ABSTRACT

Title of Dissertation:	MUCOSAL DELIVERY OF INFLUENZA VACCINE ANTIGENS
	Susan Soo Park Ochsner, Doctor of Philosophy, 2018
Dissertation directed by:	Professor, Xiaoping Zhu, Department of Veterinary Medicine

Most pathogens infect humans and animals at mucosal surfaces, yet few mucosal vaccines are available to provide protection at these sites. Though influenza virus initiates its infection in the respiratory mucosal epithelium, currently approved influenza vaccines are administered by parenteral routes, which do not offer effective respiratory immunity. A successful mucosal influenza vaccine should induce both local and systemic immunity, however, the respiratory epithelium is an imposing barrier that prevents vaccine antigens to effectively traverse the airway. The neonatal Fc receptor (FcRn) mediates transport of IgG across the epithelial cell monolayer lining mucosal surfaces. To exploit this antibody transfer pathway for antigen delivery, I produced a soluble fusion protein that fused the monomeric Fc portion of IgG to an influenza hemagglutinin (HA) antigen harboring the T4 fibritin trimerization domain. Intranasal innoculation of the HA-Fc protein along with CpG adjuvant induced high levels of durable mucosal and systemic adaptive immune

responses and, importantly, generation of lung-resident memory T cells. FcRn-dependent antigen delivery was corroborated when substantial protection characterized by significantly increased survival and reduced pulmonary pathology was observed in the HA-Fc-immunized wild-type (wt) mice. In contrast, control groups of wt and FcRn-deficient mice immunized with HA-Fc, a mutant version of HA-Fc that lacks FcRn binding capacity, HA alone, or PBS, experienced substantial morbidity, mortality, and lung damage. As the influenza nucleoprotein (NP) is highly conserved among strains, it is an attractive vaccine target. Thus I produced soluble NP-Fc fusion proteins as potential influenza vaccines. The preliminary study demonstrated that intranasal immunization of NP-Fc with CpG resulted in FcRn-mediated delivery of NP-Fc protein across the respiratory barrier and the induction of high levels of antibody titer compared to groups of control mice. Immunization with NP-Fc may be further explored for developing a universal mucosal influenza vaccine. Taken together, for the first time, my results prove that FcRn can effectively deliver an influenza antigen across the respiratory epithelial barrier, providing substantial protection against lethal respiratory infection. This study further suggests FcRn-mediated mucosal vaccination could be used to deliver a universal influenza vaccine antigen or protective antigens from other common respiratory pathogens.

MUCOSAL DELIVERY OF INFLUENZA VACCINE ANTIGENS

by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2018

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Dedication

This dissertation is dedicated to my parents, Joanna and James, my sisters, Austin and Rosa, and my husband, Evan. I could not have achieved any of this without your love and support. Thank you for your patience and for believing in me.

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Xiaoping Zhu, for his support and mentorship over the years. His passion and perseverance for science and research is unparalleled. He has taught me how to ask questions and search for answers in a methodical and measured manner. He serves as an inspiration for myself and many other scientists. I would also like to thank my dissertation committee members for their support and guidance over the years: Dr. Wenxia Song, Dr. Jeff DeStefano, Dr. Georgiy Belov, and Dr. Kenneth Frauwirth. I have strived to use your knowledge and expertise to the fullest extent.

I would like to thank the members of the Zhu laboratory for all of their help with my dissertation research. Of note, I would not have been successful without the help of Dr. Weizhong Li, Dr. Senthilkumar Palaniyandi, and Dr. Arunraj Mekhemadhom Rajendrakumar. I would also like to thank Xiaoyang Liu for providing his expertise to my research.

I would also like to thank the faculty and staff of the Department of Veterinary Medicine and the BISI office for their support and help over the years. Finally, I would like to thank my family and my husband for being my foundation and my rock so that I could accomplish all that I have during my time at the University of Maryland, College Park.

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List of Abbreviations

ACIP: Advisory Committee on Immunization and Practices

ADCC: antibody-dependent cell-mediated cytotoxicity

ADCP: antibody-dependent cell-mediated phagocytosis

APC: antigen-presenting cells

ASC: antibody-secreting cells

BAL: bronchoalveolar lavage

BALT: bronchus-associated lymphoid tissue

bNAb: broadly neutralizing monoclonal antibodies

BS^3: bis[sulfosuccinimidyl] suberate

CDC: complement-dependent cytolysis

cHA: chimeric HA

CMIS: common mucosal immune system

cRNA: complementary RNA

CTL: cytotoxic T lymphocytes

CVV: candidate vaccine virus

DC: dendritic cells

dLN: draining lymph nodes

ELISA: enzyme-linked immunosorbent assay

ELISPOT: enzyme-linked immunosorbent spot assay

FAE: follicular associated epithelium

FcRn: neonatal Fc receptor

Fd: T4 fibritin foldon

GALT: gut-associated lymphoid tissue

GC: germinal center

HA: hemagglutinin

HAI: HA inhibition

HPAI: highly pathogenic avian influenza

HSV: herpes simplex virus

IFN-α/β/γ: interferon-alpha/beta/gamma

IIV: inactivated influenza vaccines

IRF: IFN-regulatory factor

ISG: interferon stimulated gene

LAIV: live attenuated influenza vaccine

LLPC: long-lived plasma cell

M1: matrix 1

M2: matrix 2

MALT: mucosa-associated lymphoid tissue

MDCK: Madin-Darby Canine Kidney

MeLN: mediastinal lymph nodes

MHC: major histocompatibility complex

MIP-1: macrophage inflammatory protein 1

NA: neuraminidase

nAbs: neutralizing antibodies

NALT: nasal-associated lymphoid tissues

NEP/NS2: non-structural protein 2

NF-κB: nuclear factor-κB

NK: natural killer cell

NLR: NOD-like receptor

NLRP3: NLR family pryin domain containing 3

NLS: nuclear localization signals

NOD: nucleotide binding oligomerization domain

NP: nucleoprotein

NS1: non-structural protein 1

PB1: polymerase basic protein 1

PB2: polymerase basic protein 2

PB1-F2: polymerase basic protein 1-F2

PA: polymerase acid protein

PAMP: pathogen associated molecular patterns

pIgA: polymeric, IgA

pIgR: polymeric immunoglobulin receptor

PKR: protein kinase R

RIG-1: retinoic acid inducible gene I

RIV3: recombinant influenza vaccine

RNP: ribonucleoprotein

RSV: respiratory syncytial virus SA: sialic acid

SC: secretory component

SLO: secondary lymphoid organs

TCM: central memory T cell

TCR: T cell receptor

TEM: effector memory

Tfh: follicular T helper cell

Th: helper CD4+ T cell

TLO: tertiary lymphoid organs

TLR: Toll-like receptor

TNF: tumor necrosis factor

Treg: regulatory T helper cell

TRM: tissue-resident memory T cell

VLP: viral like particle

vRNA: viral RNA

WHO: World Health Organization

Chapter 1: Introduction and Specific Aims

Influenza virus

Characterization and Replication

Influenza viruses are members of the family *Orthomyxoviridae* and consist of generally spherical, enveloped virions that enclose a segmented, single-stranded negative-sense RNA genome. There are four genera of influenza virus, A, B, C, and D, though only A and B are considered major human and animal pathogens. Viral nomenclature is as follows: virus type/host of origin (if nonhuman)/geographical location/strain number/isolate year/hemagglutinin (HA) subtype and neuraminidase (NA) subtype (for influenza A viruses). For example, the virus primarily used in the following dissertation is A/Puerto Rico/8/1934/H1N1 [1] [2]. Influenza A viruses are known to infect a broad range of animals, most notably aquatic birds, which serve as natural reservoirs, and humans and swine. Influenza A virus is characterized by its HA and NA subtypes. There are currently 18 HA subtypes, which are separated into two phylogenetically distinct groups based on antigenic variations of HA subtypes: Group 1: H1, H2, H5, H6, H8, H9, H12, H11, H13, H16, H17, H18; and Group 2: H3, H4, H7, H10, H14, and H15. There are 11 characterized NA subtypes, which are phylogenetically divided into two groups: Group 1: N1, N4, N5, and N8; and Group 2: N2, N3, N6, N7, and N9; while N10 and N11 are two new subtypes found only in bats and seem to be more distantly related to N1-9 and cannot currently be included

in either Group 1 or 2 [3] [4]. Influenza B viruses, which consists of two distinct lineages, Victoria and Yamagata, and C viruses primarily infect humans, in addition to a small number of other animals, while influenza D virus has primarily been found to infect cattle and swine [2] [5]. Influenza A virus, the most prominently characterized and clinically relevant virus type, is responsible for seasonal epidemics and occasional pandemics, while influenza B virus causes seasonal epidemics, and influenza C virus produces mainly asymptomatic infection in humans. The genomes of influenza A and B viruses consist of eight RNA segments that code for eleven proteins, while influenza C and D viruses have a genome of seven RNA segments which code for nine proteins. The eleven proteins expressed by influenza A viruses are as follows: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NS1), non-structural protein 2 (NEP/NS2), polymerase acid protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2); and polymerase basic protein 1-F2 (PB1-F2) [1].

The host-derived viral membrane contains HA and NA glycoproteins, in addition to several M2 proteins. HA is responsible for viral attachment to sialic acids in glycoproteins or glycolipids and fusion to host cells (Table I.I). NA cleaves sialic acids to release newly replicated virions from the host cell membrane. Underneath the viral membrane, M1 forms a rigid matrix that engages ribonucleoprotein (RNP) complexes. RNP complexes are composed of viral RNA (vRNA) segments coated with NP, along with the polymerase proteins, PB1, PB2, and PA. M2 is a transmembrane protein that forms proton channels within the viral envelope, mediating the release of RNP complexes from M1 proteins. NS1 is critical for viral

Table I.I: Influenza viral proteins

Influenza viral protein	Abbreviation	Function
Hemagglutinin	HA	Surface glycoprotein that binds to sialic acid receptors on host cells and fuses with host membrane
Neuraminidase	NA	Surface glycoprotein that cleaves sialic acids to release new virions
Matrix 1	M1	Internal structural protein that coats inside of viral membrane and binds to viral RNA with RNP
Matrix 2	M2	Transmembrane ion channel that maintains internal pH for release of RNP into cytoplasm
Nucleoprotein	NP	Internal structural protein that coats viral RNA within RNP
Non-structural protein 1	NS1	Internal protein that acts as an IFN antagonist
Non-structural protein 2	NEP/NS2	Internal protein that mediates nuclear export of new RNP
Polymerase acid protein	PA	Internal protein that is a part of the polymerase complex
Polymerase basic protein 1	PB1	Internal protein that is a part of the polymerase complex
Polymerase basic protein 2	PB2	Internal protein that is a part of the polymerase complex
Polymerase basic protein 1-F2	PB1-F2	Internal protein that is a virulence factor and regulates polymerase activity

defense against the host immune response, acting as an interferon (IFN) antagonist that can allow the virus to evade the innate immune response. NEP/NS2 mediates the nuclear export of newly synthesized RNP complexes. PB1-F2 protein has been implicated in regulating polymerase activity and is considered an important virulence factor [1] [2] [6].

In humans, influenza A virus replication initially occurs in the epithelial cells lining the respiratory tract (Figure 1.1). Viral entry is initiated by receptor-mediated endocytosis, where HA proteins bind to sialic acid-containing glycoproteins or glycolipids present on epithelial cell surfaces. Within the acidifying endosome, HA undergoes a conformational change that allows for the fusion between viral and endosomal membranes. Mediated by the ion channel activity of M2, acidification of the virus allows RNP complexes containing single-stranded vRNA to dissociate from M1 and exit into the cytoplasm through pores created by membrane fusion. RNP complexes are transported into the nucleus, mediated by nuclear localization signals (NLS) expressed within the RNP complex proteins, notably NP. Once the vRNA is transported into the nucleus, error-prone RNA-dependent RNA polymerase proteins initiate replication and transcription. The polymerase protein complex processes the initial vRNA in two distinct ways. First, vRNA is transcribed into capped and polyadenylated messenger RNA (mRNA) to be used for subsequent translation. Second, vRNA is replicated into new full-length positive-sense vRNA, called complementary RNA (cRNA), which is used as a template to make negative-sense vRNA segments for new virions. The proteins that are part of RNP complexes, NP and the polymerase proteins, are synthesized early in the viral replication cycle. Later

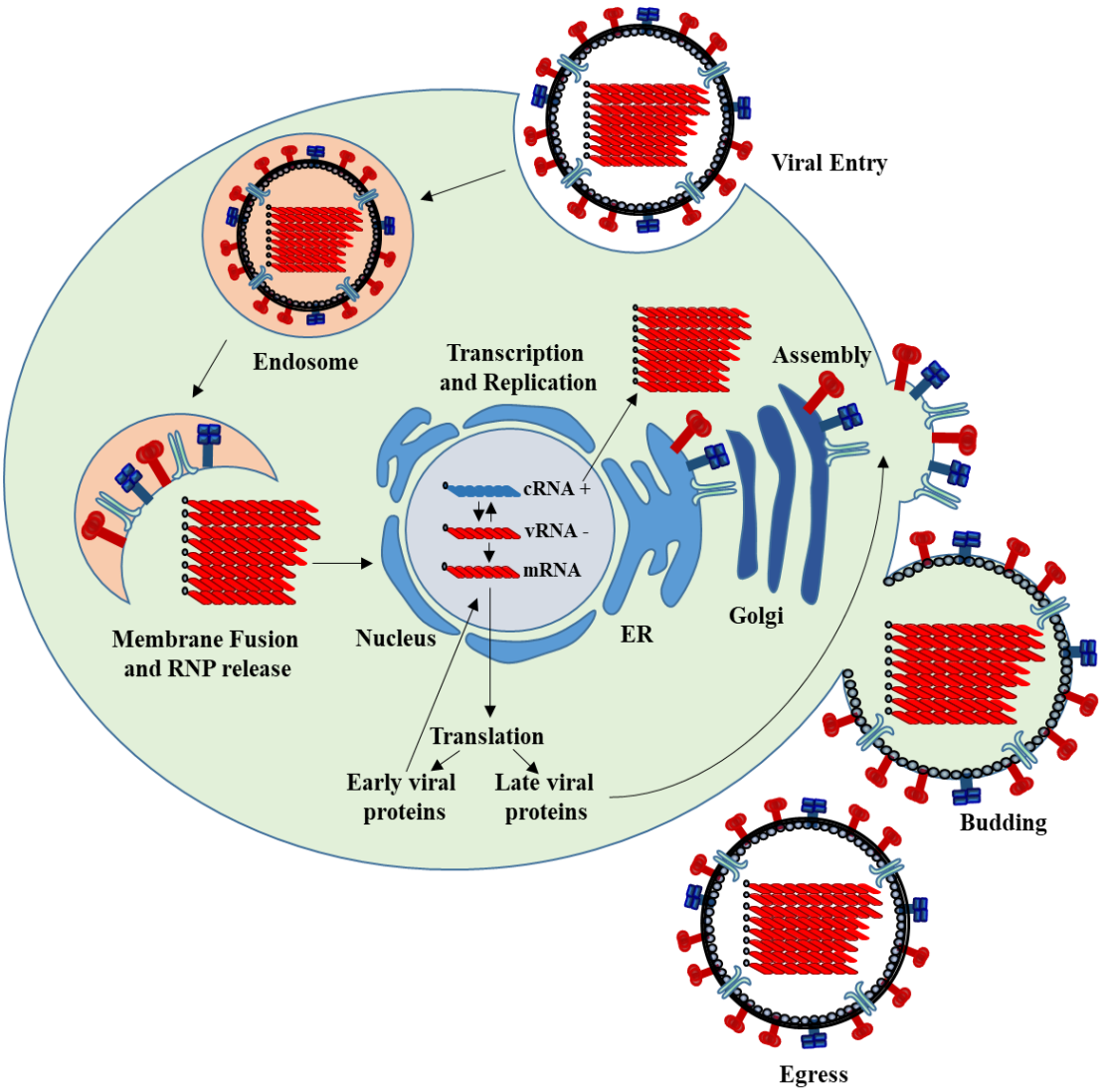


Figure 1.1. Influenza virus replication cycle.

Schematic of influenza viral replication cycle. Influenza virus binds sialic acid receptors on host cell and enters via receptor-mediated endocytosis. Upon acidification of the endosome, which is maintained by ion channel activity of M2, HA fuses the viral and endosomal membranes. RNP complexes are released into the cytoplasm and transported to the nucleus. Viral replication and transcription occur in the nucleus, where mRNA and cDNA are produced from vRNA. mRNA is exported into the cytoplasm for translation of early and late viral proteins and cRNA is used to produce new vRNA for RNP complexes. Membrane proteins, HA, NA, and M2, pass through the endoplasmic reticulum (ER) and Golgi stack for post-translational modifications before assembling at the plasma membrane. The newly formed RNP complexes, M1, and the remaining viral proteins also assemble at the plasma membrane to bud into new virions. New virions are released from the cell membrane by NA.

in the cycle, the M1 and the membrane proteins, HA, NA, and M2e, are produced. Newly made NP, PB1, PB2, PA, and M1 are imported into the nucleus to assemble new RNP complexes. The new RNP complexes are transported out of the nucleus via M1 and NEP/NS2 interaction with cellular nuclear export machinery. Newly expressed virion components assemble at the apical membrane of the polarized epithelial cell monolayer for viral assembly. The membrane proteins, HA, NA, and M2, first pass through the endoplasmic reticulum (ER) and Golgi stack for post-translational modifications. HA also undergoes cleavage from pre-cleavage HA (HA0) into subunits HA1 and HA2, which is a requirement for infectivity Through a mechanism that is still unclear, the newly formed RNP complexes, M1, and the remaining viral proteins also reach the plasma membrane to be incorporated into new virions. It is widely believed that the segment-specific genome-packaging signal sequences within the 3' and 5' termini of each vRNA mediate correct packaging of all eight genomic segments within a virus particle. Budding of virus particles is mediated by M1 protein, causing the host cell membrane to extrude until the plasma membrane fuses at the base of new virus particles, enclosing the viral contents. New virus particles are tethered to the cell surface by HA interaction with host sialic acids. The release of virus particles is accomplished by NA, which cleaves sialic acid, both on the host cell and the virus particle. This allows for virus egress while also preventing virus aggregation. New virus particles are released from the apical side of the infected cells, which allows for the increase of transmissible virus within the respiratory tract [1] [7].

Influenza Genetics

Influenza virus is continuously evolving and employs two main mechanisms for genetic variation and evolution: antigenic drift and shift. Antigenic drift transpires because of point mutations in the HA and NA glycoproteins of influenza A and B viruses due to the error-prone viral RNA polymerase complex. To evade host immunity, escape mutations occur in response to selective pressure by neutralizing antibodies (nAbs). Both HA and NA contain a number of antigenic sites where nAbs are directed against, promoting the accumulation of point mutations in those sites over time [8] [9] [10] [11]. This can lead to enough genetic variation from the parental virus strain so that previously nAbs to the parental virus can no longer recognize this newly drifted virus variant. The drifted strains can lead to seasonal epidemics and generally are in circulation for 2 to 5 years, before the strains undergo antigenic drift again, continuously creating new viruses. Currently, H1N1 and H3N2 influenza A viruses from Groups 1 and 2, respectively, are co-circulating within the human population and cause seasonal epidemics.

While existing viruses undergo antigenic drift, entirely new viruses are created during antigenic shift. Antigenic shift can occur due to interspecies reassortment of viruses, such as avian and human, allowing for the introduction of a new HA or NA subtype into the population, which is immunologically naïve to these newly shifted viruses. Because of the segmented property of the influenza genome, reassortment can occur within cells that are infected with more than one virus strain, where gene segments from one virus can combine with the gene segments of the other viruses present, producing new viruses. Reassortment events can have catastrophic

implications because they generate viruses with pandemic potential, where no pre-existing immunity is present in the human population. Reassortment has been observed within each genus of influenza, but not yet between genera [2] [12].

The successful production of infectious influenza virus *in vitro* was required to further understand the complex genetics of influenza virus. Because influenza virus contains negative-sense RNA, the genomic RNAs are noninfectious in a cell culture system. This necessitated the development of genetic engineering systems that can produce infectious virus. In 1999, two different groups developed a plasmid-based reverse genetics system for isolating newly synthesized viruses *in vitro,* which revolutionized influenza research in terms of virus characterization and vaccine development. The initial reverse genetics systems were composed of the cDNA of each RNA gene segment individually cloned into 8 plasmids for the production of vRNA. Each cDNA was cloned in the negative-sense orientation between cellular RNA polymerase I promoter and terminator sequences, which allowed for the transcription of vRNAs that lacked a 5'-cap and 3'-polyA tail. In addition, the gene segments required for viral replication, NP, PB1, PB2, and PA, were cloned into 4 different plasmids, and protein production was driven by RNA polymerase II. These 12 plasmids were transfected into Vero or 293T cells, mammalian cell lines that are approved for production of human biologics, and resulted, for the first time, in the artificial generation of infectious influenza virus [13] [14].

Subsequent advancements reduced the plasmid number from 12 to 8, where an RNA polymerase I/II expression system was employed. The cDNA of each gene segment was cloned in the negative-sense orientation between RNA polymerase I

promoter and terminator sequences. In turn, this gene cassette was cloned in the positive-sense orientation between an RNA polymerase II promoter sequence and a polyadenylation sequence. This allowed for the transcription of vRNA, the translation of mRNA, and the production of viral proteins, without the previous requirement of 4 additional plasmids for viral replication [15]. The use of reverse genetics for engineering influenza viruses was further refined by requiring only 3 plasmids. In one plasmid, the cDNA of all gene segments were cloned in tandem within an RNA polymerase I expression plasmid for the expression of vRNA. The second and third plasmids contained the gene segments of the viral polymerase subunits, PB1, PB2, and PA, and the gene segment for NP, respectively, between RNA polymerase II and polyadenylation sequences for viral protein expression. Transfection with only 3 plasmids allowed for higher transfection levels in cell culture systems that previously showed suboptimal transfection efficiencies, such as Vero cells [16].

In addition to reducing the overall number of plasmids required, there was a transition to other cell culture systems with more robust transfection efficiency levels, including 293T cells and Madin-Darby Canine Kidney (MDCK) cells. Both cell lines, which are approved for human use, produced even higher levels of replication, allowing for improved recovery of infectious virus. The establishment of the reverse genetics of influenza virus elucidated previously unknown mechanisms of pathogenesis. It was possible to observe the contribution and function of individual genes, host range restrictions, and host-virus interactions. In addition, it has facilitated the introduction of selective point mutations in viruses, and the rapid production of

reassorted viruses for viral vectors and vaccines, such as live-attenuated viruses and highly pathogenic avian influenza virus vaccines [17].

Influenza Virus Pathology and Host Immunity in the Respiratory Tract

After initial exposure, the incubation period of influenza virus can range from 1 to 5 days, depending on the amount of virus transmitted. Virus replication peaks at 48 hours, with little to no virus-shedding evident by 6 days after infection. Clinical manifestations of influenza infection in humans include high fever, dry cough, malaise, myalgia, headache, fatigue, and inflammation of the respiratory tract. Symptoms can persist from 7 to 10 days, though general weakness and chest pains can persist for several weeks. There are variations in clinical outcomes depending on the age of the patient. Infected adults can present with no symptoms or complications, or progress to viral pneumonia of varying degrees of severity. Children can have high fevers and frequent incidences of ear infections and croup. The elderly are more prone to complications with either viral or opportunistic bacterial pneumonia. In general, seasonal influenza-induced disease is most severe in infants, the elderly, and immuno-compromised individuals. Disease outcomes include inflammation that occurs within the lower and upper respiratory tract, damaging the tracheal, bronchial and alveolar epithelium. This can result in edema, leukocyte infiltration, leakage of blood in the airways, destruction of protective ciliation, and other perturbations of the epithelial cell layer [18] [19].

To defend against influenza virus infection and pathology, the host employs a multi-faceted approach to first prevent initial infection after exposure, and subsequently, resist further illness. The first line of defense is non-specific innate

immunity, which helps initiate virus-specific adaptive immunity, while memory responses can protect against reinfection. Within the innate immune system, physical and biochemical components of the respiratory tract prevent initial attachment and entry of influenza virus. This includes secretion of antiviral peptides and pulmonary clearance by the mucociliary apparatus, composed of the mucous layer and ciliated epithelial cells [20] [21] [22] [23]. Once these initial defenses are overcome, influenza virus can replicate in the respiratory epithelium, initiating of a cascade of intracellular responses. Infected cells utilize several pattern recognition receptors (PRR) to sense different pathogen-associated molecular patterns (PAMP) within the virus. Recognition of a PAMP subsequently activates the cells to direct downstream antiviral activity, including the production of Type I interferon (IFN-α/β), pro-inflammatory cytokines, and chemokines. Three main groups of PRRs have been implicated in influenza virus recognition (Table I.II). Retinoic acid inducible gene I (RIG-I) recognizes 5' triphosphate uncapped double stranded or single stranded RNA (dsRNA or ssRNA). Toll-like receptors, TLR3 and TLR7, recognize dsRNA and ssRNA, respectively. Nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family pryin domain containing 3 (NLRP3) exists in cytoplasmic signaling complexes called inflammasomes and are activated by ssRNA, viral protein M2 ion channel activity, and PB1-F2 aggregation [21] [22].

RIG-I activation contributes to a robust antiviral response. Within the cytosol of infected cells, RIG-I recognizes newly synthesized viral RNA, genomic RNA, and subgenomic RNA that contain 5'triphosphate. This results in the activation of transcription factors nuclear factor-κB (NF-κB) or IFN-regulatory factor 3 (IRF3), via

Table I.II: Pattern Recognition Receptors

PRR	PAMP	Function
RIG-I	**5’ triphosphate uncapped dsRNA and ssRNA**	**Activates NF-κB for production of pro-inflammatory cytokines and IRF3 for production of Type I IFN**
TLR3 and TLR7	**dsRNA and ssRNA**	**Activates NF-κB for production of pro-inflammatory cytokines and Type I IFN**
NLRP3	**ssRNA, M2 activity, and, PB1-F2 aggregation**	**Mediates production of IL-1β and IL-18**

signaling adaptor protein mitochondrial antiviral signaling protein (MAVS), also known as interferon-beta promoter stimulator 1 (IPS-I). One study has suggested that RIG-I detects viral RNA by infiltrating antiviral stress granules which contain viral RNA and interferon-stimulated gene (ISG) products, such as the serine/threonine kinase protein kinase R (PKR) [24]. RIG-I activation and MAVS-mediated signaling induce the production of pro-inflammatory cytokines via activation of NF-κB, and Type I IFN via IRF3 activation. In addition, RIG-I activation leads to the transcription of interferon-stimulated genes (ISGs). ISG products support the antiviral response by targeting the viral replication cycle, while also promoting IFN and ISG production or activity [25].

Though TLR3 recognizes endosomal dsRNA, the influenza viral replication cycle does not produce a known dsRNA intermediate in endosomes. It is believed that TLR3 recognizes a yet-to-be elucidated ligand within phagocytosed virally-infected cells. TLR3 activation is followed by activation of NF-κB. NF-κB mediates the production of pro-inflammatory cytokines, such as TNF-α. TNF-α can limit viral replication while also paradoxically increasing disease severity by promoting immune cell infiltration in the lungs [26] [27]. TLR7 binds endosomal ssRNA in infected cells, most notably in plasmacytoid dendritic cells (pDCs), a CD11c- subset of DCs that produces large amounts of Type I IFN in response to many viruses.

Activation of TLR7 leads to subsequent activation of transcription factors NF-κB or IFN-regulatory factor 7 (IRF7), via adaptor protein MYD88. NF-κB activation stimulates the production of pro-inflammatory cytokines, such as IL-12 and IL-6. IRF7 activation results in Type I IFN production and maintenance via positive

feedback, inducing an antiviral state in infected and neighboring cells. In a mouse model, TLR7 also has been shown to have an important role in regulating the B cell response, promoting antibody production, while dispensable for T cell response [21] [28].

The third main PRR, NLRP3, is a component of a caspase-1-activating signaling complex, the NLRP3 inflammasome, which also includes adaptor protein apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), and pro-caspase 1. The NLRP3 inflammasome recognizes influenza-specific PAMPs, such as ssRNA, proton-influx via M2 protein, and PB1-F2 accumulation. Once activated, the inflammasome mediates the autoproteolytic cleavage of pro-caspase 1 into the active form, caspase-1. Caspase-1 can then mediate cytokine production by cleaving pro-IL-lβ into the secreted form, IL-1β, and pro-IL-18 into secreted IL-18. Pro-inflammatory cytokine IL-1β mediates immune cell recruitment, which enhances local inflammation and damage. In contrast, it has also been found to have a role in increased survival after infection. IL-18 has been shown to be important for enhancing cytokine production in CD8+ T cells. In addition to cytokine production, NLRP3 inflammasome activation enhances survival and disease tolerance, recruits leukocytes to the airway, promotes respiratory tissue repair, and enhances B and T cell responses [29] [30].

Overall, activation of PRRs initiates a cascade of signaling pathways that result in the production of pro-inflammatory cytokines and IFN. In addition, PRR activation can induce chemokine gradients, such as monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, MIP-2, MIP-3α,

regulated on activation, normal T expressed and secreted (RANTES), and interferon-inducible protein 10 (IP-10) [21] [31] [32] [33] [34]. The expression of chemokines promotes the recruitment and activation of immune cells, such as macrophages, natural killer (NK) cells, and dendritic cells (DCs), to the site of infection, which can lead to viral clearance [21] [22].

Upon activation, resident and recruited immune cells continue to enhance the innate immune response. After the onset of infection in the respiratory epithelium, alveolar macrophages phagocytose infected cells. They also producing pro-inflammatory cytokines, such as TNF-α and IL-6, resulting in a local inflammation which leads to some of the clinical manifestations previously described, such as high fever, while also supporting the adaptive immune response [35] [36] [37]. NK cells promote viral clearance through two mechanisms. First, by binding to HA expressed on infected cells, and subsequently triggering cell lysis. Second, by binding to virus-specific antibodies attached to infected cells, initiating antibody-dependent cell-mediated cytotoxicity (ADCC) to lyse the cells [38] [39]. DCs are professional antigen-presenting cells (APCs) and exist as different respiratory subsets or monocyte-derived DCs. DCs serve as intermediates between innate and adaptive immunity. They sample the airway for antigens, phagocytose infected cells or are infected and migrate from the respiratory tract to the draining lymph nodes, via a CCR7-dependent chemokine gradient. During migration, they process viral proteins through the MHC class I or II pathway to present to CD8+ or CD4+ T cells, respectively, for activation. In addition to viral clearance and activation of cellular immunity, alveolar macrophages and conventional and plasmacytoid dendritic cells

(cDC and pDC) produce Type I IFN. This leads to the stimulation of hundreds of ISGs through the JAK/STAT pathway to establish an antiviral state in infected and surrounding cells. In this antiviral state, further infection is prevented by inhibiting protein synthesis within infected cells, thus limiting viral replication and spread [35] [40] [41] [42]. Production of Type II IFN (IFN-γ) has been implicated in establishing and maintaining influenza-specific effector and memory CD8+ T cells or cytotoxic T lymphocytes (CTL). Type III IFN (IFN-λ) has a role in controlling and limiting influenza virus infection [21] [22] [43].

Overall, the innate immune response employs numerous mechanisms to defend against influenza virus infection. Some of these responses seem to complement each other, where a number of cellular events can be protective, and limit the spread of the virus. Counter-productive responses are also induced that increase inflammation and pulmonary pathology. The complex nature of innate immunity highlights the importance of a balanced immune response within a host, necessitating the need of both innate and adaptive immunity. Indeed, innate immunity largely supports the induction of adaptive immunity, through the maturation of DCs, promoting an enhanced antigen presentation to T cells, and leading to the enhanced antibody and cytokine production from B and T cells, respectively.

While innate immunity defends against initial pathogen exposure, a later adaptive immune response is also mounted against viral infection. During infection, B cells are primed by antigen-presenting DCs within the marginal zone of draining lymph nodes (dLN). Mature B cells undergo proliferation and then differentiate into antibody-secreting cells (ASC) as early as 3 days post-infection, in mouse and ferret

models [44] [45] [46]. Within B cell follicles in secondary lymphoid organs (SLO), such as the lymph nodes, B cells can present antigen to follicular T helper (Tfh) cells. After activation, Tfh cells enhance and maintain B cell activity. Within SLOs, B cells are activated by Tfh cells in the GCs, stimulating proliferation and differentiation. They also undergo class switching, from IgM to IgA and IgG, and affinity maturation through somatic hypermutation which enables them to produce large amounts of high-affinity antibodies [47] [48] [49]. During the final stage of differentiation, B cells transition into two fates. They become either antigen-specific plasma cells, which produce large amounts of antibody, or antigen-specific memory B cells, which provide rapid long-lasting protection upon re-exposure of the virus or viral antigen.

The humoral response can elicit antibodies against all influenza viral proteins, both surface and internal. Antibodies against viral proteins are primarily IgM, IgA, and IgG isotypes. During primary infection, the main isotype involved in protection is IgM, which is constitutively generated, with increased production upon initial antigen exposure. IgM can bind to influenza antigens in the absence of prior antigen priming and high IgM titers have been associated with enhanced viral clearance [50]. Secretory IgA (sIgA) is secreted into the respiratory mucosa, primarily in the upper respiratory tract, providing local neutralizing protection against influenza virus before initial attachment and entry [51] [52]. During infection, serum IgG is induced at high titers and crosses the respiratory epithelium into the lower respiratory tract by IgG-receptor mediated transcytosis via the neonatal Fc receptor, FcRn, or transudation [51] [53] [54] [55].

The highest antibody titers are produced against the major surface glycoproteins, HA, and to a lesser extent, NA, during both natural infection and vaccination (Figure 1.2) [56] [57]. Most HA antibodies are directed towards the variable head domain which is under constant environmental pressure. This renders most HA antibodies to be strain-specific, which can be potently neutralizing against the infecting strain [58]. Antibodies against HA can neutralize virus in a number of ways. Primarily, HA antibodies bind to virus to prevent viral attachment and entry into the host cell. In addition, HA antibodies can opsonize virus for Fc-dependent phagocytosis, fix complement, and mediate ADCC. NA antibodies are non-neutralizing, but limit viral spread by preventing the egress of newly synthesized virions from host cells [4] [56] [59] [60]. Antibodies against the conserved HA stalk domain are naturally produced during infection, though at much lower titers compared to antibodies against HA head. HA stalk antibodies prevent fusion between the host endosomal and viral membranes. There is a growing evidence that either infection- or vaccine-induced HA stalk antibodies can neutralize against multiple subtypes within a group, and occasionally between groups [61] [62] [63]. Antibodies against NP and the extracellular domain of M2 (M2e) are non-neutralizing. They have been shown to hold promising protective properties, such as mediating ADCC, though further roles in immunity are still being characterized [64] [65].

Currently, most clinical diagnostic assays detect and quantify antibodies against the HA head domain. HA inhibition (HAI) assay is the main method to correlate protection from influenza virus infection, where the virus is tested with clinical serum samples, which is then mixed with red blood cells. As HA binds to

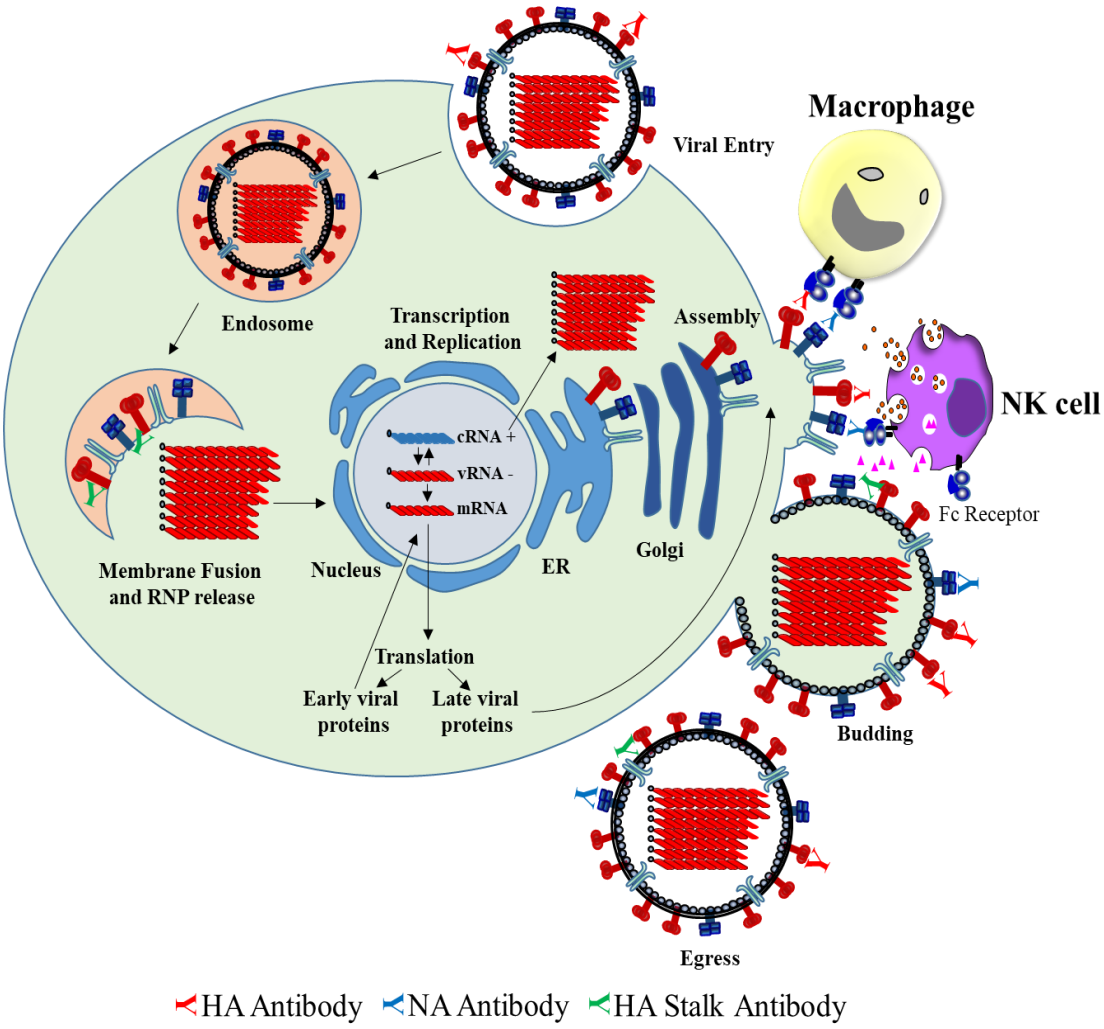


Figure 1.2. Antibody-mediated protection against influenza virus.

Model representation of function of virus-specific antibodies at different steps of the influenza virus replication cycle. Anti-HA antibody bind to virus to prevent initial attachment of virus to the cell. Anti-HA stalk antibody can bind to virus to prevent fusion and uncoating of the viral genome into the cytoplasm. Anti-NA antibody bind to newly formed virions to prevent budding and egress to reduce spread. All antibodies can bind to free virions to form immune complexes and mediate opsonization and phagocytosis of virus by macrophages and activation of antibody-dependent cell-mediated cytotoxicity performed by natural killer (NK) cells.

sialic acids on the red blood cells, causing agglutination, or clumping together of cells, the absence or presence of hemagglutination indicates if the virus has been neutralized by the antibodies in the serum. The highest dilution of serum that prevents hemagglutination is considered the hemagglutination inhibition (HAI) titer, while an HAI titer of ≥40 is protective in humans [51] [66]. HA stalk antibodies cannot be tested using standard HAI assays, as these antibodies do not prevent virus-induced agglutination of red blood cells, so HA stalk antibodies, while potentially more potent and broadly neutralizing, are more difficult to measure and standardize. There are no other standardized assays to measure antibody titers against other influenza viral proteins, although efforts to standardize NA inhibition assays are being considered [4].

Currently in clinical settings, the main correlate of protection is based on the humoral immune response. In addition, cell-mediated immunity, primarily composed of CD8+ and CD4+ T cell responses, has been shown to be an integral component of the adaptive immune response against influenza virus. While CD8+ and CD4+ T cells play an important role in defense against influenza virus, unlike nAbs, cell-mediated immune responses cannot provide sterilizing immunity (i.e., clearance of virus and prevention of productive infection of host cells) [67] [68]. Their main mechanism of protection is promoting viral clearance after the onset of infection, either through lysis of infected cells or supporting other arms of adaptive immunity [69]. Within the draining lymph nodes, naïve CD8+ and CD4+ T cells are primed by APCs. They undergo activation, proliferation, and differentiation stages in order to become

effector cells that can migrate to the site of infection, via a CCR4-dependent chemokine gradient, where they mediate antiviral activity (Table I.III) [70].

CD8+ T cells, or cytotoxic T lymphocytes (CTLs), are responsible for rapidly lysing infected cells. By recognizing specific viral peptides in the infected cell membrane via the T cell receptor (TCR), this initiates a cascade of intracellular events that result in the production of granules of perforin and granzyme B [71]. Granules are delivered via exocytosis to infected cells, which is highly regulated to ensure that neighboring uninfected cells are not subject to subsequent lytic events. Perforins are inserted into the cellular membrane, creating a pore where granzymes can enter the cell and initiate programmed cell death [72] . In addition, CTLs can also mediate apoptosis of infected cells through expression of tumor necrosis factor (TNF) ligand FasL (also known as CD95) or TNF-related apoptosis-inducing ligand (TRAIL). After activation by APCs, FasL and TRAIL expressions are upregulated. FasL and TRAIL interact with their cognate receptors, Fas and TRAIL-death receptor (TRAIL-DR), respectively, on infected cells, to initiate the apoptosis pathway, mediated by caspase 8 and 3 [73] [70]. CTLs also produce pro-inflammatory cytokines, such as IFN-γ and TNF-α, though cytolysis appears to be the primary effector function.

CD4+ T helper (Th) cells have also been shown to have cytotoxic properties, but their contribution towards lysis of infected cells is not as prominent as CD8+ T cells. Rather, the main effector functions of Th cells are cytokine production and supporting CD8+ T cell and B cell activities. Th cells can be divided into subsets with distinct functions: Th1, Th2, Th17, Tfh, and regulatory T helper (Treg). Th1 cells promote cell-mediated immunity against intracellular pathogens, such as viruses and

Table I.III: Cell-mediated immunity

Type		Function	
CD8+ T cell/CTL		**Lysis of infected cells**	Through delivery of granules of perforin and granzyme B
		Apoptosis of infected cells	Through upregulation of FasL and TRAIL expression to initiate caspase 8- and 3-mediated cell death
CD4+ T helper cell	**Th1**	Immunity against intracellular pathogens	
	Th2	Immunity against extracellular parasites; role in induction of asthma and allergy	
	Th17	Immunity against extracellular bacteria and fungi; role in promoting autoimmunity	
	Tfh	Production of co-stimulatory molecules and cytokines to support GC activation and maintenance	
	Treg	Preservation of self-tolerance to prevent autoimmunity; production of suppressive cytokines against opposing immune responses	

intracellular bacteria, through the production of IL-2, IFN-γ, and TNF-α. The Th2 immune response is directed against extracellular parasites, including helminths, and has a role in the induction of asthma and allergic inflammation. The Th2 response is highlighted by the production of pro-inflammatory cytokines, such as IL-4 and IL-5, which promote IgE class switching in B cells and recruitment of eosinophils, respectively [69] [70]. Th17 cells are responsible for induction of immunity against extracellular bacteria and fungi, via production of IL-17. IL-17 can recruit neutrophils to clear extracellular pathogens, while also inducing the production of pro-inflammatory cytokines and chemokines. The Th17 response also has a prominent role in promoting autoimmunity [75]. Tfh cells, or follicular T cells, provide unique help to B cells through the production of co-stimulatory molecules and cytokines to support the formation, maintenance, and regulation of GCs. Tfh cells are also important for supporting the differentiation of GC B cells into plasma and memory cells [48]. Regulatory T cells (Tregs) function to preserve self-tolerance to prevent autoimmunity. Tregs also provide balance to opposing immune responses through the production of suppressive cytokines, such as IL-10 and TGF-β [76].

Influenza infection results in a primarily Th1-mediated response and the main cytokines produced by both Th cells and CTLs are IFN-γ, TNF-α, and IL-10 [77] [78] [79]. Once migrated to the site of infection, T cells produce IFN- γ, which appears to be the most critical cytokine. IFN- γ has been shown to promote recruitment and activation of immune cells, mediate antibody class switching, while having minimal contribution to viral clearance [80]. T cells also produce TNF-α, although macrophages appear to be the most prominent producer of this cytokine. The role of

TNF-α requires further elucidation, as both pro- and anti-inflammatory events have been observed that may either reduce or enhance pulmonary damage [69] [81]. IL-10 is a regulatory cytokine and can reduce MHC molecule expression on immune cells [82]. Activated T cells also produce the chemokine MIP-1α, also known as CCL3. Upon binding to its receptors CCLR1, 3 or 5, MIP-1α can enhance cytokine production, leukocyte recruitment, and viral clearance [68] [69]. The various types of T cells that encompass cell-mediated immunity have essential roles in combatting influenza virus infection. These T cells mediate the cytolysis of infected cells, recruitment of supporting immune cells, and sustain the B cell response through GC formation and enhanced antibody production.

While most infection-induced antibodies are strain-specific, there are rare occurrences of induction of broadly reactive antibodies. In addition, the T cell response skews towards recognition of internal viral proteins, which allows for cross-reactive, or heterosubtypic, immunity. Heterosubtypic immunity is induced by the infecting strain but can also protect against infection from virus strains of different subtypes. Certain antibodies against HA and NA have been shown to neutralize diverse virus strains. A number of antibodies against HA stalk have the most broadly neutralizing potential against a range of virus strains. HA stalk antibodies will generally neutralize viruses within a group (Group 1 or 2), while rare antibodies against HA stalk have been found to neutralize viruses from both groups [61] [62] [63]. Within cellular immunity, a preponderance of infection-induced cross-reactive T cells has been identified that recognize epitopes derived from conserved viral proteins. CD8+ T cells primarily recognize epitopes from viral proteins NP, M1, and

PA, while antigen specificity of CD4+ T cells is still unclear [83] [84] [85] [86] [87]. While CD4+ T cells are not essential for CD8+ T cell development during the primary response, they have a more prominent role during secondary and memory responses, where CD4+ T cells contribute to enhanced CD8+ T cell and B cell responses and viral clearance [88].

The robustness of heterosubtypic immunity has been shown to rely on the strength of the memory immune response, for both T and B cell responses. Memory response is based on the ability of a primed adaptive immune system to "remember" previous antigens. Reinfection can be rapidly cleared through the establishment and activation of memory T and B cell-mediated immunity. After effector T cells migrate to the infected lung and promote viral clearance, they enter a contraction phase where 90-95% of the virus-specific T cells undergo apoptosis. The remaining 5-10% of cells transition into long-lasting memory T cells in an IL-7 and IL-15-dependent process [89] [90] [91] [92]. As memory T cells can readily recognize conserved influenza epitopes, upon re-exposure to virus, the memory T cells are poised to rapidly proliferate and differentiate into secondary effector T cells. Serving as an immediate defense against viral infection, CD8+ memory T cells rapidly proliferate to provide the effector function of lysis of infected cells. CD4+ memory cells, which do not proliferate as extensively as CD8+ memory T cells, have a prominent role supporting other immune cells, such as naïve B and CD8+ T cell responses [92] [93].

There are three main subsets of memory T cells: central memory (TCM), effector memory (TEM), and tissue-resident memory (TRM) [91]. Two of these subsets primarily circulate throughout the body. TCM cells are characterized by high

expression of the chemokine receptor, CCR7, and L-selectin, CD62L, and localization in secondary lymphoid organs (SLO). TEM cells have low levels of CCR7 and CD62L expression and circulate between nonlymphoid peripheral tissues and blood. The distinct patterns of chemokine and chemokine receptor expression dictates the migration of these memory T cells into their discrete anatomical niches. In addition, TCM generally produce Th2 cytokines and IL-2, while TEM produce Th1 cytokines [94] [95] [96].

A more recently characterized subset, TRM T cells express high levels of early activation marker, CD69, and CD103 or CD11a, for CD8+ or CD4+ T cells, respectively, and are notable for remaining at the site of infection [97] [98] [99]. TRM T cells have been found in many barrier tissues, including the skin, gut, the urogenital tract, and the lung, based on the tissue tropism of the invading pathogens [100] [101] [97] [102] [103]. It has been demonstrated that after infection, *Candida albicans*-specific TRM T cells are present in the skin. In addition, listeria monocytogenes-specific TRM T cells can reside in the gut, while herpes simplex virus (HSV)-specific TRM T cells exist in the genital tract [104] [105] [106].

Influenza infection can induce virus-specific TRM T cells that reside in the lung. These lung-resident memory T cells serve as the first line of defense against re-infection with influenza virus. They rapidly proliferate and mediate enhanced viral clearance, survival, and cross-protection against diverse virus strains [102]. In contrast to circulating memory T cells, TRM T cells appear to be in a heightened activation state, as evidenced by increased levels of CD69 expression. In addition, lung-resident memory CD8+ T cells express increased levels of the ISG product, IFN

induced transmembrane protein-3 (IFITM3), which is implicated in rendering the cells resistant to infection. The distinct protein expression patterns ensure the maintenance of these cells and elucidate one aspect of how TRM T cells can rapidly implement effector functions [107]. In addition to infection-induced lung-resident memory T cells, it has been shown that vaccination with LAIV can induce lung-resident memory T cells. It was demonstrated that they conferred heterosubtypic protection in a mouse model, offering long-lasting protection against diverse strains other than the vaccine strain [108]. It has been established that only by the intranasal route can influenza infection and vaccination induce lung-resident memory T cells. This is in contrast to other routes of administration, such as intramuscular (i.m.) route, which cannot establish lung-resident memory T cells [102].

Long-lasting protection against recurring influenza virus is also provided by memory B cell responses. B cells within GCs differentiate into two types of cells: long-lived plasma cells (LLPC) and memory B cells [109]. Plasma cells, which produce large amounts of a single type of antigen-specific antibody, migrate to the bone marrow for long-term residence. Plasma cells also maintain steady-state levels of circulating antibodies in the absence of antigen [110]. It has been shown that after influenza challenge, long-lived IgA-secreting plasma cells can be established in the respiratory tract, detectable in both the lungs and nasal-associated lymphoid tissues (NALT) [49]. Memory B cells are maintained in several organs, including the lungs, spleen, and NALT [111]. After re-exposure to viral antigen, memory B cells rapidly proliferate and differentiate into ASCs. ASCs mount a robust secondary high-affinity humoral response, providing immediate resistance to viral reinfection [112]. In

addition, certain memory B cells are activated to secrete cross-reactive antibodies, which target conserved viral proteins or epitopes such as internal proteins or HA stalk. These antibodies work in concert with memory T cell responses to provide potential heterosubtypic protection against a diverse range of virus strains [113].

Immune Evasion and Virulence Factors

The host employs several arms of immune protection to prevent infection and reduce the spread of infection. In parallel, influenza virus has evolved numerous mechanisms to evade host immunity at all stages, from innate, humoral, and cellular immune responses. Several viral proteins have evolved to counteract the innate immune response. Influenza protein NS1 blocks PRR sensing by binding to viral RNA, shielding an important viral PAMP, thus preventing cellular recognition of the virus [114]. NS1 also inhibits RIG-I function by binding to the ubiquitin ligase, tripartite motif-containing protein 25 (TRIM25), which is required for RIG-I activation. In addition, NS1 binds and inhibits RNA-dependent protein kinase (PKR), which functions to inhibit translation of viral mRNA [115]. PKR is regulated by an inhibitory complex composed of $P58^{IPK}$ and heat shock protein 40 (HSP40) [116]. Viral proteins NP and M2 have opposing effects on this complex. NP can activate it to allow for the translation of viral mRNA, while M2 inhibits the complex to induce cell death and subsequent release of new virions [117] [118]. Influenza proteins PB1-F2 and PB2 can inhibit Type I IFN production by binding and inhibiting signaling adaptor protein MAVS, limiting the availability of a prominent antiviral cytokine [119]. PB1-F2 has also been found to induce apoptosis and increase the severity of secondary bacterial infections [120].

As previously described, the influenza virus is under constant environmental pressure from humoral immunity. This is largely due to antigen-targeting antibodies, selecting the virus to undergo rapid mutations of viral proteins, primarily the head region of HA, resulting in high levels of variation within the antigenic sites [56]. The new viruses that are produced, due to antigenic drift, may not be recognized by previously established immunity against other virus strains due to infection or vaccination, thus affording little to no protection. In addition, antigenic shift results in entirely novel viruses to which the population is immunologically naïve. This can result in pandemic events characterized by high levels of morbidity and mortality [2]. Pre-existing antibodies can have little to no effect on drifted and shifted virus strains. This necessitates new ways to surmount this particular evasion strategy, primarily through advancements in vaccine development. Cell-mediated immunity is directed against internal proteins, such as NP, thus subjecting them to selective pressure. This results in the production of escape mutants of internal proteins, though not nearly to the magnitude of surface proteins, such as HA. While there are certain epitopes that can mutate in response to the environment, the virus is limited in the extent that it can alter internal proteins that are highly conserved and essential for viral fitness, thus relying on other mechanisms for immune evasion [121] [122].

There are two important virulence factors that mediate host range restriction and tissue tropism: sialic acid receptor binding activity and cleavage of different HA subtypes. Two different sialic acid expression patterns, sialic acid linked to galactose by α-2,3- or α-2,6-linkages, allow viruses to preferentially infect specific hosts. Humans generally express α-2,6-linked sialic acid in the upper respiratory tract, while

expressing α-2,3-linked sialic acid in the lower respiratory tract [123] [124] [125]. The cells constituting the upper respiratory epithelium, such as club cells (formerly known as Clara cells), secrete trypsin-like proteases that cleave the single arginine monobasic cleavage site in the HA of human influenza viruses [126] [127] [128] [129]. These viruses generally bind to α-2,6-linked sialic acid, exacting site restrictions to the infectivity of human influenza viruses to the upper respiratory tract [130]. Aquatic fowl express α-2,3-linked sialic acid within the intestine. These sialic acids serves as viral receptors for avian influenza viruses, whose multibasic cleavage site in the HA is cleaved by ubiquitously expressed furin and proprotein convertase 6 (PC6) proteases [131]. Swine express both types of sialic acids, which allow them to serve as "mixing vessels" when infected by both human and avian viruses. Occasionally, the co-infection of pigs can lead to the development of reassortant viruses, composed of avian, swine, and/or human RNA segments [132] [133]. More recently, additional reservoirs have been hypothesized as prospective mixing vessels, such as dogs and horses, with the potential to transmit the virus to humans [134] [135]. These reassortment events drive antigenic shift, introducing pandemic viruses into the population, for which there is no pre-existing immunity, which generally results in severe global morbidity and mortality.

Influenza Pandemics and Immunity

There have been four major influenza pandemic events in the 20th century which have occurred in 10 to 40 year intervals. The 1918 H1N1 pandemic, otherwise known as the "Spanish influenza", is historically recognized for its global devastation, causing 500 million infections, resulting in over 50 million deaths [136]. Though the origins

of the 1918 pandemic virus are still debated, it is generally believed to have originated from domestic and wild birds [136]. The 1957 H2N2 “Asian influenza” pandemic is a direct descendant of the 1918 H1N1 strain, where the HA and NA gene segments were replaced with H2, N2, and PB1 of avian-like origins. The 1968 H3N2 “Hong Kong influenza” pandemic supplanted the previously circulating H2N2 strain by replacing the H2 HA gene segment with H3 HA, in addition to PB1, of avian origins. Both the 1957 H2N2 and 1968 H3N2 pandemics resulted from reassortment between avian and human viruses [137] [138].

Most recently, the 2009 swine-origin H1N1 pandemic originated in Mexico, a region previously unknown to harbor pandemic potential [139] [140]. The 2009 H1N1 pandemic virus was derived by reassortment between two preexisting swine viruses, a North American swine H1N2 “triple reassortant” virus and a Eurasian H1N1 swine virus, with possible introduction of these viruses into the Mexican swine population from US and European swine trade [141] [142]. The 2009 H1N1 pandemic, or “swine flu”, was highlighted by increased disease severity in children, young adults, and pregnant women, as well as those with pre-existing lung or cardiac conditions. In contrast, there was higher resistance and protection in the elderly, due to the presence of cross-reactive antibodies in almost 30% of adults over 60 years old. Stockpiles of antivirals were administered to the affected regions, in addition to the development of a vaccine against the pandemic strain [139]. The 2009 H1N1 pandemic strain is now considered a normal human virus and circulates as a seasonal strain. Current circulating seasonal strains consist of H1 and H3 subtypes and N1 and N2 subtypes, though there is a ubiquitous prospect of the emergence of novel

pandemic viruses due to the increasing threat of highly pathogenic avian influenza (HPAI) viruses [143].

Regional epidemics of HPAI viruses have been a major cause for consternation before the 2009 H1N1 pandemic. Concern has only grown since then as these viruses could attain the potential of developing into pandemic strains if they acquire the ability of widespread transmissibility to humans. HPAI viruses can cause high rates of mortality in poultry and occasional avian-to-human transmission, which is characterized by elevated mortality in infected patients [144]. Prior to 1997, there had been rare occurrences of avian-to-human transmission. Since 1997 up to the present time, there have been a growing number of reported transmissions. In 1997, H5N1 virus transmission was first seen in Hong Kong because of direct contact and close exposure to widespread domestic fowl outbreaks in live poultry markets. H5N1 infection in humans was characterized by unusually high levels of mortality (>60%), resulting in hundreds of deaths since its identification in humans [145] [146]. In 2013, numerous human cases of H7N9 infection were documented and resulted in high levels of mortality (~30%). Though mortality was lower than what was witnessed with H5N1 infections, there appeared to be higher transmissibility to humans [147] [148]. Cases of avian-to-human transmission have been reported for H9N2, H7N7, H7N3, and H10N8 [149] [150] [151] [152] [153] [154] [155]. The continuous identification of new avian influenza viruses in domestic poultry increases the possibility of human infections.

There are growing concerns about the pandemic potential of avian viruses. Avian influenza viruses could evolve to replicate more efficiently in humans, become

highly pathogenic, and promote transmissibility. As previously described, avian viruses preferentially recognize α-2,3-linked sialic acid, which are present in the human lower respiratory tract. Mutations could occur within the receptor-binding site of HA that allow the viruses to recognize α-2,6-linked sialic acid receptors in the human upper respiratory tract. Indeed, gain-of-function studies have shown that as few as three amino acid substitutions in H5N1 virus are required to allow for respiratory droplet transmission in the ferret model, by allowing for receptor recognition, and thus infection, in the upper respiratory tract [156] [157]. A characteristic immune response resulting from an HPAI virus infection is an exacerbation of cytokine production, also known as a "cytokine storm". Cytokine storms can lead to pronounced pulmonary damage and enhanced disease severity. During a normal infection, a pro-inflammatory response is an important part of the immune response for viral clearance. Inflammation may result in some respiratory damage, but it is normally accompanied by lung repair and regeneration. Though not seen in seasonal influenza virus infections, HPAI and pandemic virus infections can result in a markedly increased production of pro-inflammatory cytokines and chemokines. Inflammation can become unmanageable, resulting in severe pulmonary pathology, such as widespread alveolar and capillary damage. In addition, the excess cytokines and chemokines can be released into the bloodstream, causing systemic damage, such as organ failure [158] [159]. The severity of disease outcome from pandemic and HPAI viruses further emphasizes the necessity of improved prevention and therapeutic treatments against these viruses.

Influenza Antivirals and Vaccines

Seasonal epidemics and the pandemic potential of reassortant viruses and evolving HPAI viruses continue to drive the need for a greater protection against infection. Antiviral drugs are the main therapeutic treatments available against influenza infection. Initially, there were two classes of antiviral therapies available, adamantanes and NA inhibitors (NAI). Previously, adamantanes were utilized as M2 inhibitors, but they could not protect against influenza B viruses. In addition, by the 2008-2009 season, high levels of adamantane resistance in circulating strains rendered them ineffective. Currently, only NA inhibitors (NAI) are licensed for use, which include zanamivir, oseltamivir, and peramivir. Both influenza A and B virus infection can be treated with oseltamivir, which is administered orally, in pill or liquid form; zanamivir, administered intranasally; and peramivir, administered intravenously. Antiviral treatment should commence within 48 hours of the onset of symptoms. Treatment has been shown to reduce symptoms by approximately one to two days and lessen infection-related complications and hospital visits in both adults and children. Presently, there appears to be transmissible resistance to NAIs, particularly oseltamivir. Current drug development is focused on developing more effective derivatives of licensed antiviral drugs to overcome such a resistance, in addition to seeking new viral protein targets [160] [161] [162].

While antiviral drugs are mainly used as post-infection therapeutics, vaccination remains the most effective way to prevent influenza infection. The main goals of vaccination are to protect against infection, reduce disease, and prevent transmission within the population by building herd immunity. The process of

selecting, producing, and manufacturing influenza virus vaccines is a massive, time-consuming, global undertaking, which is underpinned by the World Health Organization (WHO). For the past 70 years, the WHO has been tracking and monitoring global influenza epidemiology and evolution. This has been carried out by the Global Influenza Program (GIP) and the Global Influenza Surveillance and Response System (GISRS) (formerly known as the Global Influenza Surveillance Network (GISN)) [163] [164]. The GISRS is composed of 143 National Influenza Centers (NIC) in 113 countries and is responsible for surveying the global circulation of influenza virus strains. NICs are local research institutions that collect circulating virus strains and try to detect unusual strains with pandemic potential, within a country. After performing a preliminary analysis of the virus strains, NICs send representative samples of the viruses to one of six WHO Collaborating Centers (CC) where more rigorous diagnostic and genetic analyses occur. Based on the results from the local NICs sampling and the CCs analysis, the GISRS makes recommendations for the egg-grown and cell-grown candidate vaccine virus (CVV) strains. The CVV strains comprise the updated vaccine composition that would best match predominant circulating strains during the upcoming influenza season. In addition, the GISRS provides recommendations for standards in laboratory diagnostics, risk assessment activities, and serves as a global watchdog for the emergence of pandemic influenza viruses [164] [165].

In the United States, the Food and Drug Administration (FDA) reviews the WHO recommendations of vaccine strains and makes the decision for influenza vaccine compositions by late February or early March. The Advisory Committee on

Immunization and Practices (ACIP) of the Centers for Disease Control and Prevention (CDC) also makes annual recommendations for vaccinations for different groups within the population, including children, the elderly, and high-risk groups [166] [167]. Current vaccine production and manufacture of FDA-approved influenza vaccines take roughly six months, beginning just prior to the FDA's strain selections and ending production in mid-summer. Once the FDA decides the upcoming vaccine strains, the CDC and FDA provide the antigenically-verified, high-yield optimized CVVs to vaccine manufacturers, which seed the CVV strains in eggs for egg-based vaccines or MDCK cells for cell-based vaccines. There are two types of influenza vaccine composition. Trivalent vaccines contain two influenza A viruses (H1N1 strain and H3N2 strain), and one of two lineages of influenza B virus. Since 2012, quadrivalent vaccines have also been available, which are the same composition of the trivalent vaccine, in addition to the second lineage of influenza B virus [168]. Concurrent with vaccine production, the FDA produces potency reagents to standardize testing of vaccine efficacy and antigen verification across manufacturers. Both the FDA and manufacturers utilize these reagents for quality control of different lots of vaccine and to ensure that the proper amount of HA protein is included in each vaccine dose. Though NA is included in most vaccine formulations, the amount of NA is not standardized, in contrast to HA [164] [168].

Currently, there are numerous vaccine options available that differ regarding formulation, administration route, dosage amount, and presence of adjuvant. Inactivated influenza vaccines (IIV) can be formulated from embryonated eggs or, since 2016, MDCK cell culture, with the seeding of individual egg-based or cell-

based CVVs (either the three strains for trivalent or the four strains for quadrivalent) in the respective growth host. The virus in the allantoic fluid of eggs or the cell culture medium is then inactivated and treated with detergent, and the HA protein is purified and analyzed before packaging. IIVs can be administered in two injection routes: intramuscularly (i.m.), with a dosage of 15 μg of each vaccine HA antigen, 45 μg total for trivalent vaccines, and 60 μg for quadrivalent vaccines; and intradermally (i.d.), with a dose-sparing advantage over i.m. vaccination, as it requires only 9 μg of each HA antigen, for a 36 μg total for quadrivalent vaccines. This route of administration has also been shown to induce higher immunogenicity in the elderly, compared to i.m. vaccination, with no significant differences in immune responses in healthy adults [169] [170].

Since 2013, recombinant vaccines administered intramuscularly have been approved for commercial use. Recombinant vaccines do not utilize eggs or CVVs for production. Rather, they rely on an insect cell culture-baculoviral expression system and recombinant DNA technology [171]. The HA sequences are derived from the WHO recommended strains and cloned into a baculovirus vector for expression after subsequent infection of an insect cell line. The trimeric full-length HA proteins that are secreted from the insect cells are then purified and packaged for use. Recombinant vaccines contain three times the amount of HA antigen compared to egg- and cell-based vaccines, 45 μg of each HA antigen, for 135 μg or 180 μg total for trivalent or quadrivalent vaccines, respectively. The higher HA content has been shown to lead to greater immunogenicity that is longer lasting, and there is evidence that the recombinant HA vaccine can potentially be cross-protective against seasonally drifted

viruses. These vaccines do not contain egg proteins or preservatives found in some of the other IIV formulations, which makes them more accessible to certain population groups, such as those with severe egg allergies [171].

In addition, there are also specific vaccination strategies available for the elderly and children. For adults 65 years and older, there two vaccines are available. High dose trivalent IIV contains 60 μg of each vaccine HA antigen for a total of 180 μg. Trivalent IIV is adjuvanted with MF59, a squalene-based oil-in-water emulsion adjuvant, and has been available since the 2016-2017 season. Currently, children under 18 years old cannot receive recombinant vaccines, relying on the egg- or cell-based vaccines. In addition, unprimed children between 6 months to 8 years must be primed and then boosted at least 4 weeks later [169].

In addition, live attenuated influenza vaccines (LAIV) became available in the US in 2003. LAIV is administered intranasally (i.n.) and is believed to mimic natural infection by replicating in the nasal passage. Theoretically, this could induce both local and systemic immunity and provide humoral and adaptive immune responses. Attenuation of influenza virus was accomplished by successively passaging influenza A and B viruses, A/Ann Arbor/6/1960 and B/Ann Arbor/1/66, at lower temperatures until they were cold-adapted. In addition to being cold-adapted, the viruses were also temperature-sensitive, to ensure that virus replication was restricted to the upper respiratory tract. The viruses were adapted to grow at 25°C, the temperature of the nasal cavity, and could not grow at temperatures higher than 35°C, the temperature of the lower respiratory tract [172] [173]. These attenuated viruses served as master donor strains to produce seasonal LAIV. LAIV is composed of the HA and NA gene

segments of the WHO recommended vaccine strains incorporated into the backbones of the master donor strains contributing the internal proteins. LAIVs with the correct combination of HA and NA were previously produced by reassortment, and more recently, by reverse genetics, and the resultant viruses are grown in embryonated eggs. The viruses are then harvested from the allantoic fluid and packaged into the proper LAIV dosages [173]. Because of the higher risks of side effects when using a live, though attenuated, virus for vaccination, LAIV is not recommended for several population groups. These include immunocompromised individuals, pregnant women, individuals with asthma or egg allergies, and children under 2 years old [174].

Pandemic Vaccines

In the US, within weeks of the initial case of 2009 H1N1 pandemic influenza infection in April 2009, a high yield CVV was under development based on pandemic H1N1 strain, A/California/07/2009. Vaccination with monovalent IIV or LAIV was underway by October 2009. This was in time for the second wave of infection, with an initial focus on high-risk groups: pregnant women, healthcare workers, children and adults 6 months to 24 years old, and immunocompromised adults under 65. One dose was sufficient for most groups, while children under 10 years old received two doses. While clinical trials were determined to be successful in terms of safety and efficacy, a productive vaccination campaign was hindered by the delay in having the vaccines ready for market, limited supply, and very high demand [175] [176]. These are critical issues that need to be resolved for future pandemic vaccine production.

Vaccine-induced Immunity

In general, vaccination with IIV predominantly induces high levels of strain-specific neutralizing antibodies against HA and NA [177]. The rationale behind LAIV vaccination is both local and systemic immunity can be induced by simulating natural infection. In theory, the attenuated virus strains replicate in the upper respiratory tract after administration and induce protective mucosal IgA and serum IgG antibodies [177] [178]. Early clinical results with LAIV have shown higher efficacy in children 2 to 7 years old and has been generally believed to be equally effective in healthy adults compared to vaccination with IIV [179] [180] [181]. Considering more recent data, since the 2016 season, LAIV has not been recommended by the FDA and CDC because of concerns about reduced efficacy in 2- to 17-year olds, especially towards the vaccine strain, influenza A/H1N1pdm09, during the 2013-2014, 2014-2015, and 2015-2016 seasons [182]. Neither vaccination strategies induce strong T cell responses, relying primarily on antibody response. It is hypothesized that the LAIV platform should be able to induce a more robust and balanced immune response, since it mimics natural infection, though this has not been clearly demonstrated with current formulations. The strengths and limitations of current vaccinations provide a foundation to optimize existing vaccine-induced immunity and address any shortcomings.

Improving Current Vaccines and Developing Novel Influenza Virus Vaccines

Though current vaccination strategies are presently the best defense against influenza infection, there are numerous challenges to confront in designing improved vaccines. To keep up with genetic drift and produce up-to-date seasonal vaccines, a strict

surveillance and sample collection timeline must be followed in order to obtain the latest epidemiological information required to select upcoming vaccine strains. In addition, reagents necessary to test new vaccines for efficacy and potency must be produced, which makes vaccine production a costly and time-consuming endeavor [164] [183]. Any obstacles to these stages would result in reduced time to make enough vaccine to keep up with global need [184]. In addition, there is the constant necessity of making vaccines that can protect as many groups in population as possible depending on several factors such as age, allergies, and immune status. These issues are further exacerbated during times of circulating pandemic strains.

There are endeavors to either improve current seasonal vaccination platforms or develop new formulations to enhance efficacy. For current seasonal influenza vaccines, the focus is to accelerate vaccine production, improve production yields, and enhance immunogenicity to improve vaccine efficacy. These advancements are crucial to curtail vaccine shortages in times of emergency, such as pandemic events. It has been possible to rapidly increase vaccine production using several technological developments. Advanced cell culture systems are used to produce IIV, while baculovirus/insect cell expression systems produce recombinant vaccines [171] [185] [186]. Improvements to reverse genetics have facilitated the production of LAIV. In addition, the production of recombinant vaccines and certain IIV also exclude egg allergens. To improve the immunogenicity of influenza vaccines, numerous adjuvants are being explored, encouraged by the recent approval of MF59. The success of i.d. route of administration for IIV has shown how dosage sparing and alternative immunization routes can be critical factors that influence vaccine efficacy,

which is an important consideration during times of vaccine shortages [170] [187]. In addition, other vaccination platforms are being extensively developed and actively pursued, including immune complexes, DNA-based vaccines, virus-like particles (VLP), and viral vectors for vaccine antigen delivery [188] [189] [190] [191].

Commercial seasonal vaccines are currently designed to mount a robust humoral response against HA and NA, characterized by neutralizing antibodies that are specific to the vaccine subtype. Such neutralizing antibodies are the correlate of protection by which current vaccines are assessed. However, antigenic shift and drift produce alterations in these proteins, limiting protective efficacy of antibody responses and necessitating the annual production of new vaccines [164]. Developing vaccines that provide universal protection against current and emerging influenza strains remains a major public health challenge. Seasonal vaccination efficacy can be as low as 60%, with an increase in efficacy when the vaccine and circulating strains match, with a wide range of variation from season to season [192].

Current influenza virus vaccines have several shortcomings that limit their effectiveness on a year-to-year basis and over longer time periods. First, there is the requirement of the annual reformulation of the vaccines, where, due to antigenic drift, the previous year's vaccine can no longer protect against newly mutated circulating strains. In the case of a mismatch between vaccine strains and circulating strains, current vaccine formulations, which mainly induce strain-specific immunity, would not effectively protect against strains unmatched to the vaccine strain. Second, because current vaccines cannot produce broadly neutralizing or cross-protective immunity, they have limited to no efficacy against pandemic strains. Third,

depending on the vaccine formulation and route of administration, most vaccines have restrictions on who can receive any given formulation, limiting the number of vaccine types available to groups in the population, such as pregnant women, asthmatics, young children, the elderly, and the immunocompromised. Fourth, many vaccines still rely on growing vaccine strains in embryonated chicken eggs, despite increased availability of other growth systems. Harvesting vaccine strains in eggs is a time-consuming, laborious, and expensive part of the manufacturing process and can be an issue for persons with egg-allergies, who may be limited in the vaccines they can receive.

To address many of these issues, there has been a collective effort to produce universal influenza vaccines. These vaccines can protect against all strains of influenza A and B viruses, independent of HA or NA subtype, eliminating the requirement of annual reformulation and vaccination, with the potential to protect against pandemic strains. Current universal influenza vaccine approaches use a range of antigen targets, vaccine formulations, and routes of administration to induce protection mediated by a robust broadly reactive antibody response or heterosubtypic T cell immunity. Many universal vaccines that aim to induce cross-reactive antibody responses are based on the HA antigen, more specifically, the stalk region of HA. Infection-induced antibodies against HA stalk are produced at very low levels with no clear protective capacity during natural infection. Because HA stalk is relatively conserved across many influenza strains, it is an attractive antigen to induce heterosubtypic immunity through vaccination. Indeed, a growing number of broadly neutralizing monoclonal antibodies (bNAb) against HA stalk have been characterized

and shown to neutralize most viruses within groups, and occasionally between groups (group 1 and 2).

Currently, HA stalk is the most promising vaccine antigen for a universal influenza vaccine and the target of numerous vaccine platforms, in both pre-clinical and clinical stages. HA stalk vaccines are based on two main constructs: HA stalk only and chimeric HA. HA stalk only constructs completely remove the immunodominant HA head domain, so only the HA stalk remains, and is by default the major immunogen. Though it is technically difficult to develop a stable, properly folded HA stalk fragment, there have been several successes with this approach. One approach developed a trimerized HA stalk-fragment by rational design, producing an HA stalk immunogen that was stabilized by a trimerization domain and panel of mutations to simulate the prefusion conformation of the native HA. After vaccination in a mouse model, there was induction of high levels of cross-reactive antibodies that could neutralize viruses from both group 1 and 2, while providing robust heterologous and partial heterosubtypic protection after challenge [193]. Another method utilized structure-based design to develop a stable trimeric HA stalk construct that was fused to nanoparticles to increase immunogenicity. Vaccination in a mouse model with the HA stalk-nanoparticles resulted in the induction broadly neutralizing HA stalk antibodies and conferred protection against H5N1 challenge [194]. Another HA stalk design used a rational design and library approach to develop trimeric HA stalk immunogens. Vaccination in mice resulted in protection against both lethal heterologous and heterosubtypic challenges, while in a nonhuman primate model, they were able to see a reduction in disease severity after sublethal infection [195].

Several groups have been able to show cross-reactivity of HA stalk antibodies and protection against lethal heterologous challenge. Yet, HA stalk-only products can be unstable and must undergo complicated strategies to ensure proper folding to elicit appropriate immune responses, such as conformation-dependent neutralizing antibodies. Additional approaches have been explored to increase the stability of the HA stalk immunogen. One strategy is based on a series of vaccinations with chimeric HAs (cHA), where a constant HA stalk domain derived from circulating strains is fused to a variety of "exotic" HA head domains of viruses that do not circulate in humans, generally of avian origin, for serial vaccination. With the initial immunization, a host immune response is directed and primed towards the HA head domain, with relatively lower titers against the HA stalk domain. After establishing memory immune responses, the next immunization, and exposure to the same HA stalk immunogen, induces a more rapid recall response. This allows for greater induction of immunity against HA stalk, while there is a less robust primary response to the new head domain. Thus, sequential vaccinations with cHAs allow for the HA stalk domain to be the major immunogen, boosting HA stalk immunity with each immunization [196]. In both mouse and ferret models, sequential cHA vaccination has been shown to induce broadly neutralizing HA stalk antibodies, while providing high levels of protection against heterosubtypic challenge. A number of cHA vaccine formulations have been utilized, including viral vectors, DNA vaccines, and LAIV preparations. Notably, each vaccine type has been able to induce neutralizing HA stalk antibodies and provide protection against a range of viruses. Currently, clinical phases using the cHA vaccination strategy are under preparation [196] [197] [198].

Outside of HA stalk-based vaccines, there are several universal influenza vaccines under development that target other conserved viral proteins or portions of proteins, including the enzymatic site within NA, the ectodomain of M2 (M2e), NP, and M1. While NA is included in seasonal vaccine formulations, its vaccine-induced protective properties are not as well-characterized as those of HA, and NA has historically been a target for antiviral therapy. More recently, the conserved enzymatic site within the globular head of NA is being increasingly considered as an attractive universal vaccine target. While antibodies against NA cannot provide sterilizing immunity, NA antibodies do prevent viral spread, thus limiting viral load and disease severity [4]. NA antibodies have also been implicated in promoting ADCC and complement-dependent cytolysis (CDC) [199]. To refrain from placing selective pressure on the virus, thus driving mutation rates, an effective vaccine should combine NA with other viral antigens, such as HA, for a concerted immune effort. Though its role in heterologous protection has yet to be demonstrated, vaccines that utilize the conserved enzymatic site of NA could prove beneficial. At the very least, it would be prudent to regulate the amount of NA in current vaccines and develop standardized assays to determine its protective contributions.

While the M2 protein is scantly expressed within the virions, the ectodomain of M2 is highly conserved and thus, an attractive target for universal influenza vaccines. During natural infection, the primary M2e antibody response is generally weak, but there is a dramatic increase in antibody titers after a secondary viral exposure. M2e-based vaccines generally use tandem repeats of the ectodomain of M2 as the vaccine antigen. These can be fused to a large carrier protein or incorporated

into VLPs or nanoparticles to enhance immunogenicity. Vaccination with M2e induces high titers of non-neutralizing antibodies that are unable to prevent initial infection. Rather, they mediate protection through other mechanisms, such as limiting viral replication and spread, and promoting ADCC, CDC, and antibody dependent cell-mediated phagocytosis (ADCP) [200] [201] [202]. Like NA-based vaccines, an effective M2e-based vaccine would need to be administered in conjunction with additional viral antigens, such as HA or NP, to optimize vaccine efficiency and potential cross-reactivity [203] [204] [205]. It has previously been demonstrated that an adenovirus-vectored M2e- and NP-based vaccine can induce high levels of cell-mediated immunity, and a more modest humoral response while conferring heterologous protection against challenge [206].

In addition to inducing broadly reactive antibodies against conserved viral antigens, another major goal of influenza vaccine research is to create universal T-cell influenza vaccines. T-cell influenza vaccines could potentially be broadly protective by inducing cross-reactive T cells, as occurs upon natural infection. Since it has been established that the natural T cell response against influenza virus is directed towards viral proteins that are conserved across most, if not all virus strains, notably NP, and provides heterosubtypic protection, there are numerous vaccine efforts to promote cell-mediated immunity [86] [207] [208]. To induce cross-reactive T cells that recognize conserved epitopes of viral proteins, a variety of platforms have been developed. These platforms, including viral vectors, DNA vaccines, and virosomes, allow for conserved viral proteins to be delivered into the cytosol for proteasomal processing and subsequent MHC class I antigen presentation [208] [209] [210] [211]

[212]. These technologies can be expanded to developing T-cell vaccines against potential pandemic viruses, such as highly pathogenic strains of avian influenza. Since T-cell vaccines express conserved viral antigens, this would negate the necessity to continuously adapt the vaccine components to match circulating strains, as currently required. Conceivably, T-cell vaccines would be able to induce robust T cell responses where infection may not necessarily be prevented, but viral clearance would be enhanced, reducing disease severity and mortality, which would be essential during a pandemic occurrence [113].

Much of the universal T-cell vaccine effort is focused on NP. NP is the main viral antigen recognized by CTLs and thus, induction of NP-specific CTLs is considered an essential component of an effective universal T-cell vaccine [213]. Several studies have demonstrated the induction of CTLs by NP-based T-cell vaccines using DNA-based and adenoviral vector-based vaccination, which conferred protection to both homologous and heterologous challenges [214] [215]. In addition to universal NP-specific T-cell vaccines, vaccination with soluble recombinant NP has also been explored. In contrast to T-cell vaccines, this NP-based vaccine strategy allows for exogenous antigen processing through the MHC class II pathway. This allows for presentation of NP antigen to CD4+ T cells, which supports NP-specific B cell responses, resulting in the induction of high titers of anti-NP antibodies. These antibodies are non-neutralizing, but there is evidence that NP antibodies can function in conjunction with NP-specific CTLs, in addition to forming immune complexes (IC) with soluble virus-derived NP to activate host immunity, such as the complement pathway [216] [64]. Vaccine-induced NP antibodies are also believed to bind to NP

expressed on the surfaces of infected cells and mediate ADCC and CDC, though these immune events have yet to be conclusively demonstrated [217]. While the protective properties of NP alone vaccines are very promising, several vaccine strategies have demonstrated enhanced immunity and protection by combining NP with other viral proteins: M2e, as previously described; HA, where epitopes of HA and NP were administered intranasally and induced robust adaptive immunity, while also conferring high levels of protection against challenge; and M1, where a number of vaccine candidates are being pursued in phase I and II clinical trials, and have been shown to induce antigen-specific T cell responses to varying degrees of success [218] [219] [220].

Universal influenza vaccine strategies employ a number of viral targets (HA stalk, M2e, NA, NP, and M1), individually or in combination, and vaccine formulations (e.g., DNA-based vaccines, VLPs, virosomes, and viral vectors). To enhance vaccine-induced immunity, currently licensed adjuvants, including alum and MF59, and alternative adjuvants are being included in numerous experimental universal vaccine strategies. Alternative adjuvants that are being explored include TLR ligands or agonists (e.g., TLR9 agonist CpG and TLR2 agonist R_4Pam_2Cys), bacterial components (e.g., flagellin and derivatives of Cholera toxin), and various cytokines (e.g., Type I IFN and IL-1α and -β) [187] [191] [205] [221] [222] [223] [224] [225] [226] [227]. Thus, there is a concerted effort to develop more effective and broadly protective influenza vaccines that may not utilize current formulations, but rather, rely on novel antigen targets, vaccine formulations, and adjuvants to

induce a multifaceted immune response that can protect against drifted seasonal strains and shifted, potentially pandemic, strains.

Mucosal Immunity and Vaccines

It has become increasingly acknowledged that a successful influenza vaccine should induce both systemic and mucosal immunity, as influenza viruses, and most pathogens, infect at the mucosal epithelium, including the respiratory, urogenital, and intestinal tracts. Current vaccinations do not effectively induce mucosal immunity within the respiratory tract. Conceptually, LAIV should replicate within the upper respiratory tract and induce local immune responses, though this has not yet been conclusively demonstrated. The mucosal immune response, induced by infection or vaccination at mucosal surfaces, is distinct from systemic immunity, induced by parenteral routes of vaccination or by sepsis. Systemic immunity serves to keep the inner body of the host free of pathogens. In contrast, mucosal immunity, under regulation by distinct epithelial cell-derived cytokine and chemokine expression patterns, is required to differentiate between harmful pathogens and harmless self-peptides and foreign antigens or particulates, activating local immunity only when necessary to clear the former, while modulating the immune response to tolerate the latter in order to maintain mucosal homeostasis.

Mucosal Immune System Organization and Components

Mucosal immunity is initiated within a network of inductive sites and effector sites within the mucosa. Mucosal inductive sites are organized mucosal lymphoid structures, also known as mucosa-associated lymphoid tissue (MALT), that do not

contain a lymphatic system for receiving antigens [228]. They are composed of B cell follicles, T cells regions, and APCs. They include the tonsils and nasal-associated lymphoid tissue (NALT) in the upper respiratory tract, and gut-associated lymphoid tissue (GALT) and Peyer's patches (PP) in the gastrointestinal tract. Effector sites are diffused regions within the lamina propria of mucosal tissues, where antigen-specific lymphocytes migrate to after activation in inductive sites to produce sIgA and other effector molecules to mediate pathogen neutralization [228] [229] [230].

The mucosal immune system serves to produce local antigen-specific immune responses at the site of pathogen exposure or vaccination as the first line of defense against infection. A critical component of the mucosal immune system that pathogens first encounter is the mucosal epithelium, an imposing barrier that serves to prevent initial infection. The mucosal epithelial layer consists of a polarized epithelial cell layer containing intercellular tight junctions, adherent junctions, and desmosomes, with an apical membrane that faces the lumen and a basolateral membrane surface, with an underlying lamina propria [231]. The epithelial layers of the upper respiratory tract, trachea, and bronchi are composed of ciliated epithelial cells, non-ciliated mucus-secreting goblet cells, and tryptase-secreting club cells. The alveoli consist of type I alveolar cells, which largely make up the respiratory surfaces indispensable for gas exchange, and type II alveolar cells, which produce, release, and store surfactant, which relieves alveolar surface tension during respiration [231] [232] [233] [234] [235]. The mucosal epithelial layer serves as a physical barrier to prevent pathogen attachment and entry, while also actively participating in initiating local immunity to prevent further disease. Several pathogens, notably influenza virus, can overcome the

mucosal barrier by directly infecting mucosal epithelial cells, allowing for viral penetration and dissemination into the host. Within the lung, respiratory epithelial cells, in conjunction with local, resident immune cells, such as alveolar macrophages and dendritic cells, recognize the onset of infection and initiate the innate immune response as previously described, to activate the mucosal immune responses.

After exposure of a pathogen at mucosal sites, antigen-specific mucosal immune responses are generated within inductive and effector sites. At inductive sites, such as the MALT, aggregates of T and B cells form follicles underneath the epithelium which contain high endothelial venules (HEV). This induces the formation of specialized follicular associated epithelium (FAE) from the overlying epithelium. The FAE contains M cells, which are specialized epithelial cells that transport antigens from the luminal side across the epithelium to subepithelial DCs [236] [237]. In addition, intraepithelial DCs constantly sample the respiratory environment through dendrites that extend into the lumen [231]. DCs then migrate to the MALT follicles containing T and B cells to present antigen and activate local immunity. Antigen-presenting DCs also proceed to draining lymph nodes, where they initiate systemic immunity [33].

Though the roles of the FAE and M cells are well-characterized for the gut, they are also essential for initiating local immunity in the inductive sites of the respiratory tract, such as the NALT. In humans, the NALT is analogous to the Waldeyer's ring, a ring of lymphoid tissue in the pharynx that includes the tonsils and adenoids, which is considered to have an important role in the induction and modulation of mucosal immunity in the upper respiratory tract [238] [239] [240].

Bronchus-associated lymphoid tissue (BALT), composed of aggregates of lymphoid-rich follicles located within the branching of the bronchi, is an organized lymphoid tissue that has been important for respiratory mucosal immunity [241]. While some animals, such as rabbits and rats, constitutively express BALT, humans and mice do not express BALT unless it is induced by inflammatory events, such as respiratory infection or asthma. This results in the production of ectopic tertiary lymphoid organs (TLO) called inducible BALT (iBALT), which are induced at nonlymphoid sites after activation of innate immunity [242]. Unlike constitutive BALT, iBALT does not require contact with an overlying epithelium and may not always associate with an airway. Unlike SLOs such as lymph nodes (LN) and PPs, the expression of TLO like iBALT are not pre-programmed and usually occur after birth [243] [244]. The presence of iBALT has been found to be beneficial during influenza virus infection, supporting the induction of robust virus-specific CTL and Th1-dependent B cell responses, while enhancing survival after challenge [242] [245] [246].

Induction of Distinct Mucosal Immune Responses

Mucosal immunity induces both local cell-mediated and humoral immune responses. Within the respiratory mucosa, there are two distinct T cell responses. Antigen-specific CTLs are activated and proliferate to mediate clearance of infected cells [229] [247]. Within CD4+ T helper cells, Th1- and Th2-responses generally promote IgA-secreting B cell activation and maintenance [248]. Within the inductive site NALT, the humoral response is supported by local cellular immunity. It is characterized by B cell class switching from IgM to the predominant production of IgA, though there is also local production of IgG [249] [250]. The local production of

antibodies, most notably secretory IgA (sIgA) and IgG, play a crucial role in neutralizing virus within the mucosa, while IgM has an important role in limiting infection during initial exposure [251]. Within the respiratory mucosal immune system, IgA, the most prevalent antibody isotype in mucosal secretions in the upper respiratory tract, is an essential component of humoral immunity [252] [253] [254] [255]. IgA is produced by mucosal plasma cells with enhanced junction (J) chain expression, during which IgA undergoes glycosylation and dimerization mediated by the J chain. Dimeric, or polymeric, IgA (pIgA) is generated into its secretory form, sIgA, by binding to the secretory component (SC) portion of the polymeric immunoglobulin receptor (pIgR) through the J chain at the basolateral side of the epithelial layer [256] [257] [258]. After transcellular transport of pIgA via pIgR, pIgA is enzymatically cleaved from pIgR. It is then released at the apical surface of the mucosal epithelium into the lumen of the upper respiratory tract in its secreted form, sIgA. SIgA consists of pIgA and the SC of pIgR, which confers its resistance to mucosal proteases [259] [260] [261]. Pentameric IgM also uses the same pIgR epithelial transport system as sIgA, but sIgA transport is generally dominant [257] [262].

SIgA mediates mucosal immunity by several mechanisms: immune exclusion, intracellular neutralization, and antigen clearance [263]. During immune exclusion, sIgA binds to pathogens in the lumen to prevent initial attachment to the mucosal epithelium, thus inhibiting the onset of infection and viral entry across the epithelium [264]. Intracellular neutralization utilizes the natural transcellular transport that sIgA undergoes within the epithelial layer. Once a pathogen can attach to and,

subsequently, enter epithelial cells via receptor-mediated endocytosis, sIgA is able to bind the pathogen within the endosomes. This can either prevent viral replication and synthesis or mediate lysosomal degradation [265]. Once the pathogen overcomes immune exclusion and intracellular neutralization, both sIgA and locally-produced IgG are able to clear pathogens that invade the lamina propria by mediating antigen clearance. The antibodies bind to the pathogen and form IgA- or IgG-antigen complexes. The complexes are transported by pIgR-mediated transcellular transport or FcRn-mediated IgG transcytosis, respectively, back to the luminal side without damage to the epithelial cell [263] [266]. IgG within the respiratory mucosa, primarily in the lower respiratory tract, is locally produced by IgG-secreting plasma cells within the NALT. Mucosal IgG can also be serum-derived after transportation by FcRn, which is expressed by most mucosal epithelial cells, or transudation across the respiratory epithelium from circulation [55] [249] [267]. Though sIgA is considered the predominant protective mucosal antibody, it has been shown in an influenza challenge model, both IgA knockout mice and mice that express IgA have similar viral loads. This suggests that other antibody isotypes, most likely IgG, also highly contribute to mucosal immunity [268].

Mucosal Vaccine-induced Immunity and Advancements in Mucosal Vaccines

Infection or vaccination can also promote immune responses in systemic inductive sites, including the draining lymph nodes (dLN) and spleen. In these sites, mucosal DCs present antigen to lymphocytes and mediate the migration of activated immune cells to the initial mucosal site of infection or vaccination. In addition, activated immune cells can migrate to peripheral lymphoid sites through the induction of

anatomically distinct homing signal patterns, such as chemokine receptors [229]. For example, i.n. immunization with an antigen can induce mucosal and systemic immune responses in the NALT and mediastinal lymph nodes (MeLN) that drain the lungs, such as GC formation. Activation of GC formation in the lung, MeLN, and spleen allows for the production of antigen-specific antibodies at the site of vaccination in order to prevent future respiratory infection, in addition to induction of immunity in distal immune organs. It has also been demonstrated that mucosal immunization can induce long-lasting immune responses, both memory T and B cell responses, which are crucial to protecting against reinfection [229] [269]. Conversely, parenteral routes of vaccination are unable to induce robust mucosal immunity because the DCs at these sites of vaccination (e.g., muscle and subcutaneous) cannot imprint mucosa-specific homing signals on activated lymphocytes. This results in the induction of immunity only in peripheral systemic immune organs. Thus, mucosal vaccination, by inducing both durable mucosal and systemic immunity, has distinct protective advantages over parenteral vaccines, which induce systemic immune responses but not mucosal immunity [269].

Though most infections occur at mucosal surfaces, there are still very few vaccines that are administered by mucosal routes. Currently, the only licensed mucosal vaccines for humans are i.n. administered LAIV and orally administered live attenuated vaccines against poliovirus, which is no longer used in the US, cholera, rotavirus, *Salmonella* Typhi, and adenovirus, which is only available to military personnel [269] [270] [271] [272] [273] [274] [275] [276] [277]. There are several factors to consider in order to develop an effective mucosal vaccine: route of

administration, overcoming the mucosal epithelial barrier and mucosal tolerance due to low immunogenicity of antigen; requirement of safe and effective adjuvants for humans; and standardized diagnostic assays to determine vaccine efficacy and correlates of protection [229] [269] [278] [279]. These issues can make it difficult to properly induce and measure a protective mucosal immune response upon vaccination. Great strides are being made to promote mucosal vaccine research as a more effective alternative to parenterally administered vaccines. From an immunological standpoint, mucosal vaccinations can induce humoral and cell-mediated immune responses, at both mucosal and systemic levels, while also establishing memory T- and B-cell responses, which are directed to the site of vaccination, and subsequently, infection [278] [279]. Specifically, intranasal vaccination induces systemic and mucosal immune responses in several anatomical locations, primarily in the NALT, respiratory mucosa, and the genital tract. Logistically, depending on the route of administration, production could be more easily scaled-up and vaccination would be needle-free. This would require less personnel training and make vaccination more accessible for those averse to injections, with the goal of leading to higher coverage [269].

There are continuous efforts to improve upon current vaccine formulations, such as those against influenza virus. In contrast to parenterally-administered IIV, LAIV is the only mucosal influenza vaccine that is licensed in the US. Live attenuated vaccination mimics natural infection by initiating a mild infection with possible limited replication at the site of vaccination, while, ideally, avoiding induction of local inflammatory responses. Thus, live attenuated vaccines can be an

extremely effective method to induce robust mucosal and systemic immunity, but it can be difficult to balance safe attenuation and enhanced immunogenicity [280]. Though LAIV is not presently available because of concerns of reduced efficacy in children, it remains an important vaccine model. It is essential to either improve the current LAIV formulation or develop novel mucosal influenza vaccines. Indeed, there are efforts to enhance the efficacy of licensed influenza vaccines by using a combination of i.n. LAIV prime and i.m. IIV boost. Several groups have demonstrated that this vaccination strategy can induce potent cell-mediated and humoral immune responses, including high titers of neutralizing antibodies and a robust high-affinity, stalk-specific antibody response [198] [281] [282].

There are great efforts to develop novel vaccine platforms directed against important human pathogens that enhance specific components of the mucosal immune system, by exploiting certain viral or cellular factors. Several mucosal vaccine strategies focus on promoting antigen uptake of M cells and inducing NALT and sIgA production [283] [284] [285]. Novel vaccine delivery vehicles can target the mucosal epithelium, including live recombinant viral and bacterial vectors, and inactive or subunit vaccines. Viral and bacterial vectored vaccines consist of attenuated versions of the pathogen vector which can express the vaccine antigen (e.g., Adeno-associated viruses, Sendai virus, and *Salmonella* spp.). Subunit or inactive vaccines include adjuvanted soluble antigen or particulate vaccines composed of vaccine antigens and viral proteins that target M cells or are involved in attachment and entry (e.g., Sendai virus fusion glycoprotein or HIV glycoprotein gp160) which are incorporated into liposomes, VLPs, or nanoparticles [278] [286]

[287] [288] [289] [290] [291] [292] [293] [294]. Particulate vaccines are generally more immunogenic and adherent to mucosal surfaces than soluble vaccine antigens. Soluble vaccine antigens are subject to dilution and clearance at the mucus layer, with no distinct attachment mechanisms, though it has been shown that the inclusion of an adhesive adjuvant, such as chitosan, can greatly enhance adherence and immunogenicity [269] [295]. Live viral and bacterial vectored vaccines do not generally require adjuvants. Both subunit and particulate vaccines have greater efficacy when in combination with adjuvant components that have both adhesive and immunomodulating properties, such as TLR agonists [269]. It has been demonstrated that these diverse antigen delivery vehicles can target the mucosal epithelium and induce robust antigen-specific sIgA and serum IgG titers in local and distant effector sites [279] [283] [284] .

With the proper adjuvant, a mucosal vaccine can significantly enhance antigen delivery and induce immunity that overcomes mucosal tolerance, while providing protective local and systemic immunity. As previously described, there are a number of adjuvants either currently licensed in the US, such as MF59 and alum, or under development, such as TLR agonists and cytokines [296] [297] [298]. Adjuvants can either improve delivery of the vaccine antigen to APCs (e.g., oil-in-water emulsions and liposomes) or enhance the immune response (e.g., innate immune sensors, such as TLR agonists) [269] [299] [300] [301]. For mucosal vaccines, both effective vaccine delivery and overcoming immune tolerance are important considerations, thus an appropriate adjuvant could potentially resolve both issues. In addition, there are other cellular mechanisms that can be exploited to overcome the mucosal barrier,

the first obstacle that any mucosal vaccine antigen will encounter, and to improve the immunogenicity of vaccine antigens.

Neonatal Fc Receptor for IgG (FcRn)

The IgG-specific neonatal Fc receptor, FcRn, has previously been described to provide an essential role in mucosal immunity. FcRn transports locally-produced and serum-derived IgG across the mucosal epithelial barrier, but the significance of FcRn in immunity begins much earlier. FcRn was first characterized in rats, where maternal IgG in ingested milk is transported to neonates across the epithelial cells of the proximal small intestine. As later seen in humans, maternal IgG is transported to the fetus across the syncytiotrophoblasts of the placental epithelium, via FcRn-mediated transport [302] [303] [304] [305]. The main function of FcRn is pH-dependent bidirectional transport or transcytosis of IgG across a polarized epithelial cell layer, while also protecting IgG from lysosomal degradation, extending its half-life in circulation [306] [307] [308] [309]. A similar role of FcRn has been demonstrated for transporting and maintaining the serum half-life of albumin, a protein made in the liver that is essential for regulating osmotic blood pressure and transporting a number of molecular compounds, such as vitamins, drugs, and ions, throughout the body and in serum [310] [311].

Structural Analysis of FcRn-IgG Interaction

FcRn is an MHC class I-like molecule that is expressed as a heterodimer on a number of cell types, including epithelial cells, antigen-presenting cells, and endothelial cells

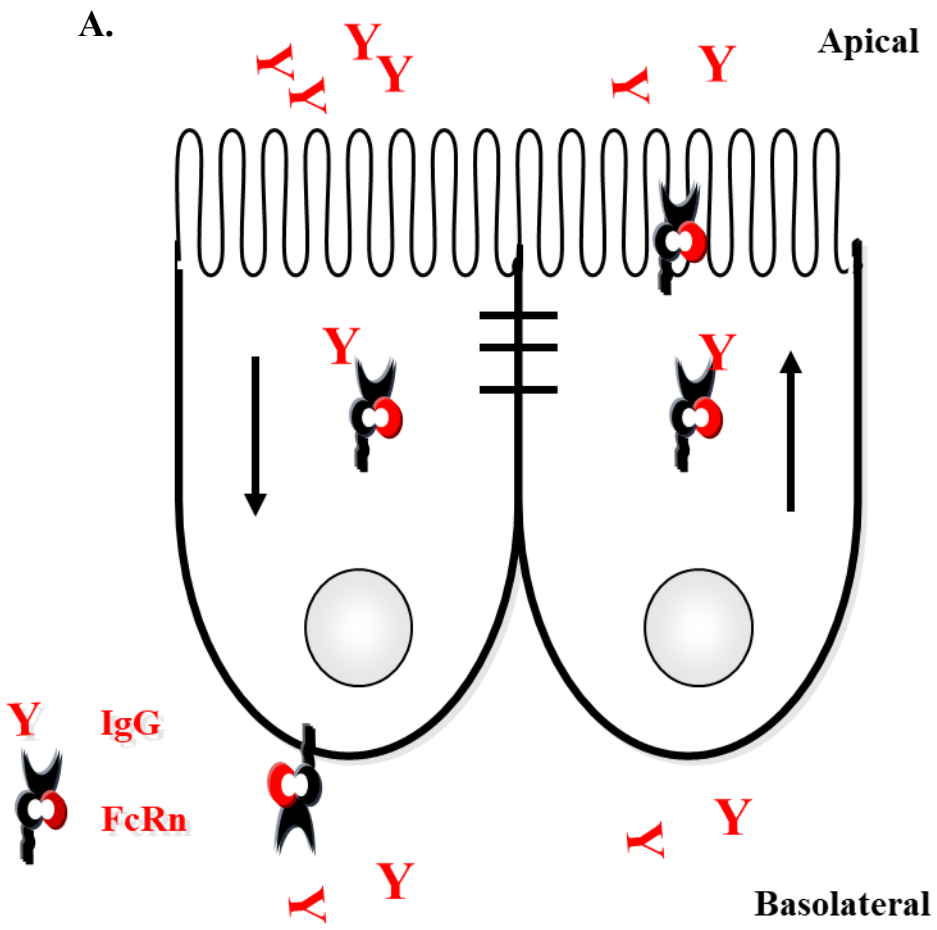
[307]. It is composed of a 50-kDa heavy chain, with 3 extracellular domains, α1, α2, and α3 helices, a transmembrane domain, and a small cytoplasmic tail. The heavy chain is non-covalently associated with a 15-kDa β_2-microglobulin (β_2m) light chain, which is required for its function, as studies in β_2m-knockout mice have shown a dysregulated IgG transport [312]. In 1994, the X-ray crystal structure of FcRn was resolved at 2.2-Å [313]. It was determined that, while classical MHC class I molecules contain a peptide-binding site, the equivalent of the binding site in FcRn is narrowed due to rearrangements of its α helices, disabling the binding and presentation functions of classical MHC class I molecules [314]. Rather, FcRn serves as an IgG-binding receptor with several essential biological roles, including IgG transport, protection, and catabolism. The interaction site between FcRn and IgG is composed of several α2 helix domains of FcRn and highly conserved amino acids within the CH2-CH3 region of the Fc portion of IgG. These amino acids include Ile253, and His310, and to a lesser, but still significant, extent, His436, which is less conserved [314] [315] [316] [317]. The hinge region has been implicated in supporting proper CH2-CH3 folding for optimal binding to FcRn [318]. The region of Fc that binds to FcRn overlaps with the binding sites to staphylococcal protein A (Protein A), streptococcal protein G (Protein G), and rheumatoid factor, but not with the binding sites to classical FcγRs or the Clq component of the complement system [319] [320] [321] [322].

As IgG naturally exists as a homodimer, there are 2 potential sites available for FcRn binding, on each subunit of the Fc portion. There are 2 proposed models of FcRn-IgG interaction, the formation of “lying-down” or “standing-up” complexes,

both of which describe a 2:1 stoichiometry. “Lying-down” complexes are composed of 2 FcRn proteins bound to 1 IgG molecule, where one FcRn molecule interacts with one binding site of IgG, and the second FcRn molecule is associated with the first FcRn through the α3 domain. “Standing-up complexes” are comprised of 2 FcRn proteins bound to 1 IgG molecule, where each FcRn binds to the 2 available sites on IgG [323]. It has been previously established that multiple FcRn molecules bind to IgG at dissimilar affinities. In addition, it has been recently demonstrated that 2 FcRn molecules independently bind to identical sites on each Fc subunit of the IgG homodimer with equal affinity, which would support the “standing up” model of FcRn-IgG [324]. Though there is evidence for both models, the stoichiometry of FcRn-IgG binding is still debated, since both 2:1 and 1:1 binding models have been demonstrated, depending on experimental conditions of studies including protein modeling and equilibrium gel filtration. It is also conceivable that a combination of both 2:1 and 1:1 stoichiometry, and formation of both “lying-down” and “standing-up” complexes, are exhibited to varying degrees in different tissue environments [307] [323].

Intracellular Trafficking and Protection of IgG by FcRn

Unlike the unidirectional transcellular transport of sIgA by pIgR, FcRn transcytoses IgG in a pH-dependent, bi-directional manner (Figure 1.3). FcRn is able to deliver IgG from tissues across a polarized epithelial layer into the lumen, in a basolateral-to-apical direction. Conversely, FcRn can also transport IgG from the luminal side into the underlying tissues, in an apical-to-basolateral route [55]. The process of pH-dependent FcRn transport of IgG varies between tissues. FcRn binds to the Fc portion


A.
Apical
Y
IgG
FcRn
Basolateral

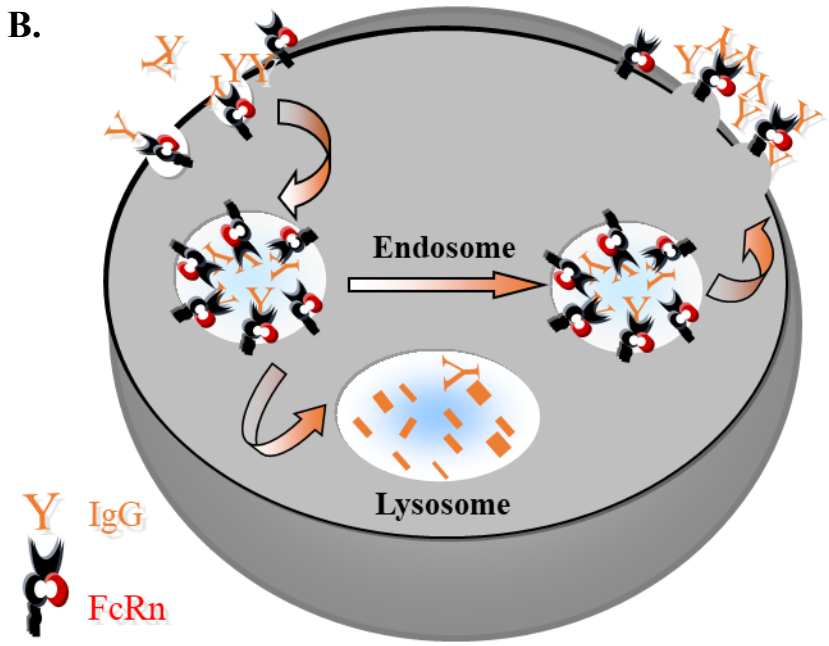


Figure 1.3. FcRn provides transport and protection to IgG antibody across polarized epithelial cell layer in a pH-dependent manner.

(A) FcRn binds to IgG either at an acidic environment at the polarized cell surface or within epithelial cells after pinocytosis or fluid-phase endocytosis. FcRn mediates the bidirectional transport of IgG across the polarized cell barrier and releases IgG upon exposure to physiological pH.

(B) FcRn binds to IgG in a pH-dependent manner, binding to IgG at acidic pH (<6.5) and releasing IgG at physiological pH (7.4) upon traversing the polarized epithelium. IgG bound to FcRn is directed away from lysosomes, in contrast to unbound protein, protecting IgG from degradation.

of IgG at slightly acidic pH levels (<6.5) and releases it at neutral or physiological pH levels (pH 7.4) [325] [326] [327] [328]. Within the acidic environment of the proximal intestinal lumen, FcRn expressed by intestinal epithelial cells is able to bind to ingested IgG at the apical cell surface, initiating endocytosis, which requires distinct dileucine- and tryptophan-based endocytosis signals, and subsequent transport of IgG, whereupon exposure to physiological pH of the underlying lamina propria, FcRn releases IgG across the intestinal epithelium [326]. Within luminal or apical surface of the vascular endothelium, in the absence of an acidic environment, IgG is taken up by cells via fluid-state pinocytosis, while FcRn is localized in apical early endosomes, and after IgG is trafficked to the apical early endosome, it can encounter FcRn within the same endocytic vesicle. After acidification of the endosomes, FcRn is able to bind to IgG and mediate its transport to the basolateral surface, whereupon exposure to physiological pH, FcRn releases IgG into the underlying tissues. FcRn-mediated basolateral-to-apical transport of IgG has been previously described in the context of mucosal immunity [307] [329]. Within the respiratory tract, local plasma cells present in the respiratory lamina propria produce IgG which can be transported across the respiratory epithelium via FcRn, resulting in the accumulation of antigen-specific IgG at the site of respiratory pathogen exposure, providing a potent local defense against infections [55] [330] [331]. In addition to the transcytosis of IgG, FcRn can recycle bound IgG back to the initial surface of entry, depending on the tissue localization and the requirements of IgG concentration within the tissue [332].

After FcRn binds to IgG, either at the acidic plasma membrane or within acidifying endosomes, the endosomes containing the FcRn-IgG complex are trafficked away from the lysosomal degradation pathway. FcRn can either recycle IgG back to the initial side of binding or transport IgG across the polarized cell layer. The outcome of IgG transport appears to be dependent on the concentration of IgG available in the local environment, which correlates to the amount of IgG in serum [306]. For example, within the vascular endothelium, at low levels of IgG, all molecules of IgG are bound to FcRn, but not all of the FcRn is bound, which allows for IgG to be recycled to the luminal side or transcytosed into the underlying tissue. In the presence of high levels of IgG, FcRn of vascular endothelial cells all interact with available IgG, but not all IgG molecules are bound. Therefore, unbound IgG is endocytosed with other molecular cargo during fluid phase endocytosis. IgG that is not bound, therefore unprotected, by FcRn, is transported to the lysosome for degradation. Thus, a prominent function of FcRn is providing protection to IgG from degradation. Through recycling, the half-life of IgG surpasses those of the other antibody isotypes (~21 days in humans, and 6-8 days in mice), while maintaining the highest concentration of IgG in serum, compared to other isotypes [306] [307] [308]. Through FcRn-mediated transcytosis, IgG is able to access underlying tissues to modulate cellular responses, such as humoral immunity, from prenatal to postnatal development, and throughout adulthood. Thus, FcRn expression ensures maintaining homeostatic levels of IgG in circulation, regulating the salvage and degradation of IgG as required, while providing humoral immunity to vulnerable tissues.

The complex mechanisms of bidirectional transport and the decision for IgG transcytosis or recycling has been characterized in several systems, including MDCK cell culture, enterocytes, and human placenta. It has been demonstrated that FcRn transport of IgG from either basolateral or apical surfaces for transcytosis or recycling involves distinct and separate cellular factors. FcRn carries IgG to recycling endosomes (RE), which contain various trafficking molecules that can execute either FcRn-mediated recycling or transcytosis of IgG. Within the RE, actin motor myosin Vb and GTPase Rab25 regulate the delivery of IgG via FcRn to the opposing membrane [332]. Conversely, within the RE, Rab4 and Rab11a mediate FcRn-directed recycling of IgG back into circulation [333]. While Rab11 has a role in mediating exocytosis of FcRn-IgG complexes, Rab4 does not appear to be required. It has also been demonstrated that the cytoplasmic tail of FcRn is crucial for optimal apical-to-basolateral transport, as mutations in this domain can lead to dysregulation of IgG transport and an increased distribution of FcRn at the apical surface [334].

FcRn Expression Patterns in Early and Adult Development

FcRn expression and transport during neonatal or fetal development have been demonstrated in many mammalian species, while the function of FcRn is not limited to early development. Numerous organs and tissues express FcRn throughout the lifetime of the host, though the expression is host-dependent. In the gut of adult humans, FcRn continues to be expressed, while adult rodents prominently reduce expression of FcRn in the gut soon after weaning, which is when pups would no longer receive milk containing maternal IgG, diminishing the necessity of FcRn in the gut [303] [335]. FcRn is highly expressed in the central nervous system (CNS), a site

of immune privilege with reduced immunity to pathogens, where tight junctions within the vascular endothelium normally exclude the diffusion of IgG, and other molecules, across the blood-brain barrier [336]. In response to certain inflammatory responses, the blood-brain barrier becomes permeable, allowing for an influx of IgG. To reduce inflammatory damage to the CNS, FcRn is believed to mediate the transcytosis of excess IgG out of the CNS and into circulation. The lungs are an important location of FcRn expression, in both epithelial cells and APCs, such as alveolar macrophages [307] [337] [338]. There are some notable differences between species in FcRn expression patterns in the lungs. In human and non-human primates, FcRn is largely expressed in the upper respiratory tract, while in rats and cows, FcRn expression is localized in the lower respiratory tract [55] [330] [339]. In adult mice, FcRn has been shown to be expressed in the epithelial cells of the bronchi, trachea, and lung [55] [340]. The pronounced expression of FcRn in the lung has spurred the development of biological compounds that can utilize the FcRn-IgG interaction to deliver a variety of therapeutics and vaccines across the respiratory mucosa [341].

Utilizing FcRn-IgG Interaction for Therapeutics and Vaccines

Fc Fusion Protein Development

FcRn-mediated transport of IgG is an essential component of host immunity during early development and throughout the lifetime of many mammalian species. This transport pathway can be exploited as a powerful tool for delivering specific biological compounds across epithelial barriers, namely, Fc fusion proteins [342]

[343]. Since FcRn mediates transport of IgG through binding of the Fc portion of IgG, therapeutic molecules that contain Fc could conceivably be delivered by FcRn across epithelial barriers. Fc fusion proteins are composed of the Fc portion of IgG fused to an effector molecule (e.g., cellular proteins, cytokines, growth factors, and viral proteins). There are numerous advantages to using FcRn-targeted Fc fusion proteins for the treatment of a variety of diseases. Administration of Fc fusion proteins can result in increased serum half-life and enhancement of effector functions compared to effector molecules alone [308] [344] [345] [346] [347]. After administration, Fc fusion proteins are effectively transported to the underlying lamina propria, which would allow them to be taken up by APCs, thus initiating immunity, resulting in improved immunogenicity of the effector molecule. It is well established that these outcomes are due to its interaction with FcRn, which mediates the stability and solubility of Fc fusion proteins *in vivo*. In addition, from a logistical standpoint, it has been demonstrated that incorporation of the Fc portion of IgG with a recombinant protein can increase protein expression and secretion, which occurs mainly in mammalian cell culture systems to ensure proper glycosylation, and affords straightforward purification via Protein A or G-based affinity chromatography [348] [349] [350].

The first Fc fusion protein for clinical purposes was developed in 1989, where the HIV glycoprotein gp120-binding domain of CD4 was fused to the Fc portion of IgG1, which could conceivably to bind to the free virus, thus preventing it from binding to its cognate receptor, CD4, and initiating infection [351]. Currently, there are 9 FDA-approved Fc fusion proteins for use as drug therapies against severe

conditions such as hemophilia, rheumatoid arthritis, and macular degeneration, where the inclusion of the Fc portion of human IgG1 is primarily to extend the serum half-life [348] [352] [353] [354] [355]. They are administered by a variety of routes, including intravenous (i.v.), subcutaneous (s.c.), intravitreal, and i.m. injection [343] [352] [354] [356]. Thus, the effectiveness and relevance of Fc fusion protein therapy to current public health cannot be minimized.

Fc fusion protein design is evolving to optimize the functionality of the Fc fragment, with the subsequent increased efficacy of the effector molecule. Initially demonstrated in IgG antibody, certain point mutations or combinations of mutations to the Fc portion of IgG (at positions Thr250, Met252, Ser254, Thr256, Thr307, Glu380, Met428, His433 and Asn434) can enhance the binding affinity between FcRn and IgG, which results in increased half-life of IgG, without altering the binding and release of IgG at physiological pH (Roopenian 2007), while additional mutations have also demonstrated enhanced IgG binding affinity to FcRn [357] [358] [359] [360] [361]. This was then confirmed using a therapeutic antibody, the licensed drug Avastin, an anti-vascular endothelial growth factor (VEGF) IgG1 antibody used for the treatment of colorectal, lung, breast, and renal cancers. The most effective VEGF antibody contained Met428Lys and Asn434S mutations, which resulted in a five-fold and three-fold increase in serum half-life in human FcRn-transgenic mice and non-human primates, respectively, compared to unmodified Avastin [346]. Thus, Fc fusion proteins containing these mutations in the Fc domain could conceivably remain in circulation longer with enhanced downstream effects of the target molecule, maximizing the therapeutic effect of the Fc fusion proteins.

Because the lung highly expresses FcRn, which directs Fc-mediated transport of IgG across the respiratory epithelium to mucosal and systemic locations, intranasal delivery of Fc fusion proteins is an attractive strategy to combat both non-infectious and infectious diseases. Though there is currently no licensed pulmonary administration of Fc fusion proteins, great strides have been made to develop the intranasal delivery of Fc fusion proteins as drug therapies. EpoFc is an Fc fusion protein based on erythropoietin, a hormone that promotes red blood cell (RBC) formation. Pulmonary administration of EpoFc resulted in a dosage-dependent retention of EpoFc and an increased stimulation of immature RBC production in non-human primates and phase I clinical trials, which has potential implications for treating conditions such as anemia [342] [362] [363]. In addition, an Fc fusion protein based on follicle-stimulating hormone (FSH), FSH-Fc, was developed for pulmonary administration in non-human primates. Recombinant FSH treatment is a common therapy for infertility, and compared to recombinant FSH alone, airway delivery of FSH-Fc resulted in increased stability in serum and an increase in ovarian weight, a marker of *in vivo* bioactivity [364].

Fc Fusion Proteins as Vaccines

While most pre-clinical and clinical Fc fusion proteins have been used as drug therapies against non-infectious disease, there is a growing effort to target Fc fusion protein treatment against infectious diseases to mediate protection as vaccines. It has been demonstrated that intranasal delivery of Fc fusion proteins based on viral antigens can promote protection against several important pathogens. This has been established using herpes simplex virus (HSV)-2 glycoprotein gD, where intranasal

vaccination with gD-Fc fusion protein in a mouse model induced high levels of both local and systemic immunity, at both the respiratory tract and the distal genital tract, which has important implications for the broad efficacy of the intranasal route [365]. Intranasal immunization with gD-Fc conferred high levels of protection against intravaginal challenge and induced robust and protective memory responses. The immunity and protection induced by i.n. vaccination with gD-Fc was demonstrated to be mediated by FcRn, as control groups that lacked FcRn/gD-Fc interaction resulted in poor induction of immunity and low survival rates. In addition, several Fc fusion protein vaccine constructs composed of HIV viral proteins have been developed that require FcRn interaction for optimal efficacy. An Fc fusion protein composed of HIV Gag protein p24 fused to Fc was able to induce high levels of long-lasting local and systemic immunity, while conferring protection against intravaginal challenge with a recombinant vaccinia virus expressing HIV Gag protein, to simulate HIV challenge in a mouse model, once again demonstrating that FcRn-mediated vaccination at the respiratory mucosa can induce protective immunity at distal mucosal sites [340]. In a non-human primate model, ENV protein gp120 fused to Fc, Env-rFc, was used for intranasal immunization and resulted in robust long-lasting gp120-specific neutralizing antibody titers [366].

Taken together, FcRn-targeted delivery of vaccine antigens can induce the high levels of local and systemic immunity and survival, positing this vaccine model as a significant platform to developing highly effective mucosal vaccines against biologically relevant pathogens. As previously known, and further verified by both HSV-2 and HIV Fc fusion proteins in mouse models, intranasal vaccination with an

antigen results in the induction of protective immunity in both local and distal mucosal sites, including the urogenital tract. This has important consequences for developing mucosal vaccines that target infections of the genital tract, but the intranasal route of administration has been well characterized to induce robust immunity within the respiratory tract, which would serve as a potent defense against respiratory infections, such as influenza virus.

Specific Aims

Vaccination is one of the most important biological achievements in the history of public health. Despite the millions of lives that vaccination has saved, there is the continuous threat of infections that can result in devastating morbidity and mortality, especially for influenza virus. Current influenza vaccination strategies struggle to keep up with rapid antigenic variations that occur in the virus, often resulting in poorly matching vaccines that provide suboptimal protection to seasonal strains. As there are no approved mucosal vaccines against influenza currently available in the US, and there are numerous advantages of mucosal vaccines over parenteral vaccines, such as IIV, there is a growing requirement for developing effective and safe mucosal influenza vaccines. One method is to utilize the natural cellular interaction that occurs as most mucosal surfaces, including the respiratory tract: FcRn-mediated transport of IgG across the mucosal epithelium through the Fc portion of the antibody. By fusing vaccine antigens to the Fc portion of IgG, it is possible to direct the vaccine antigen to the respiratory mucosa for FcRn-mediated

transcytosis across the epithelial barrier, for subsequent delivery to the underlying lamina propria, allowing the vaccine antigen to access immune cells, such as DCs, for uptake and presentation to lymphocytes in both local and peripheral lymphoid organs. This would allow for the induction of a multifaceted antigen-specific immune response: humoral and cell-mediated immunity within both mucosal and systemic immune systems. In the context of influenza virus, enhanced viral clearance and survival are dependent on durable, robust immune responses against influenza infection, specifically, neutralizing antibodies supported by the Th1 response, and CTL induction. We believe that FcRn-mediated mucosal vaccination with influenza vaccine antigens as part of Fc fusion proteins can induce long-lasting protective local and systemic immunity in a mouse model. Thus, **Specific Aim 1 is to demonstrate whether FcRn-mediated delivery of influenza vaccine antigens can protect against infection from challenge with a homologous virus strain.**

While demonstrating homologous protection is foundational for establishing a novel influenza vaccine platform, there is an urgent requirement for influenza vaccines to elicit cross-protection to reduce the necessity of annual reformulations of seasonal vaccines and to protect against potential pandemic strains. Therefore, **Specific Aim 2 is to examine whether FcRn-mediated delivery of conserved influenza vaccine antigens can protect against infection from challenge with a heterosubtypic virus strain.**

Chapter 2: FcRn-mediated Intranasal Delivery of HA-Fc Fusion Protein Confers Partial Protection Against Lethal Influenza Challenge

Abstract

Mucosal vaccination has the potential to be a successful strategy against influenza infection, but current intranasal vaccines require improvements to be safer and more protective. The neonatal Fc receptor (FcRn) for IgG mediates IgG transport across the polarized epithelial cell layer that lines mucosal surfaces, including the respiratory epithelium. It has been previously demonstrated that FcRn can transport herpesviral or HIV antigens fused to the Fc portion of IgG across mucosal barriers and engender protective immunity. To determine if this strategy can be utilized for mucosal influenza vaccines, I fused the extracellular domain of hemagglutinin to the Fc portion of IgG and produced the soluble HA-Fc/dimer fusion protein in CHO cells. C57BL/6 mice were intranasally immunized with HA-Fc along with CpG as adjuvant. Immunized mice produced higher levels of long-lived HA-specific antibody titers and induced greater percentages of tissue-resident memory (TRM) T cells in the lung compared to control and mock-vaccinated mice. Furthermore, HA-Fc/dimer immunization conferred partial protection after lethal challenge with influenza virus. My results support the model that FcRn-mediated delivery of vaccine antigens may be used to develop an influenza mucosal vaccine.

Introduction

Influenza A virus remains an important human pathogen that results in high levels of morbidity and mortality worldwide, infecting 1 billion people which cause 3 to 5 million cases of severe illness and up to 500,000 deaths every year [367]. The virus is a member of the *Orthomyxoviridae* family and contains a segmented, single-stranded negative-sense RNA genome that codes for eleven proteins, including glycoproteins hemagglutinin (HA) and neuraminidase (NA). HA is the dominant immunogen and exists as a homotrimer that is first produced in pre-cleavage form, HA0. A requirement for infectivity, HA0 is cleaved into two subunits, HA1 globular head and HA2 stalk, during viral replication. In humans, HA1 binds to sialic acids (SA) of glycoproteins or glycolipids at cell surfaces within the respiratory tract, initiating receptor-mediated endocytosis of the virus. Within the acidifying endosome, HA undergoes a conformational change that exposes HA2, mediating fusion between the viral and endosomal membranes [2].

Currently, seasonal influenza vaccines remain the most effective method to prevent infection. These include inactivated influenza vaccine (IIV), recombinant influenza vaccine (RIV3), and live-attenuated influenza vaccine (LAIV). LAIV is a mucosal vaccine composed of cold-adapted, temperature-sensitive attenuated virus strains and is administered intranasally (i.n.). LAIV has accrued a number of limitations since its inception. LAIV largely induces strain-specific immunity that provides optimal protection when the vaccine strains and the seasonal virus strains match. A number of groups in the population cannot receive LAIV, including pregnant women, children under 2 years, persons with egg allergies,

immunocompromised patients, and asthmatics. During the 2016-17 and 2017-18 seasons, a recommendation against LAIV was issued by the Advisory Committee on Immunization Practices (ACIP) due to lack of efficacy in children [167] [192].

In the US, vaccination coverage is approximately 39% of people 6 months or older, resulting in most of the country potentially unprotected [368]. Currently, all approved influenza vaccines are needle-based, administered through parenteral routes such as intramuscular (i.m.) or intradermal (i.d.), which can prevent broader acceptance of vaccination. Although the majority of pathogens target mucosal membranes (i.e. respiratory, intestinal, and urogenital), most vaccinations are administered through parenteral routes. In the US, there are few vaccines administered mucosally, including *V. cholera* and *S. typhi.* Like most pathogens, influenza virus infects at mucosal surfaces, specifically, the mucosal epithelium of the upper respiratory tract in humans. The respiratory mucosa is composed of a single epithelial layer strengthened by tight junctions that normally prevents transport of foreign antigens. A mucosal vaccine strategy that targets the epithelial cells could significantly improve antigen delivery. By transporting influenza antigens across the mucosal epithelial lining to the underlying lymphoid tissues, the antigens can access antigen-presenting cells (APCs) in order to activate innate and adaptive immunity.

Neonatal Fc receptor (FcRn) is an IgG transport receptor that is expressed in most mucosal epithelial surfaces. FcRn binds to the Fc portion of IgG within acidifying endosomes. Upon exposure to physiological pH, FcRn releases IgG to the basal side of the epithelial layer, effectively transporting IgG across the mucosal barrier, while also protecting IgG from lysosomal degradation [307]. We have

previously demonstrated that fusion of HSV-2 vaccine antigen, gD, to IgG Fc resulted in the FcRn-dependent delivery of gD-Fc fusion proteins across the respiratory mucosal epithelium, which was able to induce protective immune responses against genital infection from HSV-2 [365]. Recognizing the nature of the FcRn-IgG transfer pathway and its ability to transport viral proteins fused to IgG Fc, I reasoned that it could be used to deliver influenza vaccine antigens as part of an Fc fusion protein across the respiratory mucosa in order to activate protective against influenza infection.

In this study, I developed an influenza antigen delivery platform based on influenza viral proteins fused to IgG Fc to determine vaccine efficacy against lethal challenge in a mouse model. Influenza HA protein is the major immunogen against which natural infection and licensed vaccines mount strain-specific immunity. I utilized full-length HA for my vaccine construct, HA-Fc/dimer, which consists of monomeric HA fused to the Fc portion of IgG. In order to optimize the immunization process, I performed a dosage titration with HA-Fc/dimer. I determined the optimal dosage of i.n.-administered HA-Fc/dimer that provided significant induction of immunity and levels of protection. After i.n. vaccination with the optimized dosage, I detected high levels of long-lived HA-specific antibody titers, and importantly, the induction of an increased percentage of a newly characterized memory T cell subset, tissue-resident memory (TRM) T cells.

TRM T cells are a non-circulating subset of memory T cells that are maintained at the site of infection. Lung-resident memory T cells can provide a rapid protective and heterosubtypic immune response against influenza. They are induced

by influenza infection and have also been shown to be induced by LAIV vaccination [107] [108]. In addition, it has been shown that only the i.n. route of vaccination can induce lung-resident memory T cells, and not by i.m. injection [102]. Though FcRn-mediated vaccination with HA-Fc/dimer induced a robust and durable immune response, it could not provide full protection against lethal challenge. While initial lethal challenges resulted in 80% survival in HA-Fc/dimer-vaccinated mice, subsequent efforts to replicate high survival rates yielded an overall 45% survival rate. These results demonstrated that HA-Fc/dimer vaccination conferred partial protection to lethal challenge, while suggesting that the vaccine construct would require improvements in order to provide higher efficacy. Taken together, I established that FcRn-dependent intranasal vaccination with an Fc fusion protein based on HA, HA-Fc/dimer, induced robust immune responses, including the induction of TRM CD4+ and CD8+ T cells, and conferred partial protection against lethal challenge against influenza. With optimizations to the vaccine construct, my work would strongly support FcRn-mediated vaccination as an effective mucosal vaccine platform that is more accessible than currently available vaccines.

Materials and Methods

Cells, antibodies, and virus

Chinese hamster ovary (CHO) cells were purchased from the American Tissue Culture Collection (ATCC) and were maintained in DMEM complete medium (Invitrogen Life Technologies), supplemented with 10% FBS, 2 mM l-glutamine,

nonessential amino acids, and penicillin (0.1 μg/ml)/streptomycin (0.292 μg/ml). After transfection, stable CHO cell lines were grown in DMEM complete medium with G418 (500 μg/ml). All cells were grown at 37°C in 5% humidity. Influenza A/Puerto Rico/8/34/Mount Sinai/H1N1 (PR8) virus was generously provided by Dr. Peter Palese (Icahn School of Medicine at Mount Sinai) and was amplified in 10- to 11-day-old embryonated chicken eggs and titrated by 50% endpoint dilution assay. HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant HA was purchased from Sino Biologicals (Shanghai, China). The horseradish peroxidase (HRP)-conjugated streptavidin and anti-mouse IgG antibodies were purchased from Southern Biotech (Birmingham, Alabama).

Construction of influenza HA-Fc/dimer expression plasmids

To make the Fc fusion protein with influenza HA, the extracellular portion of PR8 HA was amplified by PCR from a plasmid containing full-length PR8 HA using the primer pair (5'-GCCGAAGCTTGCCACCATGAAGGCAAACCTACTGGTCCTGTTAAG-3', 5'-AGATCCCGAGCCACCTCCTCCGGACCCACCCCCGCCTGATCCCTGATAGATCCCCATTGATTCC-3'). A previously constructed pCDNA3 plasmid encoding the hinge, CH2 and CH3 domains of mouse IgG2a Fc served as a template for the Fc fragment, which was amplified by PCR using the primer pair (5'-GGATCAGGCGGGGGTGGGTCCGGAGGAGGTGGCTCGGGATCTGAGCCCAGAGGGCCCACAATCAAGC-3', 5'-GCCGTCTAGATTATTTACCCGGAGTCCGGGAGAAGCTC-3'). The HA antisense primer and the Fc sense primer contain complementary glycine and serine

codons to produce a 14GS linker to bridge the HA and IgG Fc fragments. In the IgG2a Fc plasmid, the Glu318, Lys320, and Lys322 residues were replaced with Ala residues to remove the complement C1q binding site [369]. In addition, in order to produce a mutant form of the HA-based Fc fusion protein that cannot bind to FcRn, the His310 and His433 residues were changed to Ala residues to eliminate FcRn binding sites [316] [318]. The HA and Fc fragments were fused by overlapping PCR and ligated into the pCDNA3 vector. All of the resultant plasmids were confirmed by double-stranded DNA sequencing to verify error-free PCR amplification and DNA cloning.

Expression and characterization of HA-Fc/dimer fusion proteins

The HA-Fc/dimer/wt and mut plasmids were transfected into CHO cells using PolyJet (SignaGen) according to the manufacturer's instructions. Stable cell lines were selected and maintained under G418 (0.5-1 mg/ml). Expression and secretion of HA-Fc/dimer fusion proteins were verified by immunofluorescence assay, SDS-PAGE and Western blotting analysis. The soluble HA-Fc/dimer proteins produced in complete DMEM containing 5% FBS with ultra-low IgG and were purified by affinity chromatography using Protein A (Thermo Scientific) and anti-mouse IgG (Rockland) conjugated agarose beads and dialyzed with PBS. Protein concentrations were determined using NanoDrop Spectrophotometer (Thermo Scientific). Molecular graphics and analyses of the structure of the HA-Fc/dimer protein were performed with Phyre 2 (Phyre2 ref) and the UCSF Chimera package (University of California, San Francisco).

Immunofluorescence assay (IFA)

Immunofluorescence was performed as previously described [370]. Briefly, cells were grown on coverslips for 48 hr. The cells were fixed with 4% paraformaldehyde (Sigma) in HBSS for 20 min and quenched with 100 mM glycine in PBS for 10 min. Cells were permeabilized with 0.2% Triton-X for 5 min and blocked with 3% normal goat serum in PBS for 30 min. Subsequent steps were performed in the dark. Cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG2a for 1 hr and with DAPI stain for the nucleus for 5 min. Cells were washed with PBS and mounted to slides with ProLong Antifade solution (Thermo Scientific). All steps were performed at room temperature. Images of the stained cells were processed using a Zeiss LSM 510 confocal fluorescence microscope and LSM Image Examiner software (Zeiss).

SDS-PAGE and Western blotting

Under reducing and non-reducing conditions, recombinant HA-Fc/dimer proteins were run on 8% SDS-PAGE gels in order to determine protein concentration and quality. SDS-PAGE gels were either stained with Coomassie blue dye or used for transferring onto nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with 5% milk in PBST (PBS and 0.05% Tween-20) and incubated overnight with anti-IgG2a-HRP (1:10,000). SuperSignal West Pico PLUS ECL substrate (Thermo Fisher) was used to visualize protein in membranes and images were developed and captured by the Chemi Doc XRS system (BioRad).

Mouse immunization and virus challenge

All mice were housed in the University of Maryland animal facilities and all of the animal experiments were performed with the approval of the Institutional Animal Care and Use Committee. Six to eight-week-old female C57BL/6 mice were purchased from Charles River Laboratory and FcRn-knockout mice in the C57BL/6 background are a kind gift from Dr. Derry Roopenian (Jackson Laboratory). C57BL/6 wild-type and FcRn-knockout mice were intranasally (i.n.) with 20 μl of 10, 5, or 1 μg HA-Fc/dimer/wt, HA-Fc/dimer/mut, recombinant HA, or PBS. Mice were also intramuscularly (i.m.) immunized with 100 μl of 5 μg HA-Fc/dimer/wt. All vaccine proteins or PBS were mixed with 10 μg of CpG ODN 1836 (Invivogen). Two weeks later, the mice were boosted with the same vaccine formulations. Two weeks after the boost, mice were i.n. infected with lethal doses (10^4 $TCID_{50}$) of the PR8 virus. For immunizations and challenge, all mice were anesthetized with intraperitoneal (i.p.) injection of 100 μl of fresh Avertin (40 mg/ml, Fisher Scientific) and laid down on their backs to allow for recovery. After infection, mice were monitored and scored daily for weight loss and for other clinical signs of illness for 14 days. Animals that lost above 30% of their body weight on the day of infection or reached a clinical score 10 or greater were euthanized.

Preparation of single-cell suspensions from tissues

The single-cell suspensions from the spleen were made by mechanical abrasion of the organs. Isolation of single cells from the lung was performed as previously described [365]. Briefly, after perfusion with 30 ml of PBS, lungs were minced and treated to enzymatic digestion in RPMI with pronase (1.5 mg/ml), Dispase (0.2%), and DNase

(0.5 mg/ml) for 40 min at 37°C with rotation. All cells from spleen and lung were filtered through a 40 μm nylon cell strainer and treated with red blood cell (RBC) lysis buffer (0.14 M NH4Cl, 0.017 M Tris-HCl at pH 7.2). All cells were washed and suspended in 2% FBS (Invitrogen) in PBS (FACS buffer) or RPMI1640 complete medium with 1-2% FBS. For each experiment, cells were pooled from 3–5 mice in each animal group.

Intravenous in vivo antibody labeling and flow cytometry

For intravenous *in vivo* labeling of circulating T cells, mice were intravenously (i.v.) injected with 3 μg of PerCP Cy5.5-conjugated anti-mouse CD3e antibody. After 10 min, lungs were collected and single-cell suspensions were made as described above. Fc block (anti-mouse CD16/CD32, BD Biosciences, 1 μg/sample) was added to the lung and spleen cell samples and incubated for 30 min at 4°C. After wash with FACS buffer, cells were incubated with fluorescently-conjugated antibodies to stain for T cell markers, CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD69 (H1.2F3), CD11a (2D7), and CD103 (M290), for 1 hr at 4°C in the dark. Isotype control antibodies were included in each experiment. After washing, cells were resuspended in 2% paraformaldehyde and analyzed using a FACSAria cytometer (BD Biosciences) and FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay (ELISA)

For the detection of HA-specific antibodies in serum, high-binding ELISA plates (Maxisorp, Nunc) were coated with 3 μg/ml of HA-Fc/dimer fusion protein in PBS and incubated overnight at 4°C. Plates were then washed three times with 0.05%

Tween 20 in PBS (PBST) and blocked with 2% BSA in PBS for 1 hr at room temperature. Samples were serially diluted in 2% BSA-PBS and were added for 2 hr incubation at room temperature. After washes, biotin-labeled anti-mouse IgG-specific Fab (1:2,000) was added and incubated for 2 hr. After washes, streptavidin-HRP (1:8000) was added. The reaction was visualized in a colorimetric assay using substrate tetramethylbenzidine (TMB) and a Victor III microplate reader (Perkin Elmer). Titers represent the highest dilution of samples showing a 2-fold increase over average OD_{450} nm values of negative controls.

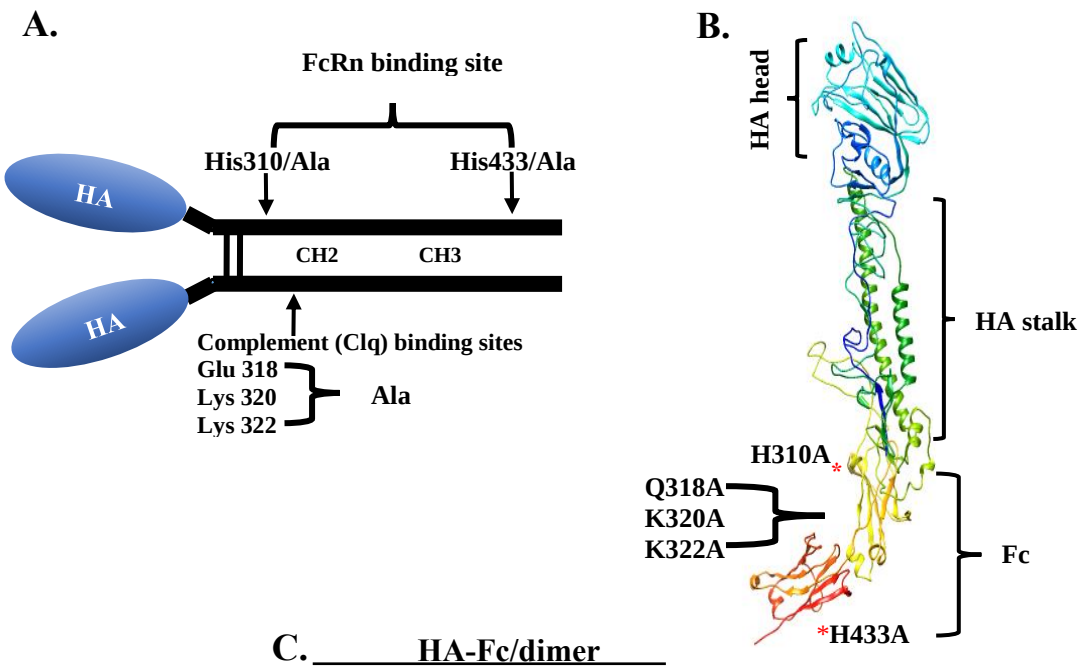
Statistics analysis

To compare the Kaplan-Meier survival curves, we used multiple Mantel-Cox tests. Differences in antibody titers were assessed by using one-way ANOVA with Tukey's multiple comparison tests. GraphPad Prism 5.01 software was used for the statistical analyses.

Results

Expression and production of HA-Fc/dimer fusion proteins

In order to demonstrate my model of FcRn-mediated delivery of influenza vaccine antigens, I produced an HA-based Fc fusion protein by cloning the external domain of HA from influenza A/Puerto Rico/8/1934/H1N1 in frame with the Fc portion of mouse IgG2a, resulting in HA-Fc/dimer/wt (Figure 2.1A-B). Mouse IgG2a was utilized because it has the highest affinity for activating FcγRI, but the lowest affinity for the inhibitory receptor, FcγRIIB. [371] [372]. Other potential isotypes, such as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FcRn binding site
His310/Ala
His433/Ala
HA
HA
CH2
CH3
Complement (Clq) binding sites
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Lys 320
Lys 322
Ala
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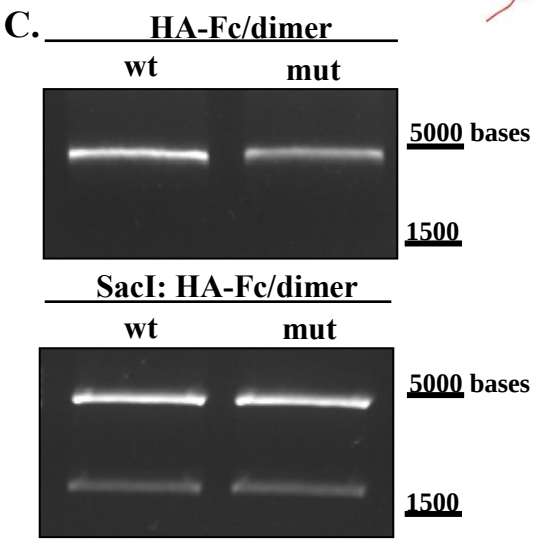
HA head
HA stalk
H310A*
Q318A
K320A
K322A
Fc
*H433A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SacI: HA-Fc/dimer
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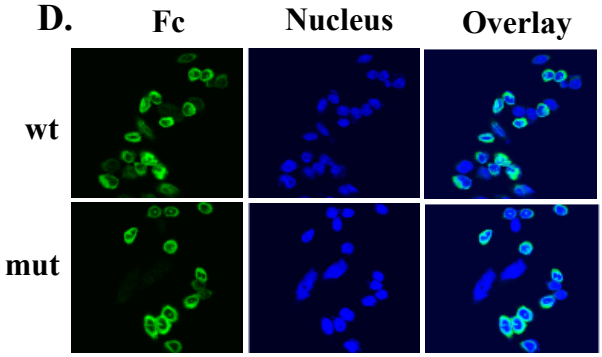
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mut

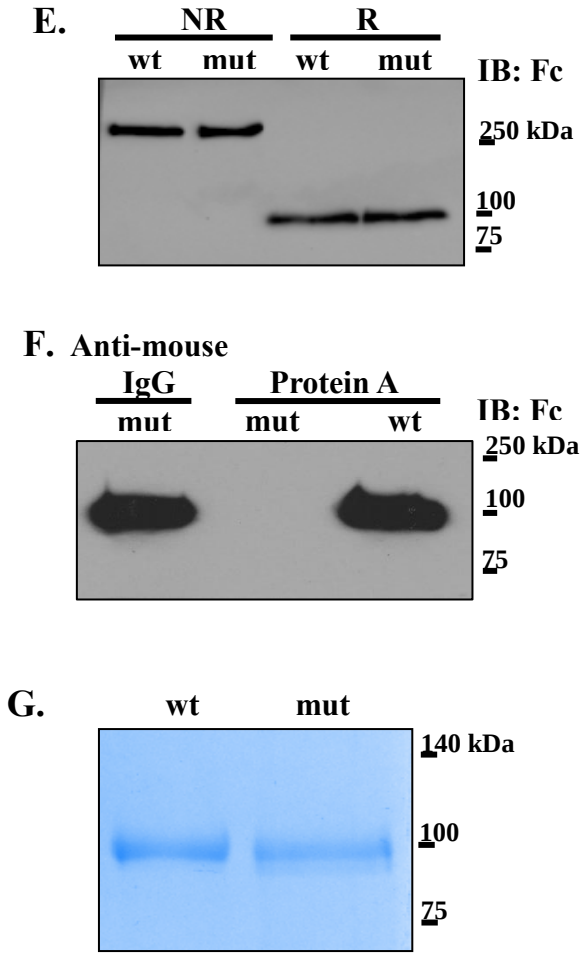


Figure 2.1. Design and expression of PR8 HA-Fc/dimer fusion protein.

(A) Schematic representation of PR8 HA fused to dimerized Fc of mouse IgG2a. Mutations were generated in the CH2 domain of Fc to abrogate the Clq binding site to prevent fixation of the complement pathway by substituting Ala at Glu318, Lys320, and Lys322. In order to produce a mutant form of HA-Fc/dimer fusion protein that cannot bind to FcRn, mutations were made to eliminate binding of Fc to FcRn by substituting Ala at His310 and His433.

(B) Predicted protein structure of HA-Fc/dimer was determined by the protein folding and recognition program, Phyre2, and modeled and visualized in the Chimera program. The HA head is in blue, the HA stalk domain is in green, and the Fc domain is depicted in yellow (CH2) and red (CH3). Mutations at the Clq binding site and FcRn binding site are noted. The following nucleotide sequences were acquired through GenBank: full-length HA: AF389118.1; IgG2a Fc: KC295246.1.

(C) The HA-Fc/dimer fusion genes (top panel) were ligated into the pCDNA3 vector. The resultant plasmids were subjected to SacI restriction enzyme digest to verify the production and size of the plasmids (bottom panel).

(D) Transfection of CHO cells with the HA-Fc/dimer plasmid produced stable cell lines of both HA-Fc/dimer/wt and mut expression. The Fc portion of HA-Fc/dimer was detected using anti-mouse IgG2a antibody.

(E) Western blot analysis of HA-Fc/dimer purified proteins in non-reducing and reducing conditions. Fc fusion proteins were probed with anti-mouse IgG2a.

(F) Pull down of HA-Fc/dimer/wt and mut by Protein A and/or anti-mouse IgG conjugated to agarose beads. 500 ng of purified protein was incubated with resin slurry for 2hr at 4°C. Boiled and eluted samples were run on SDS-PAGE. Western blot analysis of pulled-down HA-Fc/dimer/wt and mut samples was performed by probing with anti-mouse IgG2a antibody.

(G) HA-Fc/dimer fusion proteins secreted from stable CHO cell lines were purified using affinity chromatography, and quality and concentration of purified proteins were assessed using SDS-PAGE**.**

mouse IgG1, are unable to bind to FcγRI, an important activating FcγR expressed on APCs [373]. In addition, I produced a version of the HA-Fc/dimer plasmid that contained point mutations in the Fc gene at positions His310Ala and His433Ala in order to prevent binding to FcRn. This fusion construct, HA-Fc/dimer/mut, served as a control to determine the role of FcRn-mediated vaccine efficacy. Both plasmids, HA-Fc/dimer/wt and HA-Fc/dimer/mut, contained substitutions at positions Glu318, Lys320, and Lys322 to Ala residues to remove the complement C1q binding site in order to prevent the fixation of the complement pathway (Figure 2.1A-B). The plasmids were produced by overlapping PCR of the HA gene excluding the transmembrane domain and cytoplasmic tail, with the Fc portion of mouse IgG2a. After restriction enzyme digest with SacI, I determined that the plasmid was the correct size, giving an indication of an error-free nucleotide sequence, which was verified after DNA sequencing (Figure 2.1C). The resultant plasmids were transfected into CHO cells to produce stable cell lines that expressed high levels of HA-Fc/dimer/wt and mut proteins (Figure 2.1D). Using SDS-PAGE and Western Blot analysis, the secreted fusion proteins were recognized by an antibody against Fc and existed as dimers and monomers in non-reducing and reducing conditions, respectively (Figure 2.1E).

The binding site of FcRn on IgG2a Fc overlaps with the binding site of *Staphylococcus aureus* Protein A [321]. A pull-down assay with Protein A-conjugated beads can determine if an Fc fusion protein will interact with FcRn. After incubation with Protein A-conjugated agarose beads, HA-Fc/dimer/wt interacted with Protein A, while the mutant protein did not interact with Protein A as determined by

SDS-PAGE and Western Blot analysis (Figure 2.1F). As expected, HA-Fc/dimer/mut was pulled down by anti-mouse IgG-conjugated beads (Figure 2.1F). The HA-Fc/dimer/wt and mut fusion proteins were purified from cell culture supernatants by affinity chromatography using Protein A-conjugated beads and anti-mouse IgG-conjugated beads, respectively. Using SDS-PAGE and Coomassie blue staining, both Fc fusion proteins were shown to be purified with no evidence of degradation and at high concentrations (Figure 2.1G). Taken together, HA-Fc/dimer fusion proteins were produced and purified at high levels, while HA-Fc/dimer/wt interaction with Protein A strongly suggests that the vaccine antigen will be able to interact with FcRn *in vivo*.

Intranasal vaccination with HA-Fc/dimer induces a dosage-dependent HA-specific humoral immune response

In order to demonstrate if FcRn could mediate the delivery of HA-Fc/dimer across the respiratory mucosa, I intranasally (i.n.) immunized FcRn-competent (WT) or FcRn-knockout mice (KO) with HA-Fc/dimer and boosted two weeks later. WT mice were immunized with HA-Fc/dimer/wt, HA-Fc/dimer/mut, HA alone, or PBS, while FcRn-KO mice were immunized with HA-Fc/dimer/wt (Table II.I). While HA-Fc/dimer/wt is expected to be transported via FcRn in WT mice, thus activating downstream immunity, it is anticipated that FcRn-KO mice will not be able to transport HA-Fc/dimer/wt. HA-Fc/dimer/mut proteins should not interact with FcRn expressed by WT mice, thus these latter two treatment groups served as important controls for determining FcRn-mediated induction of immunity and protection.

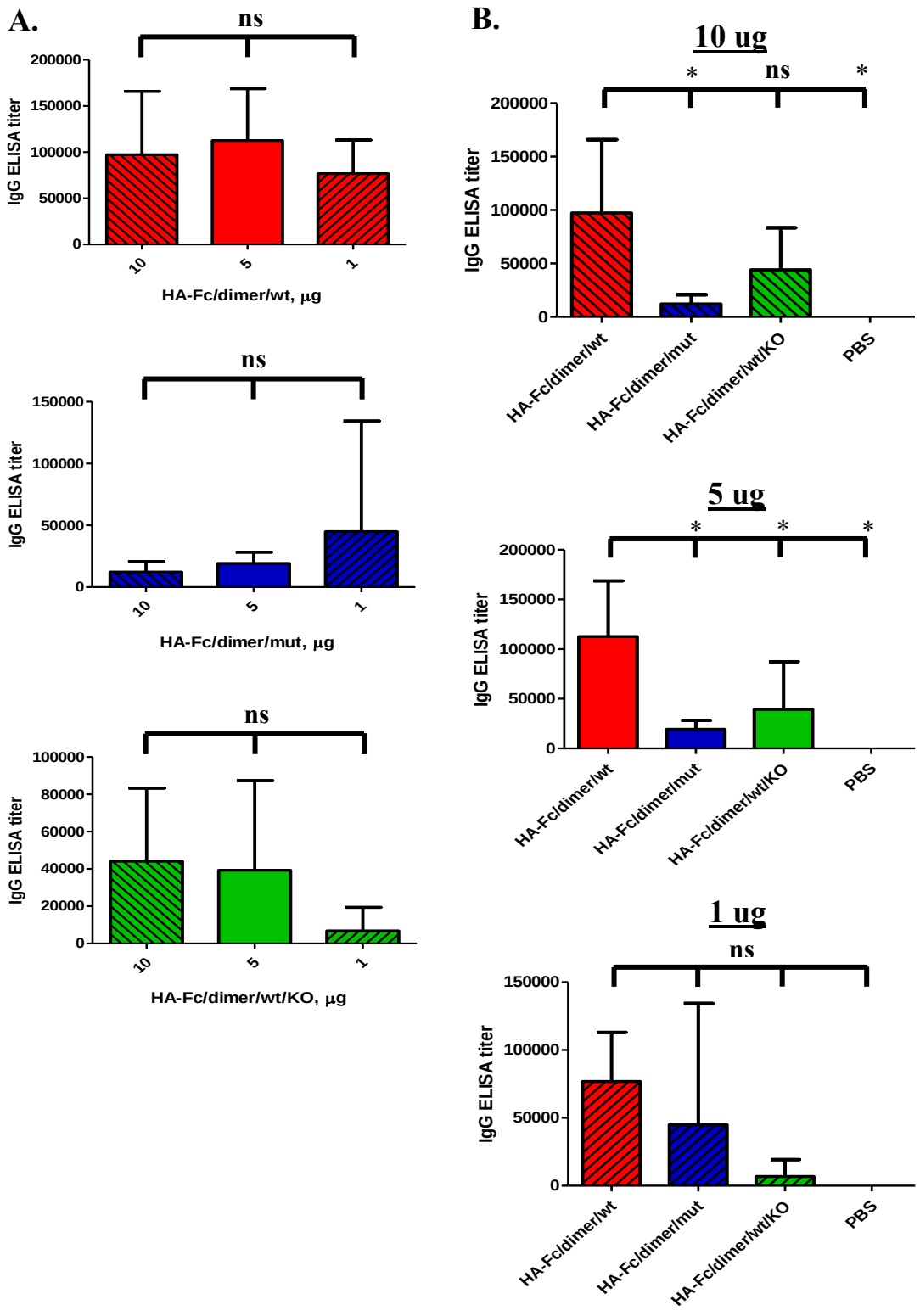
I utilized three dosages to determine the optimal amount of antigen for induction of immunity and conferral of protection. I initially intranasally administered

Table II.I: HA-Fc/dimer immunization and treatment conditions

Treatment	Mouse type	Interact with FcRn?
HA-Fc/dimer/wt	C57BL/6	Y
HA-Fc/dimer/mut	C57BL/6	N
HA-Fc/dimer/wt/KO	C57BL/6 FcRn KO	N
HA	C57BL/6	N
PBS	C57BL/6	N

10, 5, or 1 μg of HA-Fc/dimer/wt or mut, in combination with 10 μg of immunostimulatory ligand, CpG, as an adjuvant [225] [299]. Two weeks later, I boosted the mice with the same vaccine formulations. Two weeks after the boost, I collected serum from each mouse to determine HA-specific antibody levels. By ELISA, I observed that within each group, there was little appreciable difference in HA-specific antibody titers at each dosage. Importantly, at each dosage, WT mice immunized with HA-Fc/dimer/wt produced consistently higher levels of HA-specific antibody compared to the other treatment groups (Figure 2.2A). One mouse within the 1 μg dosage HA-Fc/dimer/mut-vaccinated group produced a titer of 204800, while the other mice produced antibody titers of 12800 or lower, resulting in highly skewed range of titers. Compared to the other dosages, there was no significant average difference between the dosage groups (Figure 2.2A, bottom panel). Mice mock-vaccinated with PBS had antibody titers below 100 and were not included in Figure 2.2A.

When comparing the antibody titers of all four groups within a dosage regimen, WT mice immunized with either 10 or 5 μg HA-Fc/dimer/wt induced significantly higher titers of HA-specific antibody compared to the majority of the other treatment groups (Figure 2.2B, top and middle panels). WT mice vaccinated with 1 μg of HA-Fc/dimer/wt produced higher HA-specific antibody titers on average, but there were no significant differences in titers between groups at this dosage (Figure 2.2B, bottom panel). Overall, I determined that an intranasally administered dosage of either 10 or 5 μg of HA-Fc/dimer/wt elicited robust titers of HA-specific antibody.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100000
50000
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10
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HA-Fc/dimer/mut, µg
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20000
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HA-Fc/dimer/wt/KO, µg
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HA-Fc/dimer/mut
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HA-Fc/dimer/mut
HA-Fc/dimer/wt/KO
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50000
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HA-Fc/dimer/wt
HA-Fc/dimer/mut
HA-Fc/dimer/wt/KO
PBS

Figure 2.2. Intranasal immunization with HA-Fc/dimer induces dosage-dependent HA-specific humoral immunity.

Groups of 4-5 mice were intranasally administered with purified HA-Fc/dimer/wt or mut fusion proteins at 10, 5, or 1 μg dosages or mock-vaccinated with PBS. Two weeks after initial immunization, mice were boosted with the same regimen. All treatments included 10 μg of CpG.

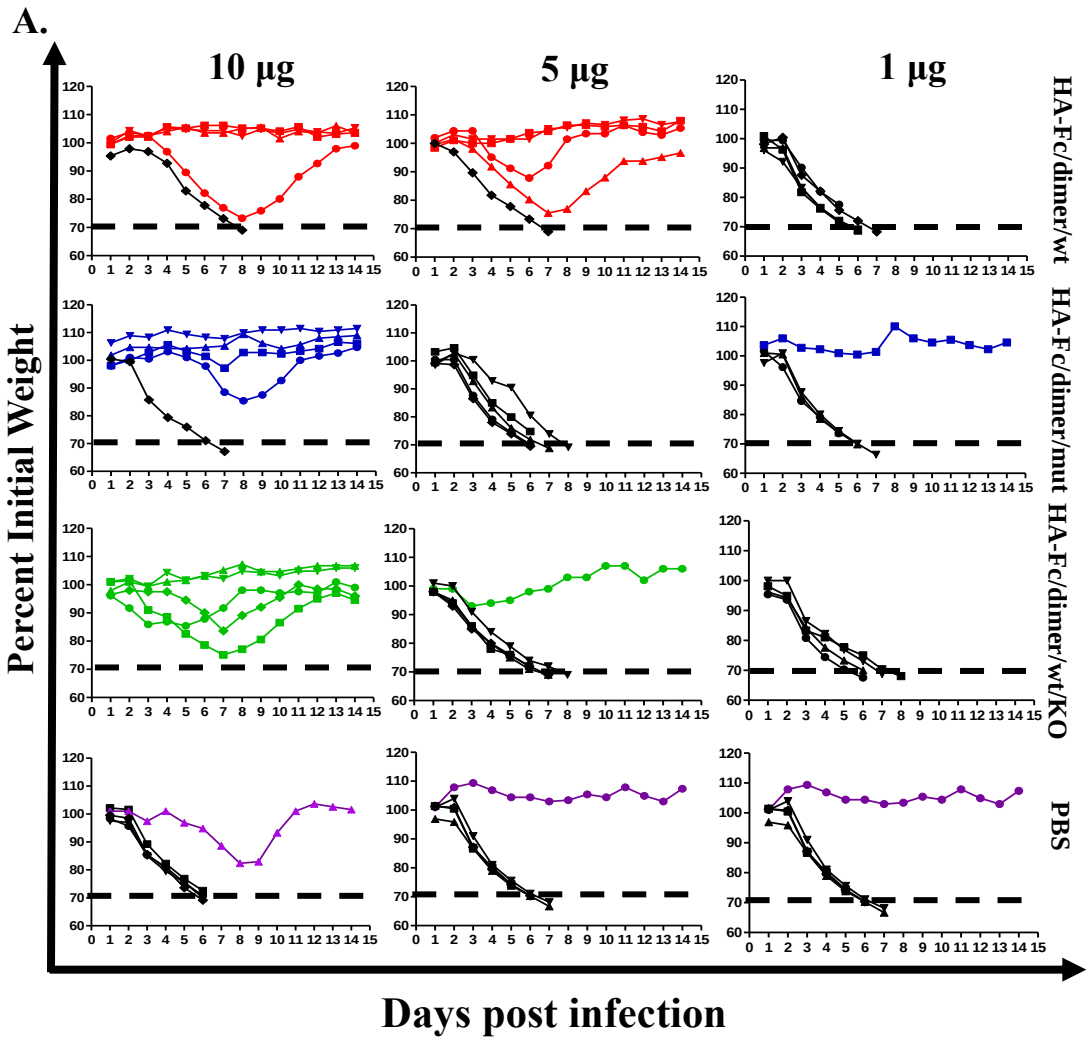
(A) Sera were collected two weeks after boost and HA-specific antibody titers were detected by ELISA. Dosage-dependent antibody titers within each group were compared. Antibody titers within the PBS mock-vaccinated group were below the level of detection and not included.

(B) HA-specific antibody titers across groups within a specific dosage were compared. Statistical differences were determined by one-way ANOVA with Tukey's multiple comparison test and values were marked as followed with asterisks in this and subsequent figures: *P<0.05, **P<0.01, ***P<0.001.

Intranasal vaccination with HA-Fc/dimer confers dosage-dependent protection against lethal challenge

In order to assess the protective efficacy of each vaccine dosage, I challenged each group within each dosage treatment with a lethal dose of PR8 (10^4 $TCID_{50}$ or 5 MLD_{50}) two weeks after a boost with the respective treatments. Mice were weighed and monitored daily. A 30% reduction in initial weight was the weight loss cutoff that necessitated euthanasia. After immunization with 10 μg of vaccine antigen, WT and FcRn-KO mice treated with either HA-Fc/wt or mut experienced relatively low levels of weight loss, with one mouse each in the HA-Fc/dimer/wt and mut-immunized groups reaching the weight loss limit by day 8 and 7, respectively. The remaining mice across the other control groups either lost weight and then recovered to initial body weight or lost minimal weight (Figure 2.3A). After immunization with 5 μg of HA-Fc/dimer, the HA-Fc/dimer/wt-vaccinated mice sustained lower levels of weight loss, while within the control groups, the majority of mice succumbed to infection or reached the weight loss cutoff between days 6 and 8 after infection. Intranasal administration of 1 μg of HA-Fc/dimer resulted in high levels of weight loss for each treatment group, with one mouse in the HA-Fc/dimer/mut-immunized group experiencing no weight loss. This mouse produced high levels of serum antibody against HA, as previously described in Figure 2.2A (Figure 2.3A).

The differences in weight loss across the treatment groups were reflected in survival rates of the mice after lethal influenza challenge. Both WT and FcRn-KO mice immunized with 10 μg HA-Fc/dimer/wt or mut had high survival rates, been 80 to 100% (Figure 2.3B, left panel). Within the 5 μg dosage treatment, WT mice


A.
10 µg
5 µg
1 µg
HA-Fc/dimer/wt
HA-Fc/dimer/mut
HA-Fc/dimer/wt/KO
PBS
Percent Initial Weight
Days post infection

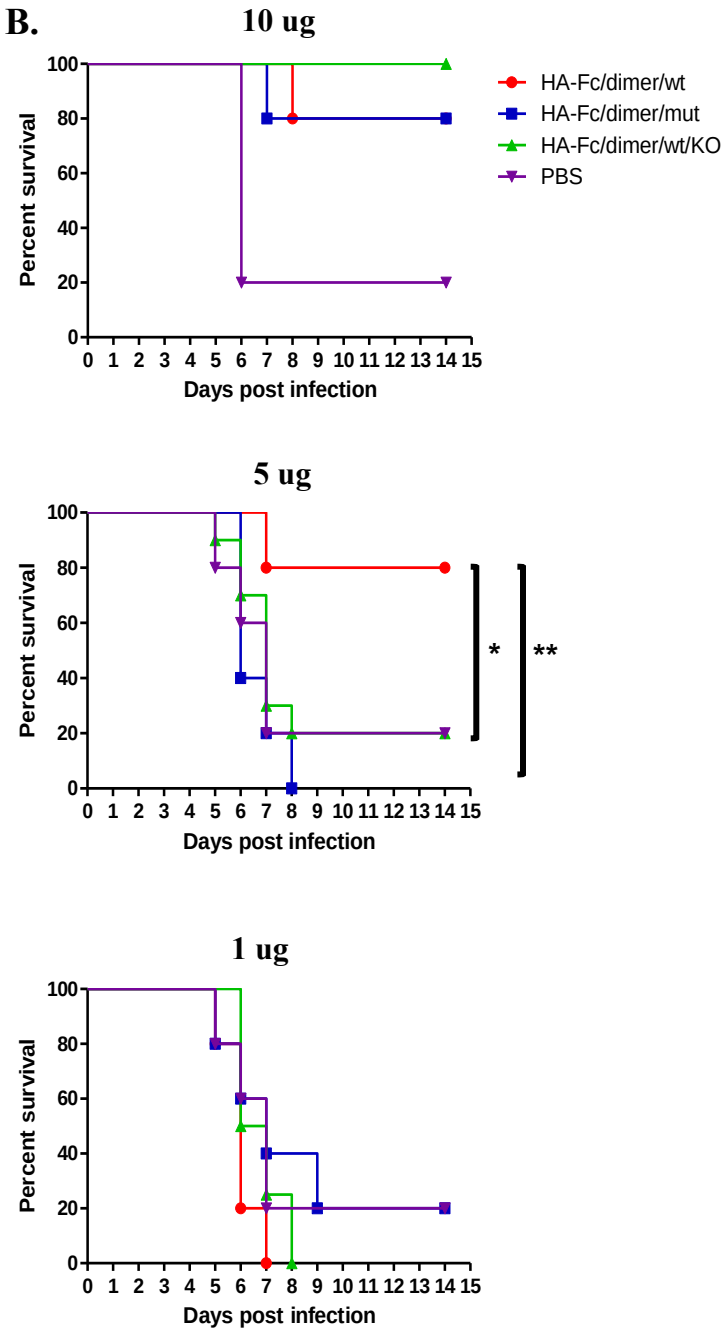


Figure 2.3. Dosage-dependent immunization with HA-Fd/dimer reduces morbidity and mortality after lethal challenge

Groups of 4-5 mice were intranasally administered with purified HA-Fc/dimer/wt or mut fusion proteins at 10, 5, or 1 μg dosages or mock-vaccinated with PBS. Two

weeks after initial immunization, mice were boosted with the same regimen. All treatments included 10 μg of CpG. Two weeks after boost, mice were infected with a lethal challenge dose of 10^4 $TCID_{50}$ or 5 MLD_{50} PR8 virus and weighed daily for fourteen days. Mice were euthanized when body weight loss reached a 30% endpoint.

(A) The individual weight loss of immunized mice in each group at each dosage.

(B) At each dosage, the Kaplan-Meier survival curves show percent survival after challenge (n=4-5). Statistical differences were determined using multiple Mantel-Cox tests.

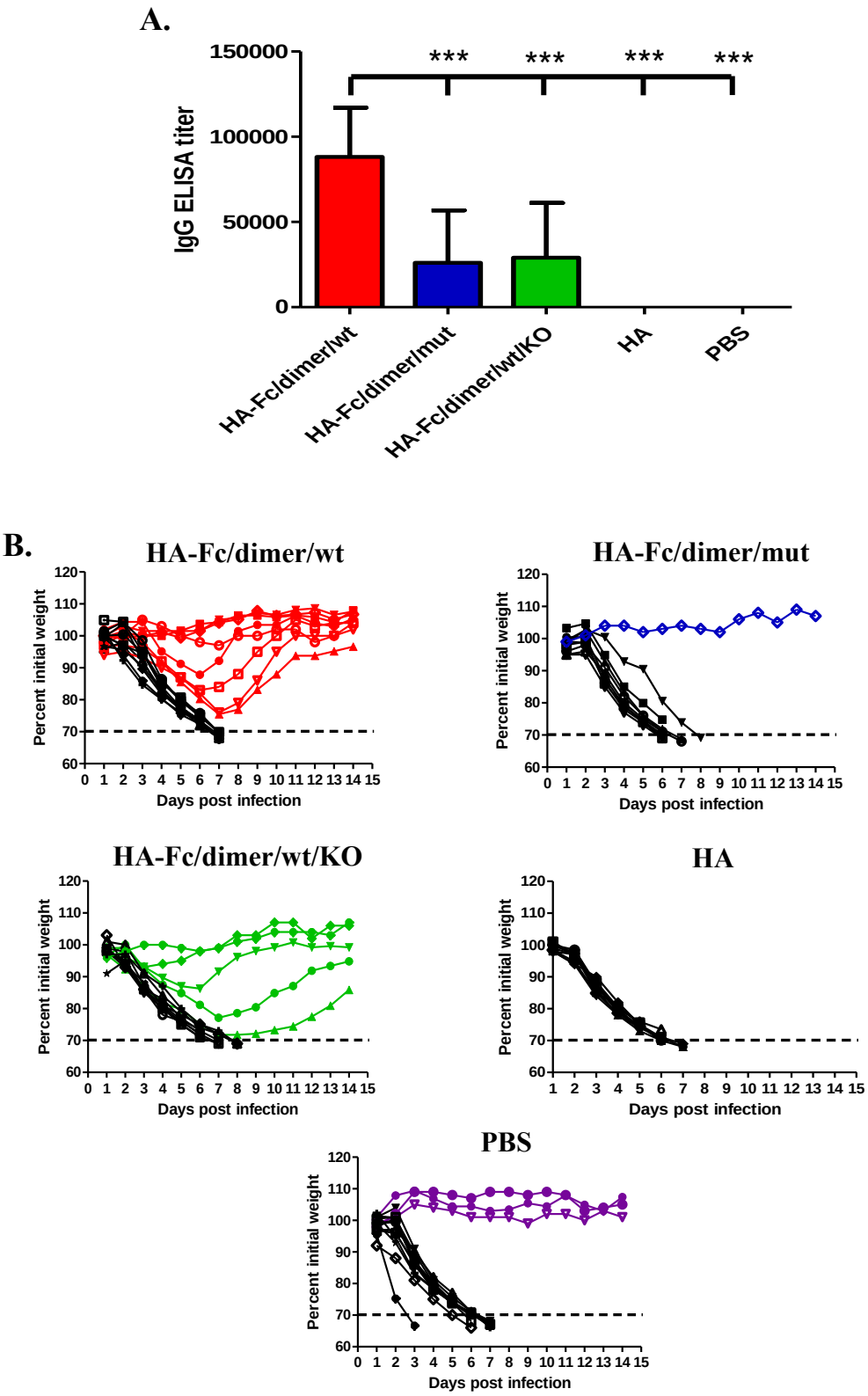
immunized with HA-Fc/dimer/wt survived at a significantly high rate, 80% survival, compared to 0 to 20% survival of the control groups (Figure 2.3B, middle panel). Mice that were immunized with 1 μg of HA-Fc/dimer sustained high levels of mortality, with survival rates of 0 to 20% for all treatment groups (Figure 2.3B, right panel). Across all dosage regimens, the majority of PBS mock-treated mice succumbed to infection or reached the 30% weight loss cutoff within 6 to 7 days after infection, though one mouse in each dosage regimen sustained weight loss and recovered or did not lose weight (Figure 2.3A). For each dosage treatment, 20% of PBS mock-treated mice survived a lethal challenge from PR8 (Figure 2.3B). Taken together, my results strongly supported 5 μg of HA-Fc/dimer as the optimal dosage for intranasal immunization that induced high levels of HA-specific antibody titers, and reduced morbidity and mortality after lethal PR8 challenge, conferring protection to 80% of HA-Fc/dimer/wt-vaccinated mice. My findings also suggest that the induction of immunity and protection are mediated by FcRn, as the control groups, where FcRn interaction with HA-Fc/dimer was impaired, had reduced levels of HA-specific antibody titers and protection against challenge.

FcRn-mediated immunization with optimized dosage of HA-Fc/dimer elicits humoral immunity and partial protection against lethal challenge

Based on my preliminary results, I moved forward to larger-scale animal studies in order to verify my findings that FcRn-mediated i.n. administration of 5 μg of HA-Fc/dimer/wt induced high levels of protection against lethal PR8 challenge compared to the control groups. While initial immunization and challenge studies utilized 5 mice per treatment group, I expanded the sample number for a total of 39 HA–

Fc/dimer/wt-immunized mice; 10 HA-Fc/dimer/mut-immunized mice; 14 HA–Fc/dimer/wt/KO-immunized mice; and 40 PBS mock-treated mice. For these animal studies, I included an additional treatment group, where 10 mice were immunized with recombinant HA that does not include the IgG Fc portion. HA alone would not be expected to interact with FcRn, providing additional insight on the potential of FcRn-independent immunity and protection. After measuring serum antibody levels two weeks after a boost, I observed that HA-Fc/dimer/wt-immunized mice had significantly higher levels of HA-specific antibody titers compared to the other treatment groups, including HA-immunized mice, which produced negligible amounts of HA-specific antibody (Figure 2.4A). These results were comparable to my previous findings, validating my premise that FcRn-mediated mucosal immunization with 5 μg HA-Fc/dimer/wt elicited high levels of HA-specific antibody titers.

The mice were then lethally challenged with PR8 as previously described and weighed daily. In contrast to my previous findings, I saw higher reductions in body weight for all groups, including HA-Fc/dimer/wt-immunized mice. Overall, 55% of the HA-Fc/dimer/wt-immunized mice succumbed to infection or reached the weight loss limit by day 7 after infection; 90% of HA-Fc/dimer/mut-immunized mice succumbed to infection or reached the weight loss endpoint between days 6 and 8; 64% of HA-Fc/dimer/wt/KO-immunized mice between days 7 and 8; 100% of HA-immunized mice between days 6 and 7; and 82% of PBS mock-immunized mice between days 3 and 7 (Figure 2.4B). I also observed a reduction in survival rates compared to my previous animal study: 45% survival of HA-Fc/dimer/wt-immunized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HA-Fc/dimer/wt
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Percent initial weight
Days post infection

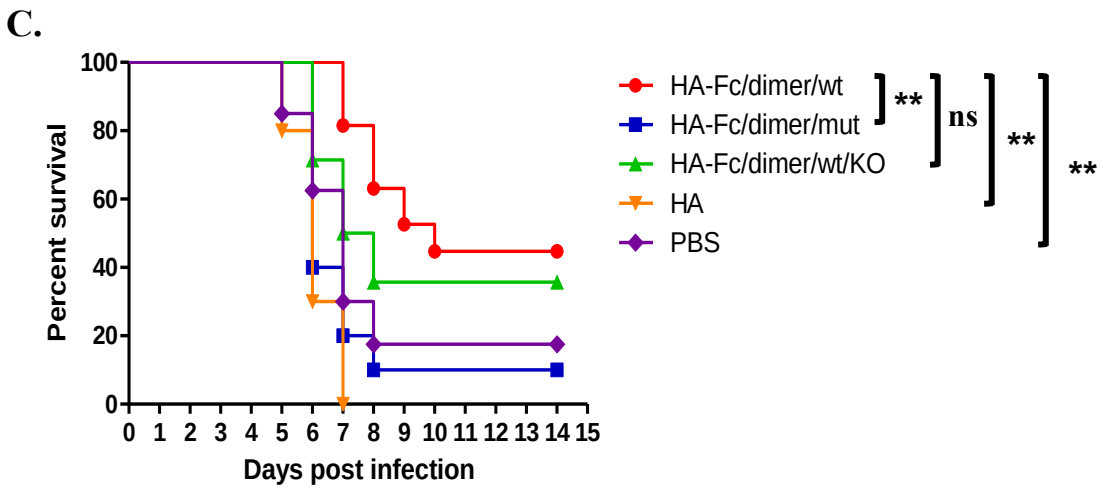


Figure 2.4. FcRn-mediated immunization with optimized dosage of HA-Fc/dimer induces robust HA-specific antibody response and confers partial protection against lethal challenge

Mice were intranasally administered with 5 μg purified HA-Fc/dimer/wt or mut fusion proteins or recombinant HA protein or mock-vaccinated with PBS. Two weeks after initial immunization, mice were boosted with the same regimen. All treatments included 10 μg of CpG.

(A) Two weeks after a boost, sera were collected and HA-specific antibody titers were detected by ELISA. HA-specific IgG antibody titers were compared between each treatment group (n=10-40). Statistical differences were determined by one-way ANOVA with Tukey's multiple comparison test.

(B) Two weeks after a boost, mice were infected as previously described and weighed daily for fourteen days. Mice were euthanized when body weight loss reached a 30% endpoint. The individual weight loss of immunized mice in each group at each dosage is shown (n=10-40).

(C) The Kaplan-Meier survival curve shows percent survival of each treatment group after lethal challenge (n=10-40). Statistical differences were determined using multiple Mantel-Cox tests.

mice, 10% of HA-Fc/dimer/mut-immunized mice, 36% HA-Fc/dimer/wt/KO-immunized mice, 0% of HA-immunized mice, and 18% survival of PBS mock-treated mice (Figure 2.4C).

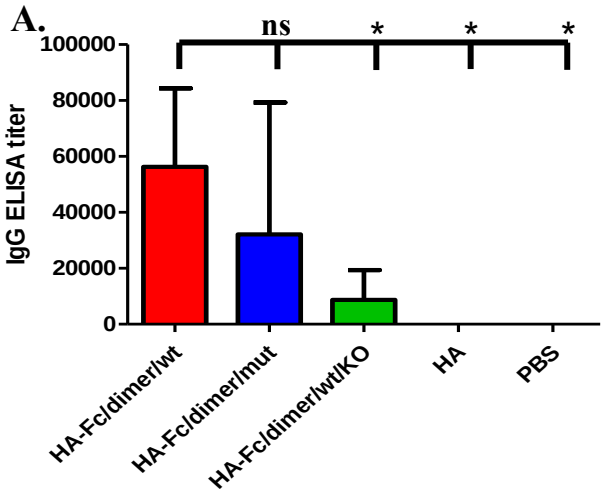
In larger-scale mouse studies, I was unable to reproduce my initial results of higher levels of protection for HA-Fc/dimer/wt-immunized mice. Though the final survival rate determined for HA-Fc/dimer/wt-immunized mice, 45%, is suboptimal for vaccine efficacy, FcRn-mediated delivery of HA-Fc/dimer/wt did confer partial protection to treated mice. The majority of the control groups experienced significantly lower survival rates after lethal challenge with influenza virus. Overall, FcRn-mediated delivery of HA-Fc/dimer allows for the induction of a robust immune response, while reducing morbidity and mortality after lethal challenge. My existing HA-Fc/dimer fusion protein does not confer acceptable levels of protection, suggesting that modifications are required to improve vaccine efficacy.

Vaccination with HA-Fc/dimer elicits robust memory immunity

Although I was unable to replicate my initial protection results after expanding my animal studies, I determined several preliminary findings on the induction of memory immunity after i.n. immunization with HA-Fc/dimer. Memory B and T cell responses established by natural infection or vaccination defend against reinfection or primary infection, respectively. During influenza infection, memory T cell responses target conserved viral protein epitopes, allowing for the potential of heterosubtypic immunity [122]. A recently identified memory T cell subset, tissue-resident memory (TRM) T cells, have been implicated in mediating enhanced protection against influenza reinfection. TRM T cells are non-circulating memory T cells that remain at

the site of infection, providing a rapid response against site-specific infections in tissues such as the lung, gut, skin, and brain. TRM T cells are characterized by expression of specific cell surface markers, the early-activation marker CD69 in concert with CD11a for CD4+ T cells and CD103 for CD8+ T cells. Lung-resident memory T cells promote viral clearance and mediate heterosubtypic protection and survival against lethal influenza challenge [107]. Induction of lung-resident memory T cells occurs after natural influenza infection. Recently, it has been demonstrated that lung-resident memory T cells can be induced by LAIV vaccination, establishing long-term, virus-specific lung-resident memory T cells in a mouse model [108]. In addition, it has been shown that only the i.n. vaccination route can elicit lung-resident memory T cells, which cannot be induced by parenteral routes, such as i.m. vaccination [102].

I determined if FcRn-mediated immunization with HA-Fc/dimer induced long-lived humoral and T cell responses. First, I characterized the memory humoral response by measuring long-lived antibody titers. Eight weeks after vaccine boost, I detected significantly higher levels of HA-specific serum antibody titers in HA-Fc/dimer/wt-immunized mice compared to the majority of the control groups (Figure 2.5A). Then, I determined if FcRn-mediated immunization with HA-Fc/dimer/wt induced lung-resident memory T cells. An intravenous (i.v.) *in vivo* antibody labeling procedure was used to differentiate circulating memory T cells from lung-resident memory T cells. Using this method, memory T cells in circulation are labeled with a fluorescently conjugated anti-T cell antibody, while T cells within the lung are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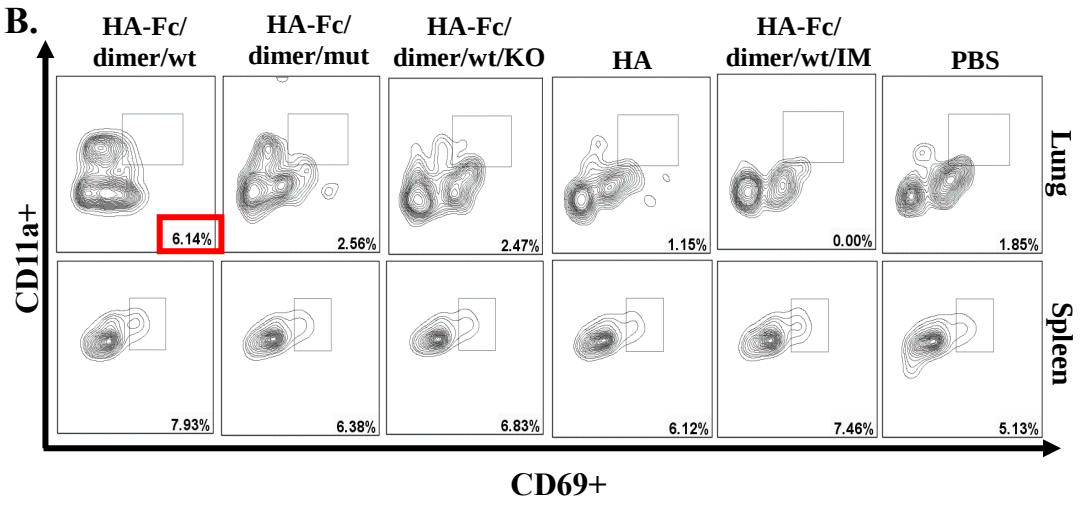
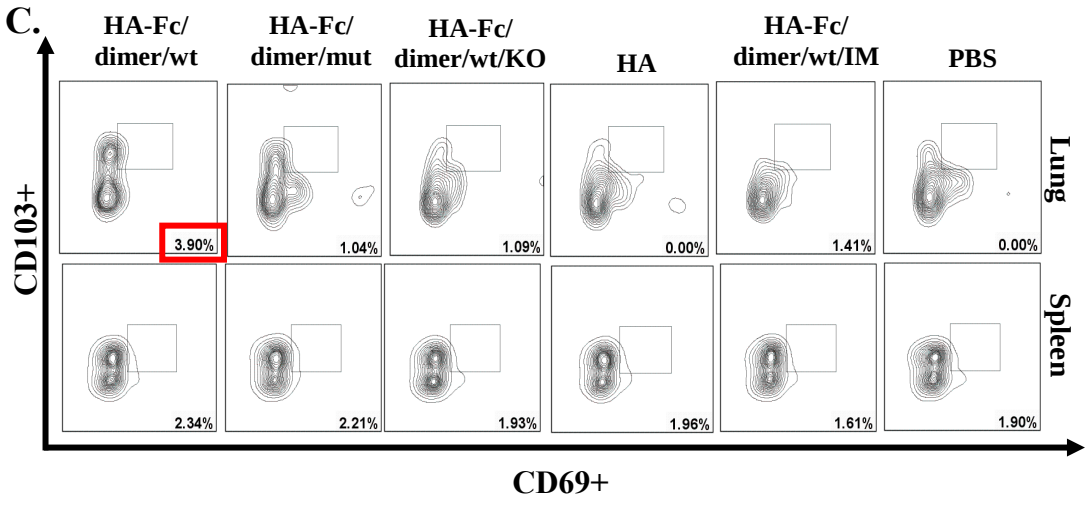

B.
HA-Fc/ dimer/wt
HA-Fc/ dimer/mut
HA-Fc/ dimer/wt/KO
HA
HA-Fc/ dimer/wt/IM
PBS
Lung
Spleen
CD11a+
6.14%
2.56%
2.47%
1.15%
0.00%
1.85%
7.93%
6.38%
6.83%
6.12%
7.46%
5.13%
CD69+


C.
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HA-Fc/ dimer/mut
HA-Fc/ dimer/wt/KO
HA
HA-Fc/ dimer/wt/IM
PBS
Lung
Spleen
CD103+
3.90%
1.04%
1.09%
0.00%
1.41%
0.00%
2.34%
2.21%
1.93%
1.96%
1.61%
1.90%
CD69+

Figure 2.5. FcRn-targeted delivery of HA-Fc/dimer elicits robust long-lasting adaptive immunity

Mice were intranasally administered with 5 μg purified HA-Fc/dimer/wt or mut fusion proteins or recombinant HA protein or mock-vaccinated with PBS. An additional control group was included, C57BL/6 WT mice intramuscularly (i.m.) vaccinated with HA-Fc/dimer/wt. Two weeks after initial immunization, mice were boosted with the same regimen. All treatments included 10 μg of CpG. Eight weeks after a boost, sera were collected from mice in each treatment group and induction of lung-resident memory T cells was determined. For detection of lung-resident memory T cells, circulating lung T cells were labeled intravenously (i.v.) with anti-CD3. Lungs cells were then stained for T cell markers CD3, CD4, and CD8 and for tissue-resident memory T cell markers CD69, CD11a and CD103.

(A) HA-specific serum IgG antibody titers were measured by ELISA to detect the long-lasting humoral response. Statistical differences were determined by one-way ANOVA with Tukey's multiple comparison test (n=4-5)

(B) Flow cytometry plots display percentages of lung-resident CD4+ T cells as CD4+CD69+CD11a+ (left panel). Percentages of circulating splenic T cells were also measured (right panel) (n=4-5).

(C) Flow cytometry plots display percentages of lung-resident CD8+ T cells as CD8+CD69+CD103+ (left panel). Percentages of circulating splenic T cells were also measured (right panel) (n=4-5).

protected from labeling. Eight weeks after vaccine boost, mice received an i.v. infusion of a fluorescently labeled anti-CD3 antibody and lungs were collected and stained for lung-resident memory CD4+ and CD8+ T cells. For this experiment, I included an additional control treatment group, WT mice i.m. immunized with HA-Fc/dimer/wt, in order to verify that parenteral routes of vaccination cannot induce lung-resident memory T cells. Importantly, I observed that i.n. administration with HA-Fc/dimer/wt promoted the induction of TRM T cells in the lungs of WT mice. There was a measurable increase in the percentages of both CD4+CD69+CD11a+ T cells (Figure 2.5B, top panel) and CD8+CD69+CD103+ T cells (Figure 2.5C, top panel) in the lungs of HA-Fc/wt-immunized mice. In contrast, the mice in the control groups, including i.m.-administered HA-Fc/wt, induced lower percentages of lung-resident memory T cells. There was minimal difference in circulating memory T cells in the spleen of immunized mice between groups, as expected (Figure 2.B and C, bottom panels). Taken together, I have demonstrated that FcRn-targeted immunization with an influenza vaccine antigen can induce lung-resident memory T cells and intranasal administration is required for this induction.

Discussion

Influenza vaccination is an important cornerstone of human health, where licensed vaccines maintain approximately 60% efficacy against circulating strains during any given season [192]. Though influenza vaccination is currently the most effective method to prevent infection, efforts are being made to improve vaccine efficacy. In order to provide an alternative strategy to current mucosal influenza

vaccines, I developed a mucosal delivery system of influenza vaccine antigens that cross the respiratory mucosa via FcRn, using HA as a model vaccine antigen. Here, I assessed the potential of HA-Fc/dimer as an FcRn-mediated mucosal vaccine against influenza virus. I determined that intranasal vaccination with HA-Fc/dimer induced robust humoral immunity and local memory T cell responses in a dosage-dependent manner while conferring partial protection to lethal challenge.

My study established that an HA-based Fc fusion protein can be delivered across the respiratory mucosa via FcRn, and promote the induction of robust HA-specific immunity. This offers FcRn-mediated delivery of influenza vaccine antigens as an attractive strategy to develop mucosal subunit influenza vaccines. In contrast to intramuscular or intradermal influenza vaccines, my influenza vaccine model, HA-Fc/dimer, is designed to cross mucosal barriers through the interaction of the Fc portion of HA-Fc/dimer with FcRn expressed at the respiratory mucosal epithelium. Fc fusion to protein-based ligands (e.g., cytokines, hormones, and enzymes) and their interaction to FcRn have been well characterized to enhance immunogenicity and serum half-life, and improve stability and solubility [349]. Previous studies have exploited the potential of Fc fusion proteins as therapeutics for various medical conditions (e.g., EpoFc, based on erythropoietin, a hormone that promotes red blood cell formation; and rFIXFc, composed of clotting protein, Factor IX) [374]. In addition, we and others have shown the efficacy of Fc fusion proteins based on viral proteins to promote protection against a number of important pathogens, including HSV-2 glycoprotein gD; HIV Gag protein p24 and ENV protein gp120; and SARS-CoV spike protein receptor binding domain (RBD). Fc fusion proteins targeting

influenza have been used as both an IL-7-based prophylactic and as subunit vaccines [340] [365] [366] [375] [376].

Building upon our previous work developing a mucosal vaccine against HSV-2, I developed an HA-based Fc fusion protein, HA-Fc/dimer, for FcRn-mediated induction of protective immunity against influenza infection. Previous studies have used HA-based fusion proteins for vaccination against influenza virus. These strategies generally utilized the highly variable HA1 head domain, or portions of it, as the vaccine antigen. They were largely administered through parenteral routes such as subcutaneous (s.c.)., and required an additional boost as part of the vaccination regimen [377] [378] [379] [380] [381]. One study utilized an HA1 head-based Fc fusion protein for intranasal immunization and detected local and systemic antibody production, but was unable to show protection against challenge [382]. These studies acknowledge the advantages of including the Fc portion of IgG in their vaccine construct, though, as most of these constructs were administered parenterally, they do not seem to emphasize the role of FcRn in mediating antigen delivery.

In contrast, I propose that delivery of HA-Fc/dimer across the respiratory mucosa to the underlying lamina propria is mediated by FcRn, leading to the activation of long-lasting humoral and cell-mediated immunity. In immunized mice, I detected significantly higher levels of durable HA-specific antibody titers in HA-Fc/dimer/wt-treated mice, which initially was reflected in lower levels of morbidity and mortality after lethal challenge. In FcRn-competent or KO mice immunized with 10 μg of HA-Fc/dimer/wt or mut, survival rates were 80 to 100%, regardless of treatment, suggesting an FcRn-independent mechanism for vaccine antigen delivery.

The respiratory tract expresses sialic acids, which are the receptors for HA. It is possible that the vaccine antigen concentration was high enough to allow for attachment and uptake by respiratory epithelial cells via sialic acid receptor-mediated endocytosis. Although high levels of survival were achieved by both HA-Fc/dimer/mut- and HA-Fc/dimer/wt/KO-immunized mice within the 10 μg dosage regimen, the production of HA-specific antibody titers was still lower than that of HA-Fc/dimer/wt-immunized mice. Within the 5 μg dosage treatment groups, only WT mice immunized with 5 μg of HA-Fc/dimer/wt experienced high levels of survival that were comparable to the 10 μg dosage groups. Thus, I believed it was reasonable to proceed with a 5 μg dosage regimen over a 10 μg dosage. In addition, from a production perspective, when developing vaccines, dosage-sparing is an important consideration, where a reduction in the amount of antigen required reduces both cost and time of production. Furthermore, the lowest dosage that can offer optimal efficacy allows for the production of greater numbers of total vaccine dosages, since each dose uses less vaccine antigen, which is a significant advantage in times of vaccine shortages.

Ultimately, after expanding animal studies using 5 μg of HA-Fc/dimer to include larger numbers of mice, I was unable to replicate the initial survival rates after lethal challenge, resulting in partial protection against infection. Based on these results, HA-Fc/dimer immunization could not serve as a reliable mucosal vaccine in its current form. It is conceivable that pursuing further animal studies with higher dosages, such as 10 μg, would shed some insight on the inconsistencies of the previous animal studies with the 5 μg dosage regimen. Though increasing the vaccine

dosage is an option to improving vaccine efficacy, my future strategy will first rely on making structural modifications to the HA-based Fc fusion protein, while maintaining the 5 μg dosage in order to preserve the dose-sparing advantage if feasible, to determine if such alterations can improve vaccine efficacy.

Although immunized mice were unable to be fully protected against lethal challenge, I did demonstrate that i.n. immunization with HA-Fc/dimer was able to induce memory immunity, both the production of long-lived HA-specific antibodies and the induction of TRM T cells. TRM T cells are induced by natural infection and in response to vaccination against a number of pathogens, including live attenuated *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) against tuberculosis (TB); and murine cytomegalovirus (MCMV) vector expressing respiratory syncytial virus (RSV) matrix (M) protein [107]. The protective benefits of lung-resident memory CD4+ and CD8+ T cells include the promotion of rapid viral clearance at the site of influenza infection and enhanced protection and survival against homologous and heterosubtypic influenza challenge [98]. Recently, it has been demonstrated that i.n. vaccination with LAIV can induce protective lung-resident T cells in mice, in contrast to i.m. vaccination with IIV or LAIV, which confirms a previous report that lung-resident T cells are induced solely via intranasal vaccination, and not by parenteral routes [108]. I have demonstrated for the first time that after intranasal vaccination with an Fc fusion protein-based influenza vaccine, FcRn-competent mice immunized with HA-Fc/dimer/wt had a measurable induction of lung-resident memory T cells. Importantly, i.m. immunization with HA-Fc/dimer/wt was unable to induce

appreciable percentages of lung-resident memory T cells, confirming that only the intranasal route of vaccination allows for lung-resident memory T cell induction.

In summary, I investigated the potential of an FcRn-targeted delivery platform of influenza vaccine antigens to promote protective immunity. Although I establish that intranasal vaccination with HA-Fc/dimer elicits durable B- and T-cell responses in a dosage- and FcRn-dependent manner, vaccine-induced immunity could not fully protect against lethal challenge. Moving forward, modifications to the HA-based Fc fusion protein in both the HA and the Fc subunits are necessary in order to improve the vaccine efficacy. In addition, a thorough analysis of both local and systemic immune responses is required to determine the contributions of both arms of immunity. During influenza infection, local and systemic immune responses inhibit and control infection. Secreted IgA antibodies prevent initial viral infection in the upper respiratory tract. Systemic and locally produced IgG antibodies neutralize the newly generated virus and prevent spread in the lower respiratory tract. In addition, the induction of germinal centers in local and secondary immune sites enhance local immunity. In contrast to parenteral vaccination, mucosal vaccination induces immune responses both at the site of immunization and in peripheral immune organs [229]. Thus, a successful mucosal vaccine model should induce both local and systemic immunity. I have demonstrated that HA-based Fc fusion proteins induce robust HA-specific antibody titers in serum, which is an important component in systemic immunity, and promote the induction of lung-resident memory T cells, which has been shown to be significant in local memory response. I will determine if FcRn-targeted delivery of HA-Fc fusion proteins can induce other facets of durable local

and systemic immunity while improving vaccine-mediated protection. By establishing my model of FcRn-targeted delivery of influenza antigens as capable of promoting long-lasting protection, I will be able to expand my platform to other antigens that have the broader potential for enhanced protection against both homologous and heterologous strains of influenza virus.

Chapter 3: FcRn-targeted Intranasal Delivery of Trimeric Hemagglutinin Protects Mice from Influenza Infection

Abstract

Influenza virus infects humans through the respiratory tract, thus an effective and safe mucosal vaccination strategy is currently needed to prevent its airway infection and transmission, especially for high-risk or vulnerable persons. The neonatal Fc receptor (FcRn) for IgG mediates IgG antibody transport across the polarized epithelial cells lining mucosal surfaces, including the respiratory epithelium. By capitalizing on this natural IgG transfer pathway, in this study, I fused a monomeric Fc portion of IgG to soluble hemagglutinin (HA) that carries a T4 fibritin trimerization domain. The trimeric HA-Fc/wt proteins were characterized by binding to FcRn in comparison with a mutant version, HA-Fc/mut, which had its FcRn binding sites removed. Intranasal (i.n.) immunization with the trimeric HA-Fc/wt proteins in mice conferred significant protection and reduced viral loads in the lungs after challenge, in comparison with wild-type mice immunized by HA-Fc/mut, HA alone, and PBS or FcRn knockout mice immunized with the HA-Fc/wt protein. The significant levels of protection in the mice immunized with HA-Fc/wt protein were attributed to the production of higher levels of neutralizing antibody, robust local immunity, including IgA production and activation of germinal centers, and long-lasting B- and T-cell responses, including the induction of HA-specific memory and plasma cells, and tissue-resident memory (TRM) T cells in the lung. Taken together, my results prove that by targeting the IgG transfer pathway, FcRn is able to

deliver trimeric influenza HA vaccine antigens into the airway which elicits a protective immunity against influenza infection in the respiratory tract. Since trimeric HA-Fc/wt protein also interacts with conformation-dependent Group 1 and 2 HA stalk antibodies, this study further suggests that an FcRn-mediated mucosal vaccination strategy could be used for delivering a universal influenza vaccine antigen.

Introduction

The respiratory tract is a site of frequent exposure to numerous pathogens. The respiratory tract can resist infection and facilitate the clearance of invading pathogens through a variety of mechanisms, including the formation of the airway barrier that is lined by polarized epithelial cells and the development of a variety of innate and adaptive immune responses. The adaptive immune responses, including effector and memory T and B lymphocytes and local and circulating antibodies, can prevent or decrease the severity of primary or subsequent respiratory infections. For example, tissue-resident memory (TRM) T cells that reside in the lung are recently appreciated subset of memory T cells and are required for optimal protection against previously encountered pathogens [98]. Presently, most vaccines against respiratory infections are designed for delivery via parenteral routes, including the muscle or skin, for protection against infections in the lung. However, they elicit relatively poor mucosal immune responses in the respiratory tract although they often induce robust systemic immune responses. A partial attribution to this failure is that systemic vaccination fails to induce strong mucosal antibody and cell-mediated immunity,

including TRM T cells that reside in the lung mucosa and are readily accessed in the event of re-exposure of a pathogen. An ideal strategy to prevent respiratory infections is to develop a mucosal vaccine that mimics natural respiratory infections by engendering beneficial immune responses in the lung. This goal can only be achieved when vaccine antigens are directly administered via the respiratory route, inducing an effective lung immune response. However, our ability to safely and effectively deliver vaccine antigens across the respiratory mucosal barrier is very limited. First, the mucosal vaccine must avoid inducing excessively robust inflammatory responses that may lead to lung damage and exacerbate other chronic diseases, such as asthma or chronic obstructive pulmonary disease (COPD). Second, since respiratory infections more commonly affect the young and elderly individuals, this restricts the implementation of certain types of mucosal vaccines, such as live attenuated vaccines [279]. Given the high impact of respiratory infections in the public health, the development of an effective and safe mucosal vaccination strategy preventing the most common respiratory infections is greatly needed as an urgent global health priority.

Epithelial monolayers lining the respiratory, intestinal, and genital tracts, as well as the placenta, polarize into apical and basolateral plasma membrane domains, which are separated by intercellular tight junctions. The neonatal Fc receptor (FcRn), an MHC class I-related receptor for IgG, is expressed in these epithelial monolayers and mediates the bilateral transfer of IgG antibody across the polarized epithelial cells. By transcytosing IgG across the epithelium, FcRn provides a line of humoral defense at mucosal surfaces, in addition to seeding maternal immunity during

neonatal life. A hallmark of FcRn is its interaction with IgG antibody in a pH-dependent manner, binding IgG at acidic pH (6.0 – 6.5) and releasing IgG at neutral or higher pH. FcRn mostly resides within low pH endosomes and binds IgG through the Fc region. Normally, IgG enters epithelial cells via pinocytotic vesicles that fuse with acidic endosomes. IgG bound to FcRn then enters a non-degradative vesicular transport pathway within epithelial cells. Bound IgG is transported to the apical or basolateral surface and released into the lumen or submucosa [307]. Evidence of IgG transport across the respiratory epithelia by FcRn suggest that FcRn might also transport a vaccine antigen from a respiratory pathogen, if fused with the Fc portion of IgG, across the respiratory mucosal barrier.

In order to test this possibility, I used a model virus, influenza A virus, a globally important respiratory pathogen which causes a high degree of morbidity and mortality annually and is a significant burden to public health. Hemagglutinin (HA), a major surface envelope glycoprotein of the virus, primarily mediates the interaction of influenza virions via cell surface sialic acid receptors. After binding, the virions are internalized through endocytic pathways to infect epithelial cells. The HA protein consists of the membrane-distal immunodominant HA1 globular head domain and the membrane-proximal HA2 stalk domain. The head domain shows high structural plasticity which is strongly affected by antigenic drift; in contrast, the stalk domain exhibits a high degree of conservation [2]. Because of its critical role in the early steps of viral infection and as the major antigen for eliciting both humoral and cellular immunity, I determined the ability of FcRn to deliver the viral protein, HA, across the respiratory epithelial barrier for the induction of protective immunity. I have

previously utilized an HA-based fusion protein, HA-Fc/dimer, for FcRn-mediated respiratory antigen delivery and observed that intranasal immunization with HA-Fc/dimer elicited HA-specific immunity and conferred partial protection, but further improvements were necessary to enhance vaccine efficacy. In this study, I made several significant modifications to the HA-based Fc fusion protein to produce HA-Fc fusion protein for immunization. I further defined protective local and systemic immune responses and mechanisms relevant to this delivery of mucosal vaccine antigens in the lung in a mouse model. These data suggest that FcRn-mediated intranasal delivery of influenza HA antigen, in the context of the HA-Fc fusion protein, induces a robust and multifaceted immunity, characterized by high levels of long-lived local and serum antibody and T-cell responses, including TRM T cells in the lung, thus providing a strong frontline resistance to lethal influenza infections. Immunization with HA-Fc also conferred high levels of protection against lethal challenge and resulted in reduced viral loads and pulmonary pathology. By using a model influenza HA antigen, these data suggest that FcRn-targeted delivery of influenza vaccine antigens in the respiratory tract represents a platform technology that allows us to further develop novel mucosal vaccines, including universal influenza vaccines, against seasonal and emerging pandemic influenza virus.

Materials and Methods

Cells, antibodies, and virus

Madin-Darby Canine Kidney Epithelial (MDCK) cells were obtained from Dr. Pamela Bjorkman (California Institute of Technology) and Chinese hamster ovary

(CHO) cells were purchased from the American Tissue Culture Collection (ATCC). MDCK cells were maintained in Opti-MEM complete medium (Invitrogen Life Technologies) and CHO cells were maintained in DMEM complete medium (Invitrogen Life Technologies), both supplemented with 10% FBS, 2 mM l-glutamine, nonessential amino acids, and penicillin (0.1 μg/ml)/streptomycin (0.292 μg/ml). Stably recombinant CHO cell lines were grown in a complete medium with G418 (500 μg/ml). All cells were grown at 37°C in 5% humidity. Influenza A/Puerto Rico/8/34/Mount Sinai/H1N1 (PR8) virus was generously provided by Dr. Peter Palese (Icahn School of Medicine at Mount Sinai) and was amplified in 10-to 11-day-old embryonated chicken eggs and titrated by 50% endpoint dilution assay. The horseradish peroxidase (HRP)-conjugated streptavidin and anti-mouse IgG, IgG1, IgG2b, and IgG2c were purchased from Southern Biotech (Birmingham, Alabama). HA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or generously provided by Dr. Florian Krammer (Icahn School of Medicine at Mount Sinai) and Dr. Jeffrey Boyington (National Institutes of Health). Recombinant HA was purchased from Sino Biologicals (Shanghai, China) or obtained from Biodefense and Emerging Infections Research Resources Repository (BEI Resources**,** Manassas, VA).

Construction of influenza HA-Fc expression plasmids

To make an IgG Fc fusion protein, a pCDNA3 plasmid encoding the hinge, CH2 and CH3 domains of mouse IgG2a Fc that was previously constructed served as a template for the Fc fragment. The rationale for using mouse IgG2a is because it has the highest affinity for activating FcγRI, but the lowest affinity for inhibitory

FcγRIIB. In this plasmid, the Glu318, Lys320, and Lys322 residues were replaced with Ala residues to remove the complement C1q binding site. In addition, in order to produce a mutant form of IgG Fc fusion protein that cannot bind to FcRn, the His310 and His433 residues were changed to Ala residues to eliminate FcRn binding sites. To make a monomeric Fc fragment, we converted the Cys224, Cys227, and Cys229 residues to the Ser residues with a DNA mutagenesis kit (Clontech). The first 20 amino acids of the signal peptide from T cell surface glycoprotein CD5 was included before the Fc nucleotide sequence, in order to direct the protein for translocation into the ER to allow for secretion from the cell [383]. To make HA-Fc fusion genes, the extracellular portion of PR8 HA was amplified by PCR from a plasmid containing full-length PR8 HA using the primer pair (5'-GGATCAGGCGGGGGTGGGTCCGGAGGAGGTGGCTCGGGATCTG ACA CAATATGTATAGGCTACCATGC-3', 5'-CCTCTGGGCACCAGGCTTCTTGATCCTGAGCCT GATCCCTGATAGATCCCCATTGATTCC-3'). The IgG Fc antisense primer and the HA sense primer contain complementary glycine and serine codons to produce a 14GS linker to bridge the IgG Fc and HA fragments. A protein trimerization domain was amplified from a plasmid containing the T4 fibritin foldon sequence provided by Dr. Daniel Perez (University of Georgia). Similarly, the HA antisense primer and the foldon sense primer contain complementary glycine and serine codons to introduce a 6GS linker between the HA and foldon fragments. The Fc, HA, and foldon fragments were fused by overlapping PCR and ligated into the pCDNA3 vector. All of the

resultant plasmids were confirmed by double-stranded DNA sequencing to verify the fidelity of PCR amplification and DNA cloning.

Expression and characterization of PR8 HA-Fc fusion proteins

The different HA-Fc plasmids were transfected into CHO cells using PolyJet (SignaGen) according to the manufacturer's instructions. Stable cell lines were selected and maintained under G418 (0.5-1 mg/ml). Expression and secretion of HA-Fc fusion proteins were determined by immunofluorescence assay, SDS-PAGE and Western blotting analysis. The soluble HA-Fc proteins were produced by culturing CHO cells in complete medium containing 5% FBS with ultra-low IgG. The proteins were purified by affinity chromatography using Protein A- (Thermo Scientific) and anti-mouse IgG- (Rockland) conjugated agarose beads and dialyzed with PBS. Protein concentrations were determined using NanoDrop Spectrophotometer (Thermo Scientific). Molecular graphics and analyses of the structure of the HA-Fc proteins were performed with Phyre 2 (Phyre2 ref) and the UCSF Chimera package (University of California, San Francisco).

The trimerization of HA-Fc was determined by the bis[sulfosuccinimidyl] suberate (BS^3, Thermo Scientific) cross-linker method according to the manufacturer's instructions. Briefly, HA-Fc proteins (0.1 mg) were incubated with BS^3 in 50-fold molar excess for 2 hr on ice. The reaction was then quenched by adding 1M Tris-HCl, pH 7.5 to a final concentration of 50 mM Tris-HCl and further incubated for 15 min at room temperature. The protein samples were subjected to electrophoresis under reducing and denaturing conditions and subsequently analyzed by Western blotting analysis with anti-HA and anti-mIgG2a antibodies.

Immunofluorescence assay (IFA)

Immunofluorescence was performed as previously described [370]. Briefly, cells were grown on coverslips for 48 hr. The cells were rinsed with HBSS and fixed with 4% paraformaldehyde (Sigma) in HBSS for 20 min and quenched with 100 mM glycine in PBS for 10 min. Cells were permeabilized with 0.2% Triton-X in HBSS for 5 min and incubated with blocking solution (3% normal goat serum in PBS) for 30 min. Cells were incubated with anti-HA antibodies diluted in blocking solution for 2 hr in the dark. After washing, Alexa Fluor 555-conjugated anti-mouse IgG1 and Alexa Fluor 488-conjugated anti-mouse IgG2a secondary antibodies were added to 1 hr in the dark. All steps were performed at room temperature. Cells were washed with PBS and mounted to slides with ProLong Antifade solution (Thermo Scientific). Images of the stained cells were processed using a Zeiss LSM 510 confocal fluorescence microscope and LSM Image Examiner software (Zeiss).

SDS-PAGE gel and Western blotting

Protein concentration and quality were assessed by 8-12% SDS-PAGE gels under reducing and non-reducing conditions. Protein in gels was either stained with Coomassie blue dye or used for transferring onto nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with 5% milk in PBST (PBS and 0.05% Tween-20) and incubated overnight with anti-IgG2a-HRP (1:10,000) or anti-HA antibodies (1:2000). For HA probing, membranes were further incubated with the anti-mouse IgG1-HRP antibody (1:5,000) for 2 hr. SuperSignal West Pico

PLUS ECL substrate (Thermo Fisher) was used to visualize protein in membranes and images were developed and captured by the Chemi Doc XRS system (BioRad).

Mouse immunization and virus challenge

All mice were housed in the University of Maryland animal facilities and all animal experiments were performed with the approval of the Institutional Animal Care and Use Committee. FcRn knockout mice in the C57BL/6 background is a kind gift from Dr. Derry Roopenian (Jackson Laboratory). Six to eight-week-old female C57BL/6 mice (Charles River Laboratory) and FcRn knockout mice were intranasally (i.n.) immunized with 20 μl of 5 μg HA-Fc/wt, HA-Fc/mut, recombinant HA, or PBS. All vaccine proteins or PBS were mixed with 10 μg of CpG ODN 1836 (Invivogen). Two weeks later, the mice were boosted with the same vaccine formulations. Two weeks after the boost, mice were i.n. infected with either lethal doses (10^4 $TCID_{50}$) or sublethal doses (400 $TCID_{50}$) of the PR8 virus. For immunizations and challenge, all mice were anesthetized with intraperitoneal (i.p.) injection of 100 μl of fresh Avertin (40 mg/ml, Fisher Scientific) and laid down on their backs to allow for recovery. After infection, mice were monitored daily for weight loss and other clinical signs of illness for 14 days. Animals that lost 25% or over of their body weight on the day of infection or had become grossly moribund were euthanized.

Collections of bronchoalveolar lavage (BAL) and nasal wash fluids and preparation of single-cell suspensions from tissues

BAL and nasal wash fluids were collected as previously described [238] [365]. Briefly, a small incision was made in the trachea. A syringe with a thin tube inserted

at the tip was filled with PBS. The syringe was inserted first into the trachea towards the lungs and PBS was carefully injected into the lungs for the collection of BAL. For sampling the nasal wash, the syringe was similarly inserted into the trachea but towards the nasal cavity. PBS was carefully injected into the nasopharynx and collected when it flowed from the nares. BAL and nasal wash fluids were then subjected to low-speed centrifugation and the supernatants were retained.

The single-cell suspensions from the mediastinal lymph nodes (MeLN) or spleen were made by mechanical abrasion of the organs. For isolation of cells from bone marrow, tibias and femurs were removed and the ends were clipped. The bone marrow was flushed out with RPMI1640. Isolation of single cells from the lung was performed as previously described (ref Nat bio). Briefly, after perfusion with 30 ml of PBS, lungs were minced and treated to enzymatic digestion in RPMI with pronase (1.5 mg/ml), Dispase (0.2%), and DNase (0.5 mg/ml) for 40 min at 37°C with rotation. All cells from the MeLN, spleen, bone marrow and lung were filtered through a 40 μm nylon cell strainer and treated with red blood cell (RBC) lysis buffer (0.14 M NH4Cl, 0.017 M Tris-HCl at pH 7.2). All cells were washed and suspended in 2% FBS (Invitrogen) in PBS (FACS buffer) or RPMI1640 complete medium with 1-2% FBS. For each experiment, cells were pooled from 3–5 mice in each animal group.

Intravenous in vivo antibody labeling and flow cytometry

For intravenous *in vivo* labeling of circulating T cells, mice were intravenously injected with 3 μg of PerCP-Cy5.5-conjugated anti-mouse CD3e antibody. After 10 min, lungs were perfused with 30 ml of PBS and the single-cell suspensions were

made as described above. Fc block (anti-mouse CD16/CD32, BD Biosciences, 1 μg/sample) was added to the lung and spleen cell samples and incubated for 30 min at 4°C. After wash with FACS buffer, cells were incubated with fluorescently-conjugated antibodies to stain for T cell markers, CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD69 (H1.2F3), CD11a (2D7), and CD103 (M290), for 1 hr at 4°C in the dark. For detection of activated germinal centers, MeLN and spleen cells were incubated with fluorescently-conjugated antibodies to stain for activated germinal center B cell markers, B220/CD45R (RA3-6B2), CD19 (1D3), FAS/CD95 (Jo2), and PNA, for 1 hr at 4°C in the dark. Isotype control antibodies were included in each experiment. After washing, cells were resuspended in 2% paraformaldehyde and analyzed using a FACSAria cytometer (BD Biosciences) and FlowJo software (Tree Star).

Intracellular cytokine staining

For determining T cell-derived cytokine levels, intracellular cytokine staining was performed as previously described [365]. Briefly, single-cell suspensions from the lungs were stimulated with 2 μg of recombinant HA for 5 hr at 37°C. Cells were then incubated with GolgiStop (BD Biosciences) for an additional 5 hr. After wash, cells were incubated with Fc block and then stained with fluorescently-conjugated antibodies for T cell surface markers, CD3, CD4, and CD8. Cells were fixed and permeabilized by incubating with BD CytoFix/Perm. After FACS buffer wash, cells were stained with antibodies against cytokines IFN-γ and TNF-α. All blocking, antibody incubation and permeabilization steps were performed in the dark for 20 min

at 4°C. After FACS buffer wash, cells were resuspended in 2% paraformaldehyde and analyzed by flow cytometry as described above.

Virus titration and pulmonary pathology

Viral titers were determined by the Reed-Muench method of 50% endpoint dilution assay and hemagglutination assay as previously described [384] [385] [386] [387]. Briefly, after PR8 challenge, mouse lungs were collected six days after sub-lethal infection or four days after lethal infection. Individual lungs were homogenized in the TissueLyser LT (Qiagen). After centrifuging the homogenates, the supernatants were serially diluted and incubated on MDCK cells for 1 hr. The supernatants were removed from cells and replaced with serum-free Opti-MEM with 1 μg/ml TPCK-trypsin. After incubation at 37°C for three days, an equal ratio of the supernatant was mixed with chicken RBCs and incubated for 35 min. Samples were scored for agglutination and virus titers were calculated by the Reed-Muench method.

To examine the lung pathology during the PR8 challenge, lungs were removed from three mice in each group and photographed to observe gross pathology. Lungs were then fixed in 10% formalin solution. The lungs were sectioned by American HistoLabs (Gaithersburg, MD) and stained with Hematoxylin and Eosin (H & E). To determine the level of pulmonary inflammation, the lung inflammations were scored as previously described in a blind manner by an independent collaborator [388]. Briefly, the scores assigned were as follows: 0, no inflammation; 1, mild, inflammatory cell infiltrate of the perivascular/peribronchiolar compartment; 2, moderate, inflammatory cell infiltrate of the perivascular/peribronchiolar space with

modest extension into the alveolar parenchyma; and 3. severe, inflammatory cell infiltrate of the perivascular/peribronchiolar space with a greater magnitude of inflammatory foci found in the alveolar parenchyma.

Enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISPOT), and microneutralization assay

For the detection of HA-specific antibodies in serum, BAL fluid, and nasal washes, high-binding ELISA plates (Maxisorp, Nunc) were coated with 3 μg/ml of the HA-Fc fusion protein or recombinant HA protein in PBS and incubated overnight at 4°C. For determination of the interaction between the HA-Fc and HA stalk-specific antibodies, plates were coated with serially diluted mAbs, starting from 3 μg /ml. Plates were then washed three times with 0.05% Tween 20 in PBS (PBST) and blocked with 2% BSA in PBS for 1 hr at room temperature. Samples serially diluted in 2% BSA-PBS, or HA-Fc (0.5 μg/well) diluted in 2% BSA-PBS, were added for 2 hr incubation at room temperature. After washes, HRP-conjugated rabbit anti-mouse IgG antibody (1:2000, Pierce) or anti-mouse subclass-specific antibodies (1:5000, Southern Biotech) were added. For detection of HA-specific antibody in serum and BAL, biotin-labeled goat anti-mouse IgG-specific Fab (1:2,000) was added for 2 hr incubation, and streptavidin-HRP (1:8000) was added as secondary antibody. All secondary antibodies were added to cells for a 1 hr incubation at room temperature. The reaction was visualized in a colorimetric assay using substrate tetramethyl benzidine (TMB) and a Victor III microplate reader (Perkin Elmer). Titers represent

the highest dilution of samples showing a 2-fold increase over average OD_{450} nm values of negative controls.

For measuring HA-specific antibody-producing plasma cells, 96-well ELISPOT plates (Millipore) were pre-wetted with 35% ethanol and washed with PBS. The plates were then coated with 5 μg/ml of recombinant HA protein overnight at 4°C and blocked with RPMI complete medium with 10% FBS for 2 hr at 37°C and 5% CO_2. Serial dilutions of single-cell suspensions from bone marrow were prepared in RPMI and added to the coated wells for 24 hr at 37°C in 5% CO_2. After cells were removed, the plates were washed 5 times with PBST, then incubated with biotin-labeled goat anti-mouse IgG-specific Fab antibody (1:2000) for 2 hr. After washing with PBST, HRP-conjugated streptavidin (1:3000) was added and incubated for 1 hr. The samples were developed with AEC substrate (BD Biosciences) in the dark. After washing with deionized water, the plates were stored upside down in the dark to dry overnight at room temperature. Spots were counted with ELISPOT Reader and analyzed by ZellNet Consulting (New Jersey).

Neutralizing antibodies were measured by a standard microneutralization assay on MDCK cells as previously described [389]. Briefly, RDE-treated serum samples were serially diluted in PBS with 1x antibiotics/antimycotics. Then, 100 $TCID_{50}$ of the PR8 virus was added to each well and incubated at 37°C for 1 hr. MDCK cells were incubated with the serum/virus mixture for an additional 1 hr at 37°C. After removing the mixture, serum-free Opti-MEM containing 1 μg/ml TPCK-trypsin was added to each well and incubated for 3 days at 37°C. Cytopathic effects (CPE) were observed daily and the presence of virus was determined by HA assay as

described above. Neutralizing antibody titers were determined as the reciprocal of the highest serum dilution preventing the 50% appearance of CPE. Each assay was done in triplicate. The average neutralizing antibody titer was determined for each treatment group.

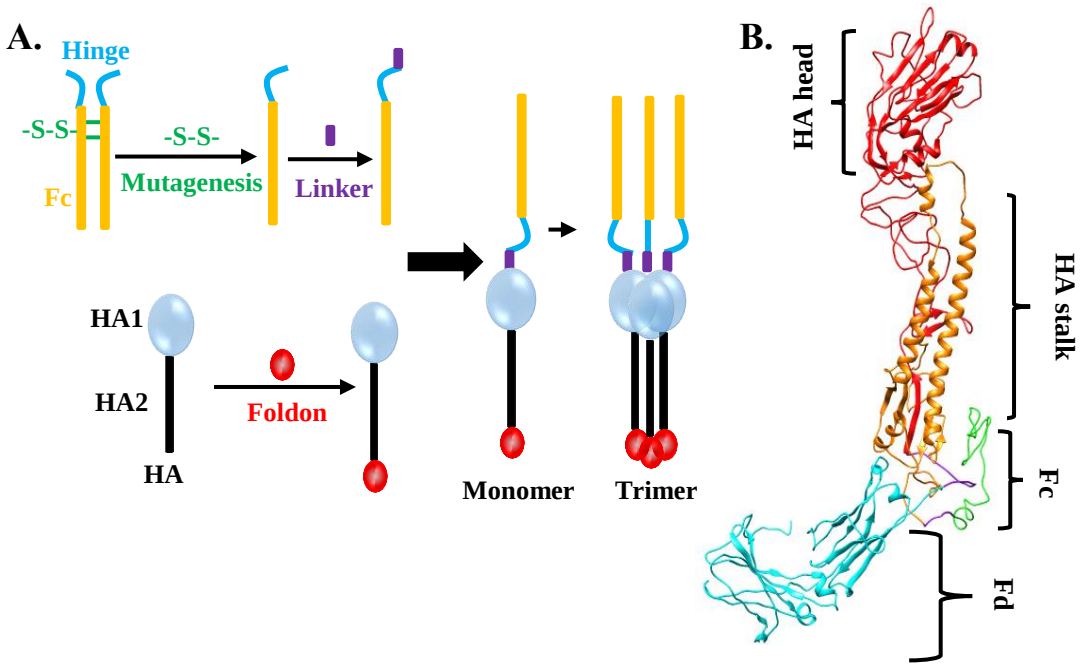
Statistics analysis

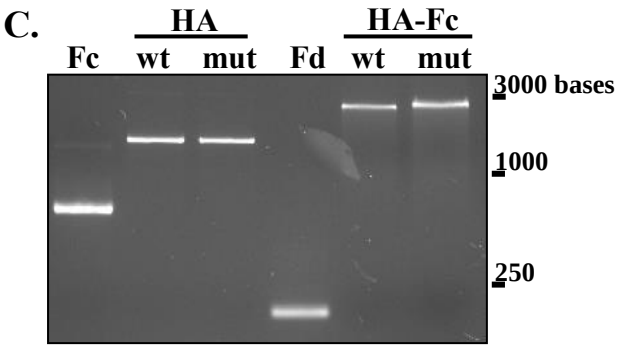
To compare the Kaplan-Meier survival curves, we used multiple Mantel-Cox tests. Differences in antibody titers, cytokine percentages, virus titers, inflammation scores, and IgG-secreting cell numbers were assessed by using paired Student's two-tailed t-test or one-way ANOVA with Tukey's multiple comparison test. GraphPad Prism 5.01 software was used for the statistical analyses.

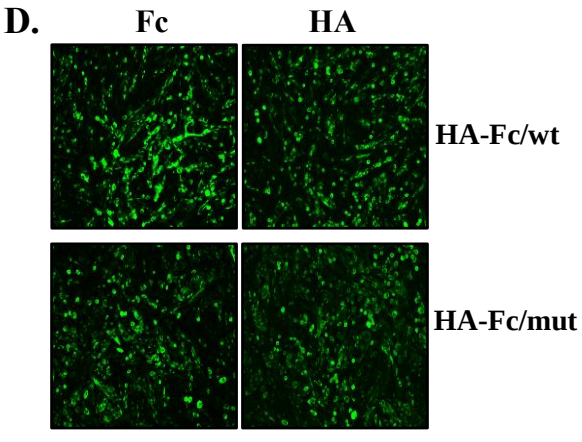
Results

Construction and expression of trimeric PR8 HA-Fc

Based on my previous findings of partial protection provided by immunization with HA-Fc/dimer, further improvements were necessary to optimize vaccine efficacy of my HA-based fusion proteins. I introduced several key modifications in each component of the fusion protein. The HA viral protein exists as a trimer within the virion, thus a trimeric HA antigen would mimic native viral HA and result in a more immunogenic vaccine antigen. Addition of the foldon (Fd) domain from T4 bacteriophage fibritin facilitates trimerization of recombinant HA [392] [393]. The


A.
Hinge
-S-S-
Fc
-S-S-
Mutagenesis
Linker
HA1
HA2
HA
Foldon
Monomer
Trimer
B.
HA head
HA stalk
Fc
Fd


C.
HA
HA-Fc
Fc
wt
mut
Fd
wt
mut
3000 bases
1000
250


D.
Fc
HA
HA-Fc/wt
HA-Fc/mut

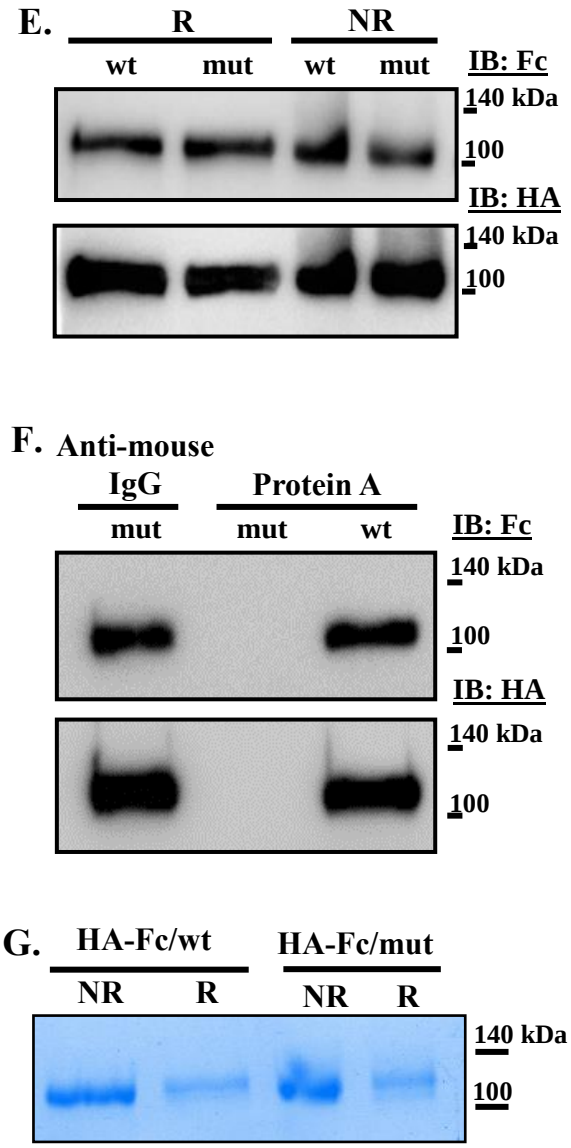


Figure 3.1. Characterization and expression of PR8 HA-Fc fusion protein.

(A) Schematic representation of PR8 HA fused to mouse IgG2a Fc monomer and T4 fibritin foldon domain (Fd). The following mutations were made in Fc portion of IgG2a: To abolish Fc dimerization, Cys224, Cys227, and Cys229 were replaced with Ser residues; to remove the complement C1q binding site, Glu318, Lys320, and Lys322 were replaced with Ala residues; and to eliminate FcRn binding sites for HA-Fc/mut fusion protein, His 310 and His 433 were replaced with Ala residues.

(B) Predicted protein structure of HA-Fc. The HA head domain is depicted in red, the HA stalk domain is depicted in orange, the monomeric IgG2a Fc domain is in blue, and the Fd domain is in green. The following nucleotide sequences were acquired

through GenBank: full-length HA: AF389118.1; IgG2a Fc: KC295246.1; and Fd: X12888.1 (AY266304.1). The final image was generated and modeled through Phyre2 (Imperial College London) and Chimera (University of California, San Francisco).

(C) Cloning HA-Fc fusion gene for transfection. Each PCR product (Fc fragment, HA fragment, and Fd fragment) were fused together using overlapping PCR to produce the final fusion gene, HA-Fc, for insertion into pCDNA3 vector to produce wild-type (wt) and mutant (mut) versions of HA-Fc plasmids.

(D) Expression of HA-Fc in stable CHO cell line. HA-Fc/wt and mut plasmids were stably transfected into CHO cells. Anti-HA and mouse IgG2a antibodies were used to detect each subunit of HA-Fc.

(E) Western blot analysis of non-reduced and reduced HA-Fc/wt and mut probed with Fc in the top panel and HA in the bottom panel.

(F) Pull down of HA-Fc/wt and mut by Protein A and/or anti-mouse IgG conjugated to agarose beads. 500 ng purified protein was incubated with resin slurry for 2h at 4°C. Boiled and eluted samples were run on SDS-PAGE. Western blot analysis of pulled-down HA-Fc/wt and mut samples probed with anti-Fc antibody in the top panel and anti-HA antibody in the bottom panel.

(G) HA-Fc/wt and HA-Fc/mut proteins were purified by affinity chromatography. Quality and concentration of purified HA-Fc were determined by SDS-PAGE analysis of HA-Fc/wt and mut in non-reducing (NR) and reducing (R) conditions.

optimized HA-Fc construct included the Fd domain at the C terminus of the fusion gene to mediate trimerization of the HA-Fc fusion protein (Figure 3.1A-B).

Upon trimerization of the HA-Fc fusion protein, the dimeric structure of the Fc portion could potentially impose steric hindrance that prohibits optimal binding to FcRn [342] [348] [390]. To eliminate dimerization of IgG Fc and potentially increase the bioavailability of the HA-based Fc fusion protein, the disulfide bond within the hinge region was disrupted by three substitutions at positions 224, 227, and 229, where each cysteine was changed to a serine, allowing the Fc portion to exist in the monomeric form [391]. I fused the monomeric IgG Fc and HA portions to the Fd trimerization domain and transfected the resultant plasmids in CHO cells for stable expression and secretion of the fusion proteins (Figure 3.1C-D). The signal peptide from the T cell surface glycoprotein CD5 was cloned before the Fc gene to mediate secretion [383]. As previously described, a mutant version of HA-Fc was produced and the C1q binding sites were disrupted with the previously described mutations. The optimized vaccine fusion proteins composed of monomeric IgG Fc and trimeric HA, HA-Fc/wt and mut, were purified from the cellular supernatant by affinity chromatography and then assessed for protein quality.

In both reducing and non-reducing conditions, soluble HA-Fc/wt existed as a monomer, which was recognized by Fc and HA antibodies as assessed by Western blotting, with similar results for HA-Fc/mut (Figure 3.1E). As previously described, I determined the ability of HA-Fc to interact with Protein A. HA-Fc/wt interacted with Protein A, while HA-Fc/mut did not, but was pulled down by anti-mouse IgG (Figure 3.1F). After protein purification, I determined the quality of purified HA-Fc by SDS-

PAGE. The HA-Fc proteins were the correct size with no evidence of degradation (Figure 3.1G). Overall, the soluble HA-Fc fusion proteins were expressed and secreted at high levels, with no degradation. HA-Fc/wt interacted with Protein A, strongly suggesting that HA- Fc/wt would interact with FcRn, while HA-Fc/mut would not be able to bind to FcRn.

HA-Fc is expressed and secreted as a trimer and can maintain trimeric conformation

Since the trimeric native state of HA is important for its immunogenicity, I determined if HA-Fc/wt maintained its trimeric conformation after expression and secretion. Using HA stalk-reactive antibodies, 6F12 and KB2, for IFA, I observed that HA-Fc proteins expressed in stable cell lines were recognized by both HA antibodies which recognize conformational epitopes in the trimerized stalk domain (Figure 3.2A). In addition, I cross-linked HA-Fc/wt with BS^3, a hydrophilic, 11 ångström cross-linker that covalently links proteins and has been demonstrated to trimerize recombinant HA [393] [394]. After cross-linked HA-Fc/wt samples were run on a reducing, denaturing SDS-PAGE gel, HA-Fc/wt formed trimers with an approximate molecular weight of 330 kDa, in addition to monomers (approximately 110 kDa) (Figure 3.2B). The reactivity of a panel of broadly neutralizing conformational HA stalk antibodies to HA-Fc/wt was also determined. Conformational antibodies against both group 1 (CR6121, FI6v3, and 6F12) and group 2 (CR8020) HAs recognized HA-Fc/wt in a concentration-dependent manner (Figure 3.2C). Taken together, I determined that the Fd domain was able to

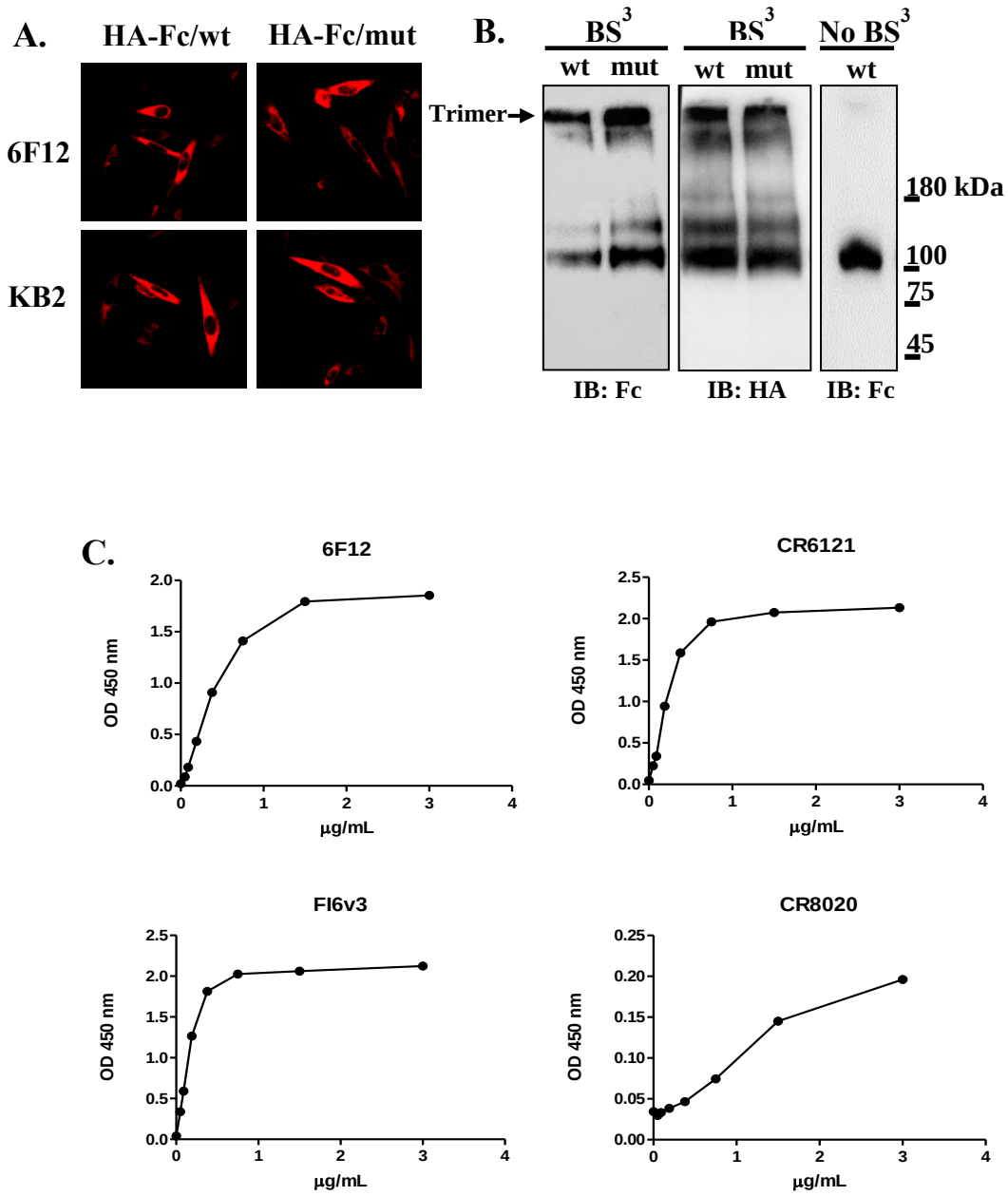


Figure 3.2. HA-Fc is expressed and secreted as a stable trimer.

(A) Stable CHO cell lines expressing HA-Fc/wt and mut were probed with conformational HA antibodies, 6F12 in the top panel, and KB2 in the bottom panel.

(B) Western blot analysis of HA-Fc cross-linked with BS^3 probed with anti-Fc antibody in the left panel, anti-HA antibody in the middle panel, compared to Fc probe of HA-Fc that had not been cross-linked.

(C) Binding of stalk-reactive conformational antibodies to HA-Fc was demonstrated with a panel of anti-HA antibodies against group 1 (6F12, CR6121, and FI6v3) and group 2 (CR8020) by ELISA.

stabilize trimerization of HA-Fc, in order to maintain a trimeric conformation throughout expression and purification.

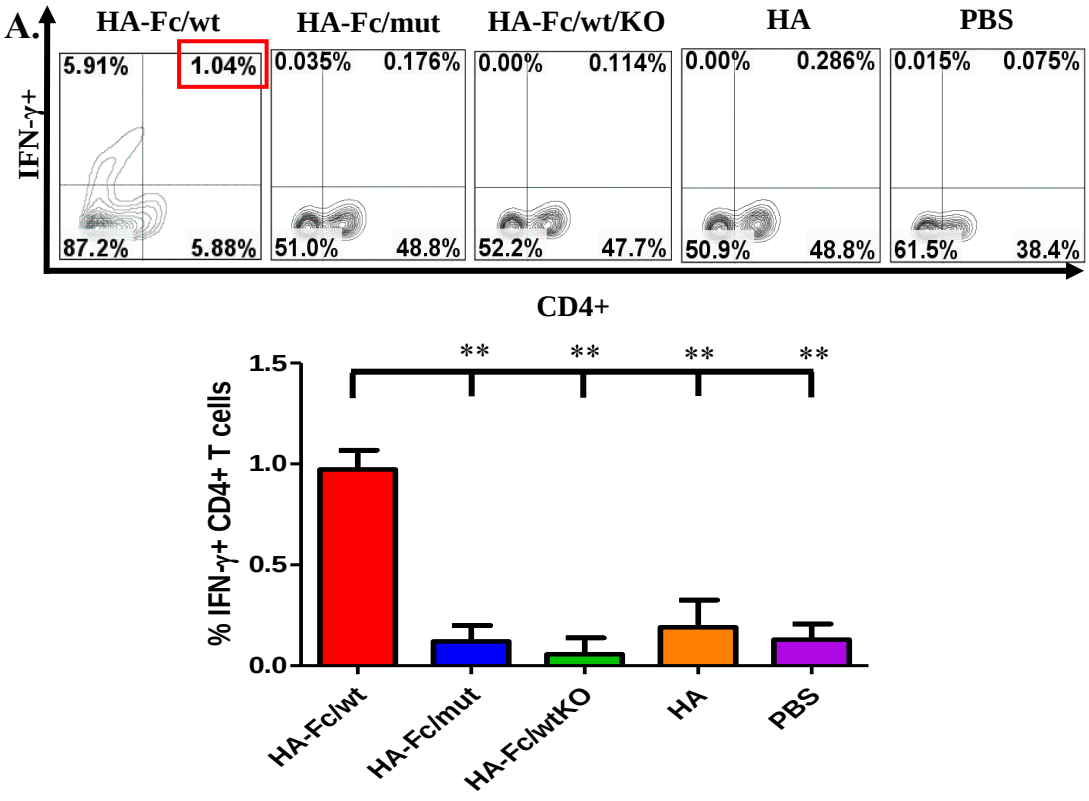
FcRn-mediated vaccination with HA-Fc induces enhanced HA-specific cellular and antibody responses

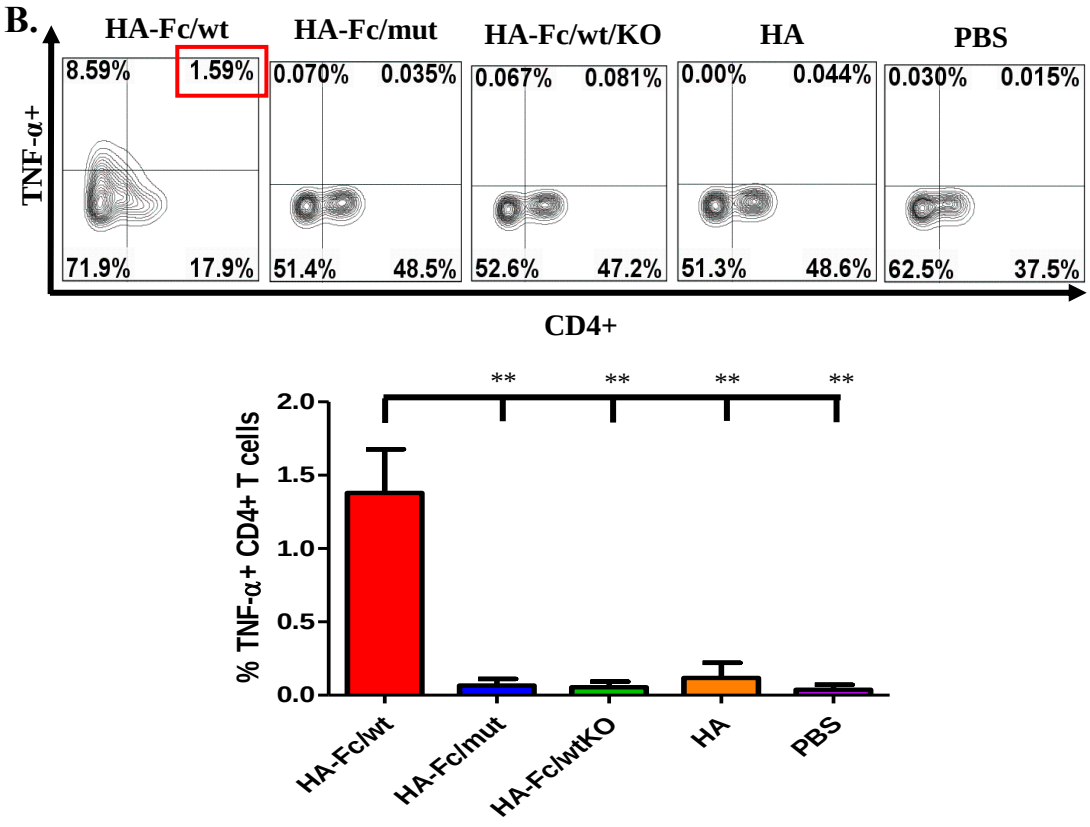
In order to evaluate protective immunity induced by FcRn-mediated HA-Fc delivery, WT or FcRn-KO mice were intranasally (i.n.) primed, and boosted two weeks later, with PBS or 5 μg of HA-Fc/wt, HA-Fc/mut, or HA, in addition to 10 μg of CpG, as previously described (Table III.I). In order to determine induction of cell-mediated immune responses, lungs from immunized mice were collected one week after a boost and subjected to intracellular cytokine staining. In HA-Fc/wt-immunized mice, I observed significantly higher percentages of both IFN-γ- and TNF-α-secreting CD4+ and CD8+ T cells in the lungs after HA stimulation, compared to the control groups (Figure 3.3A-D).

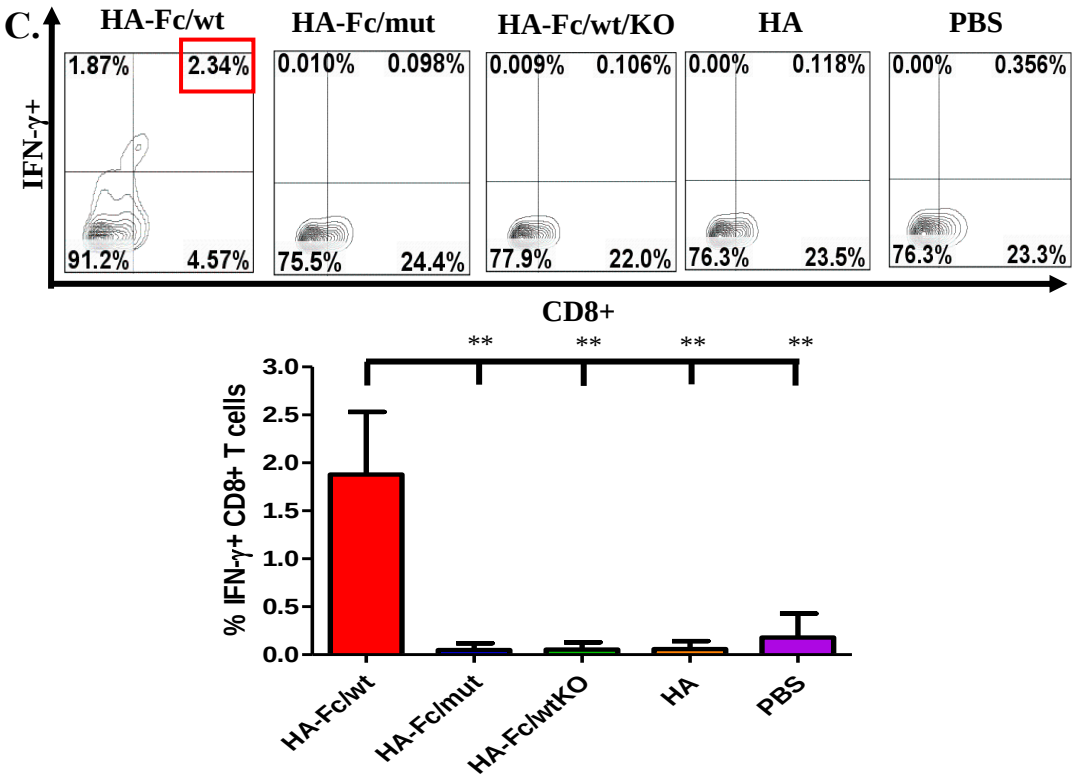
In addition, to evaluate the humoral immune response, I collected serum two weeks after a boost and measured serum IgG antibody concentrations and neutralizing antibody titers. Compared to a majority of the control groups, I detected significantly increased levels of serum IgG antibody titers in WT mice immunized with HA-Fc/wt when measuring the titers of total IgG and individual isotypes, including IgG1, IgG2b, and IgG2a (Figure 3.3E). The increased antibody response measured in HA-Fc/wt-immunized mice was further demonstrated by significantly higher levels of neutralizing antibody titers, relative to control groups (Figure 3.3F). My data demonstrate that HA-Fc delivered by FcRn can induce significantly high levels of a robust adaptive immune response.

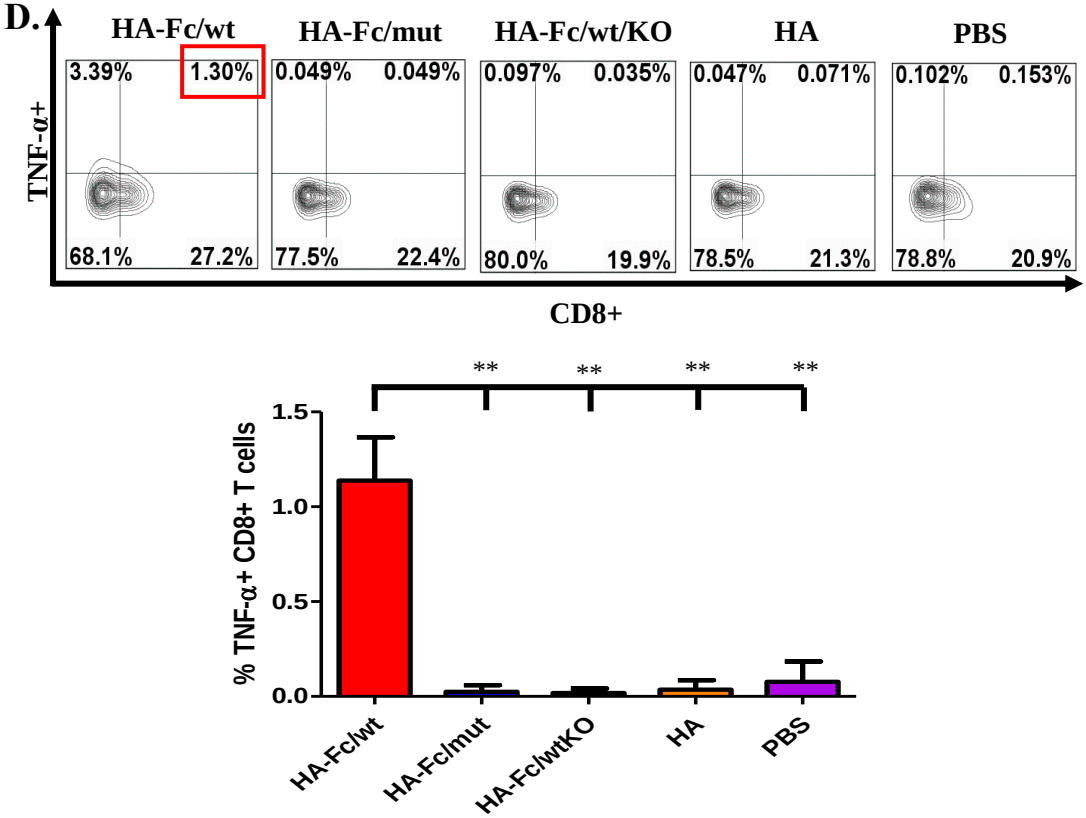
Table III.I: HA-Fc immunization and treatment conditions

Treatment	Mouse type	Interact with FcRn?
HA-Fc/wt	C57BL/6	Y
HA-Fc/mut	C57BL/6	N
HA-Fc/wt/KO	C57BL/6 FcRn KO	N
HA	C57BL/6	N
PBS	C57BL/6	N


A.
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
IFN-γ+
5.91%
1.04%
87.2%
5.88%
0.035%
0.176%
51.0%
48.8%
0.00%
0.114%
52.2%
47.7%
0.00%
0.286%
50.9%
48.8%
0.015%
0.075%
61.5%
38.4%
CD4+
% IFN-γ+ CD4+ T cells
1.5
1.0
0.5
0.0
**
**
**
**
HA-Fc/wt
HA-Fc/mut
HA-Fc/wtKO
HA
PBS


B.
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
TNF-α+
8.59%
1.59%
71.9%
17.9%
0.070%
0.035%
51.4%
48.5%
0.067%
0.081%
52.6%
47.2%
0.00%
0.044%
51.3%
48.6%
0.030%
0.015%
62.5%
37.5%
CD4+
% TNF-α+ CD4+ T cells
2.0
1.5
1.0
0.5
0.0
**
**
**
**
HA-Fc/wt
HA-Fc/mut
HA-Fc/wtKO
HA
PBS


C.
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
IFN-γ+
1.87%
2.34%
91.2%
4.57%
0.010%
0.098%
75.5%
24.4%
0.009%
0.106%
77.9%
22.0%
0.00%
0.118%
76.3%
23.5%
0.00%
0.356%
76.3%
23.3%
CD8+
% IFN-γ+ CD8+ T cells
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
HA-Fc/wt
HA-Fc/mut
HA-Fc/wtKO
HA
PBS


D.
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
TNF-α+
3.39%
1.30%
68.1%
27.2%
0.049%
0.049%
77.5%
22.4%
0.097%
0.035%
80.0%
19.9%
0.047%
0.071%
78.5%
21.3%
0.102%
0.153%
78.8%
20.9%
CD8+
% TNF-α+ CD8+ T cells
1.5
1.0
0.5
0.0
**
**
**
**
HA-Fc/wt
HA-Fc/mut
HA-Fc/wtKO
HA
PBS

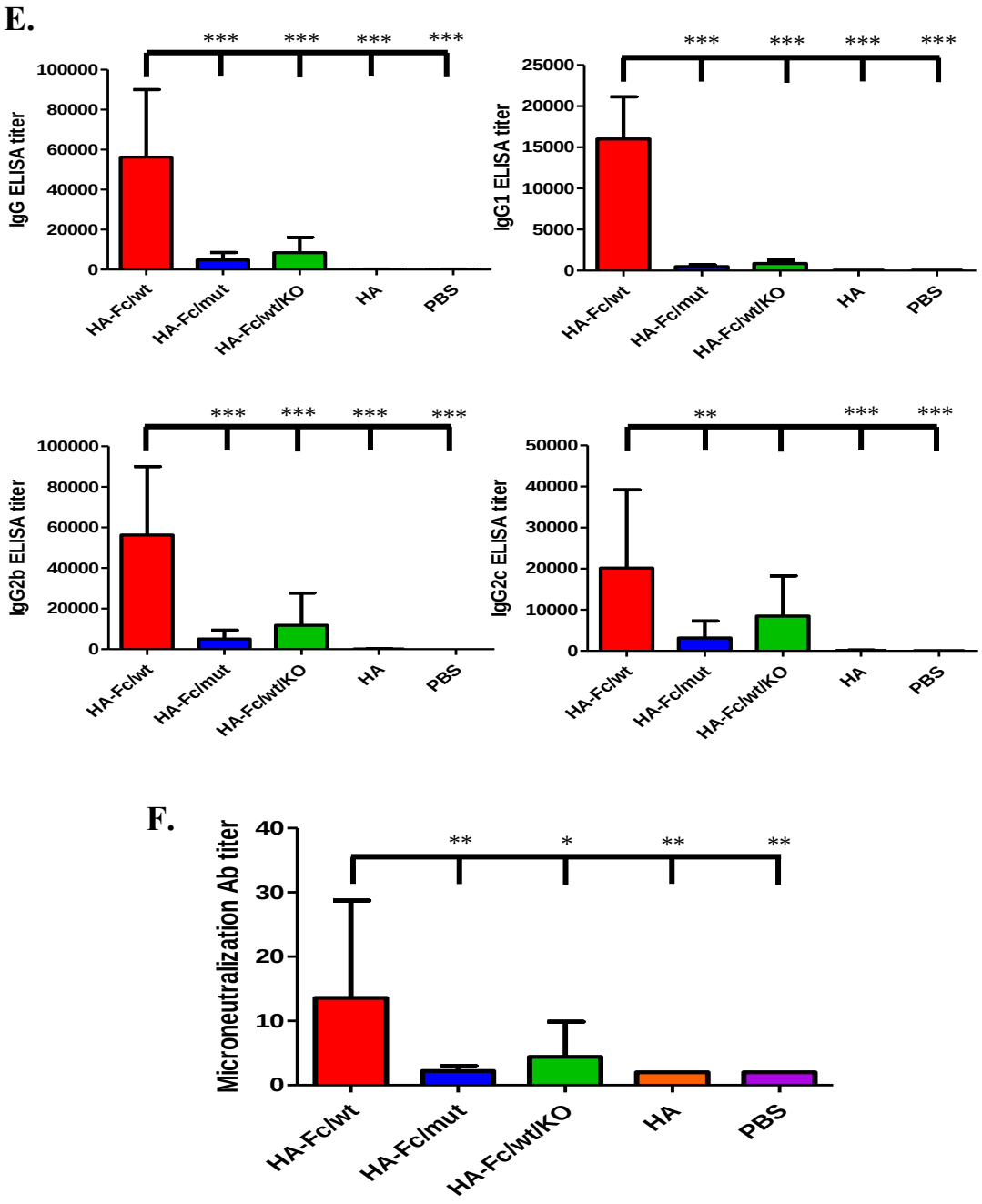


Figure 3.3. FcRn-mediated i.n. vaccination with HA-Fc induces HA-specific T cell and antibody responses.

PBS or 5 μg of HA-Fc/wt, HA-Fc/mut, or HA were i.n. administered to wild-type or FcRn knockout (KO) mice along with 10 μg of CpG. Two weeks post prime, the same conditions were i.n. administered to the respective groups for boost.

(A-D) One week post boost, lung cells from immunized mice were stimulated with recombinant HA or medium control for 12 hr and stained for surface markers CD3, CD4, and CD8. Intracellular cytokine staining was performed to detect IFN-γ and TNF-α+ secretion.

(A-B) Representative flow cytometry plots show the percentage of cells with CD4+IFN-γ+ **(A)** or CD4+TNF-α+ phenotypes **(B)**, with column graphs of the average percentage of each condition displayed underneath the respective plots.

(C-D) Representative flow cytometry plots show the percentage of cells with CD8+IFN-γ+ **(C)** or CD8+TNF-α+ phenotypes **(D)**, with the average percentage displayed as a column graph underneath the respective plots. Immunization conditions are described at the bottom of graphs. For **(A-D)**, flow cytometry plots are representative of two independent experiments with 4 immunized mice pooled in each group. Graphical data is the average percentage of the two experiments.

(E) Measurement of anti-HA IgG antibody titers. IgG titers in serum of immunized mice 2 weeks after boost were determined for total IgG, and individual isotypes: IgG1, IgG2b, and IgG2c in 10 representative mouse sera as determined by endpoint titer.

(F) Testing of the neutralizing activity of HA-Fc immunized sera. Two weeks post boost, sera were RDE-treated and heat inactivated, then diluted two-fold in PBS with 1x antibiotics/antimycotics. 100 $TCID_{50}$ of the PR8 virus was added to each serum dilution and incubated at 37°C for 1 hr. The mixture was added to MDCK cells and incubated at 37°C for 1 hr. The mixture was removed and serum-free Opti-MEM containing 1 μg/ml TPCK-trypsin was added to cells. After incubation at 37°C for 3 days, HA assay was performed with the supernatant to determine neutralization antibody titers, with 13 to 20 individual mouse serum per group used.

Statistical differences were determined by one-way ANOVA with Tukey's multiple comparison test **(A-D and F)** or paired two-tailed Student's *t*-tests **(E)** and values were marked as followed with asterisks in this and subsequent figures: *P<0.05, **P<0.01, ***P<0.001

Intranasal vaccination with HA-Fc induces robust FcRn-dependent local immunity

In contrast to parenteral vaccination, mucosal vaccination induces immune responses both at the site of immunization and in peripheral immune organs. I determined if intranasal vaccination with HA-Fc induced local immune responses that prevent and control influenza infection. Ten days after boost, I observed that HA-Fc/wt-immunized mice produced a significant increase in both secretory IgA antibody titer in nasal wash (NW) and IgG titer in bronchoalveolar lavage (BAL) fluid, from the upper and lower respiratory tracts, respectively. In contrast, there were little appreciable amounts of either antibody detected in the control groups (Figure 3.4A). In addition, I detected another facet of local humoral immunity, the induction of activated germinal centers (GC) in the mediastinal lymph nodes (MeLN). Ten days after boost, I determined the number of activated B cells in GCs by measuring B220+CD19+ B cells for expression of FAS and increased levels of peanut agglutinin (PNA)-positive regions, which are characteristic markers of activated GCs [396] [397]. HA-Fc/wt-immunized mice produced the highest percentages of FAS+ PNA^{high} cells in the MeLN, indicating that FcRn-mediated delivery of HA-Fc induced GC activation to support local humoral immunity (Figure 3.4A, left panel). Within the spleen, there was a modest increase in activated germinal center B cells compared to most of the control groups (Figure 3.4A, right panel). Induction of local immunity is an important aspect of mucosal vaccination, and my data strongly suggest that FcRn is required to induce mucosal immunity through the delivery of HA-Fc to local immune sites, such as the lamina propria underlying the respiratory epithelium.

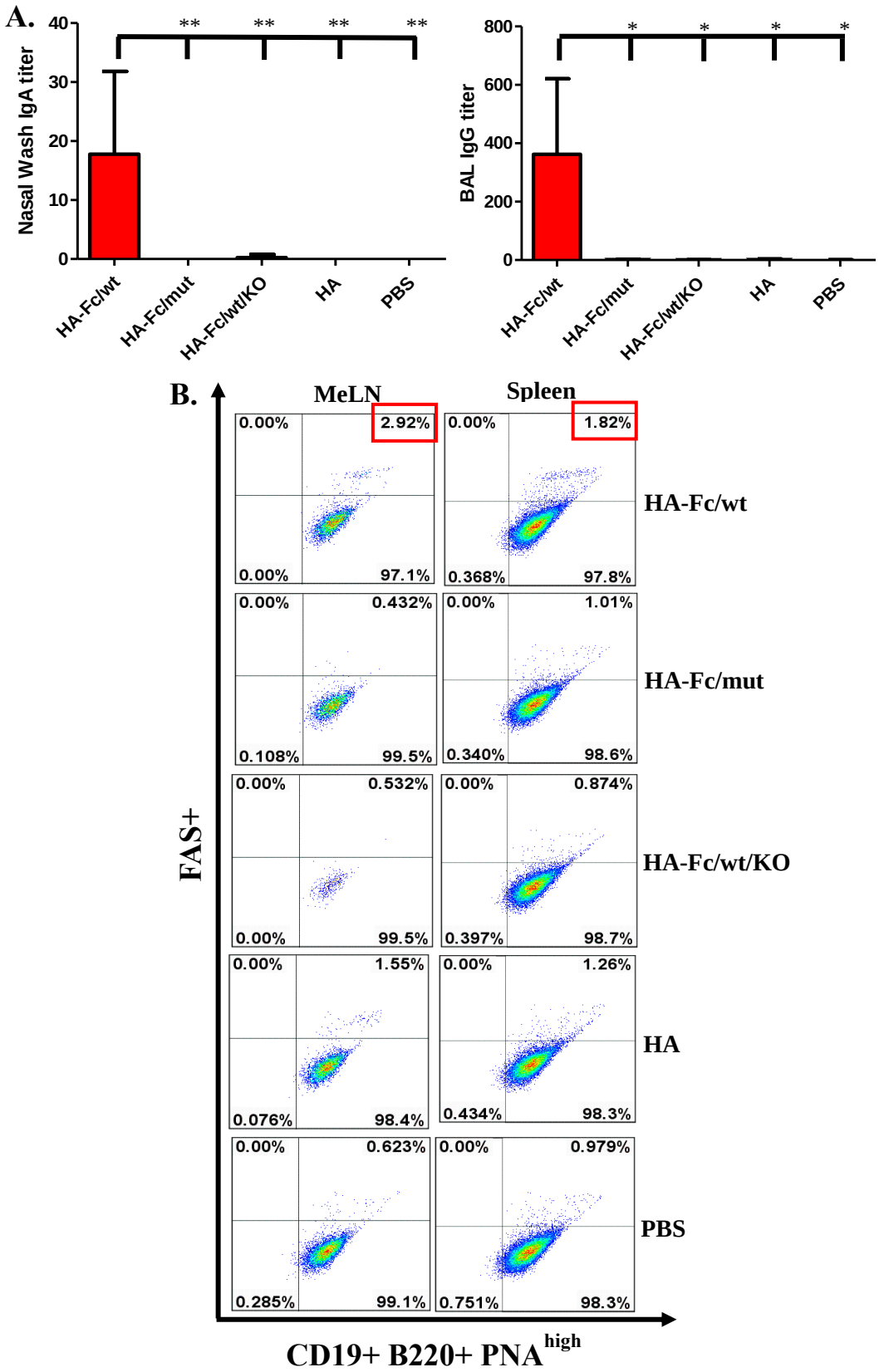

A.
Nasal Wash IgA titer
40
30
20
10
0
**
**
**
**
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
BAL IgG titer
800
600
400
200
0
*
*
*
*
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
B.
MeLN
Spleen
0.00%
2.92%
0.00%
97.1%
0.00%
1.82%
0.368%
97.8%
HA-Fc/wt
0.00%
0.432%
0.108%
99.5%
0.00%
1.01%
0.340%
98.6%
HA-Fc/mut
0.00%
0.532%
0.00%
99.5%
0.00%
0.874%
0.397%
98.7%
HA-Fc/wt/KO
0.00%
1.55%
0.076%
98.4%
0.00%
1.26%
0.434%
98.3%
HA
0.00%
0.623%
0.285%
99.1%
0.00%
0.979%
0.751%
98.3%
PBS
FAS+
CD19+ B220+ PNA high

Figure 3.4. FcRn-mediated intranasal immunization with HA-Fc induces local immunity.

Ten days post boost, nasal wash (NW), bronchoalveolar lavage (BAL), mediastinal lymph nodes (MeLN), and spleen were collected from immunized mice.

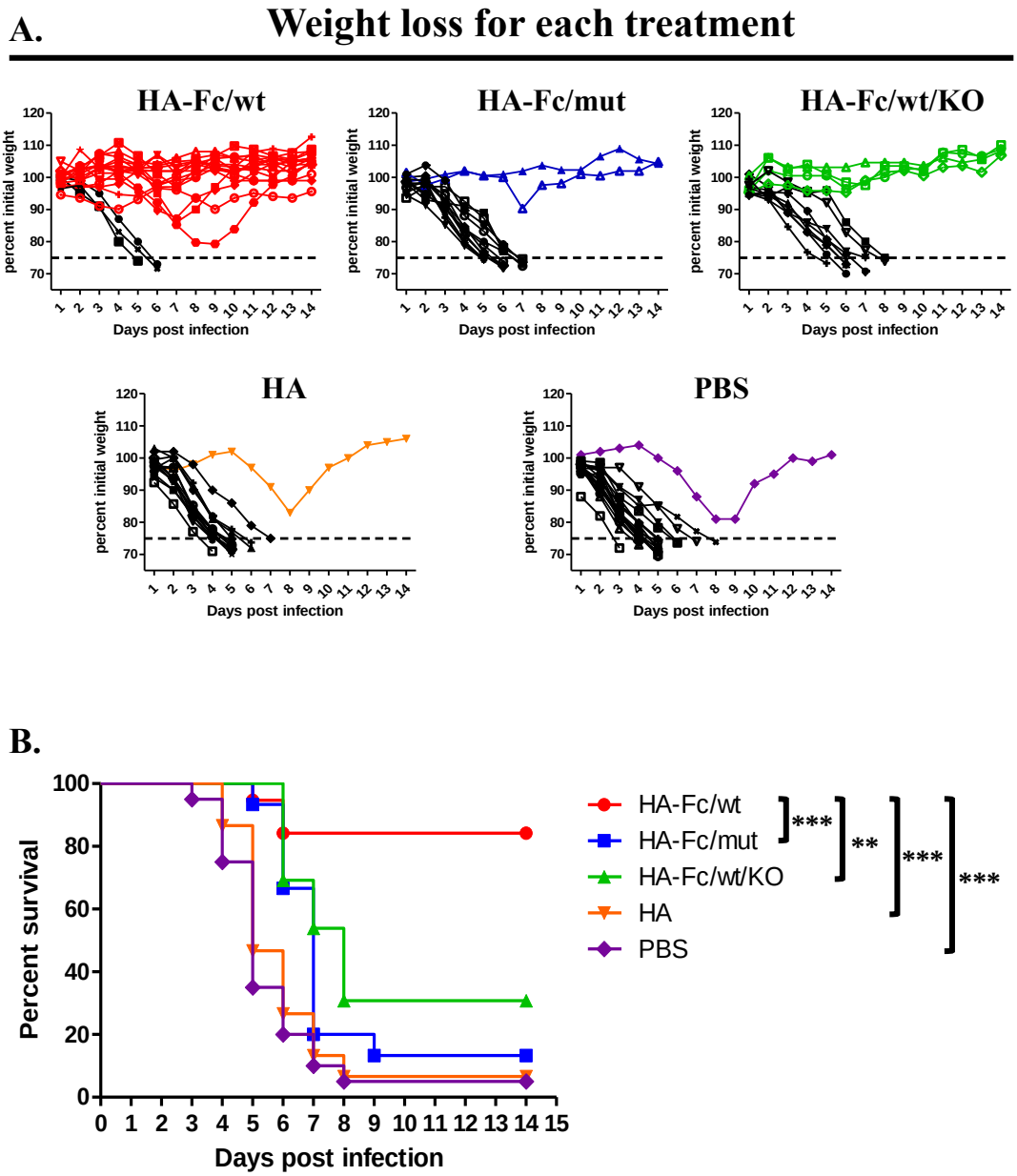
(A) Induction of local humoral immunity. Titers of HA-specific secretory IgA in NW (n=4-5) and IgG in BAL (n=3) were measured by ELISA as determined by endpoint titer.

(B) Induction of activated B cells in germinal centers. MeLN and spleen cells were stained for markers for activated B cells in germinal centers, including B220/CD45R, CD19, FAS/CD95, and PNA. FAS+ PNA^{high} cells were gated from CD19+ B220+ cells and represent the percentage of activated germinal center B cells in each treatment group. Flow cytometry plots are based on 4-5 immunized mice pooled in each treatment group.

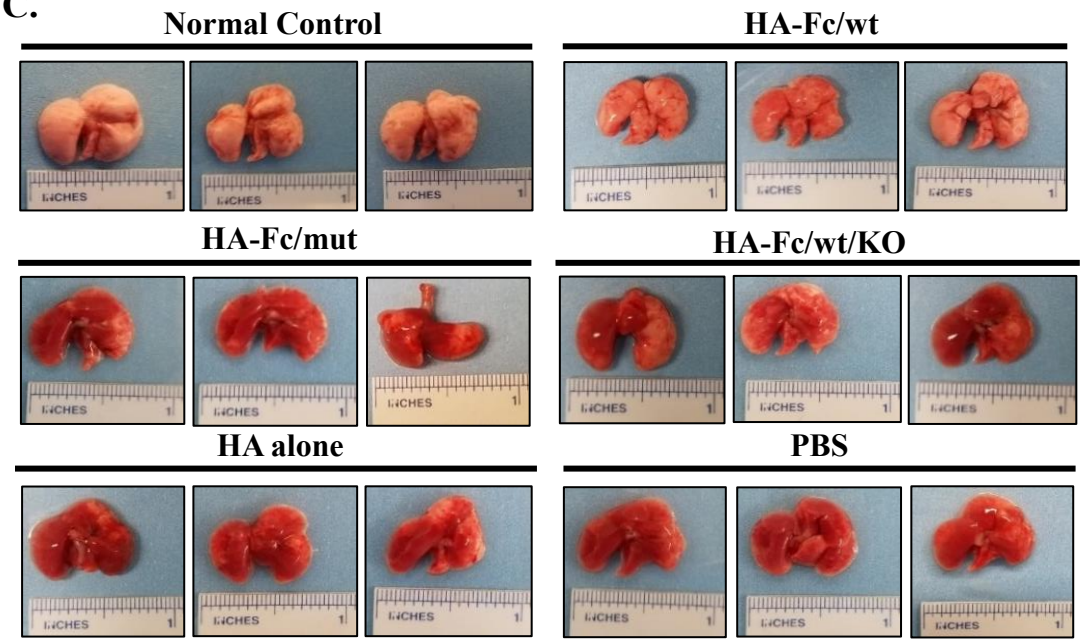
FcRn-targeted mucosal vaccination provides increased protection and survival and reduced pathology after challenge

After observing robust local and systemic immune responses in HA-Fc/wt-immunized mice, the protective potential of immunization with HA-Fc was determined. Two weeks after a boost, mice were challenged with a lethal dose of 5 MLD_{50} PR8 (10^4 $TCID_{50}$) and monitored daily as described previously. The majority of the control groups had severe weight loss (≥ 25%) within eight days after challenge (Figure 3.5A) and succumbed either to infection or were euthanized. In contrast, three of the nineteen mice in the HA-Fc/wt-immunized group sustained 25% body weight loss, with an additional mouse losing approximately 20% body weight, before fully recovering to initial body weight (Figure 3.5A). The remaining fifteen mice experienced minimal weight loss, resulting in an 84% survival rate for WT mice immunized with HA-Fc/wt, a significantly higher survival rate compared to the control groups (Figure 3.5B).

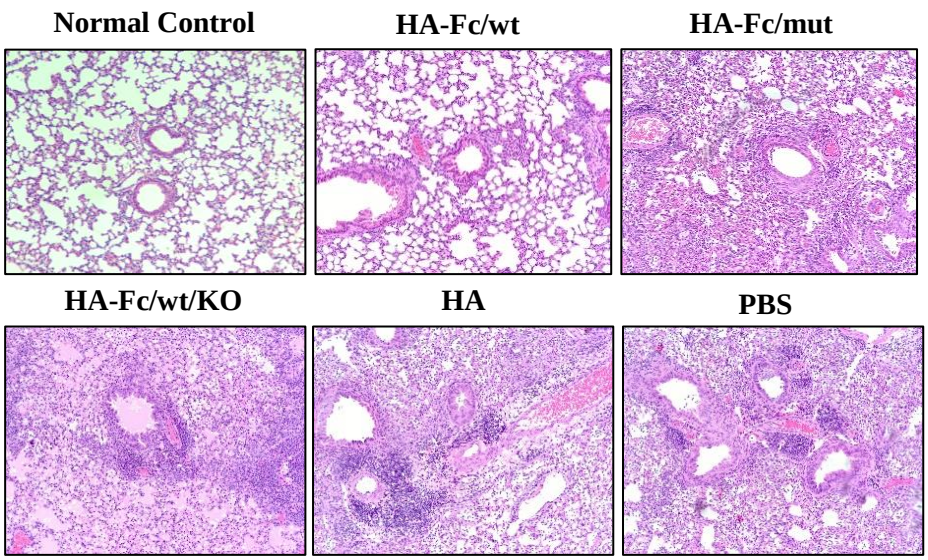
The pathogenesis of influenza infection in the lungs of immunized mice confirmed the morbidity and mortality results. The lungs of uninfected mice showed no signs of pulmonary pathology, while the lungs of HA-Fc/wt-immunized mice presented minimal damage (Figure 3.5C). In contrast, the control groups exhibited higher levels of pulmonary damage, with severe lesions, pulmonary edema and hemorrhage evident in the lungs of each group (Figure 3.5C). The individual lung sections of the mice were examined and the level of inflammation was determined. HA-Fc/wt-immunized mice had reduced inflammatory symptoms, while within the control groups, there was a discernable increase of pulmonary edema and


A.
Weight loss for each treatment
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
percent initial weight
Days post infection
B.
Percent survival
Days post infection
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
***
**
***
***

C.


Normal Control
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA alone
PBS

D.


H&E Stain: 10x
Normal Control
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS

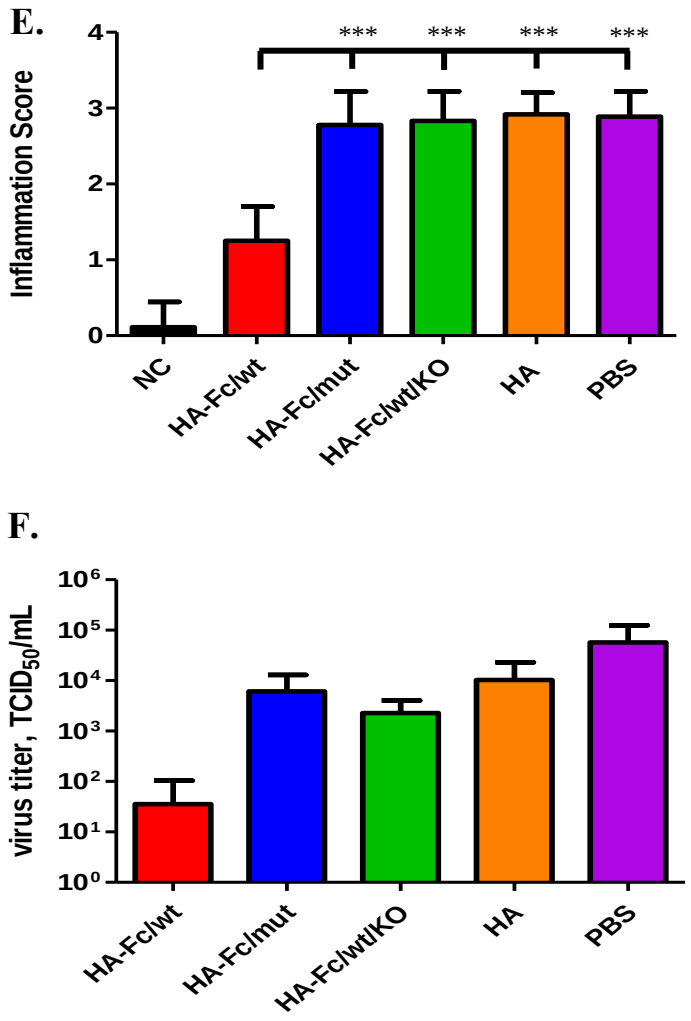


Figure 3.5. HA-Fc vaccination provides high levels of protection and survival against PR8 challenge.

Two weeks after a boost, mice were infected with a lethal challenge dose of 5 MLD_{50} PR8 virus, and weighed daily for 14 days. Mice were euthanized when body weight loss reached a 25% endpoint.

(A) The individual weight loss of immunized mice in each treatment group over 14 days is shown, with surviving mice displayed in corresponding colors (n=13-20).

(B) The Kaplan-Meier survival curve shows percent survival after challenge (n=13-20). Statistical differences were determined using multiple Mantel-Cox tests.

(C) Gross pathology of lungs from immunized and PR8 challenged mice. Lungs were collected 6 to 14 days post challenge, based on the 25% body weight loss endpoint, with uninfected mouse lungs included as a normal lung control (n=3).

(D-E) Pulmonary histopathology of representative lung sections from **(C).** Hematoxylin and eosin (H&E) stain was used to determine the level of inflammation in the lungs (10x, **D**) and inflammation scores for each group were determined by an independent pathologist **(E).** Statistical differences were determined by one-way ANOVA with Tukey's multiple comparison test.

(F) Lung virus titers of immunized mice were determined 4 days after lethal challenge (bottom panel) (n=4-5). Supernatants of lung homogenates were incubated on MDCK cells and lung titer was measured by 50% endpoint dilution assay in conjunction with HA assay.

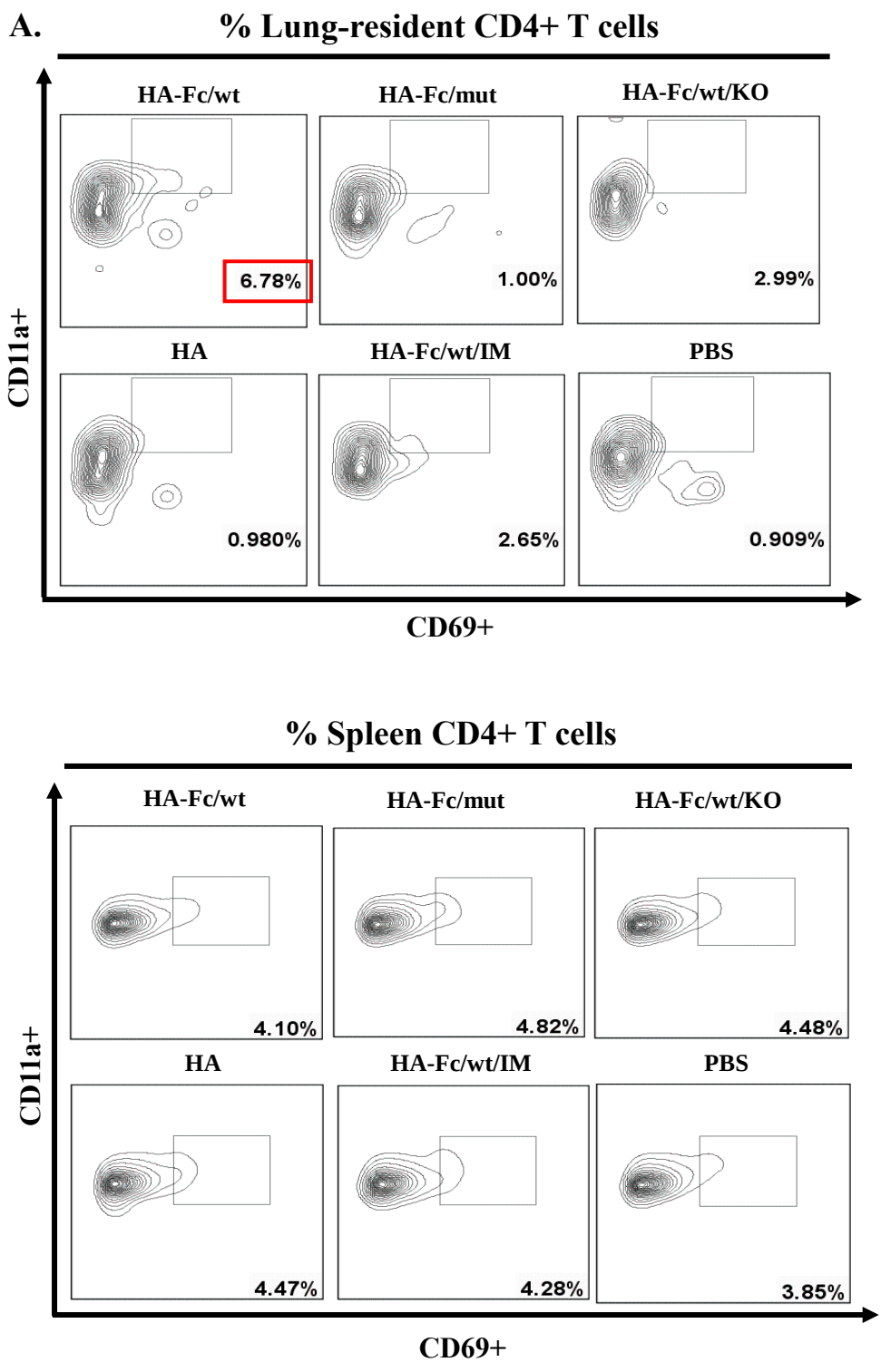
hemorrhage, and monocyte infiltration, resulting in a significantly lower inflammation score for mice immunized with HA-Fc/wt, compared to the control groups (Figure 3.5D-E). In addition, each group was assessed for viral replication in the lungs 4 days after lethal challenge. I observed markedly lower levels of virus in the lungs of HA-Fc/wt-immunized mice. When compared to HA-Fc/wt-immunized mice, there was 1.5 log to 3 log increase in virus titer across the control groups (Figure 3.5F). Collectively, these findings demonstrate that FcRn-mediated delivery of HA-Fc/wt confers protection against lethal PR8 challenge, resulting in decreased mortality, pulmonary damage, and viral replication.

Mucosal vaccination with HA-Fc induces protective memory immunity

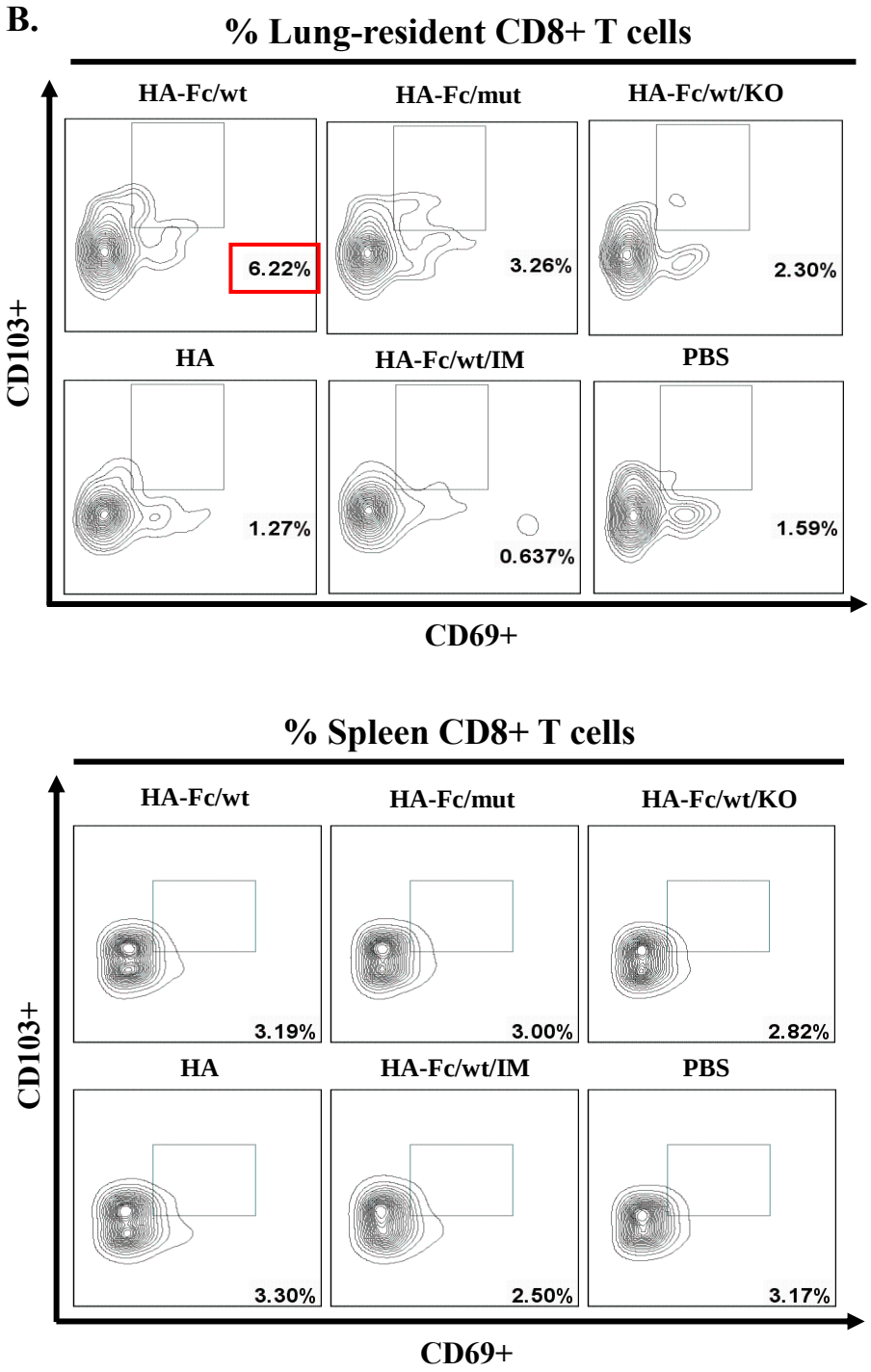
In addition to protective local and systemic immune responses, a successful influenza vaccine should induce protective memory immunity, which provides long-lasting defenses against influenza infection. Memory CD4+ and CD8+ T cells provide cross-reactive and heterosubtypic protection against influenza in mouse models, while plasma cells produce large amounts of antibodies, and memory B cells rapidly proliferate and differentiate after re-exposure to the virus [91] [398] [399]. As previously described, a recently characterized subset of memory T cells, tissue-resident memory (TRM) T cells, are non-circulating memory T cells that remain at the site of infection, in contrast to other memory T cell subsets that circulate within SLO, nonlymphoid peripheral tissues, and blood. TRM T cells provide a rapid response against site-specific infections in tissues such as the lung and have been implicated in mediating enhanced protection against influenza reinfection, promoting viral clearance and mediating heterosubtypic protection and survival against lethal

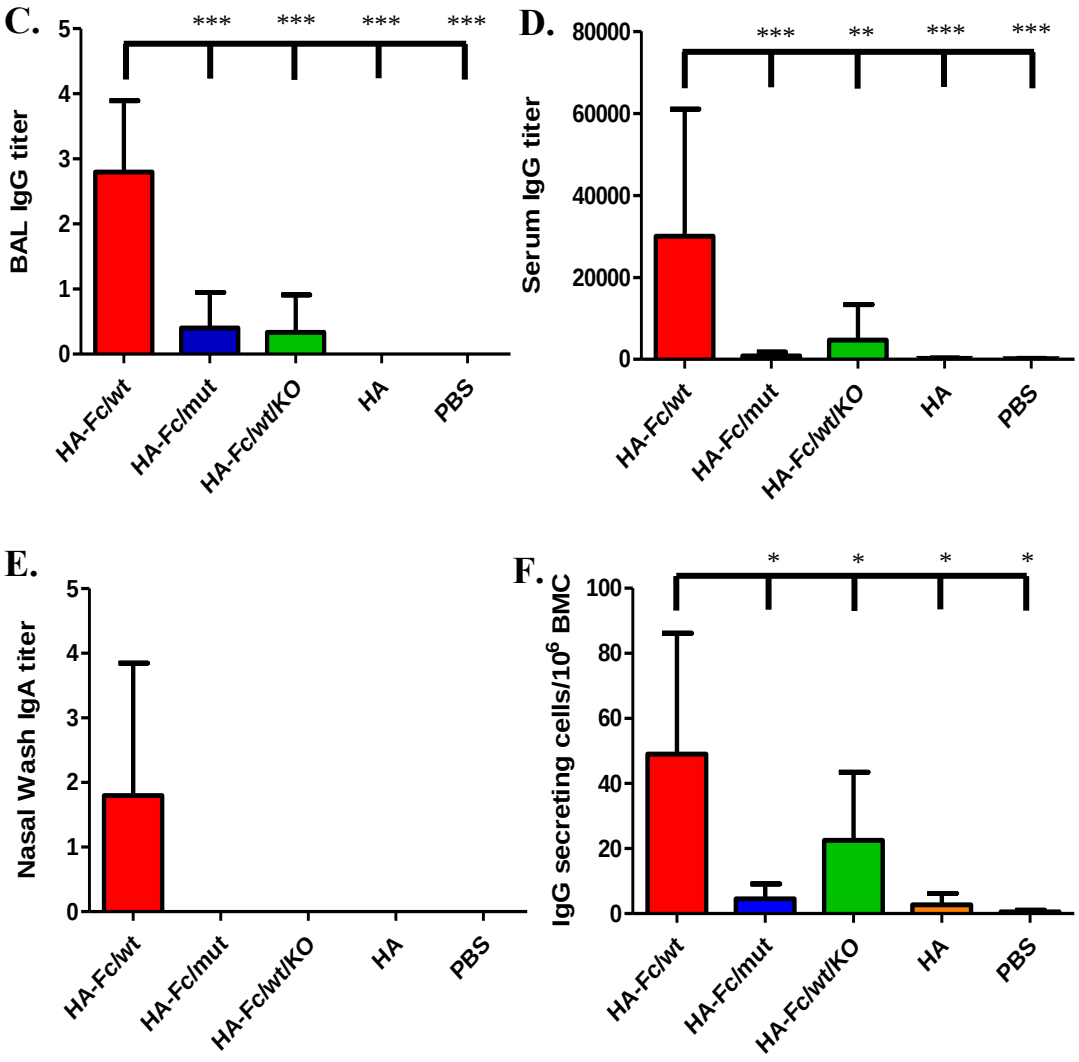
influenza challenge [98]. TRM T cells can be induced by natural infection and lung-resident memory T cells can be induced by certain i.n. vaccination strategies, including LAIV, and as previously shown in this dissertation, FcRn-mediated vaccination with other HA-Fc fusion proteins, but not by parenteral routes.

After observing high levels of protective local immunity, I determined if FcRn-mediated vaccination with HA-Fc promoted an effective memory immune response. I characterized several aspects of memory response, looking at both T cell- and B cell-mediated immunity. First, I determined if FcRn-mediated immunization with HA-Fc/wt could induce lung-resident memory T cells. As previously described, eight weeks post boost, mice were treated with an i.v. infusion of a fluorescently labeled anti-CD3 antibody. Lungs were collected and stained for lung-resident memory CD4+ and CD8+ T cells. In the lungs of HA-Fc/wt-immunized mice, there was a measurable increase in CD4+CD69+CD11a+ T cells (Figure 3.6A) and CD8+CD69+CD103+ T cells (Figure 3.6B), in contrast to the control groups, including i.m. administered HA-Fc/wt. Each control treatment induced lower percentages of lung-resident memory CD4+ and CD8+ T cells. As expected, there was minimal difference in circulating memory T cells between groups in the spleen of immunized mice (Figure 3.6A-B, bottom panels). Taken together, I have demonstrated that FcRn-mediated intranasal administration of HA-Fc fusion proteins induce lung-resident memory T cells, which has only been previously published using LAIV immunization, while supporting my previous results that observed induction of lung-resident memory T cells after HA-Fc/dimer immunization.

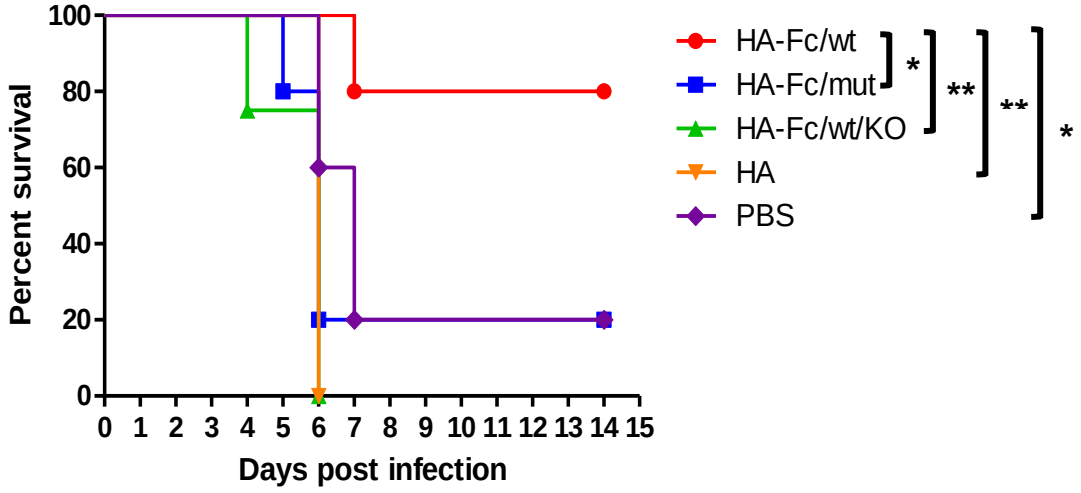

A.
% Lung-resident CD4+ T cells
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
6.78%
1.00%
2.99%
HA
HA-Fc/wt/IM
PBS
0.980%
2.65%
0.909%
CD11a+
CD69+
% Spleen CD4+ T cells
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
4.10%
4.82%
4.48%
HA
HA-Fc/wt/IM
PBS
4.47%
4.28%
3.85%
CD11a+
CD69+

B.


% Lung-resident CD8+ T cells
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
6.22%
3.26%
2.30%
HA
HA-Fc/wt/IM
PBS
1.27%
0.637%
1.59%
CD103+
CD69+
% Spleen CD8+ T cells
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
3.19%
3.00%
2.82%
HA
HA-Fc/wt/IM
PBS
3.30%
2.50%
3.17%
CD103+
CD69+


C.
BAL IgG titer
***
***
***
***
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
D.
Serum IgG titer
***
**
***
***
E.
Nasal Wash IgA titer
F.
IgG secreting cells/10^6 BMC
*
*
*
*
G.
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS

H.


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post infection
HA-Fc/wt
HA-Fc/mut
HA-Fc/wt/KO
HA
PBS
*
**
**
*

I.

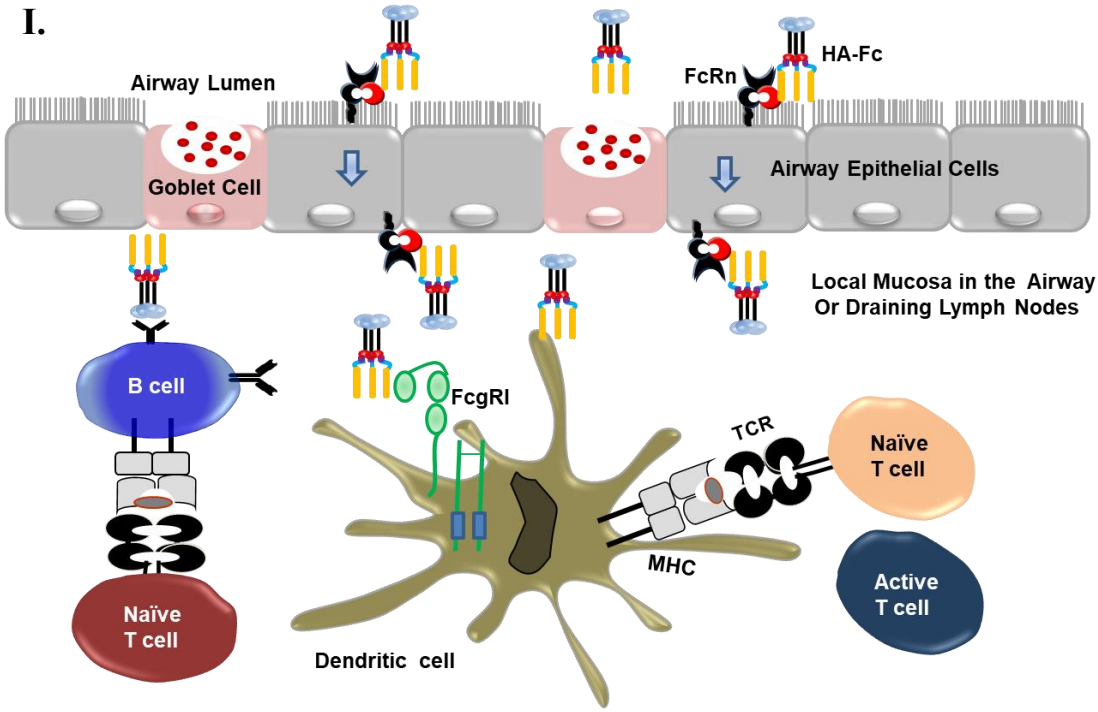

Airway Lumen
Goblet Cell
FcRn
HA-Fc
Airway Epithelial Cells
Local Mucosa in the Airway
Or Draining Lymph Nodes
B cell
Naïve
T cell
FcgRI
TCR
MHC
Naïve
T cell
Active
T cell
Dendritic cell

Figure 3.6. FcRn-mediated vaccination induces robust memory responses.

(A-B) Lung-resident memory T cells in mice 8 weeks after a boost with HA-Fc. Lung T cells were labeled intravenously (i.v.) with anti-CD3. Lungs cells were then stained for T cell markers CD3, CD4, and CD8, and for tissue resident memory T cell markers CD69, CD11a and CD103. Representative flow cytometry plots display the percentages of lung-resident CD4+ T cells as CD4+CD69+CD11a+ **(A)** and lung-resident CD8+ T cells as CD8+CD69+CD103+ **(B)**. An additional control group was included in this assay, C57BL/6 wild-type mice intramuscularly (i.m.) vaccinated with HA-Fc/wt. The induction of spleen memory CD4+ and CD8+ T cells were also measured as a control (respective bottom panels). Flow cytometry plots are representative of two independent experiments with 4 immunized mice pooled in each group.

(C-E) Longevity of HA-specific IgA in nasal wash (NW) **(C)** and IgG responses in BAL **(D)** and sera **(E)**. HA-specific IgA and total IgG titers were measured from NW, BAL, and sera of immunized mice 8 weeks after boosting by ELISA (for **(C-D)**, n=3-5 and for **(E)**, n=8-10).

(F-G) Long-lived HA-specific plasma cells from bone marrow. Bone marrow cells (BMC) were collected from the tibias and femurs of immunized mice 8 weeks after a boost. BMCs were plated on HA-coated plates and quantified by ELISPOT analysis to detect HA-specific IgG-secreting plasma cells. Data were pooled from two separate experiments with 5 immunized mice pooled in each group. Graphs are based on average ELISPOT data for four replicate wells for each experiment **(F)**. ELISPOT images from each group are representative of the two experiments **(G)**.

(H) Mean survival following influenza challenge 8 weeks following the boost. The immunized mice were i.n. challenged with 5 MLD_{50} of influenza PR8 and weighed daily for 14 days. Mice were humanely euthanized if more than 25-30% of initial body weight was lost or succumbed to infection. Percentage of mice protected on the indicated days is calculated as the number of mice surviving divided by the number of mice in each group (n=5), as shown by Kaplan-Meier survival curve. Statistical differences were determined using multiple Mantel-Cox tests.

(I) Proposed model of FcRn-mediated respiratory immunization. The Fc-fused HA antigens are transported by FcRn and targeted to the antigen presenting cells (APCs), such as dendritic cells. Antigen is taken up by pinocytosis or FcγRI-mediated endocytosis in APCs, then processed and presented to T cells in the lung or draining lymph nodes.

Statistical differences were determined by one-way ANOVA with Tukey's multiple comparison test **(D-E)** or multiple paired two-tailed Student's *t*-tests **(F)**.

Next, I characterized the memory response mediated by B cells. I detected significantly higher HA-specific IgG titers in both BAL (Figure 3.6C) and serum (Figure 3.6D) eight weeks after a boost in HA-Fc/wt-immunized mice, while there was a modest increase in HA-specific IgA titers in NW of HA-Fc/wt-immunized mice (Figure 3.6E). The enhanced antibody response is corroborated when measuring HA-specific plasma cells. Immunization with HA-Fc/wt resulted in markedly higher numbers of bone marrow-derived HA-specific plasma cells, compared to the control groups eight weeks after boost (Figure 3.6F-G). In order to determine if the vaccine-induced memory response provided protection, eight weeks after a boost, I challenged the mice with a lethal dose of PR8 virus, as previously described. HA-Fc/wt-immunized mice had a significantly higher survival rate of 80%, compared to the control groups, which experienced 0 to 20% survival (Figure 3.6H). Taken together, I demonstrated that FcRn-targeted delivery of HA-Fc/wt was required for eliciting robust memory immune responses, both local and systemic, including induction of lung-resident memory CD4+ and CD8+ T cells, HA-specific IgA and IgG, and HA-specific plasma cells. The vaccine-induced memory response conferred significant protection against a lethal challenge up to eight weeks after boost.

Discussion

Influenza virus continues to be a serious threat to global health and seasonal vaccination remains the most effective measure against infection. The necessity for more protective influenza vaccines has been an omnipresent influence on the field of

influenza research. Concurrently, there is increased interest in improving mucosal vaccination strategies to contend with the onslaught of pathogens that we encounter [229]. In addition, the use of Fc fusion proteins for mucosal application of therapeutic proteins has been well-characterized and encouraging for present and future treatments [349]. Here, I evaluate my efforts to merge these undertakings by developing an FcRn-mediated mucosal influenza vaccine that improved upon previous efforts of an HA-based fusion protein. I demonstrate that the combination of a mucosal vaccination route and FcRn-mediated vaccine delivery allows for high levels of protection against challenge through induction of robust long-lived local and systemic immune responses. I establish that FcRn-mediated transport of trimeric HA-Fc/wt is required to induce a multi-faceted and robust immune response composed of (i) local and systemic antibodies, both IgA and IgG, hallmarks of mucosal immunity and sterilizing protection, respectively, (ii) HA-specific IFN-γ and TNF-α-secreting CD4+ and CD8+ lung T cells, (iii) induction of germinal centers in MeLN, (iv) long-lived HA-specific IgA and IgG antibody titers, (v) HA-specific IgG-secreting plasma cells, and importantly, (vi) induction of lung-resident memory CD4+ and CD8+ T cells.

This translated to significantly higher levels of survival and decreased morbidity in HA-Fc/wt-vaccinated mice. In contrast, treatments that lacked functional FcRn-Fc interactions resulted in poor induction of immunity and increased levels of pulmonary pathology, which resulted in overall lower protection as evidenced by higher mortality rates. Taken together, my study has demonstrated the role of FcRn in facilitating intranasal delivery of protective influenza vaccine antigens across the

respiratory mucosa, highlighting a novel method for formulating and producing influenza vaccines that stimulate long-lasting, protective local and systemic immunity (Figure 3.6I).

My study contributes important advances towards influenza vaccine research: development of a novel mucosal subunit vaccine that induces long-lasting protective mucosal and systemic immune responses in a mouse model; ease of manufacture and scalability in an FDA-approved mammalian cell culture system; and broader coverage with ease of needle-free administration and accessibility to population groups previously restricted from LAIV vaccination, such as people with severe egg allergies, pregnant women, children under two years, and children and adults with asthma. I propose FcRn-mediated vaccination as an attractive alternative to current methods of influenza vaccination.

Present vaccinations induce mainly systemic immune responses that can be short-lived. Parenteral vaccines, such as IIV, are designed to raise high levels of strain-specific neutralizing antibodies to HA and, to a lesser extent, NA, but are poor inducers of mucosal immunity. Mucosal immunity is primarily induced at the site of infection or vaccine administration of mucosal surfaces and is crucial for clearance of pathogens which largely infect at mucosal barriers. Current efforts to optimize commercial influenza vaccines have focused on improving induction of long-lasting immunity, with growing interest in enhancing mucosal immunity.

As previously described, Fc fusion proteins have established their success as therapeutics, where numerous molecules have been fused to the Fc portion of IgG for treatment of important human diseases, although currently there are no licensed Fc

fusion proteins for use against infectious diseases. In addition, while traditional Fc fusion proteins have been based on homodimeric Fc, Fc fusion protein design has evolved to optimize the functionality of the Fc fragment, resulting in a recent shift to monomeric Fc fragments, either alone or fused to biological targets. Monomeric Fc fragments have been shown to further improve Fc fusion protein half-life, enhance tissue penetration, and allow access to sterically restricted binding sites while retaining binding capabilities to FcRn [390] [400] [401].

Expanding upon the previously described HA-Fc/dimer construct, I introduced several modifications to optimize the efficacy of my HA-based fusion protein, to produce HA-Fc as a promising vaccine candidate to protect against influenza infection. Trimerization of HA is mediated by the Fd trimerization domain of T4 fibritin to mimic the native viral protein conformation that is critical for a productive immune response. The use of Fd as a trimerization domain has been established for a number of pathogenic proteins outside of influenza HA, including HIV ENV protein gp41, RSV fusion (F) glycoprotein, and rabies virus glycoprotein (G) [402] [403] [404] [405]. In addition, the Fc fragment of mouse IgG contains mutations to abolish Fc dimerization in order to create an Fc monomer with one set of Hinge-CH2-CH3 domains that is still capable of binding to FcRn. To my knowledge, I have developed for the first time a new generation of monomeric Fc fusion proteins as FcRn-mediated vaccines against pathogenic agents. In this study, I utilize HA-Fc as a mucosal vaccine against influenza virus, where homotrimerization of HA-Fc via the Fd domain does not appear to diminish binding to FcRn, while the trimeric

conformation of HA allows for downstream activation of protective immunity against influenza infection.

While previous studies have used trimerized HA-based Fc fusion proteins for vaccination, these constructs were based on dimeric Fc and used various vaccination routes, predominantly parenteral (e.g., i.m., s.c, i.p., i.n.). Using injectable administration routes, others have shown that vaccination with full-length or portions of H1, H3, and H5 HA fused to Fc, with or without trimerization domains or adjuvant, can induce high levels of serum IgG and neutralizing antibody levels [377-382]. There is growing evidence that HA trimerization can greatly enhance immunogenicity of HA-based vaccines, which was confirmed by most of these studies. One group was able to detect local IgA production from lung lavage fluid after s.c. vaccination with an Fc fusion protein based on a trimerized conserved portion of H5 HA1, although it is unclear how a parenteral immunization route could induce a mucosal antibody response [379]. While a number of these groups did not determine vaccine-induced protection and survival after challenge, others were able to demonstrate varying levels of protection against infection using parenteral immunization routes, including one group that showed protection against different clades within the same strain type. Indeed, most parenterally administered HA-based vaccines, including IIV, have been well-characterized to induce strain-specific immunity and protection. In addition, several of these studies employed portions of the HA1 domain fused to trimerization domains as vaccines, which may not represent the native conformation of the epitopes of interest. Previous reports of an intranasal HA-based Fc fusion protein vaccine showed high levels of neutralizing antibody titer

but failed to demonstrate vaccine-induced protection after challenge. While the utility of the Fc fragment in the context of Fc fusion proteins and its interaction with other FcγRs has been stated in these reports, it does not appear that the interaction between FcRn and Fc fusion proteins was taken into consideration in these studies.

In contrast, I establish that our mucosal HA-Fc/wt vaccine results in high levels of protection as measured by increased survival and decreased pulmonary pathology and viral lung titers, and this protection is mediated by FcRn. Though the exact mechanism driving the efficacy of my vaccine strategy remains to be determined, I believe that the reduced size of HA-Fc/wt fusion protein leads to a decrease in steric hindrance of the Fc portion upon trimerization, permitting unhindered binding to FcRn at the respiratory epithelium, while also allowing for the proper conformational folding of trimeric HA, as evidenced by the recognition of HA-Fc/wt by conformation-dependent HA antibodies. Exposure to correctly folded HA is critical for developing appropriate T cell and neutralizing antibody responses that mirror those induced by exposure to natural infection. I have demonstrated that FcRn-mediated i.n. immunization with HA-Fc can induce T and B cell immunity similar to a natural infection.

Historically, HA-specific neutralizing antibody titer has been the gold standard for determining sterilizing protection against influenza infection. There is growing work that show both B cell- and T cell-mediated immune responses are important for optimal protection, in the context of systemic and mucosal immunity. Influenza-specific adaptive immune responses limit viral replication and clear infected cells. HA-Fc/wt-immunized mice elicited higher levels of neutralizing IgG

titers and serum IgG titers. In addition, in vaccinated mice, lung CD4+ and CD8+ T cells were shown to secrete IFN-γ and TNF-α, antiviral cytokines that work in concert towards viral clearance, in contrast to low to undetectable levels of cytokine secretion in the control groups.

Local humoral immune response is characterized by the secretion of IgA in the upper respiratory tract, the presence of activated germinal centers (GC), and the induction of bronchus-associated lymphoid tissue (iBALT) in lymphoid and non-lymphoid tissues [64] [69] [70] [241] [255]. Intranasal immunization with HA-Fc/wt induced a robust local immune response, characterized by increased titers of sIgA in nasal wash and IgG in BAL, which can originate from both local sites and serum, and the induction of germinal centers as evidenced by a higher percentage of activated B cells within the MeLN. FcRn-mediated vaccination with HA-Fc/wt promotes potent antiviral humoral and cell-mediated immune responses. This immunity contributed towards the significant protection and reduced pathology in HA-Fc/wt-vaccinated and challenged mice. In contrast, challenged mice from the control groups sustained increased morbidity, mortality, pulmonary inflammation, and lung viral loads.

For unknown reasons, on average, immunized FcRn KO mice experienced intermediate levels of immunity and mortality, compared to the other control and WT mice immunized with HA-Fc/wt. Individual FcRn KO-immunized mice occasionally produced higher immune responses compared to the other control groups, such as total IgG, IgG2b and IgG2c titers, and HA-specific plasma cell production. Individual serum samples containing high antibody titers may skew the average titer. Pooling organs within each group, such as lungs, spleen, and bone marrow, also introduces

contributions from individual mice that may harbor higher than average immunity, which may skew the average of the results for a given experiment. On average, measured immunity induced by FcRn KO-immunized mice did not reach the higher levels induced by HA-Fc/wt immunization in WT mice, as elevated immunity was observed in a few individual FcRn KO mice. The majority of the immunized FcRn KO mice elicited lower immune responses and experienced increased mortality and pathology. Previous studies have demonstrated that pIgR knockout mice have a disrupted respiratory mucosal epithelium, resulting in leaking of serum proteins in saliva [406]. It is possible that FcRn knockout mice may experience a similar compromised respiratory epithelium, though our unpublished data suggests that FcRn KO mice maintain epithelial integrity.

In addition to primary effector immune responses, induction of influenza-specific memory responses is crucial for protection against infection or reinfection, while prolonged protection could diminish the requirement of seasonal vaccinations. A multifaceted memory response encompassing both mucosal and systemic immunity, including virus-specific plasma cells, maintenance of antibody titers, and memory T cells, can provide long-lasting protection. FcRn-mediated vaccination with HA-Fc/wt induced higher levels of long-lived HA-specific antibodies, both IgA and IgG, and HA-specific plasma cells. In contrast, control groups did not induce a robust memory humoral response. As previously described, the induction of TRM, or lung-resident memory, T cells has important implications in controlling influenza virus infection. It has been established that lung-resident memory T cells are critical for rapid and enhanced viral clearance within the lung and mediating cross-protection

against diverse virus strains [98] [103]. In my study, I confirm my previous results based on i.n. vaccination with HA-Fc/dimer, by demonstrating that mice i.n. vaccinated with HA-Fc/wt induce higher levels of lung-resident memory CD4+ and CD8+ T cells, compared to i.n. immunized control mice and mice i.m. vaccinated with HA-Fc/wt. The presence of lung-resident memory T cells strongly suggests the potential of long-lasting vaccine-induced protection. Further studies are needed to verify that these TRM T cells are virus-specific and contribute towards long-term survival.

Taken together, I have established that FcRn mediates the intranasal transport of protective influenza vaccine antigens, providing an elegant platform for mucosal vaccine delivery. In future studies, I envision the use of FcRn-targeted influenza vaccination as a model mucosal delivery system of viral proteins that induce broadly reactive and long-lasting protection. There is a great effort to develop a universal influenza vaccine, a vaccine that can protect against all strains of influenza virus, eliminating the need for seasonal vaccination, with the potential to protect against pandemic strains. Numerous vaccine strategies are being explored, including subunit vaccines, various viral vectors, DNA vaccines, and virus-like particles (VLP). Many of these approaches utilize conserved influenza viral proteins, including the stalk domain of HA, HA2, internal viral protein nucleoprotein (NP), the ectodomain of matrix 2 protein (M2e), and neuraminidase (NA) [407]. There is an ongoing characterization of antibodies induced by these conserved proteins or subunits which have been shown to be broadly neutralizing, in the case of HA2 and NA, or broadly reactive, in the case of NP and M2e.

It has been previously demonstrated that influenza-specific T cells can recognize conserved viral proteins, which has important implications in cross-reactivity. Targeting enhanced cell-mediated immunity, in addition to broadly neutralizing antibody induction, has been considered major goals of new vaccination strategies [113]. I have demonstrated that our vaccine platform induces mucosal and systemic adaptive immunity that is both protective and long-lasting. It is reasonable to suggest that by employing FcRn-mediated delivery of highly conserved viral proteins as vaccine antigens, such immune responses could be broadly neutralizing and reactive against multiple strains of the virus, providing a platform for developing universal mucosal influenza vaccines.

In conclusion, I have developed a noninvasive, mucosal influenza vaccine model that is highly adaptable to confront the challenges of the rapidly mutating influenza virus. My study establishes that FcRn can efficiently deliver influenza viral proteins at the respiratory epithelia through the FcRn-IgG transport pathway and confer protection, highlighting the potential of our FcRn-mediated strategy as an effective mucosal influenza vaccine. Importantly, I demonstrate that intranasal administration of an HA-based Fc fusion protein can establish lung-resident memory T cells. My findings suggest that FcRn-targeted mucosal immunization may be an effective strategy for developing a universal influenza vaccine based on highly conserved viral antigens that can induce long-lasting, protective mucosal and systemic immunity.

Chapter 4: FcRn-mediated Delivery of Influenza NP Provides Partial Protection Against Lethal Influenza Challenge in Mice

Abstract

Current influenza vaccines rely on the induction of strain-specific immunity primarily against glycoproteins HA and NA, resulting in a sterilizing protection against the vaccine strain. In cases of mismatches between the vaccine and circulating viral strains, protection can be greatly reduced. In light of this narrow range of immunity produced by the licensed vaccines, there are growing efforts to develop influenza vaccines based on conserved viral proteins with the potential to induce broadly protective immunity against diverse virus strains. The development of a universal influenza vaccine that can protect against all influenza A and B viruses, irrespective of HA and NA subtype, is a major goal of influenza vaccine research. The internal structural protein, nucleoprotein (NP), is an attractive vaccine antigen target as NP retains >90% conservation across all influenza A strains. I previously demonstrated that FcRn delivers influenza HA antigens across the respiratory barrier and produces protective immunity. In this study, I determined if FcRn can effectively deliver an NP-based vaccine antigen across the respiratory mucosal epithelium and engender protective immunity against influenza infections. Intranasal immunization of mice with NP fused to IgG Fc (NP-Fc) plus CpG adjuvant induced a robust NP-specific humoral immune response and conferred partial protection against lethal influenza challenge. Thus, it is possible that FcRn-targeted delivery of NP-Fc across the respiratory epithelium could mediate the processing and presentation of antigenic

peptides that are characteristic of naturally occurring NP epitopes recognized by immune cells, allowing for the activation of potentially cross-protective immunity.

Introduction

Influenza virus remains an important human pathogen that causes significant illness during seasonal epidemics, with increased morbidity and mortality during pandemic events. During natural infection and after influenza virus vaccination, immune responses primarily consist of strain-specific neutralizing antibody production against the major immunogens, HA, and to a lesser extent, NA. Though influenza-specific immunity is currently characterized by HA antibody titers, humoral immunity does elicit the induction of antibodies against all of the viral proteins, including high levels of antibody against the internal structural protein, nucleoprotein (NP). NP is a key component of the ribonucleoprotein (RNP) complex, consisting of viral RNA coated in oligomers of NP, which are associated with a polymerase complex. NP allows for the nuclear import of the RNP via nuclear localization signals and is required for viral RNA transcription, replication, nuclear export, and packaging. NP has several known regions of high conservation across influenza A, B, and C viruses, with >90% sequence conservation among influenza A virus subtypes [1] [408]. After natural infection, the immune response against NP consists mainly of induction of an NP-specific CTL response, which is considered largely responsible for cross-protection from multiple influenza virus strains, and production of high titers of NP-specific antibodies [409] [410]. Though NP-specific antibodies are non-neutralizing, they have been demonstrated to mediate protection against homologous

and heterologous viruses through the activation of ADCC and CDC by NP-specific antibodies binding to NP epitopes expressed on the surface of infected cells [64] [217].

Because of the highly conserved structure of NP, it is an attractive vaccine target for the development of influenza vaccines capable of inducing broadly reactive protection, or universal vaccines. Based on my previous findings utilizing HA-based Fc fusion proteins as vaccine targets for FcRn-mediated intranasal delivery across the respiratory mucosal epithelium, in this study, I determined if I could expand our vaccine strategy to transport a conserved influenza viral protein to engender protective immunity. After intranasal immunization of mice with NP-based Fc fusion protein, NP-Fc, along with the CpG adjuvant, I observed that FcRn efficiently delivered NP-Fc across the respiratory mucosa into circulation. This allowed for the induction of a robust NP-specific humoral immune response and the conferral of partial protection to lethal homologous influenza infection in mice. Taken together, FcRn-mediated mucosal delivery of NP-Fc may be used to develop a universal mucosal influenza vaccine, although the immunogenic efficacy of the NP-Fc proteins would require further optimization through modifications to the NP and Fc subunits.

Materials and Methods

Cells, antibodies, and virus

Chinese hamster ovary (CHO) cells were purchased from the American Tissue Culture Collection (ATCC). CHO cells were maintained in DMEM complete medium

(Invitrogen Life Technologies), supplemented with 10% FBS, 2 mM l-glutamine, nonessential amino acids, and penicillin (0.1 µg/ml)/streptomycin (0.292 µg/ml). Stable CHO cell lines expressing fusion genes were grown in a complete medium with G418 (500 µg/ml). All cells were grown at 37°C in 5% humidity. Influenza A/Puerto Rico/8/34/Mount Sinai/H1N1 (PR8) virus was generously provided by Dr. Peter Palese (Icahn School of Medicine at Mount Sinai) and was amplified in 10-to 11-day-old embryonated chicken eggs and titrated by 50% endpoint dilution assay. Horseradish peroxidase (HRP)-conjugated streptavidin and anti-mouse IgG antibodies were purchased from Southern Biotech (Birmingham, Alabama). Anti-NP antibodies and recombinant NP were purchased from Novus Biologicals (Littleton, Colorado).

Construction of PR8 NP-Fc expression plasmids

To make a fusion gene for expression of NP-Fc protein, full-length PR8 NP was amplified from whole genome cDNA using the primer pair (5'-GCGCGAATTCACCGCCATGCCCATGGGGTCTCTGCAACCGCTGGCCACCTTGTACCTGCTGGGGATGCTGGTCGCTGCGTCTCAAGGCACCAAACG-3', 5'-AGATCCCGAGCCACCTCCTCCGGACCCACCCCCGCCTGATCCATTGTCGTACTCCTCTGCATTGTCTCCGAAG-3'). The first 20 amino acids of the signal peptide from T cell surface glycoprotein CD5 was included before the NP nucleotide sequence, in order to direct the protein for translocation into the ER to allow for secretion from the cell. To produce the Fc portion, a previously constructed pCDNA3 plasmid encoding the Hinge, CH2 and CH3 domains of mouse IgG2a Fc served as a template. Mouse IgG2a isotype was chosen because it has the highest affinity for

activating FcγRI, but the lowest affinity for inhibitory FcγRIIB. In this plasmid, the Glu318, Lys320, and Lys322 residues were replaced with Ala residues to remove the complement C1q binding site. In order to produce a mutant form of IgG Fc fusion protein that cannot bind to FcRn, the His310 and His433 residues were changed to Ala residues to eliminate FcRn binding sites. The NP antisense primer and the IgG Fc sense primer contain complementary glycine and serine codons to produce a 14GS linker to bridge the NP and IgG Fc fragments. The NP and Fc PCR fragments were fused by overlapping PCR and ligated into the pCDNA3 vector. All of the resultant plasmids were confirmed by double-stranded DNA sequencing to verify the fidelity of PCR amplification and DNA cloning.

Expression and characterization of NP-Fc fusion proteins

The NP-Fc/wt and mut plasmids were transfected into CHO cells using PolyJet (SignaGen) according to the manufacturer's instructions. Stable cell lines expressing NP-Fc were selected and maintained under G418 (0.5-1 mg/ml). Expression and secretion of NP-Fc fusion proteins were determined by immunofluorescence assay, SDS-PAGE and Western blotting analysis. The soluble NP-Fc proteins were produced by culturing CHO cells in complete medium containing 5% FBS with ultra-low IgG. The proteins were purified by affinity chromatography using Protein A- (Thermo Scientific) and anti-mouse IgG- (Rockland) conjugated agarose beads. Protein concentrations were determined using NanoDrop Spectrophotometer (Thermo Scientific). Molecular graphics and analyses of the structure of the NP-Fc proteins were performed with Phyre 2 (Phyre2 ref) and the UCSF Chimera package (University of California, San Francisco).

Immunofluorescence assay (IFA)

Immunofluorescence was performed as previously described (2008 ref). Briefly, cells were grown on coverslips for 48 hr. The cells were rinsed with HBSS and fixed with 4% paraformaldehyde (Sigma) in HBSS for 20 min and quenched with 100 mM glycine in PBS for 10 min. Cells were permeabilized with 0.2% Triton-X in HBSS for 5 min and incubated with blocking solution (3% normal goat serum in PBS) for 30 min. Cells were incubated with anti-NP antibodies diluted in blocking solution for 2 hr in the dark. After washing with PBS, Alexa Fluor 555-conjugated anti-rabbit F(ab')2 frag and Alexa Fluor 488-conjugated anti-mouse IgG2a secondary antibodies were added to 1 hr in the dark. All steps were performed at room temperature. After washing with PBS, cells were mounted to slides with ProLong Antifade solution (Thermo Scientific). Images of the stained cells were obtained using a Zeiss LSM 510 confocal fluorescence microscope and LSM Image Examiner software (Zeiss).

SDS-PAGE gel and Western blotting

Protein concentration and quality were assessed by 8% SDS-PAGE gels under reducing and non-reducing conditions. Protein in gels was either stained with Coomassie blue dye or transferred onto nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with 5% milk in PBST (PBS and 0.05% Tween-20) and incubated overnight with anti-IgG2a-HRP (1:10,000). SuperSignal West Pico PLUS ECL substrate (Thermo Fisher) was used to visualize protein in membranes and images were developed and captured by the Chemi Doc XRS system (BioRad).

Mouse immunization and virus challenge

All mice were housed in the University of Maryland animal facilities and all the animal experiments were performed with the approval of the Institutional Animal Care and Use Committee. FcRn knockout mice in the C57BL/6 background is a kind gift from Dr. Derry Roopenian (Jackson Laboratory). Six to eight-week-old female C57BL/6 mice (Charles River Laboratory) and FcRn knockout mice were intranasally (i.n.) immunized with 20 μl of 10 μg NP-Fc/wt, NP-Fc/mut, recombinant NP, or PBS. All vaccine proteins or PBS were mixed with 10 μg of CpG ODN 1836 (Invivogen). Two weeks later, the mice were boosted with the same vaccine formulations. Two weeks after a boost, mice were i.n. infected with 2.5 MLD_{50} PR8 virus ($5x10^3$ $TCID_{50}$). For immunizations and challenge, all mice were anesthetized with intraperitoneal (i.p.) injection of 100 μl of fresh Avertin (40 mg/ml, Fisher Scientific) and laid down on their backs to allow for recovery. After infection, mice were monitored daily for weight loss for 14 days. Animals that lost above 30% of their body weight on the day of infection were euthanized.

Enzyme-linked immunosorbent assay (ELISA)

For detection of NP-specific antibodies in serum 2 weeks after a boost, high- binding ELISA plates (Maxisorp, Nunc) were coated with 3 μg/ml of recombinant NP protein in PBS. For detection of NP-Fc in serum 8 hours after immunization, plates were coated with 3 μg /ml of Rabbit anti-NP polyclonal antibody. Plates were then incubated overnight at 4°C. The next day, plates were washed three times with 0.05% Tween 20 in PBS (PBST) and blocked with 2% BSA in PBS for 1 hr at room temperature. Samples were serially diluted in 2% BSA-PBS and were added to plates

for 2 hr incubation at room temperature. After washes, for detection of NP-specific antibodies, HRP-conjugated rabbit anti-mouse IgG antibody (1:7000, Pierce) was added to plates for 1 hr, and for detection of NP-Fc, HRP-conjugated Goat anti-mouse IgG2a (1:5000, Southern Biotech) was added for 1 hr. The reaction was visualized in a colorimetric assay using substrate tetramethyl benzidine (TMB) and a Victor III microplate reader (Perkin Elmer). Titers represent the highest dilution of samples showing a 2-fold increase over average OD_{450} nm values of negative controls.

Statistics analysis

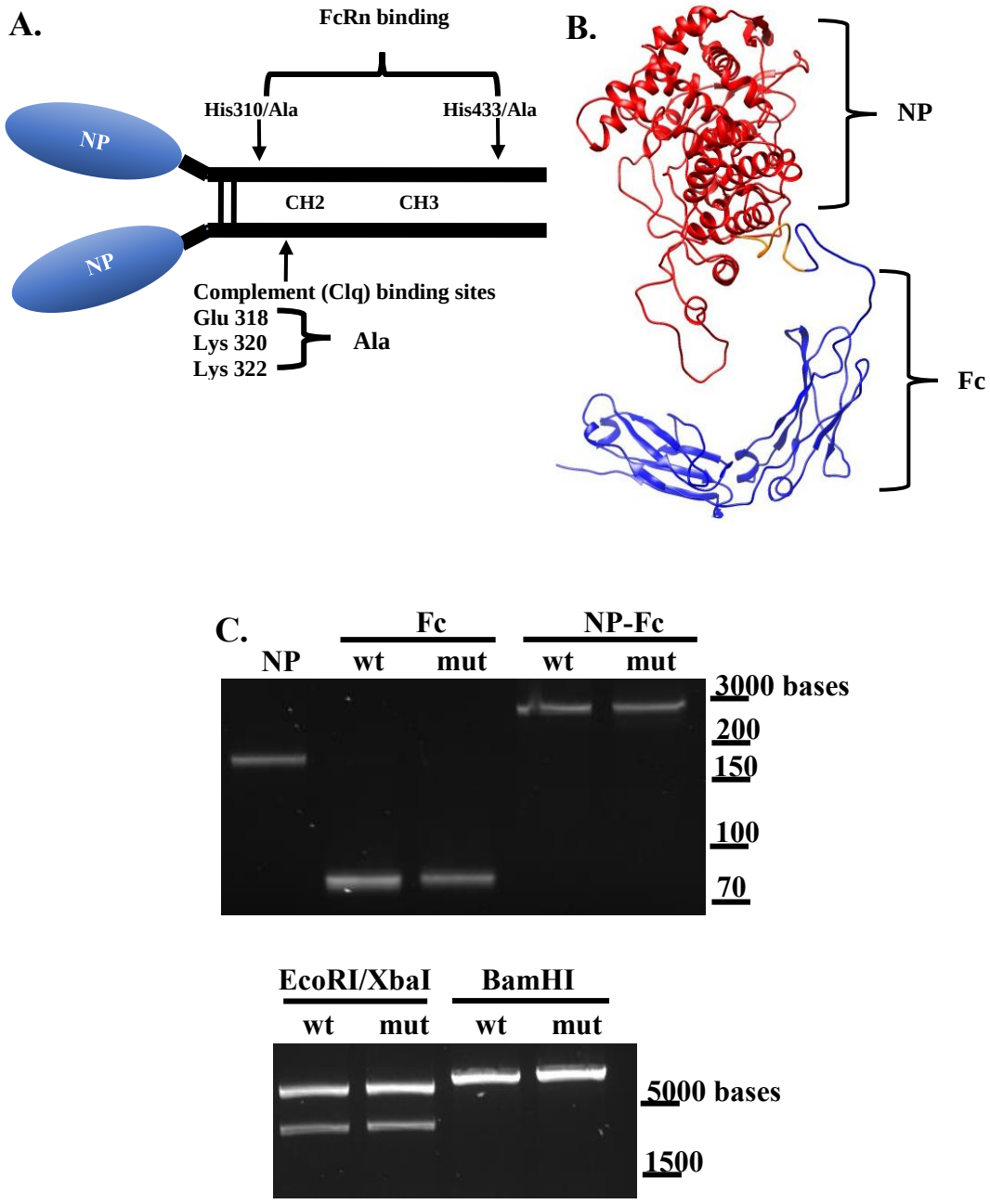
Differences in serum NP-Fc and antibody titers were assessed by using paired Student's two-tailed t-test or one-way ANOVA with Tukey's multiple comparison test. GraphPad Prism 5.01 software was used for the statistical analyses.

Results

Characterization and production of NP-Fc fusion proteins

I previously demonstrated that FcRn-mediated intranasal vaccination with HA-based vaccine antigens induced high levels of long-lasting local and systemic immunity while conferring significant protection against homologous lethal challenge. I determined if my vaccine delivery platform could be expanded to deliver conserved viral proteins that have greater potential for inducing cross-reactive immunity, such as NP. In order to demonstrate my model of FcRn-mediated delivery of conserved

influenza viral proteins, I expressed an NP-based Fc fusion protein by cloning full-length NP from influenza A/Puerto Rico/8/1934/H1N1 in frame with the Fc portion of mouse IgG2a, resulting in NP-Fc. As NP is an internal viral protein, the signal peptide from the T cell surface glycoprotein CD5 was included before the NP nucleotide sequence to mediate secretion of NP-Fc. I included the Fc portion of mouse IgG2a isotype, which has the highest affinity for activating FcγRI, but the lowest affinity for the inhibitory receptor, FcγRIIB. In addition, a mutant version of the plasmid was produced that contained point mutations in the Fc gene at positions His310Ala and His433Ala in order to prevent binding to FcRn, which served as a control to determine the role of FcRn in mediating vaccine-induced immunity. Both NP-Fc/wt and mut plasmids contained mutations to remove the complement C1q binding site in order to prevent the fixation of the complement pathway (Figure 4.1A-B). Overlapping PCR was used to fuse the NP and Fc portions of the fusion genes, which were then ligated into pCDNA3 vectors to produce the NP-Fc/wt and mut plasmids. I verified the plasmids' size through restriction enzyme digests with EcoRI/XbaI and BamHI (Figure 4.1C). This suggested that cloning resulted in error-free production of the fusion genes, which was confirmed by nucleotide sequencing of both plasmids. The NP-Fc/wt and mut plasmids were transfected into CHO cell lines and highly-expressing stable cell lines were selected and maintained for NP-Fc protein purification. Using IFA to stain stable cell lines expressing NP-Fc/wt and mut, both the NP and Fc domains were recognized by antibodies against NP and mouse IgG2a, respectively, verifying that NP-Fc proteins were properly and homogeneously expressed (Figure 4.1D).


A.
FcRn binding
His310/Ala
His433/Ala
NP
NP
CH2
CH3
Complement (Clq) binding sites
Glu 318
Lys 320
Lys 322
Ala
B.
NP
Fc
C.
Fc
NP-Fc
NP
wt
mut
wt
mut
3000 bases
200
150
100
70
EcoRI/XbaI
BamHI
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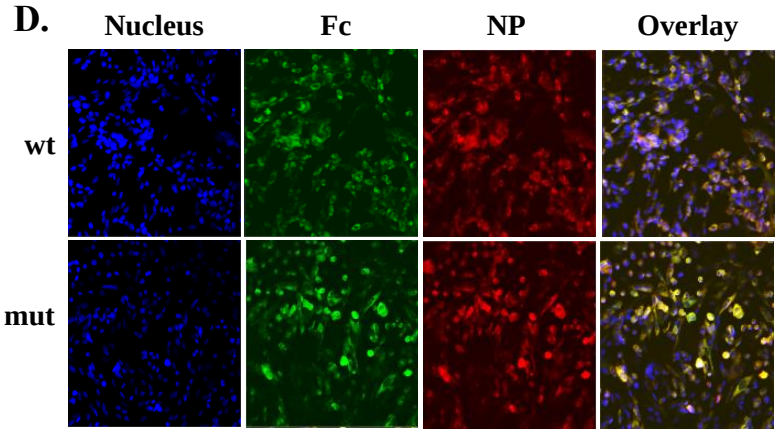
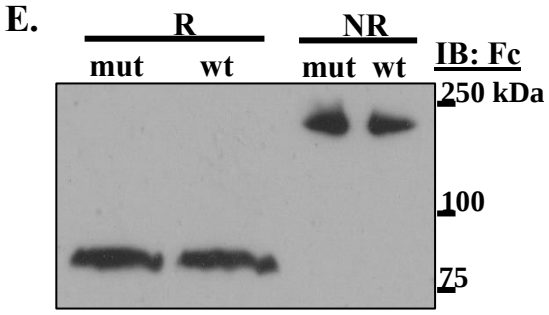
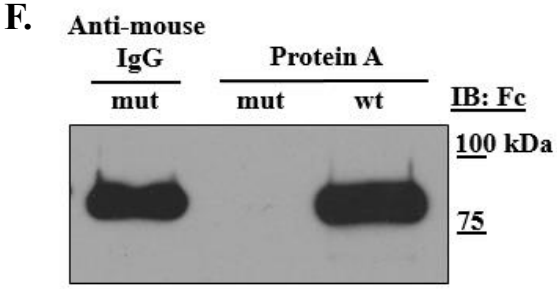
5000 bases
1500


D.
Nucleus
Fc
NP
Overlay
wt
mut


E.
R
NR
mut
wt
mut
wt
IB: Fc
250 kDa
100
75


F.
Anti-mouse IgG
Protein A
mut
mut
wt
IB: Fc
100 kDa
75

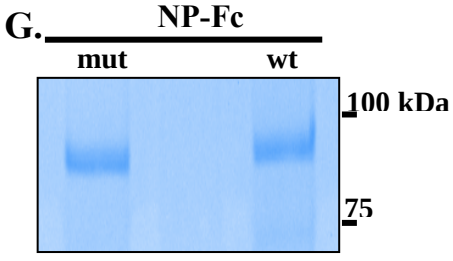

G.
NP-Fc
mut
wt
100 kDa
75

Figure 4.1. Design and characterization of PR8 NP-Fc fusion protein.

(A) Schematic representation of PR8 NP fused to Fc portion of mouse IgG2a. The following mutations were made in Fc portion of IgG2a: To remove the complement C1q binding site, Glu318, Lys320, and Lys322 were replaced with Ala residues; and to eliminate FcRn binding sites for NP-Fc/mut fusion protein, His 310 and His 433 were replaced with Ala residues.

(B) Predicted protein structure of NP-Fc. The NP domain is depicted in red and the IgG2a Fc domain is in blue. The following nucleotide sequences were acquired through GenBank: NP: CY105938.2; IgG2a Fc: KC295246.1. The final image was generated and modeled by Phyre2 (Imperial College London) and Chimera (University of California, San Francisco).

(C) Cloning NP-Fc fusion gene for transfection into CHO cell line. Components of fusion gene (NP portion and Fc portion) were fused together using overlapping PCR to produce the final fusion gene, NP-Fc, for insertion into a pCDNA3 vector to produce wild-type (wt) and mutant (mut) versions of NP-Fc plasmids.

(D) Stable expression of NP-Fc in CHO cell line. NP-Fc/wt and mut plasmids were stably transfected into CHO cells. Anti-mouse IgG2a antibody was used to stain for Fc portion of NP-Fc.

(E) Western blot analysis of non-reduced and reduced NP-Fc/wt and mut probed with Fc.

(F) Pull down of NP-Fc/wt and mut by Protein A and/or anti-mouse IgG conjugated to agarose beads. 500 ng purified protein was incubated with resin slurry for 2h at 4°C. Boiled and eluted samples were run on SDS-PAGE. Western blot analysis of pulled-down NP-Fc/wt and mut samples probed with anti-Fc antibody

(G) NP-Fc/wt and NP-Fc/mut proteins were purified by affinity chromatography. Quality of purified protein was determined by SDS-PAGE analysis of NP-Fc/wt and mut.

Based on SDS-PAGE and Western Blot, in non-reducing and reducing conditions, NP-Fc/wt and mut were produced as dimers and monomers, respectively, which were recognized by anti-IgG2a antibody (Figure 4.1E). As previously described, I confirmed the ability of NP-Fc fusion proteins to bind to FcRn using a Protein A pull-down assay. I observed that NP-Fc/wt was pulled down by Protein A-conjugated beads, while NP-Fc/mut protein was pulled down by anti-mouse IgG, but not Protein A (Figure 4.1F). NP-Fc/wt and mut proteins were purified from the cellular supernatant by affinity chromatography and high levels of protein quality and concentration were verified by SDS-PAGE and Coomassie blue staining (Figure 4.1G). Taken together, I established that NP-Fc fusion proteins were expressed and purified at high levels, and only NP-Fc/wt interacts with Protein A, which strongly suggests the ability of NP-Fc/wt to interact with FcRn *in vivo*.

Intranasal vaccination with NP-Fc induces an NP-specific humoral immune response

I determined if FcRn effectively delivered NP-Fc across the respiratory mucosal epithelium in order to mediate the induction of NP-specific immunity. I intranasally (i.n.) immunized WT or FcRn KO mice with 10 μg NP-Fc/wt, NP-Fc/mut, or NP alone. In order to demonstrate that FcRn can deliver NP-Fc/wt across the respiratory mucosa into circulation, I measured the amount of NP-Fc in serum 8 hrs after initial immunization. I observed an increase in transcytosis of NP-Fc/wt in the sera of WT mice, with decreased concentrations of NP-Fc/wt detected in sera of FcRn KO-immunized mice, and even fewer amounts of NP-Fc/mut and NP alone in the sera of

immunized WT mice (Figure 4.2A). This demonstrates that NP-Fc/wt is able to efficiently cross the respiratory epithelial barrier following i.n. immunization.

I then determined if FcRn-mediated delivery of NP-Fc induced a robust immune response. Two weeks after initial immunization, mice were boosted with the same vaccine formulations, and all prime and boost treatments included 10 μg of CpG as an adjuvant. Two weeks after a boost, I measured the level of NP-specific antibody titers in serum by ELISA. I observed that NP-Fc/wt-immunized mice elicited significantly higher titers of NP-specific total IgG in serum, while the control treatment groups produced reduced titers of anti-NP antibody (Figure 4.2B). Overall, I concluded that after i.n. immunization with NP-Fc, FcRn is required to mediate the transport of NP-Fc/wt across the respiratory mucosa in order to initiate a robust humoral immune response.

FcRn-mediated immunization with NP-Fc confers partial protection against PR8 challenge

Based on my preliminary results of NP-specific antibody induction, I determined the extent of protection that was conferred by NP-Fc vaccination against lethal challenge with PR8. Two weeks after prime and boost with the previously described vaccine antigen treatments, mice were i.n. challenged with 2.5 MLD_{50} PR8 virus ($5x10^3$ $TCID_{50}$) and weighed daily for 14 days, with a 30% weight loss cut off as the parameter for euthanasia. Within the NP-Fc/wt-immunized group, 3 mice lost weight by day 7 after infection, of which 2 mice fully recovered to initial body weight and the remaining mouse had a more modest weigh recovery, while one mouse had minimal weight loss over the course of infection. The remaining 6 mice reached 30%

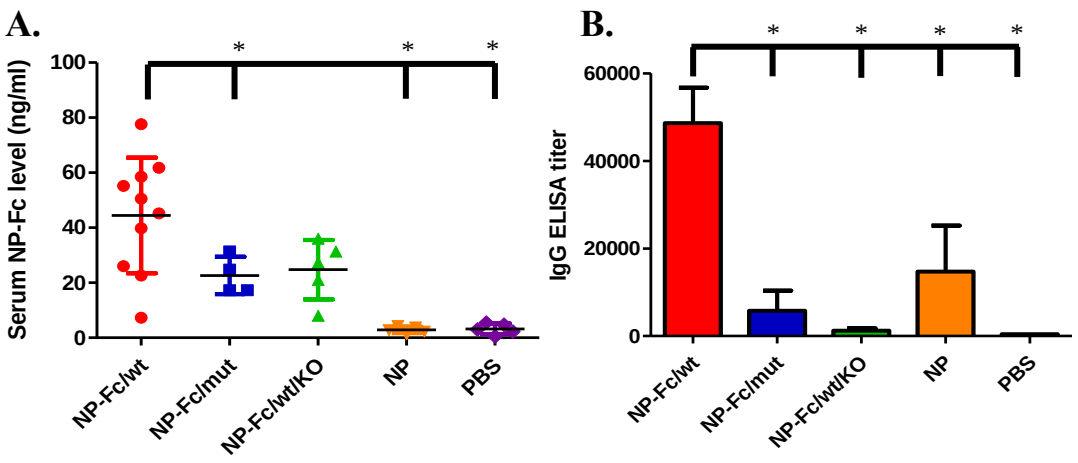


Figure 4.2. In vivo transcytosis of NP-Fc and induction of humoral immunity.

(A) PBS or 10 μg of NP-Fc/wt, NP-Fc/mut, or NP were i.n. administered to wild-type or FcRn knockout (KO) mice along with 10 μg of CpG. Eight hours after immunization, sera were collected and amount of NP-Fc that transported from the respiratory tract into serum was measured by ELISA.

(B) Two weeks post prime, the same conditions were i.n. administered to the respective groups for boost. Two weeks post boost, sera were collected and total IgG antibody titers against NP were measured by ELISA.
Statistical differences were determined by paired two-tailed Student's *t*-tests **(A)** or one-way ANOVA with Tukey's multiple comparison test **(B)** and values were marked as followed with asterisks in this and subsequent figures: *P<0.05, **P<0.01, ***P<0.001.

weight loss or succumbed to infection between days 7 and 8 (Figure 4.3A). Within the control groups, the majority of mice experienced high levels of weight loss primarily within days 6 to 8 after infection (Figure 4.3A).

Overall, after PR8 challenge, i.n. immunization resulted in a survival rate of 40% for NP-Fc/wt-immunized mice, compared to 20% survival of NP-Fc/mut- and NP-Fc/wt/KO-immunized mice, and 0% of NP-immunized mice (Figure 4.3B). Though my preliminary findings demonstrate a 40% survival rate of NP-Fc/wt-immunized mice which is inadequate for vaccine purposes, my results do indicate that FcRn-mediated delivery of NP-Fc/wt was able to confer partial protection to treated mice, while the control groups experienced lower survival rates after PR8 challenge. Overall, FcRn-mediated delivery of NP-Fc/wt allows for the induction of a robust humoral immune response, while reducing morbidity and mortality after lethal challenge. While the NP-Fc/wt fusion protein in its current iteration does not confer satisfactory levels of protection against homologous challenge, I believe that the vaccine antigen can be optimized for enhanced protective immunization. Thus, taken together with my previous results that utilized HA as a protective vaccine antigen, I believe that mucosal delivery of influenza vaccine antigens, including conserved proteins, via FcRn can serve as an alternative model to current mucosal influenza vaccine strategies.

Discussion

The development of universal influenza vaccines based on conserved viral proteins is an area of critical relevance. Currently licensed vaccines are designed to protect against seasonal epidemics. This requires costly and time-consuming annual

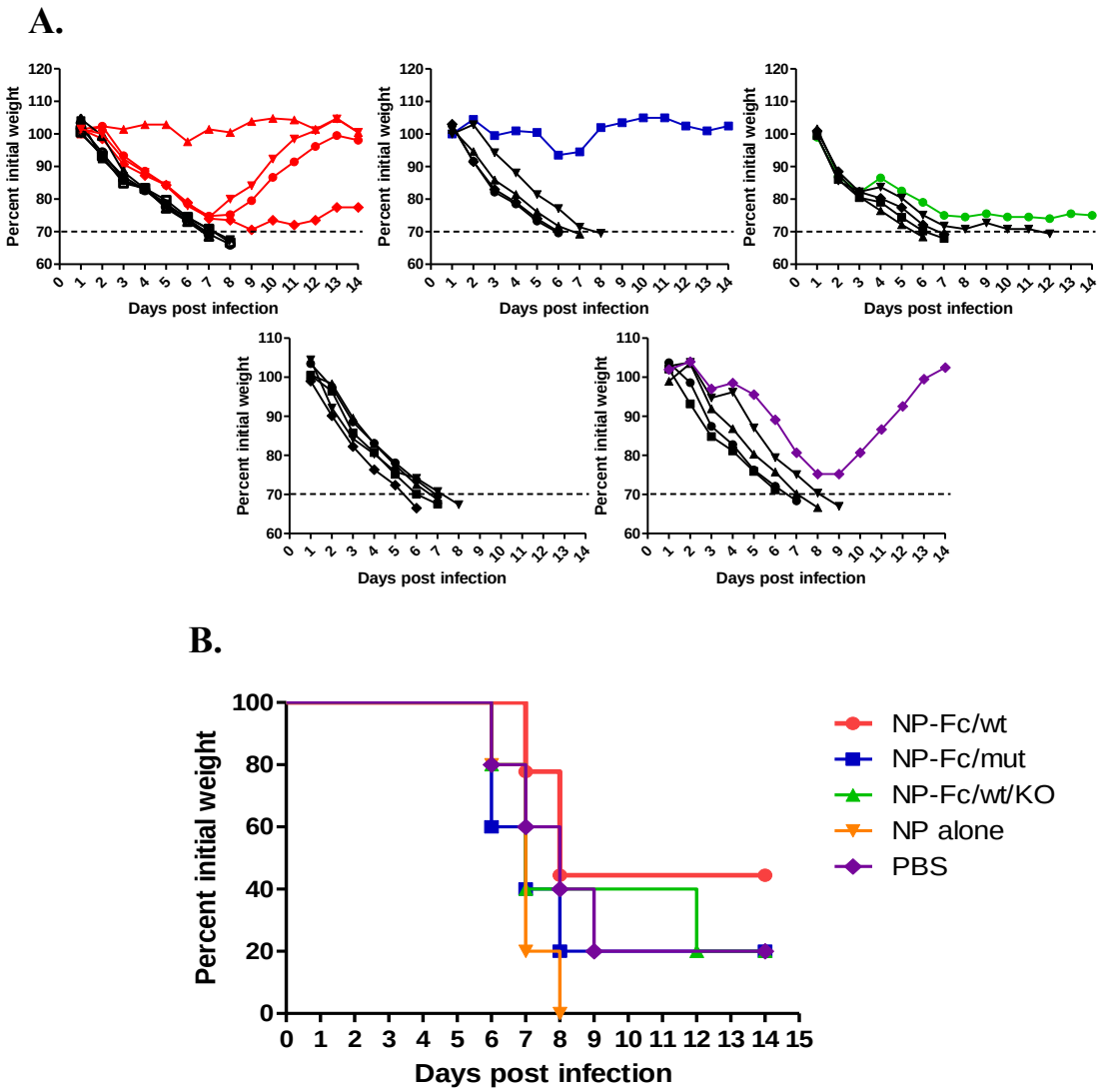


Figure 4.3. FcRn-mediated delivery of NP-Fc confers partial protection against PR8 challenge.

Two weeks after a boost, mice were infected with a challenge dose of 2.5 MLD_{50} PR8 virus ($5x10^3$ $TCID_{50}$) and weighed daily for 14 days. Mice were euthanized when body weight loss reached a 30% endpoint.

(A) The individual weight loss of immunized mice in each treatment group over 14 days is shown, with surviving mice displayed in corresponding colors (n=5-10).

(B) The Kaplan-Meier survival curve shows percent survival after challenge (n=5-10).

reformulations of influenza vaccines. In addition, based on present formulations, licensed vaccines primarily elicit strain-specific immunity that do not have the potential to protect against novel pandemic strains. In contrast, universal influenza vaccine candidates utilize conserved proteins or protein subunits as vaccine antigens. This allows for the induction of immunity with the potential to protect against diverse influenza virus strains. Current influenza vaccines rely on parenteral routes of administration that induce mainly systemic immunity. An effective universal influenza vaccine should induce a multifaceted protective immune response that includes induction of long-lasting mucosal and systemic immunity, composed of both humoral and cell-mediated immune response.

I have previously demonstrated that FcRn mediates the mucosal delivery of Fc fusion proteins based on HA, resulting in the induction of robust protective antigen-specific local and systemic immunity against homologous challenge. I determined if our model of FcRn-mediated delivery of viral proteins could be expanded to transport conserved viral proteins, such as NP, for the induction of protective immunity. I first demonstrated the efficacy of i.n. vaccination with NP-Fc against homologous challenge, which resulted in high levels of NP-specific antibody titers and partial protection against lethal PR8 challenge. As I observed similar preliminary results with immunization with HA-Fc/dimer, it is possible to improve vaccine efficacy with structural modifications to the NP-Fc construct, to both the NP and Fc portions.

Within the influenza virion, NP monomers oligomerize onto viral ssRNA. In the absence of RNA, purified NP exists in a range of binding states, from monomers to small oligomers. NP mediates oligomerization through the domain exchange loop,

a flexible tail loop formed by residues 402– 428 within NP that inserts into the binding groove of neighboring NP molecules [411]. As NP epitopes recognized by CTLs and NP-specific antibodies are primarily derived from monomeric NP, it is essential that NP vaccine antigens are also monomeric [410] [412]. As RNA-free recombinant NP molecules can readily form oligomers, it is possible the current NP-Fc fusion proteins interact to oligomers, though this would need to be verified by electron microscopy. Thus if NP-Fc does oligomerize, then it is conceivable that this conformation of NP-Fc is not optimal for antigen uptake and processing, resulting in NP epitopes that are not immunodominant.

In order to resolve this issue, two point mutations in the domain exchange loop can eliminate oligomerization, allowing for maintenance of the native monomeric conformation of NP that is processed and presented to immune cells. It has been previously shown that mutations at residues Arg416 and Glu339 abrogate oligomerization of NP molecules. Arg416, a residue within the tail loop, forms an inter-subunit salt bridge with Glu339, thus changes at either or both of these residues to Ala would destabilize NP-NP interactions, allowing NP to remain monomeric [411]. To further ensure that oligomerization does not occurs, the optimized NP-Fc vaccine protein would contain monomeric Fc fused to monomeric NP. I have previously demonstrated that monomeric Fc activates robust immunity in an FcRn-dependent manner in the context of an HA-based vaccine. It would be of interest to determine if an NP-based monomeric Fc fusion protein strategy also induces enhanced immunity and protection against influenza infection.

NP-specific T cell-mediated immunity is an important part of natural infection and has been demonstrated to confer heterosubtypic protection. A number of NP-based vaccine candidates utilize DNA or viral vectors expressing NP in order to mediate MHC class I antigen presentation primarily for the activation of NP-specific CTLs [208] [209]. Though the majority of NP-based T cell vaccines attempt to induce an NP-specific CTL response, there are also studies that demonstrate the utility of NP-specific antibodies during the course of influenza infection. One study demonstrated that i.p. injection with recombinant NP as a subunit vaccine, leading to the induction of the MHC class II pathway, mediated immunity and protection against a sublethal homologous challenge by a chiefly humoral response, while poorly inducing CTL activation [64]. We and others have demonstrated that the i.n. route of administration has a number of significant advantages over parenteral vaccination routes, including the induction of local immunity and lung-resident memory T cells. In addition, in my previous study, I observed that FcRn-mediated i.n. immunization with HA-Fc was able to induce both CTL and CD4+ T cell responses. Thus, in the future, after optimizing the NP-Fc construct, I would determine the effects of i.n. immunization with NP-Fc to see if modifications to make both NP and Fc monomeric in structure can enhance vaccine efficacy for improved protection.

Taken together, I believe that by modifying the NP and Fc subunits of NP-Fc, vaccine efficacy can be improved to the levels of robust long-lasting protective immunity as induced by FcRn-mediated immunization with HA-Fc. With optimizations to enhance immunity and protection against homologous challenge, it would then be reasonable to demonstrate if FcRn-targeted delivery of NP-Fc can

confer protection against diverse virus strains: first, across different clades; second, across subtypes within a group; and third, virus strains from groups 1 and 2. This level of cross-protection is an essential requirement in developing a universal influenza vaccine. In the future, I will determine if FcRn-mediated immunization of influenza vaccine antigens is capable of inducing broadly-reactive protection and characterize the mechanisms by which protection is achieved.

Chapter 5: Summary and Future Perspectives

In summary, I have demonstrated that FcRn efficiently mediates the mucosal delivery of influenza vaccine antigens, resulting in the induction of antigen-specific immunity and the conferral of protection against lethal homologous challenge. In Chapter 2, I established influenza HA as a model influenza antigen that can induce robust long-lasting HA-specific immunity, including the induction of lung-resident memory T cells. After observing partial protection against homologous challenge, it was necessary to improve HA-based vaccine efficacy for enhanced protection.

In Chapter 3, I optimized FcRn-mediated vaccination with HA-based Fc fusion proteins with modifications to the HA-Fc construct, including trimerization of HA and monomerization of Fc fragment. I observed significantly improved vaccine efficacy with i.n. immunization with the HA-Fc resulting in both robust immunity and protection. I established that the interaction between FcRn and HA-Fc was required for the enhanced immunity and protection conferred by immunization. FcRn competent mice immunized with HA-Fc/wt induced high levels of long-lived local and systemic HA-specific antibody titers, HA-specific plasma cells, and lung-resident memory T cells, while reducing inflammation and pulmonary pathology, allowing for 84% survival of mice lethally challenged with PR8.

In Chapter 4, I expanded my mucosal delivery platform to a conserved viral protein, NP, as a vaccine antigen that can, first, be demonstrated to protect against homologous challenge. Though I was able to observe robust induction of NP-specific humoral immunity, i.n. vaccination with NP-Fc conferred partial protection to lethal

challenge, requiring modifications to the vaccine construct in order to improve vaccine efficacy. As the immunogenic form of NP is monomeric, I believe that by introducing point mutations in both the NP and Fc portions to render them monomeric will allow for greater immunogenicity of the NP-Fc vaccine protein. In the future, the potential of optimized NP-Fc to protect against both homologous and heterologous virus strains will be assessed.

Developing a universal influenza vaccine that can protect against almost all strains of influenza A and B viruses, regardless of HA and NA subtype, is the gold standard of current influenza vaccine research. Most universal influenza vaccine candidates are based on viral proteins or subunits that are conserved across many, if not all, virus strains, employing the rationale that immunity induced against conserved epitopes have the potential to protect against all viruses that express these immunogenic domains. Induction of broadly protective immunity could eliminate the requirement of annual vaccine reformulations, while also achieving the potential to protect against novel pandemic strains.

Several viral proteins and protein domains have been exemplified for having the greatest potential for induction of broadly-reactive immunity: the stalk domain of HA or HA2, NP, and the ectodomain of M2 (M2e). A number of studies in pre-clinical and clinical stages have used these antigens in various platforms to induce broadly neutralizing antibodies and/or heterosubtypic T cell immunity. Several studies have demonstrated success with universal vaccines based on HA stalk, which relies on the induction of broadly neutralizing antibodies to mediate heterosubtypic protection. Developing and stabilizing the HA stalk domain as an immunogenic

vaccine antigen that retains proper conformation is still challenging. The use of rationale- and computer-based design has aided in optimizing the mutations required to best stabilize a trimeric form of HA stalk. In addition, the production of chimeric HA vaccine antigens, a series of immunizations with HA proteins engineered to deliver a constant HA stalk domain in the context of various exotic HA head domains, has allowed for the production of a stable full-length HA, while enhancing the immunogenicity of HA stalk.

Universal vaccine strategies based on M2e or NP rely on the induction of non-neutralizing, but broadly-reactive antibodies that have been shown to mediate important innate and adaptive immune events, such as ADCC and CDC. In addition, most T-cell vaccines under development target internal proteins, notably NP, in order to elicit heterosubtypic immunity based on NP-specific CTL activation, in addition to broadly-reactive antibody production. It has been demonstrated that combining several of these antigens into one vaccine is effective at providing heterosubtypic protection, and may be the most promising strategy, compared to vaccination with single antigens.

In this dissertation, I have demonstrated the advantages of an FcRn-mediated mucosal vaccine platform for protection against influenza virus. There are several remaining issues whose resolution would advance influenza vaccine research specifically, and mucosal vaccine research in general. First, there are other conserved viral proteins that have potential as universal vaccine candidates, including NA, M2e, and M1. Studies have employed these proteins in a wide range of formulations, from DNA and viral vectors, nanoparticles, and VLPs, to varying levels of success in terms

of immunity and heterosubtypic protection, but none have done so as an intranasal Fc fusion protein-based vaccine, where the vaccine antigen is fused to monomeric Fc for i.n. immunization. It would be of interest to determine the effects of FcRn-dependent intranasal immunization with other conserved viral antigens as potential universal influenza vaccine candidates, either as single antigens or, more likely, in combination.

Second, one of the main functions of FcRn is to protect IgG from lysosomal degradation. It has been previously shown that the presence of Fc in Fc fusion proteins can extend its half-life in serum [358] [359] [361]. In the context of vaccination, endurance of vaccine antigens in mucosal tissues and circulation can be advantageous, especially for influenza vaccines. It is conceivable that prolonged vaccine antigen exposure could necessitate fewer influenza vaccinations, in contrast to the current obligation of annual vaccinations. Studies that detail the binding kinetics of HA-Fc/wt to FcRn can determine the longevity of influenza viral antigen-based Fc fusion proteins in serum. In addition, *in vivo* pharmacokinetic studies can determine if FcRn binding improves the half-life of HA-Fc/wt in serum.

The durable presence of a vaccine antigen can also enhance the induction of antigen-specific memory responses. One important memory response is the induction TRM T cells, which I have demonstrated can be promoted by FcRn-mediated i.n. vaccination with both HA-Fc/dimer and trimeric HA-Fc. It would be beneficial to expand the characterization of lung-resident memory T cells induced by our mucosal vaccine platform. I have demonstrated that FcRn-targeted delivery of HA-based vaccine antigens increases the percentage of induced lung CD4+ and CD8+ T cells

characterized to be resident memory T cells. It would be valuable to confirm that these TRM T cells are HA-specific. In addition, it would be of interest to determine the durability of TRM T cell induction. I detected lung-resident memory T cells 8 weeks after boost, but sampling at longer intervals could prove to be more informative. It has been previously demonstrated that LAIV vaccination-induced TRM T cells enhanced heterosubtypic protection. Treating immunized and challenged mice with the sphingosine 1-phosphate receptor-1 agonist FTY720 confines circulating T cells within the secondary lymphoid tissues, while lung-resident memory T cells remain undisturbed. It was observed that FTY720 treated mice that were i.n. immunized with LAIV survived against infection with two virus strains distinct from the vaccine strain [108]. By employing FTY720 treatment, it would be possible to determine if FcRn-mediated induction of TRM T cells mediates protection against diverse virus strains.

Third, although influenza virus remains a significant human pathogen that causes respiratory disease, our model of FcRn-mediated delivery of vaccine antigens can be expanded to protect against other important respiratory pathogens. Respiratory syncytial virus (RSV) causes highly contagious acute lower respiratory tract infections and is the leading cause of hospitalizations for infants. The main immunogen is the fusion glycoprotein (RSV F), which elicits high levels of neutralizing antibodies [413]. By fusing RSV F protein to the Fc portion of IgG, it is reasonable to conceive that FcRn-targeted delivery of RSV F-Fc can induce immunity against RSV, much like i.n. immunization with HA-Fc, highlighting the versatility of our platform. By employing and broadening the scope of our platform of FcRn-

mediated delivery of vaccine antigens, I believe that we can provide an effective alternative vaccine model that advances both mucosal and Fc fusion protein-based vaccines, in order to protect against important human respiratory pathogens, including, but not limited to, influenza virus.

Bibliography

[1] P. Palese and M. L. Shaw, “Orthomyxoviridae: the viruses and their replication,” in *Fields Virology*, Philadelphia: Lippincott Williams & Wilkins, 2007, pp. 1647–1689.

[2] P. F. Wright, G. Neuman, and Y. Kawaoka, “Orthomyxoviruses,” in *Fields Virology*, Philadelphia: Lippincott Williams & Wilkins, 2007, pp. 1691–1739.

[3] “Influenza Type A Viruses | Avian Influenza (Flu),” 19-Apr-2017. [Online]. Available: https://www.cdc.gov/flu/avianflu/influenza-a-virus-subtypes.htm. [Accessed: 14-Dec-2017].

[4] T. J. Wohlbold and F. Krammer, “In the Shadow of Hemagglutinin: A Growing Interest in Influenza Viral Neuraminidase and Its Role as a Vaccine Antigen,” *Viruses*, vol. 6, no. 6, pp. 2465–2494, Jun. 2014.

[5] B. M. Hause *et al.*, “Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family,” *mBio*, vol. 5, no. 2, pp. e00031-00014, Mar. 2014.

[6] E. J. A. Schrauwen, M. de Graaf, S. Herfst, G. F. Rimmelzwaan, A. D. M. E. Osterhaus, and R. a. M. Fouchier, “Determinants of virulence of influenza A virus,” *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 33, no. 4, pp. 479–490, Apr. 2014.

[7] N. M. Bouvier and P. Palese, “The biology of influenza viruses,” *Vaccine*, vol. 26 Suppl 4, pp. D49-53, Sep. 2008.

[8] C. K. Paniker, “Serological relationships between the neuraminidases in influenza viruses,” *J. Gen. Virol.*, vol. 2, no. 3, pp. 385–394, May 1968.

[9] G. M. Air and W. G. Laver, “The neuraminidase of influenza virus,” *Proteins*, vol. 6, no. 4, pp. 341–356, 1989.

[10] R. G. Webster and W. G. Laver, “Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance,” *Virology*, vol. 104, no. 1, pp. 139–148, Jul. 1980.

[11] D. C. Wiley, I. A. Wilson, and J. J. Skehel, “Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation,” *Nature*, vol. 289, no. 5796, pp. 373–378, Jan. 1981.

[12] R. G. Webster, W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka, “Evolution and ecology of influenza A viruses,” *Microbiol. Rev.*, vol. 56, no. 1, pp. 152–179, Mar. 1992.

[13] E. Fodor, L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, and A. García-Sastre, “Rescue of influenza A virus from recombinant DNA,” *J. Virol.*, vol. 73, no. 11, pp. 9679–9682, Nov. 1999.

[14] G. Neumann *et al.*, “Generation of influenza A viruses entirely from cloned cDNAs,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. 16, pp. 9345–9350, Aug. 1999.

[15] E. Hoffmann, G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster, “A DNA transfection system for generation of influenza A virus from eight plasmids,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 11, pp. 6108–6113, May 2000.

[16] G. Neumann, K. Fujii, Y. Kino, and Y. Kawaoka, “An improved reverse genetics system for influenza A virus generation and its implications for vaccine production,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 46, pp. 16825–16829, Nov. 2005.

[17] G. Neumann and Y. Kawaoka, “Reverse genetics of influenza virus,” *Virology*, vol. 287, no. 2, pp. 243–250, Sep. 2001.

[18] J. K. Taubenberger and D. M. Morens, “The Pathology of Influenza Virus Infections,” *Annu. Rev. Pathol.*, vol. 3, pp. 499–522, 2008.

[19] N. L. La Gruta, K. Kedzierska, J. Stambas, and P. C. Doherty, “A question of self-preservation: immunopathology in influenza virus infection,” *Immunol. Cell Biol.*, vol. 85, no. 2, pp. 85–92, Mar. 2007.

[20] M. A. Mall, “Role of Cilia, Mucus, and Airway Surface Liquid in Mucociliary Dysfunction: Lessons from Mouse Models,” *J. Aerosol Med. Pulm. Drug Deliv.*, vol. 21, no. 1, pp. 13–24, Mar. 2008.

[21] K. L. Oslund and N. Baumgarth, “Influenza-induced innate immunity: regulators of viral replication, respiratory tract pathology & adaptive immunity,” *Future Virol.*, vol. 6, no. 8, pp. 951–962, Aug. 2011.

[22] A. Iwasaki and P. S. Pillai, “Innate immunity to influenza virus infection,” *Nat. Rev. Immunol.*, vol. 14, no. 5, pp. 315–328, May 2014.

[23] J. H. C. M. Kreijtz, R. a. M. Fouchier, and G. F. Rimmelzwaan, “Immune responses to influenza virus infection,” *Virus Res.*, vol. 162, no. 1–2, pp. 19–30, Dec. 2011.

[24] K. Onomoto *et al.*, “Critical role of an antiviral stress granule containing RIG-I and PKR in viral detection and innate immunity,” *PloS One*, vol. 7, no. 8, p. e43031, 2012.

[25] A. Pichlmair *et al.*, “RIG-I-mediated antiviral responses to single-stranded RNA bearing 5’-phosphates,” *Science*, vol. 314, no. 5801, pp. 997–1001, Nov. 2006.

[26] A. K. Heer *et al.*, “TLR Signaling Fine-Tunes Anti-Influenza B Cell Responses without Regulating Effector T Cell Responses,” *J. Immunol.*, vol. 178, no. 4, pp. 2182–2191, Feb. 2007.

[27] R. Le Goffic *et al.*, “Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells,” *J. Immunol. Baltim. Md 1950*, vol. 178, no. 6, pp. 3368–3372, Mar. 2007.

[28] V. Jeisy-Scott, J. H. Kim, W. G. Davis, W. Cao, J. M. Katz, and S. Sambhara, “TLR7 Recognition Is Dispensable for Influenza Virus A Infection but Important for the Induction of Hemagglutinin-Specific Antibodies in Response to the 2009 Pandemic Split Vaccine in Mice,” *J. Virol.*, vol. 86, no. 20, pp. 10988–10998, Oct. 2012.

[29] T.-D. Kanneganti, “Central roles of NLRs and inflammasomes in viral infection,” *Nat. Rev. Immunol.*, vol. 10, no. 10, pp. 688–698, Oct. 2010.

[30] M. D. Tate *et al.*, “Reassessing the role of the NLRP3 inflammasome during pathogenic influenza A virus infection via temporal inhibition,” *Sci. Rep.*, vol. 6, p. srep27912, Jun. 2016.

[31] Z. Xing *et al.*, “Host immune and apoptotic responses to avian influenza virus H9N2 in human tracheobronchial epithelial cells,” *Am. J. Respir. Cell Mol. Biol.*, vol. 44, no. 1, pp. 24–33, Jan. 2011.

[32] M. C. W. Chan *et al.*, “Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells,” *Respir. Res.*, vol. 6, p. 135, Nov. 2005.

[33] C. J. Sanders, P. C. Doherty, and P. G. Thomas, “Respiratory epithelial cells in innate immunity to influenza virus infection,” *Cell Tissue Res.*, vol. 343, no. 1, pp. 13–21, Jan. 2011.

[34] S. Matsukura *et al.*, “Expression of RANTES by normal airway epithelial cells after influenza virus A infection,” *Am. J. Respir. Cell Mol. Biol.*, vol. 18, no. 2, pp. 255–264, Feb. 1998.

[35] W. Duan and M. Croft, “Control of regulatory T cells and airway tolerance by lung macrophages and dendritic cells,” *Ann. Am. Thorac. Soc.*, vol. 11 Suppl 5, pp. S306-313, Dec. 2014.

[36] D. M. Mosser and J. P. Edwards, “Exploring the full spectrum of macrophage activation,” *Nat. Rev. Immunol.*, vol. 8, no. 12, pp. 958–969, Dec. 2008.

[37] M. D. Tate, D. L. Pickett, N. van Rooijen, A. G. Brooks, and P. C. Reading, “Critical role of airway macrophages in modulating disease severity during influenza virus infection of mice,” *J. Virol.*, vol. 84, no. 15, pp. 7569–7580, Aug. 2010.

[38] S. Schultz-Cherry, “Role of NK cells in influenza infection,” *Curr. Top. Microbiol. Immunol.*, vol. 386, pp. 109–120, 2015.

[39] C. Paget *et al.*, “Potential role of invariant NKT cells in the control of pulmonary inflammation and CD8+ T cell response during acute influenza A virus H3N2 pneumonia,” *J. Immunol. Baltim. Md 1950*, vol. 186, no. 10, pp. 5590–5602, May 2011.

[40] X. Hao, T. S. Kim, and T. J. Braciale, “Differential response of respiratory dendritic cell subsets to influenza virus infection,” *J. Virol.*, vol. 82, no. 10, pp. 4908–4919, May 2008.

[41] B. Piqueras, J. Connolly, H. Freitas, A. K. Palucka, and J. Banchereau, “Upon viral exposure, myeloid and plasmacytoid dendritic cells produce 3 waves of distinct chemokines to recruit immune effectors,” *Blood*, vol. 107, no. 7, pp. 2613–2618, Apr. 2006.

[42] M. Larsson, J. Berge, A. G. Johansson, and U. Forsum, “Human dendritic cells handling of binding, uptake and degradation of free and IgG-immune complexed dinitrophenylated human serum albumin in vitro,” *Immunology*, vol. 90, no. 1, pp. 138–146, Jan. 1997.

[43] N. A. Jewell *et al.*, “Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo,” *J. Virol.*, vol. 84, no. 21, pp. 11515–11522, Nov. 2010.

[44] G. D. Victora and M. C. Nussenzweig, “Germinal centers,” *Annu. Rev. Immunol.*, vol. 30, pp. 429–457, 2012.

[45] G. D. Victora and P. C. Wilson, “Germinal center selection and the antibody response to influenza,” *Cell*, vol. 163, no. 3, pp. 545–548, Oct. 2015.

[46] S. M. Shinall, M. Gonzalez-Fernandez, R. J. Noelle, and T. J. Waldschmidt, “Identification of Murine Germinal Center B Cell Subsets Defined by the Expression of Surface Isotypes and Differentiation Antigens,” *J. Immunol.*, vol. 164, no. 11, pp. 5729–5738, Jun. 2000.

[47] E. E. Waffarn and N. Baumgarth, “Protective B Cell Responses to Flu—No Fluke!,” *J. Immunol.*, vol. 186, no. 7, pp. 3823–3829, Apr. 2011.

[48] S. Crotty, “Follicular helper CD4 T cells (TFH),” *Annu. Rev. Immunol.*, vol. 29, pp. 621–663, 2011.

[49] A. W. Boyden, K. L. Legge, and T. J. Waldschmidt, “Pulmonary Infection with Influenza A Virus Induces Site-Specific Germinal Center and T Follicular Helper Cell Responses,” *PLOS ONE*, vol. 7, no. 7, p. e40733, Jul. 2012.

[50] S. Fernandez Gonzalez, J. P. Jayasekera, and M. C. Carroll, “Complement and natural antibody are required in the long-term memory response to influenza virus,” *Vaccine*, vol. 26 Suppl 8, pp. I86-93, Dec. 2008.

[51] C. Chiu and P. J. Openshaw, “Antiviral B cell and T cell immunity in the lungs,” *Nat. Immunol.*, vol. 16, no. 1, pp. 18–26, Jan. 2015.

[52] M. B. Mazanec, C. L. Coudret, and D. R. Fletcher, “Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies,” *J. Virol.*, vol. 69, no. 2, pp. 1339–1343, Feb. 1995.

[53] R. Ito *et al.*, “Roles of anti-hemagglutinin IgA and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia,” *Vaccine*, vol. 21, no. 19–20, pp. 2362–2371, Jun. 2003.

[54] K. B. Renegar, P. A. Small, L. G. Boykins, and P. F. Wright, “Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract,” *J. Immunol. Baltim. Md 1950*, vol. 173, no. 3, pp. 1978–1986, Aug. 2004.

[55] G. M. Spiekermann *et al.*, “Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung,” *J. Exp. Med.*, vol. 196, no. 3, pp. 303–310, Aug. 2002.

[56] W. Gerhard, “The role of the antibody response in influenza virus infection,” *Curr. Top. Microbiol. Immunol.*, vol. 260, pp. 171–190, 2001.

[57] R. Sealy, S. Surman, J. L. Hurwitz, and C. Coleclough, “Antibody response to influenza infection of mice: different patterns for glycoprotein and nucleocapsid antigens,” *Immunology*, vol. 108, no. 4, pp. 431–439, Apr. 2003.

[58] W. Gerhard, K. Mozdzanowska, M. Furchner, G. Washko, and K. Maiese, “Role of the B-cell response in recovery of mice from primary influenza virus infection,” *Immunol. Rev.*, vol. 159, pp. 95–103, Oct. 1997.

[59] S. Jegaskanda, P. C. Reading, and S. J. Kent, “Influenza-specific antibody-dependent cellular cytotoxicity: toward a universal influenza vaccine,” *J. Immunol. Baltim. Md 1950*, vol. 193, no. 2, pp. 469–475, Jul. 2014.

[60] J. J. Skehel and D. C. Wiley, “Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin,” *Annu. Rev. Biochem.*, vol. 69, pp. 531–569, 2000.

[61] D. C. Ekiert *et al.*, “Antibody recognition of a highly conserved influenza virus epitope,” *Science*, vol. 324, no. 5924, pp. 246–251, Apr. 2009.

[62] D. Corti *et al.*, “A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins,” *Science*, vol. 333, no. 6044, pp. 850–856, Aug. 2011.

[63] J. Sui *et al.*, “Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses,” *Nat. Struct. Mol. Biol.*, vol. 16, no. 3, pp. 265–273, Mar. 2009.

[64] D. M. Carragher, D. A. Kaminski, A. Moquin, L. Hartson, and T. D. Randall, “A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus,” *J. Immunol. Baltim. Md 1950*, vol. 181, no. 6, pp. 4168–4176, Sep. 2008.

[65] L. Deng, K. J. Cho, W. Fiers, and X. Saelens, “M2e-Based Universal Influenza A Vaccines,” *Vaccines*, vol. 3, no. 1, pp. 105–136, Feb. 2015.

[66] A. Morokutti *et al.*, “Validation of the modified hemagglutination inhibition assay (mHAI), a robust and sensitive serological test for analysis of influenza virus-specific immune response,” *J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol.*, vol. 56, no. 4, pp. 323–330, Apr. 2013.

[67] D. J. Topham and P. C. Doherty, “Clearance of an influenza A virus by CD4+ T cells is inefficient in the absence of B cells,” *J. Virol.*, vol. 72, no. 1, pp. 882–885, Jan. 1998.

[68] P. C. Doherty, D. J. Topham, R. A. Tripp, R. D. Cardin, J. W. Brooks, and P. G. Stevenson, “Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections,” *Immunol. Rev.*, vol. 159, pp. 105–117, Oct. 1997.

[69] M. M. Hufford, T. S. Kim, J. Sun, and T. J. Braciale, “The Effector T Cell Response to Influenza Infection,” *Curr. Top. Microbiol. Immunol.*, vol. 386, pp. 423–455, 2015.

[70] T. J. Braciale, J. Sun, and T. S. Kim, “Regulating the adaptive immune response to respiratory virus infection,” *Nat. Rev. Immunol.*, vol. 12, no. 4, pp. 295–305, Mar. 2012.

[71] D. J. Topham, R. A. Tripp, and P. C. Doherty, “CD8+ T cells clear influenza virus by perforin or Fas-dependent processes,” *J. Immunol. Baltim. Md 1950*, vol. 159, no. 11, pp. 5197–5200, Dec. 1997.

[72] M. M. Hufford, T. S. Kim, J. Sun, and T. J. Braciale, “Antiviral CD8+ T cell effector activities in situ are regulated by target cell type,” *J. Exp. Med.*, vol. 208, no. 1, pp. 167–180, Jan. 2011.

[73] S. Herold *et al.*, “Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand,” *J. Exp. Med.*, vol. 205, no. 13, pp. 3065–3077, Dec. 2008.

[74] E. L. Brincks, A. Katewa, T. A. Kucaba, T. S. Griffith, and K. L. Legge, “CD8 T cells utilize TRAIL to control influenza virus infection,” *J. Immunol. Baltim. Md 1950*, vol. 181, no. 7, pp. 4918–4925, Oct. 2008.

[75] D. J. Campbell and M. A. Koch, “Phenotypical and functional specialization of FOXP3+ regulatory T cells,” *Nat. Rev. Immunol.*, vol. 11, no. 2, pp. 119–130, Feb. 2011.

[76] J. Surls, C. Nazarov-Stoica, M. Kehl, S. Casares, and T.-D. Brumeanu, “Differential effect of CD4+Foxp3+ T-regulatory cells on the B and T helper cell responses to influenza virus vaccination,” *Vaccine*, vol. 28, no. 45, pp. 7319–7330, Oct. 2010.

[77] D. M. Brown, A. M. Dilzer, D. L. Meents, and S. L. Swain, “CD4 T Cell-Mediated Protection from Lethal Influenza: Perforin and Antibody-Mediated Mechanisms Give a One-Two Punch,” *J. Immunol.*, vol. 177, no. 5, pp. 2888–2898, Sep. 2006.

[78] D. M. Brown, E. Román, and S. L. Swain, “CD4 T cell responses to influenza infection,” *Semin. Immunol.*, vol. 16, no. 3, pp. 171–177, Jun. 2004.

[79] E. Román *et al.*, “CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function,” *J. Exp. Med.*, vol. 196, no. 7, pp. 957–968, Oct. 2002.

[80] M. J. Killip, E. Fodor, and R. E. Randall, “Influenza virus activation of the interferon system,” *Virus Res.*, vol. 209, pp. 11–22, Nov. 2015.

[81] S. H. Seo and R. G. Webster, “Tumor Necrosis Factor Alpha Exerts Powerful Anti-Influenza Virus Effects in Lung Epithelial Cells,” *J. Virol.*, vol. 76, no. 3, pp. 1071–1076, Feb. 2002.

[82] B. Koppelman, J. J. Neefjes, J. E. de Vries, and R. de Waal Malefyt, “Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling,” *Immunity*, vol. 7, no. 6, pp. 861–871, Dec. 1997.

[83] J. H. C. M. Kreijtz *et al.*, “Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice,” *Vaccine*, vol. 25, no. 4, pp. 612–620, Jan. 2007.

[84] A. C. M. Boon *et al.*, “Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8+ T lymphocytes,” *J. Immunol. Baltim. Md 1950*, vol. 172, no. 4, pp. 2453–2460, Feb. 2004.

[85] L. Y.-H. Lee *et al.*, “Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals,” *J. Clin. Invest.*, vol. 118, no. 10, pp. 3478–3490, Oct. 2008.

[86] C. Gianfrani, C. Oseroff, J. Sidney, R. W. Chesnut, and A. Sette, “Human memory CTL response specific for influenza A virus is broad and multispecific,” *Hum. Immunol.*, vol. 61, no. 5, pp. 438–452, May 2000.

[87] F. Gotch, J. Rothbard, K. Howland, A. Townsend, and A. McMichael, “Cytotoxic T lymphocytes recognize a fragment of influenza virus matrix protein in association with HLA-A2,” *Nature*, vol. 326, no. 6116, pp. 881–882, May 1987.

[88] B. J. Laidlaw *et al.*, “CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection,” *Immunity*, vol. 41, no. 4, pp. 633–645, Oct. 2014.

[89] R. M. Welsh and J. M. McNally, “Immune deficiency, immune silencing, and clonal exhaustion of T cell responses during viral infections,” *Curr. Opin. Microbiol.*, vol. 2, no. 4, pp. 382–387, Aug. 1999.

[90] E. S. Razvi, Z. Jiang, B. A. Woda, and R. M. Welsh, “Lymphocyte apoptosis during the silencing of the immune response to acute viral infections in normal, lpr, and Bcl-2-transgenic mice,” *Am. J. Pathol.*, vol. 147, no. 1, pp. 79–91, Jul. 1995.

[91] K. D. Zens and D. L. Farber, “Memory CD4 T cells in influenza,” *Curr. Top. Microbiol. Immunol.*, vol. 386, pp. 399–421, 2015.

[92] S. L. Swain *et al.*, “CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza,” *Immunol. Rev.*, vol. 211, pp. 8–22, Jun. 2006.

[93] D. M. Jelley-Gibbs, D. M. Brown, J. P. Dibble, L. Haynes, S. M. Eaton, and S. L. Swain, "Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation," *J. Exp. Med.*, vol. 202, no. 5, pp. 697–706, Sep. 2005.

[94] S. M. Kaech and E. J. Wherry, "Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection," *Immunity*, vol. 27, no. 3, pp. 393–405, Sep. 2007.

[95] F. Sallusto, D. Lenig, R. Förster, M. Lipp, and A. Lanzavecchia, "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions," *Nature*, vol. 401, no. 6754, pp. 708–712, Oct. 1999.

[96] D. Masopust, V. Vezys, A. L. Marzo, and L. Lefrançois, "Preferential localization of effector memory cells in nonlymphoid tissue," *Science*, vol. 291, no. 5512, pp. 2413–2417, Mar. 2001.

[97] J. R. Teijaro, D. Turner, Q. Pham, E. J. Wherry, L. Lefrançois, and D. L. Farber, "Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection," *J. Immunol. Baltim. Md 1950*, vol. 187, no. 11, pp. 5510–5514, Dec. 2011.

[98] D. L. Turner *et al.*, "Lung niches for the generation and maintenance of tissue-resident memory T cells," *Mucosal Immunol.*, vol. 7, no. 3, pp. 501–510, May 2014.

[99] S. N. Mueller, T. Gebhardt, F. R. Carbone, and W. R. Heath, "Memory T cell subsets, migration patterns, and tissue residence," *Annu. Rev. Immunol.*, vol. 31, pp. 137–161, 2013.

[100] J. M. Schenkel and D. Masopust, "Tissue-Resident Memory T Cells," *Immunity*, vol. 41, no. 6, pp. 886–897, Dec. 2014.

[101] D. Masopust *et al.*, "Dynamic T cell migration program provides resident memory within intestinal epithelium," *J. Exp. Med.*, vol. 207, no. 3, pp. 553–564, Mar. 2010.

[102] T. Wu *et al.*, "Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection," *J. Leukoc. Biol.*, vol. 95, no. 2, pp. 215–224, Feb. 2014.

[103] C. O. Park and T. S. Kupper, "The emerging role of resident memory T cells in protective immunity and inflammatory disease," *Nat. Med.*, vol. 21, no. 7, pp. 688–697, Jul. 2015.

[104] C. O. Park *et al.*, "Staged development of long-lived T-cell receptor αβ TH17 resident memory T-cell population to Candida albicans after skin infection," *J. Allergy Clin. Immunol.*, Nov. 2017.

[105] T. Gebhardt, L. M. Wakim, L. Eidsmo, P. C. Reading, W. R. Heath, and F. R. Carbone, "Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus," *Nat. Immunol.*, vol. 10, no. 5, pp. 524–530, May 2009.

[106] P. A. Romagnoli, B. S. Sheridan, Q.-M. Pham, L. Lefrançois, and K. M. Khanna, “IL-17A-producing resident memory γδ T cells orchestrate the innate immune response to secondary oral Listeria monocytogenes infection,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 30, pp. 8502–8507, 26 2016.

[107] H. Shin and A. Iwasaki, “Tissue-resident memory T cells,” *Immunol. Rev.*, vol. 255, no. 1, pp. 165–181, Sep. 2013.

[108] K. D. Zens, J. K. Chen, and D. L. Farber, “Vaccine-generated lung tissue–resident memory T cells provide heterosubtypic protection to influenza infection,” *JCI Insight*, vol. 1, no. 10, Jul. 2017.

[109] M. G. McHeyzer-Williams and R. Ahmed, “B cell memory and the long-lived plasma cell,” *Curr. Opin. Immunol.*, vol. 11, no. 2, pp. 172–179, Apr. 1999.

[110] M. K. Slifka, R. Antia, J. K. Whitmire, and R. Ahmed, “Humoral Immunity Due to Long-Lived Plasma Cells,” *Immunity*, vol. 8, no. 3, pp. 363–372, Mar. 1998.

[111] Y. Takahashi, “Memory B Cells in Systemic and Mucosal Immune Response: Implications for Successful Vaccination,” *Biosci. Biotechnol. Biochem.*, vol. 71, no. 10, pp. 2358–2366, Oct. 2007.

[112] H. M. Joo, Y. He, and M. Y. Sangster, “Broad dispersion and lung localization of virus-specific memory B cells induced by influenza pneumonia,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 9, pp. 3485–3490, Mar. 2008.

[113] S. Sridhar, “Heterosubtypic T-Cell Immunity to Influenza in Humans: Challenges for Universal T-Cell Influenza Vaccines,” *Front. Immunol.*, vol. 7, May 2016.

[114] A. García-Sastre, “Identification and characterization of viral antagonists of type I interferon in negative-strand RNA viruses,” *Curr. Top. Microbiol. Immunol.*, vol. 283, pp. 249–280, 2004.

[115] A. Rückle, E. Haasbach, I. Julkunen, O. Planz, C. Ehrhardt, and S. Ludwig, “The NS1 Protein of Influenza A Virus Blocks RIG-I-Mediated Activation of the Noncanonical NF-κB Pathway and p52/RelB-Dependent Gene Expression in Lung Epithelial Cells,” *J. Virol.*, vol. 86, no. 18, pp. 10211–10217, Sep. 2012.

[116] M. W. Melville, W. J. Hansen, B. C. Freeman, W. J. Welch, and M. G. Katze, “The molecular chaperone hsp40 regulates the activity of P58IPK, the cellular inhibitor of PKR,” *Proc. Natl. Acad. Sci.*, vol. 94, no. 1, pp. 97–102, Jan. 1997.

[117] K. Sharma *et al.*, “Influenza A Virus Nucleoprotein Exploits Hsp40 to Inhibit PKR Activation,” *PLOS ONE*, vol. 6, no. 6, p. e20215, Jun. 2011.

[118] Z. Guan *et al.*, “Interaction of Hsp40 with influenza virus M2 protein: implications for PKR signaling pathway,” *Protein Cell*, vol. 1, no. 10, pp. 944–955, Oct. 2010.

[119] Z. T. Varga *et al.*, "The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein," *PLoS Pathog.*, vol. 7, no. 6, p. e1002067, Jun. 2011.

[120] G. M. Conenello, J. R. Tisoncik, E. Rosenzweig, Z. T. Varga, P. Palese, and M. G. Katze, "A single N66S mutation in the PB1-F2 protein of influenza A virus increases virulence by inhibiting the early interferon response in vivo," *J. Virol.*, vol. 85, no. 2, pp. 652–662, Jan. 2011.

[121] J. T. Voeten, T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan, "Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes," *J. Virol.*, vol. 74, no. 15, pp. 6800–6807, Aug. 2000.

[122] G. F. Rimmelzwaan, J. H. C. M. Kreijtz, R. Bodewes, R. A. M. Fouchier, and A. D. M. E. Osterhaus, "Influenza virus CTL epitopes, remarkably conserved and remarkably variable," *Vaccine*, vol. 27, no. 45, pp. 6363–6365, Oct. 2009.

[123] R. J. Connor, Y. Kawaoka, R. G. Webster, and J. C. Paulson, "Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates," *Virology*, vol. 205, no. 1, pp. 17–23, Nov. 1994.

[124] G. N. Rogers and J. C. Paulson, "Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin," *Virology*, vol. 127, no. 2, pp. 361–373, Jun. 1983.

[125] G. N. Rogers and B. L. D'Souza, "Receptor binding properties of human and animal H1 influenza virus isolates," *Virology*, vol. 173, no. 1, pp. 317–322, Nov. 1989.

[126] N. S. Heaton, R. A. Langlois, D. Sachs, J. K. Lim, P. Palese, and B. R. tenOever, "Long-term survival of influenza virus infected club cells drives immunopathology," *J. Exp. Med.*, vol. 211, no. 9, pp. 1707–1714, Aug. 2014.

[127] E. Böttcher-Friebertshäuser *et al.*, "Cleavage of Influenza Virus Hemagglutinin by Airway Proteases TMPRSS2 and HAT Differs in Subcellular Localization and Susceptibility to Protease Inhibitors," *J. Virol.*, vol. 84, no. 11, pp. 5605–5614, Jun. 2010.

[128] O. P. Zhirnov, M. R. Ikizler, and P. F. Wright, "Cleavage of Influenza A Virus Hemagglutinin in Human Respiratory Epithelium Is Cell Associated and Sensitive to Exogenous Antiproteases," *J. Virol.*, vol. 76, no. 17, pp. 8682–8689, Sep. 2002.

[129] D. A. Steinhauer, "Role of hemagglutinin cleavage for the pathogenicity of influenza virus," *Virology*, vol. 258, no. 1, pp. 1–20, May 1999.

[130] D. van Riel *et al.*, "Seasonal and pandemic human influenza viruses attach better to human upper respiratory tract epithelium than avian influenza viruses," *Am. J. Pathol.*, vol. 176, no. 4, pp. 1614–1618, Apr. 2010.

[131] T. Horimoto, K. Nakayama, S. P. Smeekens, and Y. Kawaoka, “Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses,” *J. Virol.*, vol. 68, no. 9, pp. 6074–6078, Sep. 1994.

[132] W. Ma, R. E. Kahn, and J. A. Richt, “The pig as a mixing vessel for influenza viruses: Human and veterinary implications,” *J. Mol. Genet. Med. Int. J. Biomed. Res.*, vol. 3, no. 1, pp. 158–166, Nov. 2008.

[133] J. Hass, S. Matuszewski, D. Cieslik, and M. Haase, “The role of swine as ‘mixing vessel’ for interspecies transmission of the influenza A subtype H1N1: a simultaneous Bayesian inference of phylogeny and ancestral hosts,” *Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.*, vol. 11, no. 2, pp. 437–441, Mar. 2011.

[134] C. R. Parrish, P. R. Murcia, and E. C. Holmes, “Influenza Virus Reservoirs and Intermediate Hosts: Dogs, Horses, and New Possibilities for Influenza Virus Exposure of Humans,” *J. Virol.*, vol. 89, no. 6, pp. 2990–2994, Mar. 2015.

[135] K. R. Short *et al.*, “One health, multiple challenges: The inter-species transmission of influenza A virus,” *One Health*, vol. 1, no. Supplement C, pp. 1–13, Dec. 2015.

[136] D. M. Morens and A. S. Fauci, “The 1918 influenza pandemic: insights for the 21st century,” *J. Infect. Dis.*, vol. 195, no. 7, pp. 1018–1028, Apr. 2007.

[137] C. Scholtissek, W. Rohde, V. Von Hoyningen, and R. Rott, “On the origin of the human influenza virus subtypes H2N2 and H3N2,” *Virology*, vol. 87, no. 1, pp. 13–20, Jun. 1978.

[138] Y. Kawaoka, S. Krauss, and R. G. Webster, “Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics,” *J. Virol.*, vol. 63, no. 11, pp. 4603–4608, Nov. 1989.

[139] M. P. Girard, J. S. Tam, O. M. Assossou, and M. P. Kieny, “The 2009 A (H1N1) influenza virus pandemic: A review,” *Vaccine*, vol. 28, no. 31, pp. 4895–4902, Jul. 2010.

[140] “Past Pandemics | Pandemic Influenza (Flu) | CDC,” 22-Sep-2017. [Online]. Available: https://www.cdc.gov/flu/pandemic-resources/basics/past-pandemics.html. [Accessed: 24-Sep-2017].

[141] J. K. Taubenberger and D. M. Morens, “Influenza: The Once and Future Pandemic,” *Public Health Rep.*, vol. 125, no. Suppl 3, pp. 16–26, 2010.

[142] I. Mena *et al.*, “Origins of the 2009 H1N1 influenza pandemic in swine in Mexico,” *eLife*, vol. 5.

[143] “CDC Novel H1N1 Flu | The 2009 H1N1 Pandemic: Summary Highlights, April 2009-April 2010.” [Online]. Available: https://www.cdc.gov/h1n1flu/cdcresponse.htm. [Accessed: 12-Nov-2017].

[144] R. G. Webster and E. A. Govorkova, “Continuing challenges in influenza,” *Ann. N. Y. Acad. Sci.*, vol. 1323, no. 1, pp. 115–139, Sep. 2014.

[145] R. G. Webster and E. A. Govorkova, “H5N1 influenza--continuing evolution and spread,” *N. Engl. J. Med.*, vol. 355, no. 21, pp. 2174–2177, Nov. 2006.

[146] Y. Guan and G. J. D. Smith, “The emergence and diversification of panzootic H5N1 influenza viruses,” *Virus Res.*, vol. 178, no. 1, pp. 35–43, Dec. 2013.

[147] K. Yiu Lai *et al.*, “Human H7N9 avian influenza virus infection: a review and pandemic risk assessment,” *Emerg. Microbes Infect.*, vol. 2, no. 8, p. e48, Aug. 2013.

[148] V. Wiwanitkit, “H7N9 Influenza: The Emerging Infectious Disease,” *North Am. J. Med. Sci.*, vol. 5, no. 7, pp. 395–398, Jul. 2013.

[149] I. Capua and D. J. Alexander, “Avian influenza and human health,” *Acta Trop.*, vol. 83, no. 1, pp. 1–6, Jul. 2002.

[150] R. A. M. Fouchier *et al.*, “Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 5, pp. 1356–1361, Feb. 2004.

[151] S. Puzelli *et al.*, “Human infection with highly pathogenic A(H7N7) avian influenza virus, Italy, 2013,” *Emerg. Infect. Dis.*, vol. 20, no. 10, pp. 1745–1749, Oct. 2014.

[152] Centers for Disease Control and Prevention (CDC), “Notes from the field: Highly pathogenic avian influenza A (H7N3) virus infection in two poultry workers--Jalisco, Mexico, July 2012,” *MMWR Morb. Mortal. Wkly. Rep.*, vol. 61, no. 36, pp. 726–727, Sep. 2012.

[153] M. Liu *et al.*, “Genetic diversity of avian influenza A (H10N8) virus in live poultry markets and its association with human infections in China,” *Sci. Rep.*, vol. 5, p. 7632, Jan. 2015.

[154] Z. Wang, L. Loh, L. Kedzierski, and K. Kedzierska, “Avian Influenza Viruses, Inflammation, and CD8+ T Cell Immunity,” *Front. Immunol.*, vol. 7, Mar. 2016.

[155] F. Krammer and P. Palese, “Advances in the development of influenza virus vaccines,” *Nat. Rev. Drug Discov.*, vol. 14, no. 3, pp. 167–182, Mar. 2015.

[156] M. Imai *et al.*, “Experimental adaptation of an influenza H5 haemagglutinin (HA) confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets,” *Nature*, vol. 486, no. 7403, pp. 420–428, May 2012.

[157] S. Herfst *et al.*, “Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets,” *Science*, vol. 336, no. 6088, pp. 1534–1541, Jun. 2012.

[158] M. D. de Jong *et al.*, “Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia,” *Nat. Med.*, vol. 12, no. 10, pp. 1203–1207, Oct. 2006.

[159] M. B. A. Oldstone, J. R. Teijaro, K. B. Walsh, and H. Rosen, “Dissecting influenza virus pathogenesis uncovers a novel chemical approach to combat the infection,” *Virology*, vol. 435, no. 1, pp. 92–101, Jan. 2013.

[160] U. D. Allen, F. Y. Aoki, and H. G. Stiver, “The Use of Antiviral Drugs for Influenza: Recommended Guidelines for Practitioners,” *Can. J. Infect. Dis. Med. Microbiol.*, vol. 17, no. 5, pp. 273–284, 2006.

[161] G. Stiver, “The treatment of influenza with antiviral drugs,” *CMAJ Can. Med. Assoc. J.*, vol. 168, no. 1, pp. 49–57, Jan. 2003.

[162] A. Kamali and M. Holodniy, “Influenza treatment and prophylaxis with neuraminidase inhibitors: a review,” *Infect. Drug Resist.*, vol. 6, pp. 187–198, Nov. 2013.

[163] N. J. Cox, T. L. Brammer, and H. L. Regnery, “Influenza: global surveillance for epidemic and pandemic variants,” *Eur. J. Epidemiol.*, vol. 10, no. 4, pp. 467–470, Aug. 1994.

[164] P. K. Tosh, R. M. Jacobson, and G. A. Poland, “Influenza vaccines: from surveillance through production to protection,” *Mayo Clin. Proc.*, vol. 85, no. 3, pp. 257–273, Mar. 2010.

[165] C. A. Russell *et al.*, “Influenza vaccine strain selection and recent studies on the global migration of seasonal influenza viruses,” *Vaccine*, vol. 26 Suppl 4, pp. D31-34, Sep. 2008.

[166] J. P. Weir and M. F. Gruber, “An overview of the regulation of influenza vaccines in the United States,” *Influenza Other Respir. Viruses*, vol. 10, no. 5, pp. 354–360, 2016.

[167] “ACIP Influenza Vaccine Recommendations | CDC,” 13-Oct-2017. [Online]. Available: https://www.cdc.gov/vaccines/hcp/acip-recs/vacc-specific/flu.html. [Accessed: 17-Dec-2017].

[168] V. C. Huber, “Influenza vaccines: from whole virus preparations to recombinant protein technology,” *Expert Rev. Vaccines*, vol. 13, no. 1, pp. 31–42, Jan. 2014.

[169] L. A. Grohskopf, “Prevention and Control of Seasonal Influenza with Vaccines,” *MMWR Recomm. Rep.*, vol. 65, 2016.

[170] P. Tsang *et al.*, “Immunogenicity and safety of Fluzone(®) intradermal and high-dose influenza vaccines in older adults ≥65 years of age: a randomized, controlled, phase II trial,” *Vaccine*, vol. 32, no. 21, pp. 2507–2517, May 2014.

[171] M. M. J. Cox and J. R. Hollister, “FluBlok, a next generation influenza vaccine manufactured in insect cells,” *Biol. J. Int. Assoc. Biol. Stand.*, vol. 37, no. 3, pp. 182–189, Jun. 2009.

[172] H. F. Maassab, “Biologic and immunologic characteristics of cold-adapted influenza virus,” *J. Immunol. Baltim. Md 1950*, vol. 102, no. 3, pp. 728–732, Mar. 1969.

[173] H. Jin and Z. Chen, “Production of live attenuated influenza vaccines against seasonal and potential pandemic influenza viruses,” *Curr. Opin. Virol.*, vol. 6, pp. 34–39, Jun. 2014.

[174] L. A. Grohskopf, “Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices — United States, 2017–18 Influenza Season,” *MMWR Recomm. Rep.*, vol. 66, 2017.

[175] A. J. Broadbent and K. Subbarao, “Influenza Virus Vaccines: Lessons from the 2009 H1N1 pandemic,” *Curr. Opin. Virol.*, vol. 1, no. 4, pp. 254–262, Oct. 2011.

[176] “WHO | Production and availability of pandemic (H1N1) 2009 vaccines,” *WHO*. [Online]. Available: http://www.who.int/csr/disease/swineflu/frequently_asked_questions/vaccine_preparedness/production_availability/en/. [Accessed: 17-Dec-2017].

[177] D. F. Hoft *et al.*, “Comparisons of the Humoral and Cellular Immune Responses Induced by Live Attenuated Influenza Vaccine and Inactivated Influenza Vaccine in Adults,” *Clin. Vaccine Immunol.*, vol. 24, no. 1, pp. e00414-16, Jan. 2017.

[178] K. G.-I. Mohn, I. Smith, H. Sjursen, and R. Cox, “Immune responses after live attenuated influenza vaccination,” *Hum. Vaccines Immunother.*, vol. 0, no. ja, pp. 00–00, Sep. 2017.

[179] R. B. Belshe, C. S. Ambrose, and T. Yi, “Safety and efficacy of live attenuated influenza vaccine in children 2-7 years of age,” *Vaccine*, vol. 26 Suppl 4, pp. D10-16, Sep. 2008.

[180] C. S. Ambrose, M. J. Levin, and R. B. Belshe, “The relative efficacy of trivalent live attenuated and inactivated influenza vaccines in children and adults,” *Influenza Other Respir. Viruses*, vol. 5, no. 2, pp. 67–75, Mar. 2011.

[181] A. C. Tricco *et al.*, “Comparing influenza vaccine efficacy against mismatched and matched strains: a systematic review and meta-analysis,” *BMC Med.*, vol. 11, p. 153, Jun. 2013.

[182] “CDC Press Releases,” *CDC*, 01-Jan-2016. [Online]. Available: https://www.cdc.gov/media/releases/2016/s0622-laiv-flu.html. [Accessed: 17-Dec-2017].

[183] C. Gerdil, “The annual production cycle for influenza vaccine,” *Vaccine*, vol. 21, no. 16, pp. 1776–1779, May 2003.

[184] W. A. Orenstein and W. Schaffner, “Lessons learned: role of influenza vaccine production, distribution, supply, and demand--what it means for the provider,” *Am. J. Med.*, vol. 121, no. 7 Suppl 2, pp. S22-27, Jul. 2008.

[185] N. R. Hegde, “Cell culture-based influenza vaccines: A necessary and indispensable investment for the future,” *Hum. Vaccines Immunother.*, vol. 11, no. 5, pp. 1223–1234, 2015.

[186] J. A. Tree, C. Richardson, A. R. Fooks, J. C. Clegg, and D. Looby, “Comparison of large-scale mammalian cell culture systems with egg culture for the production of influenza virus A vaccine strains,” *Vaccine*, vol. 19, no. 25–26, pp. 3444–3450, May 2001.

[187] A. Domnich, L. Arata, D. Amicizia, J. Puig-Barberà, R. Gasparini, and D. Panatto, "Effectiveness of MF59-adjuvanted seasonal influenza vaccine in the elderly: A systematic review and meta-analysis," *Vaccine*, vol. 35, no. 4, pp. 513–520, Jan. 2017.

[188] S. Jones *et al.*, "DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial," *Vaccine*, vol. 27, no. 18, pp. 2506–2512, Apr. 2009.

[189] C.-J. Wei *et al.*, "Induction of broadly neutralizing H1N1 influenza antibodies by vaccination," *Science*, vol. 329, no. 5995, pp. 1060–1064, Aug. 2010.

[190] J. Steel *et al.*, "Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain," *mBio*, vol. 1, no. 1, pp. e00018-10-e00018-10, May 2010.

[191] P. C. Soema, R. Kompier, J.-P. Amorij, and G. F. A. Kersten, "Current and next generation influenza vaccines: Formulation and production strategies," *Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft Pharm. Verfahrenstechnik EV*, vol. 94, pp. 251–263, Aug. 2015.

[192] "CDC Press Releases," *CDC*, 01-Jan-2016. [Online]. Available: https://www.cdc.gov/media/releases/2016/flu-vaccine-60-percent.html. [Accessed: 17-Dec-2017].

[193] V. V. A. Mallajosyula *et al.*, "Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 25, pp. E2514-2523, Jun. 2014.

[194] H. M. Yassine *et al.*, "Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection," *Nat. Med.*, vol. 21, no. 9, pp. 1065–1070, Sep. 2015.

[195] A. Impagliazzo *et al.*, "A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen," *Science*, vol. 349, no. 6254, pp. 1301–1306, Sep. 2015.

[196] R. Hai *et al.*, "Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes," *J. Virol.*, vol. 86, no. 10, pp. 5774–5781, May 2012.

[197] F. Krammer, N. Pica, R. Hai, I. Margine, and P. Palese, "Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies," *J. Virol.*, vol. 87, no. 12, pp. 6542–6550, Jun. 2013.

[198] R. Nachbagauer *et al.*, "A universal influenza virus vaccine candidate confers protection against pandemic H1N1 infection in preclinical ferret studies," *Npj Vaccines*, vol. 2, no. 1, p. 26, Sep. 2017.

[199] T. J. Wohlbold *et al.*, "Vaccination with Adjuvanted Recombinant Neuraminidase Induces Broad Heterologous, but Not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice," *mBio*, vol. 6, no. 2, pp. e02556-14, May 2015.

[200] J. Fan *et al.*, “Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys,” *Vaccine*, vol. 22, no. 23–24, pp. 2993–3003, Aug. 2004.

[201] M. De Filette *et al.*, “Universal influenza A vaccine: optimization of M2-based constructs,” *Virology*, vol. 337, no. 1, pp. 149–161, Jun. 2005.

[202] J.-M. Song, N. V. Rooijen, J. Bozja, R. W. Compans, and S.-M. Kang, “Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus,” *Proc. Natl. Acad. Sci.*, vol. 108, no. 2, pp. 757–761, Jan. 2011.

[203] M. Zheng, J. Luo, and Z. Chen, “Development of universal influenza vaccines based on influenza virus M and NP genes,” *Infection*, vol. 42, no. 2, pp. 251–262, Apr. 2014.

[204] Q. Chen *et al.*, “Comparing the ability of a series of viral protein-expressing plasmid DNAs to protect against H5N1 influenza virus,” *Virus Genes*, vol. 38, no. 1, pp. 30–38, Feb. 2009.

[205] G. E. Price *et al.*, “Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses,” *PloS One*, vol. 5, no. 10, p. e13162, Oct. 2010.

[206] D. Zhou *et al.*, “A Universal Influenza A Vaccine Based on Adenovirus Expressing Matrix-2 Ectodomain and Nucleoprotein Protects Mice From Lethal Challenge,” *Mol. Ther.*, vol. 18, no. 12, pp. 2182–2189, Dec. 2010.

[207] J. H. C. M. Kreijtz, G. de Mutsert, C. A. van Baalen, R. a. M. Fouchier, A. D. M. E. Osterhaus, and G. F. Rimmelzwaan, “Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus,” *J. Virol.*, vol. 82, no. 11, pp. 5161–5166, Jun. 2008.

[208] G. F. Rimmelzwaan, R. A. Fouchier, and A. D. Osterhaus, “Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development,” *Curr. Opin. Biotechnol.*, vol. 18, no. 6, pp. 529–536, Dec. 2007.

[209] M. Liniger, A. Zuniga, and H. Y. Naim, “Use of viral vectors for the development of vaccines,” *Expert Rev. Vaccines*, vol. 6, no. 2, pp. 255–266, Apr. 2007.

[210] J. B. Ulmer *et al.*, “Heterologous protection against influenza by injection of DNA encoding a viral protein,” *Science*, vol. 259, no. 5102, pp. 1745–1749, Mar. 1993.

[211] R. Glück, R. Mischler, B. Finkel, J. U. Que, B. Scarpa, and S. J. Cryz, “Immunogenicity of new virosome influenza vaccine in elderly people,” *Lancet Lond. Engl.*, vol. 344, no. 8916, pp. 160–163, Jul. 1994.

[212] R. Glück, K. G. Burri, and I. Metcalfe, “Adjuvant and antigen delivery properties of virosomes,” *Curr. Drug Deliv.*, vol. 2, no. 4, pp. 395–400, Oct. 2005.

[213] E. Grant *et al.*, “Nucleoprotein of influenza A virus is a major target of immunodominant CD8+ T-cell responses,” *Immunol. Cell Biol.*, vol. 91, no. 2, pp. 184–194, Feb. 2013.

[214] S. L. Epstein *et al.*, “Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein,” *Vaccine*, vol. 23, no. 46–47, pp. 5404–5410, Nov. 2005.

[215] T. K. Berthoud *et al.*, “Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 52, no. 1, pp. 1–7, Jan. 2011.

[216] M. W. LaMere *et al.*, “Regulation of Antinucleoprotein IgG by Systemic Vaccination and Its Effect on Influenza Virus Clearance ▿,” *J. Virol.*, vol. 85, no. 10, pp. 5027–5035, May 2011.

[217] M. W. LaMere *et al.*, “Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus,” *J. Immunol. Baltim. Md 1950*, vol. 186, no. 7, pp. 4331–4339, Apr. 2011.

[218] S. S. Rao *et al.*, “Comparative efficacy of hemagglutinin, nucleoprotein, and matrix 2 protein gene-based vaccination against H5N1 influenza in mouse and ferret,” *PloS One*, vol. 5, no. 3, p. e9812, Mar. 2010.

[219] M. Matsui *et al.*, “A CTL-based liposomal vaccine capable of inducing protection against heterosubtypic influenza viruses in HLA-A*0201 transgenic mice,” *Biochem. Biophys. Res. Commun.*, vol. 391, no. 3, pp. 1494–1499, Jan. 2010.

[220] W.-S. Cheong, J. Reiseger, S. J. Turner, R. Boyd, and H.-J. Netter, “Chimeric virus-like particles for the delivery of an inserted conserved influenza A-specific CTL epitope,” *Antiviral Res.*, vol. 81, no. 2, pp. 113–122, Feb. 2009.

[221] S. B. Mizel and J. T. Bates, “Flagellin as an Adjuvant: Cellular Mechanisms and Potential,” *J. Immunol. Baltim. Md 1950*, vol. 185, no. 10, pp. 5677–5682, Nov. 2010.

[222] B. Y. Chua *et al.*, “Inactivated Influenza Vaccine That Provides Rapid, Innate-Immune-System-Mediated Protection and Subsequent Long-Term Adaptive Immunity,” *mBio*, vol. 6, no. 6, pp. e01024-01015, Oct. 2015.

[223] S. Tamura, H. Funato, T. Nagamine, C. Aizawa, and T. Kurata, “Effectiveness of cholera toxin B subunit as an adjuvant for nasal influenza vaccination despite pre-existing immunity to CTB,” *Vaccine*, vol. 7, no. 6, pp. 503–505, Dec. 1989.

[224] E. Proietti *et al.*, “Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model,” *J. Immunol. Baltim. Md 1950*, vol. 169, no. 1, pp. 375–383, Jul. 2002.

[225] C. L. Cooper *et al.*, “Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine,” *Vaccine*, vol. 22, no. 23–24, pp. 3136–3143, Aug. 2004.

[226] M. Asif, K. A. Jenkins, L. S. Hilton, W. G. Kimpton, A. G. Bean, and J. W. Lowenthal, “Cytokines as adjuvants for avian vaccines,” *Immunology and Cell Biology*, 01-Dec-2004. [Online]. Available: https://www.nature.com/articles/icb200493. [Accessed: 15-Nov-2017].

[227] H. Kayamuro *et al.*, “Interleukin-1 Family Cytokines as Mucosal Vaccine Adjuvants for Induction of Protective Immunity against Influenza Virus,” *J. Virol.*, vol. 84, no. 24, pp. 12703–12712, Dec. 2010.

[228] J. R. McGhee, “Mucosa-Associated Lymphoid Tissue (MALT),” in *Encyclopedia of Immunology (Second Edition)*, P. J. Delves, Ed. Oxford: Elsevier, 1998, pp. 1774–1780.

[229] J. Holmgren and C. Czerkinsky, “Mucosal immunity and vaccines,” *Nat. Med.*, vol. 11, no. 4 Suppl, pp. S45-53, Apr. 2005.

[230] P. Brandtzaeg *et al.*, “Regional specialization in the mucosal immune system: what happens in the microcompartments?,” *Immunol. Today*, vol. 20, no. 3, pp. 141–151, Mar. 1999.

[231] M. R. Neutra, N. J. Mantis, and J.-P. Kraehenbuhl, “Collaboration of epithelial cells with organized mucosal lymphoid tissues,” *Nat. Immunol.*, vol. 2, no. 11, pp. 1004–1009, Nov. 2001.

[232] H. E. Ward and T. E. Nicholas, “Alveolar type I and type II cells,” *Aust. N. Z. J. Med.*, vol. 14, no. 5 Suppl 3, pp. 731–734, Oct. 1984.

[233] M. C. Williams, “Alveolar type I cells: molecular phenotype and development,” *Annu. Rev. Physiol.*, vol. 65, pp. 669–695, 2003.

[234] H. Fehrenbach, “Alveolar epithelial type II cell: defender of the alveolus revisited,” *Respir. Res.*, vol. 2, no. 1, pp. 33–46, 2001.

[235] M. Weitnauer, V. Mijošek, and A. H. Dalpke, “Control of local immunity by airway epithelial cells,” *Mucosal Immunol.*, vol. 9, no. 2, p. mi2015126, Dec. 2015.

[236] W. Pankow and P. von Wichert, “M cell in the immune system of the lung,” *Respir. Int. Rev. Thorac. Dis.*, vol. 54, no. 4, pp. 209–219, 1988.

[237] J. P. Kraehenbuhl and M. R. Neutra, “Epithelial M cells: differentiation and function,” *Annu. Rev. Cell Dev. Biol.*, vol. 16, pp. 301–332, 2000.

[238] E. D. Cisney, S. Fernandez, S. I. Hall, G. A. Krietz, and R. G. Ulrich, “Examining the Role of Nasopharyngeal-associated Lymphoreticular Tissue (NALT) in Mouse Responses to Vaccines,” *J. Vis. Exp. JoVE*, no. 66, Aug. 2012.

[239] H. Kiyono and S. Fukuyama, “NALT- versus PEYER’S-patch-mediated mucosal immunity,” *Nat. Rev. Immunol.*, vol. 4, no. 9, p. nri1439, Sep. 2004.

[240] C. F. Kuper *et al.*, “The role of nasopharyngeal lymphoid tissue,” *Immunol. Today*, vol. 13, no. 6, pp. 219–224, Jun. 1992.

[241] T. D. Randall, “Bronchus-associated lymphoid tissue (BALT) structure and function,” *Adv. Immunol.*, vol. 107, pp. 187–241, 2010.

[242] J. E. Moyron-Quiroz *et al.*, “Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity,” *Nat. Med.*, vol. 10, no. 9, pp. 927–934, Sep. 2004.

[243] T. Sminia, G. J. van der Brugge-Gamelkoorn, and S. H. Jeurissen, “Structure and function of bronchus-associated lymphoid tissue (BALT),” *Crit. Rev. Immunol.*, vol. 9, no. 2, pp. 119–150, 1989.

[244] S. Y. Foo and S. Phipps, “Regulation of inducible BALT formation and contribution to immunity and pathology,” *Mucosal Immunol.*, vol. 3, no. 6, p. mi201052, Sep. 2010.

[245] K. N. Gibson-Corley, A. W. Boyden, L. Tygrett, and T. J. Waldschmidt, “Kinetics and Implications of Germinal Center Formation in Induced Bronchus-Associated Lymphoid Tissue During Influenza Infection,” *FASEB J.*, vol. 30, no. 1 Supplement, p. 700.5-700.5, Apr. 2016.

[246] K. Neyt, C. H. GeurtsvanKessel, K. Deswarte, H. Hammad, and B. N. Lambrecht, “Early IL-1 Signaling Promotes iBALT Induction after Influenza Virus Infection,” *Front. Immunol.*, vol. 7, Aug. 2016.

[247] A. Lamichhane, T. Azegami, and H. Kiyono, “The mucosal immune system for vaccine development,” *Vaccine*, vol. 32, no. 49, pp. 6711–6723, Nov. 2014.

[248] J. R. Mora *et al.*, “Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells,” *Science*, vol. 314, no. 5802, pp. 1157–1160, Nov. 2006.

[249] D. Burnett, “Immunoglobulins in the lung,” *Thorax*, vol. 41, no. 5, pp. 337–344, May 1986.

[250] P. Brandtzaeg, “The role of humoral mucosal immunity in the induction and maintenance of chronic airway infections.,” *Am. J. Respir. Crit. Care Med.*, vol. 151, no. 6, pp. 2081–2086, Jun. 1995.

[251] G. Falero-Diaz *et al.*, “Transmission of IgA and IgG monoclonal antibodies to mucosal fluids following intranasal or parenteral delivery,” *Int. Arch. Allergy Immunol.*, vol. 122, no. 2, pp. 143–150, Jun. 2000.

[252] A. J. Macpherson, K. D. McCoy, F.-E. Johansen, and P. Brandtzaeg, “The immune geography of IgA induction and function,” *Mucosal Immunol.*, vol. 1, no. 1, pp. 11–22, 2008.

[253] C. Czerkinsky *et al.*, “IgA antibody-producing cells in peripheral blood after antigen ingestion: evidence for a common mucosal immune system in humans,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 84, no. 8, pp. 2449–2453, Apr. 1987.

[254] P. C. Montgomery, J. Cohn, and E. T. Lally, “The induction and characterization of secretory IgA antibodies,” *Adv. Exp. Med. Biol.*, vol. 45, no. 0, pp. 453–462, 1974.

[255] J. M. Woof and M. A. Kerr, “The function of immunoglobulin A in immunity,” *J. Pathol.*, vol. 208, no. 2, pp. 270–282, Jan. 2006.

[256] C. S. Kaetzel, “The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces,” *Immunol. Rev.*, vol. 206, pp. 83–99, Aug. 2005.

[257] P. Brandtzaeg and H. Prydz, “Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins,” *Nature*, vol. 311, no. 5981, pp. 71–73, Sep. 1984.

[258] F. E. Johansen, R. Braathen, and P. Brandtzaeg, “The J chain is essential for polymeric Ig receptor-mediated epithelial transport of IgA,” *J. Immunol. Baltim. Md 1950*, vol. 167, no. 9, pp. 5185–5192, Nov. 2001.

[259] W. R. Brown, R. W. Newcomb, and K. Ishizaka, “Proteolytic degradation of exocrine and serum immunoglobulins,” *J. Clin. Invest.*, vol. 49, no. 7, pp. 1374–1380, Jul. 1970.

[260] J. Shuster, “Pepsin hydrolysis of IgA--delineation of two populations of molecules,” *Immunochemistry*, vol. 8, no. 5, pp. 405–411, May 1971.

[261] B. J. Underdown and K. J. Dorrington, “Studies on the structural and conformational basis for the relative resistance of serum and secretory immunoglobulin A to proteolysis,” *J. Immunol. Baltim. Md 1950*, vol. 112, no. 3, pp. 949–959, Mar. 1974.

[262] P. Brandtzaeg and F.-E. Johansen, “Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties,” *Immunol. Rev.*, vol. 206, no. 1, pp. 32–63, Aug. 2005.

[263] M. B. Mazanec, J. G. Nedrud, C. S. Kaetzel, and M. E. Lamm, “A three-tiered view of the role of IgA in mucosal defense,” *Immunol. Today*, vol. 14, no. 9, pp. 430–435, Sep. 1993.

[264] C. R. Stokes, J. F. Soothill, and M. W. Turner, “Immune exclusion is a function of IgA,” *Nature*, vol. 255, no. 5511, pp. 745–746, Jun. 1975.

[265] M. B. Mazanec, C. S. Kaetzel, M. E. Lamm, D. Fletcher, and J. G. Nedrud, “Intracellular neutralization of virus by immunoglobulin A antibodies,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 89, no. 15, pp. 6901–6905, Aug. 1992.

[266] K. E. Mostov and D. L. Deitcher, “Polymeric immunoglobulin receptor expressed in MDCK cells transcytoses IgA,” *Cell*, vol. 46, no. 4, pp. 613–621, Aug. 1986.

[267] M. Robert-Guroff, “IgG surfaces as an important component in mucosal protection,” *Nat. Med.*, vol. 6, no. 2, pp. 129–130, Feb. 2000.

[268] I. N. Mbawuike, S. Pacheco, C. L. Acuna, K. C. Switzer, Y. Zhang, and G. R. Harriman, “Mucosal immunity to influenza without IgA: an IgA knockout mouse model,” *J. Immunol. Baltim. Md 1950*, vol. 162, no. 5, pp. 2530–2537, Mar. 1999.

[269] N. Lycke, “Recent progress in mucosal vaccine development: potential and limitations,” *Nat. Rev. Immunol.*, vol. 12, no. 8, pp. 592–605, Jul. 2012.

[270] H. B. Greenberg and M. K. Estes, “Rotaviruses: from pathogenesis to vaccination,” *Gastroenterology*, vol. 136, no. 6, pp. 1939–1951, May 2009.

[271] M. F. Pasetti, J. K. Simon, M. B. Sztein, and M. M. Levine, “Immunology of gut mucosal vaccines,” *Immunol. Rev.*, vol. 239, no. 1, pp. 125–148, Jan. 2011.

[272] G. M. Ruiz-Palacios *et al.*, “Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis,” *N. Engl. J. Med.*, vol. 354, no. 1, pp. 11–22, Jan. 2006.

[273] J. D. Clemens *et al.*, “Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up,” *Lancet Lond. Engl.*, vol. 335, no. 8684, pp. 270–273, Feb. 1990.

[274] D. DeRoeck, R. L. Ochiai, J. Yang, D. D. Anh, V. Alag, and J. D. Clemens, “Typhoid vaccination: the Asian experience,” *Expert Rev. Vaccines*, vol. 7, no. 5, pp. 547–560, Jul. 2008.

[275] “Vaccine Information Statement | Typhoid | VIS | CDC.” [Online]. Available: https://www.cdc.gov/vaccines/hcp/vis/vis-statements/typhoid.html. [Accessed: 17-Dec-2017].

[276] S. Shin, S. N. Desai, B. K. Sah, and J. D. Clemens, “Oral vaccines against cholera,” *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.*, vol. 52, no. 11, pp. 1343–1349, Jun. 2011.

[277] “Vaccine Information Statement | Adenovirus | VIS | CDC.” [Online]. Available: https://www.cdc.gov/vaccines/hcp/vis/vis-statements/adenovirus.html. [Accessed: 17-Dec-2017].

[278] K. A. Woodrow, K. M. Bennett, and D. D. Lo, “Mucosal vaccine design and delivery,” *Annu. Rev. Biomed. Eng.*, vol. 14, pp. 17–46, 2012.

[279] M. R. Neutra and P. A. Kozlowski, “Mucosal vaccines: the promise and the challenge,” *Nat. Rev. Immunol.*, vol. 6, no. 2, pp. 148–158, Feb. 2006.

[280] H. Jin and K. Subbarao, “Live attenuated influenza vaccine,” *Curr. Top. Microbiol. Immunol.*, vol. 386, pp. 181–204, 2015.

[281] J. Li, M. T. Arévalo, Y. Chen, S. Chen, and M. Zeng, “T-cell-mediated cross-strain protective immunity elicited by prime–boost vaccination with a live attenuated influenza vaccine,” *Int. J. Infect. Dis. IJID Off. Publ. Int. Soc. Infect. Dis.*, vol. 27, pp. 37–43, Oct. 2014.

[282] J. L. Hallilley *et al.*, “High-Affinity H7 Head and Stalk Domain–Specific Antibody Responses to an Inactivated Influenza H7N7 Vaccine After Priming With Live Attenuated Influenza Vaccine,” *J. Infect. Dis.*, vol. 212, no. 8, pp. 1270–1278, Oct. 2015.

[283] M. Yamamoto, D. W. Pascual, and H. Kiyono, “M cell-targeted mucosal vaccine strategies,” *Curr. Top. Microbiol. Immunol.*, vol. 354, pp. 39–52, 2012.

[284] E. van Riet, A. Ainai, T. Suzuki, and H. Hasegawa, “Mucosal IgA responses in influenza virus infections; thoughts for vaccine design,” *Vaccine*, vol. 30, no. 40, pp. 5893–5900, Aug. 2012.

[285] T. Suzuki, A. Ainai, and H. Hasegawa, “Functional and structural characteristics of secretory IgA antibodies elicited by mucosal vaccines against influenza virus,” *Vaccine*, vol. 35, no. 39, pp. 5297–5302, Sep. 2017.

[286] J. G. Nedrud, X. P. Liang, N. Hague, and M. E. Lamm, “Combined oral/nasal immunization protects mice from Sendai virus infection.,” *J. Immunol.*, vol. 139, no. 10, pp. 3484–3492, Nov. 1987.

[287] T. L. Le, E. Mironova, D. Garcin, and R. W. Compans, “Induction of Influenza-Specific Mucosal Immunity by an Attenuated Recombinant Sendai Virus,” *PLOS ONE*, vol. 6, no. 4, p. e18780, Apr. 2011.

[288] M. Li, Y. Jiang, T. Gong, Z. Zhang, and X. Sun, “Intranasal Vaccination against HIV-1 with Adenoviral Vector-Based Nanocomplex Using Synthetic TLR-4 Agonist Peptide as Adjuvant,” *Mol. Pharm.*, vol. 13, no. 3, pp. 885–894, Mar. 2016.

[289] S. K. Kochi, K. P. Killeen, and U. S. Ryan, “Advances in the development of bacterial vector technology,” *Expert Rev. Vaccines*, vol. 2, no. 1, pp. 31–43, Feb. 2003.

[290] D. M. Hone *et al.*, “Optimization of live oral Salmonella-HIV-1 vaccine vectors for the induction of HIV-specific mucosal and systemic immune responses,” *J. Biotechnol.*, vol. 44, no. 1–3, pp. 203–207, Jan. 1996.

[291] D. Felnerova, J.-F. Viret, R. Glück, and C. Moser, “Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs,” *Curr. Opin. Biotechnol.*, vol. 15, no. 6, pp. 518–529, Dec. 2004.

[292] A. de Haan, H. J. Geerligs, J. P. Huchshorn, G. J. van Scharrenburg, A. M. Palache, and J. Wilschut, “Mucosal immunoadjuvant activity of liposomes: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with an influenza subunit vaccine and coadministered liposomes,” *Vaccine*, vol. 13, no. 2, pp. 155–162, Feb. 1995.

[293] J. Kunisawa *et al.*, “Sendai virus fusion protein mediates simultaneous induction of MHC class I/II-dependent mucosal and systemic immune responses via the nasopharyngeal-associated lymphoreticular tissue immune system,” *J. Immunol. Baltim. Md 1950*, vol. 167, no. 3, pp. 1406–1412, Aug. 2001.

[294] G. Sakaue *et al.*, “HIV Mucosal Vaccine: Nasal Immunization with gp160-Encapsulated Hemagglutinating Virus of Japan-Liposome Induces Antigen-Specific CTLs and Neutralizing Antibody Responses,” *J. Immunol.*, vol. 170, no. 1, pp. 495–502, Jan. 2003.

[295] I. M. van der Lubben, J. C. Verhoef, G. Borchard, and H. E. Junginger, “Chitosan for mucosal vaccination,” *Adv. Drug Deliv. Rev.*, vol. 52, no. 2, pp. 139–144, Nov. 2001.

[296] T. R. Ghimire, “The mechanisms of action of vaccines containing aluminum adjuvants: an in vitro vs in vivo paradigm,” *SpringerPlus*, vol. 4, Apr. 2015.

[297] S. Khurana *et al.*, “MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines,” *Sci. Transl. Med.*, vol. 3, no. 85, p. 85ra48, Jun. 2011.

[298] D. T. O'Hagan, R. Rappuoli, E. De Gregorio, T. Tsai, and G. Del Giudice, "MF59 adjuvant: the best insurance against influenza strain diversity," *Expert Rev. Vaccines*, vol. 10, no. 4, pp. 447–462, Apr. 2011.

[299] A. M. Harandi and D. Medaglini, "Mucosal adjuvants," *Curr. HIV Res.*, vol. 8, no. 4, pp. 330–335, Jun. 2010.

[300] S. Liang and G. Hajishengallis, "Heat-labile enterotoxins as adjuvants or anti-inflammatory agents," *Immunol. Invest.*, vol. 39, no. 4–5, pp. 449–467, 2010.

[301] C. Maisonneuve, S. Bertholet, D. J. Philpott, and E. De Gregorio, "Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 34, pp. 12294–12299, Aug. 2014.

[302] F. W. Brambell, "The transmission of immunity from mother to young and the catabolism of immunoglobulins," *Lancet Lond. Engl.*, vol. 2, no. 7473, pp. 1087–1093, Nov. 1966.

[303] N. E. Simister and A. R. Rees, "Isolation and characterization of an Fc receptor from neonatal rat small intestine," *Eur. J. Immunol.*, vol. 15, no. 7, pp. 733–738, Jul. 1985.

[304] J. L. Leach, D. D. Sedmak, J. M. Osborne, B. Rahill, M. D. Lairmore, and C. L. Anderson, "Isolation from human placenta of the IgG transporter, FcRn, and localization to the syncytiotrophoblast: implications for maternal-fetal antibody transport," *J. Immunol. Baltim. Md 1950*, vol. 157, no. 8, pp. 3317–3322, Oct. 1996.

[305] N. E. Simister, C. M. Story, H. L. Chen, and J. S. Hunt, "An IgG-transporting Fc receptor expressed in the syncytiotrophoblast of human placenta," *Eur. J. Immunol.*, vol. 26, no. 7, pp. 1527–1531, Jul. 1996.

[306] E. S. Ward, J. Zhou, V. Ghetie, and R. J. Ober, "Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans," *Int. Immunol.*, vol. 15, no. 2, pp. 187–195, Feb. 2003.

[307] D. C. Roopenian and S. Akilesh, "FcRn: the neonatal Fc receptor comes of age," *Nat. Rev. Immunol.*, vol. 7, no. 9, pp. 715–725, Sep. 2007.

[308] D. C. Roopenian *et al.*, "The MHC class I-like IgG receptor controls perinatal IgG transport, IgG homeostasis, and fate of IgG-Fc-coupled drugs," *J. Immunol. Baltim. Md 1950*, vol. 170, no. 7, pp. 3528–3533, Apr. 2003.

[309] T. Rath *et al.*, "The immunologic functions of the neonatal Fc receptor for IgG," *J. Clin. Immunol.*, vol. 33 Suppl 1, pp. S9-17, Jan. 2013.

[310] K. M. K. Sand, M. Bern, J. Nilsen, H. T. Noordzij, I. Sandlie, and J. T. Andersen, "Unraveling the Interaction between FcRn and Albumin: Opportunities for Design of Albumin-Based Therapeutics," *Front. Immunol.*, vol. 5, Jan. 2015.

[311] C. Chaudhury, C. L. Brooks, D. C. Carter, J. M. Robinson, and C. L. Anderson, "Albumin binding to FcRn: distinct from the FcRn-IgG interaction," *Biochemistry (Mosc.)*, vol. 45, no. 15, pp. 4983–4990, Apr. 2006.

[312] E. J. Israel, D. F. Wilsker, K. C. Hayes, D. Schoenfeld, and N. E. Simister, "Increased clearance of IgG in mice that lack β2-microglobulin: possible protective role of FcRn," *Immunology*, vol. 89, no. 4, pp. 573–578, Dec. 1996.

[313] W. P. Burmeister, A. H. Huber, and P. J. Bjorkman, "Crystal structure of the complex of rat neonatal Fc receptor with Fc," *Nature*, vol. 372, no. 6504, pp. 379–383, Nov. 1994.

[314] A. H. Huber, R. F. Kelley, L. N. Gastinel, and P. J. Bjorkman, "Crystallization and stoichiometry of binding of a complex between a rat intestinal Fc receptor and Fc," *J. Mol. Biol.*, vol. 230, no. 3, pp. 1077–1083, Apr. 1993.

[315] M. Raghavan, M. Y. Chen, L. N. Gastinel, and P. J. Bjorkman, "Investigation of the interaction between the class I MHC-related Fc receptor and its immunoglobulin G ligand," *Immunity*, vol. 1, no. 4, pp. 303–315, Jul. 1994.

[316] J. K. Kim, M. Firan, C. G. Radu, C. H. Kim, V. Ghetie, and E. S. Ward, "Mapping the site on human IgG for binding of the MHC class I-related receptor, FcRn," *Eur. J. Immunol.*, vol. 29, no. 9, pp. 2819–2825, Sep. 1999.

[317] C. Medesan, D. Matesoi, C. Radu, V. Ghetie, and E. S. Ward, "Delineation of the amino acid residues involved in transcytosis and catabolism of mouse IgG1," *J. Immunol. Baltim. Md 1950*, vol. 158, no. 5, pp. 2211–2217, Mar. 1997.

[318] J. K. Kim, M. F. Tsen, V. Ghetie, and E. S. Ward, "Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor," *Eur. J. Immunol.*, vol. 24, no. 10, pp. 2429–2434, Oct. 1994.

[319] J. P. Derrick and D. B. Wigley, "Crystal structure of a streptococcal protein G domain bound to an Fab fragment," *Nature*, vol. 359, no. 6397, pp. 752–754, Oct. 1992.

[320] A. L. Corper *et al.*, "Structure of human IgM rheumatoid factor Fab bound to its autoantigen IgG Fc reveals a novel topology of antibody-antigen interaction," *Nat. Struct. Biol.*, vol. 4, no. 5, pp. 374–381, May 1997.

[321] J. Deisenhofer, "Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution," *Biochemistry (Mosc.)*, vol. 20, no. 9, pp. 2361–2370, Apr. 1981.

[322] E. E. Idusogie *et al.*, "Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc," *J. Immunol. Baltim. Md 1950*, vol. 164, no. 8, pp. 4178–4184, Apr. 2000.

[323] S. Popov, J. G. Hubbard, J.-K. Kim, B. Ober, V. Ghetie, and E. S. Ward, "The stoichiometry and affinity of the interaction of murine Fc fragments with the MHC class I-related receptor, FcRn," *Mol. Immunol.*, vol. 33, no. 6, pp. 521–530, Apr. 1996.

[324] Y. N. Abdiche *et al.*, “The neonatal Fc receptor (FcRn) binds independently to both sites of the IgG homodimer with identical affinity,” *mAbs*, vol. 7, no. 2, pp. 331–343, Feb. 2015.

[325] R. Rodewald, “pH-dependent binding of immunoglobulins to intestinal cells of the neonatal rat,” *J. Cell Biol.*, vol. 71, no. 2, pp. 666–669, Nov. 1976.

[326] E. A. Jones and T. A. Waldmann, “The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat,” *J. Clin. Invest.*, vol. 51, no. 11, pp. 2916–2927, Nov. 1972.

[327] M. Raghavan, V. R. Bonagura, S. L. Morrison, and P. J. Bjorkman, “Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants,” *Biochemistry (Mosc.)*, vol. 34, no. 45, pp. 14649–14657, Nov. 1995.

[328] M. Raghavan, L. N. Gastinel, and P. J. Bjorkman, “The class I major histocompatibility complex related Fc receptor shows pH-dependent stability differences correlating with immunoglobulin binding and release,” *Biochemistry (Mosc.)*, vol. 32, no. 33, pp. 8654–8660, Aug. 1993.

[329] J. Borvak *et al.*, “Functional expression of the MHC class I-related receptor, FcRn, in endothelial cells of mice,” *Int. Immunol.*, vol. 10, no. 9, pp. 1289–1298, Sep. 1998.

[330] M. Sakagami *et al.*, “Expression and transport functionality of FcRn within rat alveolar epithelium: a study in primary cell culture and in the isolated perfused lung,” *Pharm. Res.*, vol. 23, no. 2, pp. 270–279, Feb. 2006.

[331] K.-J. Kim, T. E. Fandy, V. H. L. Lee, D. K. Ann, Z. Borok, and E. D. Crandall, “Net absorption of IgG via FcRn-mediated transcytosis across rat alveolar epithelial cell monolayers,” *Am. J. Physiol. Lung Cell. Mol. Physiol.*, vol. 287, no. 3, pp. L616-622, Sep. 2004.

[332] S. Tzaban *et al.*, “The recycling and transcytotic pathways for IgG transport by FcRn are distinct and display an inherent polarity,” *J. Cell Biol.*, vol. 185, no. 4, pp. 673–684, May 2009.

[333] E. S. Ward, C. Martinez, C. Vaccaro, J. Zhou, Q. Tang, and R. J. Ober, “From Sorting Endosomes to Exocytosis: Association of Rab4 and Rab11 GTPases with the Fc Receptor, FcRn, during Recycling,” *Mol. Biol. Cell*, vol. 16, no. 4, pp. 2028–2038, Apr. 2005.

[334] K. M. McCarthy *et al.*, “Effects of mutations in potential phosphorylation sites on transcytosis of FcRn,” *J. Cell Sci.*, vol. 114, no. Pt 8, pp. 1591–1598, Apr. 2001.

[335] E. J. Israel *et al.*, “Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells.,” *Immunology*, vol. 92, no. 1, pp. 69–74, Sep. 1997.

[336] F. Schlachetzki, C. Zhu, and W. M. Pardridge, “Expression of the neonatal Fc receptor (FcRn) at the blood-brain barrier,” *J. Neurochem.*, vol. 81, no. 1, pp. 203–206, Apr. 2002.

[337] Y. Zhang and W. M. Pardridge, “Mediated efflux of IgG molecules from brain to blood across the blood-brain barrier,” *J. Neuroimmunol.*, vol. 114, no. 1–2, pp. 168–172, Mar. 2001.

[338] R. Deane *et al.*, “IgG-assisted age-dependent clearance of Alzheimer’s amyloid beta peptide by the blood-brain barrier neonatal Fc receptor,” *J. Neurosci. Off. J. Soc. Neurosci.*, vol. 25, no. 50, pp. 11495–11503, Dec. 2005.

[339] B. Mayer, Z. Kis, G. Kaján, L. V. Frenyó, L. Hammarström, and I. Kacskovics, “The neonatal Fc receptor (FcRn) is expressed in the bovine lung,” *Vet. Immunol. Immunopathol.*, vol. 98, no. 1–2, pp. 85–89, Mar. 2004.

[340] L. Lu *et al.*, “A Neonatal Fc Receptor-Targeted Mucosal Vaccine Strategy Effectively Induces HIV-1 Antigen-Specific Immunity to Genital Infection ▿ ,” *J. Virol.*, vol. 85, no. 20, pp. 10542–10553, Oct. 2011.

[341] T. T. Kuo *et al.*, “Neonatal Fc receptor: from immunity to therapeutics,” *J. Clin. Immunol.*, vol. 30, no. 6, pp. 777–789, Nov. 2010.

[342] A. J. Bitonti and J. A. Dumont, “Pulmonary administration of therapeutic proteins using an immunoglobulin transport pathway,” *Adv. Drug Deliv. Rev.*, vol. 58, no. 9, pp. 1106–1118, Oct. 2006.

[343] T. Rath *et al.*, “Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics,” *Crit. Rev. Biotechnol.*, vol. 35, no. 2, pp. 235–254, Jun. 2015.

[344] V. Ghetie and E. S. Ward, “Multiple roles for the major histocompatibility complex class I- related receptor FcRn,” *Annu. Rev. Immunol.*, vol. 18, pp. 739–766, 2000.

[345] M.-Y. Zhang, Y. Wang, M. K. Mankowski, R. G. Ptak, and D. S. Dimitrov, “Cross-reactive HIV-1-neutralizing activity of serum IgG from a rabbit immunized with gp41 fused to IgG1 Fc: possible role of the prolonged half-life of the immunogen,” *Vaccine*, vol. 27, no. 6, pp. 857–863, Feb. 2009.

[346] J. Zalevsky *et al.*, “Enhanced antibody half-life improves *in vivo* activity,” *Nat. Biotechnol.*, vol. 28, no. 2, p. nbt.1601, Jan. 2010.

[347] T. Suzuki *et al.*, “Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR,” *J. Immunol. Baltim. Md 1950*, vol. 184, no. 4, pp. 1968–1976, Feb. 2010.

[348] T. Rath *et al.*, “The immunologic functions of the neonatal Fc receptor for IgG,” *J. Clin. Immunol.*, vol. 33 Suppl 1, pp. S9-17, Jan. 2013.

[349] P. J. Carter, “Introduction to current and future protein therapeutics: a protein engineering perspective,” *Exp. Cell Res.*, vol. 317, no. 9, pp. 1261–1269, May 2011.

[350] S. Ghose, B. Hubbard, and S. M. Cramer, “Evaluation and comparison of alternatives to Protein A chromatography Mimetic and hydrophobic charge induction chromatographic stationary phases,” *J. Chromatogr. A*, vol. 1122, no. 1–2, pp. 144–152, Jul. 2006.

[351] D. J. Capon *et al.*, “Designing CD4 immunoadhesins for AIDS therapy,” *Nature*, vol. 337, no. 6207, pp. 525–531, Feb. 1989.

[352] D. Levin, B. Golding, S. E. Strome, and Z. E. Sauna, “Fc fusion as a platform technology: potential for modulating immunogenicity,” *Trends Biotechnol.*, vol. 33, no. 1, pp. 27–34, Jan. 2015.

[353] C. for B. E. and Research, “Fractionated Plasma Products - ELOCTATE.” [Online]. Available: https://www.fda.gov/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/ucm399909.htm. [Accessed: 17-Dec-2017].

[354] C. for B. E. and Research, “Fractionated Plasma Products - ALPROLIX.” [Online]. Available: https://www.fda.gov/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/ucm390993.htm. [Accessed: 17-Dec-2017].

[355] C. for D. E. and Research, “Postmarket Drug Safety Information for Patients and Providers - Nplate (romiplostim) Information.” [Online]. Available: https://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm280200.htm. [Accessed: 17-Dec-2017].

[356] J. R. de Oliveira Dias, G. C. de Andrade, E. A. Novais, M. E. Farah, and E. B. Rodrigues, “Fusion proteins for treatment of retinal diseases: aflibercept, ziv-aflibercept, and conbercept,” *Int. J. Retina Vitr.*, vol. 2, p. 3, Feb. 2016.

[357] R. L. Shields *et al.*, “High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R,” *J. Biol. Chem.*, vol. 276, no. 9, pp. 6591–6604, Mar. 2001.

[358] S. B. Petkova *et al.*, “Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: potential application in humorally mediated autoimmune disease,” *Int. Immunol.*, vol. 18, no. 12, pp. 1759–1769, Dec. 2006.

[359] P. R. Hinton *et al.*, “Engineered human IgG antibodies with longer serum half-lives in primates,” *J. Biol. Chem.*, vol. 279, no. 8, pp. 6213–6216, Feb. 2004.

[360] C. Vaccaro, J. Zhou, R. J. Ober, and E. S. Ward, “Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels,” *Nat. Biotechnol.*, vol. 23, no. 10, pp. 1283–1288, Oct. 2005.

[361] W. F. Dall’Acqua, P. A. Kiener, and H. Wu, “Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn),” *J. Biol. Chem.*, vol. 281, no. 33, pp. 23514–23524, Aug. 2006.

[362] A. J. Bitonti *et al.*, “Pulmonary delivery of an erythropoietin Fc fusion protein in non-human primates through an immunoglobulin transport pathway,” *Proc. Natl. Acad. Sci.*, vol. 101, no. 26, pp. 9763–9768, Jun. 2004.

[363] J. A. Dumont, A. J. Bitonti, D. Clark, S. Evans, M. Pickford, and S. P. Newman, “Delivery of an erythropoietin-Fc fusion protein by inhalation in humans through an immunoglobulin transport pathway,” *J. Aerosol Med. Off. J. Int. Soc. Aerosols Med.*, vol. 18, no. 3, pp. 294–303, 2005.

[364] S. C. Low, S. L. Nunes, A. J. Bitonti, and J. A. Dumont, “Oral and pulmonary delivery of FSH-Fc fusion proteins via neonatal Fc receptor-mediated transcytosis,” *Hum. Reprod. Oxf. Engl.*, vol. 20, no. 7, pp. 1805–1813, Jul. 2005.

[365] L. Ye, R. Zeng, Y. Bai, D. C. Roopenian, and X. Zhu, “Efficient mucosal vaccination mediated by the neonatal Fc receptor,” *Nat. Biotechnol.*, vol. 29, no. 2, pp. 158–163, Feb. 2011.

[366] Z. Shubin *et al.*, “An HIV Envelope gp120-Fc Fusion Protein Elicits Effector Antibody Responses in Rhesus Macaques,” *Clin. Vaccine Immunol. CVI*, vol. 24, no. 6, Jun. 2017.

[367] “Estimates of Deaths Associated with Seasonal Influenza --- United States, 1976--2007.” [Online]. Available: https://www.cdc.gov/mmwr/preview/mmwrhtml/mm5933a1.htm. [Accessed: 18-Dec-2017].

[368] “Fewer Than Half of Americans Report Having Gotten A Flu Vaccine this Season | Seasonal Influenza (Flu) | CDC,” 07-Apr-2017. [Online]. Available: https://www.cdc.gov/flu/news/half-of-americans-received-flu-vaccine.htm. [Accessed: 18-Dec-2017].

[369] A. R. Duncan and G. Winter, “The binding site for C1q on IgG,” *Nature*, vol. 332, no. 6166, pp. 738–740, Apr. 1988.

[370] L. Ye *et al.*, “The MHC class II-associated invariant chain interacts with the neonatal Fc gamma receptor and modulates its trafficking to endosomal/lysosomal compartments,” *J. Immunol. Baltim. Md 1950*, vol. 181, no. 4, pp. 2572–2585, Aug. 2008.

[371] J. V. Ravetch and S. Bolland, “IgG Fc receptors,” *Annu. Rev. Immunol.*, vol. 19, pp. 275–290, 2001.

[372] J. V. Ravetch, “Fc receptors,” *Curr. Opin. Immunol.*, vol. 9, no. 1, pp. 121–125, Feb. 1997.

[373] P. Bruhns, “Properties of mouse and human IgG receptors and their contribution to disease models,” *Blood*, vol. 119, no. 24, pp. 5640–5649, Jun. 2012.

[374] D. M. Czajkowsky, J. Hu, Z. Shao, and R. J. Pleass, “Fc-fusion proteins: new developments and future perspectives,” *EMBO Mol. Med.*, vol. 4, no. 10, pp. 1015–1028, Oct. 2012.

[375] Y. He, H. Lu, P. Siddiqui, Y. Zhou, and S. Jiang, “Receptor-binding domain of severe acute respiratory syndrome coronavirus spike protein contains multiple conformation-dependent epitopes that induce highly potent neutralizing antibodies,” *J. Immunol. Baltim. Md 1950*, vol. 174, no. 8, pp. 4908–4915, Apr. 2005.

[376] M. C. Kang *et al.*, “Intranasal Introduction of Fc-Fused Interleukin-7 Provides Long-Lasting Prophylaxis against Lethal Influenza Virus Infection,” *J. Virol.*, vol. 90, no. 5, pp. 2273–2284, Dec. 2015.

[377] L. Du *et al.*, “A recombinant vaccine of H5N1 HA1 fused with foldon and human IgG Fc induced complete cross-clade protection against divergent H5N1 viruses,” *PloS One*, vol. 6, no. 1, p. e16555, Jan. 2011.

[378] S. Loureiro *et al.*, “Adjuvant-free immunization with hemagglutinin-Fc fusion proteins as an approach to influenza vaccines,” *J. Virol.*, vol. 85, no. 6, pp. 3010–3014, Mar. 2011.

[379] Y. Li *et al.*, “A recombinant protein containing highly conserved hemagglutinin residues 81-122 of influenza H5N1 induces strong humoral and mucosal immune responses,” *Biosci. Trends*, vol. 7, no. 3, pp. 129–137, Jun. 2013.

[380] L. Du *et al.*, “A critical HA1 neutralizing domain of H5N1 influenza in an optimal conformation induces strong cross-protection,” *PloS One*, vol. 8, no. 1, p. e53568, 2013.

[381] B. He *et al.*, “Putative suppressing effect of IgG Fc-conjugated haemagglutinin (HA) stalk of influenza virus H7N9 on the neutralizing immunogenicity of Fc-conjugated HA head: implication for rational design of HA-based influenza vaccines,” *J. Gen. Virol.*, vol. 97, no. 2, pp. 327–333, Feb. 2016.

[382] F. Yu *et al.*, “Intranasal vaccination of recombinant H5N1 HA1 proteins fused with foldon and Fc induces strong mucosal immune responses with neutralizing activity: Implication for developing novel mucosal influenza vaccines,” *Hum. Vaccines Immunother.*, vol. 11, no. 12, pp. 2831–2838, 2015.

[383] A. C. Dalton and W. A. Barton, “Over-expression of secreted proteins from mammalian cell lines,” *Protein Sci. Publ. Protein Soc.*, vol. 23, no. 5, pp. 517–525, May 2014.

[384] A. Frey, J. Di Canzio, and D. Zurakowski, “A statistically defined endpoint titer determination method for immunoassays,” *J. Immunol. Methods*, vol. 221, no. 1–2, pp. 35–41, Dec. 1998.

[385] L. J. Reed and H. Muench, “A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12,” *Am. J. Epidemiol.*, vol. 27, no. 3, pp. 493–497, May 1938.

[386] G. K. Hirst, “THE AGGLUTINATION OF RED CELLS BY ALLANTOIC FLUID OF CHICK EMBRYOS INFECTED WITH INFLUENZA VIRUS,” *Science*, vol. 94, no. 2427, pp. 22–23, Jul. 1941.

[387] G. K. Hirst, “The Quantitative Determination of Influenza Virus and Antibodies by Means of Red Cell Agglutination,” *J. Exp. Med.*, vol. 75, no. 1, pp. 49–64, Jan. 1942.

[388] J. P. Buchweitz, P. W. F. Karmaus, J. R. Harkema, K. J. Williams, and N. E. Kaminski, “Modulation of airway responses to influenza A/PR/8/34 by Delta9-tetrahydrocannabinol in C57BL/6 mice,” *J. Pharmacol. Exp. Ther.*, vol. 323, no. 2, pp. 675–683, Nov. 2007.

[389] T. Rowe *et al.*, “Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays,” *J. Clin. Microbiol.*, vol. 37, no. 4, pp. 937–943, Apr. 1999.

[390] J. A. Dumont, S. C. Low, R. T. Peters, and A. J. Bitonti, “Monomeric Fc fusions: impact on pharmacokinetic and biological activity of protein therapeutics,” *BioDrugs Clin. Immunother. Biopharm. Gene Ther.*, vol. 20, no. 3, pp. 151–160, 2006.

[391] H. Liu and K. May, “Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function,” *mAbs*, vol. 4, no. 1, pp. 17–23, Feb. 2012.

[392] Y. Tao, S. V. Strelkov, V. V. Mesyanzhinov, and M. G. Rossmann, “Structure of bacteriophage T4 fibritin: a segmented coiled coil and the role of the C-terminal domain,” *Struct. Lond. Engl. 1993*, vol. 5, no. 6, pp. 789–798, Jun. 1997.

[393] F. Krammer, I. Margine, G. S. Tan, N. Pica, J. C. Krause, and P. Palese, “A carboxy-terminal trimerization domain stabilizes conformational epitopes on the stalk domain of soluble recombinant hemagglutinin substrates,” *PloS One*, vol. 7, no. 8, p. e43603, 2012.

[394] W. C. Weldon, B.-Z. Wang, M. P. Martin, D. G. Koutsonanos, I. Skountzou, and R. W. Compans, “Enhanced immunogenicity of stabilized trimeric soluble influenza hemagglutinin,” *PloS One*, vol. 5, no. 9, Sep. 2010.

[395] J. Charles A Janeway, P. Travers, M. Walport, and M. J. Shlomchik, “The mucosal immune system,” 2001.

[396] Z. Hao *et al.*, “Fas Receptor Expression in Germinal-Center B Cells Is Essential for T and B Lymphocyte Homeostasis,” *Immunity*, vol. 29, no. 4, pp. 615–627, Oct. 2008.

[397] Y. Naito *et al.*, “Germinal Center Marker GL7 Probes Activation-Dependent Repression of N-Glycolylneuraminic Acid, a Sialic Acid Species Involved in the Negative Modulation of B-Cell Activation,” *Mol. Cell. Biol.*, vol. 27, no. 8, pp. 3008–3022, Apr. 2007.

[398] R. Ahmed and D. Gray, “Immunological memory and protective immunity: understanding their relation,” *Science*, vol. 272, no. 5258, pp. 54–60, Apr. 1996.

[399] D. L. Turner and D. L. Farber, “Mucosal resident memory CD4 T cells in protection and immunopathology,” *Front. Immunol.*, vol. 5, p. 331, 2014.

[400] T. Ishino *et al.*, “Engineering a monomeric Fc domain modality by N-glycosylation for the half-life extension of biotherapeutics,” *J. Biol. Chem.*, vol. 288, no. 23, pp. 16529–16537, Jun. 2013.

[401] R. T. Peters *et al.*, “Prolonged activity of factor IX as a monomeric Fc fusion protein,” *Blood*, vol. 115, no. 10, pp. 2057–2064, Mar. 2010.

[402] G. Gao *et al.*, “Designing a Soluble Near Full-length HIV-1 gp41 Trimer,” *J. Biol. Chem.*, vol. 288, no. 1, pp. 234–246, Jan. 2013.

[403] K. A. Swanson *et al.*, “A monomeric uncleaved respiratory syncytial virus F antigen retains prefusion-specific neutralizing epitopes,” *J. Virol.*, vol. 88, no. 20, pp. 11802–11810, Oct. 2014.

[404] L. Sissoëff, M. Mousli, P. England, and C. Tuffereau, “Stable trimerization of recombinant rabies virus glycoprotein ectodomain is required for interaction with the p75NTR receptor,” *J. Gen. Virol.*, vol. 86, no. Pt 9, pp. 2543–2552, Sep. 2005.

[405] G. B. E. Stewart-Jones *et al.*, “A Cysteine Zipper Stabilizes a Pre-Fusion F Glycoprotein Vaccine for Respiratory Syncytial Virus,” *PLOS ONE*, vol. 10, no. 6, p. e0128779, Jun. 2015.

[406] F. E. Johansen *et al.*, “Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice,” *J. Exp. Med.*, vol. 190, no. 7, pp. 915–922, Oct. 1999.

[407] F. Krammer, P. Palese, and J. Steel, “Advances in universal influenza virus vaccine design and antibody mediated therapies based on conserved regions of the hemagglutinin,” *Curr. Top. Microbiol. Immunol.*, vol. 386, pp. 301–321, 2015.

[408] A. Portela and P. Digard, “The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication,” *J. Gen. Virol.*, vol. 83, no. Pt 4, pp. 723–734, Apr. 2002.

[409] J. W. Yewdell, J. R. Bennink, G. L. Smith, and B. Moss, “Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 82, no. 6, pp. 1785–1789, Mar. 1985.

[410] A. Wahl, F. Schafer, W. Bardet, R. Buchli, G. M. Air, and W. H. Hildebrand, “HLA class I molecules consistently present internal influenza epitopes,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 2, pp. 540–545, Jan. 2009.

[411] Q. Ye, R. M. Krug, and Y. J. Tao, “The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA,” *Nature*, vol. 444, no. 7122, pp. 1078–1082, Dec. 2006.

[412] N. L. Varich *et al.*, “Location of antigenic sites recognized by monoclonal antibodies in the influenza A virus nucleoprotein molecule,” *J. Gen. Virol.*, vol. 90, no. Pt 7, pp. 1730–1733, Jul. 2009.

[413] J. S. McLellan, W. C. Ray, and M. E. Peeples, “Structure and Function of RSV Surface Glycoproteins,” *Curr. Top. Microbiol. Immunol.*, vol. 372, pp. 83–104, 2013.