Novel Double-Layered Protein Nanoparticles Conjugating Truncated Flagellin Induces Improved Mucosal and Systemic Immune Responses Conferring Influenza Cross-protection

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by

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Under the Direction of Baozhong Wang, Ph.D.

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ABSTRACT

Influenza remains a persistent global health challenge. One of the weaknesses of current seasonal flu vaccines is their limited efficacy against drifted influenza strains. Our study focused on developing a novel self-adjuvanted double-layered protein nanoparticle vaccine that will be intranasally delivered and cross-protective. These nanoparticles consisted of an influenza nucleoprotein (NP) core encapsulated by hemagglutinin (HA) and a truncated form of bacterial flagellin (tFliC). Immunizations with these double-layered nanoparticles that included tFliC as a mucosal adjuvant, which activates toll-like receptor 5 (TLR5), induced significant mucosal and systemic immune responses, conferring cross-protection against influenza in mice. Compared to traditional vaccines, the double-layered nanoparticles induced higher levels of antigen-specific IgA and IgG in mucosal samples and serum and robust T-cell responses. In addition, the nanoparticles demonstrated robust immune responses in pre-infected mice, demonstrating that prior exposure to a heterologous influenza strain synergizes the vaccine's efficacy. To further optimize the vaccine's effectiveness, we employed a slow delivery method for the prime dose, spreading the administration over several days. This strategy markedly enhanced germinal center reactions and T-cell activation in lung-draining lymph nodes, resulting in superior protective efficacy against homologous and heterologous H3N2 influenza challenges. Our results demonstrate that the tFliC-adjuvanted, double-layered protein nanoparticles can be developed into a highly effective universal influenza vaccine. This novel intranasal vaccine formulation provides robust and broad protection and highlights a promising approach to improving

influenza vaccine efficacy through simplified intranasal immunization, such as nasal drops.

INDEX WORDS: Influenza Vaccine, Double-Layered Protein Nanoparticles, Self-Adjuvanted Nanoparticles, Hemagglutinin (HA), Nucleoprotein (NP), Truncated Flagellin (tFliC), Intranasal Administration, Slow Delivery Vaccination Strategy

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DEDICATION

I would like to express my deepest gratitude to my parents and family, whose unwavering support has been a constant source of strength throughout my academic journey. I am also deeply thankful to my husband, Paul, for his enduring encouragement and understanding. Thank you all of you for your invaluable contributions to my journey.

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List of Abbreviations

Antibody-Secreting Cells	ASCs
Antigen-Presenting Cells	APCs
Bone Marrow Dendritic Cell	BMDC
Broadly Neutralizing Antibodies	bnAbs
Enzyme-Linked Immunosorbent Assay	ELISA
Enzyme-Linked Immunosorbent Spot	ELISPOT
Germinal Center	GC
Hemagglutinin	HA
Hemagglutinin Inhibition	HAI
HA3/NP nanoparticles	HN
HA3/NP nanoparticles and soluble tFliC	sTHN
HA3-tFliC/NP nanoparticles	THN
Horseradish Peroxidase	HRP
Inactivated Influenza Vaccine	IIV
Influenza A Virus	IAV
Influenza B Virus	IBV
Interferon-Gamma	IFN-γ
Interleukin-4	IL-4
Intramuscularly	I.M.
Intranasal	I.N.

Live Attenuated Influenza Vaccines	LAIVs
Matrix Proteins 1	M1
Matrix Proteins 2	M2
Mediastinal Lymph Nodes	mLNs
Neuraminidase	NA
Nucleoprotein	NP
Original Antigenic Sin	OAS
Polydispersity Index	PdI
Scanning Electron Microscopy	SEM
Secretory IgA	sIgA
Sequential Prime Immunization with THN	THN-S
Somatic Hypermutation	SHM
T Follicular Helper	T_{FH}
Tissue-Resident Memory T Cell	T_{RM}
Tissue-Resident Memory B Cells	B_{RM}
Toll-Like Receptor	TLR
Truncated Flagellin	tFliC

CHATPER 1. INTRODUCTION

The annual impact of influenza on global health is considerable, with seasonal epidemic outbreaks caused by influenza A and B viruses affecting millions of people (F. Krammer et al., 2018). These viruses remain significant public health threats due to their rapid mutation rates, which facilitates the emergence of widespread epidemics. Notably, influenza A has the potential to trigger pandemics, making the occurrence of another influenza pandemic inevitable (Allen, 2006; Chandra, 2013). This underscores the urgent need for effective surveillance, preparedness, and vaccination strategies to mitigate the impact of future influenza pandemics.

1.1. OVERVIEW OF INFLUENZA

1.1.a. Classification and Characteristics of Influenza Viruses

The influenza virus, a member of the Orthomyxoviridae family, causes a highly infectious respiratory disease(Goldrick & Goetz, 2007; F. Krammer et al., 2018). Its segmented genome encodes several critical proteins, including surface glycoproteins hemagglutinin (HA), neuraminidase (NA), matrix proteins 1 (M1), matrix proteins (M2), nucleoprotein (NP), nonstructural proteins (NS1), nuclear export protein (NEP), and polymerase subunits (AbuBakar et al., 2023). Among these proteins, the HA and NA glycoproteins, located on the surface of the virus particle, exhibit the highest degree of antigenic variability(Rina Fajri Nuwarda et al., 2021). Based on sequence conservation, influenza A viruses can be classified into two major phylogenetic groups: group I (including H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16) and group II (including H3, H4, H7, H10, H14, and H15)(Mallajosyula et al., 2015).

The rapid mutation rates and genetic reassortment of HA and NA lead to new strains against which people have little or no immunity(Low et al., 2023). Seasonal influenza infections are caused by genetic drift as the influenza viruses create new antigenic epitopes to avoid the host's preexisting protective immunity(Erbelding et al., 2018b). In contrast, influenza pandemics are caused by genetic shifts when the RNA genome segments from two influenza virus strains are reassorted, leading to the emergence of a new influenza A virus strain that is antigenically distinct from previously circulating strains(Taylor et al., 2023). Pandemic influenza typically occurs every 10–50 years and has significant global health impacts(Saunders-Hastings & Krewski, 2016).

1.1.b. Global and Economic Impacts of Seasonal Influenza

According to the World Health Organization (WHO), seasonal influenza viruses result in 3 to 5 million severe illnesses and 290,000 to 650,000 deaths annually(Jha et al., 2020; WHO, 2023). These outbreaks impact individual health, significantly strain healthcare systems, and lead to considerable economic costs(Langer et al., 2024). The annual financial burden of influenza in the United States is estimated at USD 87.1 billion, with USD 56 billion explicitly allocated for treating the elderly(Kim et al., 2021).

Influenza can cause symptoms ranging from mild to severe illness and, in some cases, can lead to hospitalization or even fatality across all age groups. Specific populations are at a higher risk for severe complications, including young children, pregnant women, the elderly, and individuals with preexisting health conditions(Paules & Subbarao, 2017; Troeger CE, 2019). The broad implications of seasonal influenza underline the necessity for effective vaccination strategies to reduce the healthcare and economic burdens associated with annual outbreaks(Hughes et al., 2019).

1.1.c. Historical Impact and the Evolution of Influenza Vaccination

Influenza A viruses have caused multiple pandemics over the past century, significantly impacting global health. Among these, the Spanish Flu (1918-1919), caused by the influenza A/H1N1 virus, resulted in the highest death toll of any disease outbreak in history(Barry, 2004). It infected approximately one-third of the global population and caused an estimated 50 to 100 million fatalities(Johnson & Mueller, 2002).

The development of the first inactivated influenza vaccine (IIV) in 1945 marked a significant milestone in combating influenza. This vaccine utilized fertilized chicken eggs and was licensed in the United States(Kim et al., 2022). However, subsequent flu epidemics revealed a critical challenge: circulating influenza viruses underwent antigenic changes, rendering existing vaccines ineffective. This necessitated continuous monitoring and updating of the vaccine strains to match the circulating flu virus strains(WHO, 2024).

1.2. CHALLENGES OF CURRENT INFLUENZA VACCINES

Currently, the most effective method for preventing and controlling seasonal influenza viruses is through annually administered vaccines(Houser & Subbarao, 2015; Trombetta et al., 2022). The Food and Drug Administration (FDA) has approved three main categories of annual influenza vaccines: inactivated, live-attenuated, and subunit (Bonifacio et al., 2022; R. F. Nuwarda et al., 2021). Inactivated influenza vaccines, often referred to as “trivalent” or “quadrivalent” vaccines, contain two influenza A virus (IAV) strains, H1N1 and H3N2, along with one or two influenza B virus (IBV) strains, either from the Victoria or Yamagata lineages(Li et al., 2022). These vaccines primarily stimulate the production of hemagglutinin (HA)-specific antibodies, which provide a protective immune response by blocking viral entry into respiratory epithelial cells(Janssens et al., 2022). Live-attenuated vaccines also contain three or four influenza strains modified to limit viral replication in the upper respiratory tract(Bonifacio et al., 2022). These attenuated live viruses can induce robust B and T cell immune responses (Mohn et al., 2015). In 2013, the FDA approved the use of recombinant subunit influenza vaccines. These vaccines are composed of HA proteins selected from various influenza strains each year to elicit strain-specific HA head-neutralizing antibody responses(Krammer, 2019). Overall, current seasonal influenza vaccines target the globular HA head domain and offer effective protection against strains that closely match the vaccine components.

1.2.a. Reduced Effectiveness

Current vaccine strategies primarily target the highly antigenically variable regions of the globular HA head domain to elicit neutralizing antibodies that block the attachment of sialic acid to the HA receptor binding sites or inhibit conformational changes associated with fusion(Pascha et al., 2024; Zhang et al., 2019). New vaccine formulations are produced yearly based on pre-seasonal surveillance and predictions due to antigenic drift in surface antigens(Boni, 2008; Thrane et al., 2020). However, vaccine efficacy is often compromised, typically between 10% and 60% among the general population, especially when the globular head HA domain of the

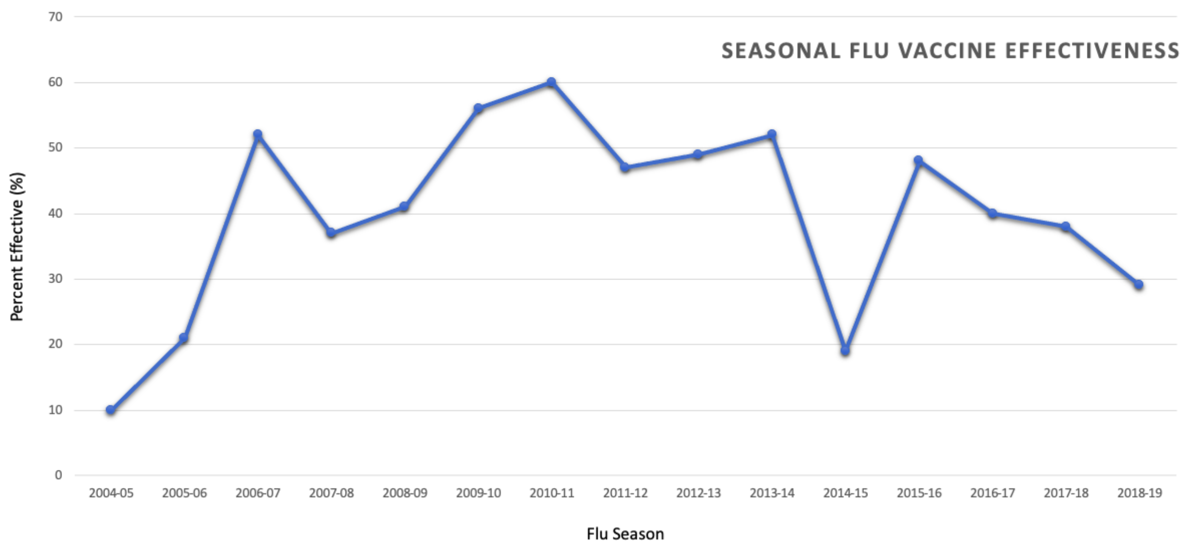

Figure 1: The Effectiveness of Current Seasonal Influenza Vaccines.
Note. The vaccine effectiveness estimates from the 2004-2019 Influenza seasons. Adapted from https//www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm

circulating virus strain is antigenically mismatched from that of the vaccine strain as viruses evolve to escape antibody-mediated neutralization(Kim et al., 2018; Tricco et al., 2013; Wang et al., 2018). Even in years when the vaccine and circulating strains are well-matched, the effectiveness of the traditional licensed influenza vaccines can be further compromised by adaptive mutations in seasonal vaccine strains grown in chicken eggs(Rajaram et al., 2020). Additionally, host-related factors such as age, preexisting immunity, and chronic conditions can further diminish the efficacy of seasonal vaccines(Castrucci, 2018). Despite these ongoing challenges, vaccines remain the most effective countermeasure against influenza infection(Jones et al., 2011).

1.2.b. Original Antigenic Sin and Immune Imprinting

Every individual develops a complex immune history through repeated exposures to novel influenza strains from infections and seasonal vaccinations(Francis et al., 2019). Since the concept of original antigenic sin (OAS) was introduced in the 1960s, researchers have studied the impact of the first influenza virus strain encountered on lifelong immunity against drifted IAV strains(Francis et al., 2019). Analyzing antibody and B cell responses has provided valuable insights into the immune system's memory and adaptability(Francis et al., 2019; Koutsakos & Ellebedy, 2023). Current understanding indicates that prior influenza exposure creates a lasting immune memory of B cell responses against HA head epitopes, significantly affecting responses to subsequent strains. Current annual influenza vaccinations may not improve the antigen-specific B cell response quality due to limited somatic hypermutation (SHM) among memory B cells(Ellebedy et al., 2016). This limitation could be attributed to OAS, where existing B cells fail to recognize new epitopes, or seasonal vaccines do not sufficiently stimulate germinal center responses. Therefore, it is crucial to develop targeted vaccination strategies that enhance the available antigen and incorporate adjuvants to mitigate the impacts of immune imprinting(Kim et al., 2012).

1.2.c. Limited Mucosal Immune Responses

Currently approved influenza vaccines are administered intramuscularly (I.M.) and effectively induce systemic immune responses(Xing et al., 2024). However, these I.M. vaccines stimulate poor mucosal immunity at the respiratory tract surfaces, the primary entry point for the virus (Azzi et al., 2022; Baker et al., 2022). Since influenza viruses primarily target the respiratory tract, mucosal secretory IgA (sIgA) and tissue-resident memory T cell (T_{RM}) responses at the virus entry site are essential for reducing viral infection and transmission(Gianchecchi et al., 2019; Wilk & Mills, 2018; Zheng & Wakim, 2022); mucosal antibodies are crucial in restricting infectivity and transmission(Brandtzaeg, 2013), while T_{RM} s can initiate rapid recall responses by recognizing the cognate antigen upon re-exposure due to their strategic location(Beura et al., 2019).

To address the challenges of influenza epidemics and pandemics, there is an urgent need for safe and effective mucosal influenza vaccine formulations that can stimulate robust local mucosal immune responses at the site of infection. These formulations could significantly reduce the spread of the disease by reducing viral infection, replication, and transmission (Mostaghimi et al., 2022).

1.3. EFFORTS TOWARD A UNIVERSAL INFLUENZA VACCINE

Due to the annual circulation of diverse influenza virus strains, the general population remains vulnerable to emerging strains despite seasonal flu vaccinations. To address the limitations of current influenza vaccine strategies and mitigate the risks associated with seasonal and pandemic influenza, the US National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes for Health (NIH), along with the broader influenza research community, has prioritized the development of a universal influenza vaccine. This vaccine aims to elicit broad and durable protective immunity against multiple strains, thereby eliminating the need for annual vaccinations(Erbelding et al., 2018a; Jazayeri & Poh, 2019; Krammer et al., 2018). Despite extensive research, a universally effective influenza vaccine targeting all strains has not yet received FDA approval. However, a promising approach involves targeting highly conserved antigens such as HA, NA, and M2, as well as internal proteins like NP and M1, to induce cross-protective antibodies and T cells(Jazayeri & Poh, 2019).

1.3.a. Targeting Conserved Epitopes

Structure-guided vaccines that target conserved epitopes to induce broadly neutralizing antibodies (bnAbs) represent a promising area of research(Kim et al., 2021). This strategy aims to create effective vaccines against various influenza strains by focusing on parts of the virus that remain unchanged across different variants. Research has extensively explored the conserved regions of the hemagglutinin (HA) stalk, shared among different viral strains and exhibit a slower mutation rate, for their potential to provide cross-reactive protection against diverse influenza strains(Kirkpatrick et al., 2018). Additionally, conserved epitopes on the HA head domain have been targeted due to their dominant immunogenicity, which induces antibodies with potent reactivity (Arunachalam et al., 2021). This approach has highlighted the importance of conserved antigens and the resulting cross-reactive bnAbs in providing broad protective immunity against influenza virus infections(Fu et al., 2016; Staneková & Varečková, 2010).

Beyond bnAbs, some non-neutralizing antibodies have also been identified as broadly reactive. These antibodies bind to viral surface proteins, such as M2, and provide cross-protection through antibody—or cell-mediated immune responses. They clear virus-infected cells using mechanisms like antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytolysis (CDC)(Gao et al., 2020; Grebe et al., 2008).

Furthermore, studies have demonstrated that T-cell responses are potential correlates for broad immune responses against various influenza strains in both mice and humans (Sridhar, 2016). T cells recognize conserved peptides in internal proteins such as NP, M1, or PB1(Jansen et al., 2019). While T cell responses cannot neutralize the virus, influenza-specific T cells induce highly cross-protective immune responses, reducing the severity and duration of infection. This is achieved through the activity of $CD8^+$ T cells, which clear virus-infected cells, and $CD4^+$ T cells, which direct the immune responses (Jansen et al., 2019). In humans, $CD8^+$ T cells have been identified as providing cross-protective immune responses, protecting against the antigenically drifted seasonal, pandemic, and avian influenza A virus strains (Koutsakos et al., 2019; Z. Wang et al., 2018).

1.3.b. Protein Nanoparticle Vaccine Platform

Conserved components of the influenza virus have been reported to induce weak immune responses, and broadly neutralizing antibodies (bnAbs) that target these conserved epitopes are often immuno-subdominant(Henry et al., 2018). Moreover, current seasonal influenza vaccines do not consistently induce these antibodies. Therefore, in addition to antigen selection, choosing an appropriate vaccine platform is critical for developing a universal influenza vaccine. Various platform technologies, such as DNA, mRNA, and nanoparticle-based vaccines, have been explored to enhance the immunogenicity of conserved epitopes(Chiba et al., 2023).

For this dissertation, solid protein nanoparticles composed of various conserved influenza antigens were utilized to enhance broad protection and long-lasting immune responses. These double-layered protein nanoparticles, generated by coating conserved influenza antigens onto desolvated nanoparticle cores, did not contain foreign self-assembling sequences. As a result, they eliminated off-target immune responses while exhibiting high antigen immunogenicity and vaccine potency(Dong & Wang, 2022; Liu et al., 2023; Y. Wang et al., 2018).

Previous studies conducted in our lab have demonstrated that this nanoparticle platform significantly enhances immune responses and provides increased protection in both influenza and SARS-CoV-2 research(Ma, Wang, Dong, Gonzalez, Song, et al., 2022; Ma, Wang, Dong, Gonzalez, Zhu, et al., 2022; Song et al., 2022; Y. Wang et al., 2020; Wang et al., 2023; Wang et al., 2021; Zhu et al., 2023b). The enhanced immunogenicity of these solid protein nanoparticles can be attributed to their substantial size, high antigen density, and structured arrangement of antigens(Lopez-Sagaseta et al., 2016; Sahdev et al., 2014). These characteristics protect antigens from proteolytic degradation, extend antigen presentation by antigen-presenting cells (APCs), enhance antigen delivery, and improve the recognition of the nanoparticles by B cell receptors. Consequently, these factors elicit robust cellular and humoral immune responses(Fredriksen & Grip, 2012; Pachioni-Vasconcelos Jde et al., 2016).

1.3.c. Truncated Flagellin as an Adjuvant for Mucosal Immune Response

Mucosal Adjuvants. Mucosal surfaces in the respiratory tract are crucial entry points for pathogens like influenza, making them prime targets for vaccine strategies to prevent infection at the initial entry site (Neutra & Kozlowski, 2006). When developing mucosal vaccines that utilize conserved antigens of influenza viruses, which often induce weak or suboptimal immunity, it is essential to include adjuvants that enhance the magnitude of the immune response, extend the duration of protection, and more effectively direct immune responses (Moni et al., 2023; Pulendran et al., 2021; Wang et al., 2020).

Although several FDA-approved adjuvants are available, none are approved for human mucosal vaccines, highlighting the need for novel, biocompatible mucosal adjuvants. Pairing the nanoparticle-based platform with appropriate adjuvants that target mucosal immunity and enhance antigen-stimulated immune responses while minimizing unnecessary inflammation is critical for mucosal vaccine design. A nanoparticle platform incorporating adjuvant and antigen offers significant advantages in intranasal vaccination. Co-localizing antigens and adjuvants within nanoparticles can protect antigens from rapid degradation, allow for controlled release, improve delivery efficiency by extending exposure to APCs, enhance antigen presentation, and ensure more targeted and effective immune responses(Fan & Moon, 2015; Lamontagne et al., 2022; Song et al., 2024; Zhu et al., 2014). As a result, intranasal vaccination with well-designed antigen/adjuvant-incorporated vaccines can activate innate immune cells in the respiratory tract and generate robust systemic and mucosal immune responses, which are crucial for protection against influenza viral infection(Gianchecchi et al., 2019; Kehagia et al., 2023; Schulze et al., 2017).

Truncated Flagellin. In this study, we incorporated truncated flagellin (tFliC) as a mucosal adjuvant to improve the breadth of cross-protection against diverse influenza viruses. Truncated flagellin was developed by removing the variable D3 region from full-length flagellin, which comprises the D0, D1, D2, and D3 domains, due to safety concerns associated with the full-length flagellin(Wang et al., 2012). As a toll-like receptor 5 (TLR5) ligand, tFliC acts as a pathogen-associated molecular pattern (PAMP), initiating signaling pathways through the adapter MyD88 molecule. This activation leads to the induction of MAPK and NF-κB gene activation, influencing gene transcription in innate and adaptive immunity(Hajam et al., 2017; Khani et al., 2019). Due to these properties, flagellin and tFliC have been widely utilized as adjuvants, facilitating signal transduction pathways that modulate immune responses(López-Yglesias et al., 2019; Zhao et al., 2021).

Building on this knowledge, incorporating tFliC onto protein nanoparticles presents a promising approach to strengthening protective immunity against influenza. tFliC nanoparticles possess inherent adjuvant properties due to their ability to activate Toll-like receptor 5 (TLR5) signaling. Co-delivering tFliC with nanoparticles can further potentiate the immune response through synergistic activation of APCs and induction of pro-inflammatory cytokines (Cuadros et al., 2004). Therefore, the resulting self-adjuvanted protein nanoparticles can generate robust immune responses against influenza, offering improved protection compared to conventional vaccines.

1.3.d. Slow Delivery Immunization

Slow delivery immunization, which involves splitting a vaccine dose into multiple sequential applications, presents a promising strategy to boost antigen immunogenicity, especially for challenging pathogens like influenza(Ray et al., 2023). Research has shown that this method enhances T follicular helper (T_{FH}) cell responses and germinal center (GC) B cell populations, resulting in significantly higher levels of autologous neutralizing antibodies (nAbs) in rhesus monkeys (Cirelli et al., 2019).

This approach addresses some of the limitations faced by traditional vaccination techniques. Conventional methods often struggle to elicit broadly neutralizing antibody (bnAb) responses due to immunological obstacles such as B cell immunodominance and limitations in the quality and quantity of GCs(Cirelli et al., 2019). By modifying the B cell repertoire, gradual dose administration shifts the immune system's focus from immunodominant non-neutralizing epitopes to a broader range. This shift is essential as it potentially fosters the development of robust and broadly neutralizing antibody responses.

Moreover, slow delivery immunization mimics the kinetics of natural infection, offering sustained antigen presentation and promoting antibody affinity maturation. This sustained presentation is crucial for generating high-quality immune responses. Consequently, this method provides a viable strategy for tackling infectious diseases where conventional approaches have been insufficient(Cirelli et al., 2019; Ou et al., 2022) . By ensuring prolonged exposure to the antigen, the immune system has more time to develop a strong and effective response, ultimately enhancing the vaccine's efficacy.

1.4. INNOVATION OF THE STUDY

Our lab has joined the effort to improve the protection efficacy of current vaccine platforms. Specifically, we have extensively investigated whether novel solid protein nanoparticle delivery systems, composed of influenza antigens, could broaden and prolong the induced immune responses(Ma, Wang, Dong, Gonzalez, Song, et al., 2022; Ma, Wang, Dong, Gonzalez, Zhu, et al., 2022; Song et al., 2022; Y. Wang et al., 2020; Wang et al., 2023; Wang et al., 2021; Zhu et al., 2023b). These studies have shown that the intramuscular administration of double-layered protein nanoparticles, which utilized structure-stabilized head-removed hemagglutinin (hrHA) stalk domains and a fusion M2e protein containing four conserved variants, effectively induced in vivo immunogenicity primarily through antibody-dependent cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADPC) (Lei Deng et al., 2018).

Building on these findings, the present study offers novel insights into developing universal influenza vaccines by engineering self-adjuvanted, double-layered protein nanoparticles conjugated with truncated flagellin (tFliC). This research distinguishes itself from existing studies by integrating both influenza nucleoprotein (NP) cores, known for their ability to induce T-cell responses, and hemagglutinin (HA), the primary target antigen of conventional influenza vaccines that elicit neutralizing antibodies, into a single nanoparticle with the inclusion of tFliC as a mucosal adjuvant to effectively address safety concerns while maintaining potent immunostimulatory effects through the activation of TLR5 and NF-κB transcription factors. This strategic combination within the nanoparticles was expected to induce superior immune activation and significantly enhance vaccine efficacy.

Furthermore, these self-adjuvanted nanoparticles were administered intranasally to enhance immune protection against influenza through localized delivery. By targeting the mucosal surfaces directly, this strategy aimed to elicit robust systemic and mucosal immune responses, including producing secretory IgA (sIgA) and establishing lung-resident memory B cell populations. This localized delivery was pivotal, as mucosal immunity provides a first line of defense against respiratory pathogens such as influenza.

Additionally, this method aimed to enhance further systemic immunity by promoting germinal center reactions and expanding effector T-cell populations through the slow-delivery vaccination strategy. The slow release of the intranasally administered, self-adjuvanted nanoparticles was designed to achieve potent and durable immune responses, addressing critical gaps in the efficacy of current influenza vaccines. By ensuring prolonged antigen exposure, the immune system had extended time to develop a robust and effective response. This targeted delivery and controlled release strategy extended antigen persistence, enhanced the frequency of antigen-specific $CD4^+$ helper T cell responses, promoted germinal center B cell reactions, produced high-affinity antibodies, and increased B cell memory populations, thereby offering a comprehensive solution to enhancing immune protection against influenza.

CHATPER 2. SPECIFIC AIMS

Although annually administered egg-derived inactivated influenza virus vaccines (IIVs) and live attenuated influenza vaccines (LAIVs) can protect healthy adults against well-matched strains, seasonal influenza vaccines suffer from variable and suboptimal levels of protection, ranging from 10 to 60%(CDC, 2024; Erbelding et al., 2018b; Trombetta et al., 2022). Moreover, since these vaccines are developed against specific circulating strains, they are inadequate against antigenically divergent drifted and pandemic strains(Erbelding et al., 2018b; Wei et al., 2020). Over the past decades, significant efforts have been made to develop universal influenza vaccines capable of inducing broadly protective immune responses. Previous research has identified conserved epitopes of influenza as potential immunogens for universal vaccines, as these determinants can induce cross-protective immune responses against antigenically drifted strains (Erbelding et al., 2018a; Kim et al., 2021; Staneková & Varečková, 2010). However, because these conserved components of influenza often induce weak immune responses, various approaches, such as nanoparticle platforms targeting conserved epitopes, have been developed to enhance vaccine effectiveness further. In this study, we aimed to develop an influenza vaccine capable of inducing broadly protective systemic and mucosal immune responses against multiple influenza A virus subtypes.

2.1. OVERALL AIM AND HYPOTHESIS

This project aimed to design an immunogen capable of enhancing immune responses against influenza. To achieve this, we developed a novel vaccine formulation: intranasally delivered, self-adjuvanted double-layered protein nanoparticles. These nanoparticles consist of a core of influenza nucleoprotein (NP) enveloped by hemagglutinin (HA) and a truncated form of a mucosal adjuvant, bacterial flagellin (tFliC) to enhance both mucosal and systemic immune responses. The central hypothesis of this project was that the intranasally administered novel influenza vaccines would elicit robust mucosal and systemic immune responses, thereby contributing to the development of more robust and enduring influenza vaccination. This hypothesis was further explored through the following specific aims:

2.2. SPECIFIC AIMS

2.2.a. Specific Aim 1

Investigate the influence of early-life influenza exposure on subsequent immune responses. This aim focused on understanding how influenza exposure in early life affects immune responses triggered by subsequent vaccinations or infections. Early-life exposure to influenza viruses I’s known to shape the immune system's memory and response to later encounters with the virus. Investigating how early influenza exposure influences subsequent immune responses provides valuable insights into the development of immune memory and protection.

By studying this influence, we aimed to understand the long-term impacts of early influenza experiences on the quality and effectiveness of immune responses to later vaccinations or infections. This knowledge could be used to design vaccination strategies that better account for an individual's immune history, leading to more effective and personalized influenza vaccines. Understanding these dynamics is crucial for optimizing vaccine efficacy and improving public health outcomes.

2.2.b. Specific Aim 2

Assess the immune responses induced by intranasal administration of HA3-tFliC/NP nanoparticles. This aim assessed whether the intranasal (I.N.) administration of HA3-tFliC/NP nanoparticles can elicit strong and broadly reactive mucosal and systemic immune responses in mice. The intranasal route of vaccine delivery targets the mucosal surfaces, the primary entry point for the influenza virus. By focusing on this route, we aimed to determine if the vaccine could induce robust local immunity at the site of infection, in addition to systemic immunity. To evaluate this, we analyzed immune responses at both local and systemic levels. This included measuring the production of secretory IgA (sIgA) at the mucosal surfaces and assessing the presence of specific antibodies and T-cell responses in the systemic circulation.

By investigating these responses, we sought to understand the potential of HA3-tFliC/NP nanoparticles delivered intranasally to induce a strong and broad immune response, offering insights into the effectiveness of this strategy for influenza vaccination.

2.2.c. Specific Aim 3

Investigate the efficacy of sequential immunization with HA3-tFliC/NP nanoparticles. This aim investigated whether sequential immunization with HA3-tFliC/NP nanoparticles can induce better or comparable immune responses and protection to current vaccine strategies. Sequential immunization involves administering multiple vaccine doses over time to enhance the immune system's response. The study compared the immune responses and protective efficacy of sequential immunization with traditional vaccination methods, examining factors such as the cross-reactivity of antibody responses, T-cell activity, germinal center (GC) reactions, and $CD4^+$ populations.

By evaluating the induced immunogenicity and protective efficacy, we aimed to determine whether sequential immunization can offer advantages over traditional vaccination strategies in providing long-lasting and broad-spectrum immunity. This included analyzing the quality and durability of the immune responses, focusing on determining the potential benefits of sequential immunization in enhancing overall vaccine performance.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. THE INFLUENCE OF EARLY-LIFE INFLUENZA EXPOSURE ON SUBSEQUENT IMMUNE RESPONSES

Nanoparticle vaccines are emerging as a potential breakthrough in developing advanced influenza immunizations. Therefore, understanding the impact of prior influenza virus exposure on immune responses to these protein nanoparticle vaccines is crucial. This section explored how prior exposure to the Phili (A/H3N2) influenza virus affects the immune response to subsequent HA3/NP protein nanoparticle vaccinations. This investigation aimed to provide insights into optimizing vaccination strategies for improved efficacy. We sought to identify critical factors that could enhance the design and administration of nanoparticle-based influenza vaccines by analyzing the immune responses elicited by these novel vaccines following previous virus exposure.

3.1.a. Results

Synthesis and Characterization of HA3/NP Nanoparticles. We synthesized HA3/NP (shell/core) nanoparticles by concentrating NP into a core particle via ethanol desolvation. The NP cores were then coated with HA3 using DTSSP crosslinkers (Figure 2A). The obtained HA3/NP nanoparticles were characterized by SDS-PAGE, which showed distinct HA3 and NP bands on the gel (Figure 2B). Scanning electron microscope (SEM) analysis revealed that the nanoparticles had morphologically spherical shapes with irregular surfaces, with diameters of less than 200 nm (Figure 2C). Further characterization of the nanoparticles involved examining the zeta potential (Figure 2D). The NP cores exhibited higher zeta potentials than the HA3/NP nanoparticles, indicating that the HA3 outer layer coating enhanced particle stability.

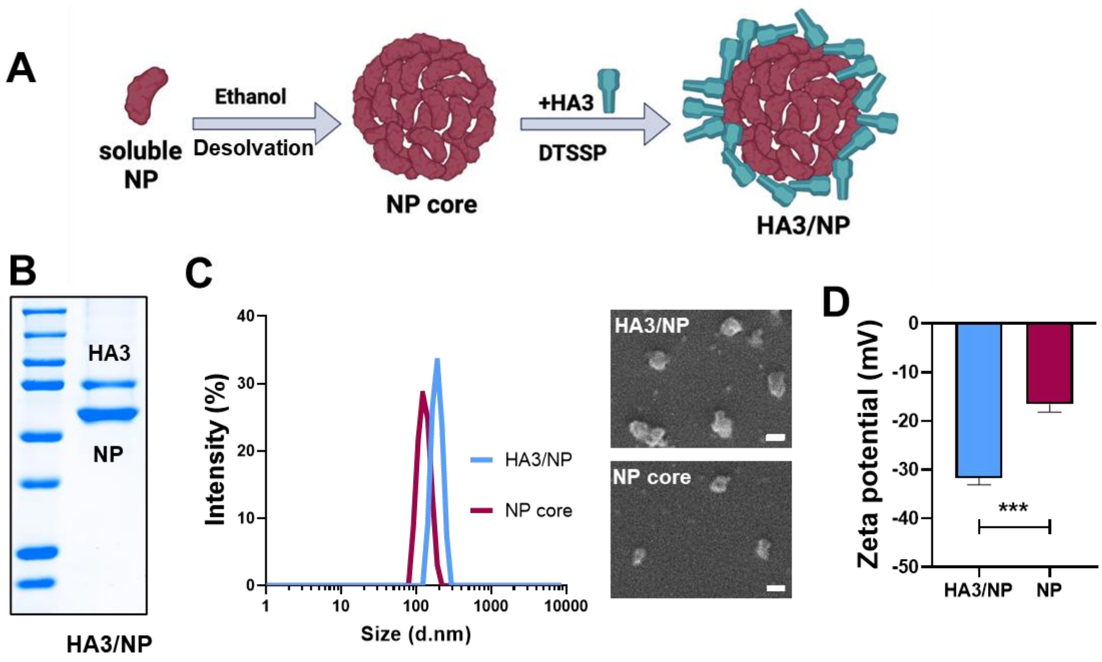

Figure 2. Fabrication and characterization of DTSSP protein-nanoparticles.
A) Fabrication of the DTSSP nanoparticles. B) Characterization of the nanoparticles by SDS-PAGE. C) Size distribution and SEM images of the NP core and the DTSSP nanoparticle. D) Zeta potential of the NP core and the DTSSP nanoparticle.

Pre-infection with Phili (A/H3N2) and Subsequent DTSSP Nanoparticle Vaccinations. Then, the mice were pre-infected with the influenza virus and received the subsequent nanoparticle vaccinations, as illustrated in Figure 3. For the imprinting process, mice were infected with sublethal doses of the Phili (A/H3N2) virus following established protocols. Naïve mice were included as controls. The mice were then immunized twice with 1µg of HA3/NP nanoparticle vaccines at weeks 16 and 20.

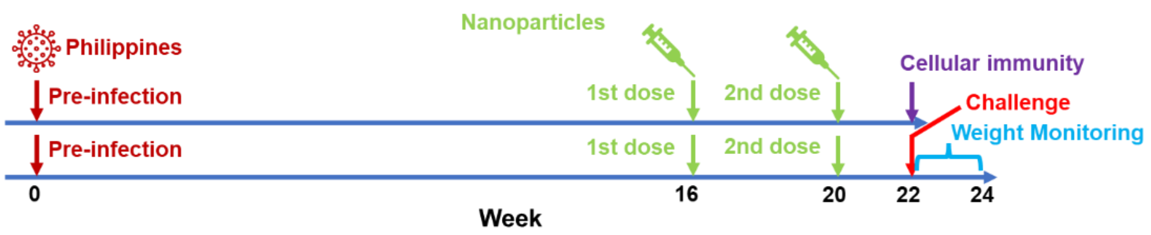

Figure 3. Schedule of pre-infection, nanoparticle immunization, and virus challenge.
Mice were pre-infected with 0.01×LD_{50} of Phili at day 0 and intramuscularly immunized twice with 1 µg of HA3/NP at weeks 16 (prime) and 20 (boost), respectively. Immune sera and spleen were collected two weeks after boost immunization.

Evaluation of Cellular Immune Responses. Two weeks post-boost-immunization, we examined IL-4 and IFN-γ secreting splenocytes using the ELISPOT assay (Figure 4). These cytokines, indicative of Th2 and Th1 immune responses(Pollard & Bijker, 2021; Zhu et al., 2010), respectively, demonstrated that the Phili-HA3/NP nanoparticle immunization group had significantly enhanced T-cell immune responses, with increased levels of IL-4 and IFN-γ secreting T-cells compared to the Phili-PBS and naïve groups. These responses were specific to HA3 and NP.

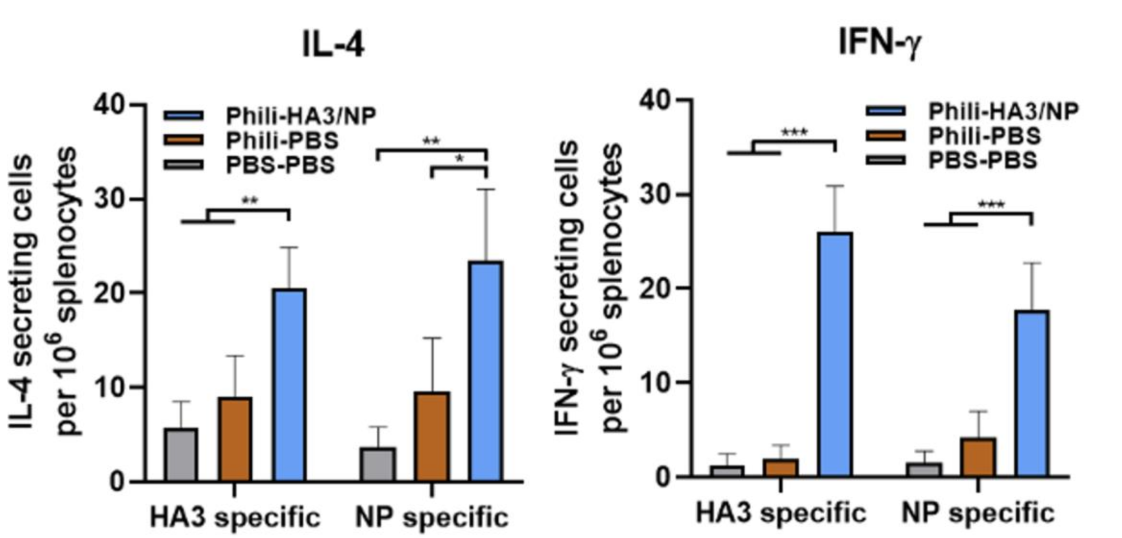

Figure 4. T Cellular immune responses.
Cellular immune responses in response to HA3 and NP proteins. HA3 and NP-specific IL-4- and IFN-γ-secreting splenocytes.

Assessment of Protective Efficacy. We then compared the protective efficacy between the Phili-PBS and Phili-HA3/NP groups, including Phili-pre-infected groups immunized with hrHA3/NP nanoparticles (Figure 5). The conserved stalk domain in influenza HA, head-removed HA (hrHA3), is a promising target for universal influenza vaccines(Impagliazzo et al., 2015; Mallajosyula et al., 2014; Yassine et al., 2015). The groups that received nanoparticle immunization demonstrated complete protection against the Aichi virus challenge, with a 100% survival rate. In contrast, the Phili-pre-infected group without immunizations showed severe weight loss and only a 40% survival rate, emphasizing the need for subsequent immunizations.

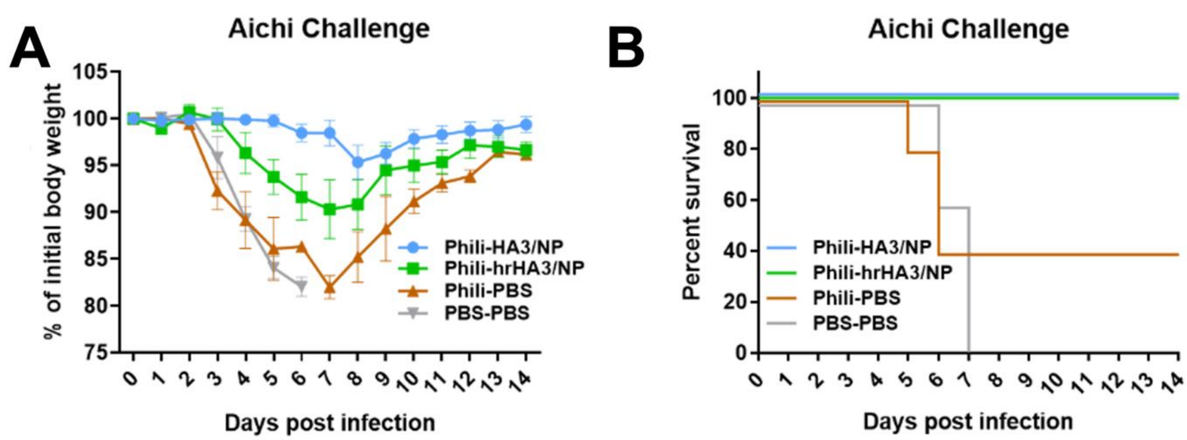

Figure 5. Body weight changes and survival rates.
Body weight changes and survival rates upon 5×LD_{50} Aichi virus challenges. Dais ta shown as mean ± SEM (n=5).

Analysis of Serum Antibody Responses. To evaluate the immune response, serum was collected two weeks post-boost-immunization, and HA3- or hrHA3- specific IgG isotypes were examined by ELISA (Figure 6). The nanoparticle immunizations significantly enhanced and balanced antigen-specific IgG, IgG1, and IgG2a antibody titers compared to the Phili-infected mice without the HA3 immunization control group. Our findings indicate that prior exposure to the heterologous Phili virus resulted in balanced HA3-specific Th1/Th2 antibody responses (IgG1 ≈ IgG2a) following nanoparticle immunization, in contrast with the Th2-biased responses previously observed from the HA3/NP nanoparticle immunizations(Ma, Wang, Dong, Gonzalez, Song, et al., 2022) (Figure 7).

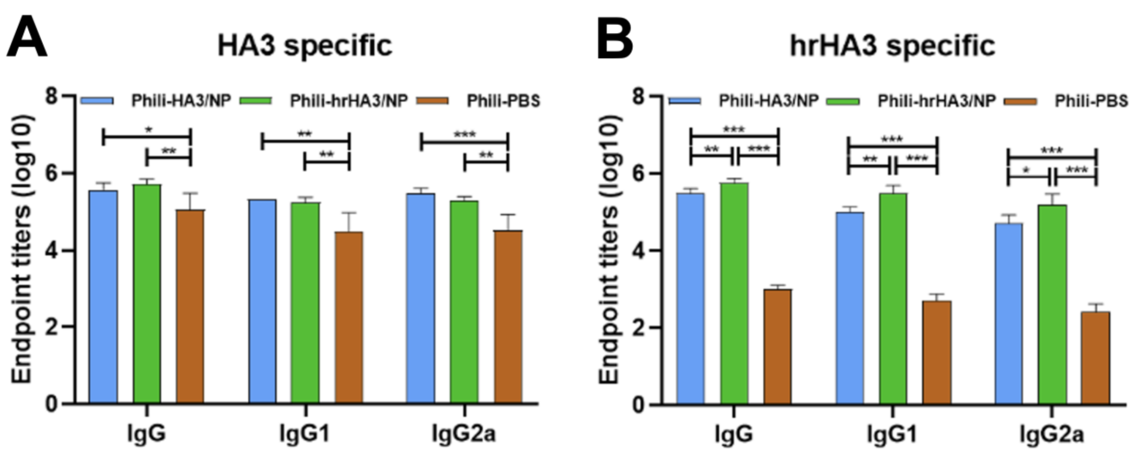

Figure 6. HA3- or hrHA3-specific IgG isotype titers.
Quantification of A) HA3 and B) hrHA3 -Specific IgG, IgG1, and IgG2a titers in mouse serum two weeks post-boost immunization. Data is shown as mean ± SEM (n=5). Statistical significance was analyzed with one-way ANOVA followed by Tukey’s test (* P < 0.05, ** P < 0.01, *** P < 0.001).

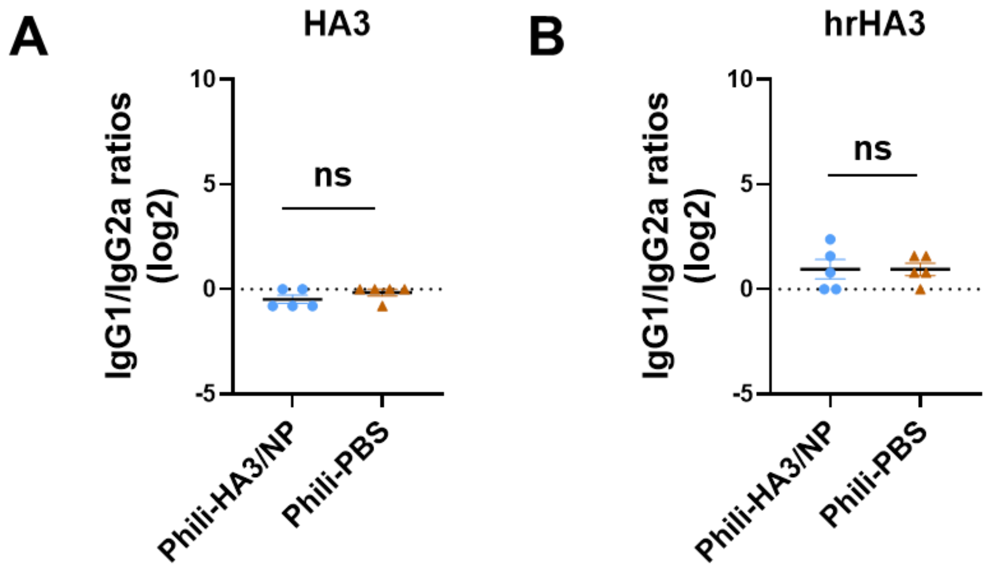

Figure 7. IgG1/IgG2a ratios in immune sera.
A) HA3- and B) hrHA3-specific IgG1/IgG2a ratios in immune sera 2 weeks post-DTSSP nanoparticle immunizations. Data are shown as mean ± SEM (n=5). Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (ns, not-significant).

Hemagglutination Inhibition. Compared to the control group, the Phili-HA3/NP group exhibited potent Aichi virus-specific hemagglutination inhibition (HAI) titers, underscoring the importance of initial exposure to a heterologous strain and subsequent immunizations in inducing Aichi-specific HAI antibodies (Figure 8A). While the antibodies produced by the Phili-hrHA3/NP group did not exhibit HAI activity against the Aichi strain, this group demonstrated higher balanced hrHA3-specific antibody titers than both the Phili-HA3/NP immunized group and the Phili-pre-infected non-immunized group (Figure 7B).

Cross-Reactivity of Serum Antibody Titers. Further evaluation of antigen-specific serum antibody titers revealed that the Phili-hrHA3/NP group had higher cross-reactivity HA4- and HA10-specific antibody titers (Figure 8B). This can be attributed to the higher sequence conservation of the HA stalk domain to HA4 and HA10 compared to HA3, as shown in Figure 9.

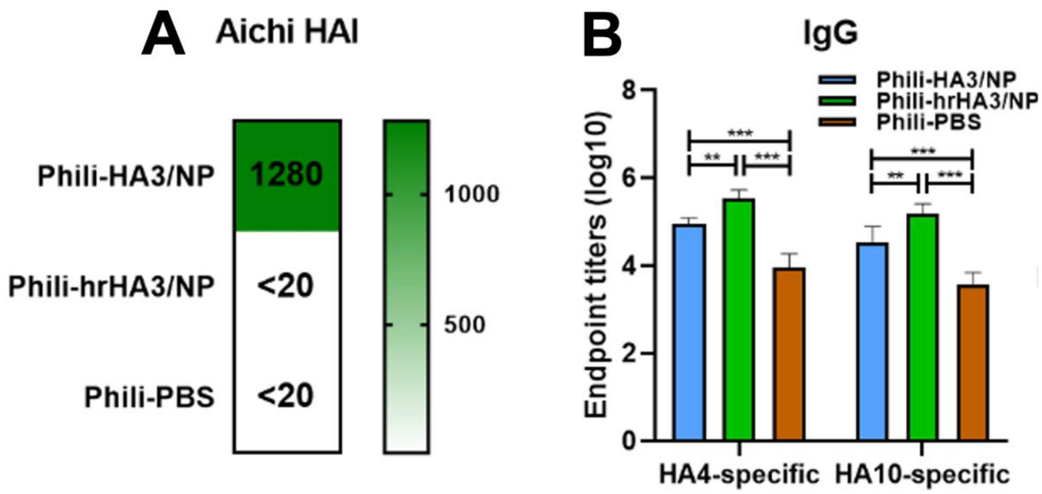

Figure 8. Serum HAI titers and cross-reactivity.
A)HAI antibody titers against Aichi virus. B) HA4 and HA10-specific cross-reactive antibody titers. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* P < 0.05, ** P < 0.01, *** P < 0.001).

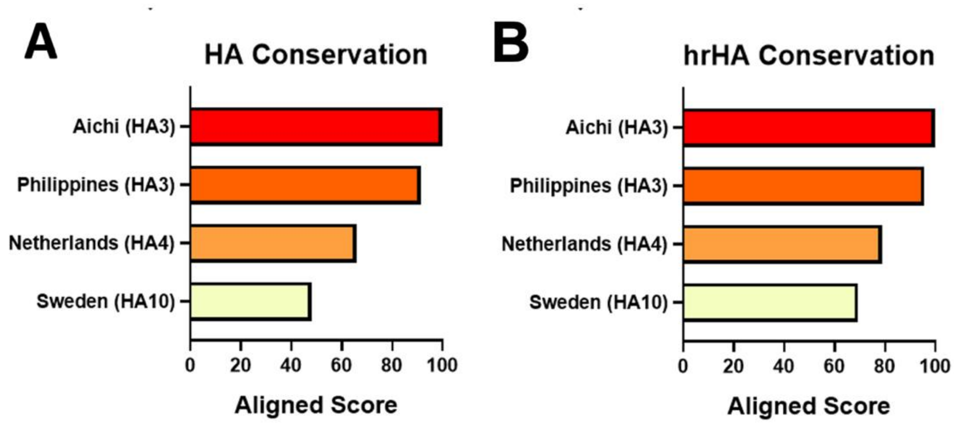

Figure 9. Sequence conservation of the HAs and hrHA.
Sequence conservation of group 2 influenza HA in comparison with Aichi A) HA3 and B) hrHA3.

3.1.b. Discussion

Examining the influence of immune history on vaccine effectiveness is crucial for creating more effective influenza vaccines. This study explored how previous virus infections shape the immune responses to HA3/NP nanoparticle influenza vaccines. Our research aimed to identify critical factors that could enhance vaccine design and efficacy by examining the interactions between past immune encounters and vaccine-induced immunity.

Our results showed that pre-infection with the Phili virus offered limited protection against Aichi virus challenges and did not result in any hemagglutination inhibition (HAI) titers against Aichi. However, when Phili pre-infection was combined with HA3/NP nanoparticle vaccination, we observed complete protection, HAI titers against Aichi, elevated antibody titers, balanced Th1/Th2 immune responses, cross-reactive immune responses, and robust IL-4 and IFN-γ secreting T-cell responses. This highlights the effectiveness of HA3/NP nanoparticle vaccines in enhancing immune responses and providing robust immune protection, particularly for Phili pre-infected groups.

Furthermore, the group that experienced pre-infection and was subsequently immunized with HA3 stalk protein nanoparticles (PNPs) exhibited complete protection against the Aichi influenza strain challenge. Although the induced antibodies lacked HAI activity, they displayed elevated titer levels and enhanced cross-reactive immunity compared to those immunized with full-length HA3 PNPs. This underscores the effectiveness and potential of HA3 stalk PNP immunization in broadening and strengthening influenza immunity. These findings emphasize the potential of using targeted immunogens, such as the HA3 stalk, to improve vaccine performance against diverse influenza strains.

3.1.c. Conclusion

In summary, this study demonstrated that the synergy between Phili pre-infection and the HA3/NP nanoparticle vaccine confers protection against the Aichi strain and induces cross-reactive immunity. The HA3/NP nanoparticle vaccine elicited robust and cross-reactive antibody and T-cell responses, suggesting its potential for cross-protection against diverse influenza strains. Additionally, this study highlighted the effectiveness of hrHA3 nanoparticles in offering complete protection against homologous virus challenges and boosting cross-reactivity to heterologous strains.

These findings emphasize the importance of considering an individual's exposure history when designing the antigen composition for influenza vaccines. Also, the study demonstrated the critical role of antigen selection in shaping the efficacy and breadth of vaccine-induced protection. By tailoring the antigen choice to account for prior exposures, it is possible to optimize the effectiveness and breadth of protective immune responses. This study provides valuable insights into optimizing influenza vaccine strategies by leveraging immune history and targeted immunogens to achieve broader and more effective immune protection.

3.2. IMMUNE RESPONSES INDUCED BY INTRA NASAL ADMINISTRATION OF HA3-tFliC/NP NANOPARTICLES

In this study, we aimed to develop a novel intranasally administered, self-adjuvanted, double-layered protein nanoparticle vaccine. This vaccine was designed to significantly enhance antigen-specific mucosal and systemic immune responses using a truncated form of bacterial flagellin (tFliC) as a mucosal adjuvant. The targeted delivery system consisted of protein nanoparticles with influenza nucleoprotein (NP) cores coated with hemagglutinin (HA).

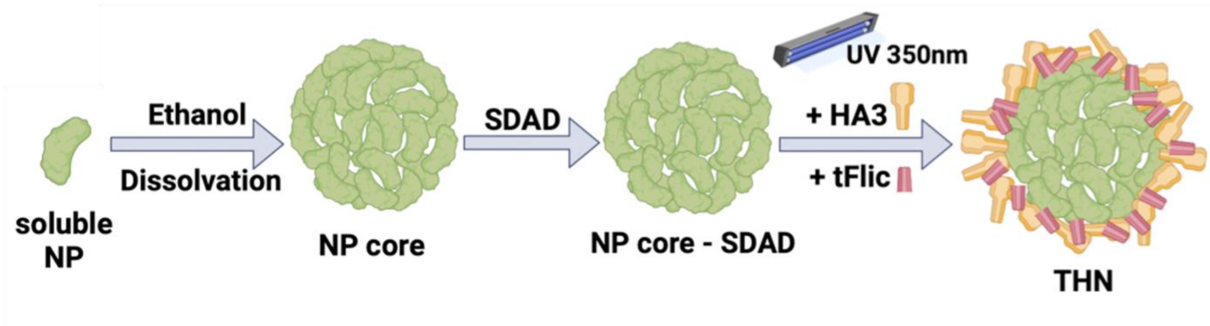

Figure 10. Fabrication of double-layered SDAD protein nanoparticles.
Diagram of SDAD protein nanoparticle generation.

3.2.a. Results

Synthesis of SDAD Protein- Nanoparticles. Protein nanoparticles were synthesized using the SDAD crosslinker to create double-layered structures comprising HA3/NP (HN) and HA3-tFliC/NP (THN) protein nanoparticles(Zhu et al., 2023a). Initially, the influenza NP nanoparticle core was fabricated via ethanol desolvation. The core was then conjugated with SDAD, resulting in an SDAD-conjugated NP core. Next, either HA3 alone or HA3 in combination with tFliC was attached to the SDAD-conjugated NP core through an ultraviolet-activated reaction at 350 nm (Figure 10).

Characterization of SDAD protein- nanoparticles. The synthesized HN and THN protein nanoparticle composition was confirmed through SDS- PAGE (Figure 11A). Dynamic light scattering (DLS) analysis determined the sizes, polydispersity index (PDI), and zeta potential of the synthesized double-layered protein nanoparticles. HN nanoparticles were 173.2 ± 19.0 nm in size, while THN measured 206.1 ± 22.4 nm (Figure 11B). Both nanoparticle groups exhibited a PDI of less than 0.2 (Figure 11C). The zeta potential values for the nanoparticles were negative, with HN at −29.57 ± 1.02 mV and THN at −35.34 ± 1.45 mV (Figure 11D). The double-layered protein nanoparticles' spherical morphology was examined by scanning electron microscopy (SEM) (Figure 11E).

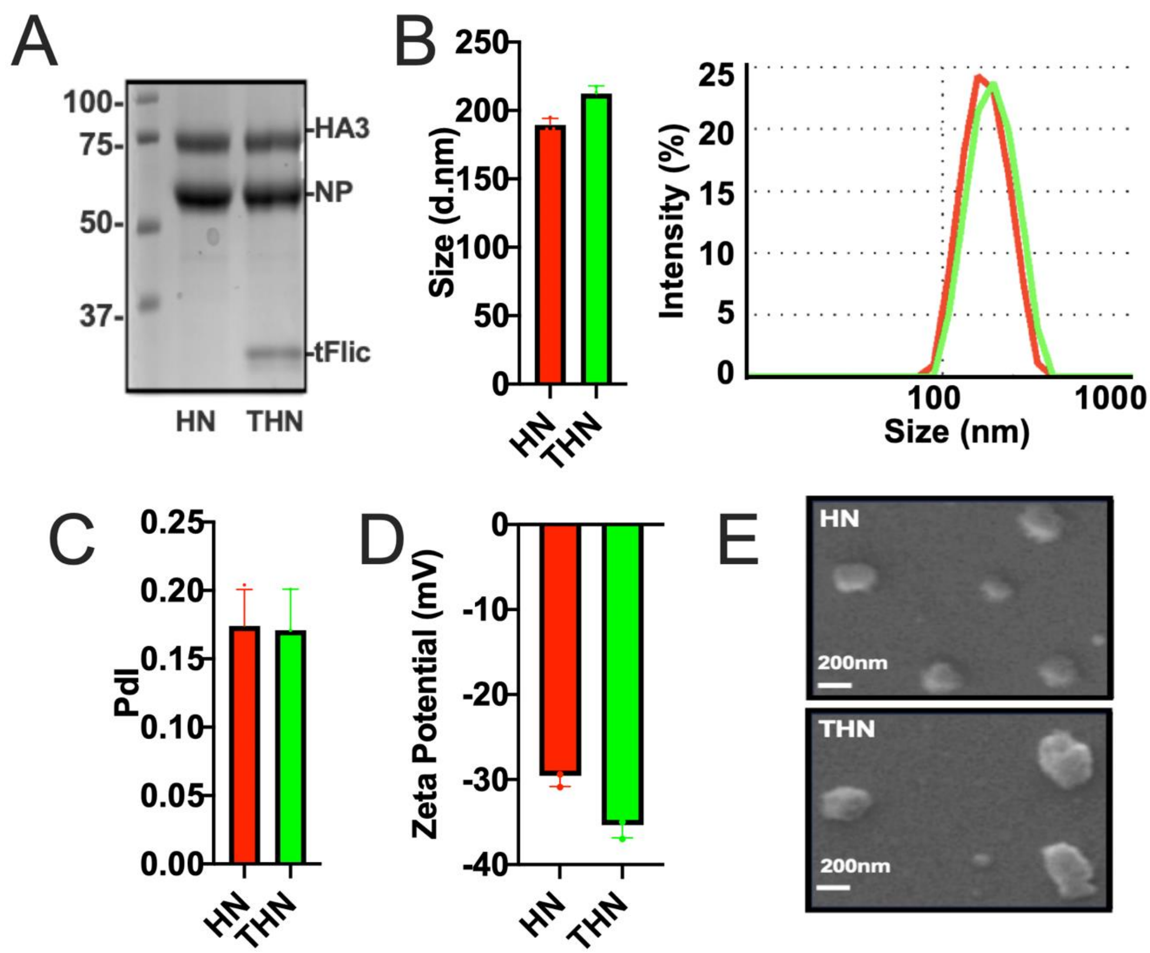

Figure 11. Characterization of SDAD protein-nanoparticles.
A) Coomassie blue staining analysis of the fabricated HN and THN nanoparticles. B) Size distribution of protein nanoparticles. C) Polydispersity index (PdI) of the nanoparticles. D) Zeta potential of nanoparticles. E) SEM images of the layered HN and THN nanoparticles. The histograms were presented as mean ± SEM.

Functional Validation of tFliC Activity. The biological activity of tFliC on the surface of the protein nanoparticles was evaluated through its capacity to activate the TLR5 innate signal in 293T cells. These cells were transfected with plasmids encoding TLR5 and NF-κB-luciferase reporter genes. Stimulation of the TLR5 pathway by both THN nanoparticles and soluble tFliC resulted in the activation of NF-κB, leading to the expression of similar levels of luciferase activity, indicating that tFliC retained its TLR5-ligand activity within the THN (Figure 12).

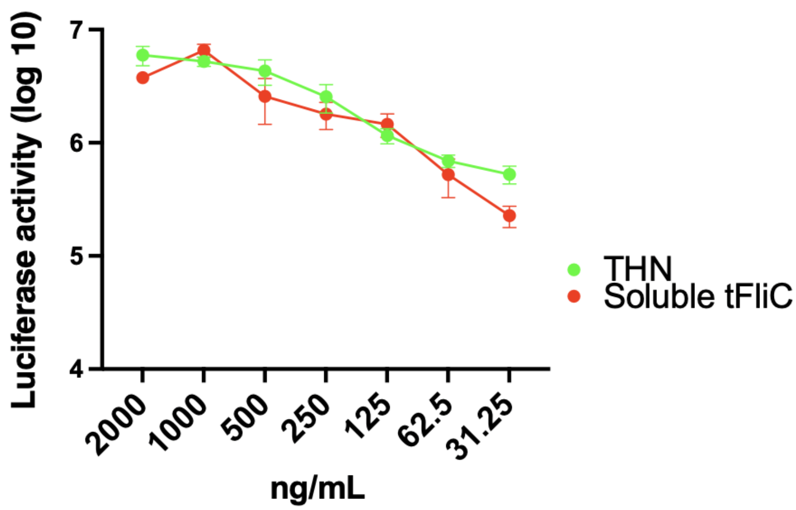

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Figure 12. Functional validation of tFliC activity.
TLR5-specific bioactivity of HA3/tFliC –NP nanoparticle and soluble tFliC.

Biodistribution of Protein Nanoparticles in Mouse Organs. We also investigated the biodistribution and the retention of the intranasally administered protein nanoparticle vaccines. The THN protein nanoparticles were fluorescently labeled and administered intranasally. After 24 hours, organs were harvested and analyzed for fluorescence using an in vitro imaging system (IVIS) (Figure 13). Ex vivo analysis revealed a significant accumulation of the nanoparticles in the lung for over 48 hours post-intranasal injection. Consistent with our previous findings, the protein nanoparticles maintained stable radiant efficiency for 48 hours post-injection(L. Deng et al., 2018). The radiant efficiency was diminished five days post-injection.

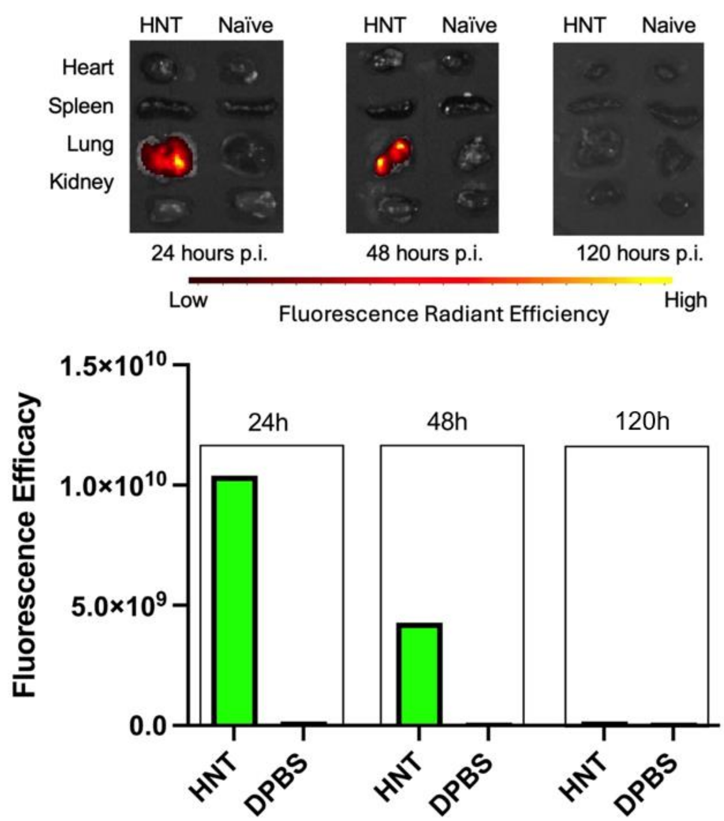

Figure 13. Biodistribution of protein nanoparticles in mouse organs.
Mice were sacrificed 24, 48, and 120 hours after administration, and organs (heart, lungs, kidneys, spleen) were collected for ex vivo imaging on Petri dishes. The Perkin-Elmer IVIS Spectrum system captured images, and Living Image software quantified the fluorescent intensity in the tissues.

Cellular Uptake of Protein Nanoparticles in JAWSII Cells. The in vitro uptake was subsequently examined using the JAWS II dendritic cells (Figure 14). Cells were exposed to Alexa Fluor 488 conjugated protein nanoparticles or soluble HA3 protein, with DBPS as a negative control. Fluorescent microscopy with the BZ-X710 (Keyence) was used to visualize the uptake, while quantitative measurements of mean fluorescence intensity per cell were obtained using the BZ-X analysis software (Keyence). The quantitative analysis showed that the mean fluorescence intensity for protein nanoparticles (PNP) was significantly higher than that of soluble proteins, indicating greater cellular uptake. The DPBS control consistently showed minimal uptake, serving as a baseline reference.

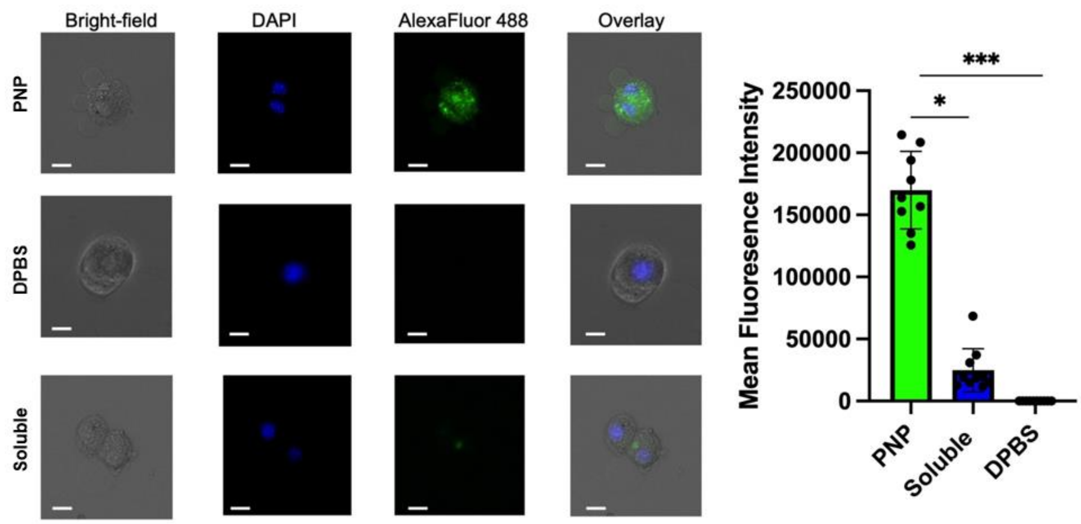

Figure 14. Cellular uptake of protein nanoparticles.
Observed under Keyence BZ-X710 fluorescent microscope with 40x magnification (Scale bars, 10μm). The fluorescence intensity of Alexa Fluor 488 was quantitatively analyzed using the BZ-X analysis software (Keyence).

BMDC Maturation and Cytokine Secretion. Subsequently, the effectiveness of protein nanoparticles in enhancing bone marrow-derived dendritic cell (BMDC) maturation and cytokine secretion was evaluated. In this section, we utilized three samples, as listed in Figure 15: HA3/NP nanoparticles (HN), HN nanoparticles with soluble tFliC(sTHN), and HA3-tFliC/NP nanoparticles (THN).

Both THN nanoparticles and HN nanoparticles with soluble tFliC significantly increased the expression of maturation markers CD80, CD40, and CD86 on CD11c+ BMDCs compared to the unstimulated control group (Figures 16 A-F). Moreover, nanoparticle treatments significantly elevated the secretion of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 from BMDCs (Figure 16 G-I), underscoring the role of protein nanoparticles in promoting DC maturation and cytokine production. The in vitro activation of BMDCs by these nanoparticles indicates their potential to induce effective immune responses in vivo, as the maturation and cytokine secretion of these innate cells are critical for antigen presentation and subsequent antigen-specific immune activation post-immunization(Zhu et al., 2023a).

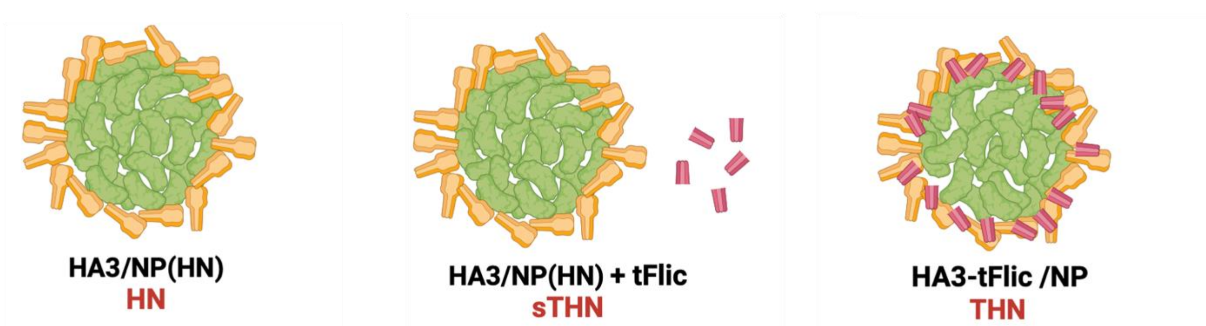

Figure 15. Schematic representation of the samples.
Schematic representation of the samples: HA3/NP nanoparticles (HN), HA3/NP nanoparticles with soluble tFliC (sTHN), and HA3-tFliC/NP nanoparticles (THN)

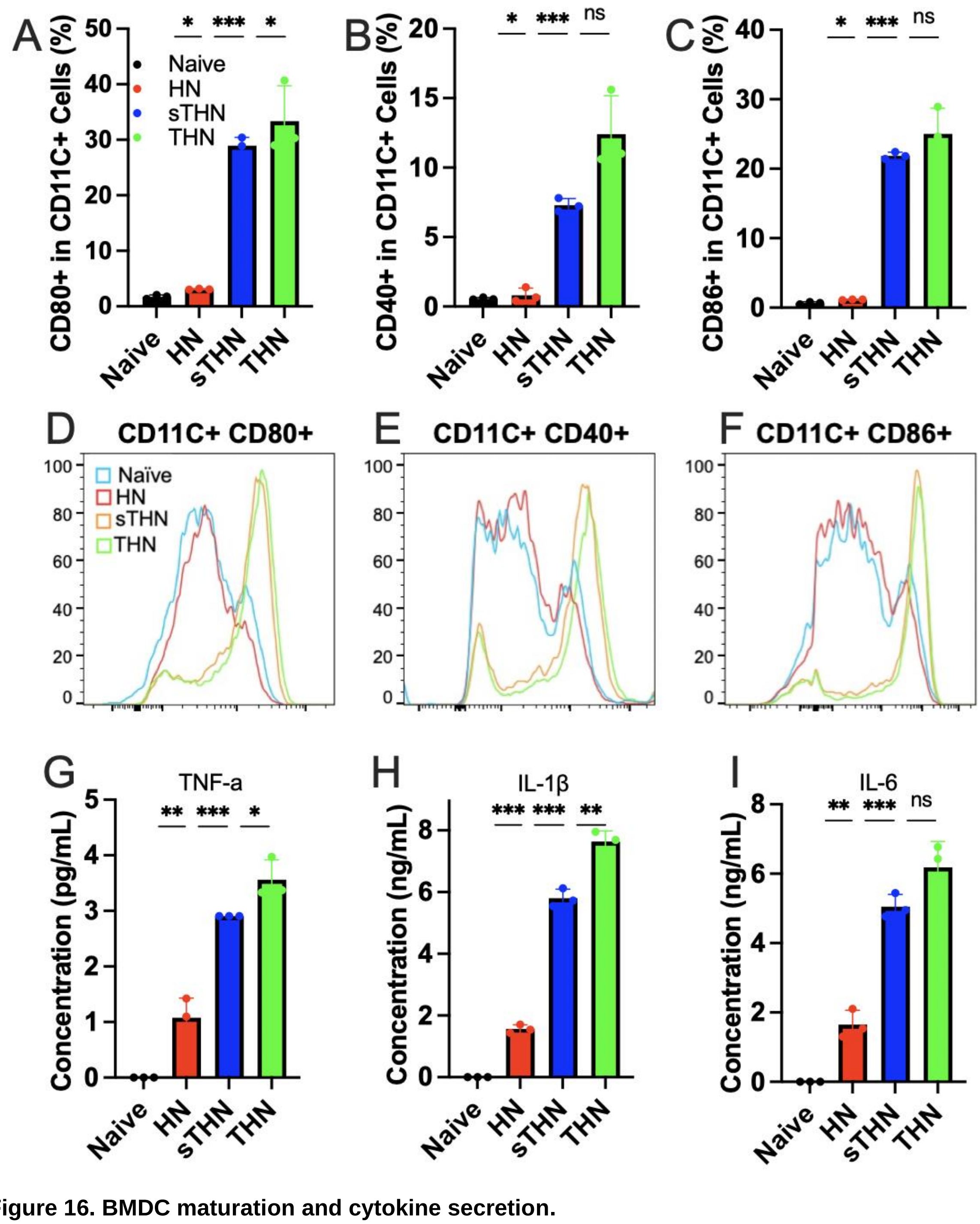

Figure 16. BMDC maturation and cytokine secretion.
Bone marrow-derived dendritic cells (BMDCs) were stimulated with protein nanoparticles with and without tFliC adjuvant. The expression of A,D) CD80, B,E) CD40, and C,F) CD86 on CD11c+ BMDCs. G) TNF-α, H) IL-1β, and I) IL-6 secretions from BMDCs cultured supernatant after stimulations with protein nanoparticles. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* P < 0.05, ** P < 0.01, *** P < 0.001).

Analysis of Serum Antibody Responses. The immunogenicity of HA3/NP nanoparticles (HN), soluble tFliC with HA3/NP nanoparticles (sTHN), and HA3-tFliC/NP nanoparticles with tFliC conjugated on their surface (THN) was examined by measuring the antigen-specific antibody responses in the serum of immunized mice four weeks after intranasal prime and boost vaccinations using ELISA. Following boost immunization, the THN and sTHN formulations resulted in significantly elevated levels of HA3 and Aichi-specific IgG, IgG1, and IgG2a compared to other groups (Figure 17). The tFliC-supplemented nanoparticles induced Th2-biased immune responses, predominantly featuring the IgG1 subtype.

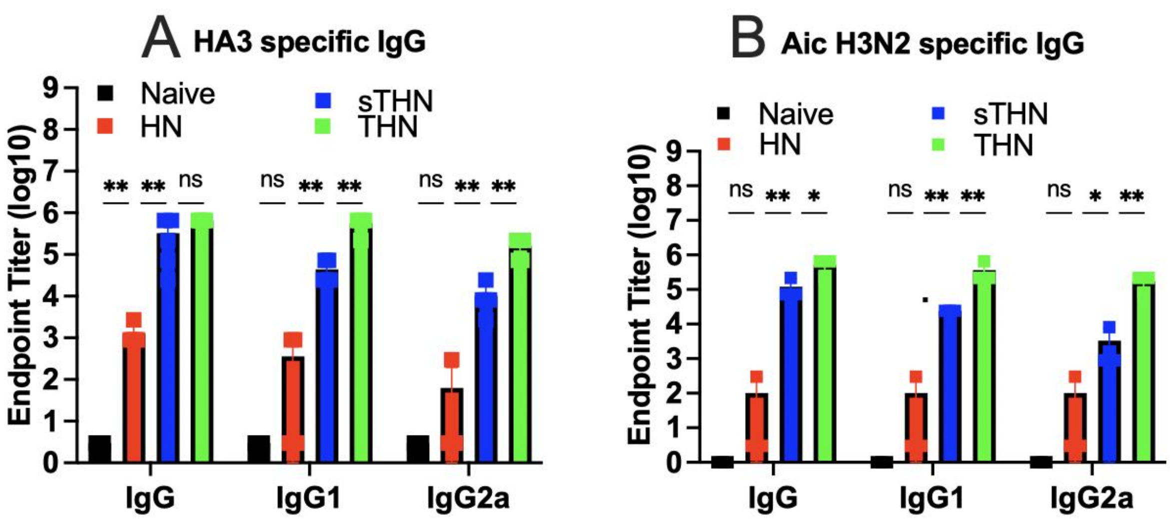

Figure 17. Analysis of serum antibody responses.
BALB/c mice (n=5 per group) were intranasally immunized twice at a 4-week interval with protein nanoparticles. Antigen-specific antibodies in the immune sera were analyzed four weeks post-boost immunization. A)HA3 and B) Aichi-specific isotype titers in serum. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Cross-Reactivity of Serum Antibody Titers. When we examined serum cross-reactivities with various influenza HA proteins and viruses from group 2, the groups immunized solely with HN nanoparticles exhibited low antibody levels (Figure 18). However, adding soluble tFliC (sTHN) improved HA4- and HA10-specific IgG levels. Mice administered tFliC-conjugated THN displayed significantly higher cross-reactive antibodies against all tested HA proteins, including HA4, HA10, and HA7.

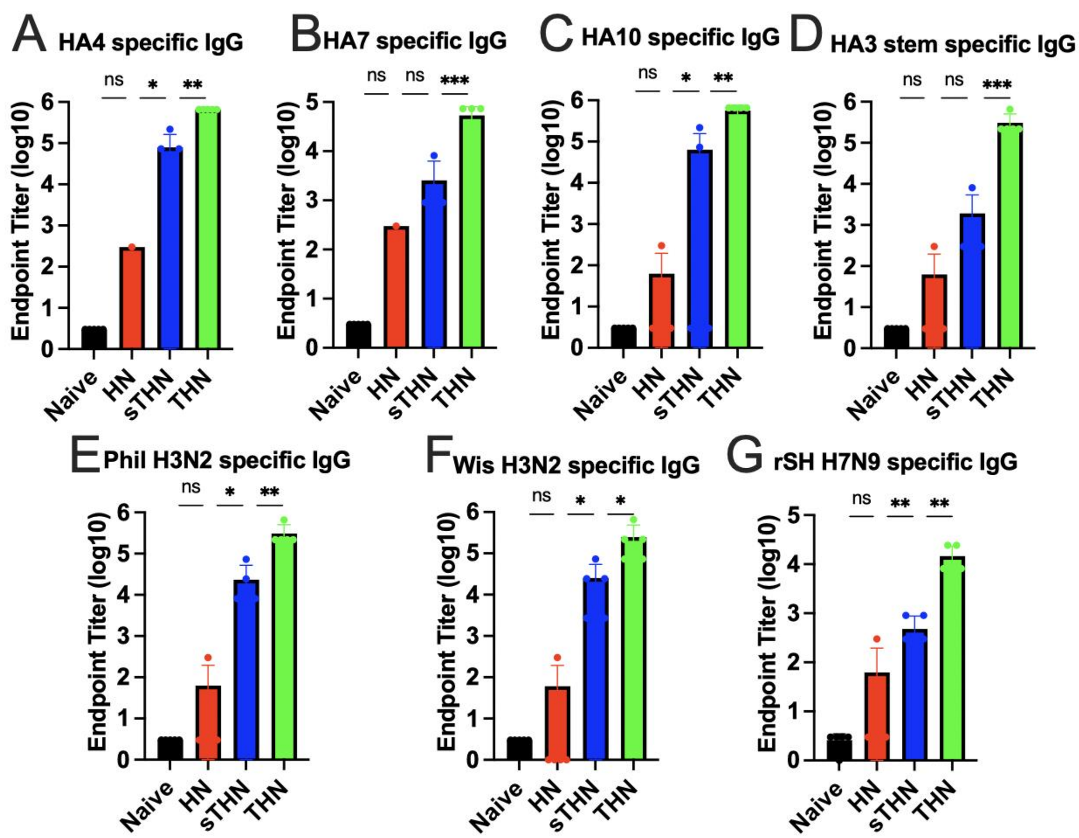

Figure 18. Cross-reactivity of serum antibody.
BALB/c mice (n=5 per group) were intranasally immunized twice at a 4-week interval with protein nanoparticles. Cross-reactivities of immune sera with A–D) various proteins (HA4, HA7, HA10, and HA3 stem) and E-G) viruses (Phili, Wis, and rSH) were measured four weeks post-boost immunization. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Notably, the THN group showed significantly elevated levels of HA3 stem-specific antibodies when the tFliC adjuvant was crosslinked on the nanoparticle surface compared to other groups. Similar findings were observed with different strains used as coating antigens, such as A/Philippine/2/82 (Phili, H3N2), A/Wisconsin/2/2013 (Wis, H3N2), and reassortant A/Shanghai/2/2013 (rSH, H7N9). Immunization with THN nanoparticles significantly increased Phili-, Wis-, and rSH-specific IgG antibody levels (Figure 18 E-G). Overall, the self-adjuvanted THN nanoparticles elicited enhanced cross-reactive antibodies against various HA proteins and the HA3 stem domain, indicating the potential for broader protection.

Assessment of Systemic Cellular Immune Responses. Enzyme-linked immunosorbent spot (ELISpot) assays were conducted to assess systemic cellular immune responses in the spleen and bone marrow four weeks after the boost intranasal immunization. The results showed a significant increase in HA3 and HA4-specific IgG-secreting B cells in the bone marrow and spleen of the THN group compared to other groups (Figure 19A-D). Additionally, the THN group exhibited a marked enhancement in the secretion of IL-4 (Figure 19E-F) and IFN-γ (Figure 19G-H) by splenocytes upon HA3 and HA4 stimulation, compared to the moderate rise in cytokine-secreting splenocytes observed with the incorporation of soluble tFliC in protein nanoparticles (sTHN, Figure 19E-H). These findings indicate that including the tFliC adjuvant on the nanoparticle surface significantly boosts systemic immune responses following intranasal immunization.

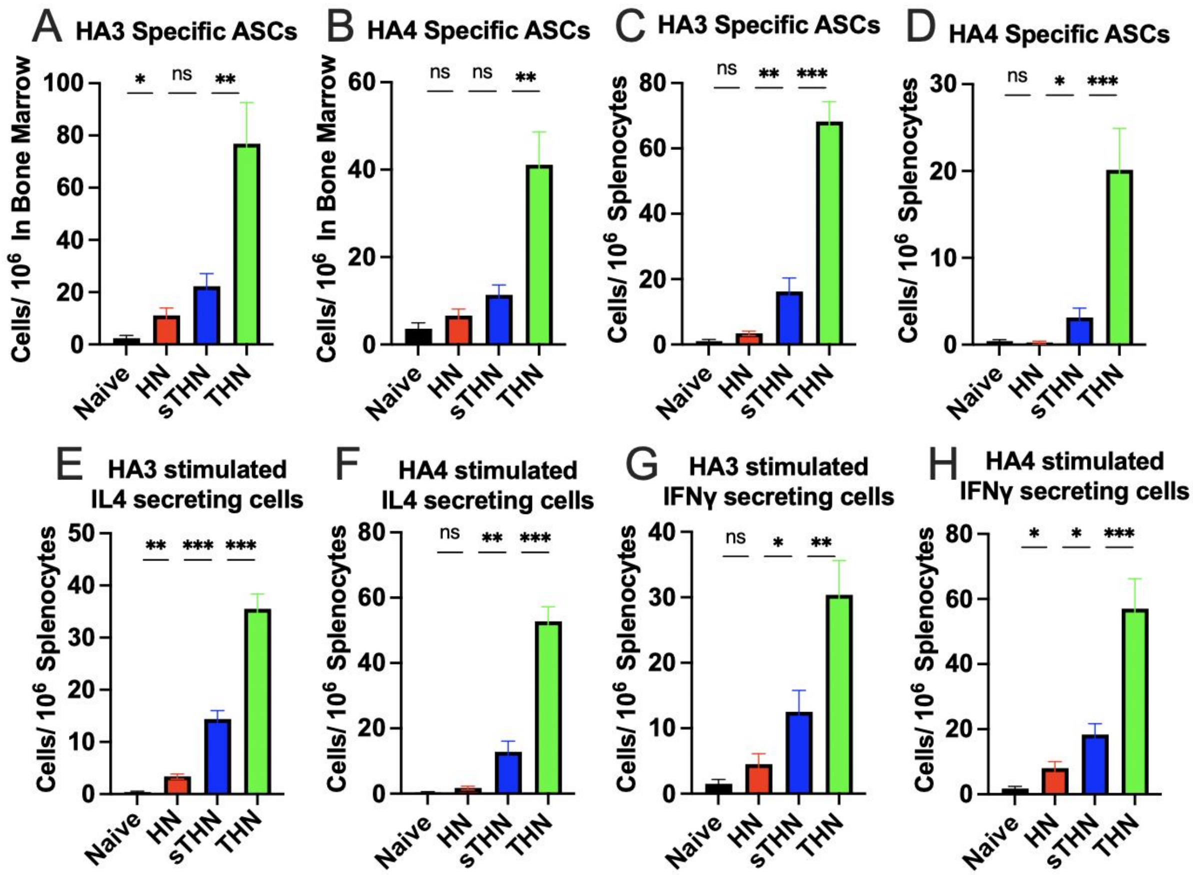

Figure 19. Cellular immune responses.
Mice were intranasally immunized twice at a 4-week interval with different protein nanoparticles. Spleens and bone marrow were collected and analyzed by ELISpot assays four weeks post-boost immunization. HA3-and HA4-specific antibody-secreting cells in the A, B) bone marrows and C, D) splenocytes were measured. E, F) IL-4 and G, H) IFN-γ and secreting splenocytes were measured after HA3 and HA4 protein stimulation. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* P < 0.05, ** P < 0.01, *** P < 0.001).

Evaluation of Protective Efficacy. The protective efficacy of the tFliC adjuvanted protein nanoparticles was examined by testing against homologous and heterologous influenza virus infections four weeks post-booster vaccination. The mice were exposed to 5x LD50 of the homologous Aichi strain (Figure 20A) or 3x LD50 of the heterologous Phili- strain (Figure 20B), with body weights tracked over 14 days following infection. In the Aichi viral challenge, the THN-immunized mice showed no weight loss and achieved a 100% survival rate. While the sTHN-immunized mice also had a 100% survival rate, they experienced considerable weight loss from day 4 to day 8. On the other hand, both HN-immunized and naïve mice displayed rapid weight loss and died between days 5 and 8 post-challenge.

In the heterologous Phili- viral challenge, both groups that received tFliC-adjuvanted vaccinations (sTHN or THN) maintained a 100% survival rate despite initial weight loss (~8% by day 5). The THN group exhibited milder symptoms and began regaining weight from day 6, returning to baseline by day 14. In contrast, the sTHN group faced significant weight loss and symptoms like decreased activity, ruffled fur, and hunched posture from days 3 to 9 (Figure 20B). Mice vaccinated with HN experienced severe weight loss with only a 20% survival rate, while the naïve mice succumbed to the infection within seven days. The observed survival rates and weight loss outcomes against both homologous and heterologous influenza virus infections indicate that incorporating the tFliC adjuvant onto the nanoparticle surface significantly enhances the protective efficacy of the intranasal vaccines.

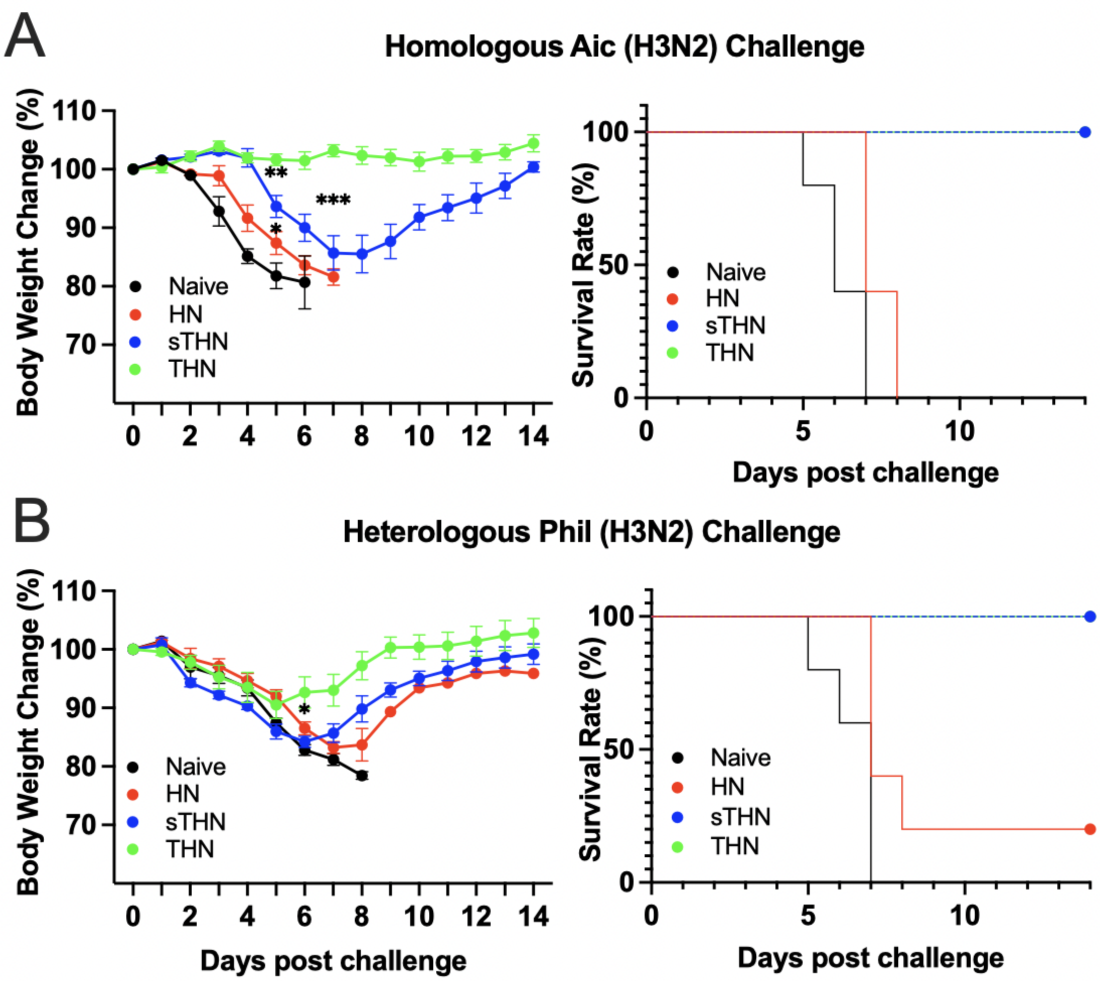

Figure 20. Protective efficacy against influenza viral challenge.
Four weeks post-boost immunization, groups of mice were challenged with homologous Aic (H3N2) and heterosubtypic Phil (H3N2) influenza viruses. Body weight changes were monitored for 14 days following the viral challenge, and survival rates were calculated. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Induction of Mucosal Immune Responses. Mucosal vaccines stimulate secretory IgA (sIgA) production, which is critical in mitigating the severity and fatality of various respiratory and gastrointestinal infections(Lavelle & Ward, 2022; Mudgal et al., 2020). Therefore, we assessed antigen-specific antibody levels in bronchoalveolar lavage fluid (BALF) and nasal wash and the presence of tissue-resident memory B cells

(B_{RM}) in the lungs four weeks post-booster vaccination to determine whether tFliC-adjuvanted nanoparticles can elicit mucosal immune responses.

BALF analysis demonstrated a significant increase in Aichi- and HA3-specific IgG and IgA antibodies in mice vaccinated with tFliC-conjugated THN nanoparticles (Figure 21A-D). Conversely, the HN group showed no such antibody response, while the sTHN group only showed a moderate rise in Aichi- and HA3-specific IgG compared to naïve controls (Figure 21A and 21C). In nasal wash samples, only the THN group exhibited elevated levels of virus- and HA3-specific IgG and IgA, distinguishing it from the rest of the groups (Figure 21E-H).

The vaccine's effect on B_{RM} populations in the lungs was also examined. B_{RM} cell populations were determined as $CD19^{+}B220^{-}IgD^{-}IgM^{-}CD38^{+}CD69^{+}$. HN and sTHN groups showed an increase in $CD38^{+}CD69^{+}$ B_{RM} cells compared to naïve mice. However, the THN group with tFliC-conjugated nanoparticles showed a significantly higher percentage of B_{RM} cells in the lungs than the HN and sTHN groups (Figure 21I). The study demonstrated that intranasal vaccination with tFliC-conjugated protein nanoparticles significantly enhances mucosal antibody responses and tissue-resident memory B cell (B_{RM}) lung populations.

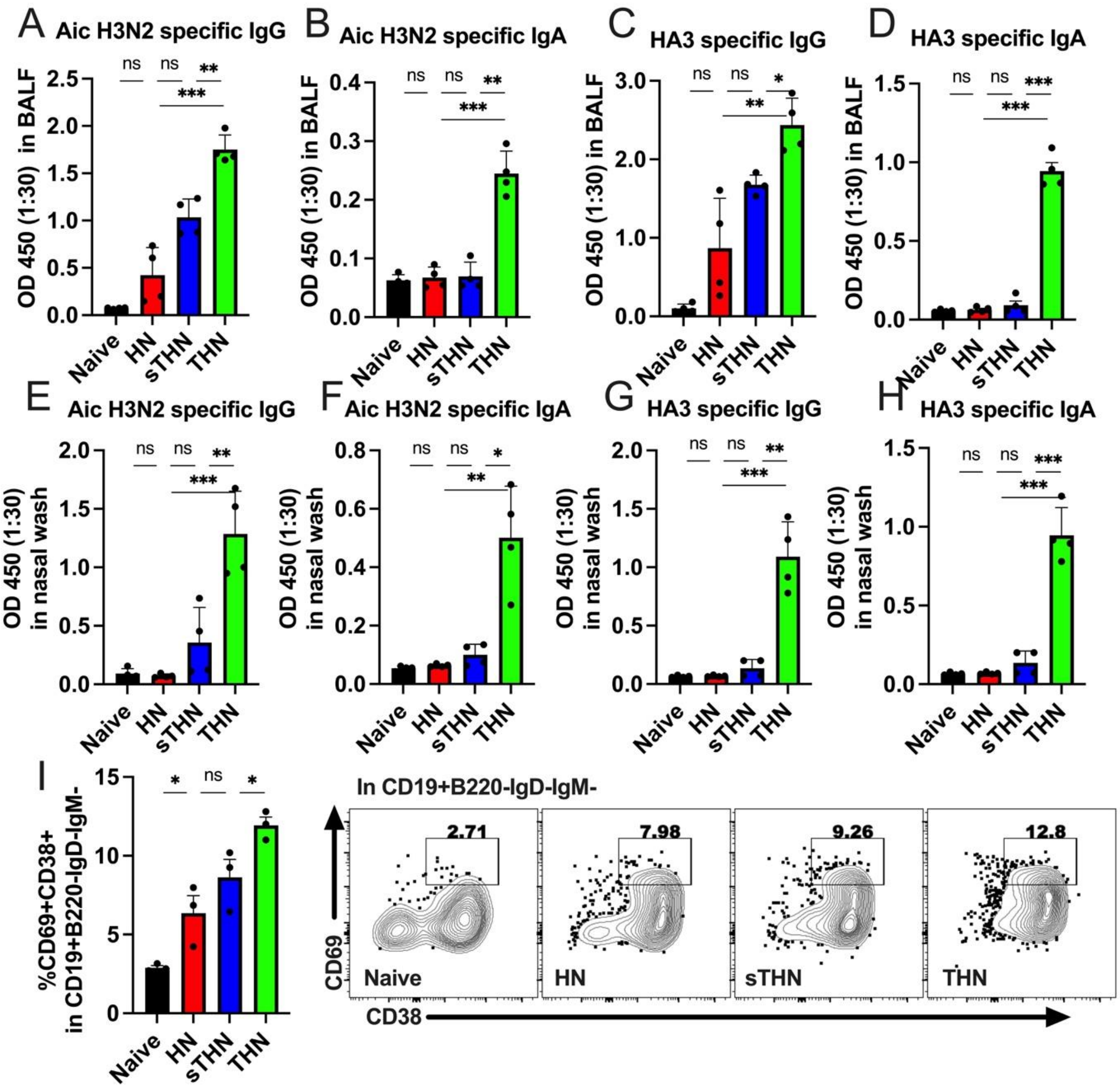

Figure 21. Mucosal immune responses and cell populations in pulmonary tissue.
Mice were intranasally immunized twice at a 4-week interval with protein nanoparticles, with and without tFliC adjuvant. One-month post-boost immunization, nasal and BALF washes were collected to determine IgA and IgG levels by ELISA. A, B) Aichi-specific IgG and IgA levels in BALF. C, D) HA3-specific IgG and IgA levels in BALF. E, F) Aichi-specific IgG and IgA levels in nasal washes. G, H) HA3-specific IgG and IgA levels in nasal washes. I) Cell populations in localized pulmonary tissue were analyzed one-month post-boost intranasal immunization, focusing on the frequencies of $CD38^+CD69^+$ populations within $CD19^+B220^-IgD^-IgM^-$ cells in the lungs. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

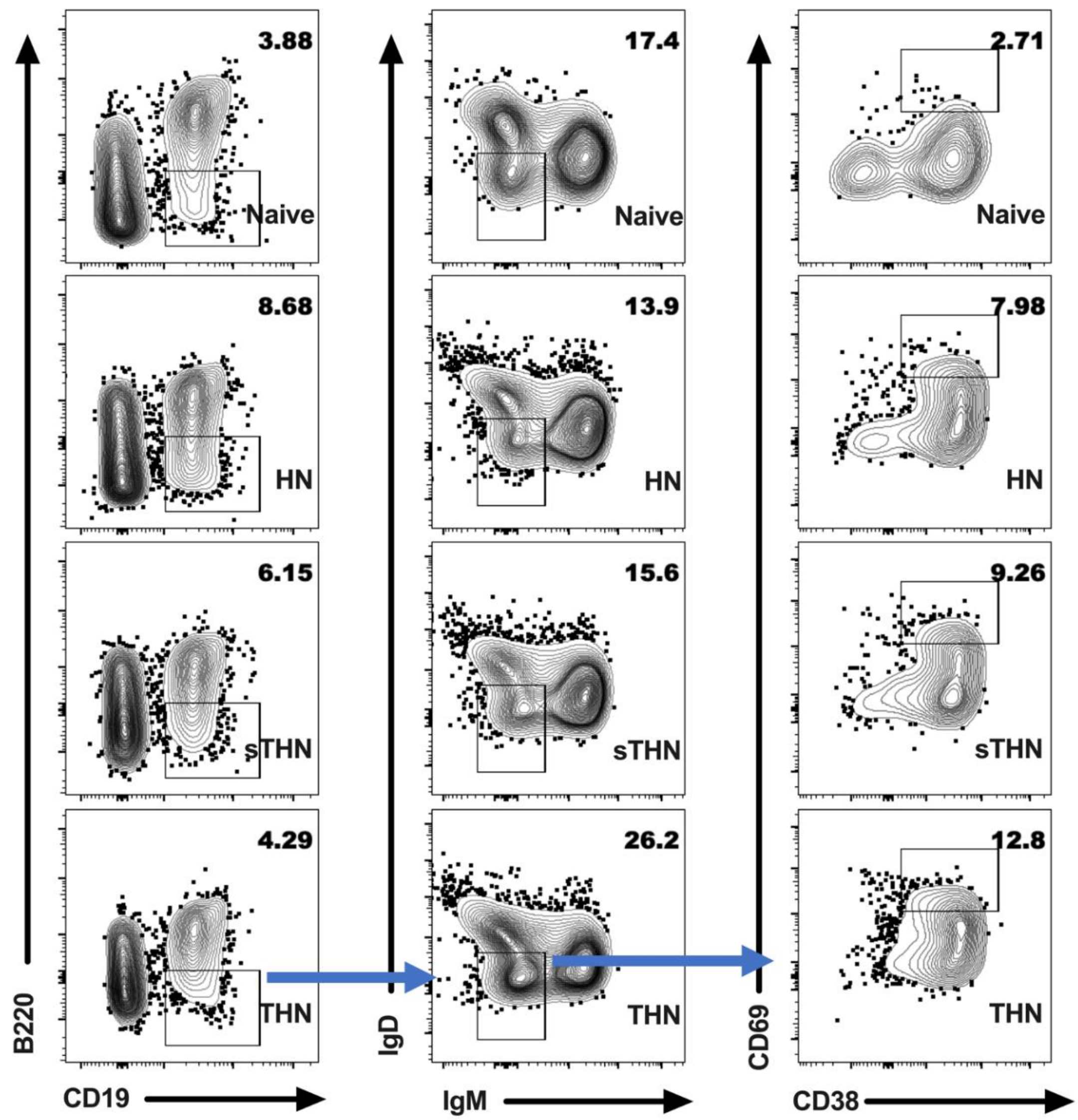

Figure 22. The gating method for tissue-resident memory B cells in the lung.
Cells were isolated from lung tissues four weeks post-boosting immunization and stained for lung tissue-resident memory B cells, designated as $CD19^{+}B220^{-}IgD^{-}IgM^{-}CD69^{+}CD38^{+}$ populations.

3.2.b. Discussion

The inclusion of NP was strategic, leveraging its ability to provoke a robust T-cell response. At the same time, HA is targeted to elicit neutralizing antibodies, which are crucial for preventing viral entry and spread. The tFliC, modified to enhance safety while maintaining its immunostimulatory properties, acted as a potent mucosal adjuvant through toll-like receptor 5 (TLR5) activation, improving the immune response.

This innovative approach yielded promising results, including significantly increased antigen-specific IgA and IgG levels in mucosal samples, crucial for first-line defense against infection. Additionally, the vaccine enhanced populations of lung-resident memory B cells, vital for long-term immune protection and rapid response to subsequent virus exposure(McGrath et al., 2022).

The development of mucosal vaccines presents significant challenges, primarily due to the restricted uptake of antigens across mucosal barriers(Tsai et al., 2023). However, these vaccines hold the potential to initiate localized immune responses at primary infection sites, such as the respiratory tract, providing an immediate and effective defense against pathogens compared to systemic vaccines(Lavelle & Ward, 2022). Nanoparticle platforms, with their high immunogenicity, targeted delivery capabilities, and controlled release properties, have emerged as promising solutions to these challenges(Dong & Wang, 2022; Y. Wang et al., 2018). These nanoparticles can protect antigens from degradation, enhance their transport across mucosal barriers, and present antigens to the immune system, improving the vaccine's effectiveness (Khalaj-Hedayati et al., 2020). In our study, we employed double-layered protein nanoparticles for these purposes.

Nanoparticles within the 20-200 nm range can efficiently traverse lymphatic endothelial cell junctions, whereas larger particles face difficulties entering lymphatic vessels. Our nanoparticles, approximately 200 nm in size with a narrow size distribution (PDI less than 0.3), were ideally suited for effective antigen trafficking. To further optimize these nanoparticles, we incorporated mucosal adjuvants to enhance antigen uptake and processing, as previous research has shown that intranasal administration of protein nanoparticles alone often results in weak immune responses(Lavelle & Ward, 2022; Zhu et al., 2023a).

We utilized a truncated version of FliC (tFliC), which excluded the hypervariable regions to mitigate potential adverse immune reactions while retaining the beneficial adjuvant properties of flagellin(Hajam et al., 2017; Rhee et al., 2023; Zhao et al., 2021). The tFliC-conjugated nanoparticles maintained the TLR5 innate signaling activity of flagellin, which is essential for promoting dendritic cell maturation, cytokine secretion, and initiating immune responses. Notably, the intranasal administration of tFliC-conjugated protein nanoparticles in mice showed no adverse effects, such as significant body weight loss (Figure 23), reduced activity, hunchback, or ruffled fur post-immunization, indicating a favorable safety profile for tFliC as a potent mucosal adjuvant.

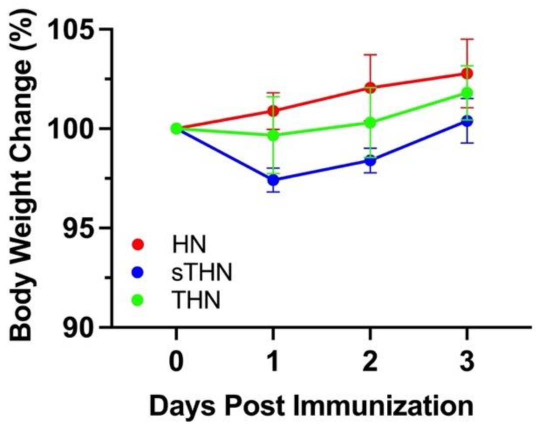

Figure 23. Body weight changes post intranasal immunization.
Mice's body weight changes were monitored in the early days post-intranasal immunization.

We then investigated the ability of the double-layered nanoparticles to elicit robust systemic and mucosal immune responses. Our results demonstrated that protein nanoparticles adjuvanted with tFliC significantly enhanced humoral immune responses. The increased levels of antigen-specific antibodies displayed a notable Th2 bias, as indicated by the predominance of the IgG1 subtype, suggesting a preference for antibody-mediated immunity over cell-mediated immunity. Additionally, the immune sera exhibited cross-reactivity with various influenza strains and targeted the conserved HA3 stem domain, indicating broad protective capability. Furthermore, evaluations of the cellular immune response revealed increased IL-4 and IFN-γ-secreting lymphocytes in mice immunized with tFliC-adjuvanted nanoparticles.

Implementing these novel nanoparticles also enhanced the mucosal immune responses, as evidenced by substantial increases in antigen-specific IgA and IgG antibody levels in BALF and nasal wash. Additionally, the vaccine formulation notably increased the populations of lung-resident memory B cells, which are essential for rapid and effective immune recall upon re-exposure to the virus, thus providing long-lasting immunity. This enhancement likely accounts for the significant cross-protection conferred by incorporating tFliC against both homologous and heterologous influenza viral challenges, as indicated by reduced body weight loss and improved survival rates compared to control groups.

Overall, incorporating tFliC into protein nanoparticles leveraged its adjuvant properties alongside the benefits of nanoparticle delivery, thereby enhancing both immunogenicity and protection through multiple mechanisms. Primarily, it activated the TLR5 signaling pathways, which stimulated the production of pro-inflammatory cytokines and enhanced the maturation of antigen-presenting cells (APCs), thereby improving antigen presentation and T-cell activation. Additionally, conjugating the adjuvant to nanoparticles ensured a controlled and stable presentation of tFliC on the nanoparticle surface, allowing sustained interaction with immune cells and extended immune stimulation. This prolonged presentation boosted the efficacy of tFliC as an adjuvant, fostering long-lasting immune responses. Moreover, this conjugation aided in targeting specific immune cells or tissues, reducing systemic side effects and improving bioavailability.

3.2.c. Conclusion

This study demonstrated the potential of double-layered protein nanoparticles to enhance antigen-specific mucosal and systemic immune responses, addressing the need for more effective influenza vaccines. By incorporating tFliC as a mucosal adjuvant, we developed a novel intranasal vaccine that leverages the benefits of nanoparticle delivery. The intranasal administration of these vaccines significantly boosted immunogenicity and protective efficacy through TLR5 activation, improved antigen presentation, stable adjuvant presentation, and targeted delivery, thereby minimizing side effects and enhancing bioavailability. These findings highlight the potential of tFliC-conjugated protein nanoparticles as a potent mucosal influenza vaccine candidate.

Future research should explore this platform further to optimize broader and more durable immunity against influenza viruses. The following section will examine how sequential immunization with these nanoparticles could further improve the induced immune responses.

3.3. EFFICACY OF SEQUENTIAL IMMUNIZATION WITH HA3-tFliC/NP NANOPARTICLES

In the previous section, we observed that intranasal vaccination with tFliC-adjuvanted protein nanoparticles improved survival rates against homologous and heterologous H3N2 viral challenges. Building on these findings, this section aimed to further enhance mucosal and systemic immune responses by administering tFliC-adjuvanted protein nanoparticles through sequential immunization. Specifically, the prime dose was delivered in five applications over eight days, utilizing the slow-delivery strategy to optimize immune responses, as outlined in Figure 24.

Figure 24. Schematic of sequential-prime immunization.
Experimental schematic. Groups of mice (n = 3) were immunized with a total of 15μg of tFliC conjugated protein nanoparticles at one time (THN) or with 3μg of the vaccine in 8 consecutive days (THN-S), respectively. The mice were boosted four weeks after the primary immunization.

3.3.a. Results

Sequential Priming Immunization. We examined the impact of extended antigen exposure through sequential intranasal immunization to enhance further the immune responses and protective efficacy of the tFliC-adjuvanted nanoparticle vaccines. We hypothesized that dividing the prime dose into five sequential administrations given every other day would improve protective immunity. In this study, one group of mice (THN-S) received five doses of 3μg of THN administered every other day. In contrast, another group (THN) received the traditional single prime immunization (15μg dose of THN), as illustrated in Figure 20. Four weeks after the initial immunization, both groups received a booster dose of 15μg of THN intranasally.

Analysis of Serum Antibody Responses. Sera were collected four weeks after the prime and boost vaccinations to compare the immune responses between the single- and sequential-prime immunizations. The sequential prime immunization resulted in higher levels of Aichi- and HA3-specific IgG levels compared to the conventional single-dose prime immunization. However, after administering a single dose of booster immunization to both groups of mice, IgG levels significantly increased compared to the naïve group, with no significant differences in antibody levels between the two immunized groups (Figure 25). Additionally, both groups displayed strong and comparable cross-reactive antibody titers against various HA proteins from HA phylogenetic group 2 (HA4, HA7, and HA10) and the conserved HA3 stem domain (Figure 26). There were no significant differences between the groups.

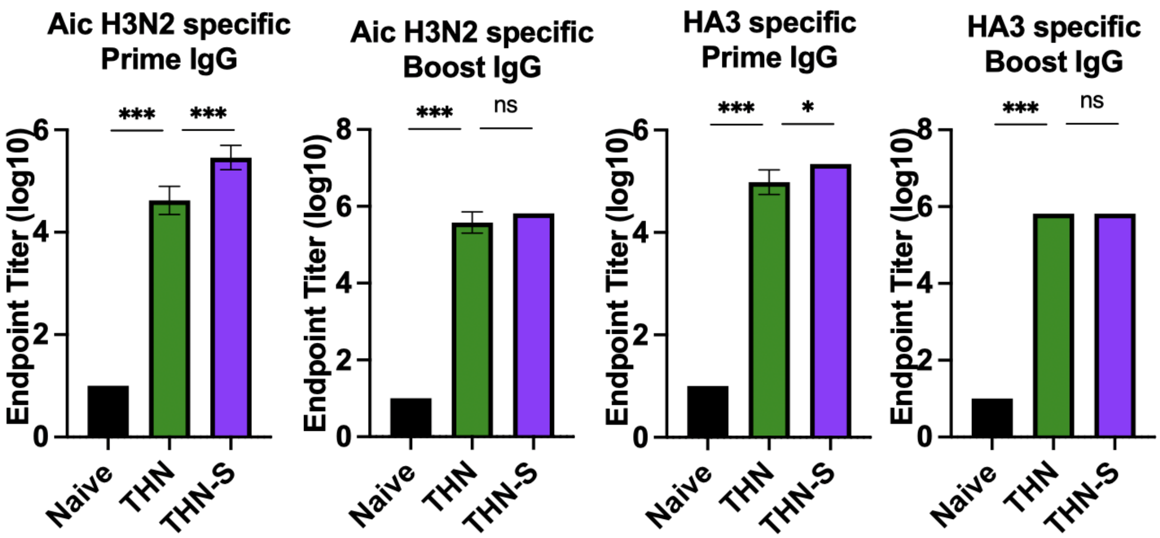

Figure 25.Aichi- and HA3-specific IgG titers of sequentially primed mice.
Mice were initially immunized with either a single dose of 15μg tFliC-conjugated protein nanoparticles (THN) or 3μg of vaccine administered over 8 consecutive days (THN-S). The mice received a booster immunization four weeks later. Aichi-specific and HA3-specific IgG titers after prime and booster vaccination were examined. *The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).*

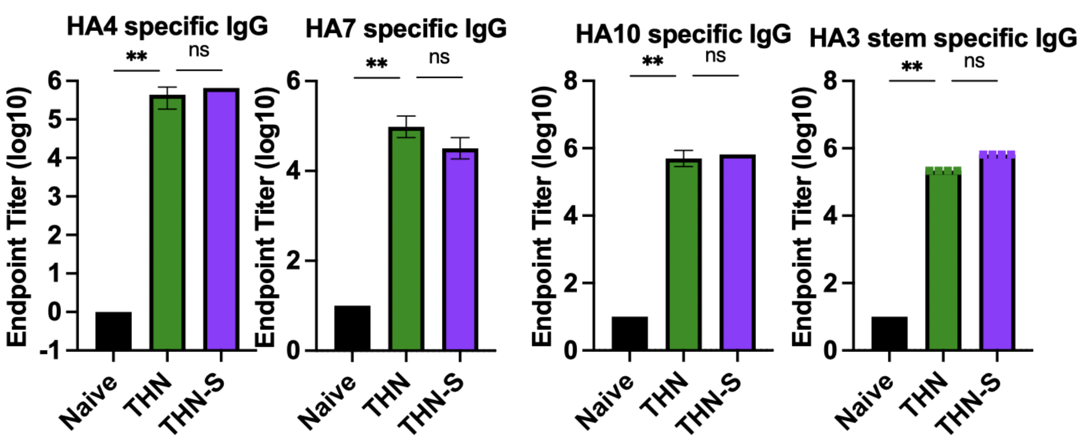

Figure 26. Cross-reactivity of serum antibody in sequentially immunized mice.
Mice were initially immunized with either a single dose of 15μg tFliC-conjugated protein nanoparticles (THN) or 3μg of vaccine administered over 8 consecutive days (THN-S). HA4, HA7, HA10, and HA3 stem-specific cross-reactivity of immune sera were examined 4 weeks post booster immunization. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Although there were no significant differences in IgG antibody levels between the two immunized groups, further analysis of the Aichi- and HA3- and specific isotype titers in serum showed that the sequential priming, significantly enhanced, and balanced antigen-specific IgG1 and IgG2a antibody titers compared to the conventional single priming immunization (Figure 27). Our findings indicate that the sequential immunization resulted in more balanced antigen-specific Th1/Th2 antibody responses following nanoparticle immunization.

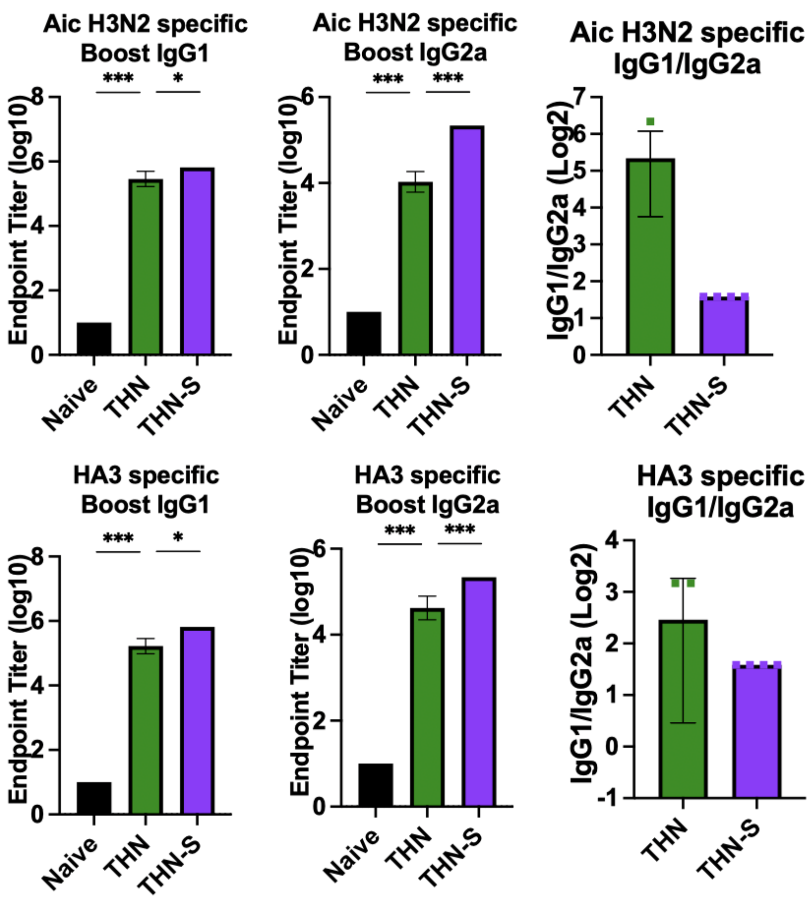

Figure 27. Aichi- or HA3-specific IgG isotype titers of sequentially primed mice.
Aichi- and HA3- specific IgG, IgG1, and IgG2 titers in mouse serum were examined 4 weeks post-booster immunization. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* P < 0.05, ** P < 0.01, *** P < 0.001).

Protective Efficacy of Sequential Immunization Against Viral Challenge.

Given that both immunization strategies induced similar antibody levels post-booster immunization, we further assessed their protective efficacy by challenging the mice with 3× LD50 of Phili- (A/H3N2) influenza virus four weeks after the booster immunization. Body weight changes and survival rates were monitored over 14 days to evaluate the outcomes (Figure 28). Naïve mice experienced significant weight loss and died within seven days. The THN group showed moderate weight loss initially but began to recover by day six, achieving a 100% survival rate. In contrast, the THN-S group exhibited complete protection against the Phili- challenge without weight loss over 14 days

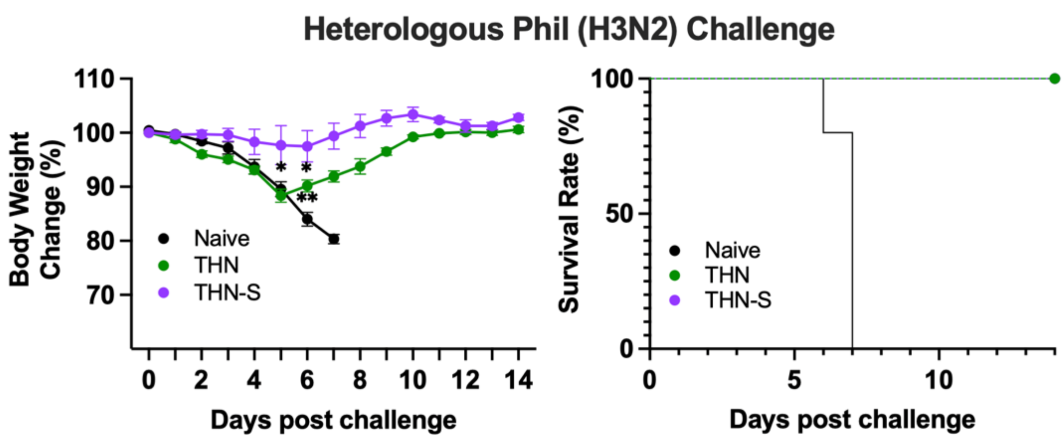

Figure 28. Body weight changes and survival rates of sequentially primed mice.
The sequential primed mice were challenged with the 3×LD_{50} Phili- virus challenge. Body weight changes and survival rates were examined for 14 days. Data is shown as mean ± SEM.

Analysis of Secreted IgA Levels. To investigate the mechanisms behind the enhanced protection observed with sequential prime immunization, we analyzed the levels of secreted IgA (sIgA) in mucosal washes two weeks after prime immunization. Mice were immunized intranasally with THN using the sequential prime strategy (THN-S) and compared to the control group receiving a single 15μg dose of THN, as outlined in Figure 29A. The THN group showed increased Aichi- and HA3-specific secretory IgA (sIgA) levels in bronchoalveolar lavage fluids (BALFs) and nasal washes (Figure 29B). Notably, the slow delivery of THN significantly boosted antigen-specific sIgA levels in mucosal washes compared to single-dose immunization.

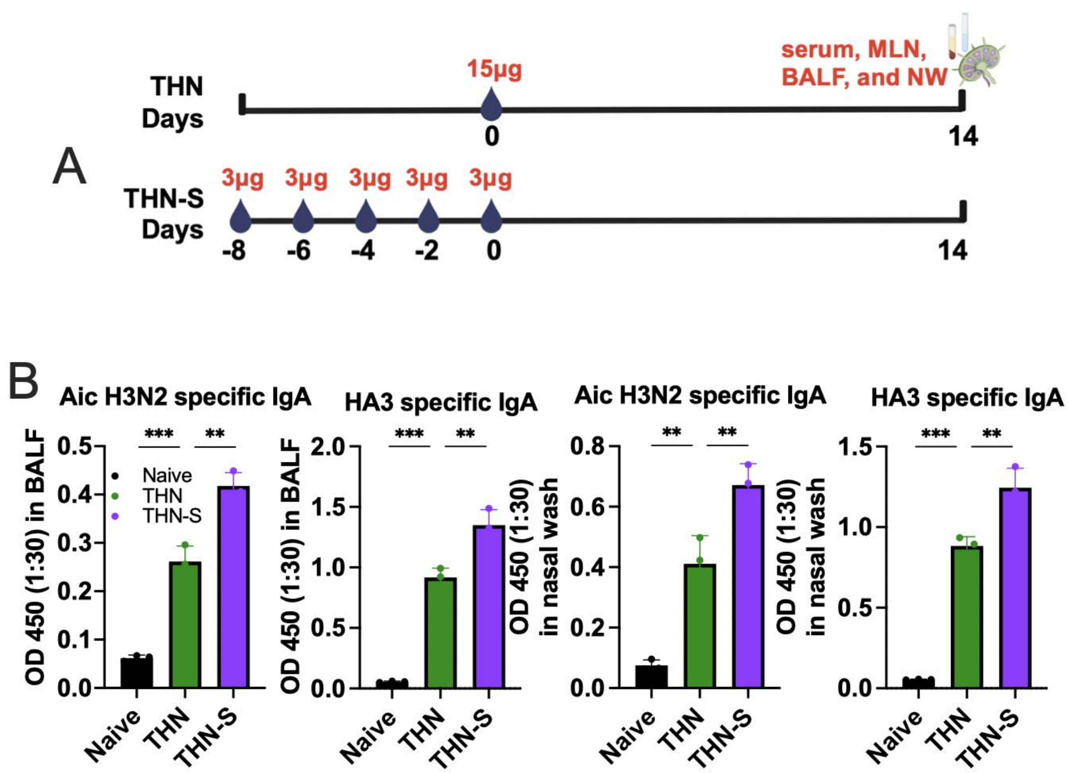

Figure 29. Mucosal immune responses induced from sequential priming.
A) Mice were intranasally immunized with THN using a sequential prime strategy (THN-S) or a single 15μg dose (THN). Serum, BALF, mLN, and nasal washes were collected two weeks post-immunization. B) Aichi- and HA3-specific secretory IgA (sIgA) levels were measured in BALFs and nasal washes. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Evaluation of Germinal Center Reactions and Effector T-Cell Populations. We also collected mediastinal lymph nodes (mLNs) to investigate further the immunological mechanisms underlying the enhanced protection observed with sequential immunization. Specifically, we analyzed germinal center (GC) reactions and effector T-cell populations in lung-draining lymph nodes. Sequential administration significantly increased the proportion of GL7 and CD95 double-positive B cell populations, indicative of robust germinal center activity, compared to the single-dose immunization strategy (Figure 30A-B).

Additionally, sequential delivery led to an increase in the percentage of $CD4^+$ T follicular helper (Tfh) cells, with a higher proportion of $CXCR5^+PD\text{-}1^+CD4^+$ populations in mLNs compared to single-dose immunization (Figure 30C-D). This enhancement suggests a more effective Tfh cell response, crucial for sustaining germinal center reactions and promoting long-lasting antibody production. Moreover, we observed elevated levels of a $CXCR5^+PD\text{-}1^-$ population, potentially representing memory Tfh (mTfh) cells, in the sequentially immunized group compared to the single-dose group (Figure 30C-D). Furthermore, sequential delivery increased the populations of $CD4^+$ effector T cells ($CD4^+$ $CD44^+$ $CD62L^-$) (Figure 30E-F).

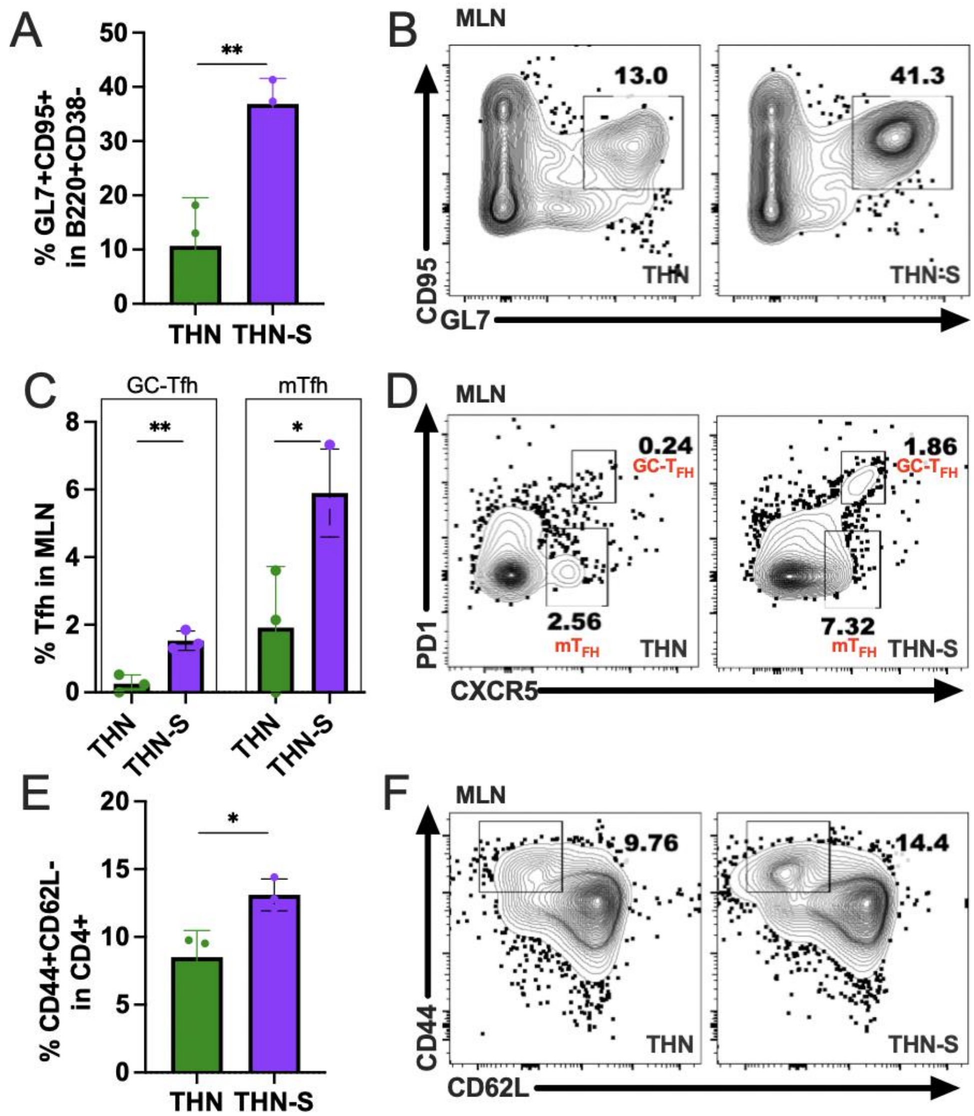

Figure 30. GC and T-cell populations induced from sequential priming immunization.
mLN draining lymph node was collected and analyzed by flow cytometry 14 days post-intranasal immunization. Flow cytometry analysis and the statistics of A, B) GC B cell ($CD95^+$ $GL7^+$), C, D) Tfh ($CXCR5^+PD1^+$) cell populations, and E, F) effector T cell ($CD44^+$CD62L-) were examined in mediastinal lymph nodes (mLN). The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Optimizing Sequential Immunization Strategies: Evaluating the Efficacy of Reduced Dosing Strategy. Initially, our immunization strategy involved administering 5 doses over 8 days, which enhanced the immune response compared to the conventional single priming immunization. However, to evaluate whether a more optimized vaccination strategy could achieve comparable or even superior outcomes, we adjusted the strategy to 3 doses over 6 days. This adjustment was driven by the potential benefits of a reduced dosing schedule, which could be more convenient for patients, cost-effective, and resource-efficient—key factors in improving patient compliance and the practicality of large-scale immunization programs.

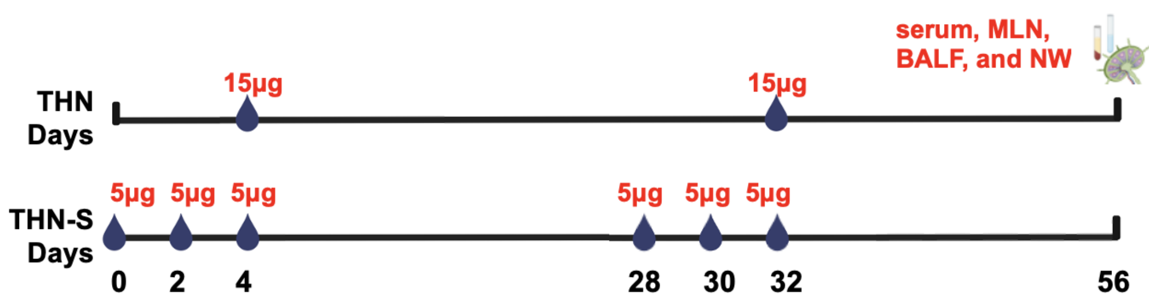

Figure 31. Schematic of optimized sequential immunization strategy.
Experimental schematic. Groups of mice were primarily primed and boosted with 15μg of protein nanoparticles at one time (THN) or with 5μg of vaccine in 6 consecutive days (THN-S), respectively.

We hypothesized that dividing the total dose (15μg) into three sequential administrations, each given every other day, would still effectively enhance protective immunity. To test this, one group of mice (THN-S) received three doses of 5μg of THN every other day. In contrast, another group (THN) received the traditional single prime immunization with a 15μg dose of THN, as illustrated in Figure 31. Three weeks after the initial immunization, mice in the THN-S groups received three doses of 5μg of THN, and mice in the THN group were given a booster dose of 15μg of THN intranasally.

Assessment of Serum Antibody Responses. The immunogenicity of the optimized sequential immunization strategy was examined by measuring the antigen-specific antibody responses in the serum three weeks after prime and boost vaccinations using ELISA. The optimized sequential immunization of THN formulations resulted in significantly elevated levels of HA3-specific IgG antibodies after the prime immunization. Furthermore, elevated IgG, IgG1, IgG2a, and IgA levels were observed compared to the conventionally immunized mice after the booster immunization (Figure 32). Additionally, the optimized sequential immunization strategy induced a more balanced antigen-specific Th1/Th2 antibody response than the conventional strategy.

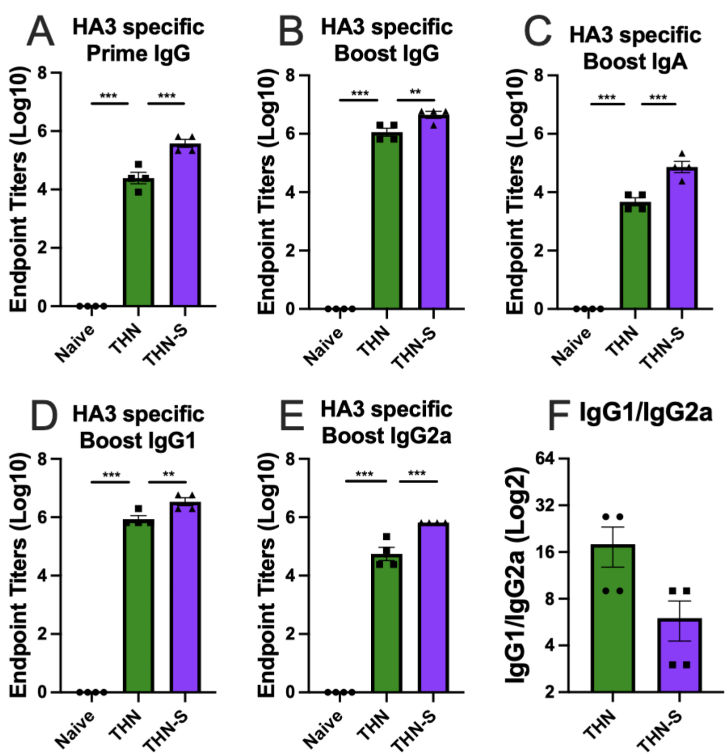

Figure 32. Serum antibody response of sequentially immunized mice.
HA3-specific antibodies in the immune sera were analyzed three weeks after A) prime and B) boost immunizations. HA3-specific C) IgA, D) IgG1, and E) IgG2a isotype titers in serum were examined. The histograms were presented as mean ± SEM. F) IgG1/IgG2a ratio was also calculated. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Analysis of Systemic Cellular Immune Responses. To comprehensively assess the systemic cellular immune responses elicited by the optimized immunization strategy, we performed ELISpot assays on spleen and bone marrow samples collected four weeks after the intranasal booster immunization. The analysis revealed a notable increase in HA3-specific IgG- and IgA-secreting B cells within both the bone marrow and spleen of the sequentially immunized group compared to the conventionally immunized and control groups (Figure 33). This significant elevation in antigen-specific B cell activity suggests that sequential immunization is critical in amplifying systemic immune responses, essential for long-term protection against the targeted antigens.

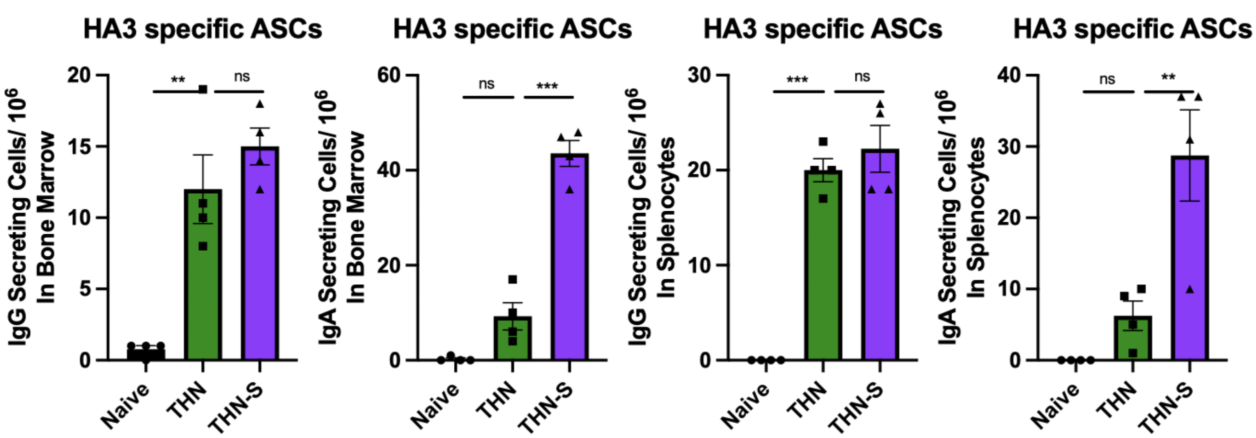

Figure 33. Cellular immune responses of sequentially immunized mice.
HA3-specific cellular immune responses in bone marrow and spleen cells were observed three weeks post-boost immunization. HA3-specific IgG- and IgA- antibody-secreting cells were measured. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Evaluation of Germinal Center Reactions and Effector T-Cell Populations.

To further investigate the immune responses elicited by the optimized immunization strategy, we collected mediastinal lymph nodes (mLNs). We analyzed the germinal center (GC) B cell and effector T-cell populations within the draining lymph nodes. Our results demonstrated that sequential priming immunization led to a significant increase in the proportion of GL7 and CD95 double-positive B cells compared to conventional single-dose immunization (Figure 34).

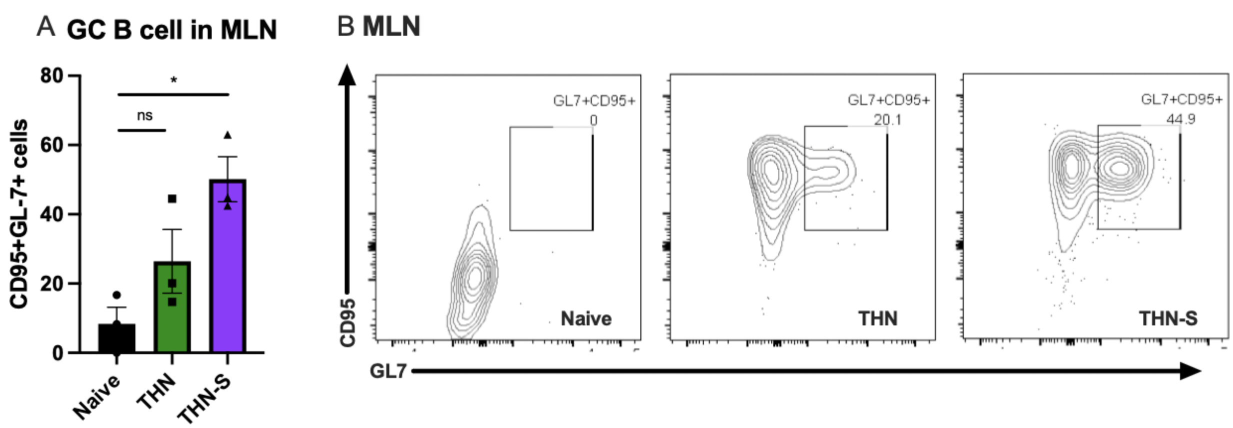

Figure 34. GC populations induced from sequentially immunized mice.
mLN draining lymph node was collected and analyzed by flow cytometry three weeks post-intranasal immunization. Flow cytometry analysis and the statistics of GC B cell ($CD95^{+}$ $GL7^{+}$). The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

In addition to enhanced germinal center activity, the optimized sequential immunization strategy also increased levels of CD4+ T follicular helper (Tfh) cells compared to the conventional immunization strategy. Specifically, we observed higher proportions of $CXCR5^{+}PD\text{-}1^{+}CD4^{+}$ Tfh cells in the mLNs of the sequentially immunized group than those receiving a single dose (Figure 35A). Moreover, the sequential strategy also increased the presence of $CXCR5^{+}PD\text{-}1^{-}$cells, potentially representing memory Tfh (mTfh) cells (Figure 35B).

These findings collectively highlight the ability of the optimized sequential immunization strategy to promote both germinal center activity and effector T-cell responses despite the reduction in the number of doses from 5 to 3. The ability to elicit strong germinal center and effector T-cell responses with fewer doses underscores the efficiency and potential practicality of this approach for broader immunization programs

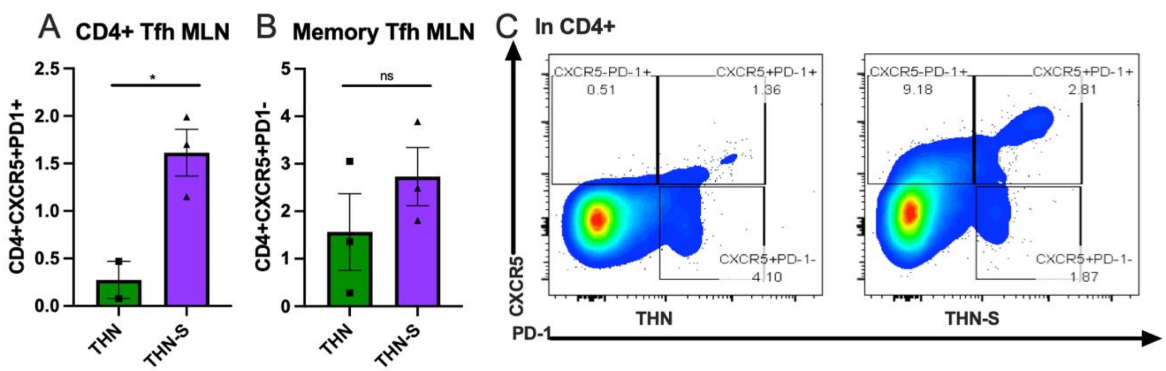

Figure 35. Tfh-cell populations in mLN of sequential immunized mice.
Flow cytometry analysis and the statistics of Tfh ($CXCR5^{+}PD1^{+}$) cell populations in mLN draining lymph nodes collected and analyzed by flow cytometry 3-weeks post-boost-immunization. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* P < 0.05, ** P < 0.01, *** P < 0.001).

Assessment of Mucosal Immune Responses. To evaluate whether the optimized sequential immunization strategy could effectively elicit mucosal immune responses, we measured antigen-specific antibody levels in bronchoalveolar lavage fluid (BALF) and nasal wash samples four weeks after the boost vaccination. ELISA analysis revealed a significant increase in Aichi- and HA3-specific IgG and IgA antibodies in the BALF and nasal wash of sequentially vaccinated mice (Figure 36A-D).

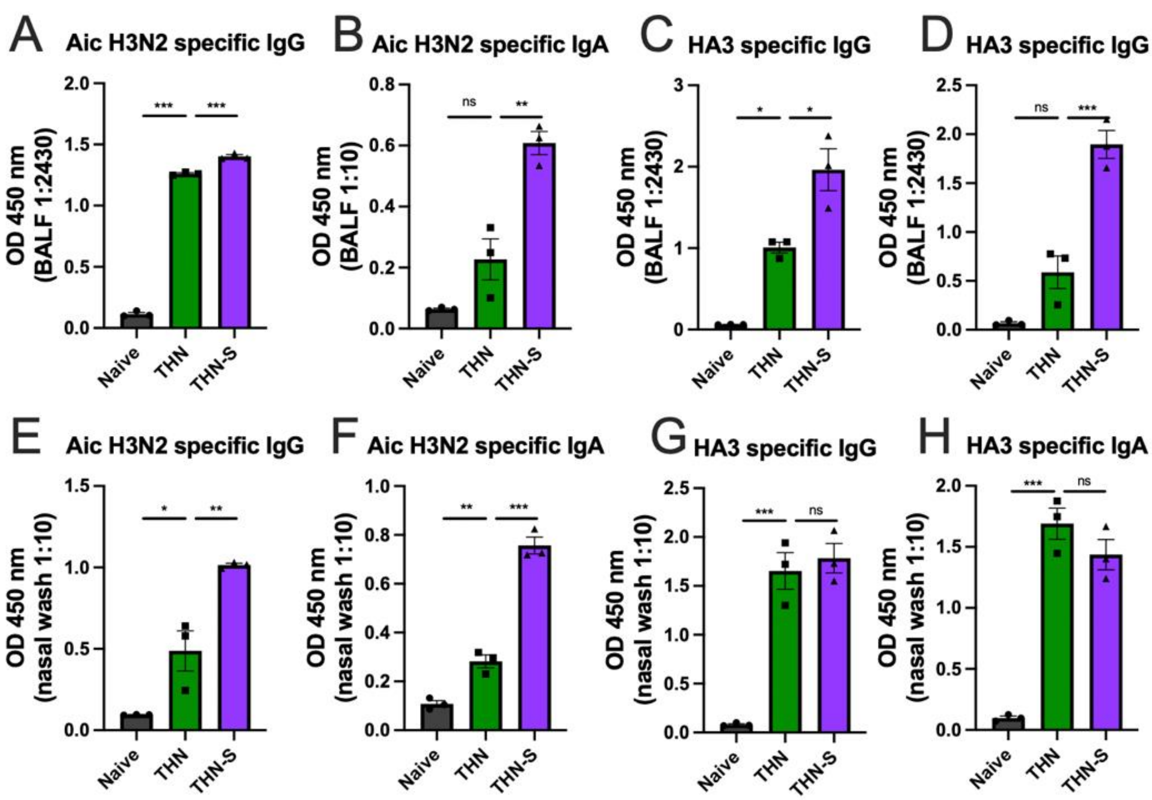

Figure 36. Mucosal immune responses in pulmonary tissue of sequentially immunized mice. Twenty-eight days post-booster immunization, nasal and BALF washes were collected to determine IgA and IgG levels by ELISA. A, B) Aichi-specific IgG and IgA levels in BALF. C, D) HA3-specific IgG and IgA levels in BALF. E, F) Aichi-specific IgG and IgA levels in nasal washes. G, H) HA3-specific IgG and IgA levels in nasal washes. The histograms were presented as mean ± SEM. Statistical significance was analyzed with one-way ANOVA followed by Tukey's test (* P < 0.05, ** P < 0.01, *** P < 0.001).

However, when comparing the sequentially immunized mice to those immunized with the conventional strategy, there was no significant difference in HA3-specific IgG and IgA antibody levels (Figures 36E and 36F). Overall, the results indicated that optimized sequential immunization could generate robust mucosal immunity, which is crucial for protection against respiratory pathogens.

3.3.b. Discussion

Recent studies have shown that extending the duration of vaccine delivery over 7–14 days through methods such as osmotic pumps or multiple small-dose injections can significantly enhance immune responses compared to traditional immunization approaches(Lee et al., 2022; Tam et al., 2016). Research on the whole inactivated virus (WIV) influenza vaccines in mice has demonstrated that prolonged antigen exposure over 28 days resulted in higher levels of high-avidity antibodies, facilitated class switching, and broadened the range of binding epitopes(Beukema et al., 2023).

Our study examined the impact of extended antigen exposure through sequential immunization via the mucosal route for the first time. By dividing the total antigen dose into multiple smaller administrations, this novel approach resulted in higher levels of antigen-specific IgA in mucosal samples and more robust germinal center (GC) reactions in lung-draining lymph nodes compared to conventional single-immunization. Sequential immunization significantly increased GC-Tfh cells, essential for B cell interactions within germinal centers, promoting B-cell antibody class switching and enhanced mucosal antibody production. Furthermore, our data indicated that sequential immunization boosted memory Tfh (mTfh) cells, typically located outside germinal centers in peripheral tissues or circulation. These mTfh cells are crucial for long-term memory responses to pathogens upon re-exposure to antigens(Gao et al., 2023). The increase in these cell populations provided superior protection against heterologous influenza viral challenges. Mice that underwent sequential immunization showed complete protection and rapid recovery from heterologous viral infections.

Also, the sequential prime immunization with five doses over eight days significantly elevated Aichi- and HA3-specific IgG antibody levels compared to conventional single-dose priming. This suggests that extended exposure to antigen through sequential administration may provide a more substantial initial immune stimulus, setting the stage for more effective booster responses. Interestingly, when a booster dose was administered, both the sequentially and conventionally primed groups exhibited comparable increases in IgG levels, indicating that the initial priming strategy was less influential in the long-term humoral response once the booster was given. However, sequential priming led to a more balanced Th1/Th2 response, as evidenced by the enhanced and balanced IgG1 and IgG2a isotype titers. This suggests that sequential immunization may better prime the immune system to produce a more balanced and comprehensive immune response as supported by the enhanced protective efficacy against heterologous influenza, with the THN-S group showing complete protection and no significant weight loss, in contrast to the moderate weight loss observed in the THN group.

Despite the apparent advantages of the five-dose regimen, we explored whether an optimized strategy with fewer doses could still yield comparable immune responses. Our results showed that the reduction from 5 to 3 doses over six days did not compromise the immune response, as the optimized sequential immunization strategy continued to promote vigorous germinal center activity and robust effector T-cell responses. The observed increase in GL7 and CD95 double-positive B cells, as well as the elevated levels of $CD4^+$ T follicular helper (Tfh) cells and potential memory Tfh cells, indicated that the optimized strategy successfully maintained the critical components of effective adaptive immunity despite the reduced dosing schedule. Furthermore, the optimized sequential strategy showed significant mucosal immune responses, with increased levels of antigen-specific antibodies in both BALF and nasal wash samples.

These findings suggest that sequential prime immunization could effectively enhance systemic and mucosal immune responses and improve vaccine efficacy against influenza and other infectious diseases. The induction of comparable immune responses with the reduced dosing strategy makes it more convenient and cost-effective and underscores its potential applicability in large-scale immunization programs where efficiency and patient compliance are critical.

3.3.c. Conclusion

In the previous section, we demonstrated that intranasal vaccination with tFliC-adjuvanted protein nanoparticles significantly improved survival rates against H3N2 viral challenges. We employed a sequential immunization strategy to enhance the induced immune responses, administering the vaccines in five applications over eight days. This approach caused a balanced Th1/Th2 response and enhanced secretory IgA (sIgA) levels, germinal center (GC) reactions, and effector T-cell populations in lung-draining lymph nodes, resulting in superior protection against heterologous influenza viral challenges. Even when the dosing strategy was significantly reduced from 5 to 3 doses, the optimized sequential immunization strategy maintained vigorous germinal center activity, effector T-cell responses, and mucosal immunity.

This method of sequential immunization shows promise in further boosting the effectiveness of tFliC-adjuvanted protein nanoparticle vaccines. It offers a potent strategy for enhanced systemic and mucosal immune responses and provides robust protection against viral challenges. Sequential immunization could be a key strategy in developing more effective and durable influenza vaccines by extending antigen exposure and promoting more comprehensive immune responses. The ability to elicit strong immune responses with fewer doses makes the strategy more practical for large-scale immunization programs and underscores its potential in developing next-generation vaccines. Future studies should continue exploring and refining these strategies, focusing on their application in different vaccine platforms and against a broader range of pathogens, ultimately contributing to advancing more effective and accessible immunization approaches.

CHAPTER 4. CONCLUSION

Influenza remains a significant global health challenge, continually exacerbated by the dynamic and rapid evolution of influenza A virus strains. Our research introduced an innovative approach to influenza vaccination by developing a novel vaccine formulation using self-adjuvanted double-layered protein nanoparticles. This study aimed to enhance influenza vaccine efficacy by understanding the impact of prior influenza exposure, developing mucosal vaccines, and employing sequential immunization.

Impact of Prior Influenza Exposure. We investigated how previous exposure to the Phili influenza virus influences immune responses to subsequent HA3/NP protein nanoparticle vaccinations. Our findings demonstrated that prior Phili infection, combined with HA3/NP nanoparticle immunization, resulted in complete protection against the Aichi virus. This combination induced balanced Th1/Th2 antibody responses, elevated HA3-specific IgG, IgG1, and IgG2a levels, and robust IL-4 and IFN-γ secreting T-cell responses. These results highlight the importance of considering previous viral exposures in designing effective vaccination strategies.

Mucosal Vaccine Development: We then developed a novel intranasal vaccine using tFliC-adjuvanted double-layered protein nanoparticles. This vaccine significantly enhanced antigen-specific mucosal and systemic immune responses. Including tFliC as a mucosal adjuvant activated TLR5 signaling, promoting dendritic cell maturation and cytokine secretion. Intranasal administration of these nanoparticles increased IgG and IgA levels in bronchoalveolar lavage fluid (BALF) and nasal washes, demonstrating strong cross-reactive antibody responses and protection against homologous and heterologous influenza viral challenges.

Sequential Immunization Strategy. We employed a sequential immunization strategy to enhance immune responses, immunizing in five applications over eight days. This approach significantly increased secretory IgA levels, germinal center (GC) reactions, and effector T-cell populations in lung-draining lymph nodes. Sequential immunization resulted in higher antigen-specific IgA levels in mucosal samples and more robust GC reactions than single-prime immunization. Additionally, it boosted memory Tfh (mTfh) cell levels, which are crucial for long-term immunity, and provided superior protection against heterologous influenza viral challenges.

In summary, this comprehensive study demonstrates that considering prior viral exposure, developing innovative mucosal vaccine formulations, and implementing sequential immunization strategies can significantly enhance immune responses and protection against influenza viruses. These findings underscore the potential of tFliC-adjuvanted protein nanoparticles as effective candidates for next-generation influenza vaccines. Future research should continue to optimize these strategies to achieve broader and more durable immunity against diverse influenza strains.

CHAPTER 5. MATERIALS AND METHODS

5.1 ETHICS

The animal studies were conducted following the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. The American Association for Accreditation of Laboratory Animal Care accredited the animal facility at Georgia State University. The Institutional Animal Care and Use Committee (IACUC) at Georgia State University approved all animal procedures under protocol A22029.

5.2. RECOMBINANT PROTEIN PURIFICATION

The nucleoprotein (NP) from Puerto Rico (A/H1N1), GCN4-stabilized trimeric hemagglutinin (HA3) from Aichi (A/H3N2), and the recombinant head-removed HA3 (hrHA3) were produced using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA). These recombinant proteins were purified using HisPur Ni-NTA Resin (Thermo Scientific, Rockford, IL, USA). The proteins were characterized by SDS-PAGE and Western blotting, with detection facilitated by an anti-His antibody (Cat. No. ab18184, Abcam).

5.3. INACTIVATED VIRUS PREPARATION

Formalin-inactivated A/Aichi viruses (FI-Aichi) were prepared as previously described(Zhu et al., 2017). The process involved inactivating purified viruses with 0.02% formalin at 37°C overnight, followed by ultracentrifugation at 30,000 rpm for 2 hours to pellet the virus. The resulting pellet was resuspended in PBS. A BCA protein assay kit (Thermo Fisher Scientific, Inc., MA, USA) was used to determine the concentration of the obtained virus.

5.4. NANOPARTICLE FABRICATION

5.4.a. DTSSP Nanoparticle Fabrication.

DTSSP nanoparticles were fabricated using the DTSSP (3,3'-dithiobis (sulfosuccinimidyl propionate)) crosslinker (Ma, Wang, Dong, Gonzalez, Song, et al., 2022). In brief, the NP protein was first desolvated with ethanol, and the resulting NP particle pellets were collected by centrifugation at 15,000 × g for 20 minutes at room temperature. These pellets were then resuspended through sonication in solutions containing HA3 or hrHA3 proteins (100 µl, 0.5 µg/µl in DPBS). Crosslinking was achieved by adding 5mM DTSSP for one hour while stirring at 4°C and quenching with 30mM Tris-HCl solution at pH 7.4 for 15 minutes. The nanoparticles were then collected by centrifugation at 15,000 × g, 4°C for 30 minutes and resuspended in 50 µl DPBS through sonication.

5.4.b. SDAD Nanoparticle Fabrication.

To create double-layered nanoparticles, we used a process adapted from earlier studies(Zhu et al., 2023a). In brief, 200µg of NP protein was combined with four times the volume of ethanol and stirred for 20 minutes. The NP-core was centrifuged at 20,000 × *g* for 20 min and resuspended in 250µL DPBS. Then, the mixture was sonicated at 40% amplitude. The resulting NP core nanoparticles were then treated with SDAD ((NHS-SS-Diazirine) (succinimidyl 2-((4,4′-azipentanamido) ethyl)- 1,3′-dithiopropionate)) for 30 minutes, followed by the addition of 15 µL Tris-HCl (1 M, PH 8.0) buffer to stop the reaction. Excess SDAD was removed by washing with DPBS and centrifuging at 20 000 × *g* for 20 min. Next, the SDAD-conjugated NP core nanoparticles were incubated with HA3 (200µg) or a combination of HA3 and tFliC (160µg and 40µg, respectively) proteins under UV light (350 nm) for 1 hour. The final nanoparticles were purified by centrifugation at 20 000 × *g* for 20 min, then resuspended and sonicated in 200μL DPBS.

The fluorescently labeled nanoparticles were prepared following the previously established protocols(L. Deng et al., 2018). Briefly, 10mg of the SDAD protein nanoparticles were dissolved in mL of 0.1M sodium bicarbonate buffer. Then, 3μL of the amine-reactive dye (Alexa Fluor 488 or Alexa Fluor 700 NHS Ester, Thermo Fisher) dissolved in DSMO at 10mg/mL was slowly added to the protein solution, followed by a 1-hour incubation at room temperature under continuous stirring. The resulting fluorescently labeled particles were combined with non-labeled nanoparticles in a 1:3 ratio.

5.5. NANOPARTICLE CHARACTERIZATION

The obtained nanoparticles were further characterized for their composition, size, surface zeta potentials, and morphology. The particle composition was examined using 10% sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel electrophoresis and Coomassie blue staining. Dynamic light scattering was performed using the Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) to determine the sizes and surface zeta potentials of the obtained nanoparticles. Scanning electron microscopy (SEM) using the Tescan Vega’s 3rd generation (TESCAN Ltd., Czech Republic) was conducted to characterize the morphology of the nanoparticles as described previously(Ma, Wang, Dong, Gonzalez, Song, et al., 2022).

5.6. TLR5 SIGNALING ACTIVITY

TLR5 signaling activity was assessed following a previously established protocol(Wang et al., 2017). In brief, HEK 293T cells were transfected with pUNO1-hTLR5 and reporter pGL4.32 plasmids using Lipofectamine 3000. After 48 hours, the transfected cells were stimulated with serial dilutions of soluble tFliC and tFliC-conjugated THN, with concentrations ranging from 2μg/mL to 31.25ng/mL. Following a 5-hour incubation, 100μL of Steady-Glo luciferase assay reagent was added to each well, and the contents were transferred to a white plate for measurement. The luciferase activity was then quantified using the Promega GloMax 96 luminometer (Promega Corporation).

5.7. BIODISTRIBUTION OF PROTEIN NANOPARTICLES

The distribution of nanoparticles administered intranasally in mice was examined using the in vivo imaging system (IVIS). Protein nanoparticles tagged with Alexa Fluor 700 were delivered intranasally, and the mice were sacrificed at specific time points (24-, 48-, and 120-hours post-administration). The heart, lungs, kidneys, and spleen were then collected and placed on transparent Petri dishes for ex vivo imaging. The images were captured using a Perkin-Elmer IVIS Spectrum system (Caliper Life Sciences), and the fluorescent intensity of the nanoparticles within the tissues was quantified by analyzing the images with Living Image software (PerkinElmer) to calculate the fluorescent radiant efficiency.

5.8. CELLULAR UPTAKE ASSAY

JAWS II cells were cultured in the alpha minimum essential medium supplemented with ribonucleosides, deoxyribonucleosides, 4mM L-glutamine, 1mM sodium pyruvate, 5ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF), and 20% fetal bovine serum (FBS). The cells were then seeded at a density of 1 $\times 10^5$ cells/well in a 24-well cell culture plate. To assess the cellular uptake, the cells were treated with fluorescently labeled protein nanoparticles at a concentration of 100μg/mL, with DPBS as the negative control. After incubating for 24 hours, the cells were washed with PBS, fixed, and permeabilized using the BD fixation/permeabilization buffer at 4 °C for 20 min. Following this, the cells were stained with DAPI at room temperature for 30 min. The uptake of protein nanoparticles and soluble HA3 proteins by JAWS II dendritic cells was analyzed using a Keyence BZ-X710 fluorescence microscope. Quantitative analysis of mean fluorescence intensity per cell was performed using the BZ-X analysis software (Keyence).

5.9. BMDC MATURATION AND CYTOKINE SECRETION

5.9.a. Flow Cytometry Analysis for the BMDC Surface Markers.

BMDC maturation and cytokine secretion were measured as previously described (Helft et al., 2015). In brief, bone marrow cells were isolated and cultured at a density of 10^6 cells/mL in complete RPMI 1640 medium (cRPMI) supplemented with 20 ng/mL GM-CSF. After two days, half of the culture medium was replaced with fresh cRPMI containing GM-CSF, and the cells were cultured for an additional day. Non-adherent and loosely attached cells were then collected by centrifugation at 250xg for 5 minutes, resuspended, and cultured in fresh GM-CSF-supplemented medium for three more days. Subsequently, the cells were harvested and seeded at a density of 10^6 cells/well in a 24-well cell culture plate. They were treated with HN, sTHN, and THN particles for 18 hours, with untreated cells as negative controls. To evaluate BMDC maturation, cells were first incubated with Zombie Aqua dye (Zombie Aqua Fixable Viability Kit, BioLegend) to differentiate live from dead cells, followed by staining with anti-mouse CD11c-APC, CD40-PE, CD80-FITC, and CD86-APC/Cy7 surface antibodies. After thorough washing, the cells were analyzed using a BD LSR Fortessa Cell Analyzer.

5.9.b. ELISA for the BMDC Cytokine Secretion.

For cytokine analysis, the TNF-α, IL-1β, and IL-6 in the supernatant of BMDC cultures were quantified using Enzyme-linked immunosorbent assay (ELISA). Plates (Corning Inc., NY, USA) were coated with 4μg/mL of cytokine-specific capture antibodies and incubated overnight at 4°C. After this, 50μL of supernatant was added to each well and incubated for 2 hours at 37°C. Biotin-conjugated detection antibodies were then added, followed by HRP-conjugated streptavidin. The absorbance was measured at 450 nm to determine the cytokine concentrations using a Reader-6000-E (Biosys).

5.10. NANOPARTICLE IMMUNIZATION AND CHALLENGE

5.10.a. DTSSP Nanoparticle I.M. Immunization and Influenza Infection.

Female BALB/c mice, aged 6–8 weeks, were divided into two experimental subsets, with five mice in each group. On day 0, all mice were pre-infected with 0.01 × LD50 of the Phili strain. One subset was intramuscularly (I.M) immunized twice with 1μg of HA3/NP nanoparticles or PBS as control at weeks 16 and 20, respectively. Immune sera were collected at week 22 for antibody analysis. Additionally, these mice were sacrificed at week 22 to collect spleens to analyze cytokine-secreting T-cell responses. The second subset of mice was I.M immunized with 1μg of double-layered protein nanoparticles (HA3/NP, hrHA3/NP, or PBS as a control) at weeks 16 and 20. These mice were subsequently challenged with 5 × LD50 Aichi at week 22 to examine the protective efficacy of the immunizations.

5.10.b. SDAD Nanoparticle I.N. Immunization and Influenza Infection.

Female BALB/c mice, aged 6–8 weeks, were divided into four experimental groups. All mice received intranasal (I.N.) priming and boosting immunizations on days 0 and 28, respectively, with 35μL of the following vaccine formulations: (1) HA3/NP nanoparticles (HN,12μg), (2) HA3/NP nanoparticles combined with soluble tFliC (sTHN, 12μg of HN and 3μg of tFliC), (3) HA3-tFliC/NP nanoparticles with tFliC adjuvanted on the protein surface (THN, 15μg), and (4) PBS as a control. Immune sera were collected at week 7 or 3 post-boost immunization. In week 8 or 4 post-boost immunization, the mice were intranasally challenged with 5× LD50 Aichi (A/H3N2) or 3× LD50 Phili (A/H3N2). Mouse body weights were monitored for 14 days following infection.

To evaluate the impact of sequential immunizations, three experimental groups of mice were immunized with 35μL of THN (15μg) intranasally. For the priming immunization, the mice received (1) PBS, (2) a single traditional prime immunization with 15μg of THN, and (3) an 8-day sequential prime immunization with THN (THN-S), consisting of five doses of 3μg each. In week 4, groups (2) and (3) received a single traditional booster immunization of 15μg THN. Four weeks after the booster immunization, the immunized mice were intranasally challenged with 3× LD50 of Phili(A/H3N2).

5.11. HUMORAL IMMUNE RESPONSE ASSAYS

5.11.a. Enzyme-Linked Immunosorbent Assay (ELISA).

Mice immunized with DTSSP-Nanoparticles. Mice immunized with DTSSP-Nanoparticles had their immune serum IgG subtypes specific to HA3, hrHA3, HA4, and HA10 examined using ELISA. ELISA plates (Corning Inc., NY, USA) were first coated with target antigens (4μg/mL) and left overnight at 4°C. The next day, plates were blocked with 2% BSA for 1 hour at 37°C. After blocking, serial dilutions of sera were added to the antigen-coated plates and incubated for 2 hours at 37°C. Subsequently, HRP-conjugated anti-mouse IgG subtype antibodies (IgG, IgG1, and IgG2a) were introduced and incubated for 1 hour at 37°C. Finally, the assay was concluded by adding the HRP substrate TMB solution (Thermo Scientific, US), stopping the reaction with 1M H2SO4, and measuring the absorbance at 450 nm using a Reader-6000-E (Biosys).

Mice immunized with SDAD-Nanoparticles. Similarly, ELISA was conducted to evaluate the IgG or IgA antibody titers specific to various proteins (HA3, HA4, HA7, HA10, and HA3 stem) or viruses (Aichi, Phili, Wis, or rSH) in sera, nasal washes, or BALF samples post-immunization. Serially diluted sera, nasal wash, and BALF samples were added to the plates pre-coated with the various protein or virus antigens (4 μg/mL). Then, HRP-conjugated anti-mouse IgG (IgG, IgG1, and IgG2a) or IgA antibodies were applied and incubated for 1 hour. The highest dilution with an OD450 value twice that of the naive group was considered the endpoint titer.

5.11.b. Hemagglutinin Inhibition (HAI) Assay.

The hemagglutinin inhibition (HAI) assay was employed to determine virus-specific HAI titers in mouse immune sera, as described (Weldon et al., 2010). In brief, the samples were treated overnight with receptor-destroying enzyme RDE II (Denka Seiken Co., Ltd) at 37°C and then heated at 56°C for 30 minutes. Subsequently, four HA units of Aichi virus were incubated with two-fold serial dilutions of the sera at room temperature for 1 hour. To determine the HAI titer, turkey red blood cells (LAMPIRE Biological Laboratories, USA) were added. The HAI titer was identified as the highest serum dilution that effectively inhibited virus hemagglutination.

5.12. CELLULAR IMMUNE RESPONSE ASSAYS

Following previously established protocols, the number of interleukin-4 (IL-4) and Interferon-gamma (IFN-γ) cytokines secreting T cells were examined by ELISPOT assays at week 22 (Y. Wang et al., 2020). Briefly, ELISPOT plates (BD Biosciences) were coated with 4 µg/mL of murine IL-4 or IFN-γ specific monoclonal antibodies overnight. After washing and blocking, one million splenocytes were added to the plates and stimulated with different stimulators for 2 days at 37°C. After incubation, the cells were removed, and biotinylated IL-4 or IFN-γ detection antibodies and horseradish peroxidase (HRP)-conjugated streptavidin were added. Spot-forming cells were then counted using a Bioreader-6000-E (Biosys, Germany).

Additionally, the antigen-specific antibody-secreting cells (ASCs) in splenocytes and bone marrow cells were examined at week 22, as previously described(Y. Wang et al., 2020). In brief, ELISPOT plates (BD Biosciences) were coated with HA proteins. After washing and blocking, bone marrow or spleen cells were added to the plates and incubated for 18 hours at 37°C. The spots were then visualized using HRP-conjugated goat anti-mouse IgG antibody and peroxidase substrate. The spot data were read and analyzed using a Reader-6000-E (Biosys, Germany).

5.13. FLOW CYTOMETRY

Following previously established protocols, mice immunized with SDAD nanoparticles were euthanized four weeks post-boosting immunization to collect the lung tissues and assess the tissue-resident memory B cell populations (Zhu et al., 2023a). Lung samples were treated with 1 mg/mL Collagenase type IV and 30 μg/mL DNase I in RPMI 1640 media for 30 minutes at 37°C. The samples were then homogenized through a 70μm cell strainer and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was discarded, and the cells were washed with FACS buffer (DPBS with 2% FBS). The cells were stained with antibodies against CD19-APC, B220-AF700, IgD-FITC, IgM-PE/Cy7, CD69-PE, CD38-Pacific Blue, CD16/32, and Zombie NIR dye.

FACS analysis was also conducted on mediastinal lymph nodes (mLNs) 14 days after the primary immunization to evaluate the impact of sequential immunizations. The mLN cells were split into two groups and stained with different antibody combinations. The first group was stained with anti-mouse B220-AF700, GL7-PerCP/Cy5.5, CD95-FITC, CD38-Pacific Blue, CD16/32 antibodies, and Zombie NIR dye. The second group was stained with anti-mouse CD4-APC, CD62L-FITC, CD44-BV421, PD1-PE/Cy7, CXCR5-PE, CD16/32 antibodies, and Zombie NIR dye. The cells were then analyzed using the BD LSR Fortessa Cell Analyzer.

5.14. STATISTICAL ANALYSIS

For humoral and cellular immune data, error bars represent the standard deviation. Data are presented as means with standard error of the mean (SEM). One-way ANOVA followed by Tukey's test, with P values < 0.05, was used to analyze the statistical significance.

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VITAE

Joo Kyung Kim was born on October 12, 1990, in Jinju, South Korea. She attended 1st High School Ambelokipon in Thessaloniki, Greece. In 2013, she earned her Bachelor of Science in Biology and Chemistry, with minors in Mathematics and Pre-professional Studies, from Mars Hill University in Mars Hill, NC. Following her undergraduate studies, she pursued a Master of Science in Biochemistry and Molecular Biology at the University of Georgia, Athens, GA. Upon completing her master's degree, she worked as a research specialist at Emory University. In 2019, she began her PhD studies at the Institute for Biomedical Sciences at Georgia State University, Atlanta, GA, under the mentorship of Dr. Baozhong Wang. She successfully completed her PhD in September of 2024 and will begin her postdoctoral training at the Food and Drung Administration in Silver Spring, MD. Her academic publications are detailed below.

- **"**Sequential priming immunization augments immunity against heterosubtypic infections in mice**"** Wandi Zhu*, **Joo Kyung Kim***, Lai Wei, Chunhong Dong, Bao-Zhong Wang. In Progress (*Co-first author)
- "Impact of influenza immune imprinting on immune responses to subsequent vaccinations in mice" Yao Ma*; Chunhong Dong*; **Joo Kyung Kim*;** Wandi Zhu; Chunhong Dong; Lai Wei; Gilbert X. Gonzalez; Muhammad Younis; Sang-Moo Kang; Bao-Zhong Wang. Under review (*Co-first author)
- "Double-layered protein nanoparticles conjugated with truncated flagellin induce improved mucosal and systemic immune responses in mice" **Joo Kyung Kim**; Wandi Zhu; Chunhong Dong; Lai Wei; Yao Ma; Timothy Denning; Sang-Moo Kang; Bao-Zhong Wang. Nanoscale Horizons; 10.1039/d4nh00287c.

- "Enhancing cross-protection against influenza by heterologous sequential immunization with mRNA LNP and protein nanoparticle vaccines" Chunhong Dong; Wandi Zhu; Lai Wei; **Joo Kyung Kim**; Yao Ma; Sang-Moo Kang; Bao-Zhong Wang. Nature Communications. 2024;15;5800
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