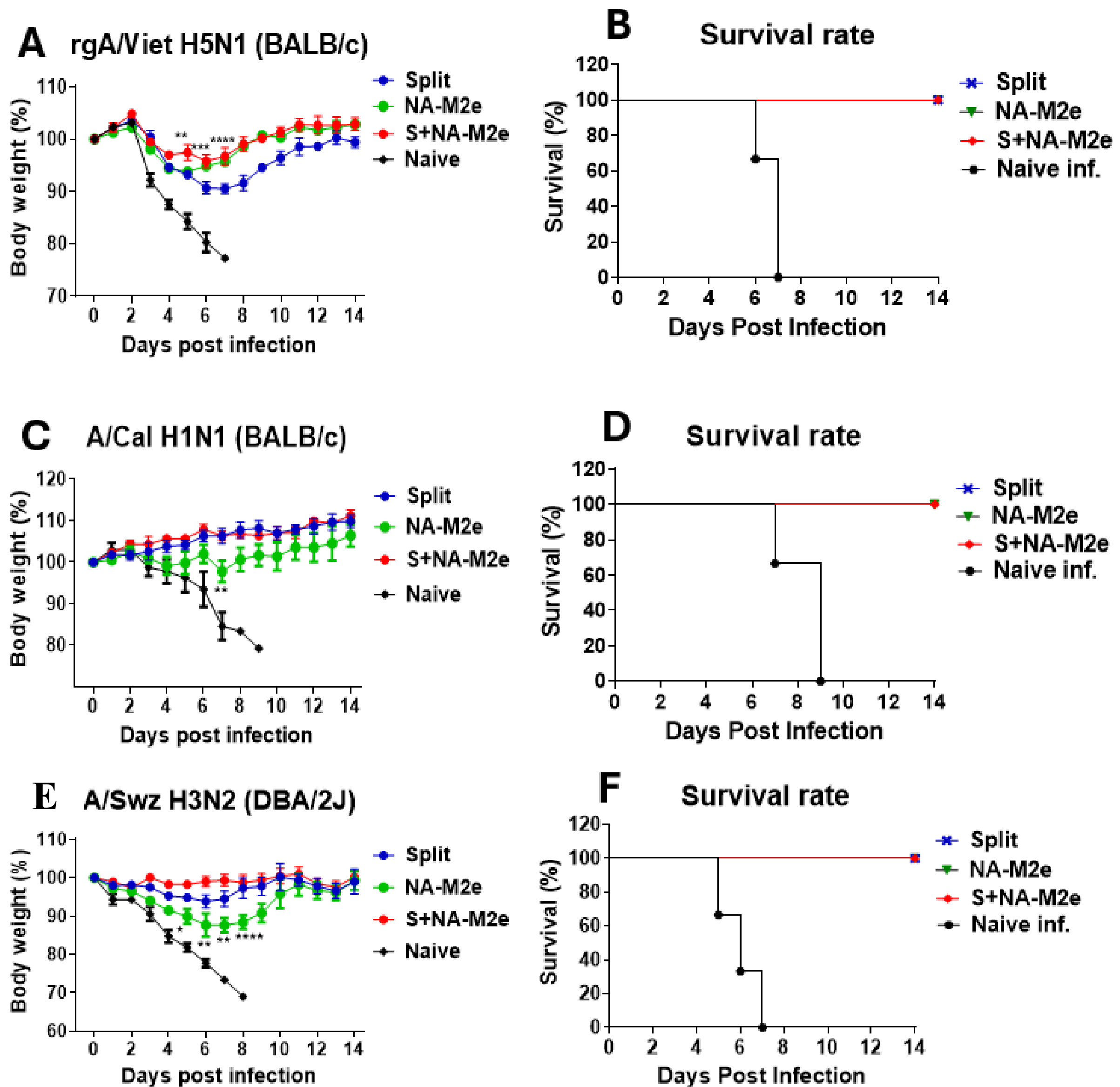


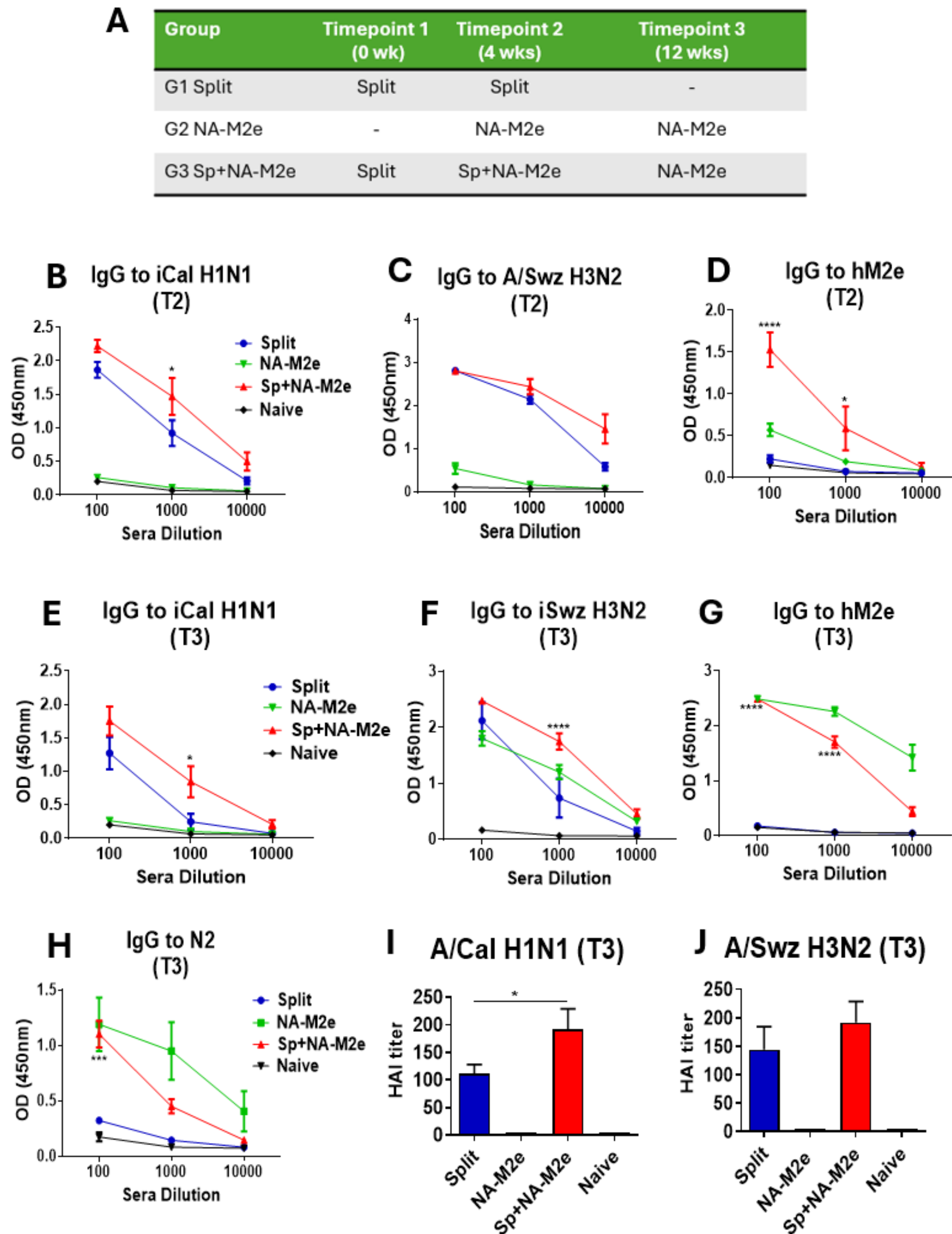
Figure 21. Boost Sera from S+NA-M2e Vaccinated Mice Protect Against Lethal Influenza Virus Challenges. Sera from boost immunized mice were mixed with lethal doses of influenza viruses and administered in naïve mice. Their body weights and survival rates were monitored for 2 weeks. (A-B) Body weight loss and survival rates in rgA/Viet/H5N1 challenge (BALB/c mice); (C-D) body weight loss and survival rates in A/Cal/H1N1 challenge (BALB/c mice); and (E-F) body weight loss and survival rates in A/Swz/H3N2 challenge (DBA/2J mice). Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with NA-M2e and S+NA-M2e groups and using two-way ANOVA followed by Tukey's posttest and indicated as *; $p< 0.05$, **; $p< 0.01$, ***; $p< 0.001$, ****; $p<0.0001$.

3.4.7 Vaccination with Split and NA-M2e in Primed Mice Induces Increased IgG Levels and Improves Protection Against Heterologous Virus

We assessed the immunogenicity after split+NA-M2e co-vaccination followed by NA-M2e only vaccine in 12 months old adult mice that were primed with bivalent split vaccine (Fig. 22A). The vaccinations were done at 4- or 8-week intervals and sera were harvested 2 weeks after each vaccination. At timepoint 2, the split group 1 had received 2 doses of split vaccine, the NA-M2e group 2 had received 1 dose of NA-M2e vaccine, and the Sp+NA-M2e group 3 had received 2 doses of split and 1 dose of NA-M2e via co-vaccination. The Split and Sp+NA-M2e groups displayed similar IgG levels to iCal H1N1 and iSwz H3N2 (Figs. 22B-C). The Sp+NA-M2e group induced increased IgG antibodies for M2e (Fig. 22D). At timepoint 3, the Sp+NA-M2e group showed higher levels of overall IgG antibodies for iCal H1N1 and iSwz H3N2, compared to those in the split group (Figs. 22E-F). The NA-M2e group showed highly increased levels of IgG antibodies for iSwz H3N2, M2e, and N2 NA antigens at T3 (Figs. 22F, G, H). Also, the Sp+NA-M2e group induced IgG antibodies for M2e and N2 NA antigens at higher levels than the split group (Fig. 22G, H). Both the split and Sp+NA-M2e groups showed protective HAI titers (>40) against A/Cal/H1N1 and A/Swz/H3N2 viruses at T3 (Figs. 22I-J).

The vaccinated mice were then challenged with a lethal dose of heterologous A/Phil/H3N2 virus (Fig. 23). The NA-M2e and Sp+NA-M2e groups were protected and recovered by day 9, whereas the split and naïve groups kept on losing body weights (Fig. 23A). At 9 days after challenge, lung viral titers were detected to be the highest levels in naïve infected mice, followed by the split group (Fig. 23B). Both the NA-M2e and Sp+NA-M2e groups showed over 10-fold reduction in lung viral titers compared to those in the split group. Consistently, NA-M2e and Sp+NA-M2e vaccinated mice displayed lower levels of IL-6, IFN-γ, and TNF-α cytokines in lung lysates

collected at 9 days after challenge (Figs. 23C-E). Taken together, these data suggest that NA-M2e vaccination in combination with split under primed immunity could be effective in inducing vaccine strain-specific neutralizing and cross-protective M2e and NA immunity.



Group	Timepoint 1 (0 wk)	Timepoint 2 (4 wks)	Timepoint 3 (12 wks)
G1 Split	Split	Split	-
G2 NA-M2e	-	NA-M2e	NA-M2e
G3 Sp+NA-M2e	Split	Sp+NA-M2e	NA-M2e

Figure 22. High Antibody Responses Elicited by Sp+NA-M2e Group in Sequential Vaccination Strategy. Groups of 12-months old adult BALB/c mice were vaccinated with either 2 doses of split (sCal H1N1 + sSwz H3N2), NA-M2e or in a split - S+NA-M2e - NA-M2e sequential order at 2 or 3 timepoints (A). Sera were harvested 2 weeks post each vaccination. IgG responses at timepoint 2 to (B) iCal H1N1; (C) iSwz H3N2; (D) hM2e. IgG responses at timepoint 3 to (E) iCal H1N1; (F) iSwz H3N2; (G) hM2e; and (H) N2. HAI titers at timepoint 3 to (I) A/Cal/H1N1 (J) A/Swz/H3N2. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with Seq-S-NA-M2e groups and using (A-G) two-way ANOVA and (H-I) one-way ANOVA followed by Dunnett's posttest and indicated as *; p< 0.05, **; p< 0.01, ***; p< 0.001, ****; p<0.0001.

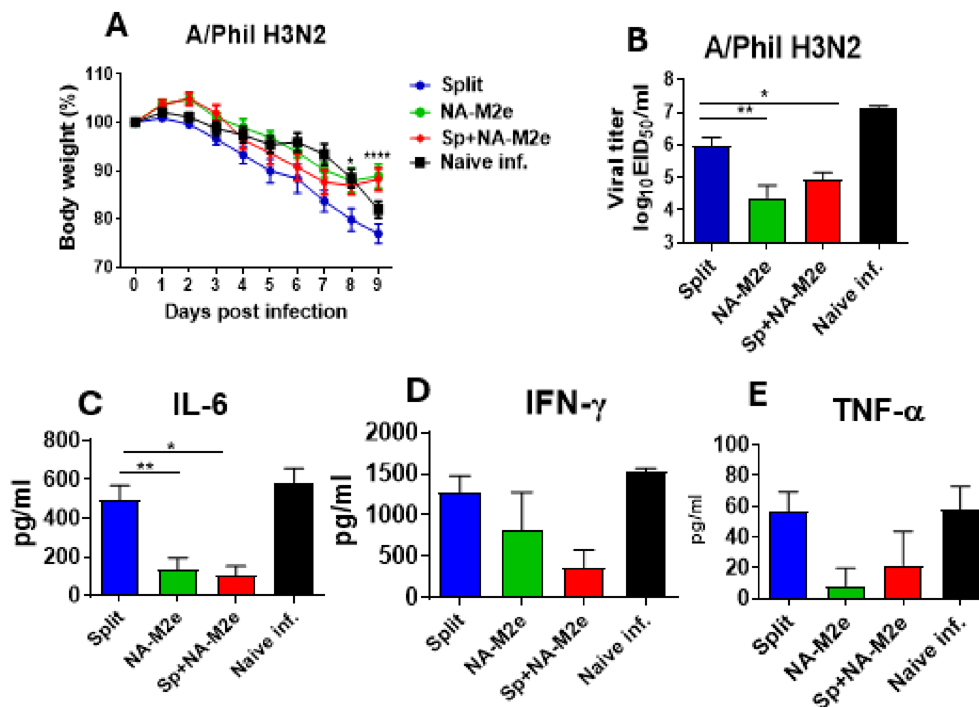


Figure 23. Improved Protection Against Heterologous A/Phil H3N2 Challenge by Split+m-cNA-M2e Sequentially Vaccinated Mice. The Split, NA-M2e or Sp+S-NA-M2e vaccinated mice were challenged intranasally with a lethal dose of heterologous A/Phil/H3N2 virus. Their body weights were monitored for 9 days before they were sacrificed, and tissues were harvested. (A) Body weight changes (B) Lung viral titers in eggs and (C) IL-6 (D) IFN-γ and (E) TNF-α cytokine levels in lung lysates at 9 dpi. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with NA-M2e and Sp+NA-M2e groups and using (A) two-way ANOVA or (H-I) one-way ANOVA followed by Dunnett's posttest and indicated as *; $p< 0.05$, **; $p< 0.01$, ***; $p< 0.001$, ****; $p<0.0001$.

3.5 Discussion

Most influenza vaccines on the market target the immunodominant hemagglutinin (HA) surface protein of the virus. However, this current vaccination strategy presents challenges for protection against variants because the HA head domain frequently mutates in response to selective pressures from immune responses and host adaptation (Hay et al., 2001). These mutations lead to antigenic drift, necessitating periodic vaccine updates (Krammer & Palese, 2014). Further complications arise when there is a mismatch between the vaccine strain and the circulating virus. To address these issues, this study investigated a strategy to supplement the bivalent (A/Cal/H1N1 + A/Swz H3N2) inactivated split virus vaccine with the NA-M2e vaccine, which has shown broad

cross-protection against multiple influenza strains in both mice and ferret models (Kim, 2024; Kim et al., 2022). This study demonstrated that NA-M2e supplemented split (Split+NA-M2e) vaccinations could elicit humoral and cellular responses at higher levels to provide enhanced protection in both homologous A/Cal/H1N1 and heterologous A/Phil/H3N2 and rgA/Viet/H5N1 lethal virus challenges, than split vaccine alone. Notably, NA-M2e alone vaccination could provide comparable homologous protection without inducing HA neutralizing antibodies as the split vaccine group and heterologous protection as the combination group. Therefore, this approach of NA-M2e supplemented vaccination has the potential to overcome the limitations of current seasonal influenza vaccines by offering broader cross-protection against potential variants emerging.

Developing a universal influenza vaccine has proven difficult due to frequent viral mutations and the low immunogenicity of conserved antigenic targets, leading to insufficient protection. Several studies have reported enhancing the cross-protective efficacy of split vaccines by incorporating NA and M2 proteins to improve their efficacy (B. E. Johansson et al., 1998; Johansson et al., 2002; Kim et al., 2014; Raha et al., 2024). While natural infections stimulate immune responses against both HA and NA, current influenza vaccines are standardized only for HA content (Chen et al., 2018). NA immunity is considered an independent correlate of protection, as they are associated with reduced lung viral titers in mice and decreased airborne transmission of influenza virus in guinea pigs (Tan et al., 2022). Previous studies in human samples reported that pre-existing NA antibodies can protect against emergent strains by reducing viral loads and shedding (Couch et al., 1974; Monto & Kendal, 1973). Preclinical studies reported that antibodies to HA provided adequate protection against homologous viruses, while antibodies to NA alone were effective in reducing lung viral titers against antigenically distant strains (Bert E. Johansson

et al., 1998; B. E. Johansson et al., 1998). A study in ferrets demonstrated that while anti-HA and anti-NA antibodies played distinct roles in mitigating the disease of influenza virus infection, optimal results were achieved when both antigens were included in the vaccination (Bosch Berend et al., 2010). Additionally, prior studies demonstrated that anti-M2e antibodies bind to M2e antigens, prevent further viral replication, and help remove infected cells via antibody-dependent cell cytotoxicity mechanisms (El Bakkouri et al., 2011). Tied together, the addition of NA-M2e to split vaccines ensures enhanced protection by inducing immune responses to HA, NA, and M2e antigens.

The enhanced protection by S+NA-M2e vaccination strategy appears to be mediated by adaptive and innate immune mechanisms. Although both split and S+NA-M2e immunized groups demonstrated similar antibody responses to inactivated viruses and HA antigens, the split group elicited significantly lower antibody responses to M2e and NA antigens after boost immunization. Furthermore, the S+NA-M2e group showed higher IgG2a/IgG1 ratios compared to the split group (Fig. 24), indicating a virus-specific Th1 skewed response. Moreover, innate immune cells, including macrophages and dendritic cells, function as antigen-presenting cells by picking up vaccine antigens, processing, and presenting antigens to T cells (Gordon & Martinez, 2010; Guermonprez et al., 2002). In our studies, we observed higher induction of CD86-expressing activated populations of monocytes, macrophages, and dendritic cell subsets in the NA-M2e and S+NA-M2e immunized groups one day after immunization. Additionally, an increased population of natural killer cells was noted, which may contribute to the secretion of cytokines such as IFN-γ and TNF-α to activate other immune cells (Vivier et al., 2008). IFN-γ expressing CD4 and CD8 T cells mediate antiviral activities to control viral replication (Kono et al., 2014; Rouse & Sehrawat, 2010). CD4 T cells stimulate B cell proliferation and differentiation to produce antigen-specific

antibodies, and activate CD8 T cells that mediate cytotoxicity by secreting granzymes and perforins for the destruction of virus-infected cells (Ueda et al., 1994). Our studies showed significantly increased IFN-γ^+ CD4 and CD8 T cell populations after homologous A/Cal/H1N1 and heterologous A/Phil/H3N2 and rgA/Viet/H5N1 challenges. Interestingly, a higher level of IFN-γ^+ CD4 compared to IFN-γ^+ CD8 T cell populations was observed. Although we did not investigate the independent roles of CD4 or CD8 T cells in conferring protection, studies suggest that CD4 T cells exhibit perforin-mediated cytotoxicity in the lungs, which may aid in faster recovery from severe infections (Brown Deborah et al., 2012; Kim et al., 2014). Moreover, we observed that NA-M2e active vaccination induced homologous protection at similar efficacy as the split vaccine group and heterologous protection as the combination group, which were supported by body weight changes and lung viral titers. This broad efficacy of NA-M2e active vaccination might have come from the effective stimulation of innate immune cells and induction of cellular immune responses, partially due to the immune-stimulating nature of NA-M2e VLPs. As expected, neutralizing antisera from split vaccine groups more effectively protected naïve mice from homologous strains than antisera of NA-M2e. However, split antisera resulted in more body weight loss during a heterologous challenge than NA-M2e and S+NA-M2e sera. The overall outcomes of this study support that the S+NA-M2e vaccination strategy can offer more effective protection against antigenically similar and diverse strains than split or NA-M2e alone.

Sequential heterologous vaccination with different vaccine types was reported to enhance cross-protection by stimulating a broad and multifaceted immune response (Dong et al., 2024; He et al., 2021). In this study, despite having different vaccine regimens, we observed a similar pattern of protection profiles with the S+NA-M2e or Sp+NA-M2e vaccine groups, demonstrating better protection compared to the split group.

This study underscores the importance of enhancing cross-protective immune responses against diverse influenza strains by supplementing split vaccines with NA-M2e. However, the dosage effects of NA-M2e vaccine should be investigated. The immune mechanisms contributing to protection in this vaccination strategy needs to be further explored to determine the immune correlates. For instance, it remains unknown whether this strategy provides long-term protection. While we analyzed innate immune responses after prime vaccination, examining the pattern of innate immune cell activation following the boost immunization could offer additional insights into the immune mechanisms and the induction of adaptive immunity that contribute to protection. Additionally, evaluating the efficacy of this approach in larger animal models, such as ferrets or non-human primates, will be necessary to gauge its relevance for human application.

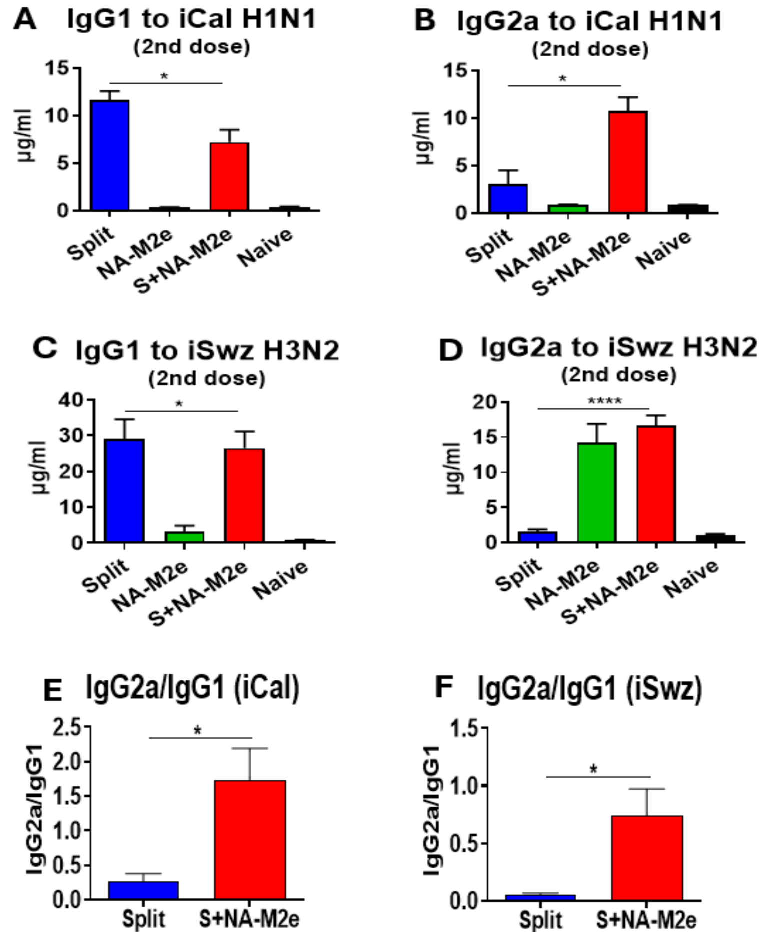


Figure 24. S+NA-M2e Vaccination Strategy Induces Higher IgG2a/IgG1 Ratios Indicating Th1 Type Immune Response. Boost (2nd dose) IgG1 and IgG2a responses to (A-B) iCal H1N1; (C-D) iSwz H3N2. Ratios of IgG2a/IgG1 to (E) iCal H1N1 (F) iSwz H3N2. The data is presented in concentrations (μg/ml). Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with S+NA-M2e group and performed by using one-way ANOVA with Dunnett's posttest and indicated as *; $p < 0.05$, and ****; $p < 0.0001$.

3.6 Conclusion

This study explored the comparative efficacy and immune mechanisms that might have contributed to the homologous and heterologous protection by a strategy of supplemented Split+NA-M2e vaccination, compared to either split or NA-M2e alone vaccine in mice. We also showed that heterologous split and NA-M2e vaccination under preexisting immunity in primed mice could enhance cross-protection. The findings in this study would have impact on developing effective vaccination strategies that could be employed to improve protection against circulating

homologous strains and newly emergent mismatch variants. It should be mentioned that there is still a significant gap in developing more cross-protective vaccines and vaccination approaches because of substantial body weight losses observed with the S+NA-M2e and Sp+NA-M2e vaccinations after heterologous virus challenges.

3.7 Acknowledgements

This study was supported by NIH/NIAID grants AI154656 (S.-M.K.). The funders had no contribution in experimental design, collection of data, analysis, decision to publish, or manuscript preparation. A/Switzerland/9715293/2013 (H3N2) virus was generously gifted by Dr. Florian Krammer. The following reagents were obtained through BEI, NIAID, NIH. H1 HA protein (NR-15749), H3 HA protein (NR- 19238), B/Florida/4/2006 (NR-9696), N1 NA protein (A/California/4/2009, NR-19234), N2 NA protein (A/Brisbane/10/2007, NR-43784), and influenza B NA proteins (B/Florida/4/2006, NR-19236). A/Hong Kong/1073/99 H9N2 influenza virus (FR- 732) was obtained from International Reagent Resource.

4. Chapter 3: Intranasal Vaccination with Multi Neuraminidase and M2 Ectodomain Virus-Like Particle Enhances Broad Protection Against Influenza

4.1 Abstract

Intramuscular injection of seasonal influenza vaccines provides strain-specific serum neutralizing antibodies not against variants and no effective mucosal immunity. Here, we report that multi-subtype neuraminidase and M2 ectodomain repeat virus-like particle vaccine (NA-M2e) confers higher efficacy of broad cross-protection via intranasal delivery than intramuscular injection. The intranasally prime-boost vaccinated mice displayed higher levels of serum and lung IgA antibodies, germinal center B cells, IFN-γ^+ CD4 and CD8 T cells, plasma cells, and resident memory B cells locally in the lungs whereas intramuscular vaccination induced strong systemic immunity. Our findings suggest that the intranasal administration of NA-M2e vaccine induces enhanced mucosal and comparable systemic immunity, offering improved efficacy of cross-protection against diverse influenza strains compared to the intramuscular route.

4.2 Introduction

Influenza viruses pose a considerable health and economic burden each year, leading to 3-5 million cases of severe illness (WHO, 2023). The influenza vaccine is a representative that requires annual updates due to frequent mutations in the global head domain of the immunodominant surface protein, hemagglutinin (HA) (Nafziger & Pratt, 2014; Zost et al., 2019). The journey of an influenza virus begins when it enters the nasal cavity of an uninfected person through aerosolized droplets and infects the epithelial cells of the mucosal surface in the upper respiratory tract (Kalil & Thomas, 2019). Approximately 80% of the body's immune cells are found in mucosal sites, particularly within the mucosa-associated lymphoid tissue which consists of specialized antigen-presenting cells (APCs), B and T cell populations (Lobaina Mato, 2019;

Yusuf & Kett, 2017). The mucosal surfaces of the respiratory tract include the nasal-associated lymphoid tissue and the bronchus-associated lymphoid tissue, which serve as inductive sites for initiating immune responses against inhaled pathogens or antigens. Accordingly, it is important to prioritize the induction of both systemic and mucosal immunity when designing vaccines against respiratory viruses such as the influenza.

Approved vaccines on the market primarily target the HA protein, but neutralizing antibodies against HA provide strain-specific protection (Edgar et al., 2023). In contrast, the M2 ectodomain protein (M2e) is conserved across strains, and the neuraminidase (NA) proteins on the influenza surface acquire relatively fewer mutations compared to the HA (Wiersma et al., 2015). Most of the approved influenza vaccines are administered intramuscularly (IM). While intramuscular administration of vaccines induces protective systemic immunity, it is invasive and carries the risk of contamination and injury, leading to safety concerns. In contrast, nasal vaccines are non-invasive, can induce both systemic and mucosal immunity, and avoid the issues of non-compliance related to needle-pricks and pain (Kehagia et al., 2023). FluMist, the only FDA-approved intranasal influenza vaccine, employs live attenuated influenza viruses and can be self-administered as a nasal spray by people up to 49 years of age (Kemp, 2024). These cold-adapted live-attenuated viruses replicate solely in the upper respiratory tract, which helps ensure safety; however, they have been found to induce variable immune responses and offer lower efficacy compared to inactivated vaccines (CDC, 2019, 2022). Previous studies have shown that IgA antibodies, found in mucosal secretions, in addition to serum IgG can induce cross-reactive antibodies, thereby providing enhanced effectiveness and breadth of protection (Lobaina Mato, 2019).

Several virus-like particle (VLP) vaccines have been approved against hepatitis B and E viruses (HBV & HEV) and human papillomaviruses (HPV) (Nooraei et al., 2021). The HeberNasvac VLP vaccine is a licensed nasal vaccine in Cuba, approved for immunization against HBV (Aguilar et al., 2022; Kehagia et al., 2023). VLP vaccine containing the tandem repeats of M2e (5xM2e) was reported to provide significant efficacy with intranasal administration (Lee et al., 2018). A previous study reported a unique (NA-M2e) VLP vaccine containing multiple subtypes of consensus NA proteins (N1 NA + N2 NA + flu B NA) plus 5xM2e repeat, which could confer broad cross-protection against different subtypes and lineage influenza A and B viruses respectively in mice after intramuscular immunization (Kim et al., 2022). The immunogenicity and efficacy of NA-M2e VLP vaccine after intranasal delivery remain unknown, in comparison with intramuscular injection. In the present study, we investigated the immunogenicity and efficacy of NA-M2e VLP vaccine after delivery via the intranasal route versus intramuscular route. In addition, profiles and distributions of innate immune responses between the mucosal and systemic sites were studied. The findings of this study underscore the significance of nasal delivery of NA-M2e vaccine in inducing mucosal and systemic immunity for the outcome of enhancing cross-protection against influenza viruses.

4.3 Methods

4.3.1 Viruses and Vaccines

For the purpose of virus challenges live viruses A/Philippines/2/1982 H3N2 (A/Phil/H3N2), A/California/04/2009 H1N1 (A/Cal/H1N1), rgA/Viet/H5N1 (containing HA and NA from A/Vietnam/1203/2004 H5N1 and a A/Puerto Rico/8/1937 (A/PR8 H1N1) backbone) (Song et al., 2011), B/Florida/4/2006 (B/FL), and A/Switzerland/9715293/2013 (A/Swz/H3N2) were used. Viruses were amplified through propagation in 10-day-old embryonated chicken eggs.

The collected allantoic fluid was centrifuged at 2,000 rpm for 20 minutes and subsequently stored at -80 °C. The NA-M2e vaccine (Fig. 1A) was produced in insect cells and prepared following procedures previously as outlined (Kim et al., 2022).

4.3.2 Immunization and Challenge

Young adult BALB/c mice (n=10, 5-8 weeks old) obtained from Jackson Laboratory (Bar Harbor, ME) were either intramuscularly (IM) or intranasally (IN) immunized with 10 μg NA-M2e vaccines in a prime-boost regimen at 3-week interval. Vaccinated and unvaccinated naïve controls were challenged with lethal doses of live viruses A/Phil/H3N2 (3xLD50, 2.3x10^2 EID_{50}), A/Cal/H1N1 (5.3xLD_{50}, 2x10^3 EID_{50},), rgA/Viet/H5N1 (3xLD_{50}, 2.6x10^4 EID_{50}), A/Switzerland/9715293/H3N2 (3xLD_{50}), and B/FL (2xLD_{50}, 5.3×10^4 EID_{50}) a month after 2nd dose immunization. The body weight changes, and survival were monitored daily for 14 days. All animal studies detailed in this manuscript received approval from the Georgia State University (GSU) Institutional Animal Care and Use Committee review boards.

4.3.3 Antibody Responses

Antibody responses in sera and lung extracts were determined by ELISA as previously outlined (Ko et al., 2018). The coating antigens were 1 µg/ml N2 NA (A/Brisbane/10/2007 H3N2), N1 NA (A/Brisbane/59/2007 H1N1), and influenza B NA (B/Florida/4/2006) and 4 µg/ml human M2e (hM2e, SLLTEVETPIRNEWGSRSN) peptide and inactivated influenza viruses A/Cal/H1N1, A/Swz/H3N2, A/Phil/H3N2, rgA/Viet/H5N1 and B/New York/PV00094/2017 (Yamagata). The levels of IgG were assessed using horseradish peroxidase (HRP)-conjugated secondary antibodies specific to mouse IgG and IgA, following the method as described (Bhatnagar et al., 2022). Quantitative concentrations of IgG were determined by converting the

respective optical density (OD) values using standard curve equations established with commercially available purified IgG antibodies from Southern Biotech, Birmingham, AL.

4.3.4 Lung Viral Titration

Diluted lung lysate samples were inoculated into 10-day-old embryonated chicken eggs, and the harvested allantoic fluid was evaluated for lung viral titers by determining hemagglutination activity as described (Wen & Wang, 1959). To determine lung viral titration in MDCK cells, the cells were seeded at a density of $4x10^4$ cells per well, and diluted lung extracts from infected mice were added to the cells followed by a 3-day incubation period. Hemagglutination inhibition (HAI) assay was performed with harvested supernatant to determine viral titers. Briefly, heat-inactivated sera were treated with receptor-destroying enzymes (RDE, Sigma-Aldrich) and mixed with 4 HA units of viruses. 0.5% chicken red blood cells were added to the mixture to determine HAI titers as described (Bhatnagar et al., 2022).

4.3.5 Cytokine Responses

Ready-Set-Go IL-6, IL-1β, TNF-α kits (eBioscience, San Diego, CA), and Mouse IFN-γ Uncoated ELISA kit from Invitrogen were used to determine cytokine levels in lung lysate samples.

4.3.6 Flow Cytometry

For intracellular cytokine staining, harvested lung or spleen tissues from day 8 post challenge were homogenized prepared as single cell suspensions. The cells were in vitro stimulated with 5 µg/ml of the following antigens hM2e peptide, N1 NA (A/Cal/04/2009 H1N1), and N2 NA (A/Brisbane/10/2007 H3N2) in the presence of Brefeldin A (20 µg/ml) at 37°C for 5 hours. Lymphocyte staining was done with anti-mouse CD3 (clone 17A2), CD4 (clone 553051), CD8 (clone 25-0081-82), and IFN-γ (clone XMG1.2) mAb. The BD Cytofix/Cytoperm Plus kit was

used ((Ko et al., 2018) for intracellular cytokine staining. The innate immune cell populations were analyzed by staining with the following: anti-CD45 (clone 30-F11), anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-CD103 (clone 2E7), anti-F4/80 (clone BM8), anti-MHCII (clone I-A/I-E), anti-B220 (clone RA3-6B2), anti-Ly6C (clone AL-21), anti-CD86 (clone PO3) and anti-CD8α (clone 53-6.7). The data were analyzed using Flowjo software (Tree Star, Inc., Ashland, OR) and expressed as absolute cell numbers. B cell populations were analyzed by staining with the following: anti-CD3, anti-IgD (clone 11.26), anti-CD138 (clone 281-2), anti-CD69 (anti-H1.2F3), anti-CD19 (clone eBio1D3), anti-CD38, anti-GL7 (clone GL7), anti-B220, and anti-MHCII. Lastly, germinal center B cells were analyzed by staining with anti-CD3, anti-CD19, anti-B220, anti-IgD and anti-GL7. Cells were blocked with Fc receptor blocker anti-CD16/32 mAb prior to staining. All data are presented as absolute cell number based on harvested cell counts.

4.3.7 Enzyme-Linked Lectin Assay (ELLA)

To determine NA inhibition (NAI) activity of immune sera, ELLA was performed as described (Pardi et al., 2022). Briefly, immune sera and virus were added to fetuin (25 μg/mL) coated 96-well plates and incubated at 37°C for 20 hours. NAI activity was measured using TMB substrate (eBiosciences) following an incubation with 1 μg/mL of HRP-labeled peanut lectin. The formula used to calculate inhibition percentage: $100 \times (OD_{\text{virus only control}} - OD_{\text{test sample}})/OD_{\text{virus only control}}$.

4.3.8 Passive Sera Transfer

For passive sera transfer experiments, sera from immunized or naïve mice were heat-inactivated at 56°C and mixed with lethal dose of viruses, A/California/04/2009 H1N1 (2xLD_{50}, 1.6x10^4 EID_{50}) in BALB/c mice, A/Switzerland/9715293/2013 H3N2 (3xLD_{50}) in DBA/2J mice, rgA/Vietnam/1203/2004 H5N1 (4xLD_{50}, 2.6x10^4 EID_{50}) in BALB/c mice and B/Florida/04/2006

($2xLD_{50}$, $5.3x10^4$ EID_{50}) in BALB/c mice. The sera-virus mixture was incubated at room temperature for 30 minutes as described (Getie-Kebtie et al., 2013), and intranasally administered to 5-8 weeks old young adult mice. The mice's body weights and survival were monitored daily for 14 days.

4.3.9 Statistical Analysis

All data presented in this study are expressed as mean ± standard error of the mean (SEM). Statistical significance for all experiments was determined by comparing IM with IN group using one-way or two-way analysis of covariance (ANOVA), followed by Dunnett's multiple comparison posttests. P values below 0.05 ($p<0.05$) were regarded as statistically significant. Data analysis was conducted using Prism software (GraphPad Software, Inc., San Diego, CA).

4.4 Results

4.4.1 Intranasal Delivery of NA-M2e Vaccine Induces IgG Antibodies at Comparable or Lower Levels as Intramuscular Route in Boost Sera

Serum antibody responses were compared in BALB/c mice after intranasal (IN) and intramuscular (IM) prime and boost dose vaccination with NA-M2e VLP (10 µg) containing multi-subtype NA (N1NA+N2NA+BNA) and M2e tandem repeat (5xM2e) (Fig. 25A). Sera were harvested 2 weeks after each prime and boost IN and IM immunization and analyzed for antibody responses (Fig. 1A). IgG responses to both M2e and N2 NA antigens were induced at high levels ($4x10^3$ titers IM) in prime sera after 1st dose IM vaccination with NA-M2e whereas IgG responses were negligible after IN prime dose (Figs. 25B). After boost NA-M2e vaccine dose, the IN group displayed significantly elevated levels of IgG antibodies for the vaccine antigens. Compared to prime IgG responses, the boost IgG responses to M2e ($6x10^4$ titers IM) and N2 NA ($2x10^4$ titers IM) increased by approximately 5 to 15 folds in IM-immunized mice, while IN immunized mice

showed an approximately 10^3- to 10^4-fold increase in IgG titers for M2e ($3x10^3$ titers IN) and N2 NA ($2x10^4$ titers IN) antigens (Fig. 25C). IM boost dose induced higher levels of IgG titers for M2e ($6x10^4$ IM vs $3x10^3$ IN) and N1 NA ($1x10^3$ IM vs $1x10^2$ IN) than IN boost dose whereas both IM and IM boost induced similar IgG titers for N2 NA ($2x10^4$ titers) and B NA ($2x10^5$ titers) antigens (Figs. 25C, D). Furthermore, IM and IN boost IgG responses in sera were determined for virus-specific antigens. IN immunized mice elicited higher antibody responses for inactivated A/Swz H3N2 antigen than the IM group ($4x10^4$ IM vs $1x10^5$ IN) but both IM and IN groups displayed similar IgG titers to inactivated A/Phil H3N2 ($5x10^2$ titers), A/Cal H1N1 ($8x10^2$ titers), rgA/Viet H5N1 ($6x10^2$ titers), and flu B/NY antigens ($1x10^3$ titers) (Figs. 25E-F). Altogether, IN immunization with NA-M2e VLP significantly boosted IgG responses after 2nd dose.

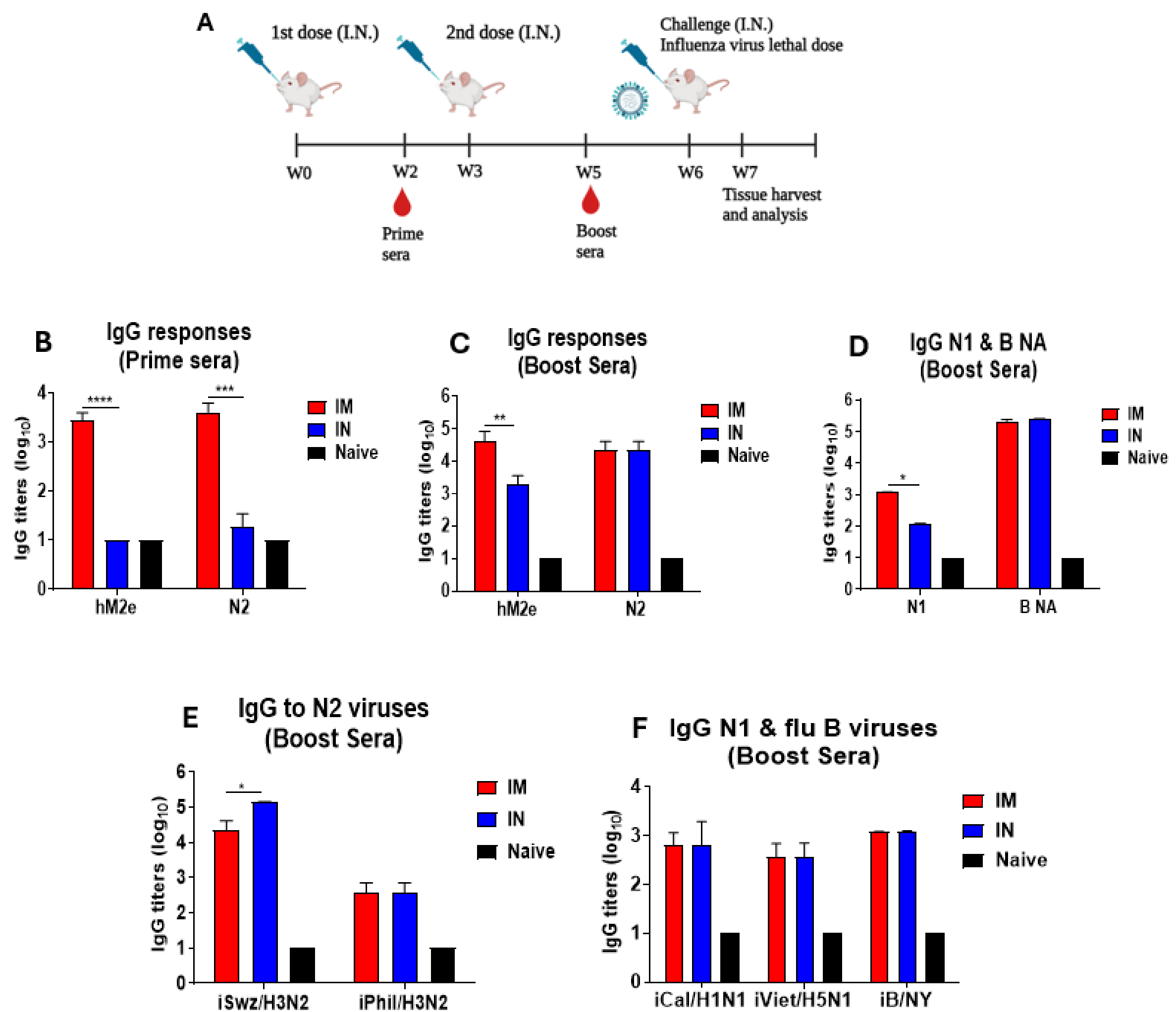


Figure 25. Higher IgG Antibody Levels to Vaccine Antigens in IN Immunized Boost Sera than in Prime Sera. Groups of 5-6 weeks old BALB/c mice (n=5) were immunized with (A) 10 µg NA-M2e (VLP containing consensus sequences: N1 NA + N2 NA + flu B NA + 5xM2e) vaccines in the intramuscular (IM) or intranasal (IN) routes of vaccine administration in a prime-boost regimen at 3-week-intervals. Prime (1st dose) sera IgG antibody responses to (B) hM2e and N2 and boost (2nd dose) sera responses to (D) N1 and B NA, (E) N2 inactivated viruses, and (F) N1 and flu B inactivated viruses were determined by ELISA. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing IM group with IN group and performed by (B-G) using one-way ANOVA with Dunnett's posttest and indicated as **; $p<0.01$, and ****; $p<0.0001$.

4.4.2 Intranasal Delivery of NA-M2e Vaccine Provides Higher Efficacy of Broad Protection than IM Route

To determine the efficacy of protection, NA-M2e IM and IN immunized mice were intranasally challenged with lethal doses of influenza A viruses. Body weight changes were monitored for 8 days (A/Phil H3N2), 7 days (A/Cal H1N1, rgA/Viet H5N1) or 6 days (A/PR8

H1N1) until sacrifice to determine lung viral titers (Figs. 26A-D). The IN groups displayed more effective prevention against weight loss and better protection against A/Philippines H3N2 (9% IN vs 15% IM), A/California H1N1 (5% IN vs 8% IM), rgA/Vietnam H5N1 (8% IN vs 13% IM), and A/PR8 H1N1 (9% IN vs 17% IM) viruses respectively than the respective IM groups. The IN groups showed significantly lower levels of lung viral titers by 100 folds than those in the corresponding IM groups. All IN vaccinated mice survived and started recovering as early as day 5, whereas IM vaccinated mice had a delayed recovery at day 6 or 7 post challenge.

Overactive innate immune responses and excessive production of cytokines can lead to severe disease during influenza virus infection. Next, we assessed the status of lung inflammation due to viral infection 8 days after A/Phil/H3N2 challenge. Both the IM and IN groups displayed lower levels of inflammatory cytokines (IL-6, IL-1β, TNF-α, IFN-γ) compared to the naïve infection group (Figs. 26E-H). The IN group more effectively lowered cytokine levels (IL-6, IFN-γ) than the IM group. Monocytes ($CD11b^{+}Ly6c^{high}F4/80^{low}$) were observed at lower levels in the lungs from the IM and IN groups than naïve infection mice whereas the IN group was more effected protected against infiltration of neutrophils ($CD11b^{+}Ly6c^{+}F4/80^{-}$) (Figs. 26I, J). Overall, these data indicate that IN delivery of NA-M2e vaccine could provide higher efficacy of protection against multiple strains of influenza A viruses than IM immunization.

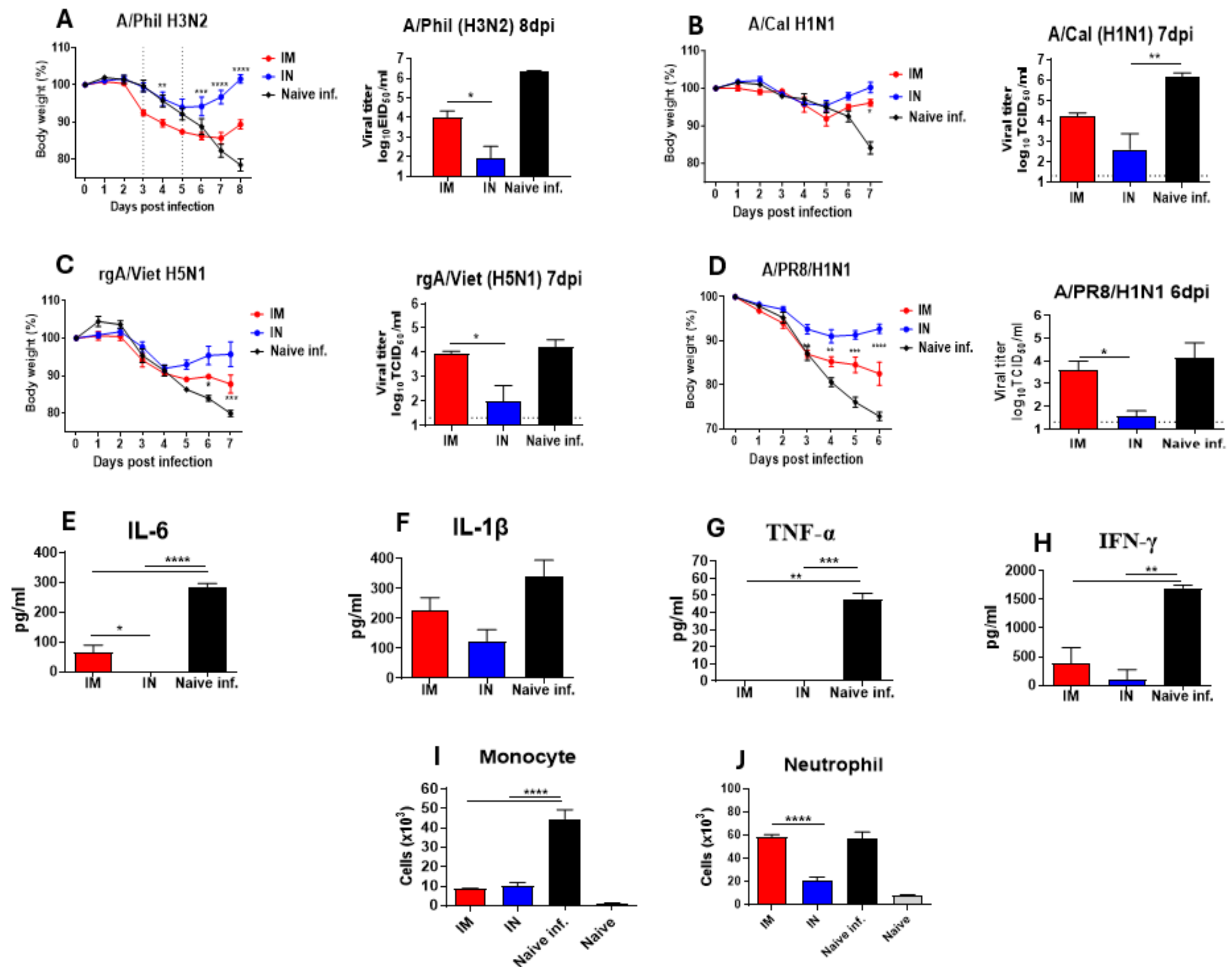


Figure 26. IN Immunized Mice Demonstrate Higher Protective Efficacy Against Influenza A Viruses Compared to IM Immunized Mice in Lethal Virus Challenge. Boost immunized IN and IM mice were challenged with lethal doses of influenza viruses and sacrificed. Their body weights were monitored for 7 dpi, or 8 dpi and tissues were harvested for further analysis. Body weight changes and lung viral titers for (A) A/Phil/H3N2 till 8 dpi (B) A/Cal/H1N1 till 7 dpi (C) rgA/Viet/H5N1 till 7 dpi (D) A/PR8/H1N1 till 6 dpi. Cytokine levels (E) IL-6, (F) IL-1β, (G) TNF-α, and (H) IFN-γ in lung extracts after challenge. Inflammatory cellular infiltrates in lungs after A/Phil/H3N2 challenge (I) Monocytes: $CD45^{+}CD11b^{+}Ly6C^{high}$ and (J) Neutrophils: $CD45^{+}CD11b^{+}F4/80^{-}Ly6C^{low}$. Error bars are represented as mean ± SEM. The statistical significance was determined by comparing IM group with IN group and performed using two-way ANOVA (A, C, E) or one-way ANOVA (B, D, F) with Dunnett's posttest.

4.4.3 Intranasal Immunization with NA-M2e Vaccine enhances IgA Antibody Responses

The IN group showed approximately two-fold higher numbers of germinal center (GC) phenotypic B cells ($B220^{+}IgD^{-}GL7^{+}$) in mediastinal lymph nodes (mLN) compared to those in the IM group on day 8 post-challenge with A/Phil/H3N2 virus (Fig. 27A). To better understand

immune correlates, we determined IgG and IgA antibodies specific to NA and M2e in lung samples and sera before and after challenge with viruses A/Cal/H1N1, rgA/Viet/H5N1, A/PR8/H1N1 and A/Phil/H3N2(Figs. 27B-I). Notably, the IM group induced higher levels of lung IgG antibodies for M2e (before challenge, Fig. 27B) and N1 NA (before and after challenge, Fig. 27C) and comparable levels of lung IgG for M2e (after challenge, Fig. 27B) and N2 NA (before and after challenge, Fig. 27D), compared to the IN group. More importantly, the IN group induced higher levels of serum IgA antibodies for M2e and N2 NA antigens (Fig. 27E, F), lung IgA for N2 NA (Fig. 27G) before and after challenge, lung IgA for M2e after challenge (Fig 27H), and lung IgA to N1 NA before challenge (Fig. 27I) than those induced by IM immunization with NA-M2e. These data support that IN delivery of NA-M2e vaccine can induce more efficient production of IgA antibodies at mucosal and systemic sites than IM immunization, suggesting a possible immune correlate of protection.

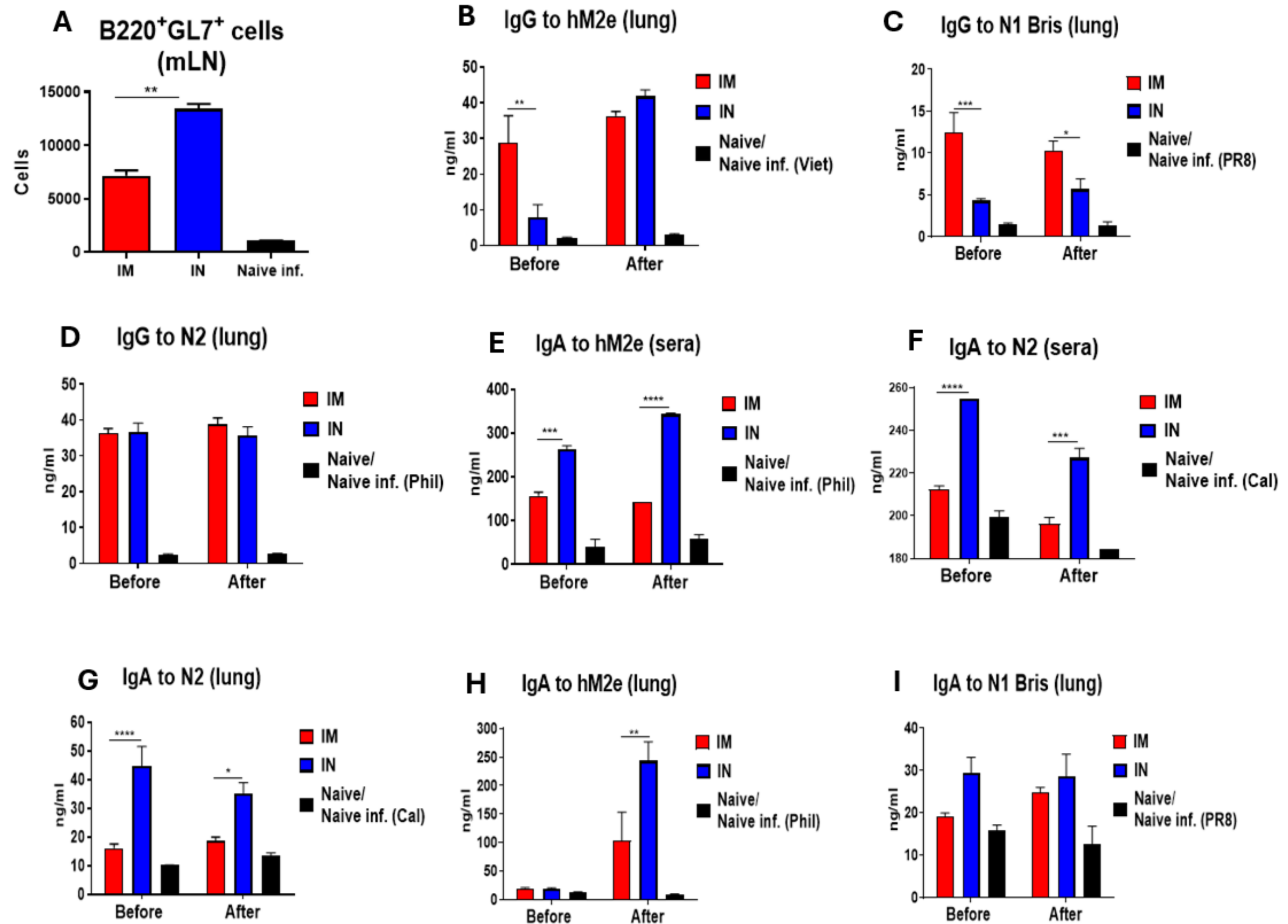


Figure 27. Elevated Levels of IgA Antibodies Induced in NA-M2e Intranasally Immunized Mice. Groups of IM and IN (n=5) prime-boost immunized mice were sacrificed before (3 weeks post boost) or after lethal virus challenge (7 or 8 dpi), and their tissues and sera were harvested. (A) B220$^+$GL7$^+$ Germinal center B cells in mLN (A/Phil/H3N2) were determined by flow cytometry. IgG antibody responses in lung extracts specific for (B) hM2e, (C) N1, and (D) N2. IgA antibody responses in sera specific for (E) hM2e, and (F) N2. IgA antibody responses in lung extracts for (G) N2, (H) hM2e and (I) N1. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing IM group with IN group and performed by using one-way ANOVA (A) or two-way ANOVA (B-G) with Dunnett's posttest and indicated as *; p<0.05, **; p<0.01, ***; p<0.001 and ****; p<0.0001.

4.4.4 Intranasal Delivery of NA-M2e Vaccine Efficiently Induces Effector T Cell Responses in the Lung Tissues

Effector T cell responses were determined 8 days post A/Phil/H3N2 challenge in lung extracts (Figs. 28A-F) and in splenocytes (Figs. 28G-I). The IN group induced 2 folds higher levels of IFN-γ^+ lung CD8 T cells with M2e or N2 NA antigen stimulation before challenge than

the IM group, where both groups showed similar levels of effector CD8 T cells after challenge (Fig. 28A, B). IFN-γ^+ CD4 T cells were observed at higher levels in the IN group than those in the IM group, where lung cells were harvested post-challenge with A/Phil/H3N2 virus and stimulated with M2e or N1 NA (Figs. 28C, D). In contrast, the IM group but not IN-immunized mice induced stronger IFN-γ^+ CD4 and CD8 T cell responses upon M2e stimulation in the splenocytes collected before and after challenge (Figs. 28G-I). We observed a higher level of N1 NA-stimulated IFN-γ^+ splenic CD4 T cells in the IN group than in the IM group after challenge (Fig. 28J). Both the IN and IM groups induced similarly high levels of IFN-γ^+ CD4 T cells and IFN-γ^+ CD8 T cells upon N2 NA stimulation of splenocytes, more prominently after challenge (Fig. 28K, L). Tied together, these findings indicate that the route of NA-M2e vaccine administration plays a crucial role in shaping the distribution and strength of T cell responses, with intranasal immunization favoring effector T cell responses in the lung while intramuscular immunization promoting stronger effector systemic T cell responses as observed in spleen tissues.

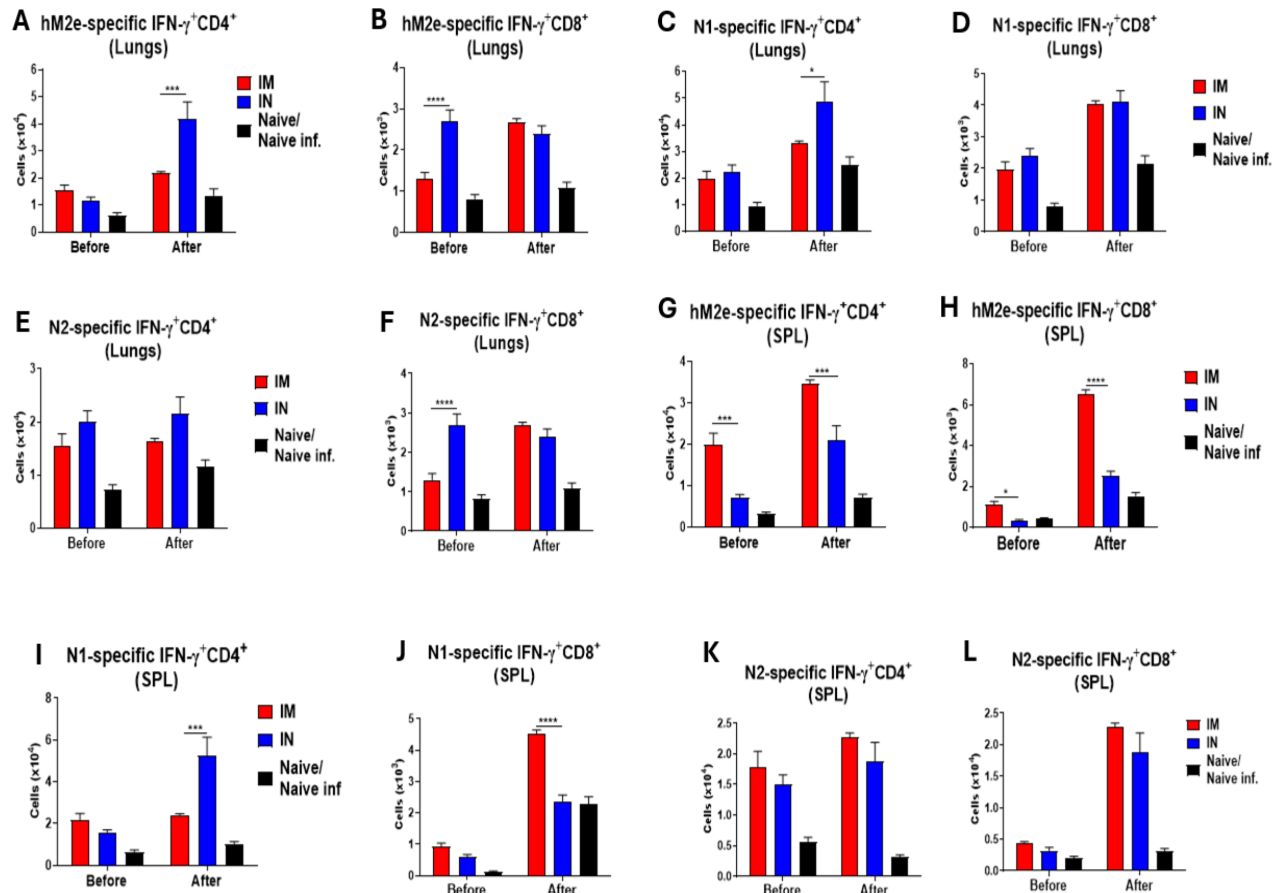


Figure 28. IN Immunized Mice Elicit Significant T Cell Responses in the Lungs. IM and IN prime-boost immunized mice were sacrificed before (3 weeks post boost) or after challenge with A/Phil/H3N2 (8 days post infection). Intracellular cytokine staining in lung cells to measure hM2e specific (A) IFN-γ^+CD4$^+$ and (B) IFN-γ^+ CD8 T cells, N1-specific (C) IFN-γ^+CD4$^+$ and (D) IFN-γ^+ CD8 T cells and N2-specific (E) IFN-γ^+CD4$^+$ and (F) IFN-γ^+ CD8 T cells. Intracellular cytokine staining in splenocytes to measure hM2e specific (A) IFN-γ^+CD4$^+$ and (B) IFN-γ^+ CD8 T cells, N1-specific (C) IFN-γ^+CD4$^+$ and (D) IFN-γ^+ CD8 T cells and N2-specific (E) IFN-γ^+CD4$^+$ and (F) IFN-γ^+ CD8 T cells. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing IM group with IN group and performed by (B-G) using two-way ANOVA with Dunnett's posttest and indicated as ***; $p<0.001$, and ****; $p<0.0001$.

4.4.5 NA-M2e Intranasal Delivery Increases B Cell Responses in the Lung

Plasma cells (PC) secrete antibodies and memory B cells (MBC), considered a precursor of plasma cells (Amanna et al., 2007; Crotty et al., 2003; Whitney E. Purtha et al., 2011). Tissue-resident memory B cells (Brm) rapidly differentiate into antibody-secreting cells and concentrate antibodies locally upon exposure to antigens, conferring long-lasting immunity (MacLean et al., 2022). B cell responses were determined 6 days after the challenge with A/PR8/H1N1 virus. PC,

MBC and Brm cell populations were significantly increased in IN immunized mice compared to IM immunized mice in lungs (Figs. 29A-C). While PC populations (consistent with IgG responses in sera) were higher in IM immunized mice in spleen, MBC and Brm populations were similar between the IN and IM groups in spleen (Figs. 29D-F). These data suggest that IN immunization with NA-M2e VLP can elevate memory B cells both in the mucosal and systemic sites.

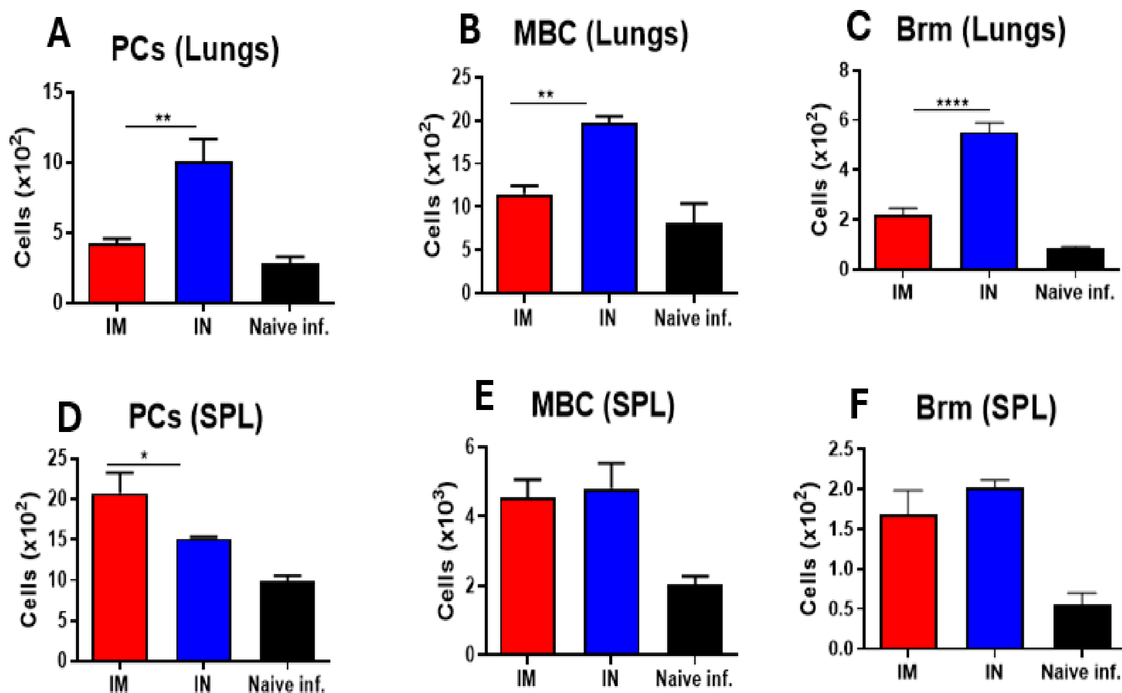


Figure 29. Higher Induction of B Cells in IN Immunized Mice. Groups of IM and IN prime-boost immunized were challenged with A/PR8/H1N1 and sacrificed 6 dpi. Plasma cells (PCs): $CD3^{-}IgD^{-}CD19^{+}B220^{+}CD138^{+}GL7^{-}$; memory B cells (MBCs): $CD3^{-}B220^{+}CD19^{+}CD138^{-}IgD^{-}$ and Resident memory B cells (Brm): $CD3^{-}B220^{+}CD19^{+}IgD^{-}CD38^{+}CD69^{+}$ in (A) lungs and (B) Spleens. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing IM group with IN group and performed by using one-way ANOVA with Dunnett's posttest and indicated as *; $p<0.05$, **; $p<0.01$, ***; $p<0.001$ and ****; $p<0.0001$.

4.4.6 IN and IM Delivery of NA-M2e Vaccine Differentially Mobilizes Innate Immune Cells in the Lung and Spleen Tissues Within a Day

Acute innate immune responses were analyzed in the lung and spleen tissues within 24 hours after priming the mice with NA-M2e VLP vaccine via the respective IN and IM routes. While serum responses did not show elevated cytokine levels, the lung extracts revealed increased MCP-1 and IL-6 levels in IN-immunized mice (Fig. 30A, B). Both monocytes and macrophages with $CD86^+$ activation marker were observed at higher levels in the lung after IN delivery whereas reverse distributions were observed in the spleen after IM delivery (Figs. 30C, D). Activated diverse $CD86^+$ DC subsets were also differentially distributed in the lung and spleen tissues, depending on the IN and IM delivery. $CD11b^+$ migratory DCs (mDC) were higher in the lung for the IN group (Fig. 30E). Whereas $CD103^+$ mDCs and pDCs were higher at both sites for the IM group (Fig. 30F. G). Furthermore, $CD11b^+$ resident DC populations were elevated at the lung and spleen for the IN group (Fig. 30H). $CD8\alpha^+$ resident DC populations were increased in the lung for the IN group and in the spleen for the IM group (Fig. 30I). Overall, IN delivery preferentially recruit activated antigen-presenting cell populations mostly in the lung meanwhile IM route in the spleen lymphoid tissues.

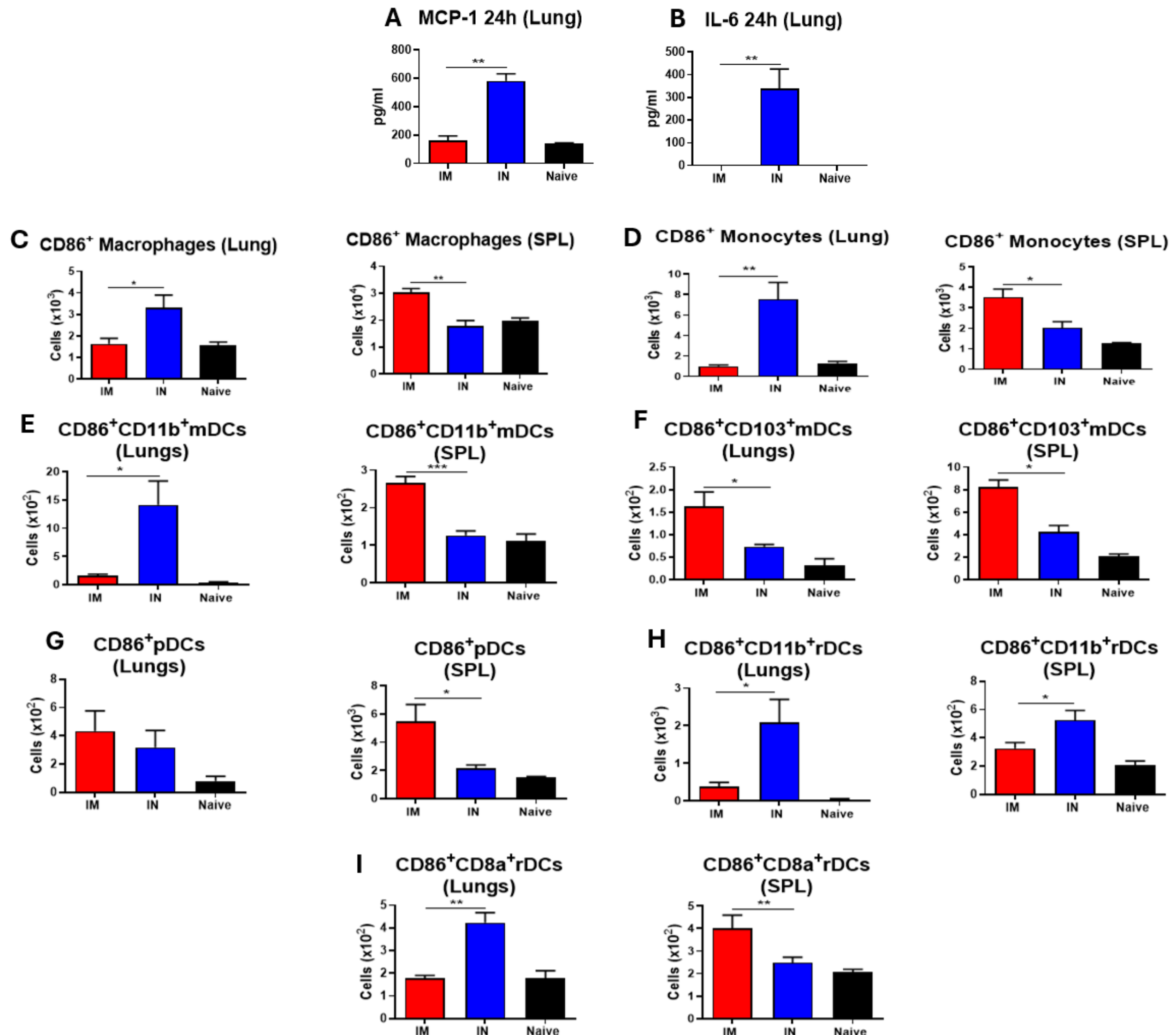


Figure 30. Activation of Innate Immune Cells in the Lungs of IN Immunized Mice 1 Day Post Prime Immunization. Prime immunized IM and IN mice were sacrificed 1 day after vaccination and their lung and spleen tissues were harvested. (A) MCP-1 chemokine (B) IL-6 cytokine responses 24 hours after prime immunization. Flow cytometry analysis of activated innate immune cell subsets in lungs and spleen (C) Monocytes: $CD45^{+}CD11b^{+}F4/80^{low}Ly6C^{high}$; (D) Macrophages: $CD45^{+}CD11b^{+}F4/80^{+}$; (E) $CD11b^{+}$mDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{high}CD103^{-}CD11b^{+}CD86^{+}$; (F) $CD103^{+}$mDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{high}CD103^{+}CD11b^{-}CD86^{+}$; (G) Plasmacytoid DC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{high}CD103^{-}CD11b^{-}220^{+}CD86^{+}$; (H) $CD11b^{+}$rDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{low}CD11b^{+}CD86^{+}$; (I) $CD8\alpha^{+}$rDC: $CD45^{+}F4/80^{-}CD11c^{+}MHCII^{low}CD8\alpha^{+}CD86^{+}$. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing split group with S+NA-M2e group using one-way ANOVA with Dunnett's posttest and indicated as *; p< 0.05, **; p< 0.01, and ***; p<0.001

4.4.7 Roles of Antisera in Conferring Protection in Naïve Mice

We determined NAI titers against A/Cal/H1N1, A/Phil/H3N2, rgA/Vietnam/H5N1 and B/Florida Yamagata in boost sera (Fig. 31A). NAI titers in IN and IM groups were similarly observed with 60-80% inhibition activities against the viruses tested. Sera from vaccinated and naïve mice were mixed with lethal doses of influenza A and B viruses and administered intranasally in naïve mice (Figs. 31B-E). Sera from both NA-M2e IM and IN immunized mice protected against lethal influenza viruses A/Cal/H1N1, A/Swz/H3N2, rgA/Viet/H5N1, and B/Florida with 100% survival rates. All mice that received naïve sera succumbed to infection with over 20% loss in body weight. Mice that received IN sera showed 5% or no loss in body weight after A/Cal/H1N1, rgA/Viet/H5N1, and B/Florida inoculation (Figs. 31B, 31C-D) and approximately 12% body weight in A/Swz/H3N2 infection (Fig. 31C). On the other hand, NA-M2e IM sera recipients lost less than 5% body weight in A/Cal/H1N1 (Fig. 31B) and B/Florida virus inoculation (Fig. 31E) and about 12% in A/Swz/H3N2 (Fig. 31C) and rgA/Viet/H5N1 virus inoculations (Fig. 31D). BALB/c mice were used as recipients of sera plus virus inoculums for all viruses except for A/Swz/H3N2 challenge. DBA/2J mouse strain was used for A/Swz/H3N2 due to their increased susceptibility to this strain of virus (Frank & Paust, 2020). Both IM and IN sera similarly protected mice from severe morbidity. In conclusion, antibodies in antisera elicited by NA-M2e IN and IM vaccination could protect against influenza viruses similarly.

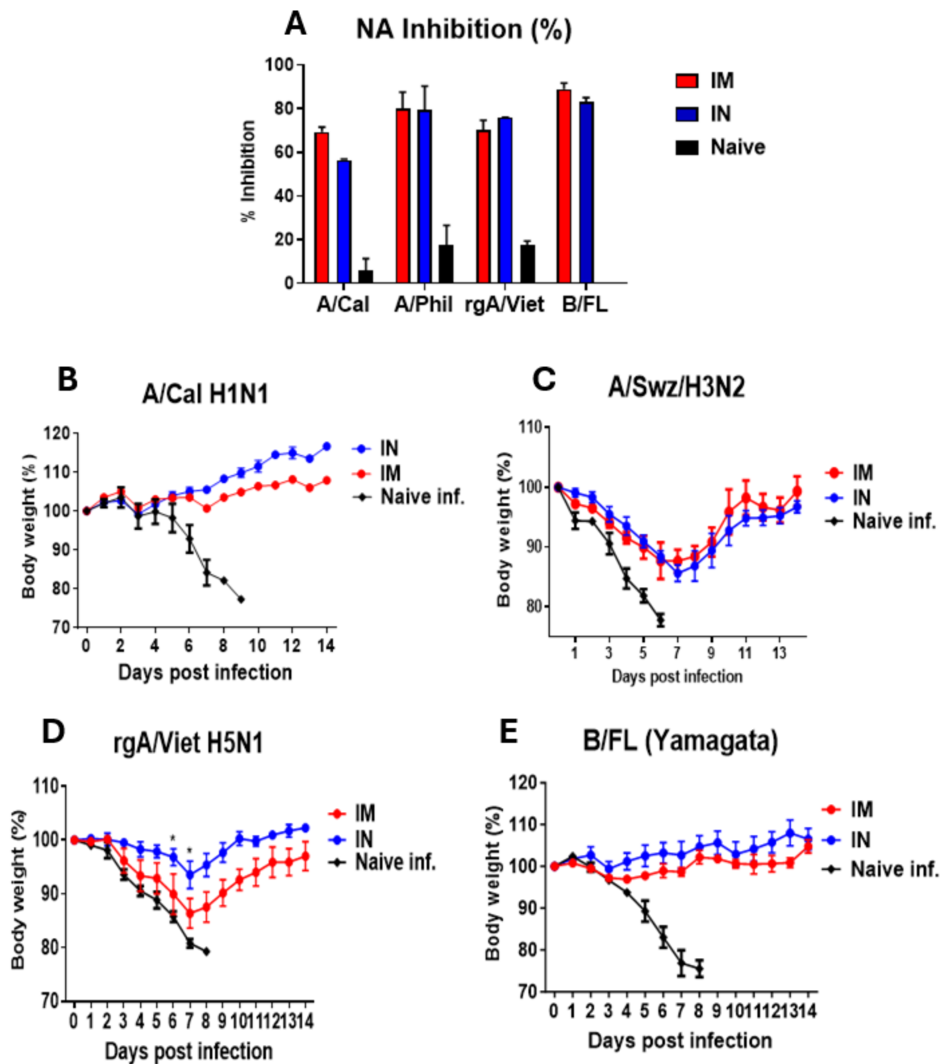


Figure 31. IM and IN boost (2nd dose) Immunized Sera Similarly Inhibit Viruses in NAI Assays and Confer Protection Against Influenza Viruses in Passive Sera Transfer Experiments. (A) Neuraminidase (NA) inhibition percentages in 20-fold diluted boost (2nd dose) immune sera against A/Cal/H1N1, A/Phil/H3N2, rgA/Viet/H5N1 and B/Fl Yamagata as determined by ELLA. Naïve mice (n=3) were intranasally inoculated with a mixture of boost antisera and lethal doses of challenge virus and monitored for 14 days daily for body weight changes. (B) A/Cal/H1N1, (C) A/Swz/H3N2, (D) rgA/Viet/H5N1, (E) B/FL (Yamagata) infection with antisera in BALB/c mice. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing IM group with IN group and performed by (A-E) using two-way ANOVA with Dunnett's posttest and indicated as *; $p<0.05$, and **; $p<0.01$.

4.5 Discussion

The strain-specificity of HA-based vaccines has prompted researchers to focus on more conserved or slower-mutating surface proteins, such as the neuraminidase and M2e, in vaccine constructs to achieve broad cross-protection against influenza viruses (Wiersma et al., 2015). Our

prior studies have demonstrated the immunogenicity and efficacy of NA-M2e VLP vaccine after IM injection in mice (Kim et al., 2022; Raha et al., 2024). The intranasal delivery without needle injection would represent an attractive and alternative vaccine delivery to induce systemic and mucosal immunity (Kehagia et al., 2023; Nakahashi-Ouchida et al., 2023). Multiple clinical studies support this route for influenza vaccines (Kiderman et al., 2001; Muszkat et al., 2000). We assessed the efficacy of intranasally administered NA-M2e VLP vaccine and explored the immune differences conferring enhanced cross-protection between IN and IM delivery.

The distinct properties of the nasal cavity pose physiological barriers to vaccine antigens. When administered intranasally, the antigens may be removed by ciliated epithelium, face absorption challenges, and be degraded by enzymes like proteases and aminopeptidases in the nasal mucosa (Dehghan, 2009; Gizurarson, 2015; Voynow & Rubin, 2009). Our study shows that IN administration with NA-M2e VLP generated significant levels of antibodies to the vaccine antigens following a booster dose but not after prime. In contrast, the IM immunized mice developed substantial antibody titers after prime, which was further increased by the boost dose. We do not understand the mechanisms by which a prime dose of IN delivery could not induce IgG antibodies at above the detection. Nonetheless, differential IgG antibodies were seen for the vaccine antigens after boost immunization. For instance, the IN group exhibited similar IgG levels to N2 and B NA but lower levels of IgG for M2e and N1 NA antigens. Lower IgG responses to M2e and N1 NA might likely be linked to the low immunogenicity of M2e and N1 NA among the NA-M2e vaccine components. Despite this, both IN and IM groups induced comparable levels of IgG titers against influenza virus antigens. Immune responses by IN delivery were further confirmed by NAI titers and protective roles of antisera in conferring protection in naïve mice, which were similar to those by IM immunization. Employing novel vaccine delivery systems and

adjuvant combinations can further enhance immune responses and boost antibody production at the mucosal sites following nasal vaccination. Careful selection of safe adjuvants is critical to avoid inflammation and adverse side effects (Sia Zachary et al., 2022).

IN-immunized mice consistently demonstrated superior protection efficacy compared to IM-immunized mice in antigenically different virus challenges. These IN-immune mice displayed lower levels of body weight loss and lung viral titers than the IM group.

We further determined the humoral and cellular immune responses induced by IN versus IM delivery. IN-immunized mice induced higher levels of GC phenotypic B cells in mucosal draining mLN and of IgA antibodies in sera and lung samples. Secretory IgA antibodies in mucosal surfaces are considered to act as the first line of defense with cross-reactive potential, and play an integral role in eliminating the invading pathogens and reducing transmission (Binsker et al., 2020). A study using recombinant NA proteins demonstrated that intranasal immunization could stimulate IgA antibodies capable of cross-reacting with homologous and heterologous strains and provide protection against virus infections. This study suggests IgA as an important correlate for higher protection efficacy, as the IM group showed lower levels of IgA in sera and lung samples, consistent with the systemic immunization and antibody production (Oh et al., 2021).

IFN-γ shifts the helper T-cell response towards a Th1 profile, which is more specialized for targeting intracellular pathogens and enhancing cell-mediated immunity (Irina et al., 2022). IFN-γ^+ T cells promote antiviral responses, enhance cytotoxic T cell (CTL) responses, and recruit immune cells to the site of infection for better control of virus replication (Kono et al., 2014; Rouse & Sehrawat, 2010). In this study, the IFN-γ+ CD4 and CD8 T cell responses in the lungs were similar or higher in the IN group compared to the IM group. A reverse pattern was observed in the spleen samples, highlighting the differences in immune responses at the distinct inductive sites

based on the route of vaccine administration. Notably the splenic IFN-γ^+ CD8 T cell responses were significantly and rapidly induced to higher levels early in the IN and IM groups after challenge compared to those before challenge. Plasma and memory B cells play a key role in long-term protection by producing antibodies in response to pathogen exposure (Inoue & Kurosaki, 2024; W. E. Purtha et al., 2011). We have studied the B cell responses in the lungs and spleen and observed significantly higher plasma and memory B cell populations induced in IN-immunized mice in the lungs compared to those in IM-immunized mice. While memory B cell populations between the IN and IM groups were similar, IM-immunized mice displayed greater plasma B cell populations in the spleen. Furthermore, differential activation of innate immune cells, especially antigen-presenting cells (APCs), was observed in the lung and spleen. The IN group demonstrated higher populations of CD86 expressing monocytes, macrophages, CD11b$^+$ migratory DCs, CD11b+ resident DCs, and CD8α resident DCs in the lung, whereas IM immunized mice displayed elevated populations of conventional CD103$^+$ migratory DCs. Apart from CD11b$^+$ resident DCs, IN-immunized mice showed a lower population of other DC subsets in spleen samples than the IM group, further demonstrating that IN delivery of vaccines has lower potency to recruit innate immune cells and APCs in the systemic lymphoid organs.

This study supports higher protective efficacy by IN delivery of NA-M2e vaccine than IM delivery, probably due to distinct immune responses at different inductive sites. After the boost dose, the IN group showed comparable levels of protection by antisera (except for rgA/Viet H5N1), serum IgG and NAI titers as compared to the IM group. Importantly, the IN group showed the higher induction of IgA antibodies, increased IFN-γ^+ T cells, GC B cells, plasma and memory B cells, as well as early activation of innate immune cells in the lungs, which are likely correlates with the enhanced protection observed in the IN group. Previous studies reported that the mucosal

surfaces of the respiratory tracts and lungs might be equally or more important than the systemic induction sites for generating immune responses against influenza viruses (MacLean et al., 2022; Weltzin & Monath, 1999). However, this study showed that IN prime dose of vaccine could not induce sufficient serum IgG responses, suggesting that mucosal-associated lymphoid organs and lungs are not as effective as systemic lymphoid organs. Therefore, both the systemic and mucosal immune systems should work together to coordinate and orchestrate protective immune responses. Additional factors such as trained innate immunity could be a factor contributing to protection (Netea et al., 2020) and should be explored. A prior study reported that IN administration of adenoviral vaccine vectors could induce trained immunity in alveolar macrophages stimulated by IFN-γ from CD8 T cells, leading to enhanced protection against subsequent challenges (Afkhami et al., 2022; D'Agostino et al., 2020; Yao et al., 2018). These alveolar macrophages further secreted IFN- γ^+ cytokines, helping recruit memory B cells to the infected lung, and elevating local antibody concentrations following a secondary exposure to the influenza virus (MacLean et al., 2022). In line with these prior reports, this study showed that the IN group induced significantly elevated levels of IFN-γ^+ CD8 T cells after boost along with increased populations of CD38+ and CD69+ expressing memory B cells and alveolar macrophages in lungs compared to IM immunized mice (Fig. 32). These findings indicate interactions among the different immune cells that might have contributed to protective immunity. Further studies need to be conducted at relevant time points after each prime and boost immunizations via IN versus IM routes to better understand the underlying mechanisms.

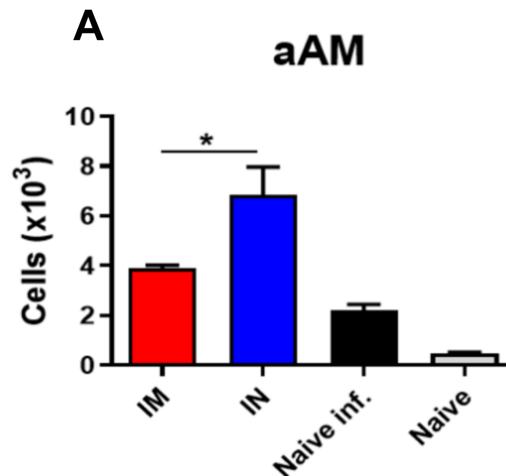


Figure 32. Significant Induction of Activated Alveolar Macrophages in IN Immunized Group. Groups of mice (n=5) were primed and boosted with NA-M2e either in the IM or IN route, challenged with a lethal dose of A/Phil/H3N2 and sacrificed on 8 dpi. (A) $CD45^{+}F4/80^{+}CD11b^{-}CD11c^{+}MHCII^{+}$ alveolar macrophages cell population was determined by flow cytometry. Error bars are represented as mean ± SEM. Statistical significance was determined by comparing IM group with IN group and performed by (A-E) using one-way ANOVA with Dunnett's posttest and indicated as *; $p<0.05$.

Despite the enhanced protective efficacy induced in the IN group, further research is warranted to assess other contributing immune parameters. This study did not determine the immune responses to NA-M2e vaccination in the upper respiratory tract, which is often a limitation in a mouse model (Sia Zachary et al., 2022) and can be an inductive site for evaluating the vaccine responses following IN immunization. Therefore, a larger animal model such as ferrets would be relevant to address some limitations in mice.

4.6 Conclusion

This study examined the cross-protective potential of the NA-M2e VLP vaccine via an IN route and compared innate and adaptive immune responses triggered by IM versus IN routes. Our findings demonstrated that IN administration of the NA-M2e VLP vaccine offered higher efficacy of protection by inducing humoral and cellular immune responses in the mucosal lung, a major site of viral replication. These results highlight the critical role of eliciting mucosal and systemic immunity to effectively control respiratory pathogens like influenza.

4.7 Acknowledgements

This study was supported by NIH/NIAID grants AI154656 (S.-M.K.). The funders had no contribution in experimental design, collection of data, analysis, decision to publish, or manuscript preparation. A/Switzerland/9715293/2013 (H3N2) virus was generously gifted by Dr. Florian Krammer. The following reagents were obtained through BEI, NIAID, NIH: B/Florida/4/2006 (NR-9696), N1 NA protein (A/California/4/2009, NR-19234), N2 NA protein (A/Brisbane/10/2007, NR-43784), and influenza B NA proteins (B/Florida/4/2006, NR-19236).

5. DISCUSSION AND FUTURE DIRECTIONS

5.1 Enhancing Cross-Protective Efficacy of Inactivated Split Vaccines by Inducing NA and M2e Immunity

Conventional inactivated split vaccines are primarily focused on optimizing HA content in vaccine formulations to induce humoral response to the immunodominant HA protein. However, selection pressure due to pre-existing immunity can cause the virus to mutate via antigenic drift mechanisms, leading to the emergence of escape mutants. Although the protective roles of NA, the second major glycoprotein on the influenza surface, are well-known, it is not standardized in vaccine preparations. Neuraminidase (NA) plays a key role in the release of nascent virions by cleaving terminal sialic acid linkages during the budding process via antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC) mechanisms (Zhang & Ross, 2024). Although anti-NA antibodies function in an infection-permissive manner, vaccination targeting NA has been associated with reduced disease severity, decreased virus shedding, and lower viral titers in the lungs of animal models (Rajendran et al., 2021). Furthermore, NA undergoes slower mutations than HA, therefore, complementing HA based split vaccines with NA could be beneficial when a strain mismatch occurs.

Human influenza infections induce a transient and weak immune response to the M2 protein, despite its expression on the surface of infected cells and exposure to the adaptive immune system (Feng et al., 2006). The anti-M2 antibodies, such as the 14C2, have been associated with restricting in vitro replication of certain IAV strains (Fu et al., 2009) and most previous studies demonstrate that M2e-specific antibodies are necessary for protection against IAV challenges. Antibodies to M2, TCN-032, have shown to reduce viral loads in humans (Bodewes et al., 2009). These findings suggest that M2e vaccination mediates protection via antibody-dependent

mechanisms. ADCC mechanisms, driven by natural killer (NK) cell activity, has been implicated in this protection. However, contrasting findings emerged from depletion experiments regarding the role of NK cells with Fc receptors in protection (L. Deng et al., 2015). Instead, alveolar macrophages with Fc receptors, which mediate antibody-dependent cellular phagocytosis (ADCP), were found to be more critical for anti-M2e IgG-mediated protection (Guilliams et al., 2014). Furthermore, CDC has been observed to play a role in anti-M2e-mediated protection, as passive transfer of anti-M2e monoclonal antibodies did not provide protection in C57BL/6 mice (Pürzel et al., 2009). Additionally, M2e-based immunizations were shown to increase IgG2a/IgG1 ratios, which are predicted to enhance virus-specific Th1 type protective immune responses. Since anti-M2e protection provides permissive immunity, it facilitates the induction of CD8+ T cell responses, which may not be possible with the neutralizing mechanisms of HA. Various strategies have been employed to enhance the immunogenicity of M2e, including M2e-presenting virus-like particles (VLP) (Kim et al., 2022; M.-C. Kim et al., 2013; M. C. Kim, J. S. Lee, et al., 2013), toll-like receptor (TLR) agonists (Mastalerz-Migas et al., 2015; Miller et al., 2020) gold nanoparticles, liposomes, and immune-stimulating complexes such as cholera toxin subunits (Calzas & Chevalier, 2019). Relative to natural infections, seasonal vaccinations induce lower immune response to NA and responses to M2 are weak in both natural infections and seasonal vaccinations. HA to NA ratio is 4:1 and HA to M2 ratio is 1:10 on the influenza surface (Bouvier & Palese, 2008), therefore, HA is more immunodominant than the other proteins due to its high abundance on influenza surface. Moreover, the small size of M2 also contributes to its poor immunogenicity. NA, a type II membrane-anchored homo-tetramer glycoprotein (Creytens et al., 2021), needs to maintain a stable conformation for full enzymatic activity, and split vaccines are inconsistent in standardizing the quantity and quality of NA in vaccine formulations (Baker & Gandhi, 1976).

To address these issues, in projects 1 and 2, I supplemented a bivalent inactivated split vaccine (H1N1+H3N2) with NA-M2e VLP vaccines in young and aged mice models. The supplementation strategy boosted IgG antibody responses against all three antigens—HA, NA, and M2e—as well as antigen-specific effector T cell responses for NA and M2e in lymph nodes. Additionally, this addressed the challenge of poor immunogenicity associated with these conserved/slowly mutating antigens and provided protection against lethal challenges in both young and aged groups. The S+NA-M2e vaccine group exhibited superior protection in both homologous and heterologous challenges compared to the split vaccine group. However, it only demonstrated better homologous protection and comparable heterologous protection as the NA-M2e group. This indicates that NA-M2e enhances the efficacy of split vaccines, while split vaccines do not contribute to the cross-protective capabilities of NA-M2e. Future studies should investigate the longevity of protection provided by the S+NA-M2e vaccination strategy and assess whether pre-existing immunity affects the cross-protective efficacy of this vaccine combination.

5.2 Bridging the Immunity Gap: Enhancing Influenza Vaccine Efficacy in the Elderly

Individuals at highest risk for a severe course of influenza infection include children under the age of 5, the elderly, pregnant women, and those with pre-existing chronic medical conditions.(Rasmussen et al., 2012). The elderly population, particularly those 65 and older, is disproportionately more susceptible and impacted by influenza-associated mortality in developed countries (Thompson et al., 2006). In population-based surveillance data, it was observed that in the 2010-11 to the 2012-13 seasons, 54-70% of hospitalizations and 71-85% of deaths occurred among individuals who were 65 or older (Reed et al., 2015). The elderly often elicit weaker immune response to influenza vaccination, which is typically around 30-40% (K. Goodwin et al., 2006). Despite this, vaccination remains the most effective tool to prevent infectious diseases such

as influenza as well as to manage other diseases in the elderly. An observational clinical study demonstrated that influenza vaccination was effective in preventing acute myocardial infarction by 15-40% (Macintyre et al., 2013). Protection against influenza viruses is also crucial in preventing secondary bacterial infections, such as pneumonia.

Several aging-related factors impair effective immunity to influenza viruses (Smetana et al., 2018). The epithelial cells provide a physical barrier to pathogens, however, the number of Langerhans' cells decreases with age (Grewe, 2001). Toll-like receptors (TLRs), crucial for recognizing foreign proteins and triggering intracellular pathways have defective expression in macrophages of the aged, and APCs are less responsive to chemokine CCL19, which is essential for migration to lymph nodes (Grewe, 2001). Furthermore, pro-inflammatory cytokines are elevated in the circulation and tissues of the aged and this state of chronic, low-grade, systemic inflammation is known as inflamm-aging (Franceschi et al., 2007). Aging-related inflammation is associated with poor health outcomes, reduced vaccine efficacy, and increased susceptibility to infectious diseases (Bowdish et al., 2015). The impairment of the innate immune cells to present antigens, and the adaptive immune system to generate de novo immune responses contributes to poor vaccine efficacy in the elderly, leading to significant influenza-related morbidity and mortality.

In Chapters 1 and 2, I evaluated the protective efficacy and adaptive immune responses to S+NA-M2e vaccination in young and aged mice. These studies revealed aging-related differences in immune responses to this vaccination strategy. Both young and aged mice survived lethal homologous and heterologous virus challenges, however, aged mice showed a delayed recovery after infection compared to young mice. Young mice vaccinated with the same split vaccines and dosages elicited at least 2 folds higher antibody responses to H1 and H3 antigens than their aged

counterparts. Additionally, the aged mice demonstrated lower HAI titers to homologous viruses A/Cal/H1N1 and rgA/Swz H3N2 than young mice. Interestingly, due to less contribution of split vaccines in the S+NA-M2e vaccination strategy, immune responses in aged mice were skewed towards T-helper type 1 (Th1) immune responses, which is more virus-specific, whereas immune responses in young mice were Th1/Th2 balanced. In experiments to determine the protective roles of antisera after vaccination, sera from aged mice sufficiently protected young mice against lethal challenges as well. However, young mice vaccinated with 2 doses of S+NA-M2e and challenged with A/Phil/H3N2 virus showed higher induction of IFN-γ^{+}CD4^{+} T cell responses compared to their aged counterparts to hM2e, N2 and N1 vaccine-specific antigens. The aged mice also demonstrated less cytokine secretion after in vitro BMDC stimulation with vaccines compared to young mice (young mice data not shown). Findings from our studies indicate that young mice are better inducers of vaccine-specific immune responses than aged mice when given the same vaccines and doses. Our studies did not compare differences in memory responses due to aging. Memory cells generated from naïve T cells in the aged show reduced cytokine secretion and proliferation upon recall responses (Haynes et al., 2003). Memory CD4+ T cells generated in young mice remained functional as the mice aged, whereas those generated in aged mice were not functional (Haynes et al., 2003). When memory CD4^{+} T cells were transferred from young mice to aged mice, the aged mice exhibited strong humoral responses (Eaton et al., 2004). Similar outcomes were observed with CD8^{+} T cells, suggesting that alterations in T cells are intrinsic and dependent on the host's age (Decman et al., 2012). Therefore, the age at primary vaccination plays a role in the functionality of memory T cells, rather than the age at recall response.

Overall health is important for optimal vaccine responses; however, effective strategies are necessary to enhance vaccine efficacy in the elderly. High-dose, quadrivalent Fluzone vaccines are

recommended for individuals who are 65 or older. Preclinical and clinical studies indicate that increasing vaccine dosages enhances vaccine-specific antibody responses in the elderly; however, the extent to which this provides protection remains unclear (Bowdish et al., 2015). Immune responses generated by the young provide long-term protection. For instance, in the 2009 H1N1 pandemic, elderly individuals with pre-existing immunity and protective antibodies demonstrated better resistance to influenza infections than young individuals (Ikonen et al., 2010). Potent adjuvants such as the oil-in-water emulsion adjuvant, MF59, have dose-sparing effects and can induce equivalent antibody titers as in young and confer protection by lowering lung viral loads (McElhaney et al., 2006). Fluad is a vaccine for the elderly that uses MF59 as an adjuvant (Podda & Del Giudice, 2003). Other potential immunostimulatory adjuvants include the lipopolysaccharide derivative 3-deacetylated lipid A (MPL-A), the saponin derived QS-2, oligodeoxynucleotides comprising CpG motifs, cytokines and TLR agonists. These adjuvants primarily function to induce innate immune responses such as cytokine secretion, antigen uptake by APCs, and their activation and migration. Lastly, nutritional interventions to reverse or manage immunosenescence as well as optimizing herd immunity against infectious pathogens could help improve influenza vaccine responsiveness in the elderly (Bowdish et al., 2015). Future studies could include various adjuvant combinations with S+NA-M2e vaccination to improve humoral and cellular responses in the aged mice.

5.3 The Role of Pre-Existing Immunity in Shaping Influenza Vaccine Responses

Most adults have some level of pre-existing immunity to influenza due to past infections or vaccinations. This pre-existing immunity can influence how the immune system responds to new influenza vaccines. Previous studies on universal vaccine candidates were often conducted in animals without prior exposure to influenza, which may not accurately reflect the real situation.

Original antigenic sin (OAS) is a phenomenon whereby secondary exposure to an antigenically drifted strain enhances the quantity and strength of antibodies elicited against primary antigens (Fazekas de St & Webster, 1966) by memory B cells. Most humans are naturally infected with influenza by the age of 7 (Bodewes et al., 2011) and continue to experience additional infections over time. Furthermore, they are immunized with seasonal vaccines containing drifted strains that correspond to the strains circulating during each respective season. Pre-existing immunity from prior infections and various vaccine combinations can influence and shape the immune response to future infections. For instance, LAIV vaccines are an attenuated form of a whole virus vaccine, which presents more antigens, including internal proteins, to which antibodies are elicited and cytotoxic $CD8^{+}$ T cell responses are generated; however, IgG antibodies induced due to LAIV are not neutralizing (Cao et al., 2014; Sasaki et al., 2007). IgA antibodies, elicited in LAIV, contribute towards mitigating influenza-related morbidity and confer protection against heterologous influenza strains (Asahi-Ozaki et al., 2004; Clements & Murphy, 1986). On the other hand, inactivated influenza vaccines mainly induce IgG and IgM antibodies to HA and variable responses to NA. Therefore, differences in primary immune responses due to various vaccine platforms and formulations could enhance or diminish immune responses against future infections/immunizations. During the 2013-2014 H1N1 influenza season, an increased susceptibility to infection was observed in the elderly, while the younger population, with immunity to non-mutated antigens, exhibited enhanced protection (Tanner et al., 2021). In contrast to that, another study demonstrated that in human subjects, pre-existing anti-NA antibodies were associated with a shortened duration of influenza A (H1N1) pandemic virus shedding and illness after natural infection (Maier et al., 2020). Previous H1N1 infection boosted humoral responses in ferrets after prime-boost vaccination with an antigenically distant influenza strain (Wei et al.,

2012). In Chapter 2, I investigated whether a sequential heterologous vaccination strategy with split and NA-M2e vaccines impacted the immune responses elicited by these vaccines. Sequential vaccination with split and NA-M2e elicited comparable IgG antibodies and conferred protection against heterologous A/Phil/H3N2 challenge, indicating similar immune responses as in S+NA-M2e vaccination strategy. It is considered that suboptimal antibodies/non-neutralizing antibodies for protection may be amplified by repeated vaccination. Using adjuvanted and/or high-dose vaccination can relieve the impacts of OAS; however, further research into the mechanistic details of B cell immunodominance is required to better understand OAS and the steps needed to overcome this.

5.4 Nasal Vaccines for Induction of Mucosal Immunity

Nasal vaccines offer a promising strategy for eliciting local mucosal immunity to combat respiratory infections. Anti-NA antibodies prevent NA action to free influenza virus particles from decoy receptors associated with mucin (Giurgea et al., 2020). A higher concentration or presence of potent antibodies in the respiratory tract, the primary entry point for influenza viruses, should allow for faster and more efficient neutralization of influenza viruses, however, inducing robust antibody responses is difficult due to mucosal tolerance. Strategies to improve nasal vaccine efficacy include the use of adjuvants. For instance, mucoadhesive chitosan and starch can aid soluble vaccine formulations to bind to mucosal membranes rather than being removed due to the constant renewal of the mucus layers (van der Lubben et al., 2001). Particulate formulations are generally more effective than soluble antigens (Peek et al., 2008) and when combined with immunomodulators, they can improve musical vaccine efficacy. Novel strategies targeting M cells using lectins and antibodies, along with DC-stimulating adjuvants, could be effective (Lycke, 2012) in enhancing the innate immune system, thereby successfully activating adaptive immunity.

Moreover, vaccine-induced memory is essential in recall responses to prevent infections. Although IgA responses are transient, long-term protection is believed to be mediated by rapid recall responses from IgA$^+$ and IgG$^+$ memory B cells following intranasal immunization (Lycke, 2012; Tengvall et al., 2010). Interestingly, it was observed that in a naïve mammalian host, influenza infection induced very weak anti-M2e antibody responses, but the primary infection elicits some degree of B cell immune memory against M2e that is significantly boosted by subsequent infections. Consistent with these findings, in Chapter 3, I observed that vaccination with NA-M2e VLP induced very low anti-M2e antibody responses after the first dose IN vaccination, which was significantly enhanced after boost immunization and challenge experiments. Despite similar or lower systemic immune responses than IM immunized mice, the IN counterparts demonstrated more effective broad cross-protection against various heterologous influenza viruses. Our study demonstrated enhanced memory B cell and alveolar macrophage populations, as well as increased IgA antibodies in serum and lung extract samples, however, further investigations into immune memory formation after every vaccination dose and challenge experiments as well as any contribution of trained immunity need to be explored to better understand the immune correlates of protection. Although M2e-based VLP vaccines have shown enhanced efficacy in several pre-clinical studies and can mediate adjuvant effects by themselves, some studies suggest inclusion of immunostimulatory molecules such as enterotoxins, PRR ligands or cytokines and chemokines in vaccine formulations can enhance vaccine efficacy, duration and breadth (Lycke, 2012). However, careful consideration of an adjuvant's potential side effects is crucial, particularly given the proximity of the nasal cavity to the olfactory bulbs, which connect to the central nervous system (R. Nakahashi-Ouchida et al., 2023). Adjuvants such as AS03 and MF59 have been linked to various adverse reactions that could severely affect sensitive mucous membranes (Halsey et al.,

2015). For example, a live attenuated influenza vaccine (LAIV) adjuvanted with E. coli heat-labile toxin was associated with cases of Bell's Palsy in recipients (Mutsch et al., 2004). Therefore, future studies on NA-M2e VLP vaccines should explore the use of adjuvants that can enhance systemic immune responses without inducing toxic side effects.

5.5 Limitations of Current Vaccines and Next-Generation Influenza Vaccines

The production of seasonal influenza vaccines, such as LAIV and inactivated vaccines, has been heavily dependent on embryonated chicken eggs for almost a century (Trombetta et al., 2019). Egg-based vaccine production raises several concerns: effective viral propagation requires that the hemagglutinin (HA) and neuraminidase (NA) genes of circulating strains be recombined with the internal genes of an egg-adapted virus, increasing the time and labor involved in developing vaccine strains. Certain strains, such as the seasonal H3N2 strains, do not propagate in eggs efficiently, causing issues with their isolation (Stevens et al., 2010). Culturing virus strains derived from humans can introduce mutations in HA head domain, that may reduce the efficacy of the vaccines (Nakowitsch et al., 2014). Alternatively, cell-culture based inactivated vaccines can also be used, but their yields are lower, and production costs are higher than egg-based vaccine production and the issues of mutations in HA remain with this method as well (Shartouny & Lowen, 2022). The mRNA vaccine technology gained widespread recognition following the success of COVID-19 vaccines, particularly Moderna's mRNA-1273 and Pfizer-BioNTech's BNT162b2. These vaccines were instrumental in preventing SARS-CoV-2-related illness, achieving high efficacy rates in clinical trials. Advantages of using mRNA vaccines include the speed of vaccine development, enhanced immunogenicity, and adaptability of mRNA vaccines in targeting different variants of the virus. Both vaccines consist of messenger RNA encoding the SARS-CoV-2 spike protein, which is encapsulated in lipid nanoparticles for efficient delivery into

cells (Pannus et al., 2022). The mRNA-based vaccines offer several advantages over traditional egg-based vaccine production. Since they do not require virus propagation in eggs, the process becomes significantly faster—cutting production time to about one-sixth or one-eighth of what is typically required for egg-based vaccines (Shartouny & Lowen, 2022). If a mismatched strain emerges, researchers can promptly alter the mRNA sequence to match the new strain. This flexibility also allows for sequence modifications to improve vaccine stability, giving scientists more control over the design process. This rapid adaptability makes mRNA technology a valuable tool for addressing emerging infectious diseases quickly and efficiently. Due to their enhanced immunogenicity, mRNA vaccines can potentially offer dose-sparing benefits (Pannus et al., 2022). In all three of my projects, a relatively high dose of 10 µg of NA-M2e VLP vaccine was employed. The reduced dosage is advantageous as high vaccine doses can often lead to adverse reactions. The ability to achieve comparable immune responses with lower doses is a significant benefit of mRNA vaccines. Several mRNA-based influenza vaccination strategies are underway. For instance, Pfizer-BioNTech is testing a COVID-19 and influenza vaccine combination which has progressed to Phase 3 clinical trials, however, this vaccine combination only met one of its two primary immunogenicity objectives. Additionally, the company is also working on a second-generation trivalent influenza vaccine that has demonstrated strong immunogenicity across all strains compared to the current market standard (Pfizer, 2024). Despite the promising advances in mRNA vaccine technology, access to these vaccines remains limited in many low-income countries, largely due to concerns over their stability and storage requirements (Shartouny & Lowen, 2022). Current mRNA vaccine approaches could be an alternative to egg-based seasonal vaccine production; however, the need for a universal influenza vaccine that provides durable protection across multiple strains remains an unmet challenge.

6. REFERENCES

Afkhami, S., D'Agostino, M. R., Zhang, A., Stacey, H. D., Marzok, A., Kang, A., Singh, R., Bavananthasivam, J., Ye, G., Luo, X., Wang, F., Ang, J. C., Zganiacz, A., Sankar, U., Kazhdan, N., Koenig, J. F. E., Phelps, A., Gameiro, S. F., Tang, S.,…Xing, Z. (2022). Respiratory mucosal delivery of next-generation COVID-19 vaccine provides robust protection against both ancestral and variant strains of SARS-CoV-2. *Cell*, *185*(5), 896-915.e819. https://doi.org/10.1016/j.cell.2022.02.005

Agarwal, D., Schmader, K. E., Kossenkov, A. V., Doyle, S., Kurupati, R., & Ertl, H. C. J. (2018). Immune response to influenza vaccination in the elderly is altered by chronic medication use. *Immunity & Ageing*, *15*(1). https://doi.org/10.1186/s12979-018-0124-9

Aguilar, J. C., Aguiar, J. A., & Akbar, S. M. F. (2022). Action Mechanisms and Scientific Rationale of Using Nasal Vaccine (HeberNasvac) for the Treatment of Chronic Hepatitis B. *Vaccines*, *10*(12), 2087. https://www.mdpi.com/2076-393X/10/12/2087

Alameh, M.-G., Tombácz, I., Bettini, E., Lederer, K., Ndeupen, S., Sittplangkoon, C., Wilmore, J. R., Gaudette, B. T., Soliman, O. Y., Pine, M., Hicks, P., Manzoni, T. B., Knox, J. J., Johnson, J. L., Laczkó, D., Muramatsu, H., Davis, B., Meng, W., Rosenfeld, A. M.,…Pardi, N. (2021). Lipid nanoparticles enhance the efficacy of mRNA and protein subunit vaccines by inducing robust T follicular helper cell and humoral responses. *Immunity*, *54*(12), 2877-2892.e2877. https://doi.org/10.1016/j.immuni.2021.11.001

Allen, J. C., Toapanta, F. R., Chen, W., & Tennant, S. M. (2020). Understanding immunosenescence and its impact on vaccination of older adults. *Vaccine*, *38*(52), 8264-8272. https://doi.org/https://doi.org/10.1016/j.vaccine.2020.11.002

Amanna, I. J., Carlson, N. E., & Slifka, M. K. (2007). Duration of Humoral Immunity to Common Viral and Vaccine Antigens. *New England Journal of Medicine*, *357*(19), 1903-1915. https://doi.org/doi:10.1056/NEJMoa066092

Antrobus, R. D., Lillie, P. J., Berthoud, T. K., Spencer, A. J., McLaren, J. E., Ladell, K., Lambe, T., Milicic, A., Price, D. A., Hill, A. V., & Gilbert, S. C. (2012). A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. *PLoS One*, *7*(10), e48322. https://doi.org/10.1371/journal.pone.0048322

Asahi-Ozaki, Y., Yoshikawa, T., Iwakura, Y., Suzuki, Y., Tamura, S. i., Kurata, T., & Sata, T. (2004). Secretory IgA antibodies provide cross-protection against infection with different strains of influenza B virus. *Journal of medical virology.*, *74*(2), 328-335. https://doi.org/10.1002/jmv.20173

Aspinall, R., & Andrew, D. (2000). *Journal of Clinical Immunology*, *20*(4), 250-256. https://doi.org/10.1023/a:1006611518223

Baker, N. J., & Gandhi, S. S. (1976). Effect of Ca++ on the stability of influenza virus neuraminidase. *Arch Virol*, *52*(1-2), 7-18. https://doi.org/10.1007/bf01317860

Berthoud, T. K., Hamill, M., Lillie, P. J., Hwenda, L., Collins, K. A., Ewer, K. J., Milicic, A., Poyntz, H. C., Lambe, T., Fletcher, H. A., Hill, A. V. S., & Gilbert, S. C. (2011). Potent CD8+ T-Cell Immunogenicity in Humans of a Novel Heterosubtypic Influenza A Vaccine, MVA−NP+M1. *Clinical Infectious Diseases*, *52*(1), 1-7. https://doi.org/10.1093/cid/ciq015

Bhatnagar, N., Kim, K. H., Subbiah, J., Park, B. R., Ko, E. J., Seong, B. L., & Kang, S. M. (2022). 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*, *197*, 105229. https://doi.org/10.1016/j.antiviral.2021.105229

Binsker, U., Lees, J. A., Hammond, A. J., & Weiser, J. N. (2020). Immune exclusion by naturally acquired secretory IgA against pneumococcal pilus-1. *The Journal of Clinical Investigation*, *130*(2), 927-941. https://doi.org/10.1172/JCI132005

Bodewes, R., de Mutsert, G., van der Klis, F. R., Ventresca, M., Wilks, S., Smith, D. J., Koopmans, M., Fouchier, R. A., Osterhaus, A. D., & Rimmelzwaan, G. F. (2011). Prevalence of antibodies against seasonal influenza A and B viruses in children in Netherlands. *Clin Vaccine Immunol*, *18*(3), 469-476. https://doi.org/10.1128/cvi.00396-10

Bodewes, R., Kreijtz, J. H., Baas, C., Geelhoed-Mieras, M. M., de Mutsert, G., van Amerongen, G., van den Brand, J. M., Fouchier, R. A., Osterhaus, A. D., & Rimmelzwaan, G. F. (2009). Vaccination against human influenza A/H3N2 virus prevents the induction of heterosubtypic immunity against lethal infection with avian influenza A/H5N1 virus. *PLoS One*, *4*(5), e5538. https://doi.org/10.1371/journal.pone.0005538

Bosch Berend, J., Bodewes, R., de Vries Robert, P., Kreijtz Joost, H. C. M., Bartelink, W., van Amerongen, G., Rimmelzwaan Guus, F., de Haan Cornelis, A. M., Osterhaus Albert, D. M. E., & Rottier Peter, J. M. (2010). Recombinant Soluble, Multimeric HA and NA Exhibit Distinctive Types of Protection against Pandemic Swine-Origin 2009 A(H1N1) Influenza Virus Infection in Ferrets. *Journal of Virology*, *84*(19), 10366-10374. https://doi.org/10.1128/jvi.01035-10

Bouvier, N. M., & Palese, P. (2008). The biology of influenza viruses. *Vaccine*, *26 Suppl 4*(Suppl 4), D49-53. https://doi.org/10.1016/j.vaccine.2008.07.039

Bowdish, D., Loukov, D., & Naidoo, A. (2015). Immunosenescence: implications for vaccination programs in the elderly. *Vaccine: Development and Therapy*, 17. https://doi.org/10.2147/vdt.s63888

Brown Deborah, M., Lee, S., Garcia-Hernandez Maria de la, L., & Swain Susan, L. (2012). Multifunctional CD4 Cells Expressing Gamma Interferon and Perforin Mediate Protection against Lethal Influenza Virus Infection. *Journal of Virology*, *86*(12), 6792-6803. https://doi.org/10.1128/jvi.07172-11

Buonaguro, L., Tornesello, M. L., Tagliamonte, M., Gallo, R. C., Wang, L. X., Kamin-Lewis, R., Abdelwahab, S., Lewis, G. K., & Buonaguro, F. M. (2006). Baculovirus-Derived Human Immunodeficiency Virus Type 1 Virus-Like Particles Activate Dendritic Cells and Induce Ex Vivo T-Cell Responses. *Journal of Virology*, *80*(18), 9134-9143. https://doi.org/10.1128/jvi.00050-06

Calzas, C., & Chevalier, C. (2019). Innovative Mucosal Vaccine Formulations Against Influenza A Virus Infections. *Front Immunol*, *10*, 1605. https://doi.org/10.3389/fimmu.2019.01605

Cao, R. G., Suarez, N. M., Obermoser, G., Lopez, S. M. C., Flano, E., Mertz, S. E., Albrecht, R. A., García-Sastre, A., Mejias, A., Xu, H., Qin, H., Blankenship, D., Palucka, K., Pascual, V., & Ramilo, O. (2014). Differences in Antibody Responses Between Trivalent Inactivated Influenza Vaccine and Live Attenuated Influenza Vaccine Correlate With the Kinetics and Magnitude of Interferon Signaling in Children. *The Journal of Infectious Diseases*, *210*(2), 224-233. https://doi.org/10.1093/infdis/jiu079

CDC. (2015, February 11, 2019). *How effective was the 2014-2015 flu vaccine?* Centers for Disease Control and Prevention. https://archive.cdc.gov/www_cdc_gov/flu/pastseasons/1415season.htm#:~:text=Top%20of%20Page,How%20effective%20was%20the%202014%2D2015%20flu%20vaccine%3F,for%20all%20ages%20was%2023%25

CDC. (2019). *Immunogenicity, Efficacy, and Effectiveness of Influenza Vaccines*. https://archive.cdc.gov/www_cdc_gov/flu/professionals/acip/immunogenicity.htm

CDC. (2022). *Live Attenuated Influenza Vaccine [LAIV] (The Nasal Spray Flu Vaccine)*. https://www.cdc.gov/flu/vaccine-types/nasalspray.html?CDC_AAref_Val=https://www.cdc.gov/flu/prevent/nasalspray.htm

CDC. (2023). *Fluzone High-Dose Seasonal Influenza Vaccine*. https://www.cdc.gov/flu/prevent/qa_fluzone.htm#:~:text=There%20are%20three%20flu%20vaccines,Fluad%20Quadrivalent%20adjuvanted%20flu%20vaccine.

CDC. (2024). *CDC Seasonal Flu Vaccine Effectiveness Studies*. https://www.cdc.gov/flu-vaccines-work/php/effectiveness-studies/?CDC_AAref_Val=https://www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm

Chan, L., Alizadeh, K., Alizadeh, K., Fazel, F., Kakish, J. E., Karimi, N., Knapp, J. P., Mehrani, Y., Minott, J. A., Morovati, S., Rghei, A., Stegelmeier, A. A., Vanderkamp, S., Karimi, K., & Bridle, B. W. (2021). Review of Influenza Virus Vaccines: The Qualitative Nature of Immune Responses to Infection and Vaccination Is a Critical Consideration. *Vaccines (Basel)*, *9*(9). https://doi.org/10.3390/vaccines9090979

Chen, J., Wang, J., Zhang, J., & Ly, H. (2021). Advances in Development and Application of Influenza Vaccines. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.711997

Chen, Y.-Q., Wohlbold, T. J., Zheng, N.-Y., Huang, M., Huang, Y., Neu, K. E., Lee, J., Wan, H., Rojas, K. T., Kirkpatrick, E., Henry, C., Palm, A.-K. E., Stamper, C. T., Lan, L. Y.-L., Topham, D. J., Treanor, J., Wrammert, J., Ahmed, R., Eichelberger, M. C.,…Wilson, P. C.

(2018). Influenza Infection in Humans Induces Broadly Cross-Reactive and Protective Neuraminidase-Reactive Antibodies. *Cell*, *173*(2), 417-429.e410. https://doi.org/10.1016/j.cell.2018.03.030

Clements, M. L., & Murphy, B. R. (1986). Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. *Journal of Clinical Microbiology*, *23*(1), 66-72. https://doi.org/doi:10.1128/jcm.23.1.66-72.1986

Cookenham, T., Lanzer, K. G., Gage, E., Lorenzo, E. C., Carter, D., Coler, R. N., Baldwin, S. L., Haynes, L., Reiley, W. W., & Blackman, M. A. (2020). Vaccination of aged mice with adjuvanted recombinant influenza nucleoprotein enhances protective immunity. *Vaccine*, *38*(33), 5256-5267. https://doi.org/10.1016/j.vaccine.2020.05.085

Corr, S. C., Gahan, C. C. G. M., & Hill, C. (2008). M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. *FEMS Immunology & Medical Microbiology*, *52*(1), 2-12. https://doi.org/10.1111/j.1574-695X.2007.00359.x

Couch, R. B., Atmar, R. L., Keitel, W. A., Quarles, J. M., Wells, J., Arden, N., & Niño, D. (2012). Randomized comparative study of the serum antihemagglutinin and antineuraminidase antibody responses to six licensed trivalent influenza vaccines. *Vaccine*, *31*(1), 190-195. https://doi.org/https://doi.org/10.1016/j.vaccine.2012.10.065

Couch, R. B., Kasel, J. A., Gerin, J. L., Schulman, J. L., & Kilbourne, E. D. (1974). Induction of Partial Immunity to Influenza by a Neuraminidase-specific Vaccine. *The Journal of Infectious Diseases*, *129*(4), 411-420. https://doi.org/10.1093/infdis/129.4.411

Cox, M. M., & Hollister, J. R. (2009). FluBlok, a next generation influenza vaccine manufactured in insect cells. *Biologicals*, *37*(3), 182-189. https://doi.org/10.1016/j.biologicals.2009.02.014

Cox, M. M., Patriarca, P. A., & Treanor, J. (2008). FluBlok, a recombinant hemagglutinin influenza vaccine. *Influenza Other Respir Viruses*, *2*(6), 211-219. https://doi.org/10.1111/j.1750-2659.2008.00053.x

Creytens, S., Pascha, M. N., Ballegeer, M., Saelens, X., & de Haan, C. A. M. (2021). Influenza Neuraminidase Characteristics and Potential as a Vaccine Target. *Front Immunol*, *12*, 786617. https://doi.org/10.3389/fimmu.2021.786617

Crotty, S. (2014). T Follicular Helper Cell Differentiation, Function, and Roles in Disease. *Immunity*, *41*(4), 529-542. https://doi.org/10.1016/j.immuni.2014.10.004

Crotty, S., Felgner, P., Davies, H., Glidewell, J., Villarreal, L., & Ahmed, R. (2003). Cutting Edge: Long-Term B Cell Memory in Humans after Smallpox Vaccination 1. *The Journal of Immunology*, *171*(10), 4969-4973. https://doi.org/10.4049/jimmunol.171.10.4969

D'Agostino, M. R., Lai, R., Afkhami, S., Khera, A., Yao, Y., Vaseghi-Shanjani, M., Zganiacz, A., Jeyanathan, M., & Xing, Z. (2020). Airway Macrophages Mediate Mucosal Vaccine-Induced Trained Innate Immunity against Mycobacterium tuberculosis in Early Stages of Infection. *J Immunol*, *205*(10), 2750-2762. https://doi.org/10.4049/jimmunol.2000532

Decman, V., Laidlaw, B. J., Doering, T. A., Leng, J., Ertl, H. C., Goldstein, D. R., & Wherry, E. J. (2012). Defective CD8 T cell responses in aged mice are due to quantitative and qualitative changes in virus-specific precursors. *J Immunol*, *188*(4), 1933-1941. https://doi.org/10.4049/jimmunol.1101098

Dehghan, M. H., Gaikwad, V.M, Dandge, B. (2009). Nasal Absorption of Drugs – Barriers and Solutions. *Research Journal of Pharmacy and Technology*, *2*(4), 634-641.

Del Campo, J., Pizzorno, A., Djebali, S., Bouley, J., Haller, M., Pérez-Vargas, J., Lina, B., Boivin, G., Hamelin, M. E., Nicolas, F., Le Vert, A., Leverrier, Y., Rosa-Calatrava, M., Marvel, J., & Hill, F. (2019). OVX836 a recombinant nucleoprotein vaccine inducing cellular responses and protective efficacy against multiple influenza A subtypes. *npj Vaccines*, *4*, 4. https://doi.org/10.1038/s41541-019-0098-4

Deng, L., Cho, K. J., Fiers, W., & Saelens, X. (2015). M2e-Based Universal Influenza A Vaccines. *Vaccines (Basel)*, *3*(1), 105-136. https://doi.org/10.3390/vaccines3010105

Deng, L., Cho, K. J., Fiers, W., & Saelens, X. (2015). M2e-Based Universal Influenza A Vaccines. *Vaccines*, *3*(1), 105-136. https://www.mdpi.com/2076-393X/3/1/105

Dong, C., Zhu, W., Wei, L., Kim, J. K., Ma, Y., Kang, S.-M., & Wang, B.-Z. (2024). Enhancing cross-protection against influenza by heterologous sequential immunization with mRNA LNP and protein nanoparticle vaccines. *Nature Communications*, *15*(1), 5800. https://doi.org/10.1038/s41467-024-50087-5

Dorrington, M. G., & Bowdish, D. M. (2013). Immunosenescence and novel vaccination strategies for the elderly. *Front Immunol*, *4*, 171. https://doi.org/10.3389/fimmu.2013.00171

Dou, D., Revol, R., Östbye, H., Wang, H., & Daniels, R. (2018). Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement. *Front Immunol*, *9*, 1581. https://doi.org/10.3389/fimmu.2018.01581

Doucet, J. D., Forget, M. A., Grange, C., Rouxel, R. N., Arbour, N., von Messling, V., & Lapointe, R. (2011). Endogenously expressed matrix protein M1 and nucleoprotein of influenza A

are efficiently presented by class I and class II major histocompatibility complexes. *J Gen Virol*, *92*(Pt 5), 1162-1171. https://doi.org/10.1099/vir.0.029777-0

Eaton, S. M., Burns, E. M., Kusser, K., Randall, T. D., & Haynes, L. (2004). Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J Exp Med*, *200*(12), 1613-1622. https://doi.org/10.1084/jem.20041395

Edgar, J. E., Trezise, S., Anthony, R. M., Krammer, F., Palese, P., Ravetch, J. V., & Bournazos, S. (2023). Antibodies elicited in humans upon chimeric hemagglutinin-based influenza virus vaccination confer FcγR-dependent protection in vivo. *Proc Natl Acad Sci U S A*, *120*(44), e2314905120. https://doi.org/10.1073/pnas.2314905120

El Bakkouri, K., Descamps, F., De Filette, M., Smet, A., Festjens, E., Birkett, A., Van Rooijen, N., Verbeek, S., Fiers, W., & Saelens, X. (2011). Universal Vaccine Based on Ectodomain of Matrix Protein 2 of Influenza A: Fc Receptors and Alveolar Macrophages Mediate Protection. *The Journal of Immunology*, *186*(2), 1022-1031. https://doi.org/10.4049/jimmunol.0902147

Fazekas de St, G., & Webster, R. G. (1966). Disquisitions of Original Antigenic Sin. I. Evidence in man. *J Exp Med*, *124*(3), 331-345. https://doi.org/10.1084/jem.124.3.331

FDA. (2024). *Vaccines Licensed for Use in the United States*. https://www.fda.gov/vaccines-blood-biologics/vaccines/vaccines-licensed-use-united-states

Feng, J., Zhang, M., Mozdzanowska, K., Zharikova, D., Hoff, H., Wunner, W., Couch, R. B., & Gerhard, W. (2006). Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. *Virol J*, *3*, 102. https://doi.org/10.1186/1743-422x-3-102

Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., Panourgia, M. P., Invidia, L., Celani, L., Scurti, M., Cevenini, E., Castellani, G. C., & Salvioli, S. (2007). Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. *Mech Ageing Dev*, *128*(1), 92-105. https://doi.org/10.1016/j.mad.2006.11.016

Frank, K., & Paust, S. (2020). Dynamic Natural Killer Cell and T Cell Responses to Influenza Infection. *Frontiers in Cellular and Infection Microbiology*, *10*. https://doi.org/10.3389/fcimb.2020.00425

Frasca, D., Landin, A. M., Lechner, S. C., Ryan, J. G., Schwartz, R., Riley, R. L., & Blomberg, B. B. (2008). Aging Down-Regulates the Transcription Factor E2A, Activation-Induced Cytidine Deaminase, and Ig Class Switch in Human B Cells. *The Journal of Immunology*, *180*(8), 5283-5290. https://doi.org/10.4049/jimmunol.180.8.5283

Fu, T. M., Freed, D. C., Horton, M. S., Fan, J., Citron, M. P., Joyce, J. G., Garsky, V. M., Casimiro, D. R., Zhao, Q., Shiver, J. W., & Liang, X. (2009). Characterizations of four monoclonal antibodies against M2 protein ectodomain of influenza A virus. *Virology*, *385*(1), 218-226. https://doi.org/10.1016/j.virol.2008.11.035

Fujii, Y., Goto, H., Watanabe, T., Yoshida, T., & Kawaoka, Y. (2003). Selective incorporation of influenza virus RNA segments into virions. *Proc Natl Acad Sci U S A*, *100*(4), 2002-2007. https://doi.org/10.1073/pnas.0437772100

Gessner, J. E., Heiken, H., Tamm, A., & Schmidt, R. E. (1998). The IgG Fc receptor family. *Ann Hematol*, *76*(6), 231-248. https://doi.org/10.1007/s002770050396

Getie-Kebtie, M., Sultana, I., Eichelberger, M., & Alterman, M. (2013). Label-free mass spectrometry-based quantification of hemagglutinin and neuraminidase in influenza virus

preparations and vaccines. *Influenza and Other Respiratory Viruses*, *7*(4), 521-530. https://doi.org/10.1111/irv.12001

Ghendon, Y. Z., Polezhaev, F. I., Lisovskaya, K. V., Medvedeva, T. E., Alexandrova, G. I., & Klimov, A. I. (1984). Recombinant cold-adapted attenuated influenza A vaccines for use in children: molecular genetic analysis of the cold-adapted donor and recombinants. *Infect Immun*, *44*(3), 730-733. https://doi.org/10.1128/iai.44.3.730-733.1984

Giurgea, L. T., Morens, D. M., Taubenberger, J. K., & Memoli, M. J. (2020). Influenza Neuraminidase: A Neglected Protein and Its Potential for a Better Influenza Vaccine. *Vaccines (Basel)*, *8*(3). https://doi.org/10.3390/vaccines8030409

Gizurarson, S. (2015). The Effect of Cilia and the Mucociliary Clearance on Successful Drug Delivery. *Biological and Pharmaceutical Bulletin*, *38*(4), 497-506. https://doi.org/10.1248/bpb.b14-00398

Goodwin, K., Viboud, C., & Simonsen, L. (2006). Antibody response to influenza vaccination in the elderly: A quantitative review. *Vaccine*, *24*(8), 1159-1169. https://doi.org/https://doi.org/10.1016/j.vaccine.2005.08.105

Goodwin, K., Viboud, C., & Simonsen, L. (2006). Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine*, *24*(8), 1159-1169. https://doi.org/10.1016/j.vaccine.2005.08.105

Gordon, S., & Martinez, F. O. (2010). Alternative Activation of Macrophages: Mechanism and Functions. *Immunity*, *32*(5), 593-604. https://doi.org/10.1016/j.immuni.2010.05.007

Gravel, C., Li, C., Wang, J., Hashem, A. M., Jaentschke, B., Xu, K. W., Lorbetskie, B., Gingras, G., Aubin, Y., Van Domselaar, G., Girard, M., He, R., & Li, X. (2010). Qualitative and quantitative analyses of virtually all subtypes of influenza A and B viral neuraminidases

using antibodies targeting the universally conserved sequences. *Vaccine*, *28*(36), 5774-5784. https://doi.org/10.1016/j.vaccine.2010.06.075

Grewe, M. (2001). Chronological ageing and photoageing of dendritic cells. *Clin Exp Dermatol*, *26*(7), 608-612. https://doi.org/10.1046/j.1365-2230.2001.00898.x

Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C., & Amigorena, S. (2002). Antigen Presentation and T Cell Stimulation by Dendritic Cells. *Annual Review of Immunology*, *20*(Volume 20, 2002), 621-667. https://doi.org/https://doi.org/10.1146/annurev.immunol.20.100301.064828

Guilliams, M., Bruhns, P., Saeys, Y., Hammad, H., & Lambrecht, B. N. (2014). The function of Fcγ receptors in dendritic cells and macrophages. *Nat Rev Immunol*, *14*(2), 94-108. https://doi.org/10.1038/nri3582

Guthmiller, J. J., Han, J., Utset, H. A., Li, L., Lan, L. Y.-L., Henry, C., Stamper, C. T., McMahon, M., O'Dell, G., Fernández-Quintero, M. L., Freyn, A. W., Amanat, F., Stovicek, O., Gentles, L., Richey, S. T., de la Peña, A. T., Rosado, V., Dugan, H. L., Zheng, N.-Y.,…Wilson, P. C. (2022). Broadly neutralizing antibodies target a haemagglutinin anchor epitope. *Nature*, *602*(7896), 314-320. https://doi.org/10.1038/s41586-021-04356-8

Halsey, N. A., Talaat, K. R., Greenbaum, A., Mensah, E., Dudley, M. Z., Proveaux, T., & Salmon, D. A. (2015). The safety of influenza vaccines in children: An Institute for Vaccine Safety white paper. *Vaccine*, *33 Suppl 5*, F1-f67. https://doi.org/10.1016/j.vaccine.2015.10.080

Hay, A. J., Gregory, V., Douglas, A. R., & Lin, Y. P. (2001). The evolution of human influenza viruses. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *356*(1416), 1861-1870. https://doi.org/10.1098/rstb.2001.0999

Haynes, L., Eaton, S. M., Burns, E. M., Randall, T. D., & Swain, S. L. (2003). CD4 T cell memory derived from young naive cells functions well into old age, but memory generated from aged naive cells functions poorly. *Proc Natl Acad Sci U S A*, *100*(25), 15053-15058. https://doi.org/10.1073/pnas.2433717100

He, Q., Mao, Q., An, C., Zhang, J., Gao, F., Bian, L., Li, C., Liang, Z., Xu, M., & Wang, J. (2021). Heterologous prime-boost: breaking the protective immune response bottleneck of COVID-19 vaccine candidates. *Emerging Microbes & Infections*, *10*(1), 629-637. https://doi.org/10.1080/22221751.2021.1902245

Henry, C., Zheng, N.-Y., Huang, M., Cabanov, A., Rojas, K. T., Kaur, K., Andrews, S. F., Palm, A.-K. E., Chen, Y.-Q., Li, Y., Hoskova, K., Utset, H. A., Vieira, M. C., Wrammert, J., Ahmed, R., Holden-Wiltse, J., Topham, D. J., Treanor, J. J., Ertl, H. C.,…Wilson, P. C. (2019). Influenza Virus Vaccination Elicits Poorly Adapted B Cell Responses in Elderly Individuals. *Cell Host & Microbe*, *25*(3), 357-366.e356. https://doi.org/10.1016/j.chom.2019.01.002

Herndler-Brandstetter, D., Landgraf, K., Tzankov, A., Jenewein, B., Brunauer, R., Laschober, G. T., Parson, W., Kloss, F., Gassner, R., Lepperdinger, G., & Grubeck-Loebenstein, B. (2011). The impact of aging on memory T cell phenotype and function in the human bone marrow. *Journal of Leukocyte Biology*, *91*(2), 197-205. https://doi.org/10.1189/jlb.0611299

Hirata, N., Yanagawa, Y., Satoh, M., Ogura, H., Ebihara, T., Noguchi, M., Matsumoto, M., Togashi, H., Seya, T., Onoé, K., & Iwabuchi, K. (2010). Dendritic cell-derived TNF-α is responsible for development of IL-10-producing CD4+ T cells. *Cellular Immunology*, *261*(1), 37-41. https://doi.org/10.1016/j.cellimm.2009.10.009

Hollingsworth, R., El Guerche-Seblain, C., Tsai, T., Vasiliev, Y., Lee, S., Bright, H., & Barbosa, P. (2021). Assessment of the benefits of seasonal influenza vaccination: Elements of a framework to interpret estimates of vaccine effectiveness and support robust decision-making and communication. *Influenza Other Respir Viruses*, *15*(1), 164-174. https://doi.org/10.1111/irv.12786

Hutchinson, E. C., & Yamauchi, Y. (2018). Understanding Influenza. *Methods Mol Biol*, *1836*, 1-21. https://doi.org/10.1007/978-1-4939-8678-1_1

Ikonen, N., Strengell, M., Kinnunen, L., Osterlund, P., Pirhonen, J., Broman, M., Davidkin, I., Ziegler, T., & Julkunen, I. (2010). High frequency of cross-reacting antibodies against 2009 pandemic influenza A(H1N1) virus among the elderly in Finland. *Euro Surveill*, *15*(5).

Impagliazzo, A., Milder, F., Kuipers, H., Wagner, M. V., Zhu, X., Hoffman, R. M. B., van Meersbergen, R., Huizingh, J., Wanningen, P., Verspuij, J., de Man, M., Ding, Z., Apetri, A., Kükrer, B., Sneekes-Vriese, E., Tomkiewicz, D., Laursen, N. S., Lee, P. S., Zakrzewska, A.,…Radošević, K. (2015). A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science*, *349*(6254), 1301-1306. https://doi.org/doi:10.1126/science.aac7263

Inoue, T., & Kurosaki, T. (2024). Memory B cells. *Nature Reviews Immunology*, *24*(1), 5-17. https://doi.org/10.1038/s41577-023-00897-3

Irina, A. R., Tat'jana, S. R., Anastasija, A. K., Goar, S. B., Andrej, D. K., Feliks, I. E., Vera, V. K. j., Tat'jana, B. K., Denis, V. B., Andrej, V. I., Ljudmila, Y. G., Valentina, G. I., Natal'ja, A. F., Alisa, I. G., Svetlana, A. B., Natal'ja, V. V. e., Julija, V. D.-S., Elena, V. S., Ekaterina, V. F., & Nadezhda, S. A. (2022). Perspective Chapter: The Role of Interferon Gamma in

Clinical Medicine. In P. Hridayesh (Ed.), *Basic and Clinical Aspects of Interferon Gamma* (pp. Ch. 5). IntechOpen. https://doi.org/10.5772/intechopen.105476

Iuliano, A. D., Roguski, K. M., Chang, H. H., Muscatello, D. J., Palekar, R., Tempia, S., Cohen, C., Gran, J. M., Schanzer, D., Cowling, B. J., Wu, P., Kyncl, J., Ang, L. W., Park, M., Redlberger-Fritz, M., Yu, H., Espenhain, L., Krishnan, A., Emukule, G.,…Mustaquim, D. (2018). Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. *The Lancet*, *391*(10127), 1285-1300. https://doi.org/10.1016/S0140-6736(17)33293-2

Janssens, Y., Joye, J., Waerlop, G., Clement, F., Leroux-Roels, G., & Leroux-Roels, I. (2022). The role of cell-mediated immunity against influenza and its implications for vaccine evaluation [Review]. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.959379

Jazayeri, S. D., & Poh, C. L. (2019). Development of Universal Influenza Vaccines Targeting Conserved Viral Proteins. *Vaccines (Basel)*, *7*(4). https://doi.org/10.3390/vaccines7040169

Jegerlehner, A., Schmitz, N., Storni, T., & Bachmann, M. F. (2004). Influenza A Vaccine Based on the Extracellular Domain of M2: Weak Protection Mediated via Antibody-Dependent NK Cell Activity. *The Journal of Immunology*, *172*(9), 5598-5605. https://doi.org/10.4049/jimmunol.172.9.5598

Jin, H., Lu, B., Zhou, H., Ma, C., Zhao, J., Yang, C. F., Kemble, G., & Greenberg, H. (2003). Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology*, *306*(1), 18-24. https://doi.org/10.1016/s0042-6822(02)00035-1

Johansson, B. E., Matthews, J. T., & Kilbourne, E. D. (1998). Supplementation of conventional influenza A vaccine with purified viral neuraminidase results in a balanced and broadened immune response. *Vaccine*, *16*(9), 1009-1015. https://doi.org/https://doi.org/10.1016/S0264-410X(97)00279-X

Johansson, B. E., Matthews, J. T., & Kilbourne, E. D. (1998). Supplementation of conventional influenza A vaccine with purified viral neuraminidase results in a balanced and broadened immune response. *Vaccine*, *16*(9-10), 1009-1015. https://doi.org/10.1016/s0264-410x(97)00279-x

Johansson, B. E., Pokorny, B. A., & Tiso, V. A. (2002). Supplementation of conventional trivalent influenza vaccine with purified viral N1 and N2 neuraminidases induces a balanced immune response without antigenic competition. *Vaccine*, *20*(11-12), 1670-1674. https://doi.org/10.1016/s0264-410x(01)00490-x

Kalil, A. C., & Thomas, P. G. (2019). Influenza virus-related critical illness: pathophysiology and epidemiology. *Crit Care*, *23*(1), 258. https://doi.org/10.1186/s13054-019-2539-x

Kang, S.-M., Yoo, D.-G., Kim, M.-C., Song, J.-M., Park, M.-K., O, E., Quan, F.-S., Akira, S., & Compans, R. W. (2011). MyD88 Plays an Essential Role in Inducing B Cells Capable of Differentiating into Antibody-Secreting Cells after Vaccination. *Journal of Virology*, *85*(21), 11391-11400. https://doi.org/10.1128/jvi.00080-11

Kehagia, E., Papakyriakopoulou, P., & Valsami, G. (2023). Advances in intranasal vaccine delivery: A promising non-invasive route of immunization. *Vaccine*, *41*(24), 3589-3603. https://doi.org/10.1016/j.vaccine.2023.05.011

Keilich, S. R., Bartley, J. M., & Haynes, L. (2019). Diminished immune responses with aging predispose older adults to common and uncommon influenza complications. *Cell Immunol*, *345*, 103992. https://doi.org/10.1016/j.cellimm.2019.103992

Kemp, A. (2024). *FluMist approved for self-administration in the US*. AstraZeneca PLC. https://www.astrazeneca.com/media-centre/press-releases/2024/flumist-approved-for-self-administration-in-the-us.html#:~:text=FluMist%20has%20been%20approved%20in,2%2D17%20years%20of%20age.

Kim, K.-H., Kwon, Y.-M., Lee, Y.-T., Kim, M.-C., Hwang, H., Ko, E.-J., Lee, Y., Choi, H.-J., & Kang, S.-M. (2018a). Virus-Like Particles Are a Superior Platform for Presenting M2e Epitopes to Prime Humoral and Cellular Immunity against Influenza Virus. *Vaccines*, *6*(4), 66. https://doi.org/10.3390/vaccines6040066

Kim, K.-H., Kwon, Y.-M., Lee, Y.-T., Kim, M.-C., Hwang, H. S., Ko, E.-J., Lee, Y., Choi, H.-J., & Kang, S.-M. (2018b). Virus-Like Particles Are a Superior Platform for Presenting M2e Epitopes to Prime Humoral and Cellular Immunity against Influenza Virus. *Vaccines*, *6*(4), 66. https://www.mdpi.com/2076-393X/6/4/66

Kim, K.-H., Lee, Y.-T., Park, S., Jung, Y.-J., Lee, Y., Ko, E.-J., Kim, Y.-J., Li, X., & Kang, S.-M. (2019). Neuraminidase expressing virus-like particle vaccine provides effective cross protection against influenza virus. *Virology*, *535*, 179-188. https://doi.org/https://doi.org/10.1016/j.virol.2019.07.008

Kim, K. H., Bhatnagar, N., Subbiah, J., Liu, R., Pal, S. S., Raha, J. R., Grovenstein, P., Shin, C. H., Wang, B. Z., & Kang, S. M. . (2024). Cross-protection against influenza viruses by chimeric M2e-H3 stalk protein or multi-subtype neuraminidase plus M2e virus-like

particle vaccine in ferrets. *Virology*, *595*, 110097. https://doi.org/https://doi.org/10.1016/j.virol.2024.110097

Kim, K. H., Lee, Y. T., Park, S., Jung, Y. J., Lee, Y., Ko, E. J., Kim, Y. J., Li, X., & Kang, S. M. (2019). Neuraminidase expressing virus-like particle vaccine provides effective cross protection against influenza virus. *Virology*, *535*, 179-188. https://doi.org/10.1016/j.virol.2019.07.008

Kim, K. H., Li, Z., Bhatnagar, N., Subbiah, J., Park, B. R., Shin, C. H., Pushko, P., Wang, B. Z., & Kang, S. M. (2022). Universal protection against influenza viruses by multi-subtype neuraminidase and M2 ectodomain virus-like particle. *PLoS Pathog*, *18*(8), e1010755. https://doi.org/10.1371/journal.ppat.1010755

Kim, M.-C., Lee, Y.-N., Ko, E.-J., Lee, J. S., Kwon, Y.-M., Hwang, H. S., Song, J.-M., Song, B.-M., Lee, Y.-J., Choi, J.-G., Kang, H.-M., Quan, F.-S., Compans, R. W., & Kang, S.-M. (2014). Supplementation of Influenza Split Vaccines with Conserved M2 Ectodomains Overcomes Strain Specificity and Provides Long-term Cross Protection. *Molecular Therapy*, *22*(7), 1364-1374. https://doi.org/10.1038/mt.2014.33

Kim, M.-C., Song, J.-M., Eunju, O., Kwon, Y.-M., Lee, Y.-J., Compans, R. W., & Kang, S.-M. (2013). Virus-like Particles Containing Multiple M2 Extracellular Domains Confer Improved Cross-protection Against Various Subtypes of Influenza Virus. *Molecular Therapy*, *21*(2), 485-492. https://doi.org/10.1038/mt.2012.246

Kim, M. C., Lee, J. S., Kwon, Y. M., O, E., Lee, Y. J., Choi, J. G., Wang, B. Z., Compans, R. W., & Kang, S. M. (2013). Multiple heterologous M2 extracellular domains presented on virus-like particles confer broader and stronger M2 immunity than live influenza A virus infection. *Antiviral Res*, *99*(3), 328-335. https://doi.org/10.1016/j.antiviral.2013.06.010

Kim, M. C., Song, J. M., O, E., Kwon, Y. M., Lee, Y. J., Compans, R. W., & Kang, S. M. (2013). Virus-like particles containing multiple M2 extracellular domains confer improved cross-protection against various subtypes of influenza virus. *Mol Ther*, *21*(2), 485-492. https://doi.org/10.1038/mt.2012.246

Kipps, T. J., Parham, P., Punt, J., & Herzenberg, L. A. (1985). Importance of immunoglobulin isotype in human antibody-dependent, cell-mediated cytotoxicity directed by murine monoclonal antibodies. *J Exp Med*, *161*(1), 1-17. https://doi.org/10.1084/jem.161.1.1

Ko, E. J., Lee, Y., Lee, Y. T., Kim, Y. J., Kim, K. H., & Kang, S. M. (2018). MPL and CpG combination adjuvants promote homologous and heterosubtypic cross protection of inactivated split influenza virus vaccine. *Antiviral Res*, *156*, 107-115. https://doi.org/10.1016/j.antiviral.2018.06.004

Kono, H., Onda, A., & Yanagida, T. (2014). Molecular determinants of sterile inflammation. *Current Opinion in Immunology*, *26*, 147-156. https://doi.org/https://doi.org/10.1016/j.coi.2013.12.004

Kovaiou, R. D., & Grubeck-Loebenstein, B. (2006). Age-associated changes within CD4+ T cells. *Immunology Letters*, *107*(1), 8-14. https://doi.org/https://doi.org/10.1016/j.imlet.2006.07.006

Krammer, F., & Palese, P. (2014). Universal influenza virus vaccines: need for clinical trials. *Nature Immunology*, *15*(1), 3-5. https://doi.org/10.1038/ni.2761

Krammer, F., Pica, N., Hai, R., Margine, I., & Palese, P. (2013). Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. *J Virol*, *87*(12), 6542-6550. https://doi.org/10.1128/jvi.00641-13

Krammer, F., Smith, G. J. D., Fouchier, R. A. M., Peiris, M., Kedzierska, K., Doherty, P. C., Palese, P., Shaw, M. L., Treanor, J., Webster, R. G., & García-Sastre, A. (2018). Influenza. *Nat Rev Dis Primers*, *4*(1), 3. https://doi.org/10.1038/s41572-018-0002-y

Laguio-Vila, M. R., Thompson, M. G., Reynolds, S., Spencer, S. M., Gaglani, M., Naleway, A., Ball, S., Bozeman, S., Baker, S., Martínez-Sobrido, L., Levine, M., Katz, J., Fry, A. M., & Treanor, J. J. (2015). Comparison of Serum Hemagglutinin and Neuraminidase Inhibition Antibodies After 2010–2011 Trivalent Inactivated Influenza Vaccination in Healthcare Personnel. *Open Forum Infectious Diseases*, *2*(1). https://doi.org/10.1093/ofid/ofu115

Le, C. T. T., Ahn, S. Y., Kang, S.-M., & Ko, E.-J. (2021). Functional NK Cell Activation by Ovalbumin Immunization with a Monophosphoryl Lipid A and Poly I:C Combination Adjuvant Promoted Dendritic Cell Maturation. *Vaccines*, *9*(10), 1061. https://doi.org/10.3390/vaccines9101061

Lee, Y.-N., Kim, M.-C., Lee, Y.-T., Kim, Y.-J., & Kang, S.-M. (2015). Mechanisms of Cross-protection by Influenza Virus M2-based Vaccines. *in*, *15*(5), 213-221. https://doi.org/10.4110/in.2015.15.5.213

Lee, Y.-N., Lee, Y.-T., Kim, M.-C., Gewirtz, A. T., & Kang, S.-M. (2016). A Novel Vaccination Strategy Mediating the Induction of Lung-Resident Memory CD8 T Cells Confers Heterosubtypic Immunity against Future Pandemic Influenza Virus. *The Journal of Immunology*, *196*(6), 2637-2645. https://doi.org/10.4049/jimmunol.1501637

Lee, Y. T., Ko, E. J., Lee, Y., Kim, K. H., Kim, M. C., Lee, Y. N., & Kang, S. M. (2018). Intranasal vaccination with M2e5x virus-like particles induces humoral and cellular immune responses conferring cross-protection against heterosubtypic influenza viruses. *PLoS One*, *13*(1), e0190868. https://doi.org/10.1371/journal.pone.0190868

Lillie, P. J., Berthoud, T. K., Powell, T. J., Lambe, T., Mullarkey, C., Spencer, A. J., Hamill, M., Peng, Y., Blais, M.-E., Duncan, C. J. A., Sheehy, S. H., Havelock, T., Faust, S. N., Williams, R. L., Gilbert, A., Oxford, J., Dong, T., Hill, A. V. S., & Gilbert, S. C. (2012). Preliminary Assessment of the Efficacy of a T-Cell–Based Influenza Vaccine, MVA-NP+M1, in Humans. *Clinical Infectious Diseases*, *55*(1), 19-25. https://doi.org/10.1093/cid/cis327

Lin, J. C., & Nichol, K. L. (2001). Excess mortality due to pneumonia or influenza during influenza seasons among persons with acquired immunodeficiency syndrome. *Arch Intern Med*, *161*(3), 441-446. https://doi.org/10.1001/archinte.161.3.441

Lobaina Mato, Y. (2019). Nasal route for vaccine and drug delivery: Features and current opportunities. *International Journal of Pharmaceutics*, *572*, 118813. https://doi.org/https://doi.org/10.1016/j.ijpharm.2019.118813

Lycke, N. (2012). Recent progress in mucosal vaccine development: potential and limitations. *Nature Reviews Immunology*, *12*(8), 592-605. https://doi.org/10.1038/nri3251

Macintyre, C. R., Heywood, A. E., Kovoor, P., Ridda, I., Seale, H., Tan, T., Gao, Z., Katelaris, A. L., Siu, H. W., Lo, V., Lindley, R., & Dwyer, D. E. (2013). Ischaemic heart disease, influenza and influenza vaccination: a prospective case control study. *Heart*, *99*(24), 1843-1848. https://doi.org/10.1136/heartjnl-2013-304320

MacLean, A. J., Richmond, N., Koneva, L., Attar, M., Medina, C. A. P., Thornton, E. E., Gomes, A. C., El-Turabi, A., Bachmann, M. F., Rijal, P., Tan, T. K., Townsend, A., Sansom, S. N., Bannard, O., & Arnon, T. I. (2022). Secondary influenza challenge triggers resident memory B cell migration and rapid relocation to boost antibody secretion at infected sites. *Immunity*, *55*(4), 718-733.e718. https://doi.org/10.1016/j.immuni.2022.03.003

Maier, H. E., Nachbagauer, R., Kuan, G., Ng, S., Lopez, R., Sanchez, N., Stadlbauer, D., Gresh, L., Schiller, A., Rajabhathor, A., Ojeda, S., Guglia, A. F., Amanat, F., Balmaseda, A., Krammer, F., & Gordon, A. (2020). Pre-existing Antineuraminidase Antibodies Are Associated With Shortened Duration of Influenza A(H1N1)pdm Virus Shedding and Illness in Naturally Infected Adults. *Clin Infect Dis*, *70*(11), 2290-2297. https://doi.org/10.1093/cid/ciz639

Martin, K., & Helenius, A. (1991). Transport of incoming influenza virus nucleocapsids into the nucleus. *J Virol*, *65*(1), 232-244. https://doi.org/10.1128/jvi.65.1.232-244.1991

Mastalerz-Migas, A., Pokorski, M., Kiliś-Pstrusińska, K., Doskocz, K., Sapilak, B. J., & Brydak, L. B. (2015). Cytokines and toll-like receptors in the immune response to influenza vaccination. *Adv Exp Med Biol*, *836*, 35-40. https://doi.org/10.1007/5584_2014_25

McElhaney, J. E., Xie, D., Hager, W. D., Barry, M. B., Wang, Y., Kleppinger, A., Ewen, C., Kane, K. P., & Bleackley, R. C. (2006). T cell responses are better correlates of vaccine protection in the elderly. *J Immunol*, *176*(10), 6333-6339. https://doi.org/10.4049/jimmunol.176.10.6333

McMahon, M., Kirkpatrick, E., Stadlbauer, D., Strohmeier, S., Bouvier, N. M., & Krammer, F. (2019). Mucosal Immunity against Neuraminidase Prevents Influenza B Virus Transmission in Guinea Pigs. *mBio*, *10*(3). https://doi.org/10.1128/mBio.00560-19

McMahon, M., O'Dell, G., Tan, J., Sárközy, A., Vadovics, M., Carreño, J. M., Puente-Massaguer, E., Muramatsu, H., Bajusz, C., Rijnink, W., Beattie, M., Tam, Y. K., Kirkpatrick Roubidoux, E., Francisco, I., Strohmeier, S., Kanekiyo, M., Graham, B. S., Krammer, F., & Pardi, N. (2022). Assessment of a quadrivalent nucleoside-modified mRNA vaccine that

protects against group 2 influenza viruses. *Proceedings of the National Academy of Sciences*, *119*(45). https://doi.org/10.1073/pnas.2206333119

Memoli, M. J., Shaw, P. A., Han, A., Czajkowski, L., Reed, S., Athota, R., Bristol, T., Fargis, S., Risos, K., Powers, J. H., Davey, R. T., & Taubenberger, J. K. (2016). Evaluation of Antihemagglutinin and Antineuraminidase Antibodies as Correlates of Protection in an Influenza A/H1N1 Virus Healthy Human Challenge Model. *mBio*, *7*(2), e00417-00416. https://doi.org/10.1128/mbio.00417-16

Mezhenskaya, D., Isakova-Sivak, I., & Rudenko, L. (2019). M2e-based universal influenza vaccines: a historical overview and new approaches to development. *Journal of Biomedical Science*, *26*(1), 76. https://doi.org/10.1186/s12929-019-0572-3

Miller, S. M., Cybulski, V., Whitacre, M., Bess, L. S., Livesay, M. T., Walsh, L., Burkhart, D., Bazin, H. G., & Evans, J. T. (2020). Novel Lipidated Imidazoquinoline TLR7/8 Adjuvants Elicit Influenza-Specific Th1 Immune Responses and Protect Against Heterologous H3N2 Influenza Challenge in Mice. *Front Immunol*, *11*, 406. https://doi.org/10.3389/fimmu.2020.00406

Moderna. (2023). *Moderna Expands the Field of mRNA Medicine with Positive Clinical Results Across Cancer, Rare Disease, and Infectious Disease*. https://investors.modernatx.com/news/news-details/2023/Moderna-Expands-the-Field-of-mRNA-Medicine-with-Positive-Clinical-Results-Across-Cancer-Rare-Disease-and-Infectious-Disease/default.aspx

Mohsen, M. O., & Bachmann, M. F. (2022). Virus-like particle vaccinology, from bench to bedside. *Cellular & Molecular Immunology*, *19*(9), 993-1011. https://doi.org/10.1038/s41423-022-00897-8

Monto, A., & Kendal, A. (1973). EFFECT OF NEURAMINIDASE ANTIBODY ON HONG KONG INFLUENZA. *The Lancet*, *301*(7804), 623-625. https://doi.org/10.1016/s0140-6736(73)92196-x

Monto, A. S., Petrie, J. G., Cross, R. T., Johnson, E., Liu, M., Zhong, W., Levine, M., Katz, J. M., & Ohmit, S. E. (2015). Antibody to Influenza Virus Neuraminidase: An Independent Correlate of Protection. *Journal of Infectious Diseases*, *212*(8), 1191-1199. https://doi.org/10.1093/infdis/jiv195

Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R. T., Linder, T., Spyr, C., & Steffen, R. (2004). Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med*, *350*(9), 896-903. https://doi.org/10.1056/NEJMoa030595

Nafziger, A. N., & Pratt, D. S. (2014). Seasonal influenza vaccination and technologies. *The Journal of Clinical Pharmacology*, *54*(7), 719-731. https://doi.org/10.1002/jcph.299

Nakahashi-Ouchida, R., Fujihashi, K., Kurashima, Y., Yuki, Y., & Kiyono, H. (2023). Nasal vaccines: solutions for respiratory infectious diseases. *Trends Mol Med*, *29*(2), 124-140. https://doi.org/10.1016/j.molmed.2022.10.009

Nakahashi-Ouchida, R., Fujihashi, K., Kurashima, Y., Yuki, Y., & Kiyono, H. (2023). Nasal vaccines: solutions for respiratory infectious diseases. *Trends in Molecular Medicine*, *29*(2), 124-140. https://doi.org/https://doi.org/10.1016/j.molmed.2022.10.009

Nakowitsch, S., Waltenberger, A. M., Wressnigg, N., Ferstl, N., Triendl, A., Kiefmann, B., Montomoli, E., Lapini, G., Sergeeva, M., Muster, T., & Romanova, J. R. (2014). Egg- or cell culture-derived hemagglutinin mutations impair virus stability and antigen content of inactivated influenza vaccines. *Biotechnol J*, *9*(3), 405-414. https://doi.org/10.1002/biot.201300225

Netea, M. G., Domínguez-Andrés, J., Barreiro, L. B., Chavakis, T., Divangahi, M., Fuchs, E., Joosten, L. A. B., van der Meer, J. W. M., Mhlanga, M. M., Mulder, W. J. M., Riksen, N. P., Schlitzer, A., Schultze, J. L., Stabell Benn, C., Sun, J. C., Xavier, R. J., & Latz, E. (2020). Defining trained immunity and its role in health and disease. *Nature Reviews Immunology*, *20*(6), 375-388. https://doi.org/10.1038/s41577-020-0285-6

Nguyen, Q. T., & Choi, Y. K. (2021). Targeting Antigens for Universal Influenza Vaccine Development. *Viruses*, *13*(6). https://doi.org/10.3390/v13060973

Nooraei, S., Bahrulolum, H., Hoseini, Z. S., Katalani, C., Hajizade, A., Easton, A. J., & Ahmadian, G. (2021). Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers. *Journal of Nanobiotechnology*, *19*(1), 59. https://doi.org/10.1186/s12951-021-00806-7

Novavax. (2020). *Novavax' NanoFlu Achieves All Primary Endpoints In Phase 3 Clinical Trial*. https://www.sec.gov/Archives/edgar/data/1000694/000110465920037764/tm2013650d1_ex99-1.htm

Oh, J. E., Song, E., Moriyama, M., Wong, P., Zhang, S., Jiang, R., Strohmeier, S., Kleinstein, S. H., Krammer, F., & Iwasaki, A. (2021). Intranasal priming induces local lung-resident B cell populations that secrete protective mucosal antiviral IgA. *Sci Immunol*, *6*(66), eabj5129. https://doi.org/10.1126/sciimmunol.abj5129

Pannus, P., Depickère, S., Kemlin, D., Houben, S., Neven, K. Y., Heyndrickx, L., Michiels, J., Willems, E., De Craeye, S., Francotte, A., Chaumont, F., Olislagers, V., Waegemans, A., Verbrugghe, M., Schmickler, M.-N., Van Gucht, S., Dierick, K., Marchant, A., Desombere, I.,…Goossens, M. E. (2022). Safety and immunogenicity of a reduced dose of the BNT162b2 mRNA COVID-19 vaccine (REDU-VAC): A single blind, randomized, non-

inferiority trial. *PLOS Global Public Health*, *2*(12), e0001308. https://doi.org/10.1371/journal.pgph.0001308

Pardi, N., Carreño, J. M., O'Dell, G., Tan, J., Bajusz, C., Muramatsu, H., Rijnink, W., Strohmeier, S., Loganathan, M., Bielak, D., Sung, M. M. H., Tam, Y. K., Krammer, F., & McMahon, M. (2022). Development of a pentavalent broadly protective nucleoside-modified mRNA vaccine against influenza B viruses. *Nature Communications*, *13*(1), 4677. https://doi.org/10.1038/s41467-022-32149-8

Paules, C. I., Marston, H. D., Eisinger, R. W., Baltimore, D., & Fauci, A. S. (2017). The Pathway to a Universal Influenza Vaccine. *Immunity*, *47*(4), 599-603. https://doi.org/10.1016/j.immuni.2017.09.007

Peek, L. J., Middaugh, C. R., & Berkland, C. (2008). Nanotechnology in vaccine delivery. *Adv Drug Deliv Rev*, *60*(8), 915-928. https://doi.org/10.1016/j.addr.2007.05.017

Perrone, L. A., Ahmad, A., Veguilla, V., Lu, X., Smith, G., Katz, J. M., Pushko, P., & Tumpey, T. M. (2009). Intranasal Vaccination with 1918 Influenza Virus-Like Particles Protects Mice and Ferrets from Lethal 1918 and H5N1 Influenza Virus Challenge. *Journal of Virology*, *83*(11), 5726-5734. https://doi.org/10.1128/jvi.00207-09

Perrone, L. A., Ahmad, A., Veguilla, V., Lu, X., Smith, G., Katz, J. M., Pushko, P., & Tumpey, T. M. (2009). Intranasal vaccination with 1918 influenza virus-like particles protects mice and ferrets from lethal 1918 and H5N1 influenza virus challenge. *J Virol*, *83*(11), 5726-5734. https://doi.org/10.1128/jvi.00207-09

Pfizer. (2024). *Pfizer and BioNTech Provide Update on mRNA-based Combination Vaccine Program Against Influenza and COVID-19 in Individuals 18-64 Years of Age*.

https://pfizer.com/news/press-release/press-release-detail/pfizer-and-biontech-provide-update-mrna-based-combination

Pielak, R. M., & Chou, J. J. (2011). Influenza M2 proton channels. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1808*(2), 522-529. https://doi.org/https://doi.org/10.1016/j.bbamem.2010.04.015

Podda, A., & Del Giudice, G. (2003). MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. *Expert Rev Vaccines*, *2*(2), 197-203. https://doi.org/10.1586/14760584.2.2.197

Powell, T. J., Peng, Y., Berthoud, T. K., Blais, M. E., Lillie, P. J., Hill, A. V., Rowland-Jones, S. L., McMichael, A. J., Gilbert, S. C., & Dong, T. (2013). Examination of influenza specific T cell responses after influenza virus challenge in individuals vaccinated with MVA-NP+M1 vaccine. *PLoS One*, *8*(5), e62778. https://doi.org/10.1371/journal.pone.0062778

Psarras, A., Antanaviciute, A., Alase, A., Carr, I., Wittmann, M., Emery, P., Tsokos, G. C., & Vital, E. M. (2021). TNF-α Regulates Human Plasmacytoid Dendritic Cells by Suppressing IFN-α Production and Enhancing T Cell Activation. *The Journal of Immunology*, *206*(4), 785-796. https://doi.org/10.4049/jimmunol.1901358

Purtha, W. E., Tedder, T. F., Johnson, S., Bhattacharya, D., & Diamond, M. S. (2011). Memory B cells, but not long-lived plasma cells, possess antigen specificities for viral escape mutants. *Journal of Experimental Medicine*, *208*(13), 2599-2606. https://doi.org/10.1084/jem.20110740

Purtha, W. E., Tedder, T. F., Johnson, S., Bhattacharya, D., & Diamond, M. S. (2011). Memory B cells, but not long-lived plasma cells, possess antigen specificities for viral escape mutants. *J Exp Med*, *208*(13), 2599-2606. https://doi.org/10.1084/jem.20110740

Pürzel, J., Schmitt, R., Viertlboeck, B. C., & Göbel, T. W. (2009). Chicken IgY binds its receptor at the CH3/CH4 interface similarly as the human IgA: Fc alpha RI interaction. *J Immunol*, *183*(7), 4554-4559. https://doi.org/10.4049/jimmunol.0901699

Raha, J. R., Kim, K. H., Bhatnagar, N., Liu, R., Le, C. T. T., Park, B. R., Grovenstein, P., Pal, S. S., Ko, E. J., Shin, C. H., Wang, B. Z., & Kang, S. M. (2024). Supplementation of seasonal vaccine with multi-subtype neuraminidase and M2 ectodomain virus-like particle improves protection against homologous and heterologous influenza viruses in aged mice. *Antiviral Res*, 105877. https://doi.org/10.1016/j.antiviral.2024.105877

Rajendran, M., Krammer, F., & McMahon, M. (2021). The Human Antibody Response to the Influenza Virus Neuraminidase Following Infection or Vaccination. *Vaccines (Basel)*, *9*(8). https://doi.org/10.3390/vaccines9080846

Ramos, E. L., Mitcham, J. L., Koller, T. D., Bonavia, A., Usner, D. W., Balaratnam, G., Fredlund, P., & Swiderek, K. M. (2015). Efficacy and safety of treatment with an anti-m2e monoclonal antibody in experimental human influenza. *J Infect Dis*, *211*(7), 1038-1044. https://doi.org/10.1093/infdis/jiu539

Rasmussen, S. A., Jamieson, D. J., & Uyeki, T. M. (2012). Effects of influenza on pregnant women and infants. *Am J Obstet Gynecol*, *207*(3 Suppl), S3-8. https://doi.org/10.1016/j.ajog.2012.06.068

Reed, C., Chaves, S. S., Daily Kirley, P., Emerson, R., Aragon, D., Hancock, E. B., Butler, L., Baumbach, J., Hollick, G., Bennett, N. M., Laidler, M. R., Thomas, A., Meltzer, M. I., & Finelli, L. (2015). Estimating influenza disease burden from population-based surveillance data in the United States. *PLoS One*, *10*(3), e0118369. https://doi.org/10.1371/journal.pone.0118369

Rehman, T. (2012). Role of the gut microbiota in age-related chronic inflammation. *Endocr Metab Immune Disord Drug Targets*, *12*(4), 361-367. https://doi.org/10.2174/187153012803832620

Rouse, B. T., & Sehrawat, S. (2010). Immunity and immunopathology to viruses: what decides the outcome? *Nature Reviews Immunology*, *10*(7), 514-526. https://doi.org/10.1038/nri2802

Russell, R. J., Haire, L. F., Stevens, D. J., Collins, P. J., Lin, Y. P., Blackburn, G. M., Hay, A. J., Gamblin, S. J., & Skehel, J. J. (2006). The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature*, *443*(7107), 45-49. https://doi.org/10.1038/nature05114

Ryder, A. B., Nachbagauer, R., Buonocore, L., Palese, P., Krammer, F., & Rose, J. K. (2015). Vaccination with Vesicular Stomatitis Virus-Vectored Chimeric Hemagglutinins Protects Mice against Divergent Influenza Virus Challenge Strains. *J Virol*, *90*(5), 2544-2550. https://doi.org/10.1128/jvi.02598-15

Sage, Peter T., Tan, Catherine L., Freeman, Gordon J., Haigis, M., & Sharpe, Arlene H. (2015). Defective TFH Cell Function and Increased TFR Cells Contribute to Defective Antibody Production in Aging. *Cell Reports*, *12*(2), 163-171. https://doi.org/10.1016/j.celrep.2015.06.015

Sakai-Tagawa, Y., Yamayoshi, S., Kawakami, C., Le, M. Q., Uchida, Y., Saito, T., Nidom, C. A., Humaira, I., Toohey-Kurth, K., Arafa, A. S., Liu, M. T., Shu, Y., & Kawaoka, Y. (2017). Reactivity and sensitivity of commercially available influenza rapid diagnostic tests in Japan. *Sci Rep*, *7*(1), 14483. https://doi.org/10.1038/s41598-017-14536-0

Sasaki, S., Jaimes, M. C., Holmes, T. H., Dekker, C. L., Mahmood, K., Kemble, G. W., Arvin, A. M., & Greenberg, H. B. (2007). Comparison of the influenza virus-specific effector and

memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccines. *J Virol*, *81*(1), 215-228. https://doi.org/10.1128/jvi.01957-06

Sasaki, S., Sullivan, M., Narvaez, C. F., Holmes, T. H., Furman, D., Zheng, N. Y., Nishtala, M., Wrammert, J., Smith, K., James, J. A., Dekker, C. L., Davis, M. M., Wilson, P. C., Greenberg, H. B., & He, X. S. (2011). Limited efficacy of inactivated influenza vaccine in elderly individuals is associated with decreased production of vaccine-specific antibodies. *J Clin Invest*, *121*(8), 3109-3119. https://doi.org/10.1172/JCI57834

Schepens, B., De Vlieger, D., & Saelens, X. (2018). Vaccine options for influenza: thinking small. *Current Opinion in Immunology*, *53*, 22-29. https://doi.org/https://doi.org/10.1016/j.coi.2018.03.024

Scheuring, U. J., Sabzevari, H., & Theofilopoulos, A. N. (2002). Proliferative arrest and cell cycle regulation in CD8+CD28− versus CD8+CD28+ T cells. *Human Immunology*, *63*(11), 1000-1009. https://doi.org/https://doi.org/10.1016/S0198-8859(02)00683-3

Schotsaert, M., Ysenbaert, T., Smet, A., Schepens, B., Fiers, W., & Saelens, X. (2015). Infection-permissive immunity provided by NA- and M2e-based vaccines protects against influenza A virus challenge and allows the induction of heterosubtypic immunity during subsequent infections. (VAC5P.1127). *The Journal of Immunology*, *194*(1_Supplement), 73.12-73.12. https://doi.org/10.4049/jimmunol.194.Supp.73.12

Shartouny, J. R., & Lowen, A. C. (2022). Message in a bottle: mRNA vaccination for influenza. *J Gen Virol*, *103*(7). https://doi.org/10.1099/jgv.0.001765

Sia Zachary, R., Chiem, K., Huang, W.-C., Seffouh, A., Teimouri Dereshgi, A., Hogan, T., Ortega, J., Davidson Bruce, A., Martinez-Sobrido, L., & Lovell Jonathan, F. (2022). Respiratory

Vaccination with Hemagglutinin Nanoliposomes Protects Mice from Homologous and Heterologous Strains of Influenza Virus. *Journal of Virology*, *96*(19), e01006-01022. https://doi.org/10.1128/jvi.01006-22

Simonsen, L., Reichert, T. A., Viboud, C., Blackwelder, W. C., Taylor, R. J., & Miller, M. A. (2005). Impact of influenza vaccination on seasonal mortality in the US elderly population. *Arch Intern Med*, *165*(3), 265-272. https://doi.org/10.1001/archinte.165.3.265

Skelton, R. M., & Huber, V. C. (2022). Comparing Influenza Virus Biology for Understanding Influenza D Virus. *Viruses*, *14*(5). https://doi.org/10.3390/v14051036

Smetana, J., Chlibek, R., Shaw, J., Splino, M., & Prymula, R. (2018). Influenza vaccination in the elderly. *Hum Vaccin Immunother*, *14*(3), 540-549. https://doi.org/10.1080/21645515.2017.1343226

Smith, G. E., Sun, X., Bai, Y., Liu, Y. V., Massare, M. J., Pearce, M. B., Belser, J. A., Maines, T. R., Creager, H. M., Glenn, G. M., Flyer, D., Pushko, P., Levine, M. Z., & Tumpey, T. M. (2017). Neuraminidase-based recombinant virus-like particles protect against lethal avian influenza A(H5N1) virus infection in ferrets. *Virology*, *509*, 90-97. https://doi.org/https://doi.org/10.1016/j.virol.2017.06.006

Song, J.-M., Van Rooijen, N., Bozja, J., Compans, R. W., & Kang, S.-M. (2011). Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus. *Proceedings of the National Academy of Sciences*, *108*(2), 757-761. https://doi.org/10.1073/pnas.1012199108

Speth, C., Bredl, S., Hagleitner, M., Wild, J., Dierich, M., Wolf, H., Schroeder, J., Wagner, R., & Deml, L. (2008). Human immunodeficiency virus type-1 (HIV-1) Pr55gag virus-like

particles are potent activators of human monocytes. *Virology*, *382* 46–58. https://doi.org/10.1016/j.virol.2008.08.043

Sridhar, S., Brokstad, K. A., & Cox, R. J. (2015). Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. *Vaccines (Basel)*, *3*(2), 373-389. https://doi.org/10.3390/vaccines3020373

Stadlbauer, D., Zhu, X., McMahon, M., Turner, J. S., Wohlbold, T. J., Schmitz, A. J., Strohmeier, S., Yu, W., Nachbagauer, R., Mudd, P. A., Wilson, I. A., Ellebedy, A. H., & Krammer, F. (2019). Broadly protective human antibodies that target the active site of influenza virus neuraminidase. *Science*, *366*(6464), 499-504. https://doi.org/10.1126/science.aay0678

Stevens, J., Chen, L. M., Carney, P. J., Garten, R., Foust, A., Le, J., Pokorny, B. A., Manojkumar, R., Silverman, J., Devis, R., Rhea, K., Xu, X., Bucher, D. J., Paulson, J. C., Cox, N. J., Klimov, A., & Donis, R. O. (2010). Receptor specificity of influenza A H3N2 viruses isolated in mammalian cells and embryonated chicken eggs. *J Virol*, *84*(16), 8287-8299. https://doi.org/10.1128/jvi.00058-10

Strohmeier, S., Amanat, F., Zhu, X., McMahon, M., Deming, M. E., Pasetti, M. F., Neuzil, K. M., Wilson, I. A., & Krammer, F. (2021). A Novel Recombinant Influenza Virus Neuraminidase Vaccine Candidate Stabilized by a Measles Virus Phosphoprotein Tetramerization Domain Provides Robust Protection from Virus Challenge in the Mouse Model. *mBio*, *12*(6), e0224121. https://doi.org/10.1128/mBio.02241-21

Subbiah, J., Oh, J., Kim, K.-H., Shin, C.-H., Park, B. R., Bhatnagar, N., Seong, B.-L., Wang, B.-Z., & Kang, S.-M. (2022). A chimeric thermostable M2e and H3 stalk-based universal influenza A virus vaccine. *npj Vaccines*, *7*(1). https://doi.org/10.1038/s41541-022-00498-6

Takai, T., Li, M., Sylvestre, D., Clynes, R., & Ravetch, J. V. (1994). FcR gamma chain deletion results in pleiotrophic effector cell defects. *Cell*, *76*(3), 519-529. https://doi.org/10.1016/0092-8674(94)90115-5

Tan, J., O'Dell, G., Hernandez, M. M., Sordillo, E. M., Kahn, Z., Kriti, D., Van Bakel, H., Ellebedy, A. H., Wilson, P. C., Simon, V., Krammer, F., & McMahon, M. (2022). Human Anti-neuraminidase Antibodies Reduce Airborne Transmission of Clinical Influenza Virus Isolates in the Guinea Pig Model. *Journal of Virology*, *96*(2). https://doi.org/10.1128/jvi.01421-21

Tanner, A. R., Dorey, R. B., Brendish, N. J., & Clark, T. W. (2021). Influenza vaccination: protecting the most vulnerable. *Eur Respir Rev*, *30*(159). https://doi.org/10.1183/16000617.0258-2020

Tarasov, M., Shanko, A., Kordyukova, L., & Katlinski, A. (2020). Characterization of Inactivated Influenza Vaccines Used in the Russian National Immunization Program. *Vaccines*, *8*(3), 488. https://www.mdpi.com/2076-393X/8/3/488

Taubenberger, J. K., & Morens, D. M. (2006). 1918 Influenza: the mother of all pandemics. *Emerg Infect Dis*, *12*(1), 15-22. https://doi.org/10.3201/eid1201.050979

Taubenberger, J. K., & Morens, D. M. (2008). The pathology of influenza virus infections. *Annu Rev Pathol*, *3*, 499-522. https://doi.org/10.1146/annurev.pathmechdis.3.121806.154316

Tengvall, S., Lundgren, A., Quiding-Järbrink, M., & Svennerholm, A. M. (2010). BAFF, stimulatory DNA and IL-15 stimulates IgA(+) memory B cells and provides a novel approach for analysis of memory responses to mucosal vaccines. *Vaccine*, *28*(33), 5445-5450. https://doi.org/10.1016/j.vaccine.2010.06.001

Thompson, W. W., Comanor, L., & Shay, D. K. (2006). Epidemiology of seasonal influenza: use of surveillance data and statistical models to estimate the burden of disease. *J Infect Dis*, *194 Suppl 2*, S82-91. https://doi.org/10.1086/507558

Tricco, A. C., Chit, A., Soobiah, C., Hallett, D., Meier, G., Chen, M. H., Tashkandi, M., Bauch, C. T., & Loeb, M. (2013). Comparing influenza vaccine efficacy against mismatched and matched strains: a systematic review and meta-analysis. *BMC Medicine*, *11*(1), 153. https://doi.org/10.1186/1741-7015-11-153

Trombetta, C. M., Marchi, S., Manini, I., Lazzeri, G., & Montomoli, E. (2019). Challenges in the development of egg-independent vaccines for influenza. *Expert Rev Vaccines*, *18*(7), 737-750. https://doi.org/10.1080/14760584.2019.1639503

Turley, C. B., Rupp, R. E., Johnson, C., Taylor, D. N., Wolfson, J., Tussey, L., Kavita, U., Stanberry, L., & Shaw, A. (2011). Safety and immunogenicity of a recombinant M2e–flagellin influenza vaccine (STF2.4xM2e) in healthy adults. *Vaccine*, *29*(32), 5145-5152. https://doi.org/https://doi.org/10.1016/j.vaccine.2011.05.041

Ueda, A., Okuda, K., Ohno, S., Shirai, A., Igarashi, T., Matsunaga, K., Fukushima, J., Kawamoto, S., Ishigatsubo, Y., & Okubo, T. (1994). NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *The Journal of Immunology*, *153*(5), 2052-2063. https://doi.org/10.4049/jimmunol.153.5.2052

van der Lubben, I. M., Verhoef, J. C., Borchard, G., & Junginger, H. E. (2001). Chitosan for mucosal vaccination. *Adv Drug Deliv Rev*, *52*(2), 139-144. https://doi.org/10.1016/s0169-409x(01)00197-1

Varga, Z. T., Ramos, I., Hai, R., Schmolke, M., García-Sastre, A., Fernandez-Sesma, A., & Palese, P. (2011). The Influenza Virus Protein PB1-F2 Inhibits the Induction of Type I Interferon

at the Level of the MAVS Adaptor Protein. *PLoS Pathogens*, *7*(6), e1002067. https://doi.org/10.1371/journal.ppat.1002067

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nature Immunology*, *9*(5), 503-510. https://doi.org/10.1038/ni1582

Voynow, J. A., & Rubin, B. K. (2009). Mucins, Mucus, and Sputum. *Chest*, *135*(2), 505-512. https://doi.org/https://doi.org/10.1378/chest.08-0412

Wang, C., & Yuan, F. (2024). A comprehensive comparison of DNA and RNA vaccines. *Advanced Drug Delivery Reviews*, *210*, 115340. https://doi.org/https://doi.org/10.1016/j.addr.2024.115340

Wang, Y., Song, T., Li, K., Jin, Y., Yue, J., Ren, H., & Liang, L. (2019). Different Subtypes of Influenza Viruses Target Different Human Proteins and Pathways Leading to Different Pathogenic Phenotypes. *Biomed Res Int*, *2019*, 4794910. https://doi.org/10.1155/2019/4794910

Wei, C. J., Yassine, H. M., McTamney, P. M., Gall, J. G., Whittle, J. R., Boyington, J. C., & Nabel, G. J. (2012). Elicitation of broadly neutralizing influenza antibodies in animals with previous influenza exposure. *Sci Transl Med*, *4*(147), 147ra114. https://doi.org/10.1126/scitranslmed.3004273

Weinstein, R. A., Bridges, C. B., Kuehnert, M. J., & Hall, C. B. (2003). Transmission of Influenza: Implications for Control in Health Care Settings. *Clinical Infectious Diseases*, *37*(8), 1094-1101. https://doi.org/10.1086/378292

Weltzin, R., & Monath, T. P. (1999). Intranasal antibody prophylaxis for protection against viral disease. *Clin Microbiol Rev*, *12*(3), 383-393. https://doi.org/10.1128/cmr.12.3.383

Wen, H. S., & Wang, S. P. (1959). Mouse adaptation of the Asian influenza virus. *J Infect Dis*, *105*(1), 9-17. https://doi.org/10.1093/infdis/105.1.9

WHO. (2018). *Influenza (Seasonal)*. https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)

WHO. (2023). *Influenza (Seasonal)*. https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)#:~:text=There%20are%20around%20a%20billion,infections%20are%20in%20developing%20countries.

Wiersma, L. C., Rimmelzwaan, G. F., & de Vries, R. D. (2015). Developing Universal Influenza Vaccines: Hitting the Nail, Not Just on the Head. *Vaccines (Basel)*, *3*(2), 239-262. https://doi.org/10.3390/vaccines3020239

Wohlbold Teddy, J., Nachbagauer, R., Xu, H., Tan Gene, S., Hirsh, A., Brokstad Karl, A., Cox Rebecca, J., Palese, P., & Krammer, F. (2015). Vaccination with Adjuvanted Recombinant Neuraminidase Induces Broad Heterologous, but Not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice. *mBio*, *6*(2), 10.1128/mbio.02556-02514. https://doi.org/10.1128/mbio.02556-14

Wohlbold, T. J., Nachbagauer, R., Xu, H., Tan, G. S., Hirsh, A., Brokstad, K. A., Cox, R. J., Palese, P., & Krammer, F. (2015). Vaccination with Adjuvanted Recombinant Neuraminidase Induces Broad Heterologous, but Not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice. *mBio*, *6*(2), e02556-02514. https://doi.org/10.1128/mbio.02556-14

Wu, Y., Wu, Y., Tefsen, B., Shi, Y., & Gao, G. F. (2014). Bat-derived influenza-like viruses H17N10 and H18N11. *Trends Microbiol*, *22*(4), 183-191. https://doi.org/10.1016/j.tim.2014.01.010

Yamayoshi, S., & Kawaoka, Y. (2019). Current and future influenza vaccines. *Nature Medicine*, *25*(2), 212-220. https://doi.org/10.1038/s41591-018-0340-z

Yang, P., Wang, W., Gu, H., Li, Z., Zhang, K., Wang, Z., Li, R., Duan, Y., Zhang, S., & Wang, X. (2014). Protection against influenza H7N9 virus challenge with a recombinant NP-M1-HSP60 protein vaccine construct in BALB/c mice. *Antiviral Res*, *111*, 1-7. https://doi.org/10.1016/j.antiviral.2014.08.008

Yao, Y., Jeyanathan, M., Haddadi, S., Barra, N. G., Vaseghi-Shanjani, M., Damjanovic, D., Lai, R., Afkhami, S., Chen, Y., Dvorkin-Gheva, A., Robbins, C. S., Schertzer, J. D., & Xing, Z. (2018). Induction of Autonomous Memory Alveolar Macrophages Requires T Cell Help and Is Critical to Trained Immunity. *Cell*, *175*(6), 1634-1650.e1617. https://doi.org/10.1016/j.cell.2018.09.042

Yassine, H. M., Boyington, J. C., McTamney, P. M., Wei, C.-J., Kanekiyo, M., Kong, W.-P., Gallagher, J. R., Wang, L., Zhang, Y., Joyce, M. G., Lingwood, D., Moin, S. M., Andersen, H., Okuno, Y., Rao, S. S., Harris, A. K., Kwong, P. D., Mascola, J. R., Nabel, G. J., & Graham, B. S. (2015). Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nature Medicine*, *21*(9), 1065-1070. https://doi.org/10.1038/nm.3927

Yusuf, H., & Kett, V. (2017). Current prospects and future challenges for nasal vaccine delivery. *Hum Vaccin Immunother*, *13*(1), 34-45. https://doi.org/10.1080/21645515.2016.1239668

Zhang, X., & Ross, T. M. (2024). Anti-neuraminidase immunity in the combat against influenza. *Expert Rev Vaccines*, *23*(1), 474-484. https://doi.org/10.1080/14760584.2024.2343689

Zheng, M., Luo, J., & Chen, Z. (2014). Development of universal influenza vaccines based on influenza virus M and NP genes. *Infection*, *42*(2), 251-262. https://doi.org/10.1007/s15010-013-0546-4

Zimmerman, R. K., Nowalk, M. P., Chung, J., Jackson, M. L., Jackson, L. A., Petrie, J. G., Monto, A. S., McLean, H. Q., Belongia, E. A., Gaglani, M., Murthy, K., Fry, A. M., & Flannery, B. (2016). 2014-2015 Influenza Vaccine Effectiveness in the United States by Vaccine Type. *Clin Infect Dis*, *63*(12), 1564-1573. https://doi.org/10.1093/cid/ciw635

Zost, S. J., Wu, N. C., Hensley, S. E., & Wilson, I. A. (2019). Immunodominance and Antigenic Variation of Influenza Virus Hemagglutinin: Implications for Design of Universal Vaccine Immunogens. *The Journal of Infectious Diseases*, *219*(Supplement_1), S38-S45. https://doi.org/10.1093/infdis/jiy696

7. VITAE

Jannatul Ruhan Raha

EDUCATION

Doctor of Philosophy in Translational Biomedical Sciences **Aug 2020 – Dec 2024**

Georgia State University | Atlanta, GA, USA | CGPA: 4.15/4.00

Bachelor of Science in Biochemistry and Biotechnology, *Summa Cum Laude* **Jan 2016 – Jan 2020**

North South University | Dhaka, Bangladesh | CGPA: 3.91/4.00

PROFESSIONAL EXPERIENCE

Research Assistant | *Georgia State University, Atlanta, GA, USA* **Jan 2021 – Present**

Responsibilities:

- Lead influenza viruses and vaccine research by leveraging project design, management, and experimental skills
- Conduct comprehensive literature reviews and prepare manuscripts for publication in peer-reviewed journals
- Revise and proofread manuscripts as requested by editors and journals
- Supervise undergraduate and master's students to perform lab experiments adhering to established lab protocols

  and regulatory standards, and assist with the preparation of scientific posters and thesis
- Collaborate with other labs to conduct research in various therapeutic areas
- Participate and present in scientific conferences, symposiums, and meetings

TEACHING EXPERIENCE

Biology Teacher | *The Aga Khan School, Dhaka, Bangladesh* **Jun 2019 – Dec 2020**

Undergraduate Teaching Assistant | *North South University, Dhaka, Bangladesh* **Sep 2018 - Jan 2019**

PUBLICATIONS

- **Raha JR**, Kim KH, Bhatnagar N, Liu R, Le CTT, Park BR, Grovenstein P, Pal SS, Ko EJ, Shin CH, Wang BZ, Kang SM. Supplementation of seasonal vaccine with multi-subtype neuraminidase and M2 ectodomain virus-like particle improves protection against homologous and heterologous influenza viruses in aged mice. Antiviral Res. 2024 Mar 30:105877. doi: 10.1016/j.antiviral.2024.105877. Epub ahead of print. PMID: 38561077.
- **Raha JR**, Kim KH, Bhatnagar N, Liu R, Park BR, Le CTT, Grovenstein P, Pal SS, Kang SM. Supplementation of seasonal vaccine with multi-subtype neuraminidase and M2 ectodomain virus-like particle improves protection against influenza viruses in young mice and under pre-existing immunity conditions (manuscript in preparation)
- **Raha JR**, Kim KH, Bhatnagar N, Liu R, Le CTT, Grovenstein P, Pal SS, Kang SM. Intranasal vaccination with multi-subtype neuraminidase and M2 Ectodomain virus-like particle enhances protection against influenza viruses (manuscript in preparation)
- Li Z, Kim KH, Bhatnagar N, Park BR, Jeeva S, Jung YJ, **Raha J**, Kang SM, Chen X. Physical radiofrequency adjuvant enhances immune responses to influenza H5N1 vaccination. FASEB J. 2022 Mar;36(3):e22182. doi: 10.1096/fj.202101703R. PMID: 35113455; PMCID: PMC8928172.
- Jarrell ZR, Lee CM, Kim KH, He X, Smith MR, **Raha JR**, Bhatnagar N, Orr M, Kang SM, Chen Y, Jones DP, Go YM. Metabolic reprogramming and increased inflammation by

cadmium exposure following early-life respiratory syncytial virus infection-the involvement of protein S-palmitoylation. Toxicol Sci. 2023 Nov 6;197(2):186–96. doi: 10.1093/toxsci/kfad112. Epub ahead of print. PMID: 37941452; PMCID: PMC10823773.

- Liu R, Natekar JP, Kim K-H, Pathak H, Bhatnagar N, **Raha JR**, Park BR, Guglani A, Shin CH, Kumar M, et al. Multivalent and Sequential Heterologous Spike Protein Vaccinations Effectively Induce Protective Humoral Immunity against SARS-CoV-2 Variants. *Vaccines*. 2024; 12(4):362. https://doi.org/10.3390/vaccines12040362
- Kim KH, Bhatnagar N, Subbiah J, Liu R, Pal SS, Raha JR, Grovenstein P, Shin CH, Wang BZ, Kang SM. Cross-protection against influenza viruses by chimeric M2e-H3 stalk protein or multi-subtype neuraminidase plus M2e virus-like particle vaccine in ferrets. Virology. 2024 Apr 25;595:110097. doi: 10.1016/j.virol.2024.110097. Epub ahead of print. PMID: 38685171

AWARDS AND EXTRACURRICULAR ACTIVITIES

Award | *Georgia Bio Life Sciences 2023 Summit | Atlanta, GA* **Nov 2023**

- Anthony Shuker Scientific Poster Award Winner

Award | *Georgia State University | Atlanta, GA* **Oct 2023**

- Poster session (3rd place) at GSU Graduate Conference for Research, Scholarship and Creative Activity

Award **|** *North South University | Dhaka, Bangladesh* **Jan 2016 –Jan 2020**

- Merit Scholarship (100% tuition waiver)