Intranasal Administration of Highly Conserved Influenza Antigens to Elicit Local Mucosal Responses and Enhance Cross-subtype Protection

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Abstract

Influenza causes approximately 650,000 deaths worldwide each year and remains one of the top ten causes of death in Canada. The public health burden of influenza is exacerbated by the highly variable year-to-year effectiveness of licensed vaccines. The unpredictable evolution of influenza surface antigens often leads to a mismatch between the circulating strain and the predicted vaccine strain. Additionally, current vaccines have limited ability to prevent early viral replication in the upper respiratory tract. Alternative strategies are needed to address these limitations in current influenza vaccines.

This thesis investigates the intranasal (IN) administration of conserved influenza antigens as a strategy to enhance heterosubtypic protection. By characterizing both systemic and mucosal immune responses elicited by this approach, I aim to advance our understanding of immunity against influenza viruses and to provide insights for the design of next-generation vaccines.

My research compared the protective mechanisms of intramuscular (IM) and IN delivery of a recombinant adenovirus encoding the highly conserved influenza nucleoprotein (NP), adjuvanted through fusion to CD40 ligand. While IM administration more effectively induced systemic cellular immune responses, IN immunization conferred superior cross-subtype protection through robust mucosal antibody production and pulmonary T cell responses. This thesis also evaluated the protective efficacy and immune responses elicited by an IN vaccine designed to express the consensus sequence of hemagglutinin subunit 2 (HA2) derived from all H3 strains. This approach provided cross-subtype protection against lethal H3N2 and H7N9 challenges. Further immunological analysis revealed that the vaccine induced antigen-specific pulmonary

cellular and humoral immunity. Notably, a C-terminal region of the HA2 consensus sequence was identified to contain immunodominant epitopes, capable of inducing potent CD4+ and CD8+ T cell responses.

In conclusion, these studies provide mechanistic insights into the protective immune responses induced by IN administered vaccines targeting conserved influenza antigens. These findings contribute to the development of next-generation mucosal vaccines against a broad range of emerging respiratory viral pathogens.

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Dedication

To my father, Baojian Zhang 张宝剑,

for guiding me through life and instilling in me the confidence to explore.

List of Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
APC	Antigen-Presenting Cell
BALF	Bronchoalveolar Lavage Fluid
BPL	β-propiolactone
BSA	Bovine Serum Albumin
CD40L	CD40 Ligand
CDC	Centers for Disease Control and Prevention
cGAMP	2′,3′-cyclic Guanosine Monophosphate–adenosine Monophosphate
CMV	Cytomegalovirus Promoter
COBRA	Computationally Optimized Broadly Reactive Antigen
COVID-19	Coronavirus Disease 2019
CPE	Cytopathic Effect
CTL	Cytotoxic T Lymphocyte
CTV	CellTrace Violet
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration

FTY720	Fingolimod
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
HA	Hemagglutinin
HA1	Hemagglutinin Subunit 1
HA2	Hemagglutinin Subunit 2
HAT	Human Airway Trypsin-like Protease
HLA	Human Leukocyte Antigen
HPAI	Highly Pathogenic Avian Influenza
IAV	Influenza A Virus
ICS	Intracellular Cytokine Staining
IEDB	Immune Epitope Database
IL	Interleukin
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
LAIV	Live Attenuated Influenza Vaccine
LRT	Lower Respiratory Tract
M1	Matrix Protein 1
M2	Matrix Protein 2
MAIT	Mucosal-Associated Invariant T cell
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
MVA	Modified Vaccinia Ankara

NA	Neuraminidase
NALT	Nasal-Associated Lymphoid Tissue
NCBI	National Center for Biotechnology Information
NEP	Nuclear Export Protein
NET	Neutrophil Extracellular Trap
NHP	Non-Human Primate
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NP	Nucleoprotein
NS1	Non-Structural Protein 1
NS2	Non-Structural Protein 2
OAS	Original Antigenic Sin
PA	Polymerase Acidic Protein
PB1	Polymerase Basic Protein 1
PB2	Polymerase Basic Protein 2
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PFU	Plaque-Forming Unit
PHAC	Public Health Agency of Canada
PRR	Pattern Recognition Receptor
QIV	Quadrivalent Influenza Vaccine
RBC	Red Blood Cell
RIV	Recombinant Influenza Vaccine

RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
STING	Stimulator of Interferon Genes
TCID	Tissue Culture Infectious Dose
TCR	T Cell Receptor
TLR	Toll-Like Receptor
TMB	Tetramethylbenzidine
TMPRSS	Transmembrane Protease Serine Subfamily
TNF	Tumor Necrosis Factor
T_{RM}	Tissue-Resident Memory T cell
TTS	Thrombosis with Thrombocytopenia Syndrome
URT	Upper Respiratory Tract
VAERD	Vaccine-Associated Enhanced Respiratory Disease
VE	Vaccine Effectiveness
VITT	Vaccine-Induced Immune Thrombotic Thrombocytopenia
vRNA	Viral Ribonucleic Acid
vRNP	Viral Ribonucleoprotein
WHO	World Health Organization

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1.1 Epidemiology and the Impacts of Influenza Viruses

1.1.1 Epidemiology of influenza

Seasonal influenza remains a significant global public health burden, causing an estimated one billion infections and up to 650,000 deaths annually[1]. In Canada, influenza infection consistently ranks among the top ten leading causes of death[2]. Influenza viruses undergo routine seasonal global migration, resulting in alternating epidemics between the northern and southern hemispheres during their respective winters. Transmission peaks during the colder months in temperate regions and occurs year-round in tropical areas, transmitting from person to person via respiratory droplets, direct contact, and fomites[3]. The annual recurrence of these seasonal epidemics drives viral evolution and complicates immunization strategies and vaccine development.

In addition to seasonal epidemics, influenza also poses a tremendous pandemic threat, which has led to devastating health and economic impacts in the past. The first recorded influenza pandemic dates back to 1918, with a global mortality estimate nearing 100 million. The 1957 H2N2 and 1968 H3N2 pandemics caused an estimated one million deaths[4]. Although the WHO reported 18,631 laboratory-confirmed deaths for the most recent 2009 H1N1 pandemic, other estimates suggest there may have been more than 395,000 respiratory deaths and an additional 83,300 cardiovascular deaths associated with the infection[5,6].

1.1.2 People at increased risk for complications

Although the overall mortality rate for influenza infection is under 0.1%[7], it is more likely to cause hospitalization and death in vulnerable populations. Age is an important determining factor for disease severity, with both the very young and the elderly at higher risk of serious illnesses. Young children under 5 years of age, especially under the age of 2, have immature immune systems. They tend to have a more suppressive immune response that biases towards the Th2 phenotype and is often producing lower levels of cytokines[8,9]. According to a meta-analysis study, in 2008 alone, there were 28,000–111,500 deaths in children younger than 5 years of age attributable to influenza infections[10]. Older adults over the age of 65 are also highly vulnerable to influenza infections due to immunosenescence, a gradual decline in immune function with age, affecting both innate and adaptive immunities. Some of the effects include downregulation of phagocytosis, fewer naïve immune cells, and more dysfunctional memory cells[11]. In addition, vaccine-induced protection does not reliably persist beyond one year in the older population[12,13].

Other high-risk populations include pregnant people, individuals with chronic illnesses or multiple comorbidities, and immunocompromised individuals[14]. While healthy older children and young adults typically mount strong immune responses against influenza infections, it is not always enough to afford protection. During the 2009 H1N1 pandemic, people between the ages of 10 and 50 represented approximately 75% of reported infections, indicating a disproportionate impact on this age group[15]. In the uninfected population, 33% of those over 60 years of age had pre-existing neutralizing antibodies, generated from previous exposures to a H1N1 strain prior to 1957[16]. These

variations in susceptibility and immune responses across distinct populations underscore the complexity and unpredictability of influenza epidemiology.

1.1.3 Emerging zoonotic influenza viruses

The challenge of influenza extends beyond its ability to infect humans. As a highly adaptable virus, it persists in a wide range of animal reservoirs, which act as ongoing sources of viral evolution. These reservoirs contribute to the unpredictable emergence of new strains with the potential for human-to-human transmission. For instance, the 2009 H1N1 pandemic strain contained genes from North American and Eurasian avian, human, and swine strains[17].

Wild aquatic birds are the natural reservoir for influenza A viruses (IAV). Their migratory behavior and overlapping habitat with other species have led to the global dissemination of influenza viruses into new host populations, including domestic poultry and wild mammals[18,19]. Furthermore, co-infection of different IAV strains in wild aquatic birds could lead to reassortment, an exchange of genetic material, increasing the likelihood of spillover to mammalian hosts[20]. It has been observed that wild birds often carry multiple hemagglutinin (HA) and neuraminidase (NA) subtypes, creating conditions that facilitate reassortment and the emergence of highly pathogenic avian influenza (HPAI) viruses[21–23]. The on-going H5N1 epizootic, which began in spring 2024, is a particularly alarming example of this phenomenon. IAVs typically do not infect cattle[20]; however, this clade 2.3.4.4b strain was most likely introduced into cattle via a single bird-to-cattle transmission event in late 2023[24]. As of July 2025, 1,074 dairy herds across 17 U.S. states have reported infections, along with 64 confirmed human cases[25]. As many scientists have expressed their concerns with the outbreak, there remains a risk that the virus may

acquire additional mutations that enhance human adaptation. In addition to close surveillance, mitigation efforts should focus on reducing viral circulations in animals, especially in pigs, as well as minimizing the risk of co-infection by vaccinating at-risk personnel[20].

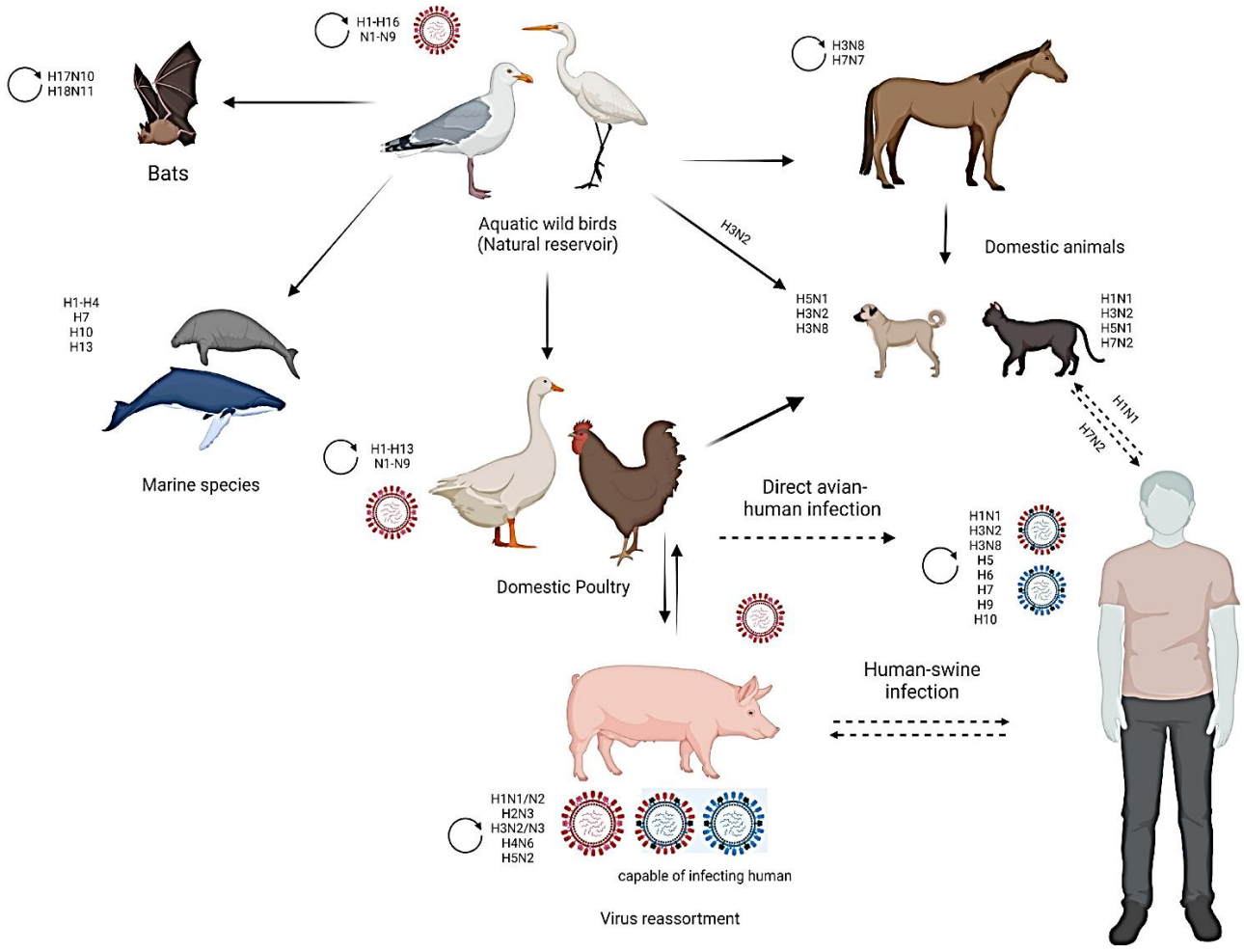


Figure 1.1 Influenza A viruses have a broad host range, with aquatic wild birds serving as their primary natural reservoir.

From these species, the viruses can cross species barriers and infect various hosts, including domestic poultry, livestock, bats, horses, pigs, and humans. The circular arrows indicate ongoing viral circulation within and between these populations. This image is Copyright © 2023 AbuBakar J. et al.19 in accordance with open access Creative Commons CC BY 4.0 license.

1.2 Virology of Influenza

1.2.1 Overview of influenza virus biology

Influenza viruses are members of the *Orthomyxoviridae* family. They are segmented, negative-stranded RNA viruses enclosed in a host-derived lipid membrane, known as an envelope. Influenza virus particles are typically spherical, measuring 80–120 nm in diameter[26]. Depending on the strain and host, some particles may also be filamentous, extending up to 20 µm. The viral genome consists of eight RNA segments, encoding transcripts for 10 essential viral proteins[27]. These include RNA polymerase subunit proteins (PB1, PB2, and PA), surface glycoproteins HA and NA, nucleoprotein (NP), matrix protein (M1), membrane protein (M2), nonstructural protein (NS1), and nuclear export protein (NEP, formally referred to as NS2).

There are four types of influenza viruses, type A, B, C, and D. Two subtypes of influenza A, H1N1 and H3N2, and two lineages of influenza B, Victoria and Yamagata, are endemic in the human population[1]. Influenza C infection, which is rare and only leads to mild symptoms, does not present public health importance. Influenza D viruses primarily infect cattle and are not known to infect humans. As previously discussed, IAVs pose a considerable threat to public health due to their ability to circulate in animal reservoirs and cause pandemic outbreaks[28]. IAVs are classified into subtypes based on their HA and NA surface glycoproteins. A total of 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11) have been identified[29]. HA subtypes are further classified into two distinct phylogenetic groups: group 1 includes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18, while group 2 consists of H3, H4, H7, H10, H14, and H15.

1.2.2 Viral replication

Influenza viruses initiate cell entry by binding to sialic acids on host cells via the head domain of HA (HA1). This interaction is a key determinant of host species specificity. Viruses with tropism for human cells bind to linear α-2,6-sialic acids, whereas avian influenza viruses preferentially bind to the bent α-2,3-sialic acids found within the avian gut and in the human lower airways[30]. Once binding occurs, the host cell internalizes the virus through endocytosis. The low pH within the endosome triggers a conformational change in HA, exposing the fusion peptide located in the HA stem (HA2). The fusion peptide inserts itself into the host membrane, facilitating fusion between the viral envelope and the endosomal membrane. The M2 channels acidify the viral interior, leading to the release of viral ribonucleoproteins (vRNPs) from M1 and into the host cytosol. Unlike most other RNA viruses, a part of the influenza virus replication process occurs within the nucleus, requiring the virus to intricately orchestrate both host and viral mechanisms for transportation[27]. Nuclear localization signals on NP enable the transport of vRNA into the nucleus[31]. Inside the nucleus, transcription begins with “cap-snatching”, a process in which the PB2/PA/PB1 complex cleaves and steals the cap structure from host pre-mRNA to prime viral RNA (vRNA) transcription[32].

Translation of viral proteins occurs in the cytoplasm using host cell machinery[33]. Progeny vRNPs are assembled in the nucleus, beginning with the association of vRNA with PA, PB1, PB2 and NP. The fully assembled vRNPs then associate with M1 and NEP, forming a complex that exits the nucleus and moves towards the cell surface. The viral membrane proteins, NA, HA, and M2, are synthesized and trafficked through the Golgi apparatus to the plasma membrane[27].

Within the Golgi apparatus, the fusion incompetent precursor HA0 is cleaved by host proteases into functional subunits HA1 and HA2. Human and low pathogenic avian strains have monobasic cleavage sites that can be cleaved by proteases such as transmembrane protease serine S-1 member 2 (TMPRSS2), human airway trypsin-like protease (HAT), and possibly TMPRSS4, which are only expressed in respiratory epithelial cells[34]. In contrast, HPAI strains contain multibasic sites that are cleaved by furin, a ubiquitously expressed endoprotease, contributing to the increased pathogenicity of these strains[35].

At the plasma membrane, vRNPs are localized to the budding site and assembled with the remaining viral components. Following budding, NA hydrolyses the sialic acid to facilitate viral release. This NA-mediated cleavage also promotes the subsequent dissemination of progeny viruses through mucus and respiratory epithelial cells, promoting additional rounds of infection[36].

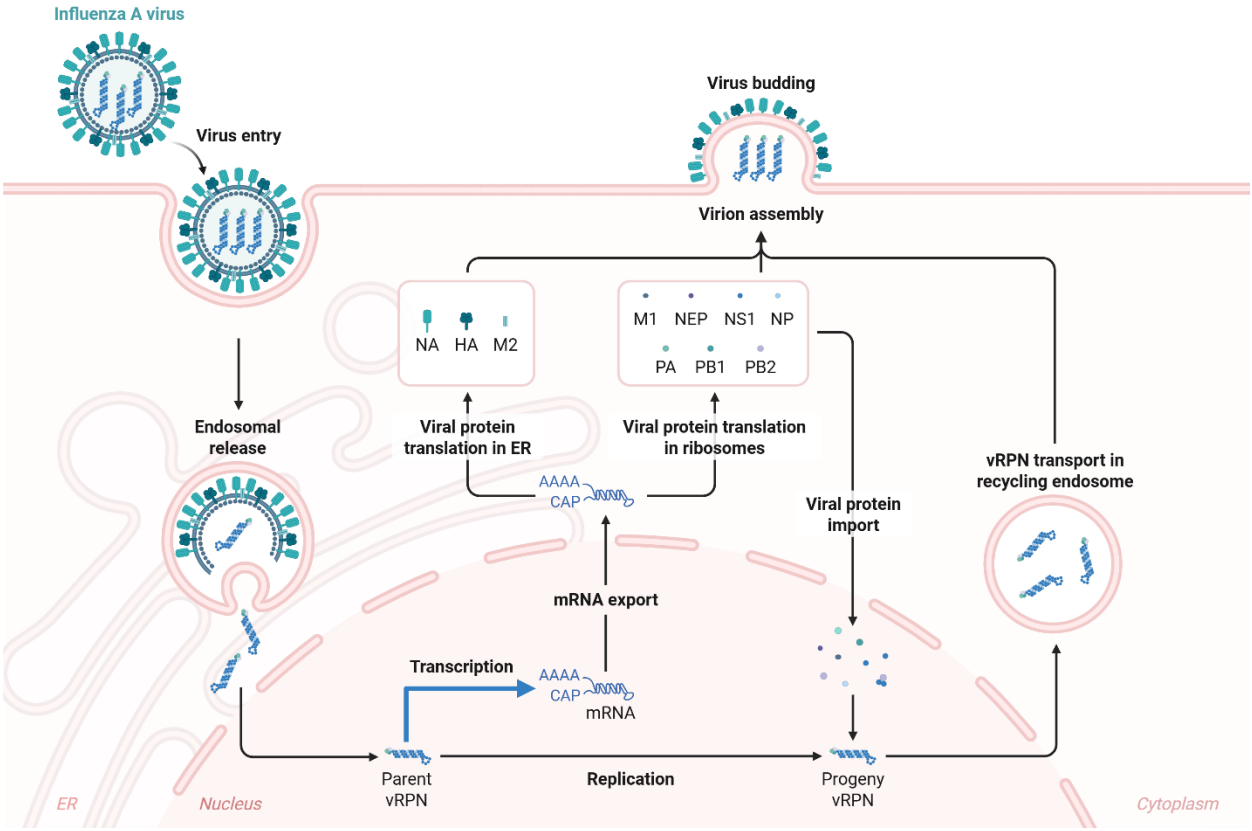


Figure 1.2 Influenza A virus replication.

Cell entry is initiated by the binding of HA to sialic acids on the host cells. The low pH within the endosome leads to the fusion between the viral envelope and the endosomal membrane. The M2 channels acidify the viral interior, leading to the endosomal release of viral ribonucleoproteins (vRNPs) from M1 and into the host cytosol. Translation of viral proteins occurs in the cytoplasm and progeny vRNPs are assembled in the nucleus. At the plasma membrane, vRNPs are localized to the budding site and assembled with the remaining viral components, and new virions are released from host cell. Figure reprinted from "Influenza Virus Life Cycle" Retrieved from https://app.biorender.com/biorender-templates[37].

1.2.3 Genetic variation and immune evasion in influenza viruses

The high genetic variability of IAV is attributed to its error-prone RNA-dependent RNA polymerase and segmented genome[38]. Through antigenic drift and shift, the virus accumulates mutations that enable it to evade pre-existing immunity and generate novel strains to which the human population is immunologically naïve. The estimated mutation rate of IAV is 9.01×10^{-5} substitutions/site, over 20-fold higher than that of severe acute

respiratory syndrome coronavirus 2 (SARS-CoV-2)[39]. This high mutation rate leads to gradual changes in HA and NA that accumulate over time and allow the virus to escape neutralizing antibodies acquired through previous infection or vaccination. These changes are known as antigenic drift, which occur naturally during viral replication[40,41]. Over time, natural selection favors antigenic variants with a fitness advantage that allows the virus to evade immunity[42,43].

In contrast to the gradual nature of antigenic drift, antigenic shift represents more sudden and dramatic changes that give rise to novel strains with pandemic potential[43]. This process relies on reassortment, an event likely to occur during co-infections, particularly within the wide range of wild animal reservoirs. A prominent example is the 2009 H1N1 pandemic strain, which emerged following multiple reassortments[17]. This genetically novel virus led to an abnormally high mortality rate in individuals under 60 years of age, who lacked cross-protective immunity[42]. However, it is important to note that reassortment is not a random process; complex RNA- and protein-based interactions impact the viability of reassortant progeny[44]. Despite 144 theoretically possible HA/NA subtype combinations, only 117 have been observed in wild birds[45]. HA and NA co-evolve to delicately balance the avidity of HA-mediated attachment and NA-mediated virion release. An extreme example of this co-evolution is an oseltamivir resistant H3N2 clinical isolate, which lacked the NA gene segment but compensated by acquiring mutations that reduced HA receptor avidity[46].

To keep pace with the constant antigenic shift and drift of the virus, public health agencies implement a range of strategies, including zoonotic surveillance and control and investments in universal vaccine research. Seasonal flu vaccines are regularly updated,

with the WHO making recommendations 6 months prior to the respective flu season for Northern and Southern hemispheres. These recommendations are made based on the Global Influenza Surveillance and Response System, which monitors circulating strains worldwide[47]. Many countries, including Canada, also stockpile vaccines and antivirals. As part of Canada's contingency planning for the on-going H5N1 outbreak, the Public Health Agency of Canada secured an initial supply of 500,000 doses of GSK's vaccine in February 2025 to protect at-risk personnel[48]. Vaccination remains the most effective tool for preventing infection and reducing the burden of disease, emphasizing the critical need for continued innovation in influenza vaccine development.

1.3 Current Influenza Vaccines

1.3.1 History of influenza vaccines

Influenza vaccination has a storied history, marked with continual innovation to match the virus's ever-evolving nature. The first influenza vaccine was developed in the 1930s–1940s, following the discovery that the influenza virus could be amplified in embryonated chicken eggs[49]. The first vaccines were produced by purifying the virus using centrifugation and subsequent inactivation with formalin[50]. With the re-emergence of an H1N1 strain in 1977, the WHO recommended the administration of trivalent vaccines, containing H1N1, H3N2, and an influenza B virus[51]. The emergence of this strain also prompted the implementation of the first surveillance system. In the 1980s, two distinct lineages of influenza B emerged, necessitating the use of quadrivalent vaccines[52]. This recommendation continued until 2023, when the WHO once again recommended trivalent vaccines due to the disappearance of the B/Yamagata strain. The evolving nature

of influenza viruses and the dynamic history of influenza vaccines highlight the need to maintain adaptable immunization strategies to ensure effective disease prevention.

1.3.2 Flu vaccines in Canada

Currently in Canada, there are three types of licensed influenza vaccines: inactivated influenza vaccine (IIV), recombinant influenza vaccine (RIV), and live attenuated influenza vaccine (LAIV). Overall, IIVs are the most widely used influenza vaccines[53], with 9 of them authorized and available for use in Canada[54]. Higher dose and adjuvanted formats of IIVs are available for specific age groups to enhance immune responses. Fluad®, an IIV containing the oil-in-water adjuvant MF59, is recommended for children 6 to 23 months and adults 65 years of age and older. The RIV, Supemtek™, contains recombinant HAs produced in baculovirus-insect cell expression systems, which does not rely on egg supplies. Lastly, the LAIV, FluMist®, is available for people 2 to 59 years of age but not recommended for pregnant or immunocompromised individuals[54]. As the only intranasal (IN) vaccine, FluMist® utilizes cold-adapted viruses to elicit mucosal immunity. LAIV contains the HA and NA genes from the WHO recommended strains and six gene segments from a cold-adapted, attenuated virus[54–56]. Because of the temperature sensitivity, the virus only replicates in the cooler upper airway, but not in the warmer, lower respiratory tract.

The National Advisory Committee on Immunization recommends adults and children 9 years of age and older to receive a dose of influenza vaccine annually[57]. Children 6 months to 9 years of age who have never received an influenza vaccine should be given two doses in the current season. In Canada, influenza vaccination coverage was 42% in 2023-2024, similar to previous seasons. While vaccination coverage among

seniors was close to the goal of 80%, only 44% of the adults aged 18-64 years with chronic medical conditions received the flu shot[58]. Interestingly, the leading reason for not getting vaccinated was the perception that the vaccine was not needed[58].

1.3.3 Challenges of the current influenza vaccines

According to the Centers for Disease Control and Prevention (CDC), the effectiveness of seasonal influenza vaccines over the last decade ranged from 19 to 56%[59]. Similarly, the Canadian Sentinel Practitioner Surveillance Network estimated that the vaccine effectiveness (VE) is approximately 50% against H1N1, 32% against H3N2, and 63% against influenza B[56]. The evidence for VE of LAIV, the only IN influenza vaccine, is less conclusive. During the 2013-2016 influenza seasons, studies in the U.S. showed mixed results. VE from those studies ranged from 0-50%, leading to suspended recommendation of LAIV for the next two seasons[60–62]. Similar studies were conducted in other countries, including Canada, where the effectiveness ranged from 31-57.6%[63]. The VE for influenza is far below that of other viral respiratory diseases. For example, a single dose of the measles, mumps, and rubella vaccine is 93%, 78%, and 97% effective against the three respective viruses[64]. The low VE underscores the challenges faced by the current influenza immunization design. While a mucosal vaccine is available, the inconsistency in performance indicates that much remains to be understood and improved. There are many factors contributing to the low influenza VE, including mismatch between the predicted vaccine strain and the circulating strain, adaptation mutations induced during vaccine production, and immune imprinting[65].

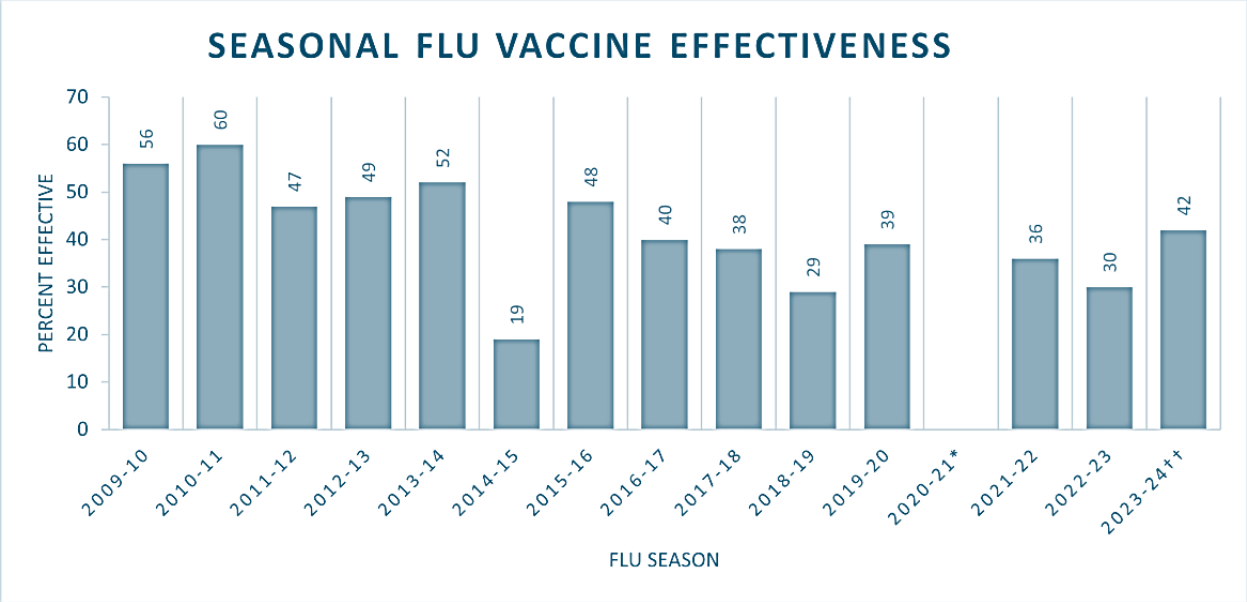


Figure 1.3 Seasonal influenza vaccine effectiveness.

Graph generated based on data from CDC[59]. * Not estimated due to low influenza virus circulation. †† VE estimates for 2024-2025 flu season are preliminary.

The current influenza vaccines take approximately six months to be manufactured, allowing time for an antigenically divergent virus to supersede the originally predicted strain. Lower VEs are consistently observed during those seasons. During the 2018-2019 influenza season, a publication by the CDC reported that the VE was less than 10% against H3N2 due to the circulation of a new strain during the latter part of the season[66]. Similarly, an antigenically drifted H1N1 strain emerged after January during the 2019-2020 season, leading to a 7% VE[67]. Those two seasons are among the many examples of this observation and the impact of constant viral evolution on immunization may not be always fully captured[65].

While viral evolution is a key challenge, many researchers have also raised their concern about our reliance on egg-based vaccine manufacturing. Since its conception 80 years ago, more than 80% of influenza vaccine production capacity still relies on

embryonated eggs[68]. This method remains popular due to its scalability, low production cost, and reliable safety and tolerability. However, there are several limitations with egg-based manufacturing. Firstly, the long manufacturing process makes it difficult to respond swiftly to strain changes or emergency situations[43]. During the 2009 H1N1 pandemic, IIVs were not available in time to prevent the second wave of the pandemic[69]. Secondly, the manufacturing of IIVs depends on the availability of embryonated eggs. This problem has been highlighted by the on-going H5N1 avian influenza pandemic. In the U.S. alone, over 174 million poultry were culled to control the spread of H5N1 since December 2021[25]. While many countries stockpile vaccines to circumvent this problem, there is a risk that pandemic strains may be genetically divergent enough to render stockpiles ineffective[43]. Lastly, “egg-adaptation” mutations acquired during the viral amplification can lead to changes in the antigenic structure of HA, inducing responses against the egg-adapted structure instead of the actual circulating viral strains[70]. Egg-adapted changes have the most impact on the immunogenicity of H3N2 strains[71] and mutations occurring near the surface of the receptor binding site could directly impact the induction of neutralizing antibodies[72].

Annual immunization against influenza is recommended due to short-lived vaccine-induced immune responses and the constant antigenic shift[73]. However, there are case studies suggesting that repeated immunization against influenza reduces VE, with the earliest observation dating back to the 1970s[74–78] . A Canadian study found that during the 2014-2015 influenza season, VE was significantly lower if patients had been vaccinated one year prior[75]. Nevertheless, it should be noted that although a reduced VE was observed, vaccination in two consecutive years still provides better protection than

not receiving the vaccine at all[79]. The immunological mechanism underlying this phenomenon is not fully understood, but it could be related to the concept of original antigenic sin (OAS) or immunological imprinting[80]. Instead of inducing *de novo* responses to the new vaccine strain, OAS causes cross-reactive antibodies that already exist to be boosted. These antibodies may be less effective or non-neutralizing against the circulating strain[81]. Most antibodies boosted by vaccination are pre-existing clonotypes, rendering newly elicited clones a smaller percentage[82].

Other factors such as the age of vaccinees[53], the lack of vaccine-induced cellular immune responses[83], and elimination of the virulence proteins by the host's antiviral immune defences[84] could also impact the overall VE of the current influenza vaccines. In summary, the limited and inconsistent VE of the current influenza vaccines reflects complex interplay between the evolution of the virus, the heterogeneity of the vaccinated population, and constraints in vaccine design and production. These multifaceted challenges illustrate the pressing need for innovative vaccine strategies that move beyond traditional platforms and aim to induce durable and broadly protective immunity.

1.4 Conserved Antigens as Targets for Universal Influenza Vaccines

1.4.1 The pursuit of universal protection

The development of a universal influenza vaccine has been and remains to be an active and pressing area of research. In 2017, the National Institute of Allergy and Infectious Diseases (NIAID) convened a workshop, where scientists and clinicians outlined the criteria for a universal influenza vaccine: be at least 75% effective; protect against group 1 and 2 IAVs, with influenza B as a secondary target; provide protection

that lasts at least one year; and be suitable for all ages[85]. Almost a decade later in 2025, the National Institutes for Health (NIH) announced a project entitled “Generation Gold Standard”, outlining the development of a universal influenza vaccine[86]. The vaccine is named BPL-1357, using a β-propiolactone (BPL)-inactivated, whole-virus platform made up of four strains of low-pathogenicity avian IAV subtypes, H1N9, H3N8, H5N1, and H7N3. In the published pre-clinical study, vaccinated mice and ferrets demonstrated protection against the 1918 H1N1 strain, HPAI H5N8 strain, and H7N9[87]. The IN formulation of the vaccine is currently in phase Ib and II/III clinical trials. Whether this experimental vaccine will come close to the criteria established in 2017 and meet the timeline of Food and Drug Administration (FDA) approval target by 2029 is unclear, given the limited data currently available.

1.4.2 Targeting conserved antigens

Universal influenza vaccine development is a global effort. There are over 200 vaccine candidates from more than 180 developers, and 40 of them are in clinical trials[88]. The primary strategy in designing a universal vaccine is to target conserved antigens that elicit broadly cross-reactive antibodies and/or T cell responses. Researchers have explored various options, including surface proteins such as NA, M2e, HA2, and internal proteins such as M1 and NP. Each antigen will be briefly reviewed in the context of vaccination, with greater emphasis placed on NP and HA2, the primary focuses of this thesis.

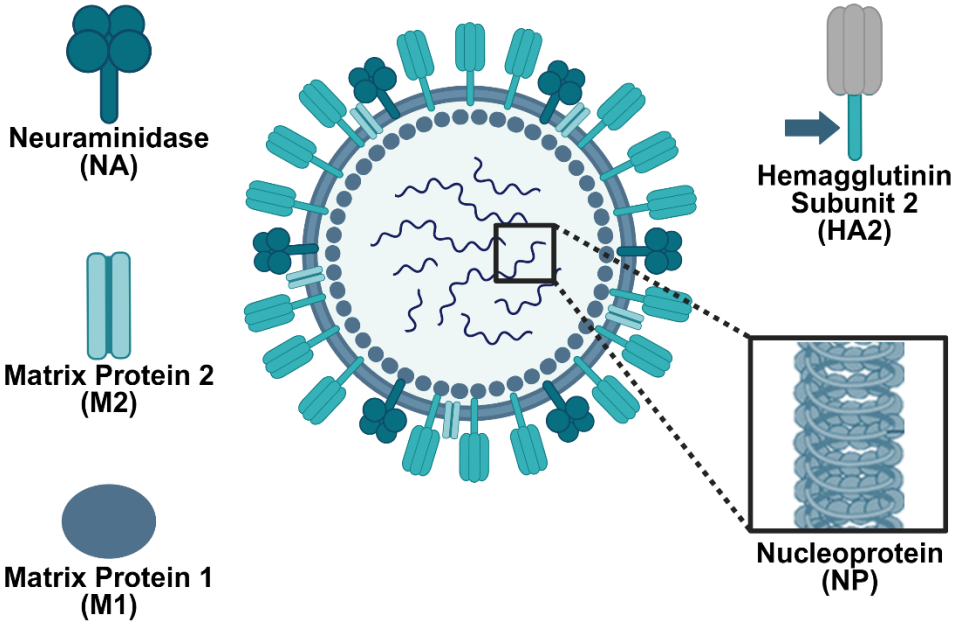


Figure 1.4 Conserved influenza virus antigens that elicit broadly cross-reactive antibodies and/or T cell responses.

NA, expressed on the surface of the viral envelope, is involved in both the entry and release of the virus. NA aids cell entry by cleaving decoy receptors in the mucus of the respiratory tract[36]. As newly formed virions are budding off from infected cells, NA cleaves sialic acids to prevent HA aggregating to the host cell membrane. NA has been an attractive target to induce broad immunity due to its slower genetic drift and its ability to induce cross-protective antibodies within a subtype. NA has been used for vaccination in the form of recombinant proteins, including as a polypeptide protein with conserved regions from other viral proteins[89], adjuvanted with CpG 1018[90], or as a multivalent computationally optimized broadly reactive antigen (COBRA)[91]. The effectiveness of NA has been demonstrated by many other studies[92–95].

M2 is another influenza surface protein, responsible for pumping protons into the virus upon activation by the acidic endosomal environment. The ectodomain of the protein,

M2e, is a popular candidate for universal vaccine designs[96]. M2e has been shown to be a reliable antigen in numerous preclinical studies[97–99]. In the 2000s, ACAM-FLU-A was tested clinically, a recombinant virus-like particle vaccine with Hepatitis B virus core as the carrier protein to present M2e[100]. Anti-M2e antibodies were detected in 90% of the participants, but there has been no further testing or development, perhaps due to the rapid decline of the antibodies[101]. Despite being clinically tested as early as the 2000s, most of the M2e based vaccines have not advanced into later stages of clinical testing[102] (NCT01181336, NCT01184976, NCT03789539). It should be noted that unlike antibodies against HA, antibodies against M2e are non-neutralizing and can still be infection permissive[103]. Therefore, M2e is often explored as an additional antigen in combination with other viral proteins or carriers to broaden or enhance the immune response.

On-going work has also focused on inducing cell-mediated immune responses against conserved internal proteins, such as the multifunctional M1. M1 is a structural component of the virion. It surrounds vRNPs and complexes with NEP. M1 also regulates other steps of the viral life cycle, such as vRNP trafficking and virion assembly, through protein-protein interactions[104]. Like other internal proteins, it is not under significant evolutionary pressure. In 2022, Evans *et al.* conducted a phase II clinical study on a Modified vaccinia Ankara (MVA) vaccine expressing NP and M1[105] . Although the vaccine was well tolerated and induced moderate T cell responses to the antigens, it did not boost the immune response of a standard quadrivalent inactivated influenza vaccine (QIV) immunization. The authors suggested that localized T cell responses would need to be enhanced by a different vaccine regimen or through IN vaccination. Other groups have developed broad-spectrum vaccines based on M1 and tested them in preclinical studies,

utilizing self-amplifying mRNA[106], recombinant protein platforms[107], or heterologous prime-boost strategies[108].

There are also vaccines targeting the conserved epitopes of multiple antigens. FLU-v is a peptide-based multiepitope vaccine derived from conserved regions of M1, M2, and NP[109]. FLU-v is administered subcutaneously with an oil-in-water adjuvant, Montanide ISA VG51. In a human challenge study, the vaccine protected against mild-to-moderate disease, defined as the presence of viral shedding and clinical symptoms (32.5% experienced mild-to-moderate disease in single-dose vaccinated versus 54.8% in placebo). The systemic cellular immune response induced by FLU-v recognized a wide range of influenza strains, including H1N1, H3N2, and H5N1[110]. Another poly-epitope vaccine, Multimeric-001, is a recombinant protein made up of conserved epitopes from M1, NP, and HA[111]. Although polyfunctional T cell responses were detected in peripheral blood mononuclear cells (PBMCs), the vaccine did not provide protection when used as a standalone vaccine. FP-01.1, a vaccine made up of 6 synthetic peptides from NP, M1, PB1, and PB2, was also tested clinically[112]. T cell responses were detected in 75% of vaccinated individuals, but no further clinical testing has been carried out.

Lastly, instead of using genetic sequences from a specific strain, computationally designed HA and NA have also been used for vaccination. COBRAs are made up of consensus sequences that target conserved regions. So far they have been tested in various vaccine platforms, including influenza virus-based[113], nucleic acid-based[114], recombinant proteins[115], virus-like particles[116], and virus-vectored[117]. In a recent study, incorporating COBRA HA and NA into LAIVs provided better protection in elderly ferrets[118]. Similarly, another vaccine that incorporated epitopes from recent circulating viruses into

various recombinant COBRA HAs, elicited antibodies against historical H3N2 strains[119]. While preclinical results are promising, it remains to be determined whether the broad reactivity will translate effectively to humans.

1.4.3 Nucleoprotein

The monomeric NP is 56 kDa, composed of a head domain, a body domain, and a flexible tail loop[120]. NP is the most abundant viral protein expressed in infected cells, accumulating in the nucleus in the early phase of infection before distributing throughout the cytoplasm during the later phase[121]. Despite being an internal protein, multiple studies have detected NP on the surface of infected cells[122–124], enabling FcγR-dependent protective immune mechanisms[125,126].

NP is a critical component of the vRNP complex. Recognized functions of NP include, but are not limited to, vRNA packing, nuclear trafficking, and vRNA transcription and replication[127]. Each influenza virion contains eight segments of vRNA, assembled as individual vRNPs, containing the viral RNA polymerase (PB1, PB2, and PA), NPs, and a single strand of vRNA. Within each vRNP, the vRNA wraps around the arginine-rich groove of multiple NP molecules. The NP molecules are linked to each other via salt bridges on the tail loop and the binding pocket on the body domain[120]. Within the vRNP complex, NP also directly interacts with the PB1 and PB2 subunits of the viral polymerase[128]. Because NP carries out critical and diverse functions, mutations within this protein could severely impair viral replication. Combined with the relatively low immunological pressure on internal proteins, this results in NP being highly conserved, making it an attractive antigen for universal influenza vaccines[127].

Hayward *et al.* found that in the unvaccinated population, about 20% of the individuals had H3N2 NP-specific T cells responses[129]. T cells isolated from these individuals showed strong cross-reactivity against NP from the 2009 pandemic H1N1. The presence of NP-specific T cells prior to the influenza season was correlated with fewer reported symptoms and reduced risk of infection. However, it should be noted that the overall T cell responses in most participants were low, if detectable at all. When administered as a standalone antigen in vaccines, NP confers limited protection. NP vaccines often require enhancement through the use of adjuvants[130,131]. One example is OVX836, a homo-heptameric recombinant NP vaccine currently undergoing clinical trials[132]. The vaccine contains seven copies of NP, each fused with a pro-immunogenic oligomerization domain. In a recently published phase II study, in addition to demonstrating significant humoral and cellular responses, the vaccine had a preliminary vaccine efficacy at around 84%[133].

There were two viral vector-based NP vaccines that went through clinical testing, but are no longer active. One was a heterologous vaccination regimen based on MVA and the chimpanzee adenovirus ChAdOx2, both expressing NP and M1[134]. T cell responses were maintained over 18 months. The second vaccine was also MVA-based, expressing NP and M1, administered intramuscularly (IM) and followed by an IN challenge with an H3N2 strain[135]. Although significant expansion of antigen-specific CD4+ and CD8+ T cells were detected in peripheral blood, it was not enough to reduce the nasopharyngeal viral load compared to the placebo group. The author suggested that a localized T cell response induced by mucosal delivery may provide better protection. This indicates that

peripheral T cell responses are not sufficient for optimal protection, emphasizing the need for a more robust mucosal immune activation.

1.4.4 Hemagglutinin subunit 2 (HA2)

HA is critical for viral entry into host cells and is a key determinant for host- and cell-tropism[136]. The globular head domain (HA1) contains the receptor binding site, which is the primary target for neutralizing antibodies. HA2, often referred to as the “stem” or “stalk” of HA, is the membrane-anchoring subunit essential for membrane fusion during viral entry. It contains an N-terminal fusion peptide, a long alpha-helix, and a transmembrane domain[137]. During viral entry, upon exposure to the acidic environment of the endosome, HA undergoes an irreversible conformational change. The HA2 subunit turns itself “inside out” into a trimer of hairpins, exposing the fusion peptide[138]. The fusion peptide inserts itself into the host endosomal membrane, allowing the viral genome to enter the host cell through a fusion pore. HA2 is highly conserved, with the potential to induce protective humoral and T cell responses. HA2-specific antibodies can provide protection through multiple mechanisms, including neutralization and antigen-dependent cellular cytotoxicity (ADCC)[139–141].

There are several strategies being investigated to target immune responses against the conserved HA2 domain[142]. One strategy, employed by the recombinant vaccine, G1 mHA, is to vaccinate with a headless HA, lacking the HA1 globular head domain[143]. Compared to a traditional trivalent influenza vaccine, G1 mHA induced significantly higher titers of stem-specific antibodies. In their latest publication, Swart *et al.* screened G1 mHA in combination with multiple adjuvants in non-human primates to further enhance the stem-specific immune responses[144]. The NIAID is also testing a self-

assembling HA2 ferritin nanoparticle (H1ssF). In a phase I dose-escalation trial, the vaccine induced homologous and heterologous binding antibodies[145]. Another strategy, chimeric HA (cHA), referring to the use of “exotic” HA head domains, which are typically from avian influenza strains that humans are not exposed to, and a conserved HA2 domain from current seasonal strains. Utilizing the principle of OAS, the anti-stem response can be boosted by additional vaccinations of cHA with a consistent stem and various head sequences[142]. In a recent phase I study, immunizing with a cHA-based inactivated influenza vaccine was found to boost T cell responses against HA2 and NP[141].

HA2 or epitopes of HA2 could be targeted alongside other viral antigens to enhance protection. For example, one of the nine conserved linear epitopes in Multimeric-001 was from HA2. This recombinant protein vaccine consisted of three copies of each epitope, and it was the first universal influenza vaccine to enter phase III clinical trial, but it did not proceed beyond that[146]. In another study, researchers used ChAdOx1 and MVA vectors expressing group 2 cHAs, NP and M1[147]. Compared to vaccines expressing only cHA or NP+M1, the combined approach showed superior protection.

While HA2 is a promising universal vaccine target, there is animal model evidence for the potential of vaccine-associated enhanced respiratory disease (VAERD). Mechanisms of VAERD are complex and not fully characterized, but it has been shown that some anti-HA2 antibodies could promote viral membrane fusion activity[148]. In a recent study, Kimble *et al.* vaccinated ferrets and pigs with a whole inactivated virus vaccine and both animal models showed elevated clinical signs post-challenge with high levels of HA2-binding antibody[149]. The complex interactions underscore the importance of characterizing the quality and functionality of HA2-specific immune responses in

preclinical and clinical studies. Furthermore, the majority of HA2 studies focus on B cell responses, and HA2-specific T responses are often studied in challenge models or in the presence of other antigens. Under those circumstances, immune responses may be directed towards less desired non-HA2 targets through epitope competition[150–152]. Evidently, in a recent clinical trial testing a cHA split-virion vaccine, low CD4+ and no CD8+ anti-HA2 T cell response was detected[153]. This further highlights the need to investigate HA2-specific T cell responses with particular focus on dissecting mucosal responses[154,155].

1.5 Adenoviral Vectors

1.5.1 Viral vector-based vaccines

Viral vector-based vaccines utilize modified viruses to deliver genetic material encoding antigen(s) from a target pathogen, stimulating immunity against the pathogen. Viral vectors naturally stimulate both innate and adaptive immune responses, eliciting robust humoral and T cell responses[156]. While conventional vaccines often require adjuvants to prime the innate immune system, viral vectors themselves can be recognized by a spectrum of pattern recognition receptors (PRRs) and serve as intrinsic adjuvants[157]. This initial activation of the innate immune system is crucial for driving the downstream adaptive immune responses. Many viral vectors can infect both immune cells and non-immune cells[158]. When non-immune cells such as muscle cells are infected, the expressed transgene fragment is captured by antigen-presenting cells (APCs), leading to the production of antibodies and T cell responses. When the transgene is expressed in immune cells such as dendritic cells (DC) and macrophages, cytotoxic T lymphocyte (CTL) responses are initiated via intracellular antigen presentation[159].

Adenovirus, a member of the family *Adenoviridae,* has garnered a lot of research interest as a vaccine delivery platform. This non-enveloped virus contains a linear double-stranded DNA genome encoding between 22 and 40 genes[160]. The virus is known for its icosahedral shape, measuring 80–100 nm in diameter. Human infection with wildtype adenoviruses is common and typically only causes mild flu-like symptoms, which can be managed with rest and over-the-counter pain relievers[161]. Adenovirus has several features that make it a desirable vector for prophylactic vaccine applications. Firstly, adenoviral vectors are non-integrating, thus the risk associated with genomic integration is avoided as the viral DNA remains extrachromosomal[162]. Secondly, the adenoviral vector genome is stable and can carry up to 36 kb of exogenous DNA[163]. Thirdly, they can be amplified to high titers in various cell lines, and the vectors are very stable and do not require ultra-cold storage[164]. Lastly, there is extensive research on the adenovirus vectors, and they display safe and effective profiles in numerous clinical trials[160,165].

Adenovirus vectors can be further modified to enhance safety and eliminate pathogenicity. The vector can be made replication-incompetent through the deletion of the viral gene E1, which encodes for proteins that activate viral transcription and manipulate host cell cycle to enhance viral DNA replication[166]. The viral genome E3 region, which encodes an immunomodulatory protein to downregulate the host immune response, is also often deleted to enhance safety and to accommodate larger transgene insertions[167].

1.5.2 Types of adenoviral vectors

Human adenoviruses exhibit substantial genetic and biological diversity, with more than 100 types having been identified. They are categorized into seven species (A–G),

and further subdivided by serotype[168,169]. Species C serotype 2 and 5 are most commonly used to make recombinant vectors[170]. Other serotypes, such as Ad26 from species D[171], Ad4 from species E and Ad7 from species B[172], have also been employed for vaccination. Ad26 was used in a single-dose Coronavirus Disease 2019 (COVID-19) vaccine developed by Janssen Vaccines, targeting the spike protein. The vaccine was granted full authorization by Health Canada in November 2021, although Janssen voluntarily withdrew its authorization in Canada in 2023, following reports of rare adverse effects[173,174]. The U.S. Food and Drug Administration licensed live, orally administered Ad4 and Ad7 vaccines in 2011 for use in military personnel against acute respiratory disease caused by these two adenovirus types[175]. It is worth highlighting that distinct serotypes of adenoviral vectors have very different biology and therefore various immunological potencies[176]. The precise determinants associated with high potency in certain serotypes of adenoviral vectors are still to be determined.

1.5.3 Application of adenoviral vector as an intranasal vaccine

Adenoviral vectors are well-suited for IN vaccination due to their natural tropism for the respiratory epithelium and their intrinsic adjuvanting properties which can enhance local immunity[176]. During the COVID-19 pandemic, the IN delivery route gained renewed research interest due to its potential in providing sterilizing immunity and reducing viral shedding. Two adenoviral vector IN vaccines received approval in India and China, BBV154 and Ad5-nCoV respectively. BBV154 utilized a chimpanzee adenoviral vector (ChAd36), encoding a perfusion-stabilized SARS-CoV-2 spike protein. In phase III of the clinical trial, BBV154 induced significant levels of circulating neutralizing antibodies and high levels of mucosal and circulating IgA[177]. Notably, no significant vaccine-induced T

cell responses were detected in PBMCs. It has been shown that IN vaccination predominantly induces local cellular immunity in the respiratory tract, which is difficult to assess by sampling peripheral immune cells in clinical studies, leading to the underestimation of cellular immune responses[178]. The other IN vaccine, Ad5-nCoV, initially developed as an IM vaccine, later received approval as an aerosolized formulation delivered via a nebuliser[179]. In their latest clinical trial, Ad5-nCoV was compared to IM administered BNT162b2 (Pfizer)[180]. BNT162b2 induced better IgG responses, but Ad5-nCoV had higher levels of IgA. Overall, the two vaccines showed similar protective efficacy with the Ad5-nCoV group reporting less adverse drug reactions. The IN administration of other adenoviral vector-based COVID vaccines were also tested but yielded mixed results. ChAdOx1 nCoV-19, developed by AstraZeneca and approved for IM use, did not have a clear boosting effect when administered IN. Researchers observed infection in 7/42 participants, suggesting suboptimal vaccine efficacy[181]. Similarly, in another clinical trial conducted by Altimmune Inc. to evaluate the safety and immunogenicity of AdCOVID, another Ad5-based vaccine, no adequate antiviral immunity was detected[182]. This led the company to discontinue any further development[183]. The inconsistency and the lack of in-depth comparative investigations highlight the knowledge gap in utilizing adenoviral vectors as a vaccine platform.

While there is no licensed adenoviral vector IN vaccines against influenza, several have undergone clinical testing. Matsuda *et al.* tested a replication-competent Ad4 vector encoding H5 HA[184]. Ad4-H5-Vtn was administered as an oral capsule or via tonsillar swab or nasal spray. Better responses were observed in the tonsillar and IN delivery, with neutralizing antibodies remaining stable up to week 26. Although the vaccine also

significantly increased H5-specific CD4+ and CD8+ T cells, there was no follow-up study after the conclusion of phase I. In the same year, another company published the results of their phase II study of an IN Ad5 vaccine, NasoVAX, designed to express H1 HA[185]. The vaccine induced mucosal antibodies, along with HA-specific T cell responses in PBMC samples. Despite the authors concluding that the vaccine demonstrated a safe and immunogenic profile, there was no further clinical testing of this vaccine. Interestingly, this study demonstrated that IN administration elicited comparable immune responses in participants regardless of their pre-existing serostatus against the vector.

1.5.4 Challenges in adenoviral vector-based vaccines

While it is one of the most popular viral vectors, adenoviral vaccines still require further optimizations to enhance their safety and efficacy. The last time an adenoviral vaccine was used for mass-immunization was from 1971 to 1999, when a replicating Ad4 was orally administered to more than 10 million military members to prevent acute infection caused by this specific adenovirus type[186]. The COVID-19 pandemic led to the approval of some adenoviral vector-based vaccines, such as Janssen's Ad26.COV2.S, Oxford-AstraZeneca's ChAdOx1 nCoV-19, and CanSino's Ad5-nCOV. Over 23 million doses of ChAdOx1[187] and more than 50 million doses of Ad26.COV2.S were administered worldwide[188]. The massive number of global administrations revealed a rare but serious complication, vaccine-induced immune thrombotic thrombocytopenia (VITT), also known as thrombosis with thrombocytopenia syndrome (TTS). The reported rates of VITT range from 1 case per 26,500 to 127,3000 after the first dose of ChAdOx1 nCoV-19 and 1 case per 518,181 after the second dose. For Ad26.COV2.S, it was estimated to be 1 case per 263,000 doses administered[189]. Although the pathogenesis of VITT and its link to

adenoviral vectors remains under investigation, high levels of autoantibodies against platelet factor 4 (PF4) often coincided with the complication[190]. These antibodies can form platelet-activating immune complexes, leading to clotting[191]. The consensus in the field is that given the rareness of VITT, the benefit-risk ratio for preventing severe COVID-19 is still favorable. Nonetheless, the risk of VITT highlights how further research refinements are needed for adenoviral vector-based vaccines[192].

Another commonly discussed challenge for adenovirus-based vaccines is anti-vector immunity. The global seroprevalence of Ad5 and Ad26 is generally high, with approximately 70% and 44% of the population having antibodies against them respectively[193]. Pre-existing neutralizing antibodies may limit the effectiveness of adenoviral vector vaccines. A common approach to avoid this issue that is already in application is utilizing alternative serotypes. For example, the ChAdOx1 nCoV-19 utilizes a chimpanzee adenovirus, which does not commonly infect the human population[194]。 Other approaches include inducing immunity through mucosal delivery[195], genetically or chemically modifying the capsid to modulate vector-host interaction[196], and employing mix-and-match heterologous immunization regimens[197,198].

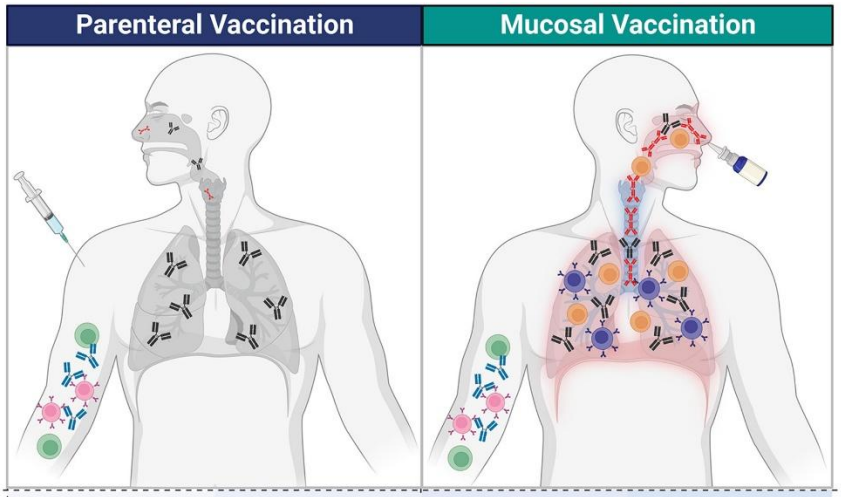
1.6 Mucosal Immunity

1.6.1 The central role of mucosal immunity in respiratory defense

The mucosal surface is approximately 200 times larger than that of the skin[199], with the lungs being the body's largest interface with the outside environment[200]. The nasopharynx-associated lymphoid tissue (NALT), located on the soft palate at the entrance to the nasopharyngeal duct in mice, is the first line of defense against inhaled pathogens in the upper respiratory tract (URT)[201]. B cells typically comprise 45-70% of

the lymphocyte population within the NALT, depending on the mouse strain[202] Upon antigen stimulation, the NALT size increases two-fold[203]. The NALT in the mouse model is considered analogous to Waldeyer's ring in humans, a series of tonsils including palatine, nasopharyngeal, and lingual[204]. Similarly to NALT in mice, most of the tonsil lymphocytes are also B cells. The T cell population includes follicular T helper cells, enhancing B cell proliferation and differentiation[205]. In a recent publication, Massoni-Badosa *et al.* characterized the proteome, epigenome, and single-cell transcriptome of human tonsils[206]. The authors identified 121 cell types and states, demonstrating the complexity and heterogeneity of this secondary lymphoid organ. The authors observed stepwise maturation of naive B cells toward the germinal center state and multiple states of plasma cell differentiation. Furthermore, they observed reduced abundance of naïve and central memory T cells in older adults, providing additional evidence of age-related immunosenescence.

The majority of vaccine trials in humans have traditionally sampled cellular responses by collecting PBMCs. With the growing recognition that mucosal and systemic immune systems encompass distinct immune populations, it is essential to devote greater effort to investigating immune mechanisms at mucosal sites[207,208].



	Parenteral Vaccination		Mucosal Vaccination		
Circulating antibody		Circulating		Circulating	Systemic Immunity
Memory B Cells		Circulating		Circulating	
Anti-viral T cells		Circulating		Circulating	
Mucosal IgA		URT		URT/LRT	Mucosal Immunity
Tissue-resident memory T cells				URT/LRT	
Tissue-resident memory B cells				LRT	
Mucosal IgG		URT/LRT		URT/LRT	

Figure 1.5 Differential immune outcomes of parenteral and mucosal vaccines.

1.6.2 Strategies to enhance mucosal immunity

Prime-and-pull, or heterologous, immunization strategies use a parenteral prime followed by a mucosal "pull" stimulus to draw activated immune cells into the respiratory tract. During the COVID-19 pandemic, Lapuente *et al.* explored this vaccination strategy by IN administering Ad5 or Ad19 vectored vaccines following a systemic plasmid DNA or mRNA prime[210]. Compared to two IM immunizations, the heterologous regimen induced higher levels of mucosal IgA and T_{RM} cells, with enhanced mucosal neutralization. Leveraging the prime-and-pull strategy to combine the well-established seasonal IM influenza vaccines with IN adenoviral vectors presents a compelling future direction to improve protective efficacy.

Researchers are also targeting niche immune cell populations unique to the mucosal environment. Mucosal-associated invariant T (MAIT) cells were first named in 2003, with their receptor and ligands characterized in 2010. Unlike other T cells, they express invariant T cell receptors, which allow them to function both like an adaptive and an innate immune cell, helping to bridge the two systems[211]. In a study published by Pankhurst and colleagues in 2023, they intranasally delivered recombinant antigen in combination with with 5-A-RU (5-amino-6-D-ribitylaminouracil) and methylglyoxal, which spontaneously form the potent MAIT cell ligand 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil)[212]. They found that this combination led to the induction of both serum and mucosal antibody responses. As sampling techniques and single-cell multi-omics technologies continue to advance, previously unrecognized immune cell

populations are being identified within the mucosal environment, which can be novel adjuvantation targets to enhance mucosal immunity.

1.6.3 Immunoglobulin A

Immunoglobulin A (IgA) is the predominant antibody isotype in the mucosa, playing a key role in protecting against a variety of respiratory pathogens[213]. IgA is especially prominent in the URT, with an IgG:IgA ratio of 1:3[214]. The ratio in the lower respiratory tract (LRT) more closely resembles that of the serum, at around 2.5:1. In a study conducted by Renegar *et al.*, the researchers compared the protective effects following intravenous administration of anti-influenza IgG or IgA in mice[215]. While they found that the passive transfer of IgA was able to prevent URT pathology and reduce viral shedding, it did not prevent pathology in the LRT. Conversely, administration of IgG prevented LRT pathology but had minimal to no effect on URT viral burden or pathology. These differing outcomes highlight the distinct role each antibody isotype plays during infection.

IgA exists in monomeric and polymeric, mostly dimeric, forms, with the monomeric IgA circulating systemically and dimeric IgA being produced at the mucosal surfaces. Unlike IgG, IgA can be transported through epithelial cells efficiently via polymeric immunoglobulin receptors[216]. Furthermore, IgA can engage with Fc alpha receptors on myeloid cells, inducing antiviral functions such as neutrophil extracellular trap (NET) programmed cell-death, phagocytosis, and the production of chemoattractants[217,218]. IgA also has greater potential to prevent IAV infection than IgGs[219]. The multivalency of IgA can increase avidity, providing better heterosubtypic immunity when affinity decreases. In addition to neutralization, non-neutralizing cross-reactive polymeric secretory IgA has been shown to be more efficient at inhibiting virus particle release and plaque formation

than IgG[220]. In a study from the 1990s, Mazanec *et al.* showed that IgA, but not IgG, could form intracellular interactions with viral antigens, thereby reducing infection[221]. The protective efficacy of IgA also extends to other respiratory viruses. For example, in a study assessing mild versus severe COVID-19 cases, some healthy participants had no serum antibodies against SARS-CoV-2, but had neutralizing IgA in mucosal fluids[222]. IgA plays a multifaceted role at the mucosal surface at the URT, mediating heterosubtypic protection against respiratory pathogens like influenza.

1.6.4 Tissue-resident memory cells

Tissue-resident memory (T_{RM}) cells are a specialized subset of memory cells that reside in non-lymphoid tissues without re-entering circulation. These cells are adapted to their local environment, and upon an infection, T_{RM} that reside in the respiratory tract provide rapid and localized immunity[223]. T_{RM} cells express signature adhesion surface markers, such as CD103 and CD69. CD103 binds E-cadherin on epithelial cells to support tissue retention, while CD69 suppresses the activity of sphingosine-1-phosphate receptor 1 (S1PR1) to limit tissue egression[224]. In an experimental human influenza challenge study, virus-specific CD8+ T cells detected in bronchoalveolar lavage fluid (BALF) displayed classic T_{RM} cell markers[225]. In addition, antigen-specific CD8+ T cells in PBMC and BALF showed distinct kinetics, phenotypes, and functional characteristics. This suggests that although cellular immunity responses in PBMCs could be linked to protection, they poorly represent the responses in the respiratory tract that directly eliminate viruses during early infection.

In a landmark paper by Wu *et al.*, the rate of viral clearance after reinfection was found to be closely associated with the presence of T_{RM} cells in the airway[226]. Despite a

large population of influenza-specific T cells in circulation, the loss of T_{RM} about 7 months post-infection led to the waning of heterosubtypic immunity. Slütter and colleagues found that the T_{RM} population exists in an equilibrium between apoptosis and reseeding from the circulatory population[227]. Over time the capacity to replenish T_{RM} cells is lost, leading to progressive loss of heterosubtypic protection. Lung T_{RM} cells are relatively short-lived compared to T_{RM} cells in other tissues, such as the skin. Given the central role of mucosal T_{RM} cells in providing protection against respiratory viruses, various vaccine strategies have been developed to optimize their induction and persistence. Uddbäck *et al.* showed that CD8+ T_{RM} cells in the lungs can be maintained for at least one year following IN vaccination with an adenovirus expressing influenza NP[228]. This maintenance was not observed following natural infection or non-respiratory vaccination. Parabiosis experiments showed that the lung T_{RM} cells were continuously replenished by circulating memory CD8+ T cells, drawn to the respiratory tract by persistent antigen expression.

CD8+ T_{RM} cell differentiation and retention within the lungs is reinforced by APCs, such as DCs[229]. Researchers have identified adjuvants that could activate such APCs, which in turn induce potent T_{RM} responses and enhance mucosal immunity. Co-delivery of strong adjuvants can activate local DCs, fostering an environment conducive for T_{RM} cell maintenance. Wang *et al.* reasoned that a stimulator of interferon genes (STING) agonist, 2′,3′-cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), could serve this purpose[230]. The adjuvant was delivered by a pulmonary surfactant–biomimetic liposome, along with IN delivered inactivated, split-virion influenza vaccine. A single dose of the adjuvanted vaccine demonstrated enhanced cross-subtype protection in both mice and ferrets, concurrent with durable lung CD8+ T_{RM} cells. Another adjuvant

NexaVant, a dsRNA toll-like receptor (TLR) 3 agonist, was also tested in combination with a commercial QIV[231]. The vaccine-induced pulmonary T_{RM} cells provided heterosubtypic protection in both mouse and ferret models. Interestingly, when the same formulation was administered IM instead of IN, it did not provide cross-protection despite inducing similar levels of splenic CD4+ T cell responses, again highlighting the importance of local responses.

1.7 Rationale, Hypothesis, and Objectives

1.7.1 Rationale

Seasonal influenza remains a persistent global health burden and a pandemic threat, with a mutation rate over 20-fold higher than that of the SARS-CoV-2 virus[39]. Although currently licensed influenza vaccines are widely accepted and available, they have several limitations. Vaccine-induced immune responses predominantly target surface antigens, relying on neutralizing antibodies. While effective against strain-matched viruses, this strategy provides limited efficacy against mismatched and novel zoonotic strains. The parenteral delivery of these vaccines also fails to elicit significant mucosal immune responses, which are essential to limit early infection and reduce transmission[232,233]. To offer broad protection against emerging influenza strains, next-generation vaccines need to elicit immunity within the respiratory mucosa and target more conserved antigens.

As one of the most conserved influenza proteins, the NP has emerged as a particularly attractive vaccine target. Due to its sequence conservation across various subtypes, NP can induce cross-reactive immune responses[234]. Over the last several decades, clinical and pre-clinical studies have explored the potential of NP-induced

immunity but have yet to give rise to an approved product. In a recent clinical study, an IM MVA-vectored NP vaccine successfully induced antigen-specific T cell responses in the peripheral blood but failed to lower the nasopharyngeal viral load upon infection[135]. This highlights the necessity of understanding and improving NP-induced mucosal immunity. An in-depth study that compares the mechanisms of protection elicited by the two routes of administration is of great need, particularly in delineating the importance of systemic and mucosal immunity.

Another conserved antigen of interest is HA2. Unlike the variable HA1 head, the HA2 stem is relatively more conserved, especially within influenza subtypes. Several vaccine design strategies targeting the stem region have been tested clinically, including chimeric HA, “headless” HA, computationally derived immunogens, and multi-epitope recombinant vaccines[235]. Among the seasonal influenza virus subtypes, H3N2 warrants particular attention due to its association with higher morbidity and mortality and historically inferior vaccine efficacy. To target the H3 subtype, a vaccine was designed based on the consensus HA2 sequence from H3 strains, a strategy that previously demonstrated success against H1 and influenza B viruses[236,237]. Most previous publications have focused on humoral responses, leaving the contribution of HA2-specific T cell responses poorly defined. Consequently, key immunodominant H3 HA2 T cell epitopes remain to be identified.

Recognizing these knowledge gaps, this thesis aims to investigate how IN vaccination targeting conserved antigens, such as NP and HA2, influences both mucosal and systemic immune responses. By directly comparing IN and IM immunization and dissecting the immunological correlates of protection, this work seeks to inform the design

of universal influenza vaccines capable of providing broad and robust protection by leveraging mucosal immunity.

1.7.2 Hypothesis

I hypothesize that intranasal administration of conserved influenza antigens will confer superior and broader protection relative to intramuscular delivery. By characterizing both humoral and cell-mediated immune responses and examining local and circulating immunity in parallel, these findings will inform the design and improvement of next-generation vaccination strategies against influenza viruses.

1.7.3 Objectives

1. Investigate intranasal administration as an alternative route to enhance cross-subtype protection conferred by conserved influenza antigen vaccines
2. Dissect the local mucosal responses elicited by intranasal vaccination, encompassing both humoral and cell-mediated immunity

2 Dissecting Immunological Mechanisms Underlying Influenza Viral Nucleoprotein-induced Mucosal Immunity Against Diverse Viral Strains

Preface: This chapter has been previously published as a research article.

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2.1 Abstract

The nucleoprotein (NP) of type A influenza virus (IAV) is highly conserved across all virus strains, making it an attractive candidate antigen for universal vaccines. While various studies have explored NP-induced mucosal immunity, here we interrogated the mechanistic differences between intramuscular (IM) and intranasal (IN) delivery of a recombinant adenovirus carrying NP fused with a bifunctional CD40 ligand. Despite being less effective than IM delivery in inducing systemic cellular immune responses and antibody-dependent cellular cytotoxicity (ADCC), IN immunization elicited superior antigen-specific recall humoral and cellular response in the nasal associated lymphoid tissue (NALT) of the upper respiratory tract, the initial site of immune recognition and elimination of inhaled pathogens. IN vaccination also induced significantly stronger pulmonary T cell responses in the lower respiratory tract than IM vaccination, in particular the CD8 T cells. Moreover, blocking lymphocyte circulation abrogated IM but not IN immunization induced protection, illustrating the critical role of local memory immune response upon viral infection. Notably, the CD40-targeted nasal delivery not only improved the magnitude but also the breadth of protection, including against lethal challenge with a newly isolated highly pathogenic avian H5N1 strain. These findings are informative for the design of universal mucosal vaccines, where the predominant mode of protection is independent of neutralizing antibodies.

2.2 Introduction

Influenza is a highly contagious respiratory virus that has caused more than six distinct pandemics and several epidemics in the past century. Every year, seasonal flu causes an estimated one billion infections and results in 290,000 to 650,000 deaths

worldwide [1]. The year-to-year efficacy of licensed vaccines is highly variable, ranging from 19% to 60% [2]. Influenza vaccine efficacy is affected by a wide range of factors, such as mismatches between predicted vaccine strains and the circulating strains, a problem exacerbated by the wide range of animal reservoirs that can lead to reassortment [3]. With the ongoing panzootic event caused by the highly pathogenic avian influenza (HPAI) and the rising number of cases in the recent flu seasons, there is an urgent need to understand and develop universal influenza vaccines that induce broadly protective immunity at the mucosal surface [2,4].

Despite emerging evidence showing enhanced vaccine efficacy via intranasal (IN) administration, routine influenza vaccination remains intramuscular (IM). The only available IN influenza vaccine, live attenuated influenza vaccine (LAIV), is less effective in adults than children and is only recommended for healthy populations aged 2 to 59 [5–7]. Furthermore, human studies rarely evaluate the underlying mechanisms of protection induced by IN vaccination due to technical limitations of tissue sampling at mucosal sites.

While the current influenza vaccines mainly target surface proteins, such as hemagglutinin (HA), the nucleoprotein (NP) remains another desirable antigen due to its abundance in infected cells and its highly conserved sequence. Unlike the surface antigens, which are under constant selective pressure exerted by neutralizing antibodies, NP is highly conserved across all influenza A subtypes. This high conservation is also due to the critical and multifunctional role of NP in RNA packaging, nuclear trafficking, and viral RNA transcription and replication [8–10]. Over the last several decades, extensive studies have explored the potential of NP as a vaccine antigen, yielding mixed results. Specifically, NP has been studied in various platforms, such as viral-vectored vaccines

[11–15], protein subunit or peptide vaccines [16–20] and mRNA vaccines [21–25]. Additionally, various T cell-based influenza vaccines were tested in clinical trials, including self-assembling nanoparticles targeting the NP [26], peptide-based constructs targeting multiple epitopes [27–29], and a Modified vaccinia Ankara (MVA)–vectored construct expressing NP and M1 [30]. The parenteral administration of these vaccines successfully induced significant antigen-specific T cell responses in peripheral blood. However, in a controlled human infection challenge model testing the MVA-vectored vaccine, the immunization had no effect on the nasopharyngeal viral load upon infection. This suggests that the peripheral T cells may not be sufficient to provide effective protection [30], which supports observations from our study. Better induction of mucosal immunity would be desirable, highlighting the necessity of understanding the mechanism of IN vaccination [31,32]. Notably, several influenza vaccine studies have demonstrated that IN administration provides better protection than IM by generating strong immune responses in the lungs [11,33–38]. Yet, more in-depth studies are needed to compare the immune responses elicited by the two routes of administration, particularly at addressing both systemic and mucosal immunities.

In this study we aim to examine the IN and IM administration of a CD40 ligand (CD40L)-adjuvanted NP vaccine. CD40L has been employed by various labs as an adjuvant, demonstrating robust protection [39–43]. It simultaneously acts as a targeting ligand to promote uptake by antigen-presenting cells (APCs) and as a molecular adjuvant that stimulates APC activation [44]. Therefore, the ectodomain of mouse CD40L was fused to the NP sequence to enhance immunogenicity and to sustain an effective immune response. Thus, in this study we used the vaccine construct, Ad-NP-CD40L, to interrogate

the differences in protective mechanisms elicited by IN and IM administration. The comparison focused on less-studied aspects, such as characterization of systemic and mucosal immunities and the memory responses in the nasal-associated lymphoid tissue (NALT), especially in the context of eliciting cross-subtype protection upon exposure to diverse strains of viruses, including a newly isolated highly pathogenic avian influenza (HPAI) H5N1.

2.3 Method

2.3.1 Mice

Six-week-old female BALB/c mice (Charles River) were used for all animal experiments. All animal procedures were performed in accordance with institutional guidelines and ethical approval was granted by the Animal Care Committee at Health Canada, Ottawa, ON, Canada and the Public Health Agency of Canada, Winnipeg, MB, Canada. Animal experiments were performed under Animal Utilization Protocol (AUP) H21-019, 2021-011, 2022-007, and 2023-004.

2.3.2 Generation of rAds

Recombinant adenoviruses constructs (rAds) were generated as previously described [33]. In brief, the Ad-NP-CD40L construct was designed to express a trimeric, secreted form of influenza A/duck/Yokohama/aq10/03 (H5N1) NP (GenBank accession #AB212281), with 23 amino acids from the human tyrosinase signal peptide (GenBank accession # AH003020) at the N terminus, fused to a 27 amino acid fragment from the bacteriophage T4 fibritin trimerization motif connected to the ectodomain of mouse CD40L (GenBank accession #NM_011616, aa 117–260) (Supp Fig 2.8). Empty vector control was used as controls. rAds were generated using AdenoVator Adenoviral Expression

System with pAdenoVator-CMV5 (Cuo)-IRES-GFP transfer vector (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions. Cloning was confirmed by DNA sequencing and restriction enzyme digestion. For vaccination, the rAds constructs were amplified in HEK-293A cells and purified by ultracentrifugation with a 30% sucrose cushion. rAd stocks were titrated using the Adeno-X Rapid Titer Kit (Takara Bio USA Inc.).

2.3.3 Weight loss and survival studies

Mice were immunized intranasally or intramuscularly with 10^9 PFU of each rAd construct in 25 µl or 50 µl, respectively. Mice were prime immunized on day 0 and boosted on day 28. Four weeks post-boost vaccination, mice were challenged intranasally with 1000 PFU of the A/Netherlands/602/09 (H1N1), 3.85 × 105 PFU of A/Hong Kong/01/68(H3N2), or 10 PFU of A/ RT.Hawk/ON/2022 (H5N1) influenza virus in 25 µl. The mice were weighed and monitored for signs of illness for 14 days post-challenge. A separate group of mice were sacrificed at the peak of illness for viral load determination. Five days post-challenge for A/Netherlands/602/09 (H1N1), 3 days for A/Hong Kong/01/68(H3N2), and 4 days for A/ RT.Hawk/ON/2022 (H5N1); necropsy days determined by previous preliminary challenge experiments. Lung and nasal turbinates were collected from 4 or 5 mice per group for viral load determination.

2.3.4 Tissue collection

Serum was collected from vaccinated mice 21 days after prime vaccination (Day 21) and 21 days after boost (Day 49) for measurement of antibody levels and antibody-dependent cellular cytotoxicity. Bronchoalveolar lavage fluid (BALF) was collected on 4 weeks post-boost (Day 56) for mucosal antibodies analysis. Spleens were collected 4 weeks post-boost (Day 56) for systemic cytokine level measurement. Nasal-associated

lymphoid tissue (NALT) was collected on Day 61, 5 days after an intranasal challenge of 1000 PFU of influenza A/Netherlands/602/09 (H1N1). The tissue was isolated and cultured as previously described [45]. Briefly, after removing NALT from the upper palate of the sacrificed mouse, it was successively washed eight times in RPMI 1640 with 10% FBS. The NALT was then transferred into a new 48-well plate containing the same media and cultured in a 5% CO2 incubator at 37 °C for 24 hours. The supernatant was collected to determine mucosal antibody and cytokine levels.

2.3.5 Viral titration

The plaque assay was performed for the H1N1 challenge as described previously [33]. Briefly, the lungs and nasal tissues were harvested 5 days post challenge and flash frozen in liquid nitrogen. Following thawing on ice, lungs and nasal tissues were homogenized with a pestle in 300µl and 250µl of PBS, respectively. After centrifugation and filtration with a 0.45um syringe filter, ten-fold serial dilutions of the homogenates were prepared in serum-free complete DMEM medium supplemented with 25mM HEPES buffer, 0.2%BSA and 2 µg/ml TPCK-treated trypsin. The homogenate inoculums incubated on confluent MDCK cells for 2 hours, at 37°C. After removing the inoculum, cells were washed and overlaid with complete DMEM medium containing 25mM HEPES buffer, 0.2% BSA, 2 µg/ml TPCK-treated trypsin and 0.8% agarose. After incubation for 4 days at 37°C/5% CO2, the cell monolayers were stained with crystal violet for plaque counting.

TCID50 assays were performed for H3N2 and H5N1 challenges as previously described [46]. Lungs and nasal turbinates were weighed and homogenized in 1 mL of MEM–0.1% BSA–L-Glu–2 penicillin-streptomycin (PS) with a 5-mm stainless steel bead

using a Bead Ruptor Elite tissue homogenizer (Omni). Cell debris was removed by centrifugation, leaving the supernatant for viral load detection. Samples were serially diluted (1:10) in MEM supplemented with 0.1% BSA, L-Glu, and TPCK-trypsin (MEM–BSA–L-Glu–trypsin). 100 µL of each dilution was added to the wells of a 96-well plate of MDCK cells in triplicate, followed by incubation at 37°C with 5% CO2 for 4 to 5 days. The presence of cytopathic effect (CPE) was observed and the TCID50 titer per milliliter or gram of tissue was determined using the Reed-Muench method [47].

2.3.6 Antibody detection by enzyme-linked immunosorbent assay (ELISA)

The end-point titers of serum, NALT supernatant, and BALF anti-NP antibodies were determined as described previously, with minor adjustments [25]. Briefly, 96-well plates were coated with 100 µl /well of 0.5 µg/ml of recombinant influenza A H1N1 (A/California/07/2009) nucleoprotein (Sino Biological Inc., 40205-V08B). Overnight incubation at 4°C was followed with PBS wash containing 0.05% Tween 20 (PBS-0.05T) and blocked for 2 hours at 37°C with 3% BSA in PBS-0.05T. After another wash, two-fold serial dilutions of the serum, NALT supernatant, or BALF in blocking buffer were added for 1 hour at 37°C. Following a wash, antibody binding was detected by HRP-conjugated anti-mouse IgG (Cytiva), anti-mouse IgG1, IgG2a, IgG2b (Jackson Immunoresearch Laboratories) or anti-mouse IgA (Life Technologies). The plates were developed by adding tetramethylbenzidine (TMB) substrate (Cell Signaling Technology) and the reaction was stopped by addition of 0.16M sulfuric acid. The absorbance recorded at 450 nm (OD450) and the end-point antibody titers were expressed as the reciprocals of the final detectable dilution. The cut-off was defined as the mean of control samples plus 3 standard deviations.

2.3.7 Antibody-dependent cellular cytotoxicity (ADCC) assay

The ADCC activity of the serum antibodies was measured with the Promega ADCC Reporter Bioassay, according to the manufacturer's instructions. Briefly, 50,000 MDCK cells were seeded into the wells of a white clear-bottom 96-well plate and grown overnight. Cells were then infected with 5 MOI of influenza A/Netherlands/602/09 (H1N1) for 24 h. Serum samples were heat-inactivated for 30 minutes at 56°C, serially diluted, and added to the infected cells. Mouse FcγRIV effector cells (Promega) were then added at 100,000 cells/well. After incubation at 37°C/5% CO2 for 5 hours, Bio-Glo™ luciferase assay substrate (Promega) was then added. Luminescence values were read in relative luminescence units (RLU) after 5 minutes. ADCC activity is expressed as fold induction, relative to a 'no antibody' control.

2.3.8 Multiplex ELISA

Spleens were collected from vaccinated animals and stimulated with 5 μg/ml of each of the selected peptides (TYQRTRALV and ASNENMETM) after homogenization [48]. Following a 48-hour incubation, the supernatant was collected for downstream measurements. NALT was collected according to the protocol described in previous sections and cultured ex vivo. The supernatant was collected for downstream measurements. Cytokine secretion in the supernatants from both tissues were measured using a ProcartaPlex Multiplex Immunoassay kit (Life Technologies). The plates were read on a Luminex 200 system (MilliporeSigma). Data analysis was performed using MILLIPLEX Analyst version 5.1 software for determining pg/ml of each cytokine.

2.3.9 Proliferation assay

The proliferation assay was performed as described previously [49], with modifications to adapt to mouse lung samples. Briefly, the lungs were collected and digested into a single-cell suspension with a lung dissociation kit according to manufacturer's instructions (Miltenyi Biotec, 130-095-927). After passing each sample through a 70 µm cell strainer, the cells were labeled using the CellTrace™ Violet Cell Proliferation Kit (Invitrogen, C34571) at a concentration of 1.0×10^6 cells/mL for 20 min at room temperature, protected from light. We then added five times the original staining volume of RPMI medium (10% FBS) to the cells for 5 min at RT to quench unbound dyes. Cells were then cultured at a concentration of 250,000 cells per well in the presence of 1 µg/mL NP peptide pool (TYQRTRALV and ASNENMETM). Stimulation with an equal concentration of DMSO in PBS was performed as a negative control. After incubation at 37 °C, 5% CO2, and 95% humidity for 72 hours, cells were washed and stained with Live/dead dye (ThermoFisher L34971), anti-CD3 (BD 564010), anti-CD4 (BD 553051), and anti-CD8 (BD 553030) for analysis by flow cytometry on FACSymphoney A1.

2.3.10 FTY720 administration and lung flow cytometry

FTY720 (Sigma SML0700) was dissolved in 0.9% NaCl and administered intraperitoneally (i.p.) daily at 1 mg/kg body weight, starting three days before challenge, and continued for five more days after challenge till necropsy. An equal volume of PBS was administered as control.

To validate the FTY720 treatment, whole blood was collected in EDTA blood tubes (BD 0265732) at necropsy. Red blood cells (RBC) were lysed with RBC lysis buffer (ThermoFisher 501129743) and quenched with RPMI (10% FBS) according to

manufacturer's instructions. After washing with media, the cells were stained with Live/dead dye (ThermoFisher L34971), anti-CD45 (BD 561874), anti-CD3 (BD 563123), anti-CD4 (BD 552775), and anti-CD8 (BD 566409). The excess antibodies were washed away with FACS buffer and cells were fixed with fixation buffer (BD 554655) for 30 minutes at 4°C prior to analysis by flow cytometry (FACSymphoney A1) the next day.

At necropsy, the lungs were collected and digested into a single-cell suspension with a lung dissociation kit according to manufacturer's instructions (Miltenyi Biotec, 130-095-927). After passing each sample through a 70 μm cell strainer and washed with PBS, the cells were stained with Live/dead dye (ThermoFisher L34971), anti-CD45 (BD 561874), anti-CD4 (BD 552775), anti-CD8 (BD 566409), anti-CD69 (562920), and anti-CD103 (BD 565529). The excess antibodies were washed away with FACS buffer and cells were fixed with fixation buffer (BD 554655) for 30 minutes at 4°C prior to analysis by flow cytometry (FACSymphoney A1) the next day.

2.3.11 Quantification and statistical Analysis

Statistical analyses were conducted using Mann-Whitney test or one-way ANOVA when appropriate. Bonferroni post-tests were used to adjust for multiple comparisons between different test groups. All statistical analyses were performed using GraphPad Prism 9 software.

2.4 Results

2.4.1 Intranasal administration of an adenovirus-based vaccine afforded better cross-subtype protection against lethal influenza challenges than intramuscular administration

To evaluate the efficacy of Ad-NP-CD40L, BALB/c mice received 10^9 PFU of the recombinant adenovirus in a prime and boost regimen four weeks apart through IN or IM administration. Four weeks post-boost, mice were challenged with a lethal dose of either H1N1, H3N2, or a HPAI H5N1 strain (Fig 2.1A). Following the H1N1 challenge, while both IN and IM vaccination provided complete protection with 100% survival, we did observe differences in morbidity and viral burden in both the upper respiratory tract (URT) and the lower respiratory tract (LRT). Specifically, while the IN vaccinated group had no weight loss upon challenge, significant bodyweight loss was observed in the IM group. This result is in agreement with the viral titration data, where IN vaccinated mice had lower viral burden than the IM group in both the nasal turbinate and lung tissues (Fig 2.1B). In vaccinated animals challenged with H3N2 (Fig 2.1C), the difference between IN and IM immunization was further accentuated. While IN administration provided complete protection (100% survival rate) against the lethal challenge with minimal weight loss (approx. 5%), IM vaccinated animals had similar disease progression to that of the control groups. Moreover, viral titration of infected tissues showed that only IN and not IM administration significantly reduced viral burden in both the nasal turbinates and the lungs (Fig 2.1C). In short, it is clear that for type A influenza virus (IAV) subtypes currently in circulation (H1N1 and H3N2), IN administration of NP vaccine provides superior protection than IM.

Given the heightening concerns with HPAI, we tested vaccine efficacy against a highly virulent avian strain that was recently isolated from a red-tailed hawk in Ontario, Canada [50]. The H5N1 strain, A/RT.Hawk/ON/2022, displayed high virulence and lethality in three commonly used mammalian models for influenza disease studies. More importantly, it was shown that it can be efficiently transmitted by direct contact between ferrets. As demonstrated by Kobasa et al., the LD50 for this strain in BALB/c model was <1 PFU [50]. As such a low challenge dosage is impractical for inoculation, animals were challenged with 10 PFU of the virus to maintain consistency across the animals with respect to inoculation dosage. Compared to the control groups at 0% survival, IN administered animals had a 40% survival and a significantly delayed disease progression, while the IM vaccination had no effect on improving the survival rate when compared to the controls (Fig 2.1D). The IN vaccinated animals also experienced significantly less weight loss on days 3, 4, and 5 post-challenge compared to the IM group. Lung viral titration results are in accordance with survival and weight loss data, with the IN group having significantly reduced viral burden. Although the difference in nasal turbinate viral burdens is not significant between IN and IM, IN administration resulted in a greater reduction of viral burden compared to the control groups. These results indicate that IN administration remains superior to IM administration with respect to protection from morbidity and mortality, when challenged with the HPAI strain. Interestingly, despite the vaccine NP sequence being highly similar to the NP from challenge virus strain, HPAI A/RT.Hawk/ON/2022 (Supp Fig 2.10), the IN vaccination failed to provide full protection, suggesting that other factors could be at play (see discussion).

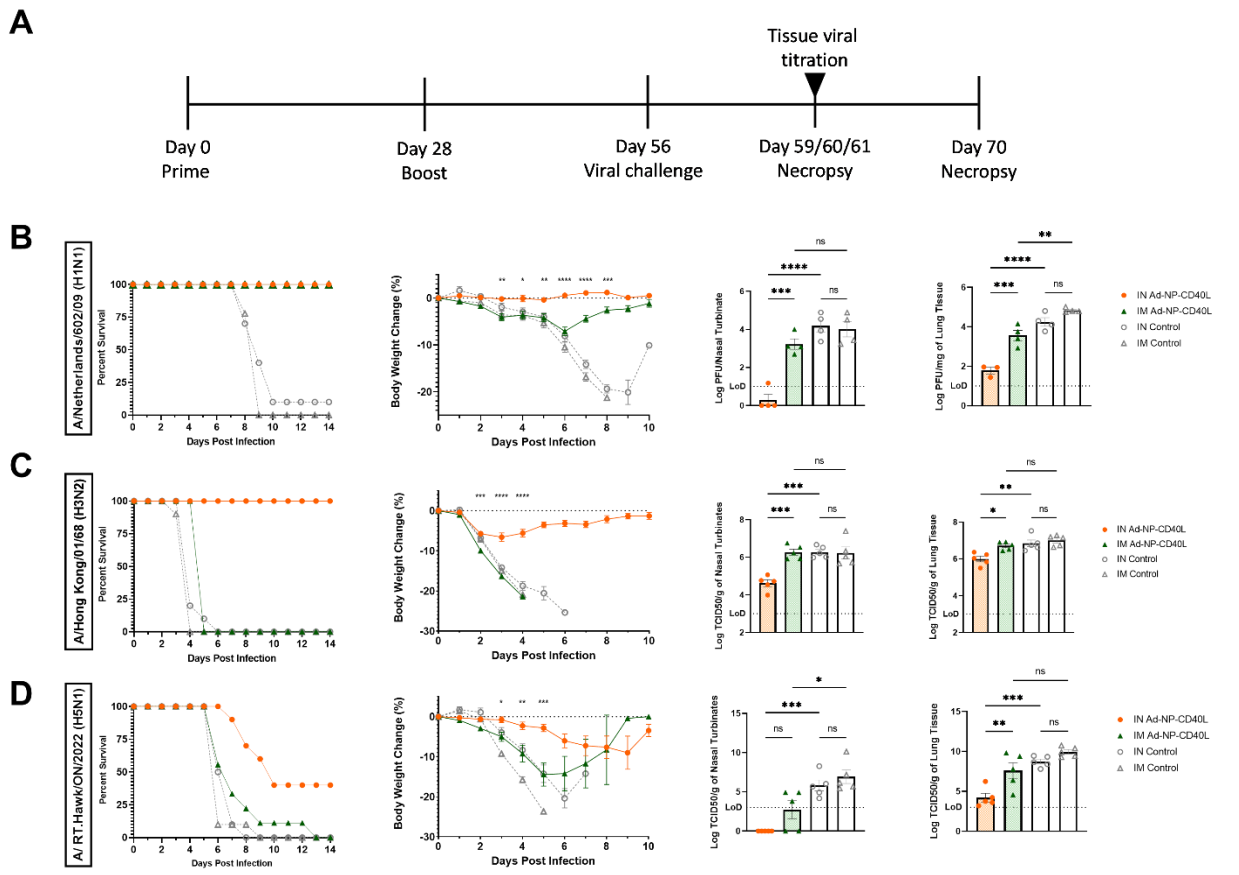

Figure 2.1 Intranasal immunization provides superior cross-subtype protection against lethal influenza challenges compared to intramuscular immunization.

(A) Schematic diagram of the immunization, viral challenge, and necropsy timeline for all three strains of IAV challenge. BALB/c mice were IN or IM administered Ad-NP-CD40L or Ad-Empty as controls with a prime/boost regimen, followed by an intranasal challenge of (B) 1000 PFU of A/Netherlands/602/09 (H1N1), (C) 3.85 × 10^5 PFU of A/Hong Kong/01/68 (H3N2), or (D) 10 PFU of A/ RT.Hawk/ON/2022 (H5N1). (Left to right) Survival (n = 10), change in body weight (n = 10) (unpaired T-test comparisons between intranasally (IN) and intramuscularly (IM) vaccinated Ad-NP-CD40L groups are shown), viral titration from infected nasal turbinates (n = 3 or 4) and lungs (n = 3 or 4) are shown. (one-way ANOVA with Bonferroni post-test). n.s. = not significant, *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001. Limit of detection (LoD).

2.4.2 Intranasal vaccination induced strong recall immune responses in the NALT

Having observed enhanced protection following IN administration, we next investigated the mucosal and systemic humoral responses by determining NP-specific antibody levels in the respiratory tract and serum. As a part of the URT, the NALT is the

initial site of recognition and elimination of inhaled pathogens. In NALTs collected post-challenge, we observed significantly higher levels of NP-specific IgG and IgA in the IN group compared to the IM group (Fig 2.2A). A similar trend was observed in the post-boost bronchoalveolar lavage fluid (BALF), which is indicative of the humoral responses in the LRT (Fig 2.2B). Interestingly, IN and IM administration induced similar levels of systemic humoral responses, as detected by enzyme-linked immunosorbent assay (ELISA) in the sera of post-boost vaccinated animals (Fig 2.2C). Notably, only IN administration induced significant levels of NP-specific IgA in the NALT, BALF, and serum (Fig 2.2A-C).

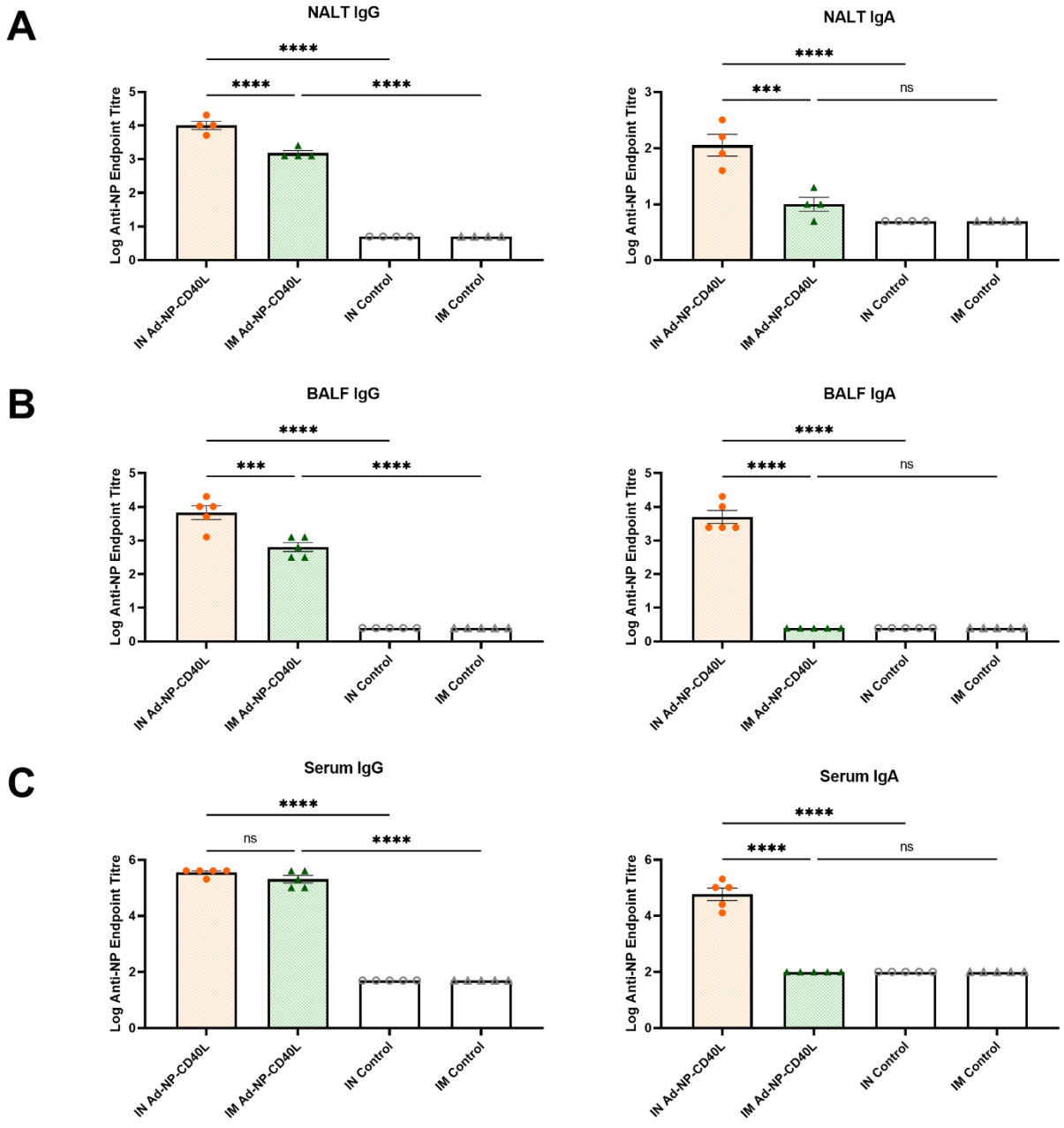

Figure 2.2 Intranasal immunization elicits stronger NP-specific IgG and IgA antibody responses along the respiratory tract compared to intramuscular immunization.

(A) Nasal-associated lymphoid tissue (NALT) was collected on Day 61, 5 days after an intranasal challenge of 1000 PFU of influenza A/Netherlands/602/09 (H1N1). After 24 hours of ex vivo culture, the supernatant was collected to determine anti-NP IgG and IgA endpoint titers (n = 4). **(B)** Bronchoalveolar lavage fluid (BALF) was collected 4 weeks post-boost (Day 56) to determine anti-NP IgG and IgA endpoint titers (n = 5). **(C)** Serum

from vaccinated mice were collected 4 weeks post-boost (Day 56) to determine anti-NP IgG and IgA endpoint titers (n=5) (one-way ANOVA with Bonferroni post-test). n.s. = not significant, ***p < 0.001, ****p < 0.0001.

2.4.3 Both administration routes induced balanced Th1/Th2 systemic responses with IM inducing enhanced antibody effector functions

Given the comparable anti-NP IgG levels in the sera, we further determined the IgG subtypes induced by the two routes of administration. IN administration induced similar levels of IgG1 and IgG2b relative to IM administration, but IN elicited a slightly higher level of IgG2a (Fig 2.3A). To characterize the type of immune response, we calculated IgG2a:IgG1 ratio. Both routes had similar ratios of just above 1.0, which indicated a balanced Th1/Th2 with a slight Th1-skew (Fig 2.3B).

As expected, antibodies induced by NP, an interior viral protein, have no neutralizing activities targeting viral entry (not shown). However, it has been shown, both *in vivo* and *in vitro*, that NP is expressed on the surface of infected cells despite being an internal viral protein [51,52]. Therefore, we next investigated the antibody-dependent cellular cytotoxicity (ADCC) effector functions of the serum antibodies. While IN administration induced significant levels of ADCC antibodies compared to the controls, IM immunization group displayed a higher level of ADCC activity compared to the IN group (Fig 2.3C). We did not observe any ADCC activity in BALF likely due to the lower antibody concentrations when compared to serum samples and/or the sensitivity of the assay.

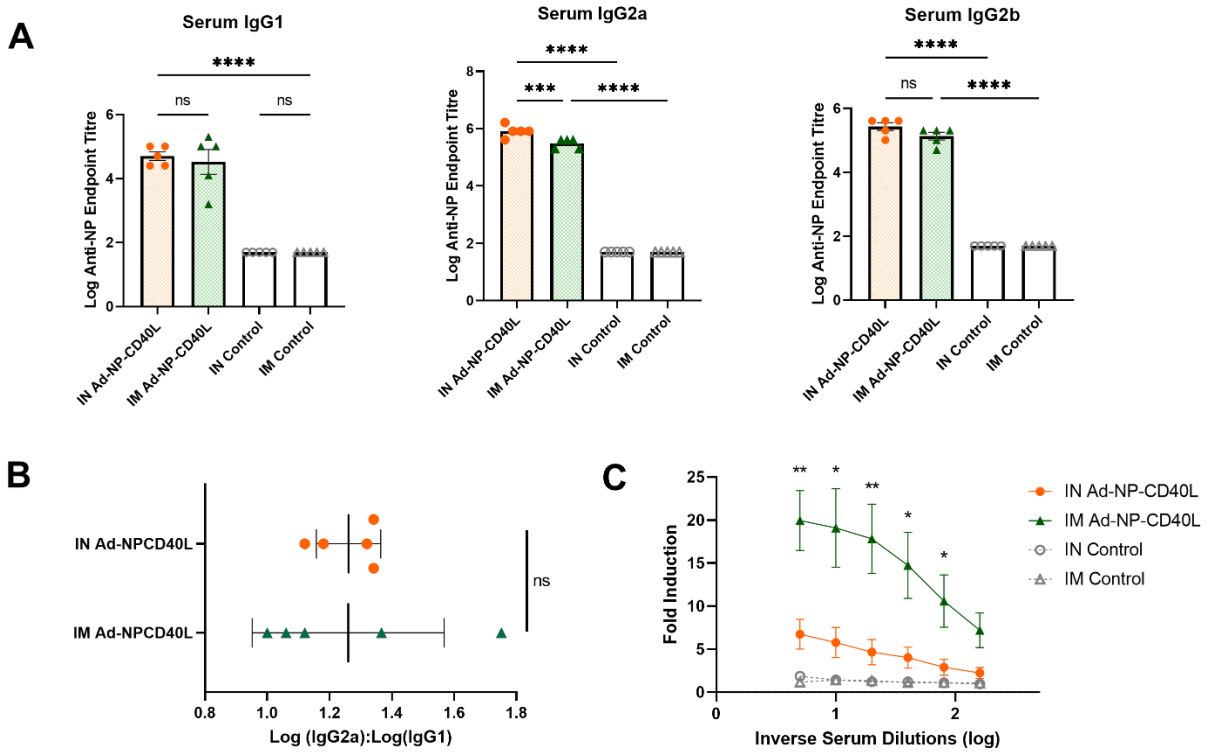

Figure 2.3 Intranasal and intramuscular immunization both elicit a balanced systemic Th1/Th2 immune response.

(A) Serum from vaccinated mice were collected 4 weeks post-boost (Day 56) to determine log of anti-NP IgG1, IgG2a, and IgG2b endpoint titers (n=5). Data shown is mean ± SEM. (one-way ANOVA with Bonferroni post-test) ns = not significant, ***p < 0.001, ****p < 0.0001. **(B)** NP-specific serum IgG2a:IgG1 ratio indicating Th2- or Th1-biased nature of the immune response (n = 5). Data shown is mean ± SEM. (unpaired T-test) ns = not significant **(C)** Post-boost serum was used to determine the antibody-dependent cellular cytotoxicity against A/Netherlands/602/09 (H1N1) (n = 4). Data shown is mean ± SEM. (unpaired T-test comparisons between IN and IM vaccinated groups are shown) *p < 0.05, **p <0.01.

2.4.4 Distinct systemic and mucosal cytokine profiles between the two routes of administration

We next investigated cytokine production following vaccination by IN or IM routes. To evaluate systemic responses, splenocytes from vaccinated animals were stimulated with immunodominant NP peptides prior to the quantification of secreted cytokines. IM administration induced about 2 to 6 folds higher cytokine production upon stimulation compared to the IN group, including Th1, Th2, and Treg cytokines (Fig 2.4). Notably,

compared to their respective controls, Th1 cytokines such as IFN gamma, TNF-alpha, and Interleukin (IL)-18 were produced at higher levels (about 6 to 10 folds higher than the control) when compared to Th2 cytokines (about 2 to 3 folds higher), such as IL-4, IL-5, and IL-10. Similarly, IN administration also induced significant levels of Th1 cytokines compared to its respective control, such as IFN gamma, TNF-alpha, and IL-18 (about 4 to 24 folds higher), although at a lower level than IM administration. The spleen cytokine profile is consistent with the systemic antibody subtypes, both administration routes demonstrating the Th1-skewed nature of the induced response.

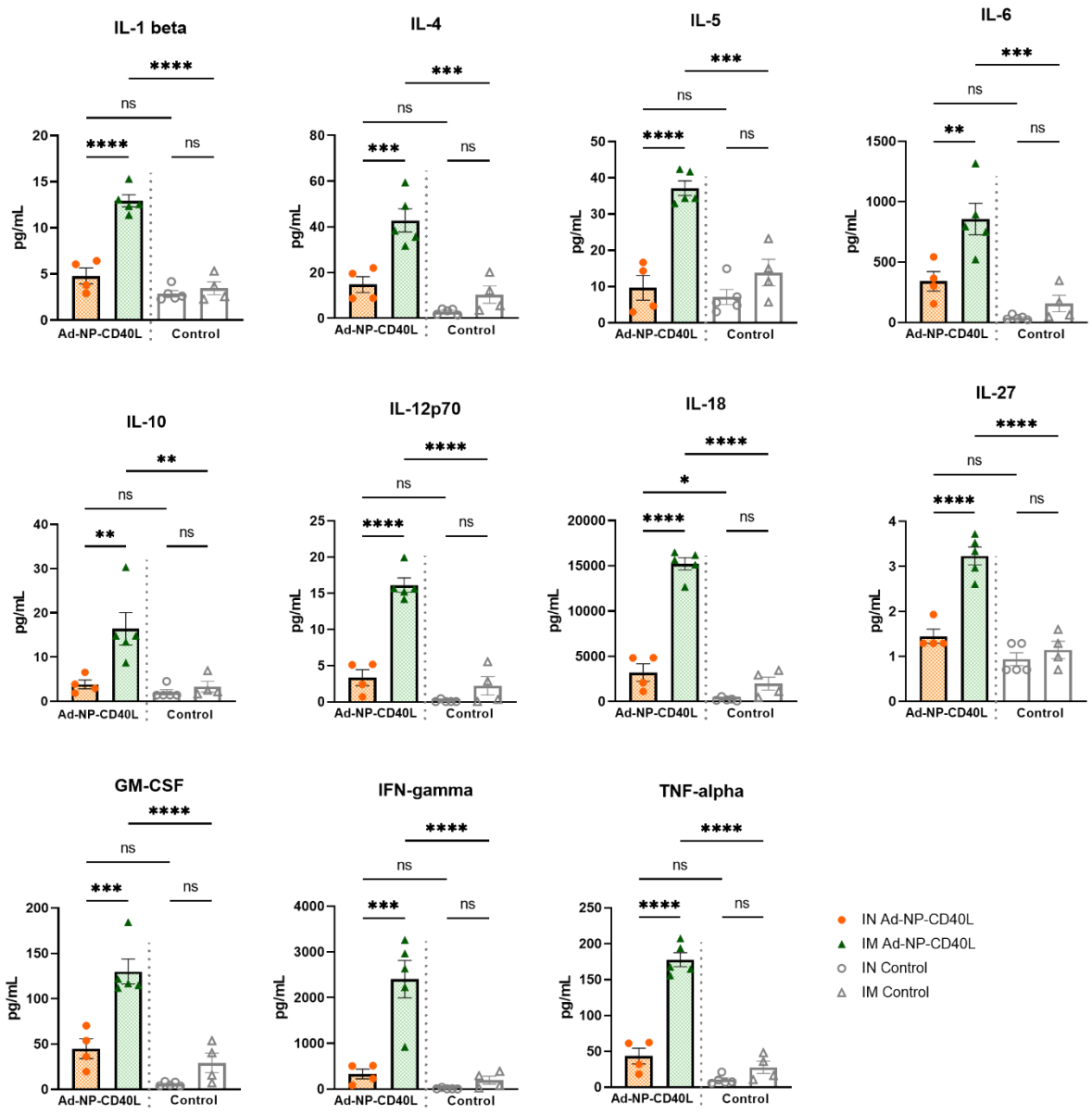

Figure 2.4 Intramuscular immunization stimulated higher cytokine responses in the spleens compared to intranasal immunization.

Splenocytes were isolated 4 weeks post-boost (Day 56) and incubated with TYQRTRALV and ASNENMETM at 5 µg/ml each (n = 4 or 5). Following 72-hour incubation, cytokines in the supernatant were quantified with a Luminex system. Data shown is mean ± SEM. (one-way ANOVA with Bonferroni post-test) ns = not significant, *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001.

Having examined the systemic cytokine production in the splenocytes, we next determined cytokine responses in the local mucosal tissues using the same cytokine panel. As shown in Fig 2.5, IN immunization induced higher levels of cytokine when compared to IM immunization in the NALT by about 1 to 2.5 folds. Furthermore, IN vaccination elevated the levels of Th1 cytokines such as TNF-alpha, IL-12p70 and GM-CSF when compared to IM administration. While like other secondary lymphoid organs, the NALT may contain a small number of circulating lymphocytes within the tissue. It is of note, however, that a stimulated NALT is predominantly composed of non-circulating activated B cells and CD4+ T cells that help B cells to respond [53]. Other than the cytokines shown in Fig 2.5, we did not find any other significantly elevated cytokines in the NALT compared to the controls. It is to be noted that the NALT is a much smaller tissue compared to the spleen, made of approximately 10^5 lymphocytes [54]. Therefore, the cytokine production levels of the two tissues cannot be directly compared. Nonetheless, these results collectively indicate that IN immunization induces a more robust mucosal immunity with a cell-mediated response profile that is distinct from that of the IM response at the local mucosal tissues.

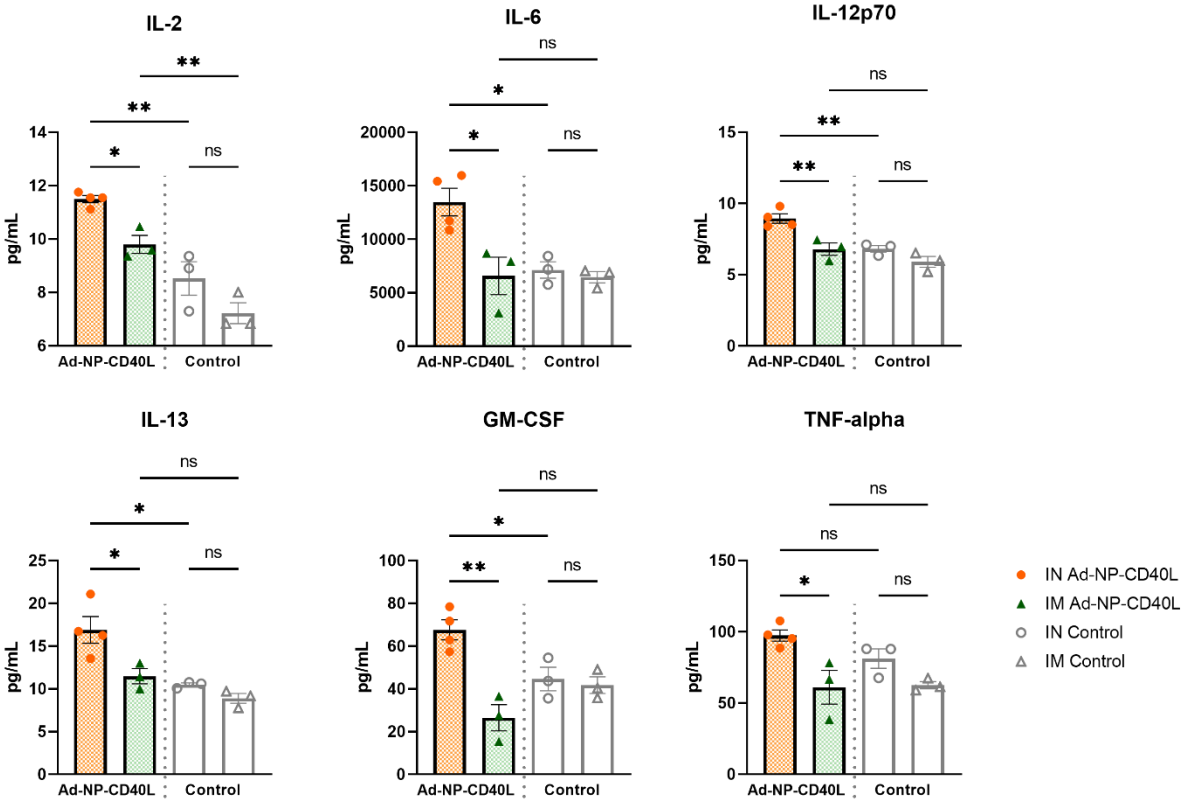

Figure 2.5 Upon infection, intranasal immunization induced stronger cytokine responses in the nasal associated lymphoid tissue (NALT) compared to intramuscular immunization.

NALTs were collected on Day 61, 5 days after intranasal challenge of 1000 PFU of influenza A/Netherlands/602/09 (H1N1). After 24 hours of ex vivo culture, a Luminex system was used to quantify cytokines in the supernatant (n = 4). Data shown is mean ± SEM. (one-way ANOVA with Bonferroni post-test) ns = not significant, *p < 0.05, **p <0.01.

2.4.5 More robust antigen-specific pulmonary T cell responses were detected after IN vaccination

Having observed a distinction in the cytokine profiles among the systemic and local mucosal immune responses following the two different routes of administration, we next investigated antigen-specific T cell proliferation in the lungs. The lung tissues from vaccinated mice were homogenized and stained using CellTrace® Violet (CTV), a fluorescent dye that diminishes as the labeled cell proliferates. Proliferation was thus measured as the percentage of cells with diminished CTV stain (Fig 2.6A). The

percentage of proliferating cells were measured for each of the CD3+, CD4+, and CD8+ T cell populations. In all three T cell populations, IN vaccinated mice had more antigen-specific T cell expansion when compared to the IM vaccinated group (Fig 2.6B-D). Notably, the fold increase in proliferating CD8+ T cells was the highest among the T-cell populations studied. These results indicate that IN immunization induces more proliferative antigen-specific T cells in the lungs than IM vaccination, which may lead to a faster recall response upon infection.

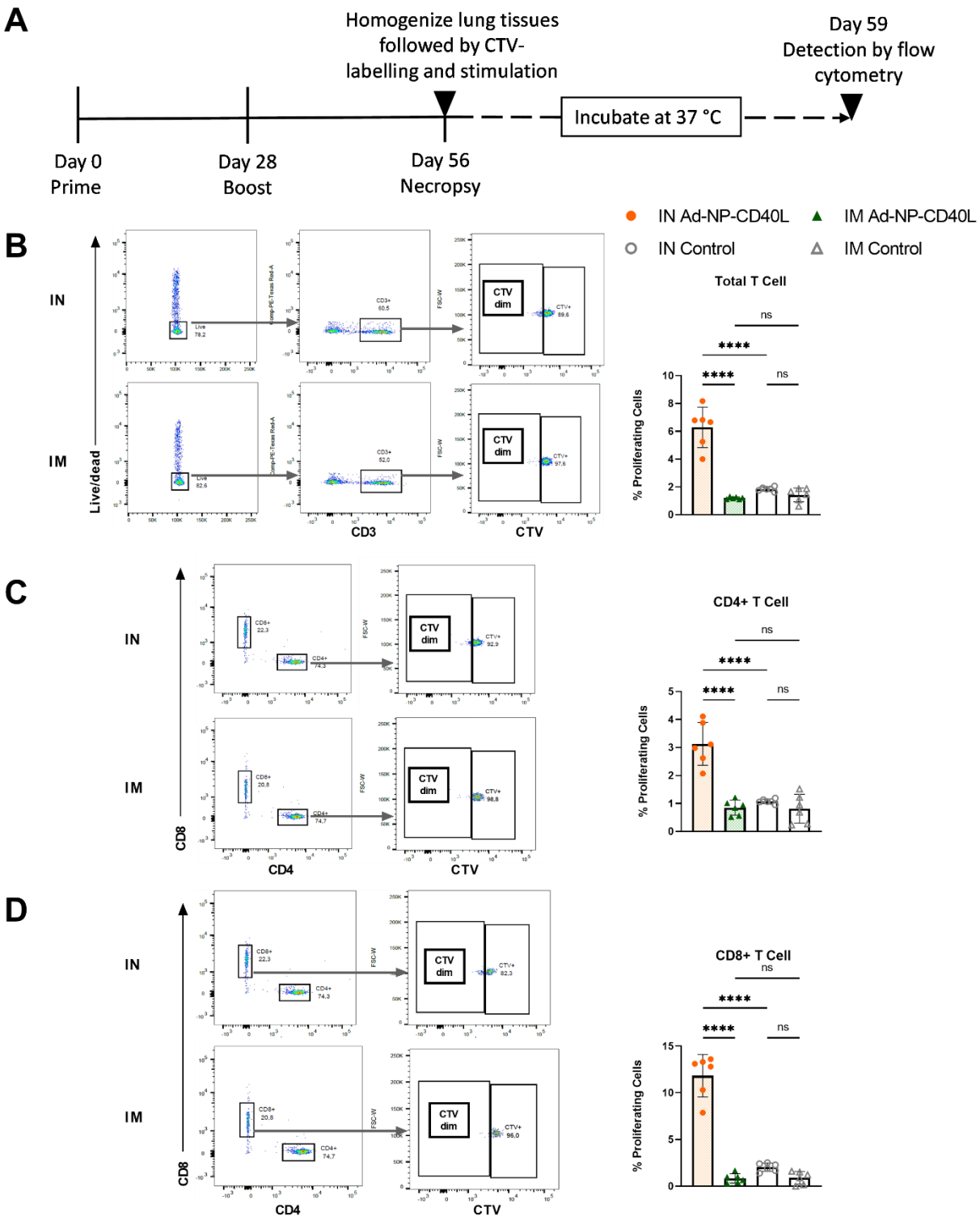

Figure 2.6 Intranasal immunization induces robust antigen-specific T cell proliferation in the lungs compared to intramuscular immunization.

(A) Schematic diagram of the immunization, necropsy, and proliferation assay timeline. BALB/c mice were intranasally (IN) or intramuscularly (IM) administered Ad-NP-CD40L or Ad-Empty (Control) with a prime/boost regimen on Day 0 and Day 28 and were necropsied on Day 56. The lung tissues were digested, homogenized, and stained using CellTrace® Violet (CTV). After quenching and washing, the lung cells were stimulated with immunogenic peptides from NP. Following 72-hour incubation, frequency of proliferating cells was measured by flow cytometry. Graphs showing representative gating strategies and raw frequency of proliferating cells in **(B)** total T cells, **(C)** CD4+, **(D)** CD8+ populations. (one-way ANOVA with Bonferroni post-test) Data shown is mean ± SEM. ns = not significant, ****p < 0.0001.

2.4.6 Blocking lymphocytes egression had no impact on IN vaccination efficacy

As elevated immune responses were detected in the respiratory tract following IN immunization, our next aim was to test whether it could confer protection in the absence of circulating lymphocytes. We utilized the drug FTY720, a sphingosine-1-phosphate receptor 1 agonist, which prevents lymphocyte egress from lymphoid tissues and bone marrow into circulation. It is an approved oral treatment for relapsing forms of multiple sclerosis [55,56]. IN or IM vaccinated mice were intraperitoneally (IP) administered with FTY720 or PBS daily, starting on Day 53 for 9 days up till necropsy. The animals were then challenged with H1N1 on Day 56 and necropsied on Day 61 (Fig 2.7A).

The experiment was designed to ensure effective blocking of lymphocyte circulation, in order to dissect the protective roles of antibody responses and T cells. We first validated the effectiveness of the FTY720 treatment by quantifying the number of T cells in whole blood via flow cytometry. Overall, there was a drastic decrease in the number of both CD4+ and CD8+ T cells in the blood after FTY720 treatment compared to the PBS groups. There were no significant differences between PBS treatment groups of IN and IM Ad-NP-CD40L vaccinated animals (Fig 2.7B). It is of note that no significant differences were detected in the anti-NP antibody levels between the treatment and non-treatment groups (Fig 2.7C). This is to be expected since B cells and antibody production

do not depend on circulating lymphocytes. Therefore, the humoral response is not a major contributor to the differences in protection that are observed.

Importantly, as evident in Fig 2.7D, the FTY720 treatment did not affect the protection conferred by IN vaccination. Irrespective of the treatment, the lung viral load was undetectable by plaque assay. In contrast, FTY720 treatment resulted in a significant increase in viral load in the IM group. To further characterize the CD4+ and CD8+ T cells in the lungs, they were stained for the co-expression of two tissue resident cells (TRM) markers, CD69 and CD103. As shown in Fig 2.7E, IN vaccination induced a higher percentage of CD69+/CD103+ cells than the IM group among Ad-NP-CD40L vaccinated animals. Interestingly, an elevation of CD69+/CD103+ cells in the IN control was also observed. This is likely due to adaptive immune responses triggered by epitopes present in the adenoviral vector, as part of its adjuvating effects as a vaccine vector [57]. In addition, compared to their no-treatment PBS controls, the percentages of CD69+/CD103+ cells were increased by the FTY720 treatment. This effect was most prominent in the IN vaccinated Ad-NP-CD40L group. Importantly, however, the number of CD69+/CD103+ cells in the IN Ad-NP-CD40L vaccinated mice was significantly higher than the IN control group. Taken together, these results demonstrate that IM vaccination relies on the circulating lymphocytes for protection, whereas the IN vaccination relies on local tissue responses that display characteristics of tissue resident cells and can provide full protection in the absence of circulating lymphocytes.

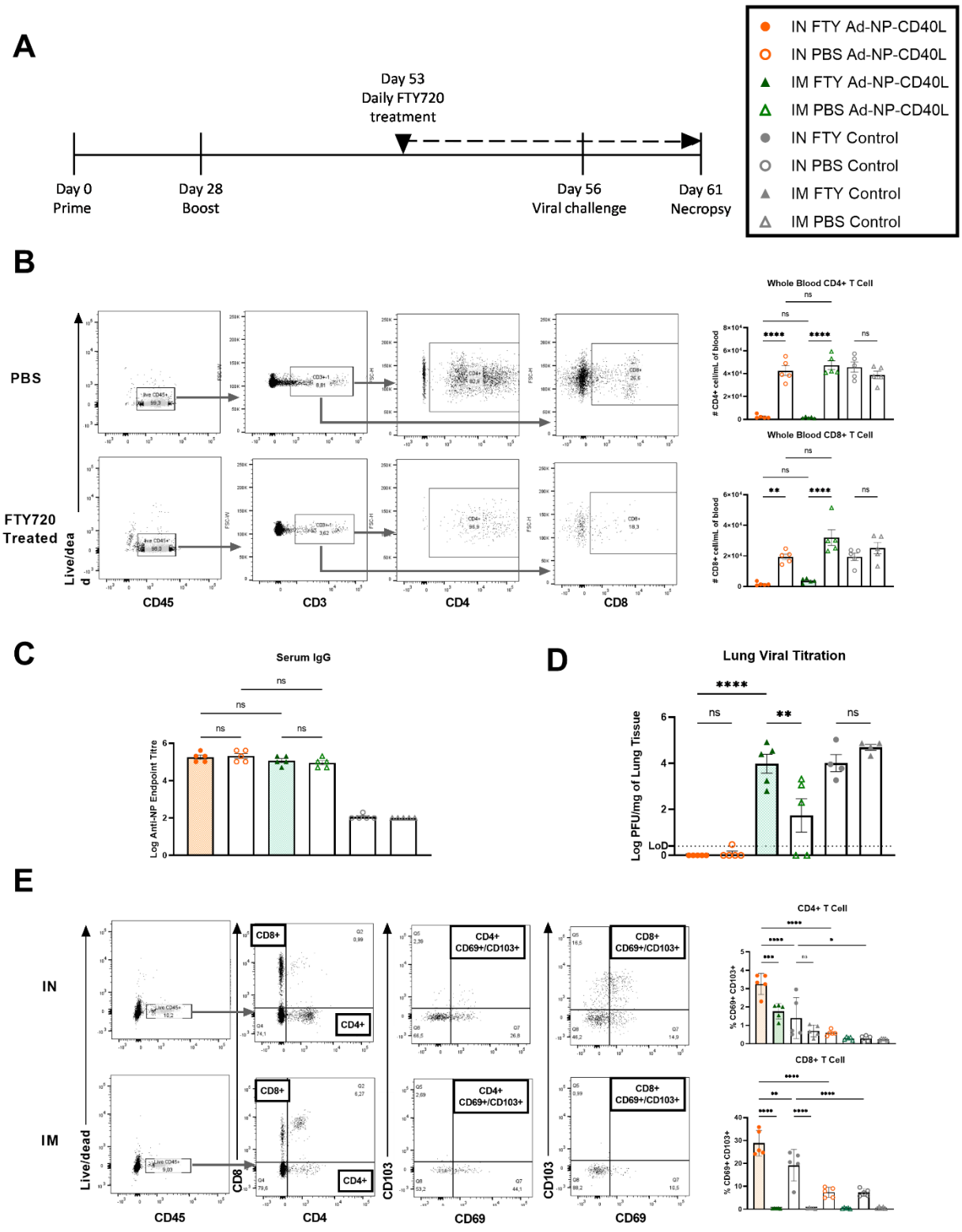

Figure 2.7 Intranasal immunization provided better protection without circulating lymphocytes than intramuscular immunization.

(A) Schematic diagram of the immunization, FTY720 treatment, viral challenge, and necropsy timeline. BALB/c mice were intranasally (IN) or intramuscularly (IM) administered Ad-NP-CD40L or Ad-Empty (Control) with a prime/boost regimen on Day 0 and Day 28, followed by daily FTY720 or PBS intraperitoneal injections starting on Day 53 for 9 days. The animals were challenged with 1000 PFU of influenza A/Netherlands/602/09 (H1N1) on Day 56 and necropsied on Day 61. **(B)** The number of CD4+ and CD8+ cells were quantified in the blood via flow cytometry to validate the FTY720 treatment (n = 5). Graph showing representative gating strategies. Data shown is mean ± SEM. **(C)** Serum was collected at necropsy to determine log of anti-NP IgG endpoint titer (n = 5). **(D)** Viral titre from infected lungs (n = 5) collected 5 days post-challenge were determined by plaque assay. Data shown is mean ± SEM. **(E)** The frequency of CD69+/ CD103+ cells in the CD4+ and CD8+ populations in the lungs were determined by flow cytometry (n = 5). Graph showing representative gating strategies. Data shown is mean ± SEM. (one-way ANOVA with Bonferroni post-test) ns = not significant, **p <0.01, ***p < 0.001, ****p < 0.0001.

2.5 Discussion

The ever-evolving nature of the influenza surface glycoproteins requires annual update to the influenza vaccines. While the development of HA-based universal vaccines has been vigorously pursued [58], substantial interests remain in exploring the potential of more conserved antigens, such as NP [59,60]. As aforementioned, a variety of vaccine platforms have been explored to deliver NP [11–25]. Most studies supported NP as a candidate target, with several studies demonstrating that mucosal delivery elicits a more robust protection compared to other routes of administration. These studies have employed various constructs and platforms including mRNA [34], polyvalent virosome [61], and co-administration of multiple recombinant vectors carrying different viral proteins [11,12,35,62]. Our current study differs from these previous studies in two main aspects, which are the nature of the NP antigen and the head-to-head comparison between IN and IM administrations. Specifically, we utilized an adenovirus vector-based vaccine expressing NP fused with the ectodomain of CD40L, with CD40L functioning as both a targeting ligand and a molecular adjuvant. As shown in our previous work, the subcutaneous (SC) delivery of this vaccine did not provide full protection against lethal

influenza challenges, with only 50-70% survival [44], suggesting the need of improvement. We aimed to answer two questions: first, would IN or IM delivery improve the efficacy of Ad-NP-CD40L? Second, if so, what underlying mechanisms mediate differences in protection induced by IN and IM administration?

To assess the breadth of protection, BALB/c mice were challenged with H1N1, H3N2, and a newly identified HPAI H5N1 strain. Irrespective of the challenge strain, IN administration provided significantly better protection than IM administration, an observation largely in agreement with other comparison studies [11,12,34,35,61,62]. Notably, while both IN and IM vaccination provided 100% survival against H1N1 challenge, IM administration failed to prevent body weight loss and had significantly higher viral burden in the URT and LRT (Fig 2.1B). The difference in protection between the two routes was more prominent following H3N2 challenge, where IM administration failed to provide any protection relative to negative controls (Fig 2.1C). These data indicate that IN vaccination is superior to both IM vaccination, as found in this study (Fig 2.1), and SC vaccination, as observed in our previous investigation [44]. In the challenge against the HPAI H5N1 strain (A/RT.Hawk/ON/2022), IN vaccination provided partial protection, while IM administration afforded no protection (Fig 2.1D). This HPAI strain was recently isolated from a red-tailed hawk and was found to have high pathogenicity in mice, with a LD50 of <1 PFU [50]. It is to be noted that the NP sequence in this H5N1 strain is 99.3% identical to that of the vaccine NP sequence (Supp Fig 2.10). Therefore, the partial protection is not only due to presence of sequence conservation, but more importantly the local immune responses, as evident by the lack of protection in IM group despite the sequence conservation. While investigations are ongoing towards better understanding of the

remarkable virulence of A/RT.Hawk/ON/2022 in mammalian animal models, our observations support the notion that IN vaccination of Ad-NP-CD40L provides superior cross-protection when compared to IM vaccination (Fig 2.1D)**.** The more virulent nature of H3N2 and H5N1 has also been reported by others that they tend to have a faster disease progression than H1N1, which could lead to more severe disease outcomes if the infections are not contained in the early phase [63–65]. However, it is also likely that differences in the lethality of challenge dosage used for the strains could be a reason for the varying degree of survival conferred by the two routes of vaccinations among the different influenza strains. Regardless, although both vaccination strategies provided protection against lethality, other parameters to measure disease progression of the H1N1 infection, such as weight and viral titer, were consistent with the trend observed in the challenge experiments with the other two influenza strains (Fig 2.1). Results from this study and others [11,12,34,35,61,62] have collectively reinforced the notion that IN vaccination induces more effective protection against wider range of viral strains, while the incorporation of the bifunctional CD40L ectodomain into antigens could also induce a broader immune response against a range of virus subtypes [44,66].

Interestingly, despite IN administration providing better protection, both routes of administration induce robust serum antibody responses, with a slight Th1-skew (Fig 2.2 & 2.3). However, IM administration induced markedly stronger antibody effector function activity (ADCC) (Fig 2.3C) and antiviral cytokine responses in the spleen (Fig 2.4). In the absence of neutralizing antibodies, such as responses induced by NP, such defense mechanisms could play important roles [33,67,68]. However, as per our observation, such superior serum humoral responses are not sufficient to afford complete protection. We

also noted a recent study by Vanderven *et al.*, where it was demonstrated that *in vitro* Fc receptor-binding anti-NP monoclonal antibodies are not sufficient to completely protect against challenge upon passive transfer [69]. Given that IN vaccination primarily induces local responses, ADCC and other effector functions at these mucosal sites are yet to be studied in depth, with the functional roles at mucosal site being largely unknown [70]. While we were unable to detect ADCC activity in the BALF (data not shown), our observations clearly indicate that a strong mucosal response is needed in addition to systemic responses to afford superior protection from challenge with influenza A. (Fig 2.6). These observations are largely in agreement with others, in particular the role of pulmonary T cell responses [62,71].

One of the less studied lymphoid tissues in influenza vaccine research is the NALT, which is the initial site of immune recognition and elimination of inhaled pathogens in the URT [72]. In our study, strong recall antigen-specific IgA and IgG were only observed in the NALT from IN-vaccinated animals upon viral infections (Fig 2.2A). Moreover, IN vaccination also induced significantly stronger cytokine responses the NALT (Fig 2.5). Notably, IL-6, markedly elevated in IN vaccinated animals, is known to be a key cytokine promoting the differentiation and proliferation of plasma cell precursors and instigates the development of IgA antibodies [73], supporting the elevated IgA expression levels detected at the mucosal sites (Fig 2.2A). IL-2, previously linked to restoring mucosal immunity in aged mice, was also detected significantly higher in the NALT [74]. Notably, the small size of NALT with low number of lymphocytes in the NALTs required *ex vivo* culturing following challenge. As shown in Figs 2.2 & 2.5, NALTs from IN and IM groups were compared under the same collection timeline and culture conditions. We found IN

immunization induced higher mucosal antibodies and cytokines (Fig 2.2 & 2.5), which is in agreement with tissues collected pre-challenge. These findings at the NALT are consistent with the protective responses detected in the BALF and lungs, supporting the observation of potent mucosal immunity at both URT and LRT induced by Ad-NP-CD40L IN vaccination.

Lastly, we interrogated the protective roles of circulating and resident lymphocytes. We found that blocking lymphocytes egression had minimal effect on the effectiveness of IN vaccination, while in contrast, increased viral burden was observed following IM immunization. These results are consistent with a study with a different experimental design, which was the IN co-administration of multiple recombinant vectors carrying HA, NP, and IL-1β [12], while ours contains only NP fused with a bifunctional CD40L. Furthermore, our observation was also supported by the increased frequency of resident CD69+/CD103+ cells in the CD4 and CD8 T cell populations in the lungs (Fig 2.7). It should be noted that multiple assays employed in this study, including cytokine quantification (Fig 2.4 & 2.5), proliferation assay (Fig 2.6), and the blocking circulation of lymphocytes experiment (Fig 2.7), allowed us to compare and dissect cellular responses induced by IN and IM administrations. Other assays such as intracellular cytokine staining and activation-induced marker assays would also have added value for future studies.

Given that the respiratory tract is the natural site of infection for adenovirus, the adenoviral vector is a desirable platform for mucosal immunization as demonstrated by various clinical studies [75–77]. During natural infection with adenovirus, pro-inflammatory cytokines, such as IL-6 and TNF-alpha, are released. However, data on the profile of cytokines released in response to mucosal vaccinations are limited [78]. A

challenge faced by adenovirus-based vector vaccines is pre-existing immunity, which could weaken the efficacy of some vectors with widespread serotypes [79]. However, several approaches have been explored with promising findings as reviewed by Zhang *et al* [80]. One of these strategies is to induce immunity through the nasal delivery to overcome pre-existing immunity [11,81,82]. Other approaches have also proven to be promising, such as utilizing nonhuman or less common serotypes [83,84], modifying the surface of the viral particle [85,86], and employing heterologous prime-boost regimens [87]. Thus, in addition to inducing a protective immune response, IN immunization could also overcome issues with pre-existing immunity to the vector, when compared to IM administration.

In conclusion, we have demonstrated that IN delivery significantly improves the efficacy of Ad-NP-CD40L compared to systemic immunization, inducing superior protection against multiple influenza A strains. This superior protection was primarily mediated by mucosal responses, while the limited protection induced by IM vaccination stems from its lack of potent local immune responses. These findings, along with other reports with different experimental approaches [12,62,71], have advanced our knowledge of the NP-induced mucosal protection and may inform the design of future cross-subtype protective vaccines against influenza, particularly, those which employ highly conserved interior viral proteins.

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Author Contributions

Wanyue Zhang: conceptualization of the project, conducting the experiments, data analysis and visualization, draft and edit manuscript

Angela Sloan & Jérémie Prévost: H5N1 animal experiments at NML, reviewing manuscript

Darwyn Kobasa & David Safronetz: supervision at NML

Levi Tamming, Sathya Raman & Annabelle Pfeifle: helping with some experiments, reviewing manuscript

Caroline Gravel & Jianguo Wu: lab management

Wangxue Chen: data analysis consulting

Xuguang Li, Anwar M Hashem, Jingxin Cao & Michael J.W. Johnston: funding acquisition

Lisheng Wang, Simon Sauve, Michael Rosu-Myles & Xuguang Li: supervision

Disclosure statement

The authors declare no conflict of interest.

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2.5.2 Supplemental Material

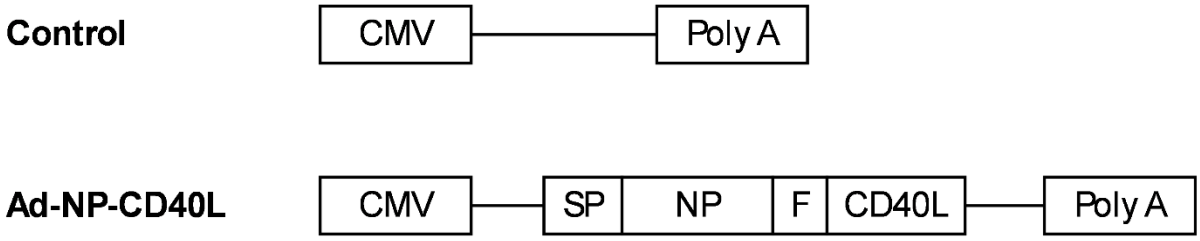

Figure 2.8 Schematic representation of the recombinant adenovirus vaccine constructs.

Ad-NP-CD40L construct was designed to express a secreted form of NP under the cytomegalovirus promoter (CMV), with the human tyrosinase signal peptide (SP) at the N terminus, fused to the bacteriophage T4 fibritin trimerization motif (F) and the ectodomain of mouse CD40L, with the polyadenalytion tail (PolyA) at the terminal.

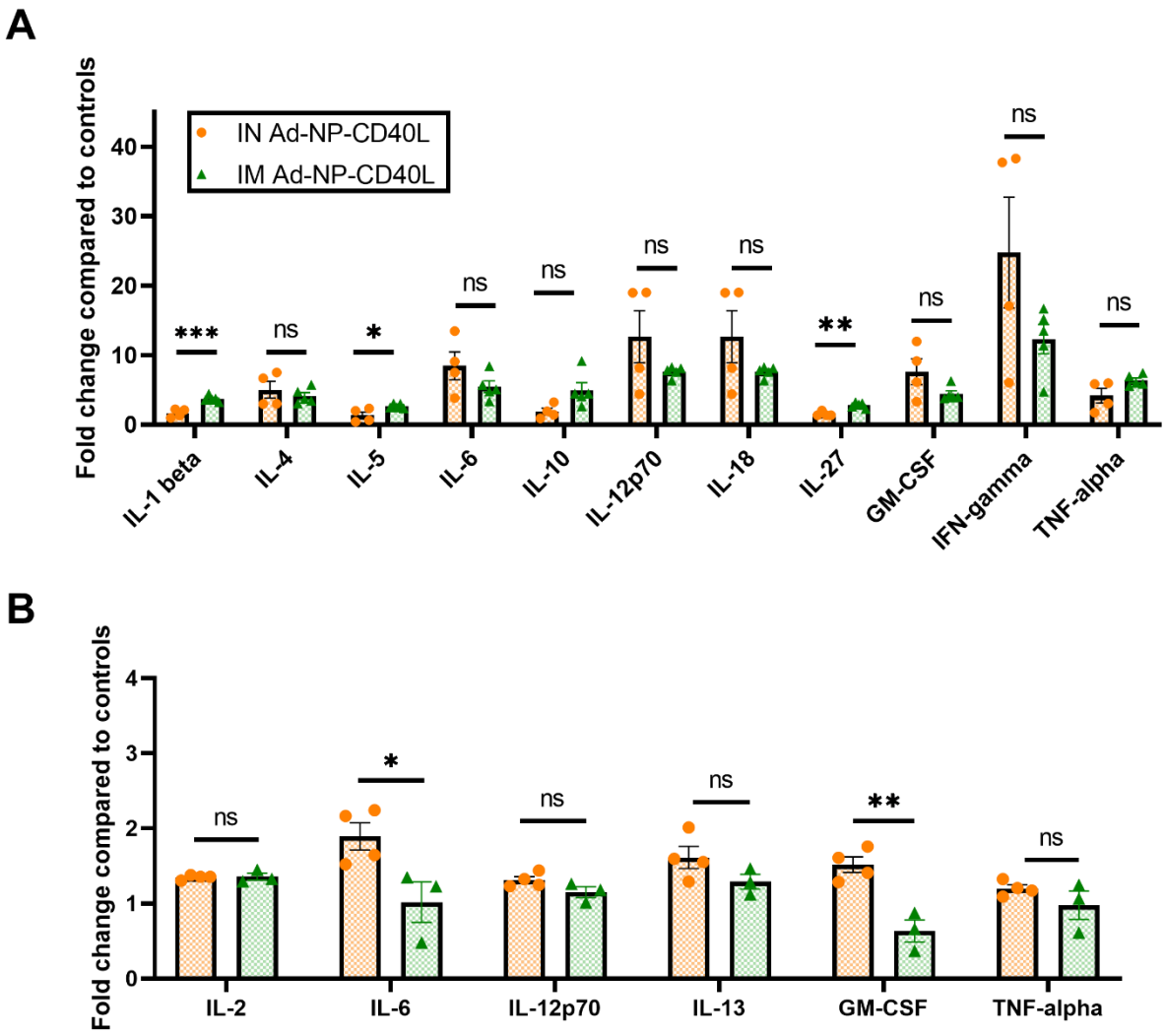

Figure 2.9 Fold change of cytokine responses in the (A) spleens and in the (B) nasal associated lymphoid tissue (NALT) compared to their respective IN or IM administered control groups.

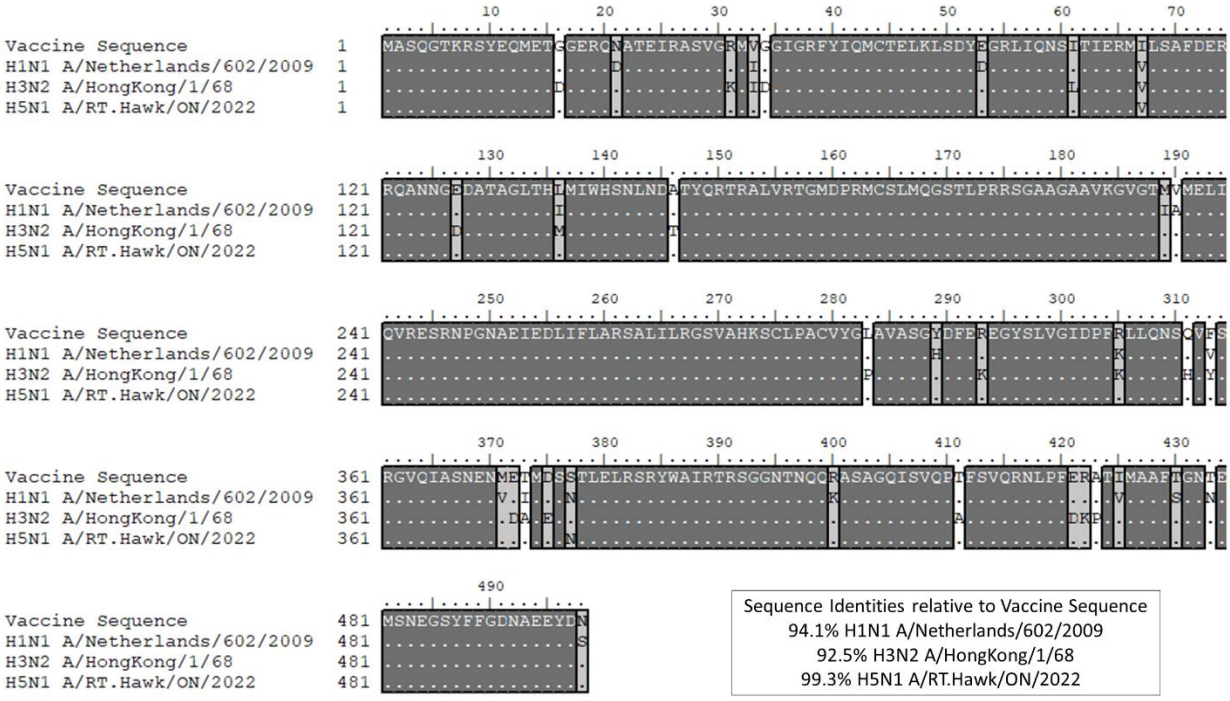

Figure 2.10 Multiple sequence alignment shows the NP sequences of the vaccine and the challenge strains.

NP protein sequences aligned by multiple sequence alignment program ClustalW using the BioEdit 7.7.1 program.

3 Intranasal Vaccine Induces Broad and Long-Lasting Immunity Against the Hemagglutinin Stem of Group 2 Influenza A Viruses

Preface: This chapter has been previously published as a research article.

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3.1 Abstract

Influenza viruses remain a major global health threat, with H3N2 posing a particular challenge due to its rapid evolution, limited vaccine efficacy, and association with more severe influenza seasons. Although the importance of T cells in vaccine-induced protection is well established, hemagglutinin stem (HA2)-specific T cell responses have been relatively understudied, especially in the absence of other antigens. We engineered an adenoviral vector vaccine (Ad-HA2) to express an H3 hemagglutinin stem consensus sequence generated through bioinformatic analysis. The vaccine provided heterosubtypic protection against lethal challenges with group 2 influenza A viruses, H3N2 and H7N9, with protection maintained for up to six months post-vaccination. The vaccine induced robust HA2-specific humoral and cell-mediated responses in the nasal-associated lymphoid tissue (NALT) of the upper respiratory tract, the first immune site to recognize and eliminate inhaled pathogens. The vaccine also elicited significant levels of antibodies and T cell responses in the lower respiratory tract and pulmonary immune sites. In addition, circulating antibodies in the serum demonstrated effective antibody-dependent cellular cytotoxicity (ADCC) activity. Finally, using a peptide pool matrix screening approach combined with in silico verification, we identified an immunogenic C-terminus region of the HA2 consensus sequence that activated CD4+ and CD8+ T cells, which warrants further investigation. Collectively, these findings are informative for the design and evaluation of mucosal influenza vaccines targeting the hemagglutinin stem.

3.2 Introduction

Influenza viruses remain a persistent global health threat, necessitating continuous development of effective vaccines. Each year, seasonal influenza results in 3–5 million cases of serious illness and 290,000–650,000 deaths worldwide (1). Despite decades of research efforts, the year-to-year efficacy of licensed vaccines is highly variable (2). A major challenge in the development of effective influenza vaccines is the need to confer immunity against continuously evolving viral strains. Current seasonal influenza vaccines primarily induce neutralizing antibodies against the highly variable hemagglutinin (HA) head domain, offering limited protection against antigenically drifted strains (3).

Among the seasonal influenza viruses, H3N2 warrants particular attention due to its epidemiological and virological characteristics. The H3 influenza subtype was first introduced into the human population during the global pandemic of 1968, leading to more than one million deaths worldwide (4). Vaccine efficacy is consistently lower for H3N2 when compared to the other circulating influenza A subtype, H1N1, especially for mismatched viruses (5,6). This disparity in vaccine efficacies can be partially explained by the egg-based vaccine production process, as H3N2 cannot propagate in eggs without acquiring adaptive mutations that alter antigenicity, rendering vaccine-induced antibodies poorly reactive to wild-type H3N2 strains (7–9). Furthermore, H3N2 is associated with higher morbidity and mortality than other influenza subtypes, as it causes more outbreaks among the elderly (10). H3N2's dominance during severe influenza seasons and the limited vaccine efficacy against this particular subtype illustrate the need for improved vaccine strategies which generate cross-protection against divergent H3N2 influenza viruses.

Influenza A viruses (IAV) have 18 known hemagglutinin subtypes, categorized into two phylogenetic groups based on their antigenic characteristics. Group 1 includes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18, while group 2 comprises H3, H4, H7, H10, H14, and H15 (11,12). In group 2, aside from H3, the H7 subtype is also a cause for concern. During 2005-2022, various highly pathogenic avian influenza (HPAI) H7 viruses led to 106 outbreaks globally, resulting in the loss of over 33 million poultry (13). H7 also has high pandemic potential, with a fatality rate exceeding 40% in reported human infections (13).

Targeting the conserved hemagglutinin stem region (HA2) offers a promising approach to generate cross-protection. Unlike HA1, the highly variable globular head which undergoes continuous antigenic drift and dominates immune responses to current vaccines, HA2 forms the stem region and evolves at a slower mutation rate due to its functional restraints and low immune pressure (14). Several vaccination strategies targeting the stem region of HA are being tested in clinical studies, including headless recombinant protein vaccines (15–19), live attenuated influenza vaccines (LAIVs) and inactivated split vaccines consisting of HA head domains from subtypes to which humans are naïve to and a conserved stem domain (20–26), stabilized stem trimers displayed on ferritin nanoparticles (27–32), and as a part of a multi-epitope recombinant protein (33–38). Many other preclinical studies also have explored various vaccine platforms for the delivery of “headless” HA (39–46). Our lab has previously successfully utilized consensus sequences of HA2 in designing vaccines against H1N1 (47) and influenza B viruses (48). Thus, we hypothesize that the same strategy can be employed for the design of a vaccine against the H3 subtype. Furthermore, HA2-specific T-cell responses have been relatively

understudied compared to humoral responses (49). Although the importance of T cells in providing heterosubtypic protection is well-established (50), HA2-specific T cell responses are often studied in challenge models, with immune responses directed towards less desired non-conserved targets through epitope competition (51–53). For instance, in a recent clinical trial testing the inactivated split vaccines consisting of “exotic” HA head domains and a conserved stem domain, the researchers did not find significant T cell responses against the stem domain (54). This finding further highlights the need for in-depth studies to investigate HA2-specific T cell responses induced by vaccination, with a focus on mucosal responses (55,56).

In this study, we designed a recombinant adenovirus expressing a consensus sequence of the HA2 subunit of the H3 subtype through bioinformatic analysis. This intranasal vaccine demonstrated long-lasting cross-subtype protection against group 2 influenza A viruses by inducing robust antigen-specific mucosal and systemic cellular and humoral immune responses. Furthermore, mapping of the synthetic sequence allowed us to identify and characterize an immunogenic region of HA2 that induced significant T cell responses.

3.3 Method

3.3.1 Ethics

All animal procedures were performed in accordance with institutional guidelines and ethical approval was granted by the Animal Care Committee at Health Canada, Ottawa, ON, Canada, the National Research Council Human Health Therapeutics Animal Care Committee, Ottawa, ON, Canada, and the Public Health Agency of Canada,

Winnipeg, MB, Canada. Animal experiments were performed under Animal Utilization Protocol (AUP) 2024-006, 2024.01, and H21-019.

3.3.2 Generation of vaccine construct

A conserved consensus sequence of the HA2 subunit of influenza A H3 was identified using a previously described bioinformatics approach (57). In summary, the consensus sequence was generated by first retrieving all Influenza A H3 subtype HA sequences from the NCBI Flu database, applying filters for Flu A, all N subtypes, all hosts, all countries, and all subtypes. The dataset was then refined by eliminating redundant and ambiguous sequences, followed by alignment using Clustal-W. The final consensus sequence was determined using the EMBOSS software package.

The recombinant adenovirus construct (Ad-HA2) was generated as previously described (48). The Ad-HA2 construct was designed to express a trimeric, secreted form of the consensus HA2 with the human tyrosinase signal peptide (GenBank accession #AH003020) at the N-terminus. To stabilize the HA2 conformation without inducing H3 HA-specific immune responses, two fragments (amino acids 16–69 and 306–361) from the HA1 subunit of influenza B/Florida/04/06 joined by a GSGSG linker, were added to the N-terminus of the HA2 consensus sequence (58). The bacteriophage T4 fibritin trimerization motif (YIPEAPRDGQAYVRKDGEWVLLSTFLG) was added to the C-terminus of the HA2 sequence, connected by another GSGSG linker. An empty vector control was used as a control (Suppl Fig 3.8). rAds were generated using the Gateway®-adapted ViraPower™ adenoviral expression system (Life Technologies), according to the manufacturer's instructions. The e pAd/CMV/V5-DEST™ vector is a human type 5 replication-incompetent adenovirus. For vaccination, the rAds constructs were amplified

in HEK-293A cells and purified by ultracentrifugation with a 30% sucrose cushion. rAd stocks were titrated using the Adeno-X Rapid Titer Kit (Takara Bio USA Inc.).

3.3.3 Weight loss and survival studies

All animal experiments utilized female BALB/c mice (Charles River) between 5-6 weeks old. Mice received intranasal vaccination of 10^9 PFU of Ad-HA2 construct or Ad-Empty control on Day 0 and Day 28. Four weeks post-boost vaccination, mice were challenged intranasally with lethal doses of mouse-adapted A/Hong Kong/1/1968 (H3N2), mouse-adapted A/Anhui/1/2013 (H7N9) (59), A/Mexico/047/2009 (H1N1), or A/RT.Hawk/ON/2022 (H5N1). Challenged mice were weighed and monitored for signs of illness for 14 days post-challenge.

3.3.4 NALT and BALF collection

Various samples were collected from mice four weeks post-boost vaccination. Serum samples were collected to evaluate antibody levels and antibody-dependent cellular cytotoxicity. Bronchoalveolar lavage fluid (BALF) was collected for mucosal antibody analysis. Nasal-associated lymphoid tissue (NALT) was isolated according to a previously described protocol with modifications (60). Briefly, NALT was carefully removed from the upper palate of euthanized mice and washed eight times with RPMI 1640 media supplemented with 10% FBS to remove blood and debris. The tissue was then transferred to a fresh 48-well plate containing the same media with HA2 consensus sequence overlapping peptides at 5 µg/ml. The tissue was cultured at 37°C in a 5% CO2 incubator for 24 h, and the supernatant was collected for analysis of mucosal antibody and cytokine levels.

3.3.5 Enzyme-linked immunosorbent assay (ELISA)

The end-point titers of anti-HA antibodies in serum, NALT supernatant, and BALF samples were assessed using ELISA, following a previously described protocol (61). Briefly, 96-well plates were coated with 100 µl/well of recombinant influenza A H3N2 (A/Texas/50/2012) hemagglutinin (Sino Biological Inc.) at 0.5 µg/ml and incubated overnight at 4°C. Plates were then washed with PBS containing 0.05% Tween 20 (PBS-0.05T) and blocked with 3% BSA in PBS-0.05T. After blocking, plates were washed again, and two-fold serial dilutions of serum, NALT supernatant, or BALF in blocking buffer were added and incubated for 1 h at 37°C. Antibody binding was detected by HRP-conjugated anti-mouse IgG (Cytiva), IgG1, IgG2a, IgG2b (Jackson Immunoresearch Laboratories) or IgA (Life Technologies). Tetramethylbenzidine (TMB) substrate (Cell Signaling Technology) was added to develop the plates, and the reaction was stopped with 0.16 M sulfuric acid. Absorbance was measured at 450 nm (OD450), and end-point antibody titers were calculated as the reciprocals of the last detectable dilution. The cutoff was defined as the mean absorbance of control samples plus three standard deviations.

3.3.6 Enzyme-linked immunosorbent spot (ELISpot) assay

Spleens from vaccinated mice were collected four weeks post-vaccination and homogenized with a gentleMACS™ Dissociator (Miltenyi Biotec). Single-cell suspensions were stimulated with 5 µg/ml of indicated peptides. Cellular immune responses were evaluated using a Mouse IFN-γ Single-Color ELISPOT kit (ImmunoSpot by Cellular Technology Limited) following the manufacturer’s protocol. The plates were analyzed with an ImmunoSpot® S6 Analyzer for quantification.

3.3.7 Antibody-dependent cellular cytotoxicity (ADCC) assay

The ADCC activity of post-boost serum antibodies was assessed using the Promega ADCC Reporter Bioassay, following the manufacturer's protocol. In brief, 50,000 MDCK cells/well were plated in 96-well plates and incubated overnight. The cells were infected with A/Hong Kong/01/68 (H3N2) at a multiplicity of infection (MOI) of 5 and incubated for 24 h. Serum samples were heat-inactivated at 56°C for 30 minutes, serially diluted, and added to the infected cells. Mouse FcγRIV effector cells (Promega) were then added at 100,000 cells per well. Following a 5-hour incubation at 37°C with 5% CO_2, Bio-Glo™ luciferase assay substrate (Promega) was added. Luminescence was measured in relative luminescence units (RLU), and ADCC activity was expressed as fold induction relative to control wells without antibodies.

3.3.8 Multiplex ELISA

Lung-draining lymph nodes were collected from vaccinated animals and homogenized with a gentleMACS™ Dissociator (Miltenyi Biotec). Single-cell suspensions were stimulated with 5 μg/ml of HA2 consensus sequence overlapping peptides. Following a 24-hour incubation, the supernatant was collected for downstream measurements. NALT was harvested and cultured ex vivo following the protocol outlined in previous sections. Cytokine secretion in the supernatants from both tissues was assessed using the ProcartaPlex Multiplex Immunoassay kit (Life Technologies). The plates were then read on a Luminex 200 system (MilliporeSigma). Data analysis was conducted using MILLIPLEX Analyst version 5.1 software to determine cytokine concentrations in pg/ml.

3.3.9 Flow cytometry

Lung-draining lymph nodes were collected from vaccinated animals and homogenized with a gentleMACS™ Dissociator (Miltenyi Biotec). The lungs were digested with a lung dissociation kit according to the manufacturer's instructions (Miltenyi Biotec). The single-cell suspensions of each tissue were stimulated with 5 µg/ml of HA2 consensus sequence overlapping peptides for 5 hours at 37°C with 5% CO2. GolgiPlug and GolgiStop Protein Transport Inhibitors (BD Bioscience) were added 45 minutes into the stimulation incubation according to the manufacturer's protocol. Stimulated cells were washed and stained with viability stain and antibodies against extracellular markers, including LIVE/DEAD™ Fixable Violet Dead Cell Stain (Thermo Fisher Scientific Inc.), anti-CD3 PerCP-Vio® 700 REAfinity™, anti-CD4 VioGreen™ REAfinity™, and anti-CD8a PE-Vio® 770 REAfinity™ (Miltenyi Biotec). The cells were permeabilized and fixed with BD cytofix/cytoperm (BD Biosciences) for 20 min, followed by perm wash and stained with anti-mouse cytokine antibodies, including anti-IFN-γ FITC REAfinity™, anti-TNF-α PE REAfinity™, and anti-IL-2 APC, REAfinity™ (Miltenyi Biotec). The excess antibodies were washed away with FACS buffer and the samples were stored at 4°C prior to analysis by flow cytometry (FACSymphoney A1) the next day. Data analysis was completed using FlowJo version 10.10.0.

3.3.10 *In silico* analysis

MHC class binding predictions were performed using NetMHCpan 4.1 (62,63) and Immune Epitope Database (IEDB) Analysis Resource (64). The human allele panels for HLA class I and II were compiled to cover most of the population (65,66).

3.3.11 Statistical analysis

The P values for percent survival were calculated using the logrank test. Antigen-specific antibody levels and cytokine levels were subjected to two-tailed unpaired t-test analysis. Results from ELISpot assay were analyzed by one or two-way analysis of variance (ANOVA) when appropriate. Bonferroni post-tests were used to adjust for multiple comparisons between different test groups. All statistical analyses were performed using GraphPad Prism 9 software.

3.4 Results

3.4.1 Immunization with H3 subtype HA2 consensus sequence provides long-term protection

A conserved consensus sequence of the HA2 subunit of influenza A H3 was generated based on all available influenza A H3 subtype HA sequences in the NCBI Flu database. A recombinant adenoviruses construct (Ad-HA2) was designed to express a trimeric secreted form of the consensus HA2 with the human tyrosinase signal peptide on the N-terminus. The consensus HA2 sequence was stabilized by two fragments from the HA1 subunit of influenza B/Florida/04/06 (amino acids 16–69 and 306–361) (58), joined to the N-terminus of the HA2 consensus sequence by GSGSG linkers.

After optimizing the vaccine dose (Suppl Fig 3.9), we first evaluated the efficacy of the vaccine within the phylogenetic group 2. BALB/c mice were vaccinated intranasally with 10^9 PFU of Ad-HA2 or an empty vector control (Ad-Empty) twice at a 4-week interval. Four weeks post-boost, mice were challenged with a lethal dose of H3N2 or H7N9, a group 2 avian influenza subtype with pandemic potential (Fig 3.1A). Following the H3N2 challenge, Ad-HA2 immunization provided complete protection with 100% survival and

minimal body weight loss compared to the Ad-Empty control (Fig 3.1B). This trend was consistent with the H7N9 challenge, where Ad-HA2 afforded a survival rate of 100% and minimized weight loss to less than 10% (Fig 3.1C).

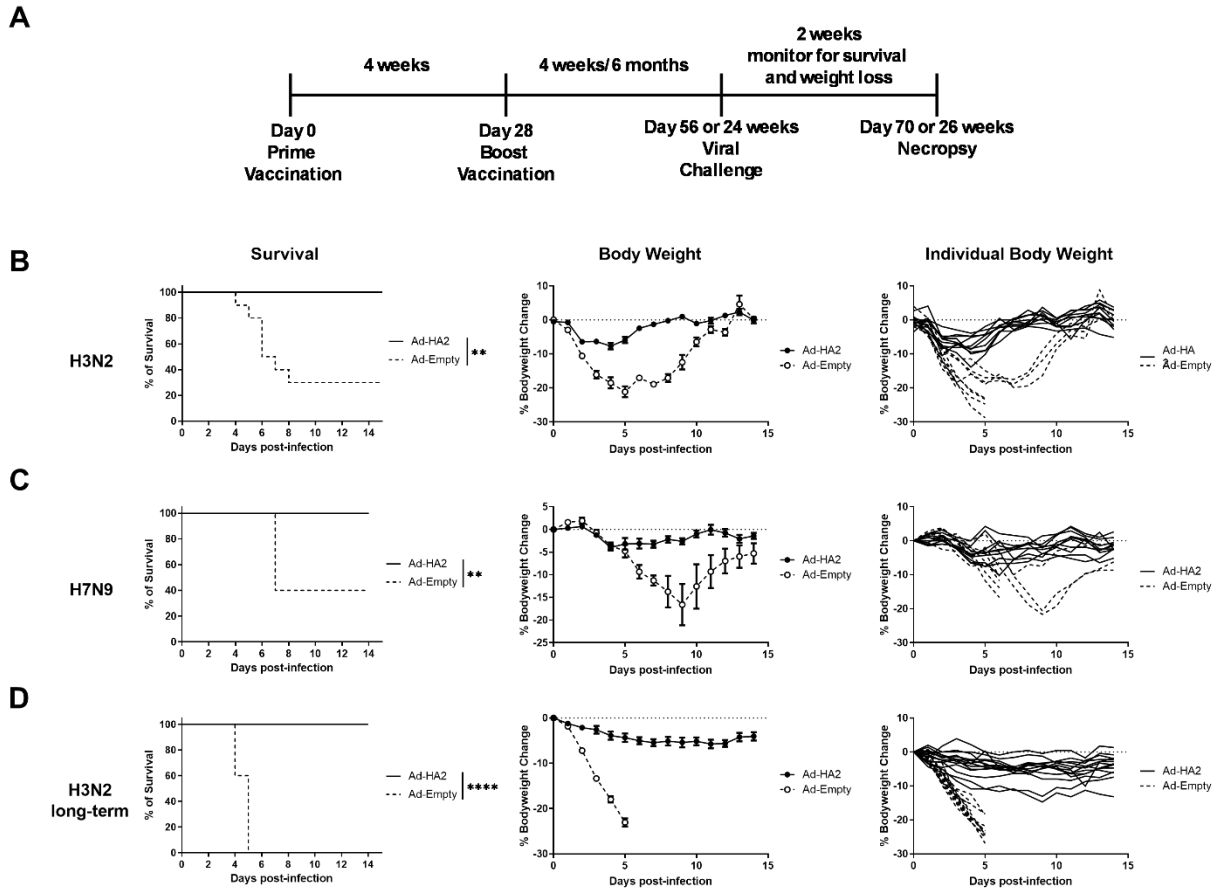


Figure 3.1 Immunization with Ad-HA2 provides short-term and long-term full protection against lethal challenges of IAV strains from group 2.

(A) Schematic diagram of the immunization, viral challenge, and necropsy timeline for short or long-term IAV challenge. BALB/c mice were intranasally administered 10^9 PFU of Ad-HA2 or Ad-Empty as a control with a prime/boost regimen, followed by an intranasal challenge of (B) H3N2 or (C) H7N9 four weeks post-boost. (D) Mice were challenged 6 months post-vaccination in the long-term protection study. (Left to right) Survival, average change in body weight, and individual body weight (n = 10) are shown. Data shown are mean ± SEM. **p < 0.01 and ****p < 0.0001.

To assess the longevity of the protective immune response induced by vaccination, an additional group of mice were challenged with H3N2 6-months after their second

vaccination. While all mice in the Ad-Empty group succumbed to infection by day 5 post-infection, Ad-HA2 immunization provided complete protection with 100% survival and minimal weight loss (Fig 3.1D). Overall, these results suggest that Ad-HA2 could provide effective long-term protection.

3.4.2 Immunization with H3 subtype HA2 consensus sequence is unable to provide protection against group 1 strains

Given the robust protection against the group 2 influenza strains and previous reports of cross-reactive antibodies (67,68), we next tested the vaccine against more phylogenetically removed group 1 IAV strains. Four weeks post-boost, vaccinated mice were challenged with a lethal dose of H1N1. Although slightly higher survival was observed in the vaccinated group, the difference was not statistically significant, and no protection was observed against weight loss (Fig 3.2A). In addition, we tested the vaccine against an HPAI H5N1 strain in clade 2.3.4.4b; the clade responsible for the ongoing avian flu outbreak. A/RT.Hawk/ON/2022 was recently isolated from a red-tailed hawk in Ontario, Canada. This strain exhibited significant virulence, lethality, and possible transmission in mammalian models (69). Similar to the trend we observed in the H1N1 challenge experiment, the vaccine did not provide significant protection against H5N1 (Fig 3.2B). Overall, these results suggest that Ad-HA2 is unable to provide significant protection against group 1 IAV strains.

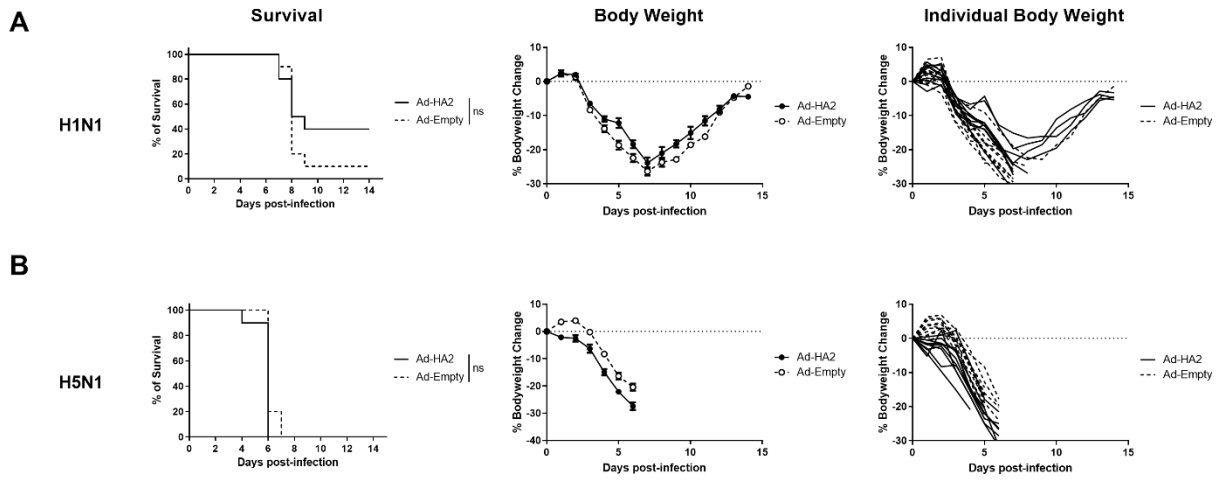


Figure 3.2 Immunization with Ad-HA2 provides no significant protection against challenges of IAV strains from group 1.

BALB/c mice were intranasally administered 10^9 PFU of Ad-HA2 or Ad-Empty as a control with a prime/boost regimen, followed by an intranasal challenge of (A) H1N1 or (B) an HPAI strain of H5N1 four weeks post-boost. (Left to right) Survival, average change in body weight, and individual body weight (n = 10) are shown. Data shown are mean ± SEM. ns = not significant.

3.4.3 Intranasal vaccination induces high levels of HA antibodies and antibody-dependent cellular cytotoxicity (ADCC)

As intranasal vaccinations are known to induce robust mucosal responses, we first investigated the humoral response generated by Ad-HA2 vaccination in the respiratory tract. As part of the upper respiratory tract, the nasal-associated lymphoid tissue (NALT) is the first site for the detection and elimination of inhaled pathogens such as IAV. In the NALTs collected four weeks post-vaccination (Fig 3.3A), without viral challenge, we observed significant levels of HA-specific IgG and IgA in the vaccinated group (Fig 3.3B). Ad-HA2 also induced significant levels of HA-specific IgG and IgA in the bronchoalveolar lavage fluid (BALF) (Fig 3.3C). These results show that intranasally administered Ad-HA2 can induce potent humoral responses in both the upper and lower respiratory tracts.

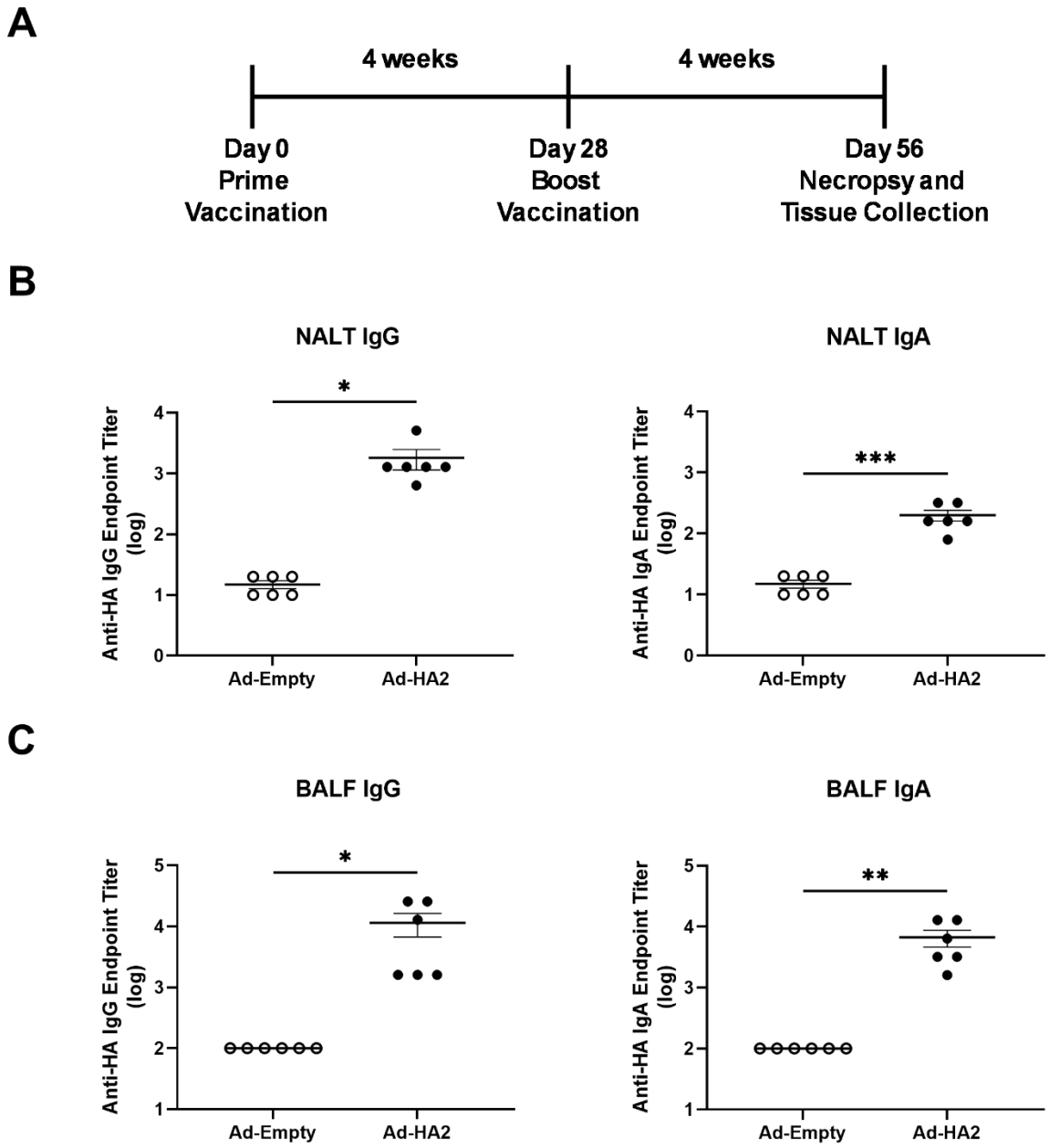


Figure 3.3 Intranasal administration of Ad-HA2 elicited strong HA-specific IgG and IgA antibody responses along the mucosal tract.

(A) Schematic diagram of the immunization and necropsy timeline for mechanistic investigations. BALB/c mice were intranasally administered 10^9 PFU Ad-HA2 or Ad-Empty as a control with a prime/boost regimen, followed by necropsy and sample collection on D56. (A) Nasal-associated lymphoid tissue (NALT) was collected and cultured *ex vivo* for 24 hours before the supernatant was collected to determine anti-HA IgG and IgA endpoint titers (n = 6). (B) Bronchoalveolar lavage fluid (BALF) was collected to determine anti-HA

IgG and IgA endpoint titers (n = 6). Data shown are mean ± SEM. *p < 0.05, **p <0.01, and ***p < 0.001.

Next, we assessed the systemic humoral response by quantifying the antibodies in the sera of vaccinated mice. We observed significant levels of HA-specific IgG and IgA antibodies in the immunized group compared to the control group (Fig 3.4A). Ad-HA2 vaccination induced significant levels of IgG1, IgG2a, and IgG2b in sera collected four weeks post-boost vaccination (Fig 3.4B). IgG2a is associated with a Th1 response, which plays an important role in inducing cell-mediated responses against intracellular pathogens, whereas IgG1 is associated with a Th2 response (70). Vaccination induced an IgG2a:IgG1 ratio slightly greater than 1.0, indicating a balanced Th1/Th2 response with a subtle Th1-skew, which is favorable for vaccines against respiratory viruses (Fig 3.4C) (71).

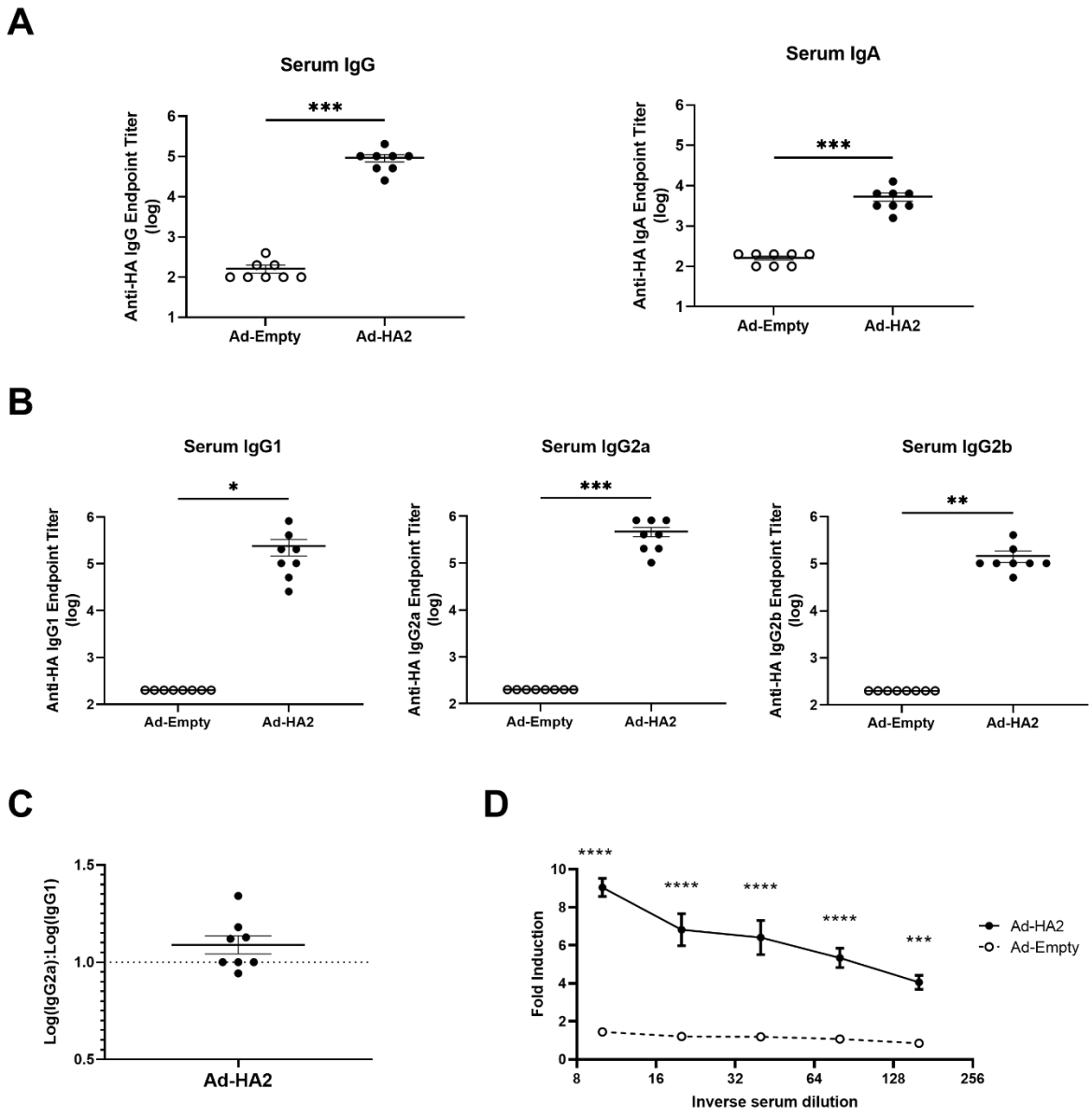


Figure 3.4 Intranasal administration of Ad-HA2 elicited strong HA-specific IgG and IgA antibody responses with balanced Th1/Th2 response and high ADCC activity.

(A) Serum from vaccinated mice were collected four weeks post-boost (Day 56) to determine log of anti-HA IgG and IgA endpoint titers (n = 8). (B) The same serum samples were collected to determine log of anti-HA IgG1, IgG2a, and IgG2b endpoint titers (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001 (C) Serum IgG2a:IgG1 ratio indicating a balanced and slightly Th1-biased immune response (n = 8). Data shown are mean ± SEM. (D) Post-boost serum was used to determine the antibody-dependent cellular cytotoxicity against A/Hong Kong/1/1968 (H3N2) (n = 8). Data shown are mean ± SEM.

IgG2a and IgG2b are recognized as highly effective IgG subclasses for inducing antibody-dependent cellular cytotoxicity (ADCC) (72). Given that significant levels of IgG2a and IgG2b antibodies were induced by the immunization, we next assessed their ADCC potential. Ad-HA2 vaccinated sera demonstrated strong ADCC activity against the same H3N2 strain used in the challenge experiments. Taken together, these results suggest that the administration of Ad-HA2 induces significant levels of mucosal and systemic humoral response, along with significant ADCC activity.

3.4.4 Ad-HA2 immunization induced balanced antiviral cytokines in the NALT and lung-draining lymph nodes

We next evaluated antigen-induced cytokine production in the NALT and lung-draining lymph nodes collected four weeks post-boost vaccination. Multiplex enzyme-linked immunosorbent assays (ELISAs) were performed to evaluate cytokine secretion after the NALT and lymph node cells were stimulated for 24h with an overlapping peptide pool covering the consensus HA2 sequence. We detected a broad increase in cytokine production by both the NALT (Fig 3.5A) and lung-draining lymph node cells (Fig 3.5B). Both Th1 cytokines IFN-γ, IL-2, IL-18, and TNF-α and Th2 cytokines IL-5 and IL-13 were secreted following antigen-stimulation, indicating that a balanced Th1/Th2 immune response was induced by intranasal administration of Ad-HA2.

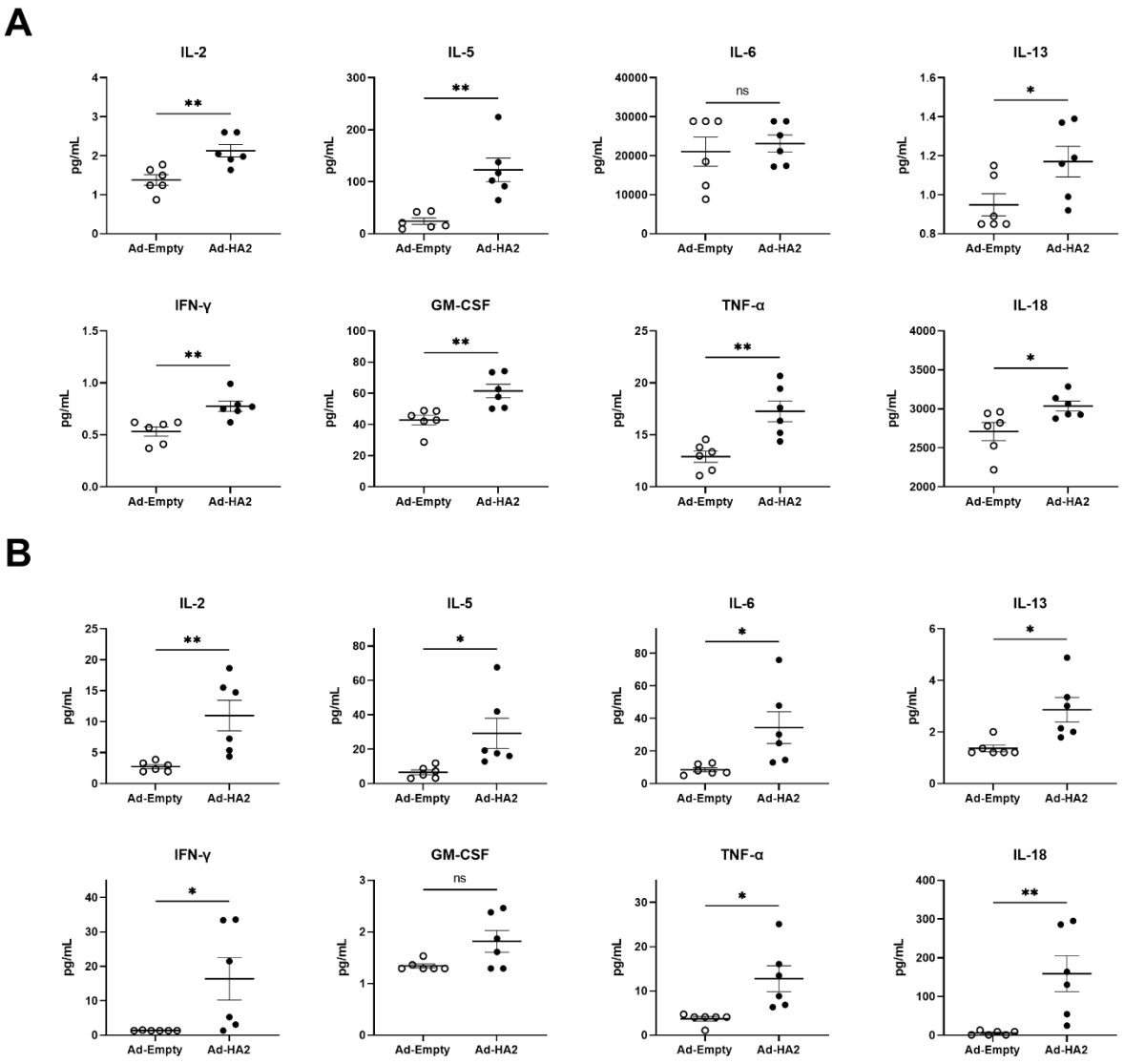


Figure 3.5 Immunization with Ad-HA2 induced antigen-specific cytokine responses in NALT and lung-draining lymph nodes.

(A) NALTs and (B) lung-draining lymph nodes were collected 4 weeks post-boost vaccination and stimulated with an overlapping peptide pool covering the consensus HA2 sequence in the vaccine (n = 6). Cytokines in the supernatant were quantified with a Luminex system. Data shown are mean ± SEM. ns = not significant, *$p < 0.05$, and **$p < 0.01$.

3.4.5 Ad-HA2 immunization induced robust pulmonary CD4+ and CD8+ T cell responses

CD4+ and CD8+ specific T-cell responses in the lungs and draining lymph nodes were further evaluated via intracellular cytokine staining (ICS) (Fig 3.6). We observed a

higher percentage of IFN-γ+ CD4+ T cells and IL-2+ CD8+ T cells in the lung tissues of vaccinated mice compared to the control group (Fig 3.5A). In the lung-draining lymph nodes, we detected a higher percentage of IFN-γ+ CD8+ T cells and higher percentages of IL-2+ and TNF-α+ CD4+ and CD8+ T cells (Fig 3.5B). Taken together, these results demonstrate that Ad-HA2 intranasal immunization induces robust pulmonary CD4+ and CD8+ T cell immune responses as evident by the antigen-induced cytokine secretion.

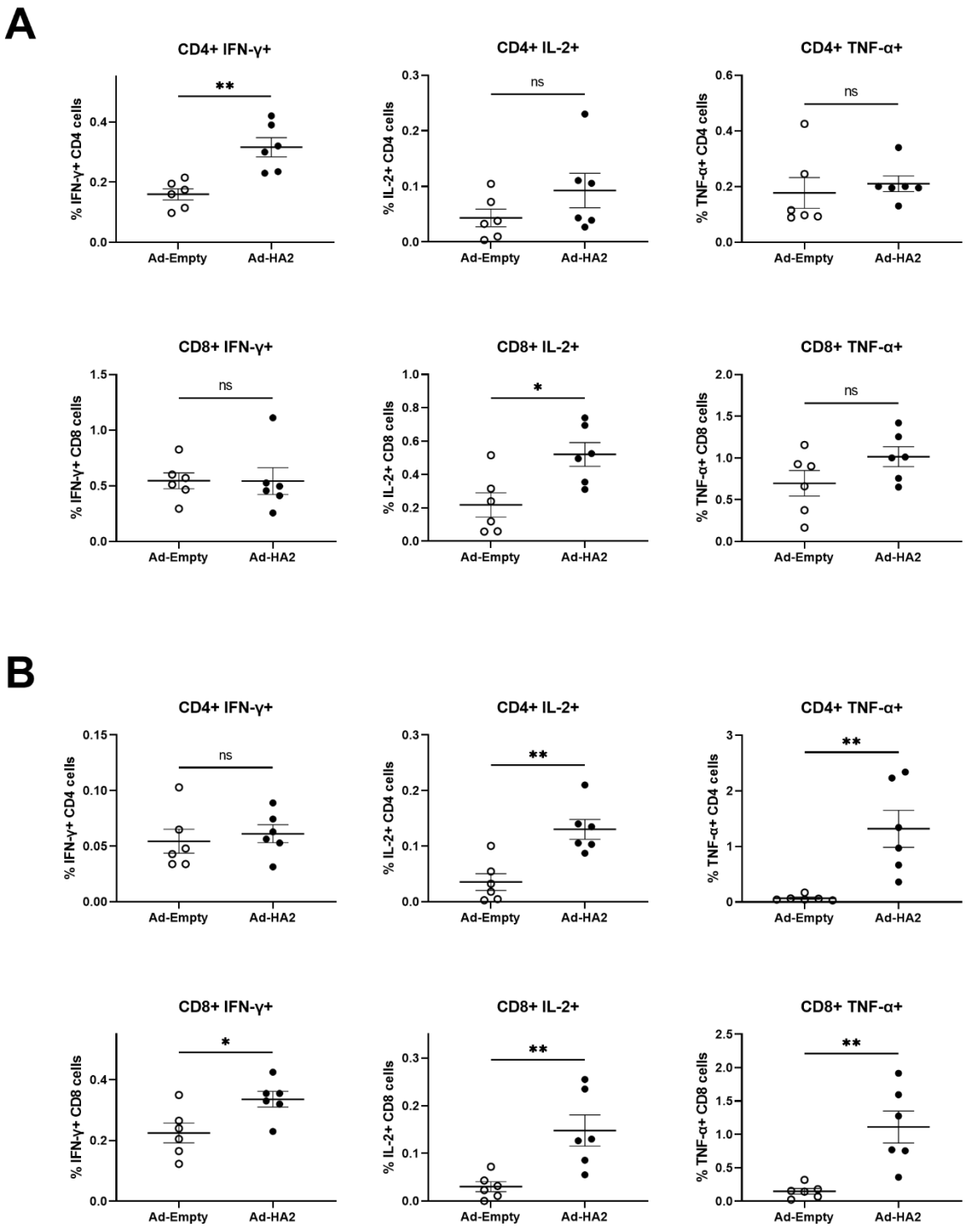


Figure 3.6 Immunization with Ad-HA2 induced antigen-specific cytokine responses in CD4+ and CD8+ T cells of the lungs and lung-draining lymph nodes.

(A) Lungs and (B) lung-draining lymph nodes were collected 4 weeks post-boost vaccination and stimulated with an overlapping peptide pool covering the consensus HA2 sequence in the vaccine (n = 6). T cell populations and cytokines were analyzed by flow cytometry, gating strategy outlined in Supp Fig 3.10. Data shown are mean ± SEM. ns = not significant, *p < 0.05, and **p < 0.01.

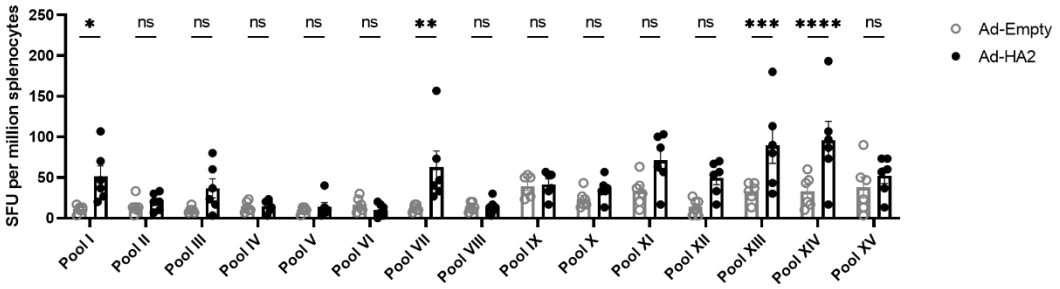
3.4.6 Identification of immunogenic epitopes on the C-terminal region of the HA2 consensus sequence

After confirming that immunization with the HA2 consensus sequences was able to induce antigen-specific cellular response, we sought to identify immunodominant regions within the sequence. We designed a peptide pool matrix with 15-mer peptides with 11-mer overlaps that covers the HA2 consensus vaccine sequence. The peptides were numbered 1 to 53; peptides 47 and 49 cannot be manufactured due to high hydrophobicity. However, the sequence within those two peptides are covered by adjacent peptides, 46, 48, and 50 with overlapping. The peptides are divided into peptide pools I to XV, as outlined in Fig 3.7B and Suppl Table 3.7.2. Compared to testing each peptide individually, this method decreases the number of assays and amount of animal tissues required (Suppl Tables 3.7.1 & 3.7.2) (73).

Four weeks post-boost vaccination, spleens were harvested and homogenized into single-cell suspensions. The splenocytes were stimulated with each of the matrix pools before cellular responses were measured by enzyme-linked immunosorbent spot (ELISpot) assay. Stimulation with pools I, VII, XIII, and XIV induced significant IFN-γ secretion in the splenocytes of vaccinated animals (Fig 3.7A). According to the matrix design, the common peptides in those four pools were peptides 36, 42, and 43 (Fig 3.7B). To confirm the immunogenicity of these candidate peptides, we performed another ELISpot assay where the splenocytes were stimulated with the individual candidate peptides, including three other peptides from the same pools as negative controls

(peptides 29, 38, and 45). No significant signal was detected in splenocytes stimulated with peptide 36, whereas peptides 42 and 43 induced significant IFN-γ compared to the unstimulated control (Fig 3.7C).

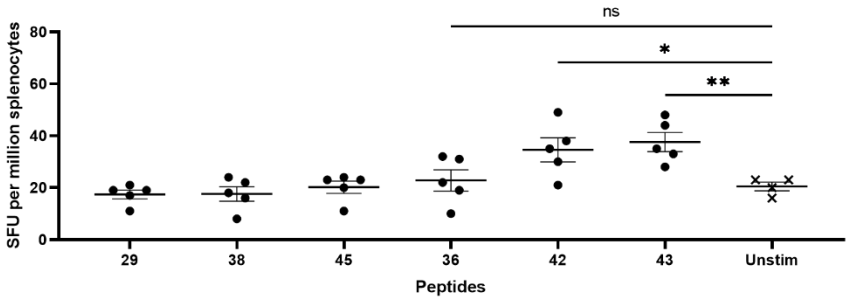
A



B

	I	II	III	IV	V	VI	VII
VIII	1	2	3	4	5	6	7
IX	8	9	10	11	12	13	14
X	15	16	17	18	19	20	21
XI	22	23	24	25	26	27	28
XII	29	30	31	32	33	34	35
XIII	36	37	38	39	40	41	42
XIV	43	44	45	46		48	
XV	50	51	52	53			

C



D

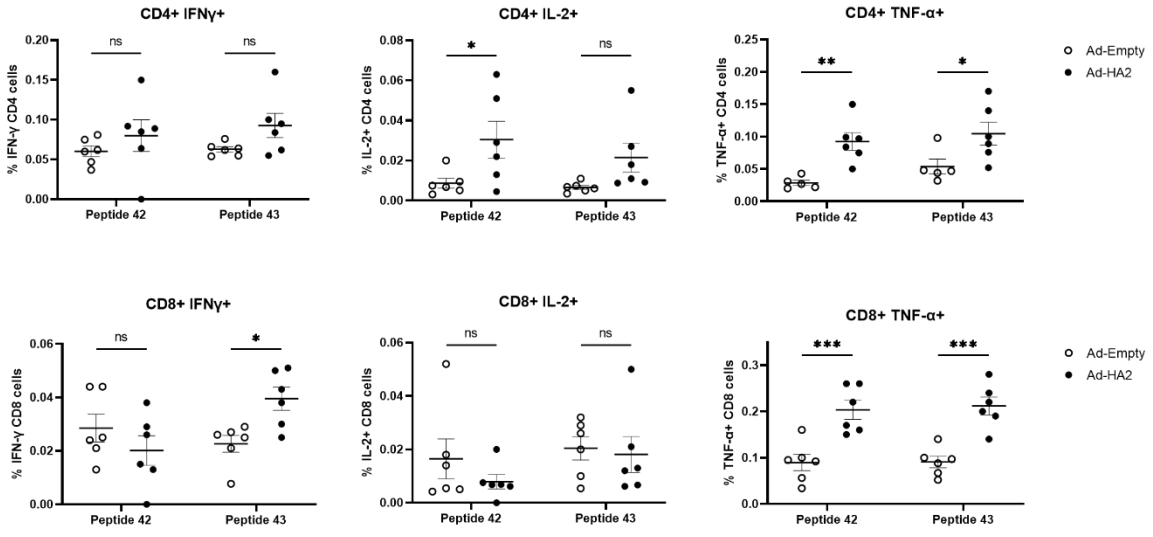


Figure 3.7 Peptide pool matrix identifies an immunogenic region on the HA2 consensus sequence that induces robust cellular responses.

(A) Splenocytes from vaccinated mice were collected four weeks post-boost (Day 56) and were stimulated with each of the peptide matrix pools (Pool I to XV) before ELISpot assays were performed. The number of IFN-γ spot-forming units were detected with an ImmunoSpot® S6 Analyzer. Data shown are mean ± SEM. ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. (B) Peptide pools that yielded positive signals are highlighted in light yellow in the peptide pool matrix, and the overlapping peptides that are potential immunogenic peptides are highlighted in bright yellow (peptides 36, 42, and 43). (C) Splenocytes from vaccinated mice were collected four weeks post-boost (Day 56) and were stimulated with each of the candidate (peptides 36, 42, or 43) or negative control (peptides 29, 38, or 45) before ELISpot assays were performed. The number of IFN-γ spot-forming units were detected with an ImmunoSpot® S6 Analyzer. Data shown are mean ± SEM. ns = not significant, *p < 0.05, and **p < 0.01. (D) Lung-draining lymph nodes from vaccinated mice were collected four weeks post-boost (Day 56) and were stimulated with peptide 42 or 43. T cell populations and cytokine production were characterized by flow cytometry, gating strategy outlined in Supp Fig 3.10. Data shown are mean ± SEM. ns = not significant, *p < 0.05, **p < 0.01, and ***p < 0.001.

We performed intracellular cytokine staining to further characterize the immune response induced by peptides 42 and 43. Single-cell suspensions of the mice lung-draining lymph nodes were stimulated with both peptides. Peptide 42 induced the expression of IL-2 and TNF-α in CD4+ T cells, as well as TNF-α expression in CD8+ T cells (Fig 3.7D). Peptide 43 induced TNF-α expression in CD4+ T cells and IFN-γ and TNF-α expression in CD8+ T cells (Fig 3.7D). The trend of cytokine production induced by the two peptides were comparable, which can be explained by the similarity in their peptide sequences. Overall, these results indicate that the region “EALNNRFQIKGVELKSGYK” is immunogenic and induces potent cellular responses (Supp Fig 3.11).

The identified immunogenic region was further validated with *in silico* major histocompatibility complex (MHC) binding affinity predictions using Immune Epitope Database (IEDB) tools (74). The predictions showed that this C-terminal proximal region

contains peptides with high binding affinity for both mouse and human T cell class I and class II MHCs (Table 3.4.1 & Supp Tables 3.7.3–6).

Table 3.4.1 Predicted epitopes derived from the synthetic HA2 consensus sequence.

The identified immunogenic peptide was bolded for each epitope. For the complete ranking of predicted epitopes, see Supp Tables 3.7.3–6.

Peptide	Allele	Median binding percentile	Eluted ligand score
T cell class I prediction for BALB/c			
SGYKDWILW	H2-Dd	0.08	0.155564
RFQIKGVEL	H2-Kd	0.2	0.328724
GYKDWILWI	H2-Kd	0.22	0.311665
KSGYKDWIL	H2-Dd	0.76	0.027577
RFQIKGVEL	H2-Dd	1.1	0.020072
T cell class II prediction for BALB/c			
ALNNRFQIKGVELKS	H2-IEd	0.11	0.334907
FQIKGVELKSGYKDW	H2-IAd	14	0.204478
TYDHDVYRD**EALNNR**	H2-IEd	26	0.009853
FQIKGVELKSGYKDW	H2-IEd	27	0.009642
T cell class I prediction for human 27 allele panel			
ALNNRFQIK	HLA-A*03:01	0.03	0.03
SGYKDWILW	HLA-B*58:01	0.08	0.08
SGYKDWILW	HLA-B*57:01	0.09	0.09
ALNNRFQIK	HLA-A*30:01	0.13	0.13
KGVELKSGY	HLA-A*30:02	0.16	0.16
T cell class II prediction for human 7 allele panel			
TYDHDVYRD**EALNNR**	HLA-DRB3*01:01	2.5	0.250132
ALNNRFQIKGVELKS	HLA-DRB5*01:01	2.7	0.319562
TYDHDVYRD**EALNNR**	HLA-DRB1*03:01	3.9	0.366681
TYDHDVYRD**EALNNR**	HLA-DRB3*02:02	6.5	0.085708
FQIKGVELKSGYKDW	HLA-DRB4*01:01	12	0.068338

3.5 Discussion

Current seasonal influenza vaccines primarily target the variable HA head domain, necessitating yearly updates to contend with the ever-evolving virus. The H3N2 subtype poses a particular challenge due to its affiliation with severe influenza seasons and poor vaccine efficacy (4,5,10). Targeting the conserved HA2 stem subunit offers a promising approach to generate cross-protection as demonstrated by numerous vaccine strategies delivering “headless” HA (15–46).

In this study, we investigated the breadth and durability of protection afforded by the intranasal administration of a recombinant adenovirus expressing a synthetic HA2 consensus sequence derived from all H3 subtypes. Given the numerous HA2 stem studies documented in literature, our present study was aimed at addressing the following uncharacterized aspects of HA2-induced immunity: we were particularly interested in the protection against HPAI subtypes, such as H7N9 and H5N1. We also aimed to characterize the pulmonary immune responses generated against HA2, with a focus on the NALT, an understudied early immune site for recognition and elimination of inhaled pathogens (61,75). Finally, we systematically delineated immunogenic HA2 T cell epitopes, which may have been overshadowed in previous studies by more dominant epitopes from the HA head or other antigens (49,52,53).

The Ad-HA2 vaccine demonstrated heterosubtypic protection, providing strong immunity to lethal challenge with both H3N2, a seasonal influenza strain with heightened disease burden, and H7N9, an avian influenza strain that causes sporadic human infections with high mortality rates (Fig 3.1B&C) (10,76). The induced immunity was also

durable, with Ad-HA2 providing complete protection against H3N2 challenge six months post-vaccination (Fig 3.1D). These results largely agree with other vaccination strategies regarding the durability and breadth of HA stem-specific immune responses (24,77). While effective protection was observed against H7N9, the vaccine was unable to afford protection against group 1 subtypes, such as H1N1 and H5N1 (Fig 3.2).This inability to protect across divergent groups has also been observed in other studies targeting HA2 (43,78). This observation highlights a possible limitation of utilizing HA2 as a target antigen for universal influenza vaccines. However, it is worth noting that universal protection may potentially be achieved through adjuvantation (79,80).

Intranasal vaccination with Ad-HA2 induced significant anti-HA IgG and IgA antibodies in both the upper and lower respiratory tract (Fig 3.3). Based on the IgG subtype titers in the serum, the vaccine induced a balanced and slightly Th1-skewed response (Fig 3.3C). This is favorable as Th2-biased immune responses have been associated with the enhancement of lung pathology and disease progression (81). Despite the presence of high antibody titers, no neutralizing activity was detected by an *in vitro* microneutralization assay (not shown). Headless HA2 delivered by other vaccine platforms such as mRNA (82) and recombinant protein (41) have also demonstrated protection in the absence of neutralizing antibodies. Broadly reactive stem-specific antibodies can provide *in vivo* protection through Fc-mediated effector functions, such as ADCC (83,84). Indeed, although the Ad-HA2 induced antibodies had no neutralizing activity, the intranasal vaccine induced high levels of ADCC activity in the sera (Fig 3.4D). These results suggest that Ad-HA2 elicits both robust mucosal and systemic humoral

responses, which contribute to protection through Fc-mediated effector functions such as ADCC.

Cytokine profiling can shed light on vaccine-induced T cell responses in the lung-draining lymph nodes and mucosal tissues. We detected significant increases in antiviral cytokines in both the NALT and lung-draining lymph nodes (Fig 3.5), including IFN-γ, TNF-α, and IL-2, indicating that cell-mediated immunity was induced at both sites. We detected the production of both Th1 and Th2 cytokines, again indicating that intranasal vaccination with Ad-HA2 induces a balanced Th1/Th2 immune response. Further analysis of T cell responses revealed induction of antigen-specific CD4+ and CD8+ T cells in the lymph nodes (Fig 3.6B). Interestingly, we did not observe any significant T cell responses in the spleen by ICS, likely due to the intranasal administration of adenovirus-vectored vaccines favouring local responses (61). Lastly, antigen-specific CD4+ and CD8+ T cells in the lungs had fewer upregulated cytokines compared to the lymph nodes, which could be due to site-specific responses or the more rapid decline of memory T cells in the lungs (85). Future longitudinal studies could be conducted to dissect the durability of T cell responses in the mucosal tissues and their interactions with and distinction from those in lymphoid organs.

As interest has grown in the HA stalk as a target for universal influenza vaccines, several studies have aimed to identify T cell epitopes within this region (86,87). In order to identify and characterize the immunogenic region in our consensus HA2 sequence, we employed a peptide pool matrix method (Fig 3.7). We found that the C-terminus region “EALNNRFQIKGVELKSGYK” was immunogenic and induced antigen-specific CD4+ and CD8+ responses (Fig 3.7D). This may partially explain the lack of protection against group

1 influenza strains, as this region is poorly conserved across the two groups, with amino acids differing in their physicochemical properties (Supp Fig 3.11). The *in silico* MHC binding affinity predictions showed that this region contains peptides with high binding affinity for both mouse and human T cell class I and class II MHCs (Table 3.4.1 & Supp Tables 3.7.3–6), corroborating our *in* vivo findings and highlighting its potential for future investigations and human applications. T cell responses specific to this C-terminus region have also been identified after natural H3N2 infection in humans (88). A portion of our identified sequence, “RFQIKGVEL”, has also been shown to generate T cell responses in mice (89,90). Recently, Yuan and colleagues utilized this short peptide along with 36 other *in silico* predicted epitopes in a broadly protective multi-epitope vaccine (91). Interestingly, the authors observed that lower levels of immune responses were generated against the H3 peptide pool compared to other subtypes (91), suggesting heterosubtypic immunodominance among the influenza strains. Our study highlights the need to identify immunogenic HA2 regions in the absence of other competing epitopes to better understand T cell responses and advance vaccine design.

Despite the well-established role of T cell responses in providing cross-protection against influenza, HA2-specific T cell responses have been relatively understudied compared to the humoral responses. In this study, we demonstrated that a synthetic sequence encoding consensus H3 HA2 provided durable cross-subtype protection with robust humoral and cell-mediated responses indicating effective pulmonary immunity, in mucosal tissues such as the NALT. Nonetheless, the failure of protection against group 1 IAV, H1 and H5 subtypes, highlights the challenge in utilizing HA2 alone as a universal vaccine candidate against both groups of IAV. This may be due not only to sequence

differences, but also the virulence associated with some HPAI strains. By employing peptide pool matrix in combination with *in silico* verification, we also revealed an immunogenic H3 HA2 region capable of eliciting strong T cell responses, which warrants further investigation to help inform the design of future influenza vaccines.

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Author contributions

Wanyue Zhang: conceptualization of the project, conducting the experiments, data analysis and visualization, draft and edit manuscript

Gary Van Domselaar: vaccine construct sequence generation

Angela Sloan & Jérémie Prévost: H3N2 animal experiments at NML, reviewing manuscript

Darwyn Kobasa & David Safronetz: supervision at NML

Levi Tamming, Sathya Raman & Annabelle Pfeifle: helping with some experiments, reviewing manuscript

Caroline Gravel: lab management

Anh Tran: animal experiment collaborator at NRC (data not included)

Xuguang Li, Wangxue Chen, Xu Zhang & Michael J.W. Johnston: funding acquisition

Lisheng Wang, Simon Sauve, Michael Rosu-Myles & Xuguang Li: supervision

Declaration of interests

The authors declare that there are no competing interests.

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3.7 Supplemental Materials

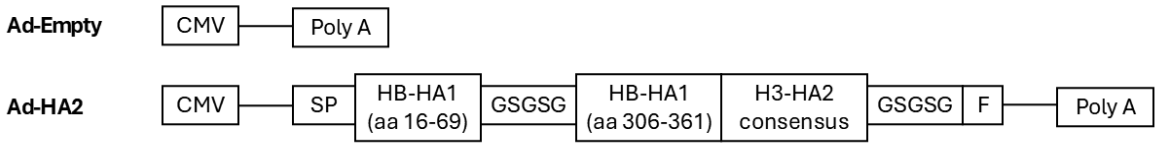

Figure 3.8 Schematic representation of the recombinant adenovirus constructs.

The constructs utilize the cytomegalovirus promoter (CMV) to drive the expression and end with a polyadenylation tail (Poly A). The Ad-Empty does not encode for any protein. The Ad-HA2 construct encodes for a human tyrosinase secretion signal peptide (SP), followed by two fragments (amino acids 16–69 and 306–361) from the HA1 subunit of influenza B/Florida/04/06 (HB-HA1) joined by a GSGSG linker to stabilize the HA2 consensus sequence (H3-HA2), and a trimerization motif from the T4 bacteriophage fibritin (F).

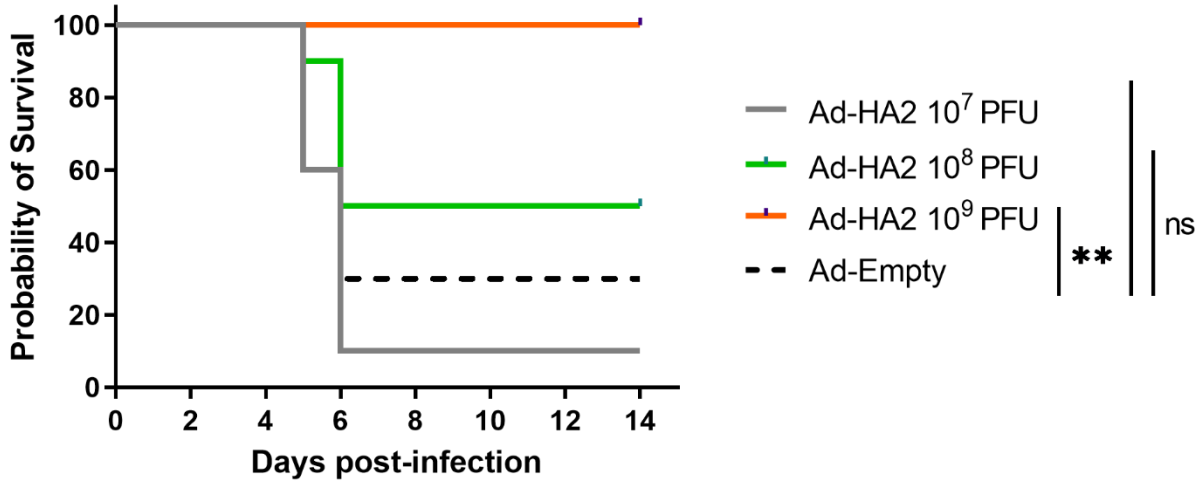

Figure 3.9 Vaccination dosage optimization challenge study determined that two dosages of 10^9 PFU of Ad-HA2 is the most effective.

BALB/c mice were intranasally administered Ad-HA2 at 10^7, 10^8, or 10^9 PFU or Ad-Empty at 10^9 PFU as a control with a prime/boost regimen, followed by a lethal challenge of H3N2 four weeks post-boost. Probability of survival is shown (n = 10) ns = not significant and **p < 0.01.

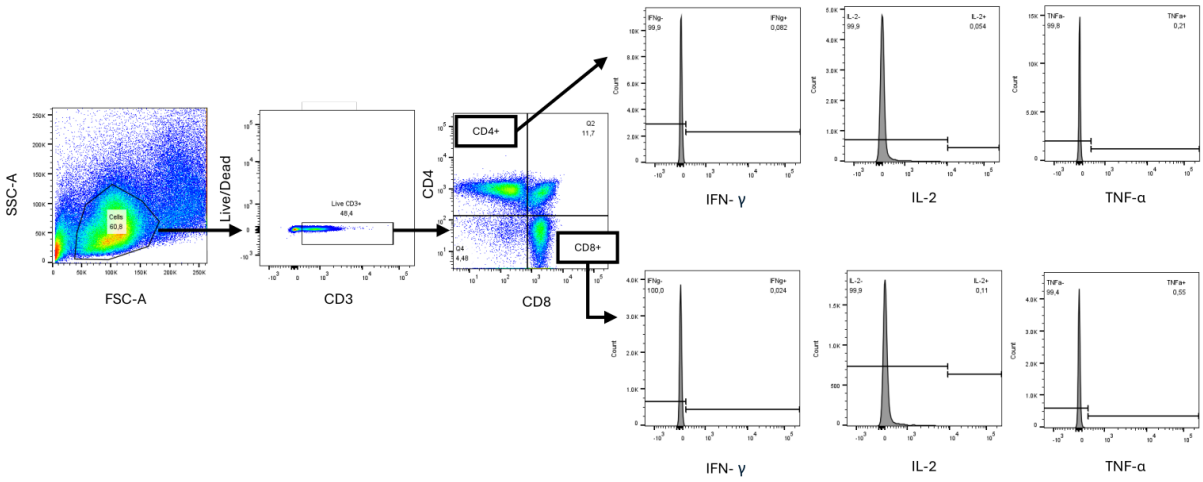

Figure 3.10 Representative gating strategy for intracellular cytokine staining flow experiments in Fig 3.5 and Fig 3.6D.

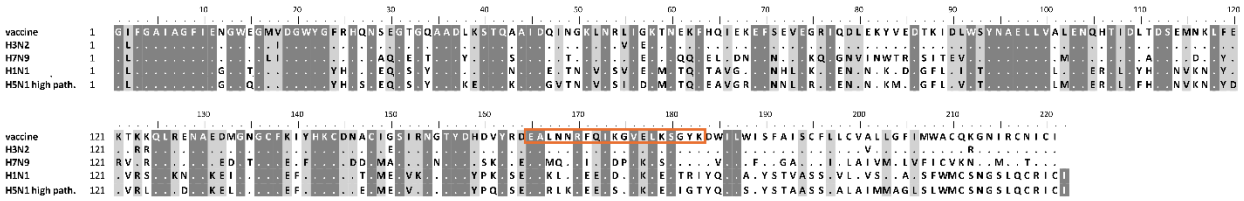

Figure 3.11 Alignment of the vaccine consensus HA2 sequence against various IAV subtypes.

The identified immunogenic region on the HA2 consensus sequence indicated with orange box.

Table 3.7.1 Sequences of 15-mer peptides with 11-mer overlaps that covers the HA2 vaccine sequence

Peptide #	Sequence	Peptide #	Sequence	Peptide #	Sequence
1	GIFGAIAGFIENGWE	19	VEGRIQDLEKYVEDT	37	DNACIGSIRNGTYDH
2	AIAGFIENGWEGMVD	20	IQDLEKYVEDTKIDL	38	IGSIRNGTYDHDVYR
3	FIENGWEGMVDGWYG	21	EKYVEDTKIDLWSYN	39	RNGTYDHDVYRDEAL
4	GWEGMVDGWYGFRHQ	22	EDTKIDLWSYNAELL	40	YDHDVYRDEALNNRF
5	MVDGWYGFRHQNSEG	23	IDLWSYNAELLVALE	41	VYRDEALNNRFQIKG
6	WYGFRHQNSEGTGQA	24	SYNAELLVALENQHT	42	EALNNRFQIKGVELK
7	RHQNSEGTGQAADLK	25	ELLVALENQHTIDLT	43	NRFQIKGVELKSGYK
8	SEGTGQAADLKSTQA	26	ALENQHTIDLTDSEM	44	IKGVELKSGYKDWIL
9	GQAADLKSTQAAIDQ	27	QHTIDLTDSEMNKLF	45	ELKSGYKDWILWISF
10	DLKSTQAAIDQINGK	28	DLTDSEMNKLFEKTK	46	GYKDWILWISFAISC

11	TQAAIDQINGKLNRL	29	SEMNKLFEKTKKQLR	47	WILWISFAISCFLLC*
12	IDQINGKLNRLIGKT	30	KLFEKTKKQLRENAE	48	ISFAISCFLLCVALL
13	NGKLNRLIGKTNEKF	31	KTKKQLRENAEDMGN	49	ISCFLLCVALLGFIM*
14	NRLIGKTNEKFHQIE	32	QLRENAEDMGNGCFK	50	LLCVALLGFIMWACQ
15	GKTNEKFHQIEKEFS	33	NAEDMGNGCFKIYHK	51	ALLGFIMWACQKGNI
16	EKFHQIEKEFSEVEG	34	MGNGCFKIYHKCDNA	52	FIMWACQKGNIRCNI
17	QIEKEFSEVEGRIQD	35	CFKIYHKCDNACIGS	53	ACQKGNIRCNICI
18	EFSEVEGRIQDLEKY	36	YHKCDNACIGSIRNG		

*Cannot be manufactured due to high hydrophobicity

Table 3.7.2 Peptide pool matrix design

Pool	**Peptide #**							
I	1	8	15	22	29	36	43	50
II	2	9	16	23	30	37	44	51
III	3	10	17	24	31	38	45	52
IV	4	11	18	25	32	39	46	53
V	5	12	19	26	33	40		
VI	6	13	20	27	34	41	48	

VII	7	14	21	28	35	42		
VIII	1	2	3	4	5	6	7	
IX	8	9	10	11	12	13	14	
X	15	16	17	18	19	20	21	
XI	22	23	24	25	26	27	28	
XII	29	30	31	32	33	34	35	
XIII	36	37	38	39	40	41	42	
XIV	43	44	45	46		48		
XV	50	51	52	53				

Table 3.7.3 IEDB T cell class I prediction for BALB/c with the identified immunogenic region on the HA2 consensus sequence highlighted

Peptide	Allele	Median binding percentile	Eluted ligand score
SGYKDWILW	**H2-Dd**	**0.08**	**0.155564**
KTNEKFHQI	H2-Dd	0.11	0.119308
VALENQHTI	H2-Dd	0.11	0.113062
RFQIKGVEL	**H2-Kd**	**0.2**	**0.328724**
GYKDWILWI	**H2-Kd**	**0.22**	**0.311665**
YNAELLVAL	H2-Dd	0.44	0.044367

VALENQHTI	H2-Kd	0.47	0.169564
VALLGFIMW	H2-Dd	0.58	0.034916
SFAISCFLL	H2-Kd	0.61	0.134408
LFEKTKKQL	H2-Kd	0.63	0.127812
VALENQHTI	H2-Ld	0.72	0.108971
KSGYKDWIL	**H2-Dd**	**0.76**	**0.027577**
KFHQIEKEF	H2-Kd	0.87	0.088075
YNAELLVAL	H2-Ld	0.88	0.087079
LWSYNAELL	H2-Kd	0.93	0.081941
SYNAELLVA	H2-Kd	0.95	0.079255
RFQIKGVEL	**H2-Dd**	**1.1**	**0.020072**

Table 3.7.4 IEDB T cell class II prediction for BALB/c with the identified immunogenic region on the HA2 consensus sequence highlighted

Peptide	Allele	Median binding percentile	Eluted ligand score
ALNNRFQIKGVELKS	**H2-IEd**	**0.11**	**0.334907**
NKLFEKTKKQLRENA	H2-IEd	0.46	0.214345
EKFHQIEKEFSEVEG	H2-IEd	5.2	0.049105
GTGQAADLKSTQAAI	H2-IAd	5.7	0.363346
ADLKSTQAAIDQING	H2-IAd	7.3	0.312512
GMVDGWYGFRHQNSE	H2-IEd	8.6	0.033318
KLNRLIGKTNEKFHQ	H2-IEd	8.8	0.032588
WYGFRHQNSEGTGQA	H2-IEd	12	0.024874
FQIKGVELKSGYKDW	**H2-IAd**	**14**	**0.204478**
RIQDLEKYVEDTKID	H2-IAd	18	0.162606

ALENQHTIDLTDSEM	H2-IAd	19	0.154933
IEKEFSEVEGRIQDL	H2-IAd	19	0.148259
DQINGKLNRLIGKTN	H2-IEd	19	0.014956
HQNSEGTGQAADLKS	H2-IAd	22	0.13029
IEKEFSEVEGRIQDL	H2-IEd	23	0.011745
TYDHDVYRDEALNNR	**H2-IEd**	**26**	**0.009853**
KTKKQLRENAEDMGN	H2-IAd	27	0.104927
LWSYNAELLVALENQ	H2-IAd	27	0.103881
FQIKGVELKSGYKDW	**H2-IEd**	**27**	**0.009642**

Table 3.7.5 IEDB T cell class I prediction for human 27 allele panel with the identified immunogenic region on the HA2 consensus sequence

Peptide	Allele	Median binding percentile	Eluted ligand score
ALNNRFQIK	HLA-A*03:01	0.03	0.03
SGYKDWILW	HLA-B*58:01	0.08	0.08
SGYKDWILW	HLA-B*57:01	0.09	0.09
ALNNRFQIK	HLA-A*30:01	0.13	0.13
KGVELKSGY	HLA-A*30:02	0.16	0.16
GYKDWILWI	HLA-A*23:01	0.25	0.25
RFQIKGVEL	HLA-B*08:01	0.29	0.29
ALNNRFQIK	HLA-A*11:01	0.31	0.31
GYKDWILWI	HLA-A*24:02	0.31	0.31
SGYKDWILW	HLA-A*32:01	0.33	0.33
RFQIKGVEL	HLA-A*24:02	0.37	0.37
RFQIKGVEL	HLA-A*23:01	0.49	0.49

SGYKDWILW	HLA-B*53:01	0.53	0.53
KGVELKSGY	HLA-B*15:01	0.69	0.69
FQIKGVELK	HLA-A*11:01	0.8	0.8
GVELKSGYK	HLA-A*11:01	0.88	0.88
GVELKSGYK	HLA-A*03:01	0.91	0.91
ALNNRFQIK	HLA-A*31:01	0.94	0.94

Table 3.7.6 IEDB T cell class II prediction for human 7 allele panel with the identified immunogenic region on the HA2 consensus sequence highlighted

Peptide	Allele	Median binding percentile	Eluted ligand score
AELLVALENQHTIDL	HLA-DRB1*15:01	2.5	0.462748
TYDHDVYRDEALNNR	**HLA-DRB3*01:01**	**2.5**	**0.250132**
DTKIDLWSYNAELLV	HLA-DRB1*15:01	2.6	0.459097
EKYVEDTKIDLWSYN	HLA-DRB3*01:01	2.6	0.248062
ALNNRFQIKGVELKS	**HLA-DRB5*01:01**	**2.7**	**0.319562**
EKYVEDTKIDLWSYN	HLA-DRB1*03:01	3.4	0.416309
KTKKQLRENAEDMGN	HLA-DRB4*01:01	3.6	0.201081
TYDHDVYRDEALNNR	**HLA-DRB1*03:01**	**3.9**	**0.366681**
NKLFEKTKKQLRENA	HLA-DRB5*01:01	4.2	0.245892
NKLFEKTKKQLRENA	HLA-DRB1*03:01	4.7	0.313489
ADLKSTQAAIDQING	HLA-DRB1*07:01	4.9	0.326067
WYGFRHQNSEGTGQA	HLA-DRB3*02:02	5.6	0.100473
IEKEFSEVEGRIQDL	HLA-DRB5*01:01	6	0.18257
TYDHDVYRDEALNNR	**HLA-DRB3*02:02**	**6.5**	**0.085708**
WYGFRHQNSEGTGQA	HLA-DRB5*01:01	8	0.141074

IEKEFSEVEGRIQDL	HLA-DRB1*07:01	8.8	0.194578
IAGFIENGWEGMVDG	HLA-DRB3*02:02	9	0.057239
SEVEGRIQDLEKYVE	HLA-DRB4*01:01	9.4	0.085703
KLNRLIGKTNEKFHQ	HLA-DRB1*15:01	9.6	0.159961
EKYVEDTKIDLWSYN	HLA-DRB4*01:01	9.8	0.081715
AELLVALENQHTIDL	HLA-DRB4*01:01	10	0.079628
SIRNGTYDHDVYRDE	HLA-DRB1*03:01	11	0.126657
DQINGKLNRLIGKTN	HLA-DRB1*15:01	12	0.123338
GTGQAADLKSTQAAI	HLA-DRB1*03:01	12	0.112379
WYGFRHQNSEGTGQA	HLA-DRB4*01:01	12	0.070495
FQIKGVELKSGYKDW	**HLA-DRB4*01:01**	**12**	**0.068338**

4 Discussion and Conclusion

Seasonal influenza remains a major global health burden, causing up to a billion infections and as many as 650,000 deaths annually[1]. Additionally, the threat of an influenza pandemic remains a constant concern due to its extensive animal reservoirs. The unprecedented HPAI H5N1 outbreaks seen in wildbirds, poultry, and dairy farms over the last few years underscore this threat[238]. The inconsistent VE of current influenza vaccines reflects their failure to keep up with the viral evolution and the inability to induce mucosal immunity[239]. To address these two limitations, this thesis utilizes IN delivery of conserved viral antigens to promote heterosubtypic protection by engaging the humoral and cell-mediated mucosal immunity.

4.1 Route of Vaccination Shapes Mechanisms of Protection

Despite growing interest in mucosal vaccination strategies against influenza, comprehensive comparisons of IN and IM administration and how they affect both local and systemic immunity remain limited. In chapter 2, I compared the IN and IM administration of an adenoviral vector-based vaccine encoding NP fused with a CD40L molecular adjuvant. Humoral and cellular responses at both the local and systemic sites were characterized, including tissue-resident T cell formation, and cytokine profile. Breadth of protection was assessed against commonly circulating subtypes and in light of the H5N1 outbreaks, the protective efficacy against a highly lethal and recently isolated HPAI H5N1 strain was also tested.

IM administration of Ad-NP-CD40L generated high levels of circulating antibodies with potent ADCC activity. It also elevated cytokine responses in the spleen, indicative of activated peripheral T cells. Despite inducing potent systemic immunity, IM administration

did not protect against heterosubtypic H3N2 and H5N1 challenge. On the other hand, IN delivery demonstrated superior cross-protective efficacy, providing full protection against H3N2 challenge and partial protection against the HPAI H5N1 strain. Although IN administration induced a lower systemic T cell response compared to IM administration, a higher response was detected in the NALT. In order to delineate the protection conferred by circulating and resident immunity, I blocked lymphocyte trafficking using FTY720, a drug that sequesters lymphocytes to lymph nodes and prevents them from entering circulation. The protective efficacy of IM vaccination was significantly compromised, revealing its dependence on circulating immune cells, while protection in the IN group was unaffected.

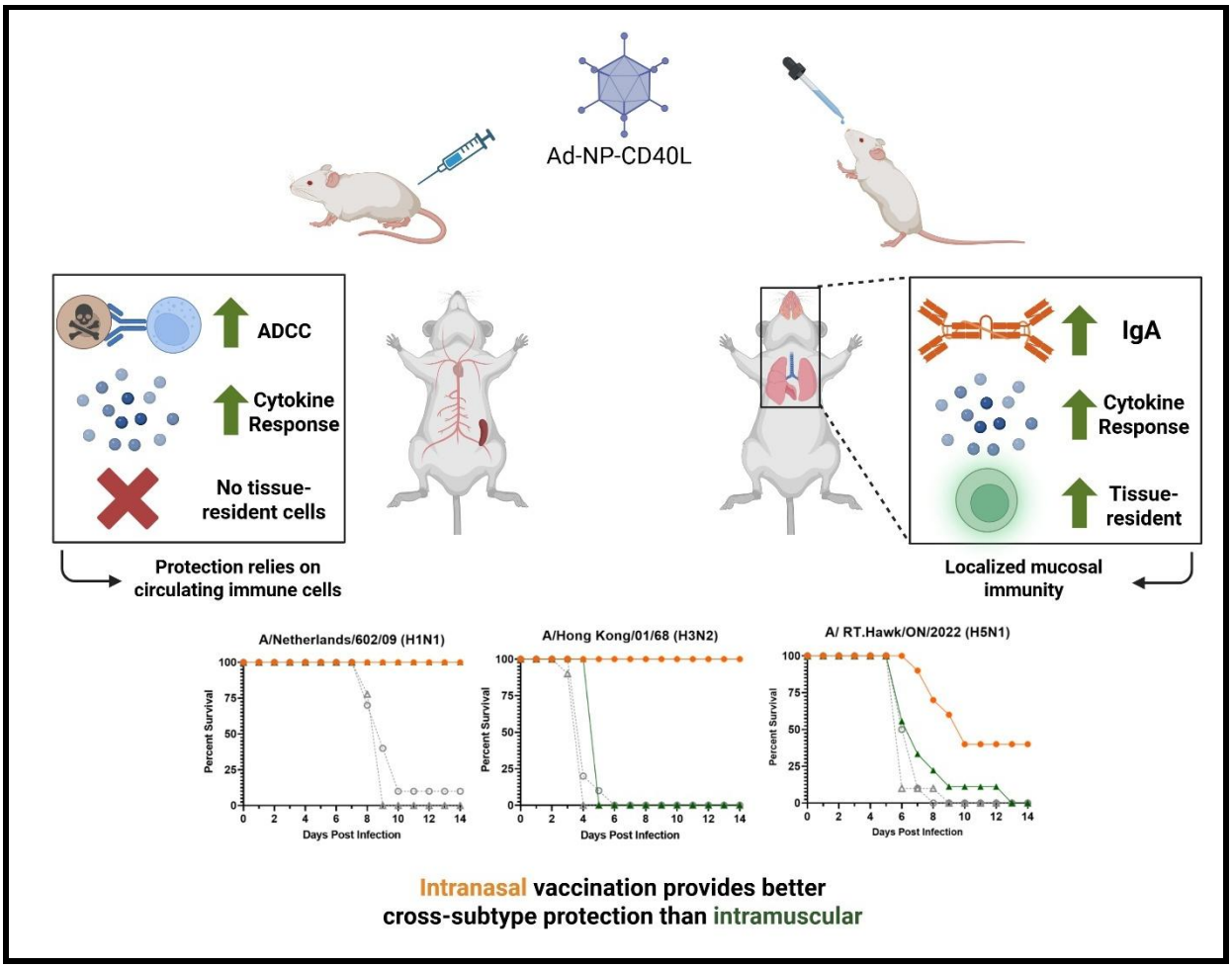

Figure 4.1 Graphic overview of Chapter 2 - Dissecting immunological mechanisms underlying influenza viral nucleoprotein-induced mucosal immunity against diverse viral strains.

This study provides compelling evidence that the route of administration fundamentally shapes the quality and localization of vaccine-induced immune responses. Despite driving strong systemic responses against a highly conserved antigen, IM vaccination failed to provide heterosubtypic protection. In contrast, IN administration of Ad-NP-CD40L not only enhanced mucosal immunity but also broadened the cross-subtype protection. In addition, both humoral and cell-mediated immune responses were characterized in the NALT, a critical yet understudied secondary lymphoid organ in the URT that serves as the first line of defense against inhaled pathogens.

4.2 Mechanistic Insight into HA2-induced Protection

Most HA2 vaccine studies have focused on eliciting humoral responses, leaving the T cell responses relatively underexplored. The majority of studies evaluating HA2-specific T cell responses do so in the presence of full-length HA or within multivalent vaccine constructs, where epitope competition can obscure their contribution. To address these gaps, the study in chapter 3 employed a recombinant adenoviral vector expressing a consensus HA2 sequence.

The vaccine conferred robust and durable protection against homologous H3N2 and heterosubtypic H7N9 challenge, but no protection was observed against the phylogenetically distant group 1 subtypes, namely H1N1 and H5N1. Robust T cell responses were observed in both the lungs and draining lymph nodes, with a balanced Th1/Th2 cytokine profile. In addition, I identified an immunodominant epitope in the C-terminal region of the HA2 consensus sequence that is capable of eliciting potent CD4+ and CD8+ T cell responses. Based on *in silico* MHC binding affinity prediction, this epitope holds potential for future human vaccine applications. Collectively, these results showed that a consensus H3 HA2 vaccine delivered intranasally elicits durable mucosal and systemic immunity against group 2 IAV, whereas the lack of protection against group 1 strains demonstrated some of the possible limitations of HA2-based vaccines.

4.3 T Cell immunity in next-generation influenza vaccines

Numerous animal studies have demonstrated the protective potential of cellular mediated responses. As early as the 1970s, adoptive transfer studies demonstrated the protective effects conferred by cytotoxic T cells[240]. In a recent human challenge study published in 2021, pre-existing influenza-specific CD8+ T cells correlated strongly with

reduced viral load[225]. The 2009 H1N1 pandemic provided a unique opportunity to study human T cell immunity in antibody-naïve individuals. It was observed across multiple studies that pre-existing T cell responses were linked with fewer symptoms and better protection against infection[129,241,242]. The cross-protective potential of T cells has also been observed against other respiratory viruses, such as SARS-CoV-2, and are gaining interest as a strategy to prevent severe disease and combat new variants[129,243].

Vaccine design strategies targeting T cell epitopes present a promising direction for universal influenza vaccine development. In this thesis, I characterized T cell responses targeting two conserved influenza antigens. In chapter 2, I conducted a proliferation assay to demonstrate that IN vaccination induces robust and proliferative pulmonary T cell responses at the site of infection. In chapter 3, I evaluated HA2-specific T cell responses through ICS. In response to antigen stimulation, I observed cytokine production in both the lungs and draining lymph nodes. I demonstrated that the two conserved antigens, one internal and one external, are both targets for humoral and cellular responses. In addition, I identified an immunodominant region within the consensus HA2 sequence and *in silico* human MHC binding affinity predictions highlight the potential of this region for future investigations and possible human applications.

A major challenge in developing T cell-based vaccines has been the diversity of human leukocyte antigen (HLA). HLA haplotypes determine which antigenic peptide get presented to T cells. Extensive screening and testing will have to be conducted for any human T cell-based vaccine to ensure sufficient population coverage[244]. As previously discussed, many experimental vaccines incorporate multiple T cell epitopes. However, this has led to an interesting phenomenon termed heterosubtypic immunodominance,

where the vaccine-induced immunity protects better against some strains but not another. For example, Yuan and colleagues tested a multi-epitope vaccine with 19 B-cell linear epitopes, 7 CD4+ T cells epitopes, and 11 CD8+ T cells epitopes of HA and NA from H1N1, H3N2, and influenza B viruses[245]. They observed lower levels of immune responses against H3 compared to other subtypes, potentially a consequence of heterosubtypic immunodominance among the influenza strains. Therefore, it is essential to evaluate antigens or epitopes independently as I did in chapter 3.

This thesis provides experimental evidence supporting the immunogenicity and functional relevance of IN delivery of NP and HA2 as T cell targets. However, challenges such as HLA diversity and heterosubtypic immunodominance remain to be investigated in future studies.

4.4 Future Directions

Mucosal vaccine development presents unique challenges compared to parenteral vaccines. For example, mucosal vaccines must overcome the epithelial barriers and ensure sufficient antigen expression and immune stimulation. Adjuvants like TLR agonists, such as CpG oligodeoxynucleotides that targets TLR9[246–248], or polyinosinic:polycytidylic acid that targets TLR3, enhance both humoral and cell-mediated responses[249,250]. There has also been a recent surge of interest in applying lipid nanoparticle (LNP) for IN immunization[251–253]. Delivery devices can also be employed to improve vaccine penetration and dispersion into the nasal passages[254,255]. However, the use of IN adjuvants is complicated by safety concerns. An enterotoxin-based adjuvant, a class of previously well-accepted component for oral administration, was found to be associated with an increased risk of Bell's palsy[256].

Another challenge that the next-generation vaccines must address is the limited efficacy in older populations. There is evidence demonstrating that age-related immunosenescence also extends to the mucosal tract. In an infection mouse model, Toapanta and Ross observed that aged mice had higher morbidity and recovered slowly following influenza infection due to delayed infiltration of APCs into the lungs[257]. This impairment ultimately affected the production of cytokines and chemokines and postponed the activation of adaptive immune response. In the elderly population, immunity induced by vaccination is weakened due to the presence of chronic inflammation, reduced migration of APCs, and reduced T cell activation[258]. Strategies such as high-dose and adjuvanted vaccination are already being implemented with the current seasonal vaccines to address this issue. Other experimental alterations to the vaccine regimen, such as additional booster immunizations[259], delayed interval vaccination[260], and intradermal injection[261] can also improve immune responses in the elderly population. Intriguingly, there are strategies aimed at enhancing the aged immune system and reverse immunosenescence, such as inhibiting chronic inflammation to restore functions like autophagy and CTL survival[258,262].

Recent advances in sampling and omics methods has provided a better understanding of our mucosal system, gaining insights that can be applied to a variety of diseases. Ramirez *et al.* were able to non-invasively sample adenoid lymphoid tissue from healthy adult donors, a tissue previously thought to only be active during childhood before atrophying as we reach adulthood[207]. Adenoid germinal center B cells were present in 70% of adults up to 68 years old. They detected an age-dependent decline in the numbers of adenoid germinal center B cells and germinal center follicular helper T cells, providing

further explanation for the increased risk of respiratory infections observed in older populations. With the advancement of single-cell sequencing technology, the limited cell number obtained from mucosal sampling is less of an issue. Long-standing questions such as immune imprinting and inconsistency in T cell responses can now be studied on an individual level. Deep learning approaches are being utilized to extract informative and compact features from noisy, heterogeneous, and high-dimensional scRNA-seq data to improve downstream analysis. Tools are being made for imputation and denoising, dimensionality reduction, batch effect removal, cell clustering, and cell type annotation[263]. These new advancements in integrating multi-omics data will facilitate the design of more targeted and more efficient mucosal vaccines.

4.5 Final Remarks

Influenza remains a major global health burden with unpredictable pandemic potential. Licensed vaccines continue to have highly variable efficacy and limited ability to prevent early infection. While IN vaccination has been proposed as a strategy to enhance protection by engaging mucosal immunity, its mechanisms of action and protective efficacy require further investigation. This thesis addressed two key objectives: first, to evaluate IN administration as an alternative route for augmenting cross-subtype protection using conserved viral antigens; and second, to characterize the mucosal immune responses elicited by IN vaccination. When assessing IN and IM immunization in parallel, we observed that IN immunization conferred superior heterosubtypic protection, with much stronger local recall responses in the respiratory tract. In a separate study, a synthetic consensus HA2 IN vaccine from the H3 subtype provided robust protection against H3N2 and H7N9, eliciting mucosal antibodies and T cell responses. A

highly immunogenic C-terminal epitope was identified, with potentials for human application. Collectively, this work provides mechanistic insight into IN delivery of conserved influenza antigens and informs the rational design of next-generation mucosal vaccines capable of providing broad protection against diverse and emerging respiratory viruses.

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